EFFECTS OF IN OVO EXPOSURE TO SODIUM PERCHLORATE ON DEVELOPMENT, GROWTH AND REPRODUCTION OF FENCE LIZARDS (*SCELOPORUS SP*.)

By

MELINDA SUZANNE REDICK-HARRIS

Bachelor of Science
Texas A&M University
College Station, TX
1997

Master of Science
University of North Texas
Denton, TX
2001

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2006

EFFECTS OF IN OVO EXPOSURE TO SODIUM PERCHLORATE ON DEVELOPMENT, GROWTH AND REPRODUCTION OF FENCE LIZARDS (*SCELOPORUS SP*.)

Dissertation Approved:

Larry G. Talent

Dissertation Adviser

David M. Janz

Joseph R. Bidwell

Karen McBee

A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGEMENTS

Although I am fortunate to hold the author spot on this dissertation, many people chipped in to make it a reality. First and foremost, I'd like to thank my advisor Larry Talent for allowing me the opportunity to work on this project. Thank you for being supportive and practical and excited about the research. Thank you, perhaps most of all, for being my friend.

I want to thank my committee members Dave Janz, Joe Bidwell and Karen McBee for their patience and straight talk. Their comments and critiques have aided my professional development and contributed greatly to my understanding.

Being able to depend on numerous people for advice and help is extremely important and was vital to this project. I am grateful for Scott Talent, Troy Talent, Jonathan Udoka and Sean Ball for taking the time to care for the lizards. Dr. Weber walked me through the technical aspect of working in the lab and Ruth Carlson and Naomi Cooper also shared lab space and ideas for which I am grateful.

My family definitely deserves a huge THANK YOU. My parents, William and Janice Redick were very supportive (once the shock of this endeavor wore off), and provided a constant ear and much needed advice. I also owe thanks to my brother John Redick for making available the occasional yet necessary diversion. My husband Lance Harris has sacrificed his time to give me enough to finish this project while providing encouragement and constant love and I am grateful and indebted. Abby Redick, my daughter, began the journey that is graduate school with me eight years ago. She has spent countless hours in the lab, moved from Denton, TX, to Stillwater, OK, and has happily thrived in graduate school poverty. Abby makes motherhood an irreplaceable joy.

Funding for this project was provided by the American Chemistry Council and Oklahoma State University Department of Zoology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter II.

Chapter III.

CHAPTER I

INTRODUCTION

Numerous reptile species are listed as endangered or threatened in the United States and the world, and environmental contaminants are thought to be at least partly responsible (Gibbons et al., 2000). Ecotoxicological studies are often performed using standard laboratory models. Because a laboratory model fails to exist for reptiles, they have been largely neglected in the realm of ecotoxicology (Hopkins, 2000). In fact, toxicology studies involving reptiles comprise only about 1.5% of published studies of which most are turtles and alligators, leaving squamate reptiles grossly underrepresented (Sparling et al., 2000). Recent studies on western fence lizards (*Sceloporus occidentalis*) and eastern fence lizards (*S. undulatus*) indicate they have potential as laboratory reptile models because they are small, easy to handle, display phenotypic and behavioral sexual dimorphism, and breed well in captivity (Talent et al., 2002; Brasfield, 2004). Therefore, fence lizards may be useful for many types of ecotoxicological studies including evaluating effects of endocrine disrupting chemicals on reptiles.

Elucidating effects of endocrine disrupting chemicals on vertebrates is one area of concentration in ecotoxicology. Of particular interest for my research are thyroid inhibiting compounds. The function and inhibition of thyroid hormones in vertebrates have received considerable attention because these hormones affect growth, metabolism, as well as influence production of other hormones (McNabb, 1983, John-Alder, 1983). Disruption of the thyroid gland and subsequent hormone production may be particularly

deleterious to vertebrates and result in permanent changes in some physiological systems. Perchlorate is a pervasive compound that interferes with the normal functioning of the thyroid gland and can cause a reduction in circulating thyroid hormones (Urbansky, 1998).

Perchlorate contamination is usually attributed to ammonium perchlorate, which is used predominantly by the government as a fuel oxidizer necessary to propel rockets and missiles at high velocities (Urbansky, 1998). In western states such as Nevada, California, and New Mexico, perchlorate has been detected in water and soil at levels high enough to generate concern regarding human health (EPA, 1998). Because perchlorate competitively inhibits iodide uptake by the thyroid gland, the amount of thyroid hormones (thyroxine, T_4, and triiodothyronine, T_3) the body can synthesize may be reduced when an animal is exposed to perchlorate. Ultimately, chronic perchlorate exposure often results in hypothyroidism in vertebrates.

Terrestrial reptiles inhabit environments where perchlorate contamination has occurred. Many reptiles burrow into the ground and are therefore in direct dermal contact with the substrate. Furthermore, reptiles ingest small amounts of soil to acquire necessary minerals (Sylber, 1988; Beyer et al,. 1994), and bury their eggs in the substrate. Because lizard eggs are porous, developing embryos may be exposed to dissolved perchlorate as pore water diffuses into the egg throughout incubation. Most species of lizards are insectivorous and may consume insects that have accumulated perchlorate by feeding on perchlorate-concentrating plants.

Although exposure of reptiles to perchlorate is likely in some habitats, almost nothing is known of the effects of perchlorate on reptiles. Furthermore, standard toxicity

tests for determining the effects of contaminated soil on vertebrates do not exist. Therefore, a need to investigate the effects of substrate perchlorate contamination on a reptile is evident.

Overall Objectives

The purpose of this study was to evaluate the effects of *in ovo* exposure to perchlorate on western (*Sceloporus occidentalis*) and eastern (*S. undulatus*) fence lizards. Specifically, the following objectives were examined:

1. Evaluate the effects of exposing embryonic western fence lizards to graded doses of perchlorate.
   a. Characterize the normal thyroid (T_3 and T_4) and corticosteroid hormone levels during the perinatal period.
   b. Determine the effects of *in ovo* exposure to sodium perchlorate on thyroid (T_3 and T_4) and corticosteroid hormone levels of hatchling fence lizards.
   c. Evaluate the effects of perchlorate exposure on oxygen consumption as a direct measurement of metabolic rate during the incubation period.
   d. Determine the effects of *in ovo* exposure to perchlorate on incubation length, hatching success, hatchling size, and subsequent growth.
2. Determine the effects of *in ovo* exposure to perchlorate on subsequent reproduction, circulating thyroxine (T_4) and testosterone (males only) of adult eastern fence lizards.

General Hypotheses

Five general hypotheses were tested during my research. The results make up the bulk of this dissertation. The hypotheses are as follows:

1. Ho: There will be no detectable increase in thyroid or glucocorticoid hormones in western fence lizard embryos preceeding hatch.

   Ha: An increase in thyroid or glucocorticoid hormones will occur preceeding the hatch of western fence lizards similar to that seen in precocial birds.

2. Ho: There will be no detectable difference in whole body thyroid hormone levels between perchlorate exposure groups and controls in hatchling western fence lizards.

   Ha: Perchlorate will cause a reduction in circulating whole body T_3 and T_4 levels in hatchling western fence lizards.

3. Ho: The number of western fence lizards that hatch will not change in perchlorate exposed vs. control groups.

   Ha: Because thyroid hormones are instrumental in the hatching process and perchlorate causes hypothyroidism, the number of western fence lizards that hatch will decline in a dose dependant manner in perchlorate exposed individuals.

4. Ho: Whole body corticosterone levels in perchlorate exposed groups of western fence lizards will not differ from control groups.

   Ha: Because the endocrine system is an integrated system, changes in T_3 and T_4 levels will cause subsequent changes in whole body corticosterone levels in western fence lizards.

5. Ho: Embryonic hypothyroidism due to *in-ovo* perchlorate exposure will not cause decreased clutch sizes of adult eastern fence lizards.

   Ha: Embryonic hypothyroidism due to *in-ovo* perchlorate exposure will cause decreased clutch sizes of adult eastern fence lizards.

LITERATURE REVIEW

Fence Lizard Eggs

Female fence lizards usually bury the entire clutch of eggs in a single location. The eggshell consists of a calcareous layer and fibrous shell membrane (Packard et al., 1982). The external calcareous layer consists mainly of calcium carbonate in the form of calcite (Ferguson, 1982; Packard et al., 1982), but this layer is extremely thin, leaving the egg porous and flexible.

Unlike avian eggs with a thick calcareous outer layer, the porous eggs of fence lizards exchange water and gasses readily with the surrounding environment (Ackerman et al., 1985). Their eggs generally contain proportionately less water at oviposition than reptiles that lay hard-shelled eggs and must absorb supplemental water from the environment for proper development (Packard and Packard, 1980) at times doubling or tripling in mass from oviposition to time of hatch (Tracy, 1980; Andrews and Sexton, 1981). Furthermore, as eggs gain water from the substrate, they also lose water vapor to the atmosphere (Ackerman et al., 1985). Therefore, an incubating egg can act as a wick which absorbs water and dissolved contaminants from the substrate and may bioaccumulate soil contaminants as water is evaporated from the egg. Talent et al. (2002) suggested that fence lizard eggs may have potential as a lizard embryo toxicology assay

for evaluating effects of soil contaminants on reptile development. Subsequently, Brasfield et al. (2004) demonstrated that incubating eastern fence lizard eggs will accumulate cadmium from the incubation substrate.

Environmental water absorption also has been directly linked with incubation time and implicated in affecting hatchling size. Eggs of the grass anole (*Anolis auratus*) (Andrews and Sexton, 1981), zebra-tailed lizard (*Callisaurus draconoides*), and the common snapping turtle (*Chelydra serpentina*) (Packard et al., 1982) incubated in substrates with a higher water potential had a longer incubation period than eggs incubated in drier substrates. Hatchlings from clutches incubated in substrates with a higher water potential were also larger, but it is unclear if this is a function of the hydric environment or factors such as length of incubation period and egg size at hatch (Packard et al., 1982).

The hatching process of fence lizards is a multi-step process although the trigger is unknown. At the onset of hatching, blood vessels near the eggshell begin to pull away from the eggshell, water beads on the outer eggshell giving the appearance of a sweating shell. During this period it is likely that the amount of oxygen available to the embryo decreases as the blood vessels pull away from the inner surface of the shell (Zug, 2001). The egg tooth, located on the reptile's snout, must slice through the leathery eggshell before the lizard begins to breathe through its nose. In this early pip stage, only the head is exposed and the remaining yolk is retracted internally through the umbilical cord. Once the yolk is fully internalized, the lizard will emerge fully from the eggshell, dig out of the substrate and disperse (Zug, 2001). The internalized yolk provides nutrients for the juvenile for several days after hatch.

Dispersal occurs in juveniles and distance of dispersal is dependent on environmental quality. Where habitat quality is high, lizard populations are large and dispersal distance increases. Males also tend to travel a greater distance away from nest sites than their female nest-mates (Zug, 2001).

As a result of nest moisture and thermal conductivity of the incubation substrate, hatchlings initially larger in size may be less prone to predation than their smaller cohorts. Because actively foraging organisms are typically less vigilant, optimal foraging models predict a direct relationship between predation and foraging time. Smaller side-blotched lizard (*Uta stansburiana*) juveniles actively forage more often and for longer periods of time than their larger conspecifics, which increases the risk of predation (Ferguson and Fox, 1984).

Most squamates must reach a minimum size before reaching sexual maturity. *Sceloporus undulatus* in a natural environment may take 1-2 years before sexual maturity is achieved (Ferguson et al., 1980). However, time to maturity can be reduced under optimal laboratory conditions. Ferguson and Talent (1993) decreased the time to sexual maturity from 1-2 years to 4-5 months in *Sceloporus undulatus garmani* and *S. u. elongatus,* respectively.

Territoriality and Related Hormones

Territory quality is also linked to body size. Sceloporine lizards have been studied extensively with regard to territoriality. According to phylogenetic mapping, territoriality in *Sceloporus* appears to stem from a common ancestor because the behavior is consistent throughout the clade (Martins, 1994). Smaller males consistently obtain and

defend inferior territories that tend to attract fewer females, which have obvious breeding implications.

To be considered territorial, three criteria must be met: 1) territory location does not vary, 2) resident organisms must defend the space, and 3) animals must retain some exclusive or priority use of space (Brown and Orians, 1970). Defense behaviors displayed by *Sceloporus* include:

- Pushups where the front legs flex, moving the head and trunk up and down.
- Nod sets or shudder bobs: Head moves from side to side rapidly interspersed with pushups (Carpenter, 1967; Martins, 1994).
- Fullshows: pushup with an arched back and laterally compressed ribcage (Martins, 1993).
- Chase
- Bite

Badges that imply social dominance include size, frequency of territorial displays, and a lighter dorsal coloration (Sheldahl and Martins, 2000). If a lizard displays these characteristics, territory is rarely lost. In fact, once territories are established, lizards can maintain residency for years suggesting that territories are established once early in life and maintained throughout maturity, in which case, larger hatchlings would have a distinct advantage (Packard et al., 1982)

Higher levels of aggression are seen when territories are being established and during the breeding season. The breeding season for *Sceloporus* is species specific. During this time testosterone levels in males are elevated, which directly affects male male aggression (Klukowski and Nelson, 1998). Similar to birds, plasma testosterone levels in *Sceloporus* are status dependent (Dufty and Wingfield, 1986; Hegner and Wingfield, 1986). Socially dominant lizards consistently have higher levels of

circulating testosterone than their submissive counterparts. Although testosterone has been directly linked with social aggression, social interactions do not precipitate rapid androgenic responses (Moore, 1987; Smith and John-Alder 1999) as is seen in birds (Wingfield et al. 1990). Rather, testosterone levels are high throughout the breeding season and declines afterwards. In August, when testosterone levels in *Sceloporus undulatus* are low, a staged prolonged challenge by another male failed to produce elevated testosterone levels or an escalated territorial response (Moore, 1987). Interestingly, after the breeding season, exogenous testosterone administration failed to produce an aggressive response in male lizards, indicating testosterone is only one variable in a complex system of hormones and behavior (Moore, 1988).

A reciprocal relationship between adrenal and gonadal steroid hormones is apparent in *Sceloporus undulatus* (Smith and John-Alder 1999). Levels of glucocorticoid hormones from the adrenal glands are an indication of the overall stress level of an organism. Animals undergoing periods of drought or starvation, display increased levels of circulating adrenal hormones, although a seasonal pattern has also been established. In squamates, annual fluctuations appear to correspond with reproductive cycles. During the breeding season, corticosterone, the predominant glucocorticoid measured in reptiles, levels are lower, even in times of stress. Because increased adrenal hormones suppress testosterone and reduce reproduction, it is thought that lowered adrenal hormones permit increased testosterone synthesis during breeding periods (Dunlap and Wingfield, 1995). After the breeding season, a prolonged challenge will not affect testosterone levels, but corticosterone levels increase (Klukowski and Nelson, 1998).

A similar trend is seen in female mountain spiny lizards (*Sceloporus jarrovi*). Females of this species aggressively defend territories from other conspecific females months before the start of the fall breeding season (Ruby, 1978) at which point, circulating levels of estradiol and testosterone are elevated. However, progesterone levels in plasma remain basal. Woodley and Moore (1999a) studied aggression in ovariectomized females with and without testosterone implants and concluded that decreased estradiol levels resulted in decreased aggression, but elevated testosterone levels produced excessive aggression. A concurrent experiment by Woodley and Moore (1999b) indicated that plasma corticosterone levels in female mountain spiny lizards are indirectly proportional to plasma testosterone and aggressive response to intruders of the same sex. These results are similar to those seen in male eastern fence lizards (*Sceloporus undulatus*) (Dunlap and Wingfield, 1995).

Focus on seasonal behaviors tends to center on annual hormone fluctuations. Although correlating hormone levels with responsive action is important in understanding function, it underestimates the multifaceted endocrine system as a whole. Hormones either act alone or in conjunction with other hormones to mediate or induce multiple functions and change in one hormone may cause a cascade of endocrine changes.

Thyroid Hormones

The two predominant thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) are hormones that affect several physiological aspects. These include: metabolism (John-Alder, 1983; Osteen, 1995), aerobic capacity (John-Alder, 1983; Chandola-Saklani and Kar, 1990), time of hatch (Daugeras et al., 1975; Decuypere et al., 1981; McNabb, 1988),

oocyte development (Ruby and Eales, 1999), sprint speed and endurance (John-Alder and Joos, 1991), gonadal function, and scale shedding (Chandola-Saklani and Kar, 1990). Furthermore, behavioral aspects such as hibernation (Sellers et al., 1982) and thermoregulation (Sinervo and Dunlap, 1995) are also affected in several taxa.

T_4, considered a prohormone in mammals, is converted by monodeiodinase to the biologically active compound, T_3 in target tissues. T_4 may be equally as important, biologically, as T_3 in birds and reptiles. For example, Chandola-Saklani and Kar (1990) examined the difference in T_3 and T_4 related to O_2 consumption, testes weight and number of scales molted in the garden lizard (*Calotes versicolor*). Molting and testis weights were affected to a greater extent by T_4 than T_3 even after lizards were treated with iopanoic acid (IOP), which arrests monodeiodination in tissue. T_4 also appears to be the active hormone in the early phase of amphibian metamorphosis (Galton et al., 1982).

To my knowledge, little data concerning thyroid hormones and oviparous reptiles exist. Therefore, bird data may offer the closest comparison. Thyroid hormones play a substantial role in developing precocial birds. Halfway through incubation, the thyroid is organized into follicles and a chick has the ability to synthesize thyroid hormones (Wentworth and Ringer, 1986). Serum thyroid hormones steadily increase from this time until just before the start of the pipping response. A spike in T_3 and T_4 can be detected from the beginning of hatch (beak penetrates the air cell); T_3 and T_4 peaks when the head is through the shell and sharply declines when the chick is one day old (McNabb, 1988). A similar increase in thyroid hormone levels was observed in altricial birds 6 days after hatch. Altricial birds differ from precocial birds in that they require significantly more parental care after hatching. The spike in thyroid hormones in both altricial and precocial

birds seems to coincide with thermoregulation. Precocial birds must thermoregulate immediately after hatching whereas altricial birds are constantly brooded for the first week (McNabb et al. 1984). Parental behavior then changes, chicks are brooded less and must regulate their own body temperature. Although Decuypere et al. (1981) did not observe an increase in hypothalamic-pituitary induced thyroid hormone release from the thyroid gland in response to rapidly cooling temperatures, monodeiodination rate of T_4 to T_3 increased in juvenile chickens (*Gallus gallus*). Increased conversion of T_4 to T_3 without increased thyroid hormone synthesis implicates that T_3 is the primary hormone responsible for thermoregulation in chickens.

Thermoregulation in reptiles is behavioral rather than metabolic but the thyroid plays a role in determining which temperatures are voluntarily selected by lizards within a thermal gradient (Wilhoft, 1966). Administration of exogenous T_4 to three populations of *Sceloporus occidentalis* caused the lizards to behaviorally thermoregulate at higher temperatures than controls and increased T_4 also increased metabolic rate of exposed lizards in the presence of a thermal gradient, but growth rates remain unchanged (Sinervo and Dunlap, 1995). However, studies in which physiologically relevant T_4 was administered, levels failed to induce an increase in resting metabolic rates in granite spiny lizards (*Sceloporus orcutti*) (John Alder, 1986), or *Sceloporus occidentalis* (John-Alder, 1990).

A general consensus is that administering physiologically relevant exogenous T_4 does not affect resting metabolic rates or growth rate of physostomatid lizards. Thyroidectomized lizards, however, display decreased metabolic and growth rates (Gerwien and John-Alder, 1992). Cardiac size (John-Alder and Joos, 1991) and oxygen

consumption (Chandola-Saklani and Kar, 1990) decrease as a result of low thyroid activity, which may lead to decreased activity levels and lower endurance levels.

Perchlorate

Perchlorate is extremely soluble and stable in water and it is difficult to remediate. It reaches the environment from a variety of sources including rocket fuels, explosive and pyrotechnic devices, fertilizers, feed additives, herbicides, and a variety of manufacturing processes (Sridhar et al., 1999). It is a prevalent component in some human water sources and is thought to be the cause of some developmental abnormalities.

Perchlorate is a powerful oxidizer and used extensively in propellants and explosives. Ammonium perchlorate is heavily relied upon as the primary fuel oxidizer by the military and aerospace industry, a major source of perchlorate pollution. The ammonium cation is readily biodegraded and replaced with sodium (Urbansky, 2000). Perchlorate is highly water-soluble, over 10,000 mg/L, and mobile throughout ecological systems. Furthermore, because chemical breakdown of the perchlorate ion occurs very slowly under normal environmental conditions, it is considered an environmentally persistent contaminant (Urbansky, 1998). Cox et al. (2000) found that certain anaerobic bacteria can reduce perchlorate by half within 30 days. However, reduction in an aerated control after the same 30 days was negligible. One subsurface transport model predicts a single concentrated release of ammonium perchlorate may act as a groundwater contaminant source for 100 years (Flowers and Hunt, 2000). Due to its physiological activity and detection in public drinking water, perchlorate has become a recent cause for concern regarding public and ecological health (EPA, 1998).

Ecological effects of perchlorate contamination have been most noticeable in aquatic organisms that undergo spontaneous metamorphosis signaling the beginning of another life phase. Sea lampreys undergo true metamorphosis when entering the free-swimming, parasitic, juvenile phase from a sedentary, filter feeding larval form. At the onset of metamorphosis, a dramatic decrease in circulating thyroid hormone can be measured (Kao et al., 1999). Sea lampreys exposed to potassium perchlorate undergo metamorphosis sooner and at temperatures that are not beneficial to free living juveniles (Manzon and Youson, 1999; Kao et al., 1999). Amphibians require an increase in thyroid hormone before the tail is resorbed, marking the end of metamorphosis (Zug, 2001). Repression of circulating thyroid hormone by environmentally relevant concentrations of ammonium perchlorate hinders tail resorption in a predictable dose dependent manner (Goleman et al., 2002).

Perchlorate is similar to iodide in both size and charge and therefore competitively inhibits the uptake of iodide into the thyroid gland. Inside the thyroid gland, perchlorate merely takes up space, which inhibits iodide absorption and reduces thyroid hormone synthesis (Saito et al., 1983). Due to its thyroid inhibiting properties, potassium perchlorate is often a medicinal treatment for an overactive thyroid and Grave's disease due to its regulatory effect of T_4 (Orgiassi and Mornex, 1990).

Almost nothing is known about the effects of perchlorate on reptiles. Reptiles, however, occur in most habitats where perchlorate contamination is present. Terrestrial reptiles occur in and around spill sites and in areas irrigated with contaminated water. They are probably exposed to perchlorate by ingesting substrate to obtain essential minerals (Sylber 1988; Beyer et al. 1994) and eating insects that have fed on perchlorate-

concentrating plants. Aquatic turtles and snakes, in particular, may be exposed to perchlorate by eating contaminated food and drinking contaminated water. Furthermore, most oviparous reptiles bury their eggs in substrate where developing embryos could be exposed to perchlorate. Nevertheless, the effect of substrate contamination on vertebrates has been almost totally neglected.

Relevance Statement

Perchlorate has recently been defined as a mobile, persistent environmental contaminant worthy of extensive investigation. The primary affect of vertebrate exposure is reduced thyroid hormone synthesis. Completion of this project will link thyroid hormone action to survival, other hormone levels, reproduction, as well as physical performance in the same organism. The variety of endpoints and exposure periods within the life cycle of the western fence lizard will serve to elucidate which stages are most susceptible to lasting exposure effects. Rarely are the effects of a compound studied at multiple levels of organization using a single organism.

LITERATURE CITED

Ackerman RA, Dmi'el R. Ar A. 1985. Energy and water vapor exchange by parchment-shelled reptile eggs. Physiological Zoology 58:129.

Andrews RM, Sexton OJ. 1981. Water relations of the eggs of *Anolis auratus* and *Anolis limifrons*. *Ecology* 62:556-562.

Beyer WN, Conner EE, Gerould S. 1994. Estimates of soil ingestion by wildlife, *Journal of Wildlife Management* 58:375-382.

Brasfield SM, Bradham K, Wells JB, Talent LG, Lanno RP, Janz DM. 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and *in ovo* effects on hatching size and thyroid function. Chemosphere 54:1643-1651.

Brown JL, Orians GH. 1970. Spacing patterns in mobile animals. *Annual Review of Ecology and Systematics* 1:239-269.

Carpenter CC. 1967. Aggression and social structure in iguanid lizards. Pp. 87-196 In W. W. Milstead (Ed) *Lizard Ecology: a Symposium*. University of Missouri Press, Columbia, Missouri. USA.

Chandola A, Bhatt D. 1982. Triiodothyronine fails to mimic the gonado-inhibitory action of thyroxine in spotted munia: Effects of injections at different times of the day. *General Comparative Endocrinology* 48:499-503.

Chandola-Saklani A, Kar A. 1990. Evidence for the role of thyroxine as a hormone in the physiology of a lizard. *General and Comparative Endocrinology* 78:173-179.

Cox EE, Edwards E, Neville S. 2000. In situ bioremediation of perchlorate in groundwater. In *Perchlorate in the Environment* (Urbansky ed.) Klewer Academic/Plenum Publishers. New York.

Decuypere E, Michels H, Kuhn ER. 1981. Thyroid-hormones before and during the hatching process in chickens - effects of low-temperature and iopanoic-acid (iop) treatment. *Annales D Endocrinologie* 42:75-76 1981

Duageras N, Brisson A, Lapointe-Boulu F, Lachiver F. 1975. Thyroidal iodine metabolism during the development of the chick embryo. *Endocrinology* 98:1321

Dufty AM, Wingfield JC. 1986. Temporal patterns of circulating LH and steroid hormones in a brood parasite, the brown-headed cowbird, *Molothrus ater* males. *Journal of Zoology* A 208:191-203.

Dunlap KD, Wingfield JC. 1995. External and internal influences on indices of physiological stress. I. Seasonal and population variation in adrenocortical secretion of free living lizards, *Sceloporus occidentalis. Journal of experimental zoology* 271: 36-46.

Environmental Protection Agency. 1998. Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization Based on Emerging Information, External Review Draft. Washington, DC. EPA Doc. No. NCEA-1-0503

Ferguson GW, Bohlen C, Woolley HP. 1980. *Sceloporus undulatus*: comparative life-history and regulation of a Kansas population. Ecology 61: 312-322.

Ferguson GW, Fox SF. 1984. Annual variation of survival advantage of large juvenile side-blotched lizards *Uta stansburiana*: its causes and evolutionary significance. Evolution 38: 342-349.

Ferguson GW, Talent LG. 1993. Life history traits of the lizard *Sceloporus undulatus* from two populations raised in a common laboratory environment. *Oecologia* 93:88-94

Ferguson MWJ. 1982. The structure and organization of the eggshell and embryonic membranes of *Alligator mississippiensis. Transactions of the Zoological Society of London.*

Flowers TC, Hunt JR. 2000. Long-Term Release of perchlorate as a potential source of groundwater contamination. In *Perchlorate in the Environment* (Urbansky ed.) Klewer Academic/Plenum Publishers. New York.

Galton VA, Cohen JS, Munak K. 1982. T_4 5' monodeiodinase: The acquisition and significance of this enzyme system in the developing *Rana catesbeiana* tadpole. Gunma Symposium 19 pp 75-90 Japan.

Gerwien RW, John-Alder HB. 1992. Growth and behavior of thyroid-deficient lizards (*Sceloporus undulatus*) *General and Comparative Endocrinology* 87:312-324.

Gibbons JW, Scott DE, Ryan TJ Buhlmann KA, Tuervulle TD, Metts BS, Greene JKL, Mills T, Leiden Y, Poppy S, Wine CT. 2000. The global decline of reptiles, deja vu amphibians. *BioScience* 50:653-666.

Goleman WL, Carr JA, Anderson TA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environmental Toxicology and Chemistry* 2(3):590-597.

Hegner RE, Wingfield JC. 1986. Behavioral and endocrine correlates of multiple brooding in the semi-colonial house sparrow, *Passer domesticus*. I. Males. *Hormones and Behavior* 20:294-312.

Hopkins WA. 2000. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. *Environmental Toxicology and Chemistry* 19:2391-2393.

John-Alder HB, Joos B. 1991. Interactive effects of thyroxine and experimental location on running endurance, tissue masses and enzyme activities in captive versus field

active lizards (*Sceloporus undulatus). General Comparative Endocrinology* 81:120-132.

John-Alder HB. 1983. Effects of thyroxine supplementation on metabolic rate and aerobic capacity in a lizard. *American Journal of physiology* 13:R659.

John-Alder HB. 1986. Effects of physiological thyroxine delivery in lizards. *American Zoologist* 26(4):A23-A23.

John-Alder HB. 1990. Effects of thyroxine on standard and total metabolic rates in the lizard *Sceloporus undulatus. Physiological Zoology* 63:837-885.

Kao Y, Manzon RG, Sheridan MA. Youson JH. 1999. Study of the relationship between thyroid hormones and lipid metabolism during $KClO_4$ induced metamorphosis of landlocked lamprey, *Petromyzon marinus. Comparative Biochemistry and Physiology* Part C 122:363-373.

Klukowski M, Nelson CE. 1998. The challenge hypothesis and seasonal changes in aggression and steroids in male northern fence lizards *(Sceloporus undulatus hyacinthinus). Hormones and Behavior* 33:197-204.

Manzon RG, Youson JH. 1999. Temperature and $KClO_4$ induced metamorphosis in the sea lamprey (*Petromyzon marinus). Comparative Biochemistry and Physiology* Part C 124:253-257.

Martins EP. 1993. Contextual use of the push-up display by the sagebrush lizard, *Sceloporus graciosis. Animal Behavior*. 45:25-36.

Martins EP. 1994. Phylogenetic perspectives on the evolution of lizard territoriality. Pp 117-144. In L. J. Vitt and E. R. Pianda, (Eds.) *Lizard Ecology: Historical and*

Experimental Perspectives. Princeton University Press, Princeton, New Jersey. USA.

McNabb AFM. 1988. Peripheral thyroid hormone dynamics in precocial and altricial avian development. *American Zoology* 28:427-440.

McNabb FMA, Lyons LJ, Hughes TE. 1984. Free thyroid-hormones in altricial (ring doves) versus precocial (japanese quail) development. *Endocrinology* 115:2133-2136

McNabb FMA. 1983 The role of serum binding proteins in determining free thyroid hormone concentrations during development in quail. *Endocrinology* 113:957.

Moore MC. 1987. Circulating steroid hormones during rapid aggressive responses of territorial male mountain spiny lizards, *Sceloporus jarrove. Hormones and Behavior* 21:511-521.

Moore MC. 1988. Testosterone control of territorial behavior: Tonic release implants fully restore seasonal and short-term aggressive responses in a free-living castrated male lizards. *General and Comparative Endocrinology* 70:450-459.

Orgiassi J, Mornex R. 1990. Hyperthyroidism In: Ma Greer (Ed.) *The Thyroid Gland*. Raven Press, New York, NY. Pp 405-495

Osteen S. 1995. Evolutionary physiology of energy use in reptiles. Ph.D. Dissertation. University of Chicago.

Packard GC, Packard MJ. 1980. Evolution of the cleidoic egg among reptilian antecedents of birds. *American Zoologist* 20:351-362.

Packard MJ, Packard GC, Boardman TJ. 1982. Structure of Eggshells and water relations of reptilian eggs. *Herpetologica.* 38:136-155.

Ruby DE. 1978. Seasonal changes in the territorial behavior of the iguanid lizard, *Sceloporus jarrove. Copeia* 1978:430-438.

Ruby SM, Eales JG. 1999. Relationship between hepatic deiodination of thyroxine and early oocyte development in rainbow trout, *Oncorhynchus mykiss. Canadian Journal of Zoology* 77:509-513.

Saito K, Yamamoto K, Takai T, Yoshida S. 1983. Inhibition of iodide accumulation by perchlorate and thiocyate in a model of the thyroid iodide transport system. *Acta Endocrinology* 104:456-461.

Sellers JC, Wit LC, Ganjam VK, Etheridge KA, Ragland IM. 1982. Seasonal plasma T_4 titers in the hibernating lizard *Cnemidophorus sexlineatus. General and Comparative Endocrinology* 46:24-28.

Sheldahl LA, Martins EP. 2000. The territorial behavior of the western fence lizard *Sceloporus occidentalis. Herpetologica* 56:469-497.

Sinervo B, Dunlap KD. 1995. Thyroxine affects behavioral thermoregulation but not growth rate among populations of the western fence lizard *(Sceloporus occidentalis). Journal of Comparative Physiology* B 164:509-517.

Smith LC, John Alder HB. 1999. Seasonal specificity of hormonal, behavioral, and coloration responses to within and between sex encounters in male lizards (*Sceloporus undulatus). Hormones and Behavior* 36:39-52.

Sparling DW, Linder G, Bishop CA, editors. 2000. *Ecotoxicology of Amphibians and Reptiles*. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC) 904 p.

Sridhar, S., Collette TW, Garrison AW, Wolfe NL, McCutcheon SC. 1999. Perchlorate Identification in Fertilizers. Environmental Science and Technology 33:3469.

Sylber CK. 1988. Feeding habits of the lizard *Saromalus varius* and *S. Hispidus* in the Gulf of California. *Journal of Herpetology* 22:413-424.

Talent LG, Dumont JN, Bantle JA, Janz DM, Talent SG. 2002. Evaluation of western fence lizards (*Sceloporus occidentalis*) and Eastern fence lizards (*Sceloporus undulatus*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 22(5):899-905.

Tracy CR. 1980. Water relations of parchment shelled lizard (*Sceloporus undulatus*) eggs. *Copeia* 3:478-482.

Urbansky ET. 1998. Perchlorate chemistry: implications for analysis and remediation. *Bioremediation Journal* 2:81-95.

Urbansky ET. 2000. *Perchlorate in the Environment.* Klewer Academic/Plenum Publishers. New York.

Wentworth BC, Ringer WJ. 1986. Circulating thyroid hormones in domestic birds. *Poultry Science* 40:1275-1276.

Wilhoft DC. 1966. Reproduction in the tropical Australian skink *Leiolopisma rhomboidalis. American Midland Naturalist* 70:442-461.

Wingfield JC, Hegner RE, Dufty AM, Ball GF. 1990. The challenge hypothesis: Theoretical implications for patterns of testosterone secretion, mating systems and breeding strategies. *American Naturalist* 136:829-846.

Woodley SK, Moore MC. 1999a. Female territorial aggression and steroid hormones in mountain spiny lizards. *Animal Behavior* 57:1083-1089.

Woodley SK, Moore MC. 1999b. Ovarian hormones influence territorial aggression in free living female mountain spiny lizards. *Hormones and Behavior* 35:205-214.

Zug GR, Vitt LJ, Caldwell JP. (Eds.) 2001. *Herpetology*. Academic Press. London.

CHAPTER II

PERINATAL CORTICOSTERONE AND THYROID HORMONE LEVELS IN WESTERN FENCE LIZARDS (*SCELOPORUS OCCIDENTALIS*) AND THE EFFECTS OF LONG TERM *IN-OVO* EXPOSURE TO PERCHLORATE

ABSTRACT

The primary objectives of this study were to characterize thyroid and corticosteroid hormones in parinatal fence lizards (*Sceloporus occidentalis*) and then determine if perchlorate would cross the eggshell membrane in absorbed pore water and affect hormone levels in hatchling lizards. Glucocorticoid and thyroid hormones increase around the time of hatch in some birds and reptiles. Perchlorate is known to cause hypothyroidism in vertebrates, which may affect levels of circulating glucocorticoid hormones. In this study, corticosterone and thyroid hormones were characterized in perinatal fence lizards. Fence lizard eggs were also incubated on perchlorate spiked substrate and whole body hormone levels in hatchling lizards were measured using ELISA. A corticosterone spike was detected the day before pipping followed by a spike in T_3 on the day of pipping. Incubation of lizard eggs on perchlorate spiked substrate caused whole body thyroid hormones to decrease, which presumably inhibited the pipping response in lizards exposed to concentrations of 1,585 μg ClO_4^-/g dry perlite and higher. Whole body corticosterone was also affected after exposure to perchlorate but not in a predictable manner. Our data indicate that thyroid and

glucocorticoid hormones may be necessary for embryonic lizards to initiate and survive hatching.

INTRODUCTION

Endocrine disrupting contaminants are ubiquitous in the environment and little is known about effects of endocrine disrupting chemicals on the hormone levels of reptiles. Especially obscure are the effects of *in ovo* exposure to a thyroid inhibiting chemical on hormone levels in hatchling lizards. In addition to being a potent endocrine disrupting chemical, perchlorate is highly water soluble, persistent and has been detected in ground water near a number of military bases throughout California and Nevada (Motzer, 2001). Ammonium perchlorate is a common fuel oxidizer used in solid fuels to propel missiles and rockets. At contaminated sites, perchlorate has been measured from 85 μg/L to as high as 3,700 mg/L in well water (Urbansky, 1998) and 1800 mg/kg in soil (Motzer, 2001).

Although little is known about the biological effects of toxicants on lizards (Hall 1980; Hall and Henry 1992; Campbell and Campbell, 2000), it seems likely that some species of lizards are exposed to perchlorate. The western fence lizard, *Sceloporus occidentalis* occurs in a diversity of habitats and would potentially be exposed to perchlorate by a number of exposure routes such as: soil ingestion at spill sites and in areas irrigated with contaminated water, eating insects that have consumed plants that bioconcentrate perchlorate (Urbansky, 98). Perhaps most interesting is that fence lizards absorb contaminants across their eggshells during incubation (Brasfield et al., 2004).

Thyroid hormone levels in organisms that have been exposed to perchlorate are typically below levels measured in control animals (McNabb et al., 2004; Goleman et al., 2002). In addition, hypothyroidism may lead to a decrease in circulating corticosterone hormone levels (Rodriguez et al., 2003) and both thyroid and glucocorticoid hormones are associated with the hatching process (Kalliecharan and Hall, 1974; Thommes and Hylka, 1977; Hulbert, 2000; Whittle et al., 2001). For example, prior to hatching, chicken embryos show increases in endogenous corticosterone and plasma T_3 (Scott, 1981). Corticosterone levels in embryonic urogenital tissues of American alligators (*Alligator mississippiensis*) also increase prior to hatch (Medler and Lance, 1998). Furthermore, around the time of hatch in birds and parturition in mammals, a surge in both glucocorticoid and thyroid hormones typically occurs (Kalliecharan and Hall, 1974; Thommes and Hylka, 1977; Hulbert, 2000; Whittle et al., 2001).

Although perinatal thyroid and glucocorticoid hormones have been studied in birds and to a limited extent in alligators, nothing has been published on perinatal hormone levels in lizards. Birds and reptiles have many similar characteristics; however the two taxa are quite variable with regard to metabolism due to differing thermoregulatory requirements. Because thyroid and glucocorticoid hormones appear to increase during the hatching process in birds, and little is known about perinatal hormone levels in reptiles, there is a need to characterize T_3, T_4, and the predominant reptilian glucocorticoid, corticosterone, during the perinatal period in all major groups of reptiles. Background knowledge of hormone trends is beneficial from a physiological as well as toxicological standpoint. With increased awareness of effects of endocrine disrupting

chemicals ubiquitous in the environment, characterizing normal hormonal cycles in lizards will provide a database for future toxicological studies.

The objectives of this study were 1) to examine whole body levels of T_3, T_4, and corticosterone during the perinatal period in western fence lizards (*Sceloporus occidentalis*) and, 2) to determine the effects of long term *in ovo* sodium perchlorate exposure on whole body thyroid and corticosterone hormone levels

METHODS AND MATERIALS

Animal housing

A laboratory population of western fence lizards was established from lizards that were collected from the San Joaquin Valley in California (Talent et al., 2002). Breeding lizards were housed on corncob substrate in 74.2-L glass aquaria that were covered with a 3-mm steel mesh lid. A 60-W incandescent light bulb was positioned over one end of each cage to permit thermoregulation across a temperature gradient of 26-40 ºC. Ambient room temperature was maintained at approximately 22 ºC and a 14:10 h light:dark light cycle was provided. Lizards were fed with house crickets, *Acheta domestica*, that had been dusted with a Herptivite® and Rep-Cal® mixture (1:1 v/v) (Rep-Cal Research Labs, Los Gatos, CA, USA) and had constant access to water. Lizards were also provided with an oviposition site consisting of a container of moist sand. Eggs were removed from containers within 12 hours of being laid.

Individual clutches were maintained in separate 600 ml plastic incubation chambers filled with 30 g vermiculite and 30 mL reagent grade water (18 mΩ). A 1 mm hole was drilled into the side to allow gas exchange throughout incubation. All eggs were incubated at a constant 28°C and monitored weekly for mortality. At 28°C embryos

hatch in approximately 49 days and when incubated together, all eggs in a clutch usually hatch within 12 hours of each other. To determine perinatal hormone levels, individual embryos were removed from the clutch and sacrificed prior to hatch. Embryonic age (days prior to hatching) was determined based on when eggs remaining in the clutch hatched. Hatchlings were placed in cages that were similar to those described for adults until they were sacrificed for determining post-hatch hormone levels.

Corticosterone Extraction

Lizards and embryos were sacrificed at -20°C at ages ranging from 4 days before hatch to 4 days after hatch. Whole body samples were individually minced with acid washed scissors in a clean glass round bottom centrifugation tube. Once minced, the samples were homogenized 3 X 20 seconds using a Tissue-Tearor™ homogenizer (BioSpec Products, Bartlesville, OK, USA) in 3 ml ice-cold reagent grade water (18 mΩ). Six ml ethyl ether was added to the homogenate, vortexed for 30 seconds and then centrifuged 10 min at 1700 X *g*. Samples were snap frozen in liquid nitrogen and ether fraction was decanted into a clean borosilicate test tube. The remaining tissue plug was resuspended in 6ml ethyl ether, vortexed, and centrifuged twice more as previously described. Supernatant from the second and third resuspension was added to the first. Test tubes containing supernatant were placed in a dry bath 45°C under a constant stream of N_2 until dry. Samples were reconstituted in 1mL sodium phosphate buffer containing gelatin (pH 7.6) and stored at -20°C until time of assay.

Thyroid Extraction

Lizard embryos and hatchlings were euthanized at –20°C within the test chambers, at ages ranging from 4 days before hatch to 10 days after hatch. Sample sizes were 10 lizards per sample day. Eggs from different clutches were marked to account for variation in hormone levels between the clutches. Specimens were allowed to thaw slightly before embryos were removed from the eggshell, separated from yolk, rinsed, dried. Internalized yolk was removed from the abdomen of post hatch lizards. Homogenization and extraction methods were modified from Kobuke et al. (1987) and Brasfield et al. (2004). Hatchlings were individually homogenized in 95% ethanol containing 1 mM 6-N-propyl-2-thiouracil (PTU, Sigma) to prevent the enzymatic conversion of T_4 to T_3. Specimens were minced in 2 ml of the ethanol/PTU mixture with acid-washed scissors. Homogenization was achieved using a Tissue-Tearor™ homogenizer (BioSpec Products, Bartlesville, OK, USA) in 50 ml conical bottom plastic centrifuge tubes (3 x 20 sec). Samples were then vortexed for 30 seconds before being centrifuged for 10 min at 1700 X *g* and 4 °C. The resulting supernatant was decanted into borosilicate glass test tubes. Pellets were re-extracted by adding 2 ml of the ethanol PTU solution and vigorously vortexing for 30 sec until resuspended and centrifuged again. Supernatant from the second extraction was mixed with the first. Extracts were evaporated at room temperature under a stream of N_2. Samples were then reconstituted in 225 µL sodium phosphate buffer containing gelatin (pH 7.6), and stored at -20°C until time of assay.

Hormone Determination

All whole body hormone levels were determined using enzyme linked immunosorbant assays (ELISA) specific for T_4, T_3 (ICN Biomedical, Costa Mesa, CA) or corticosterone (Assay Designs, Inc., Ann Arbor, Michigan) according to the manufacturer's directions. Extraction efficiencies were determined using spiked samples and were greater than 80%. Serial dilution of extracts revealed parallelism with the standard curve supplied by the manufacturer. Interassay and intraassay coefficients of variation were below 15% and 10%, respectively.

Perchlorate Exposure

We exposed eggs to sodium perchlorate in two separate replicates. Eggs from 30 clutches were placed into moist vermiculite and incubated for seven days at 28 ºC to determine which eggs were fertile and healthy. For each replica, eggs were distributed into five treatment groups and a control. An attempt was made to place no more than one egg per clutch in a treatment. Exposure chambers were prepared for each egg by placing 4 g of oven dried, size-separated perlite (<4 >0.85 mm) into acid-washed, oven-dried borosilicate cylinders (70 mm deep). Solutions of sodium perchlorate (> 99%, Sigma) were prepared in reagent grade water (18 MΩ).

Nominal concentrations of 1.95, 19.5, 195, 1,950 and 19,500 μg $NaClO_4$/g dry perlite were prepared. These concentrations of $NaClO_4$ resulted in nominal ClO_4^- concentrations of 1.58, 15.8, 158, 1,585, and 15,852 μg ClO_4^-/g dry perlite. The control was prepared by saturating perlite with an equivalent volume of reagent grade water.

Perchlorate solution or reagent grade water (control) was delivered by volumetric pipette (9 ml) into each exposure chamber.

All control and experimental eggs were placed on the perlite substrate of the exposure chambers and chambers were sealed with a plastic cap. A 1-mm hole was drilled in the center of each plastic cap for gas exchange. Egg, cap and chamber were weighed together. Every week the chambers were weighed and any difference in mass and reagent grade water was added to replace evaporated water. Chambers kept in a Precision incubator set at 28ºC ± 0.5 ºC and each egg was checked daily for mortality. Embryos were monitored until they hatched or died. Mortality of questionable embryos was verified by examination of the membrane blood vessels using back lighting.

Data Analysis

Experiments were constructed in a blocked design to account for variation within lizard clutches. Normality within each exposure group and homoscedasticity between exposure groups were determined by Kolmogorov Smirnov and Bartlett tests, respectively. All assumptions of ANOVA were met so one-way ANOVA followed by a Dunnett's *post hoc* test to compare experimental groups with control groups. A level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Perinatal Hormone Analysis

A significant spike ($p = 0.0002$) in corticosterone levels occurred on incubation day number 47, one day before pipping, at which point the mean corticosterone level reached 12.48 ng/g lizard tissue. After the corticosterone level peaked, it decreased during the pipping process to 6.79 ng/g and further dropped to 3.79 ng/g on the day of hatching (Figure 1).

We also measured a spike in embryonic (T_3) during the pipping process and through hatching. On day 47 of incubation, whole body T_3 was 0.60 ng/g tissue but increased significantly ($p = 0.006$) to 1.23 ng/g lizard tissue on the day of pipping. After embryos fully emerged from the egg, T_3 hormone levels decreased gradually until day 2 post hatch and then remained steady at 0.5 – 0.6 ng/g tissue which was slightly less than pre-hatch concentrations (Figure 2 A).

Whole body (T_4) levels were more variable than T_3. Nevertheless, a pattern in tissue levels of T_4 was apparent. On incubation day 44 tissue levels of T_4 were 6.44 ng/g. The mean levels increased and by incubation day 46, two days prior to pipping, T_4 levels were 9.57 ng/g. Thereafter, tissue levels decreased to 6.45 ng/g on the day of hatching, increased again on day one post hatch and then decreased on day two post hatch to 6.04 ng/g which was lower although not significant ($p = 0.066$) than the peak on incubation day 46 (Figure 2 B).

The T_3:T_4 ratio was lowest (0.0608) on incubation day 46, 2 days before pipping, then increased to 0.16 on the day of pipping. Thereafter, the T_3:T_4 ratio decreased to a

low of 0.089 on day one post hatch and remained around 0.11 for the next 10 days post hatch (Figure 2 C).

Hormones after exposure to perchlorate

Exposure of fence lizard eggs to sodium perchlorate resulted in significant ($p < 0.05$) mortality in the two highest exposure groups (Figure 3). Of the eggs exposed to 1,585 and 15,852 μg ClO_4^-/g, 50% and 100%, respectively, failed to hatch.

Differences in mean T_4 levels of hatchlings incubated on perchlorate-spiked substrate were affected by exposure to perchlorate (Figure 4). Whole body T_4 levels in hatchlings from the 1.58 and 15.8 μg ClO_4^-/g exposure groups were not different than levels in control lizards. However, hatchlings in the 158 and 1,585 μg ClO_4^-/g exposure groups had whole-body levels (3.8 and 2.4 ng/g, respectively) of T_4 that were significantly less ($p = 0.02$ and 0.001, respectively) than tissue levels (6 ng/g) measured in control hatchlings (Figure 4). However, tissue levels of T_4 in the embryos that did not hatch in 1,585 and 15,852 μg ClO_4^-/g exposure groups were not different from levels measured in control hatchlings

A clear dose response was observed in T_3 levels. In the eggs that hatched, there was a stepwise decrease in T_3 from a high of 1.1 ng/g in the controls to a low of 0.5 ng/g in the 1,585 μg ClO_4^-/g exposure group, which was significantly ($p = 0.016$) lower than control T_3 levels (Figure 5). Similar to the results for T_4, T_3 tissue levels in embryos that failed to hatch in the 1,585 and 15,852 μg ClO_4^-/g exposure groups were not significantly lower than levels measured in controls.

There was no apparent dose-dependant response in corticosterone levels in hatchlings from the different exposure groups (Figure 6). The 158 μg ClO_4^-/g perlite exposure group had significantly ($p < 0.05$) lower tissue levels of corticosterone (1.8 ng/g) than detected in control hatchlings (3.79 ng/g). However, the highest corticosterone levels (5.98 ng/g) were measured in hatchlings from the 1,585 μg ClO_4^-/g exposure group. Similar to trends measured in thyroid hormones, embryos exposed to 1,585 and 15,852 μg ClO_4^-/g that did not hatch had corticosterone levels that were not different from control hatchlings. The T3:T4 ratios in exposed lizards were also not different from control lizards.

There was a stepwise dose dependent increase in mortality of lizards immediately after they pipped as the perchlorate concentrations in the incubation substrate increased (Figure 7). None of the control hatchlings died while hatching. However, ten percent of the lizards in the 1.58 μg ClO_4^-/g exposure group died while hatching and the percentage of neonates that died during the hatching process increased to 22% in the 1,585μg ClO_4^-/g exposure group.

DISCUSSION

Perinatal Hormones

The objective of this study was to determine the effects of perchlorate on whole body corticosterone and thyroid hormone levels in western fence lizards after incubation on spiked substrate. However, we first needed to understand how these hormones interact around the time of hatch for these organisms. During the perinatal period of western fence lizards, whole body tissue levels of corticosterone and thyroid hormones

exhibited peaks in concentration similar to patterns previously reported for other oviparous vertebrates (Wise and Frye, 1973; Kalliecharan and Hall, 1974; Medler and Lance, 1998). Whole body corticosterone in western fence lizards began to increase on day 45 of incubation and peaked at day 47, the day before pipping. Similar trends have been recorded in urogenital tissues of alligators prior to hatch (Medler and Lance, 1998) and in chicken (*Gallus domesticus)* embryos (Wise and Frye, 1973; Kalliecharan and Hall, 1974). The range of baseline corticosterone found in other studies (10-20 ng/ml) was higher than the levels we detected but we were homogenizing an entire hatchling rather than sampling from plasma or a specific tissue. Using the entire body probably added a dilution factor to our samples. However, hatchling lizards were approximately one gram at hatch and collecting enough plasma to measure hormones was not possible.

A significant spike was also apparent for T_3 during the pipping portion of the hatching process, similar to the trend seen in Japanese quail (McNabb, 1988). A spike in thyroid hormones of an altricial bird species (*Streptopelia risoria*) occurred at a later time, suggesting that the increase is related to the onset of metabolic thermoregulation (McNichols and McNabb, 1987). Although reptiles are similar to precocial birds in that they are oviparous, terrestrial vertebrates, hatchlings do not internally thermoregulate. Thermoregulation in reptiles is primarily behavioral rather than endothermic. Administration of exogenous T_4 to three populations of western fence lizards that were provided with a thermal gradient resulted in a higher core body temperature in experimental animals compared to controls (Sinervo and Dunlap, 1995). However, when a thermal gradient was not provided, physiologically relevant levels of injected T_4 had little or no effect on metabolic rates of captive western fence lizards (Joos and John-

Alder, 1990) and granite spiny lizards (*Sceloporus orcutti*) (John-Alder, 1986). Although the predominant thyroid hormone synthesized by the thyroid gland is T_4, T_3 is considered a more biologically active form. Therefore, the prominent spike in T_3 that we observed in the pipping stage was likely related to some function other than thermoregulation.

The purpose of the spike in corticosterone on the day prior to pipping in western fence lizards is unknown. However, glucocorticoid hormones increase deiodinase activity during embryonic development in fetal lamb livers (Wu et al., 1978) and chicken embryos (Darras et al., 1996; Decuypere et al., 1982), which may increase the T_4 to T_3 conversion (Hadley, 2000). Furthermore, an increase in glucocorticoid hormones from the adrenal glands of saltwater crocodiles (*Crocodylus porosus)* caused an apparent increase in monodeiodinase enzymes (Shepherdley et al., 2002). A significant corticosterone spike in our samples occurred on day 47 concurrent with an apparent decrease in T_4. Interestingly, T_3 levels spiked one day later, on the day of pipping. Due to the observed sequence of hormone fluctuations, it is likely that corticosterone increased monodeiodinase activity in fence lizards. Although a significant decrease in the concentrations of T_4 were not apparent, T_4 levels were an order of magnitude higher than T_3 and a slight decrease in T_4 due to deiodination would result in a dramatic increase in T_3 concentrations. Therefore, it is likely that deiodination occurred during this time period and the previous corticosterone spike may have caused an increased conversion rate.

Hormones after perchlorate exposure

Exposure to perchlorate during the incubation period had a pronounced effect on whole-body tissue levels of T_3 and T_4 in hatchling western fence lizards. After long term in ovo exposure to perchlorate there was a clear dose dependent decrease in whole body T_3 levels. T_4 also decreased, as perchlorate concentrations increased in the incubation substrate. Perchlorate exposure has an anti-thyroidal effect by interfering with iodide uptake in the thyroid gland, which inhibits thyroid hormone synthesis (Urbansky, 1998). Within the thyroid gland, T_4 is the predominant thyroid hormone synthesized and to a lesser extent T_3. The thyroid gland is unique in that it is the only endocrine gland that has the ability to store hormones, namely T_4 (Hadley, 2000). Therefore, because lizards probably have the capacity to store T_4 within the colloid portion of thyroid cells, mild inhibition of glandular hormone synthesis in lower perchlorate exposure groups may not have been detected by the measurement of whole body T_4.

To our knowledge, no other studies have been conducted on the effects of perchlorate on reptiles. Therefore, we cannot compare our results with other reptile studies. However, some comparative information is available for amphibians and birds. A study by Goleman et al. (2002) found decreased whole body T_4 levels in *Xenopus laevis* tadpoles exposed to 14.14 mg/L perchlorate for 70 days. In the current study, 15.8 μg ClO_4^-/g in the substrate equaled 7.02 mg/L ClO_4^- in the pore water. At this concentration there was virtually no affect of perchlorate on the endpoints measured. It is possible that tadpoles completely immersed in perchlorate solution are influenced to a greater extent than lizard eggs incubated on perchlorate-spiked substrate. Although

perchlorate appears to affect the embryo, it is unclear at which point throughout incubation concentrations are high enough in the embryo to affect whole body T_4 levels.

Evaluating lizard sensitivity with another terrestrial rather than aquatic organism may be more appropriate. McNabb (2004) found that ammonium perchlorate concentrations below 1,000 mg/L in drinking water caused no significant decrease in circulating T_4 levels in plasma of bobwhite quail (*Colinus virginianus*) chicks. However, exposure to ammonium perchlorate concentrations equal to or higher than 1,000 mg/L did cause a decrease in circulating T_4 in the quail after two weeks of exposure. A decrease in plasma T_4 was also detected when chicks were exposed to 250 mg/L ammonium perchlorate for 8 weeks. The amount of perchlorate in 250 and 1,000 mg/L ammonium perchlorate are approximately 212.5 and 850 mg/L, respectively. We measured significant differences at 70.2 mg/L ClO_4^-, which is the equivalent of 158 μg ClO_4^-/g. Therefore, it appears that lizards may be more sensitive to perchlorate than birds. However, we examined the effects of perchlorate on developing embryos, not juveniles or adults. Because exposure routes and life stages in these studies are different, a direct comparison of sensitivity cannot be made.

In both the *Xenopus* and quail study, T_3 was not measured, presumably because T_4 is the predominant hormone leaving the thyroid gland and would intuitively be more sensitive to perchlorate exposure. T_3 is considered the more biologically active of the two hormones and is usually circulating at lower concentrations than T_4 (Hadley, 2000). Once in circulation, T_4 is enzymatically converted to T_3 in the target tissues. In a sense, T_4 is a prohormone or store for T_3, which adds another layer of protection against measurable effects attributable to exposure to thyroid disrupting chemicals.

Thyroid hormones appear to be instrumental in the hatching of vertebrates although, nothing is known about the specific trigger that initiates the pipping response in lizards. In our study, 50% and 100% of the 1,585 and 15,852 μg ClO_4^-/g exposure groups, respectively, failed to initiate hatch at the same time as control lizards. Because the mean whole body levels of T_3 and T_4 in hatchlings from the 1,585μg ClO_4^-/g exposure group were significantly lower than levels in controls, one would suspect that T_3 and T_4 levels in embryos that failed to hatch in the 1,585 and 15,852 μg ClO_4^-/g exposure groups would be even lower. However, within the 1,585 μg ClO_4^-/g exposure group, the mean tissue level of T_3 and T_4 were significantly higher in embryos that failed to hatch than in neonates that hatched. Furthermore, none of the eggs in the 15,852 μg ClO_4^-/g exposure group hatched, although T_3 and T_4 levels were not significantly lower than levels in control hatchlings. However, a comparison of T_3 and T_4 tissue levels among hatchlings from the different exposure groups and embryos that failed to hatch may not be appropriate because hormones fluctuate during the pipping and hatching process and the embryos that failed to hatch did not go through this progression from embryo too hatchling.

Glucocorticoids also appear to be influential during the parinatal period. Interactions between glucocorticoid and thyroid hormones are complicated and have been extensively studied (Liu et al., 2003; Rodriguez et al., 2003; Sullivan et al., 2002; Johnston et al., 2001; Rittenhouse and Redei, 1997; Brien, 1976). Given the interactive nature of thyroid and glucocorticoid hormones, it is not surprising that corticosterone appears at least moderately sensitive to changes in thyroid hormones caused by perchlorate exposure. Hypothyroidism hinders but does not prevent a stress induced

increase in corticosterone in rats (Rodriguez et al., 2003) presumably because low thyroid hormone levels decrease activity in the hypothalamus pituitary adrenal axis (Rittenhouse and Redei, 1997). Although corticosterone levels in stressed individuals in Rodriguez's study were lower in thyroidectomized individuals, an increase in corticosteroid still occurred at the onset of an immobilization stress trial. This indicates a corticosterone response can still be invoked when rats encounter a stressful event.

In our study, a significant decrease in whole body corticosterone hormone level was measured in the 158 μg ClO_4^-/g exposure group. This is also the lowest concentration at which whole body T_4 levels were significantly reduced and all lizards hatched without obvious difficulty. However, in hatchlings from the 1,585 μg ClO_4^-/g exposure group, we found a significant decrease in both thyroid hormone levels but an increase in tissue levels of corticosterone. *In-ovo* perchlorate exposure at this concentration hinders the hatching process and only around 50% successfully pipped through the eggshell. Those that did hatch were weak and lethargic and few were able right themselves from a dorsal position (Redick-Harris, unpubl. data). It is possible that the process of hatching was more difficult therefore more stressful for lizards exposed to 1,585 μg ClO_4^-/g compared to lizards exposed to lower perchlorate concentrations and control animals. Therefore, it is possible that the additional hatching stress caused by perchlorate induced decreases in thyroid hormones may have caused the apparent increase in corticosterone.

Changes in thyroid hormone levels affect corticosteroid levels but together glucocorticoids and thyroid hormones act synergistically on the pulmonary surfactant systems of all vertebrates (Daniels and Orgeig, 2001). Pulmonary surfactants are

necessary before the lungs are required to exchange gasses because lizard lungs completely compress upon exhalation causing contact of opposing respiratory structures. Without an anti-adhesive, inhalation is difficult or impossible (Daniels et al., 1995). Pipping for a lizard entails slicing through the egg-shell with the egg tooth. Simultaneously, the blood vessels that surround the yolk sac, which have been providing a point of gas exchange, draw away from the eggshell as the yolk sac is being retracted into the abdominal cavity of the neonate (Zug et al., 2001). At this point in the pipping process, the snout of the neonate must be through the eggshell and the lizard able to respire or it will quickly die due to suffocation. In our study, we observed a dose-dependent relationship in lizard mortality during the hatching process (between the eggtooth actually piercing the egg and the hatchling fully emerging from the shell). Although we did not measure the surfactant levels in lizards, we suspect that *in-ovo* perchlorate exposure influenced the production of surfactant. However, additional research is needed to elucidate the actual cause of death of the neonates that died during the hatching process.

In conclusion, corticosterone and thyroid hormones appear to influence the hatching process. The apparent spike in corticosterone preceding the spike in T_3 suggests corticosterone may have an effect on thyroid hormones. When embryonic lizards are exposed to perchlorate spiked substrates during incubation, whole body thyroid hormones decrease and some embryos never initiate hatch. Although corticosterone levels appear to be influenced by embryonic hypothyroidism, a clear relationship cannot be deduced from this study.

LITERATURE CITED

Brasfield SM, Bradham K, Wells JB, Talent LG, Lanno RP, Janz DM. 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. *Chemosphere* 54:1643-1651.

Brien TG. 1976. The adrenocortical status of patients with thyroid diseases, *Clinical Endocrinology* 5:97-99.

Campbell KR, Campbell TS. 2000. Lizard contaminant data for ecological risk assessment. *Reviews of Environmental Contamination and Toxicology* 165:39-116.

Daniels CB, Orgeig S. 2001. The comparative biology of pulmonary surfactant: past, present and future. *Comparative Biochemistry and Physiology Part A* 129:9-36.

Daniels CB, Orgeig S, Smits AW. 1995. The composition and function of reptilian pulmonary surfactant. *Respiration Physiology* 102:121-135.

Darras VM, Kotanen SP, Geris KL, Berghman LR, Kuhn ER. 1996. Plasma thyroid hormone levels and iodothyronine deiodinase activity following an acute glucocorticoid challenge in embryonic compared with post hatch chickens. *General and Comparative Endocrinology* 104:203-212.

Decuypere E, Kuhn ER, Clijmans B, Nouwen EJ, Hichels H. 1982. Prenatal peripheral monodeiodination in the chick embryo. *General and Comparative Endocrinology* 47:15-17.

Goleman WL, Carr JA, Anderson TA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in development *Xenopus laevis*. *Environmental Toxicology and Chemistry* 22:590-597.

Hadley ME. 2000. *Endocrinology*. Prentice-Hall Upper Saddle River, NJ.

Hall RJ. 1980. Effects of environmental contaminants on reptiles: a review. Special Science Report–Wildlife, 228. U.S. Fish and Wildlife Service.

Hall RJ, and Henry PFP. 1992. Assessing effects of pesticides on amphibians and reptiles: status and needs. *Herpetological Journal* 2:65-71.

Hulbert AJ. 2000. Thyroid hormones and their effects: a new perspective. *Biological Review* 75:519-631.

John-Alder HB. 1986. Effects of physiological thyroxine delivery in lizards. *American Zoologist* 26:A23-A23.

Johnston SD, Daniels CB, Booth DT. 2001. Development of the pulmonary surfactant system in the green sea turtle, *Chelonia mydas*. *Respiration Physiology* 126:75-84.

Joos B, John-Alder HB. 1990. Effects of thyroxine on standard and total metabolic rates in the lizard *Sceloporus undulatus. Physiological Zoology* 63:837-885.

Kalliecharan R, Hall BK. 1974. A developmental study of the levels of progesterone, corticosterone, cortisol, and cortisone circulating in the plasma of chick embryos. *General Comparative Endocrinology* 24:364-372.

Kobuke L, Specker JL, Bern HA. 1987. Thyroxine content of eggs and larvae of coho salmon, *Oncorhynchus-kisutch*. *Journal of Experimental Zoology* 242:89-94.

Liu LX, Dean CE, Porter TE. 2003. Thyroid hormones interact with glucocorticoids to affect somatatroph abundance in chicken embryonic pituitary cells in vitro. *Endocrinology* 144:3836-3841.

McNabb FMA, Larsen CT, Pooler PS. 2004. Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. *Environmental Toxicology and Chemistry* 23:997-1003.

McNabb FMA. 1988. Peripheral thyroid hormone dynamics in precocial and altricial avian development. *American Zoologist* 28:427-440.

McNichols MJ, McNabb FMA. 1988. Development of thyroid function and its pituitary control in embryonic and hatchling precocial Japanese quail and altricial ring doves. *General and Comparative Endocrinology* 69:109-118.

Medler KF, Lance VA. 1998. Sex differences in plasma corticosterone levels in alligator (*Alligator mississippiensis*) embryos. *Journal of Experimental Zoology* 280(3):238-244.

Motzer WE. 2001. Perchlorate: problems, detection, and solutions. *Environmental Forensics.* 2:301-311.

Rittenhouse PA Redei E. 1997. Thyroxine administration prevents streptococcal cell wall induced inflammatory response. *Endocrinology* 138:1434-1439.

Rodriguez TT, Albuquerque-Araujo WIC, Reis LC, Antunes-Rodrigues J, Ramalho MJ. 2003. Hypothyroidism attenuates stress induced prolactin and corticosterone release in septic rats. *Experimental Physiology.* 88:755-760.

Scott TR, Johnson WA, Satterlee DG, Gildersleeve RP. 1981. Circulating levels of corticosterone in the serum of developing chick embryos and newly hatched chicks. *Poultry Science* 60:1314-1320.

Shepherdley CA, Daniels CB, Orgeig S, Richardson SJ, Evans BK, Darras VM. 2002. Glucocorticoids, thyroid hormones, and iodothyronine deiodinases in embryonic

saltwater crocodiles. *American Journal of Physiology - Regulatory Integrative Comparative Physiology* 283:R1155-R1163.

Sinervo B, Dunlap KD. 1995. Thyroxine affects behavioral thermoregulation but not growth rate among populations of the western fence lizard *(Sceloporus occidentalis). Journal of Comparative Physiology B* 164:509-517.

Sullivan LC, Orgeig S, Daniels CB. 2002. Regulation of pulmonary surfactant secretion in the developing lizard, *Pogona vitticeps*. *Comparative Biochemistry and Physiology Part A* 133:539-546.

Talent LG, Dumont JN, Bantle JA, Janz DM, Talent SG. 2002. Evaluation of western fence lizards (*Sceloporus occidentalis*) and eastern fence lizards (*Sceloporus undulates*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 21:899-905.

Thommes RC, Hylka VW. 1977. Hypothalamo-adenohypophyseal-thyroid interrelationships in chick-embryo .1. TRH and TSH sensitivity. *American Zoologist* 17(4):900-900.

Urbansky ET. 1998. Perchlorate chemistry: implications for analysis and remediation. *Bioremediation Journal* 2:81-95.

Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, Lye LJ, Gibb W, Challis JRG. 2001. Glucocorticoid regulation of human and ovine parturition: the relationship between fetal hypothalamic-pituitary-adrenal axis activation and intrauterine prostaglandin production. *Biological Reproduction* 64:1019-1032.

Wise PM, Frye BE. 1973. Functional development of the hypothalamo-hypophyseal-adrenal cortex axis in the chick embryo *Gallus domesticus*. *Journal of Experimental Zoology* 185:277-292.

Wu SY, Klein AH, Chopra IJ, Fisher DA. 1978. Alterations in tissue thyroxine-5'-monodeiodinating activity in perinatal period. *Endocrinology* 103:235-239.

Zug GR, Vitt LJ, Caldwell JP. (Eds.) 2001. *Herpetology*. Academic Press. London

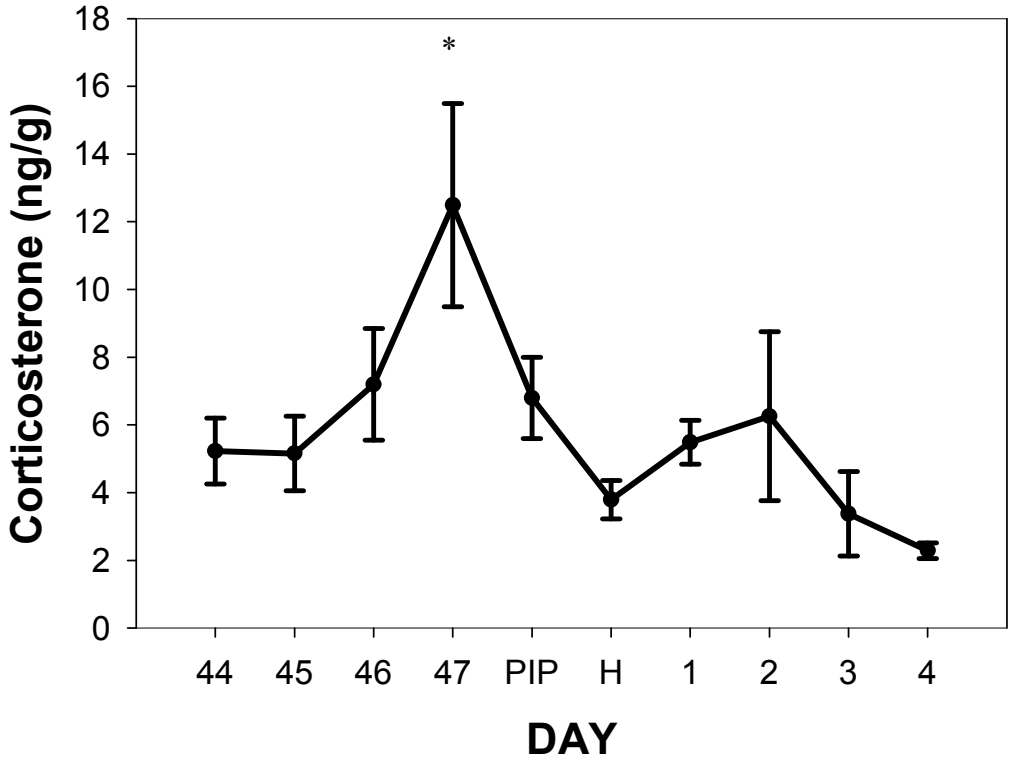
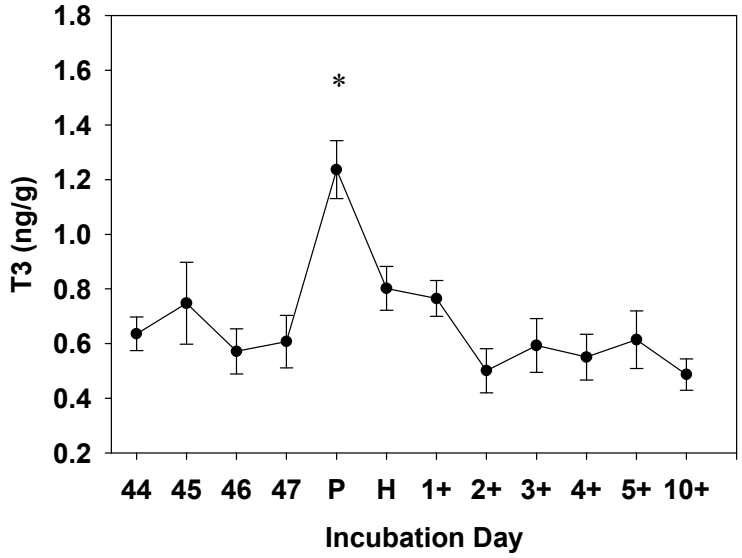
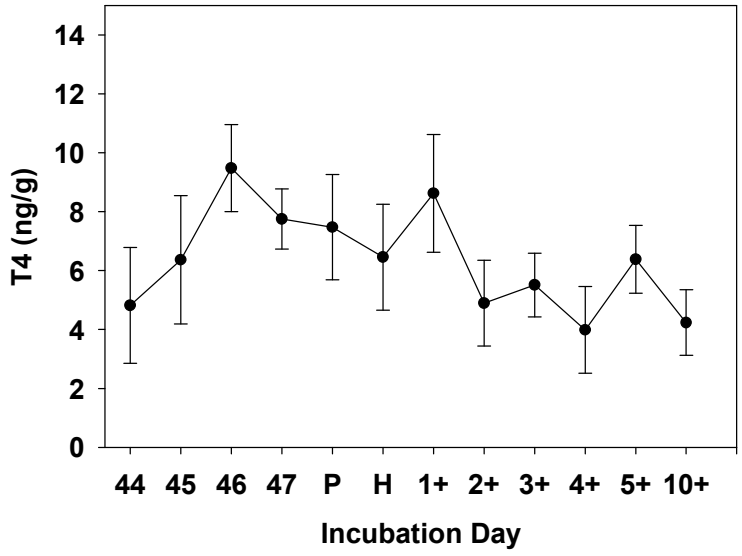


Figure 1. Whole body corticosterone hormone levels in embryonic, perinatal, and hatchling western fence lizards. Stages are designed as day of incubation (44-47), pip (egg shell penetrated), H (day of hatch), and age after hatching (1 - 4). Values are mean ± SEM. An asterisk indicates a significant difference.

A.


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
T3 (ng/g)
*
44 45 46 47 P H 1+ 2+ 3+ 4+ 5+ 10+
Incubation Day

B.


14
12
10
8
6
4
2
0
T4 (ng/g)
44 45 46 47 P H 1+ 2+ 3+ 4+ 5+ 10+
Incubation Day

C.

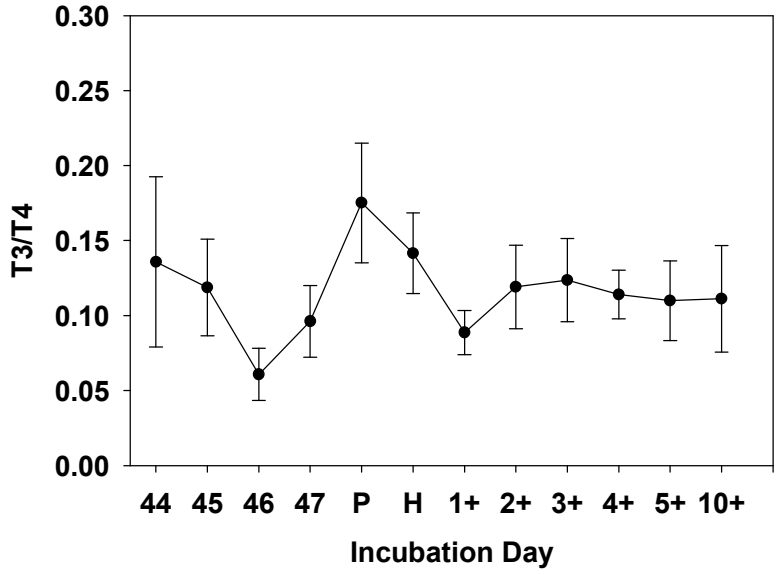


Figure 2. Whole body thyroid hormone levels in embryonic, perinatal, and hatchling western fence lizards. Stages are designed as day of incubation (44-47), pip (egg shell penetrated), H (day of hatch), and age after hatching (1+ - 10+). Values are the mean ± SEM. Sample size was 10 embryos for each sampling day A. whole tissue triiodothyronine T_3 levels (ng/g). B. whole tissue thyroxine T_4 levels (ng/g). C. Calculated T_3:T_4 ratio.

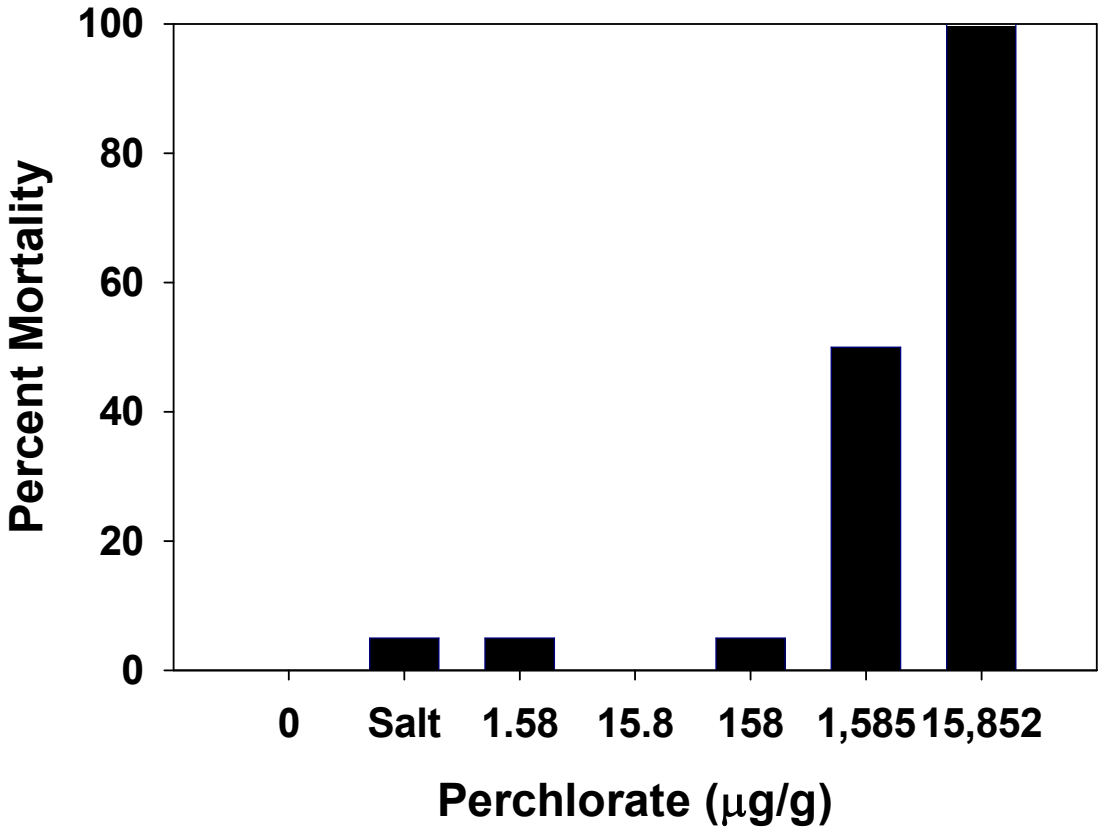


Figure 3. Percentage of western fence lizard embryos that failed to hatch after *in ovo* exposure to perchlorate-spiked perlite. Sample size is 20 for each exposure group.

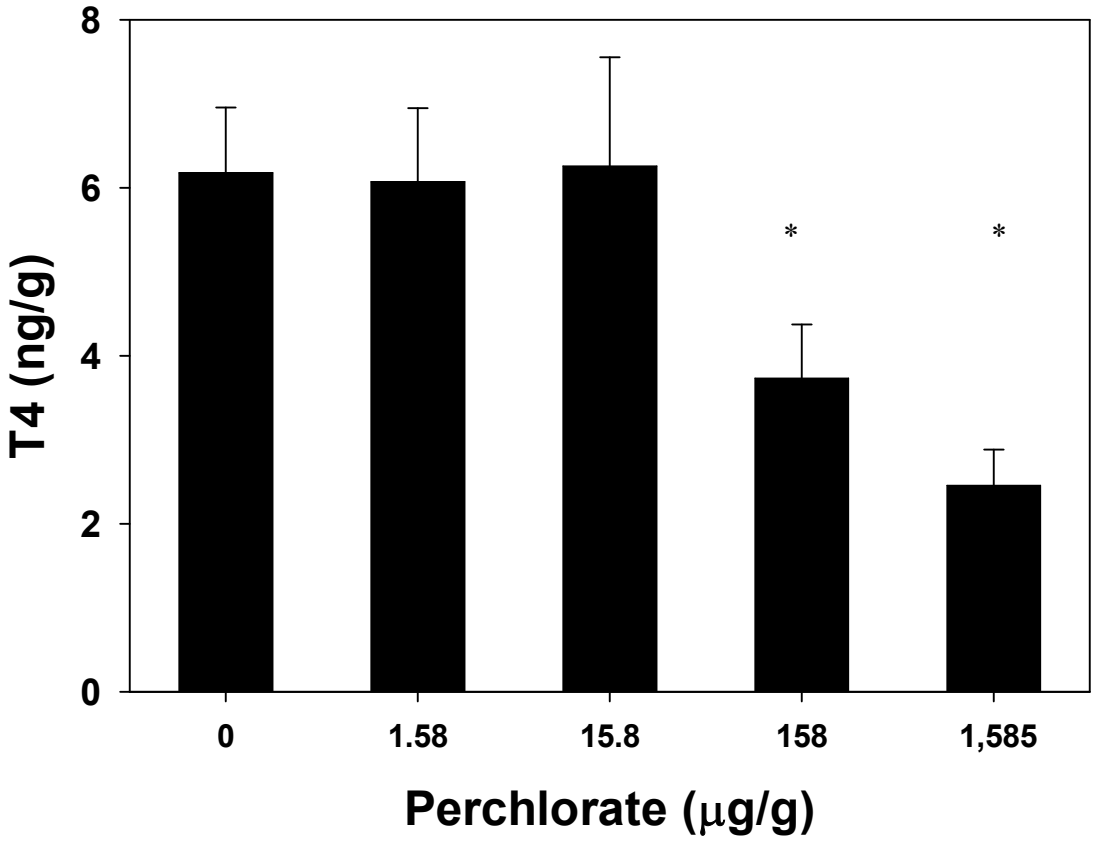


Figure 4. Whole body thyroxine (T_4) levels in tissues of hatchling western fence lizards after long term *in-ovo* exposure to perchlorate during incubation. Values are the mean ± SEM. Sample size was 10 for each concentration. Asterisks indicate significant differences between lizards in the perchlorate concentration groups and the control.

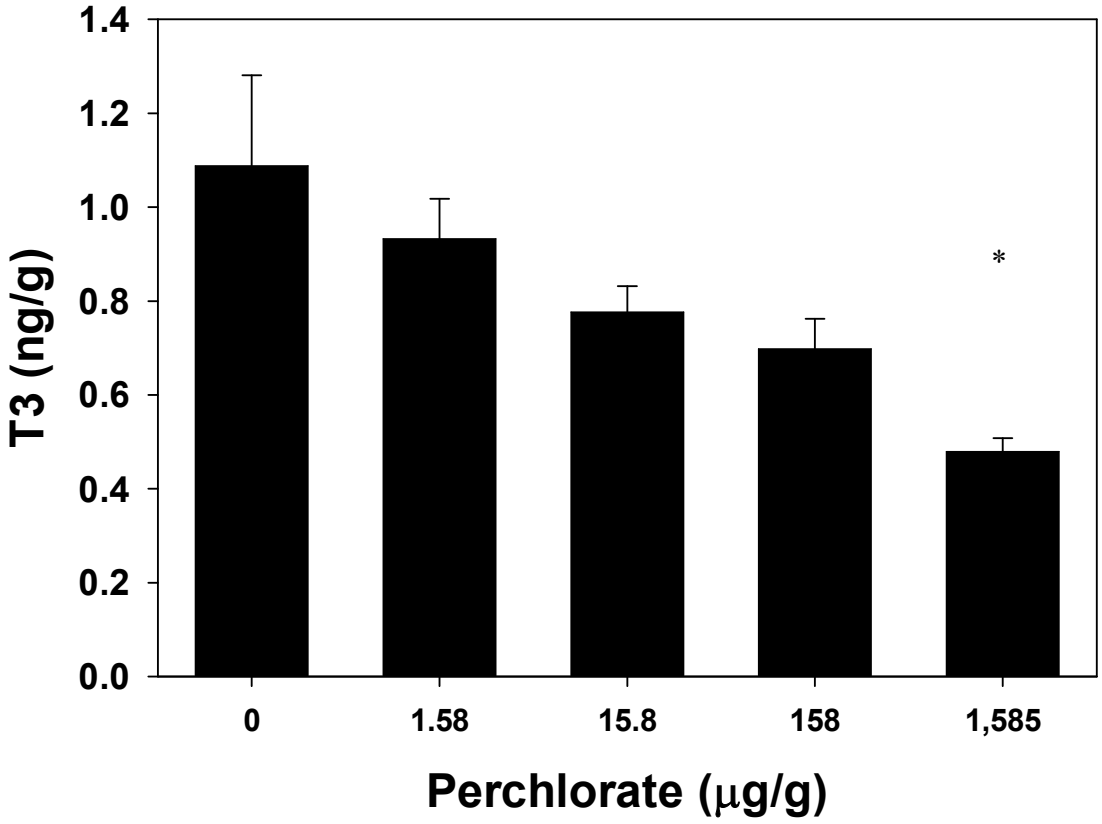


Figure 5. Whole body triiodothyronine (T_3) levels hatchling western fence lizards after long term *in-ovo* exposure to perchlorate during incubation. Values are the mean ± SEM. Sample size was 10 for each concentration. Asterisks indicate significant differences between lizards in the perchlorate concentration groups and the control.

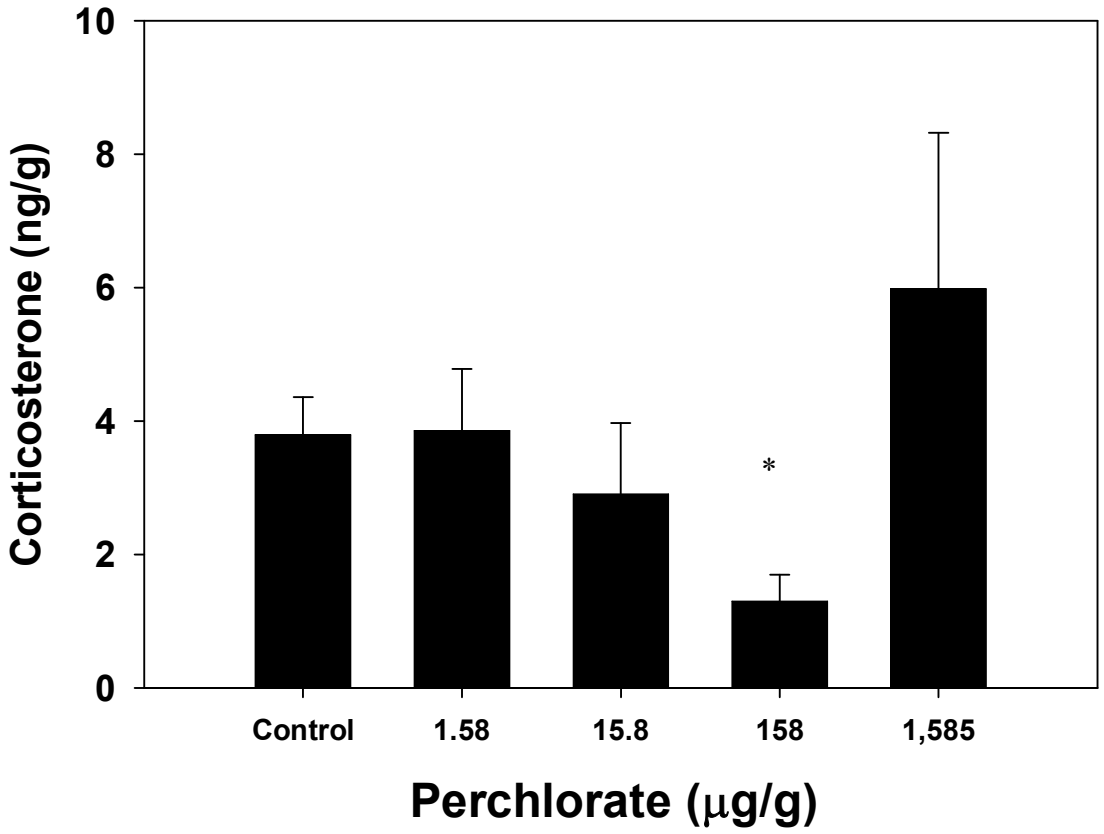


Figure 6. Whole body corticosterone levels in hatchling western fence lizards after long term *in-ovo* exposure to perchlorate during incubation. Values are the mean ± SEM. Sample size was 10 for each concentration. Asterisks indicate significant differences between lizards in the perchlorate concentration group and the control.

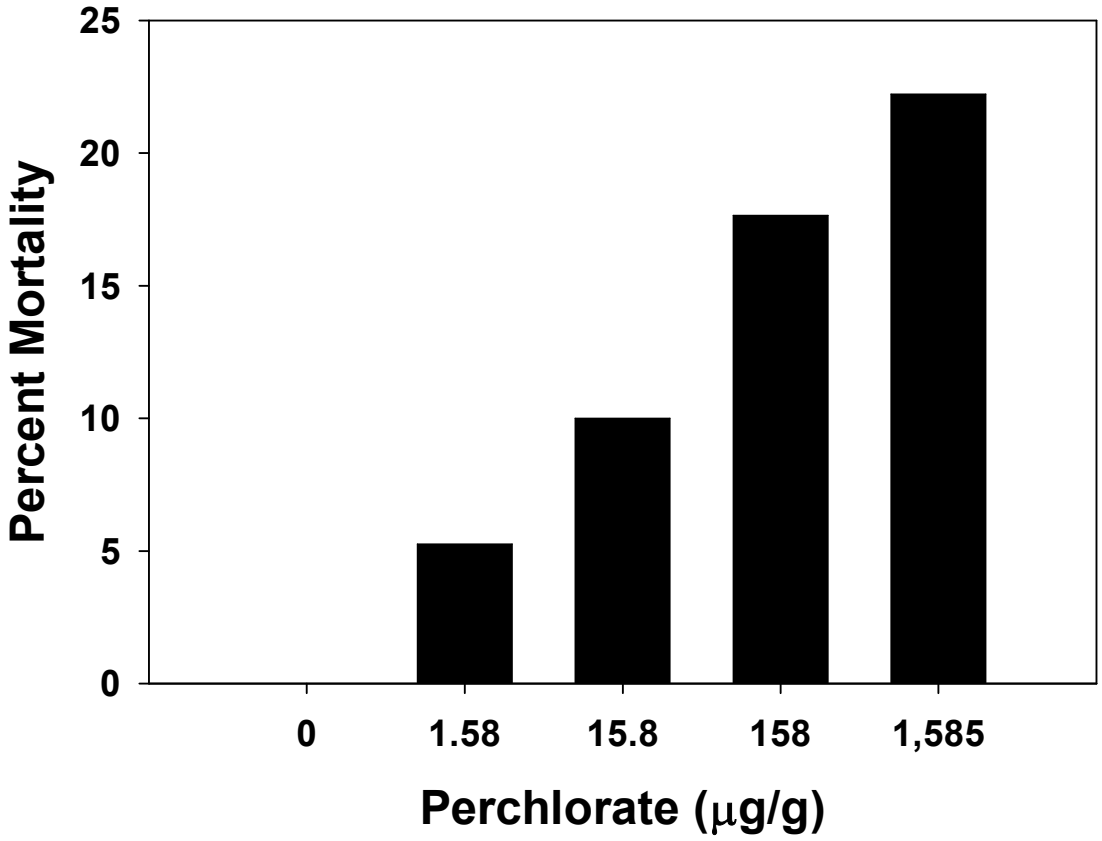


Figure 7. Percentage of western fence lizard embryos exposed to perchlorate that died after pipping but before fully emerging from the shell. Sample size was 9-20 individuals for each concentration.

CHAPTER III

EFFECTS OF *IN OVO* EXPOSURE TO PERCHLORATE ON DEVELOPMENT AND METABOLISM OF EMBRYONIC LIZARDS

ABSTRACT

Perchlorate is a thyroid hormone inhibiting chemical that has been detected in the groundwater in some states. It is soluble in water and causes hypothyroidism in hatchling lizards that have been incubated on perchlorate spiked substrate. To evaluate the effects of perchlorate on development and metabolism of lizard embryos, western fence lizard (*Sceloporus occidentalis*) embryos were exposed to perchlorate concentrations via spiked substrate during incubation and embryonic oxygen consumption, egg growth, incubation length, and hatchling mass were monitored. Incubation length appeared to be the most sensitive endpoint and increased from 49 days in control lizards to 51 days in those exposed to 158 μg ClO_4^-/g perlite. Furthermore, some embryos never initiated a hatching response although they continued to live inside eggs for a week or more past the hatching date of controls. Total embryonic oxygen consumption was decreased by perchlorate in a dose-related manner and embryos exposed to 1,585 μg ClO_4^-/g perlite consumed significantly less oxygen during development than other groups. However, a more pronounced decrease was detected in embryos that failed to hatch at identical

concentration levels. There were also no size differences in hatchlings that successfully emerged from the eggs.

INTRODUCTION

Contamination of soil and water with perchlorate has occurred in many areas of the United States primarily because of its use in pyrotechnics, explosives, and as an oxidizer in solid fuels (Motzer, 2001). The effects of perchlorate on wildlife are of concern because perchlorate salts are soluble in water, break down slowly under typical environmental conditions (Urbansky, 1998), and can interfere with the ability of the thyroid gland to utilize iodide to produce thyroid hormones (Kyung et al., 2002). Sufficient thyroid hormone levels are necessary for normal metabolism, growth, and development (Stanbury and Wyngaarden, 1952). Therefore, high levels of perchlorate contamination could have deleterious effects on wildlife populations.

Most studies that evaluate effects of perchlorate on terrestrial vertebrates are concerned with mammals. Relatively little is known about the effects of perchlorate on birds (McNabb et al., 2004) and to our knowledge; nothing has been published on the effects of perchlorate on reptiles. Terrestrial reptiles are common throughout most areas of the United States where perchlorate contamination has occurred (Urbansky, 1998). Furthermore, embryos of oviparous reptiles may be especially vulnerable to the effects of soil contaminants because many species of reptiles bury their eggs in soil and the structure of their eggshell probably increases the risk of absorbing contaminants from the surrounding substrate (Brasfield et al., 2004; Moeller, 2004). The eggs of most species of lizards in the United States have a flexible shell that is thin and highly extensible

(Packard et al., 1982). These eggs contain an insufficient amount of water to complete development and must be in contact with moist substrate where they absorb water from the surrounding soil and often more than double in mass before they hatch (Tracy, 1980; Packard et al., 1982). Therefore, water-soluble contaminants in the soil may diffuse into eggs and affect embryonic development. Because embryos undergo rapid development, they may be more sensitive to contaminant exposure than adults (Amdur et al.,1991).

The western fence lizard (*Sceloporus occidentalis*) has potential for use as a model for evaluating effects of soil contaminants on the embryonic development of reptiles because the species produces flexible-shelled eggs, is easy to maintain in captivity, and appears suitable for laboratory toxicological studies (Brasfield et al., 2002; Talent et al., 2002; Burnham et al., 2003). Furthermore, using the western fence lizard for evaluating the effects of perchlorate on lizards is environmentally relevant because the species is common over much of the western United States, especially in California and Nevada where perchlorate contamination is a problem (Motzer, 2001).

In evaluating the sensitivity of embryonic reptiles to perchlorate, we evaluated the effects of *in ovo* sodium perchlorate ($NaClO_4$) exposure on western fence lizard embryos. We examined the effects of perchlorate exposure using multiple endpoints and, in this paper; we report the effects of sodium perchlorate exposure on length of incubation, hatchling size, and oxygen consumption in embryos.

MATERIALS AND METHODS

Research animals

A laboratory population of western fence lizards was established from lizards that were collected from the San Joaquin Valley in California (Talent et al., 2002). Breeding lizards were housed on corncob substrate in 74.2-l glass aquaria that were covered with a 3-mm steel mesh lid. Heat and light were provided by a 60-W incandescent light bulb that was positioned over one end of each cage to permit thermoregulation across a temperature gradient of approximately 26-40 ºC. Ambient room temperature was maintained at approximately 22 ºC and a 14:10 h light:dark light cycle was provided. Lizards were provided with a water source and fed daily with house crickets, *Acheta domestica*, that had been dusted with a Herptivite® and Rep-Cal® mixture (1:1 v/v) (Rep-Cal Research Labs, Los Gatos, CA, USA). Lizards were also provided with an oviposition site consisting of a container of moist sand. Eggs were removed from the oviposition containers within 12 hours after being laid.

Perchlorate exposure

To avoid compromising the results relative to evaluating different endpoints, we exposed fence lizard eggs to sodium perchlorate in two replicates. Replicate number one was used to determine oxygen consumption. Replicate number two was used to evaluate egg growth during incubation, hatchling mass, hatchling snout-vent length, and incubation length. The mean incubation length for the control and each exposure group was calculated only for lizards that successfully hatched. Prior to exposing eggs to perchlorate, eggs from 64 clutches were placed into moist vermiculite within 12 hours of

oviposition and incubated for seven days at 28 ºC to determine which eggs were fertile and healthy. On day seven of incubation, 140 eggs were distributed into each of five treatment groups a salt control and a control for both replicates. No more than one egg per clutch was placed in the same treatment group of a replicate.

Exposure chambers were prepared for each egg by placing 4 g of oven dried, size-separated perlite (<4 >0.85 mm) into acid-washed, oven-dried borosilicate cylinders (70 mm deep). Solutions of sodium perchlorate (> 99%, Sigma) were prepared in reagent grade water (18 MΩ). A perchlorate solution was delivered by volumetric pipette (9 ml) into each exposure chamber. Nominal concentrations of 1.95, 19.5, 195, 1,950 and 19,500 µg $NaClO_4$/g dry perlite were prepared (Table 1). These concentrations of $NaClO_4$ resulted in nominal ClO_4^- concentrations of 1.58, 15.8, 158, 1,585, and 15,852 µg ClO_4^- /g dry perlite. The control was prepared by saturating perlite with an equivalent volume of reagent grade water. In addition, to distinguish between the effects of sodium and perchlorate on developing embryos, we set up a sodium chloride control with the sodium concentration (3,666 µg/g perlite) equal to that in our highest $NaClO_4$ concentration (19,500 µg $NaClO_4$ /g dry perlite) both replicates to ensure potential differences were not due to osmotic changes associated with a salt exposure.

All control and experimental eggs were placed on the perlite substrate of the exposure chambers and chambers were sealed with a plastic cap. A 1-mm hole was drilled in the center of each plastic cap for gas exchange. Chambers were placed into a Precision incubator that was maintained at 28 ºC ± 0.5 ºC throughout the incubation period and each egg was checked daily for mortality. Vials containing the egg, substrate and solution were weighed at the onset of the experiment and weekly thereafter. After

comparing weekly chamber weights to those recorded at the onset of the experiment, chamber water balance was returned by pipetting reagent grade water into the exposure chamber to replace any water lost due to evaporation.

Embryos were monitored until they hatched or died. Mortality of embryos was verified by examination of the membrane blood vessels using back lighting. All hatchlings were weighed to a hundredth of a gram, sex was determined, and snout-vent length (SVL) was recorded to the nearest millimeter. Incubation time was also calculated and recorded.

Oxygen consumption

Oxygen consumption (ml/g/h) was measured weekly for each egg in replicate number one beginning 7 days after initial exposure, i.e. 2 weeks after oviposition. Individual eggs were removed from exposure chambers, weighed, and placed into a metabolism chamber. Metabolism chambers were constructed from wide-mouth 118 ml polyurethane specimen jars similar to those used in Moeller (2004). Chambers were half filled with approximately 50 ml washed sand and 18 ml reagent grade water. The addition of sand and water decreased chamber volume and provided a buffer for temperature change during the egg transfer period. Prior to use, chambers were maintained in the incubators for several hours to bring their temperature up to 28 °C. After eggs were transferred into the metabolism chambers, they were returned to the incubator for at least 2 hours. Air samples were then taken from individual chambers using a modified syringe and percent oxygen was measured using a Sable Systems F C-1 O_2 analyzer (Las Vegas, NV, USA). After air samples were taken, eggs were transferred

back into the control or perchlorate exposure chambers and incubated as described above. Weekly oxygen consumption measurements continued for each egg until it hatched or died.

Changes in oxygen utilization were measured weekly and plotted to depict trends in oxygen consumption throughout incubation. To gain an understanding of the oxygen consumed throughout the entire incubation period, we used the weekly data points for oxygen consumption to estimate an integral of total oxygen consumed (Roe et al., 2005).

Data analysis

Experiments were constructed in a blocked design to account for variation within lizard clutches. Normality within each exposure group and homoscedasticity among exposure groups were determined by Kolmogorov Smirnov and Bartlett tests, respectively. Once assumptions of ANOVA were met, a one-way ANOVA followed by a Dunnett's post hoc test was performed. A level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Length of incubation period, egg growth and hatchling size

In replicate two, there were no significant differences in the incubation length of controls, sodium controls, and eggs incubated at the two lower perchlorate concentrations (1.58, and 15.8, μg ClO_4^-/g) (Fig. 1). However, the mean incubation period of hatchlings from eggs in the 158 and 1,585 μg ClO_4^-/g exposure group was significantly longer than

controls. The incubation length of embryos exposed to 15,852 μg ClO_4^-/g could not be determined because all embryos died before hatching. The embryos that did not hatch in the 1,585 and 15,852 μg ClO_4^-/g exposure groups continued to live within the egg for several weeks after controls hatched before dying without any sign that they attempted to hatch. Examination of the dead embryos indicated that they had depleted most of their yolk reserves prior to death.

During incubation of eggs in the second replicate, there were no significant differences in egg mass measured weekly among control eggs and those in the four lowest sodium perchlorate concentrations. However, eggs in the highest concentration (15,852 μg ClO_4^-/g) and the sodium controls had significantly smaller mass ($p = 0.03$) than controls from the second week of exposure throughout the remainder of the incubation period (Fig. 2). By week five of exposure, the mean mass of eggs exposed to the highest concentration (15,852 μg ClO_4^-/g) was 0.24 g lower than control eggs.

No significant differences in mean hatchling size were observed among controls, sodium controls, and exposure groups, as measured by mass, which ranged from 0.69 g to 0.73 g, or snout-vent length, ranging from 26 mm to 30 mm (Fig. 3).

Oxygen consumption

Oxygen consumption rates did not differ significantly between controls and experimental groups for the first three weeks of perchlorate exposure for eggs in replicate one (Fig. 4). However, by the fifth week of incubation (fourth week of exposure), mean oxygen consumption of the two highest perchlorate concentrations differed significantly ($p < 0.01$) from controls. By the sixth week of exposure, near the end of incubation of

controls, the mean oxygen consumption of embryos exposed to 1,585 and 15,852 μg ClO_4^-/g perlite were 0.053 ml/g/h and 0.050 ml/g/h, respectively, whereas mean oxygen consumption of controls was 0.079 ml/g/h.

To elucidate the differences in oxygen consumption of successful and unsuccessful hatches at the same exposure level, we separated the data of the 1,585 μg ClO_4^-/g exposure group into embryos that hatched and those that did not. The oxygen consumption of these two groups differed throughout most of the incubation period. Starting at week four and continuing throughout the remainder of the incubation period, oxygen consumption was significantly higher ($p<0.05$) in embryos that hatched compared to those that did not (Fig. 5). Interestingly, oxygen consumption of both groups peaked at week six of incubation similar to controls. Subsequent oxygen consumption rates decreased for eggs that did not hatch between weeks 6 and 7. In the week prior to the successful hatching of embryos exposed to 1,585 μg ClO_4^-/g, oxygen consumption was nearly 50% lower than levels observed in control lizards before they hatched.

Total oxygen consumption during the entire incubation period showed a dose-related response with oxygen consumption first increasing in low concentration exposure groups and then decreasing as perchlorate concentration increased (Fig. 6). Although not statistically significant, embryos in the 1.58 μg ClO_4^-/g exposure group consumed more oxygen than the control group whereas embryos in the 1,585 ($p = 0.0087$) and 15,852 ($p = 0.001$) μg ClO_4^-/g exposure groups consumed significantly less oxygen than the controls.

DISCUSSION

Length of incubation period, egg growth and hatchling size

One of the most sensitive endpoints evident in this study was the length of incubation in the controls and lizards exposed to higher perchlorate concentrations. *In ovo* exposure to perchlorate increased the length of incubation in the 158 and 1,585μg ClO_4^-/g perlite exposure groups by 2 and 4.7 days, respectively. Little is known about the actual trigger mechanism(s) that initiates the hatching process in reptiles but the process was delayed in embryos exposed to 158 and 1,585 μg ClO_4^-/g. One possible explanation for the delay in hatching is that perchlorate exposure delayed development. Developmental delay has been linked to perchlorate-induced hypothyroidism (Goleman et al., 2002; McNabb et al., 2004). However, if the only effect of perchlorate exposure was to delay development due to hypothyroidism, it is unclear why the unsuccessful embryos in the 1,585 μg ClO_4^-/g exposure groups continued to grow in the eggs but did not hatch even after reaching full development. Therefore, we suspect that perchlorate toxicity caused multiple effects instead of just reducing embryonic development rate due to hypothyroidism.

Because eggs were exposed via pore water absorption, it is unclear at which point perchlorate concentrations within the egg were high enough to influence developing embryos. The hydric environment surrounding flexible shelled reptile eggs determines the extent to which they will initially absorb pore water during the first half of incubation and lose it during the second half (Packard et al., 1982). All of the eggs in our study increased in mass from the day of oviposition to week 4 of exposure. At this point the mass of controls and all concentrations except for the 15,852 μg ClO_4^-/g treatment group

reached a plateau for the last 2 weeks of incubation before hatch similar to trends seen by Angilletta et al. (2000). The 15,852 μg ClO_4^-/g exposure group, which weighed significantly less throughout incubation, continued to increase in mass until week 5 and then decreased dramatically at week 6. Nevertheless, of the eggs that hatched normally, we saw no difference in hatchling mass or snout-vent length between the controls, salt controls, or any treatment group.

Because we exposed lizards to sodium perchlorate, the effects of sodium concentrations in the exposure groups were a concern. Eggs in the salt control, although weighing less than control eggs throughout much of incubation, weighed significantly more than eggs exposed to the highest concentration of sodium perchlorate. Incubation length and hatchling size were both unaffected by sodium chloride. Thus, it appears that the reduction in egg growth in the 15,852 μg ClO_4^-/g exposure group was at least partially related to perchlorate exposure.

Oxygen consumption

Contaminant exposure can be metabolically costly. However, a dose dependant pattern in oxygen consumption as a direct measurement of metabolic rate in reptiles is difficult to generalize. Metabolic rates are reported to increase (Hopkins et al., 1999), decrease (Nagle et al. 2001), and remain steady (Moeller, 2004) under various contaminant assaults. In the present study oxygen consumption, was affected within four weeks of exposure to perchlorate in the incubation substrate. The exposure duration necessary for perchlorate to accumulate in levels high enough to produce a measurable effect was unclear. Had the eggs been exposed within 24 hours of oviposition, we likely

would have seen differences in oxygen consumption earlier. It is also important to note that we accounted for egg mass in our oxygen consumption calculations because egg size was affected by exposure to perchlorate. All of our eggs lost mass within the last week of incubation. Had we not included mass in our calculations, we would have seen a more prominent decrease in oxygen consumption just prior to hatch, forming more of a sigmoid curve as seen with other lizards (Thompson and Russell, 1999). We found that the metabolic rate of all embryos increased at the same rate until week four at which point the 1,585 and 15,852 μg ClO_4^-/g perlite exposure groups began to lag behind. By week five and six these exposure groups consumed significantly less oxygen than the control eggs and their respiration rate did not increase following this time. If the only effect of perchlorate toxicity was to reduce developmental rate, it seems likely that the developmentally delayed lizards would eventually catch up and respire at the same rate as the controls before they hatched. This assumption is supported by research conducted on *Sceloporus undulatus* by Angilletta et al. (2000). Development was delayed through temperature manipulation and found that metabolic rate was decreased when compared on a direct time interval but when graphed as a percentage of incubation, metabolism across temperatures was not different suggesting that lizards at the same level of maturation will metabolize at the same rate. However, in the present study the embryos in replicate one did not ever consume oxygen at the same rate as controls although they continued to live until they reached full development. Therefore, the reduced oxygen consumption in lizards that were exposed to the highest dose of perchlorate appears to have been caused by other factors in addition to delayed development rate.

Incubation length was extended and percent hatch decreased by half for the embryos exposed to 1,585 μg ClO_4^-/g perlite. We were therefore able to compare those that hatched later or not at all to the oxygen consumption of the control group. We found differences in oxygen consumption beginning at week four and continuing throughout the remainder of incubation in these groups. However, when the control eggs hatched at six weeks of exposure, rather than catching up as we expected, oxygen consumption in the 1,585 μg ClO_4^-/g group that hatched decreased over the next two weeks to oxygen consumption rates seen at week four for control eggs. The average length of incubation for the eggs that finally hatched in this concentration was 53 days. Eggs exposed to 1,585 μg ClO_4^-/g that failed to hatch displayed an increase in oxygen consumption rates only to week three at which point they leveled off and remained low throughout an incubation that ended unsuccessfully. Overall, the eggs that did not hatch were respiring at a lower level than the controls throughout much of incubation. Decreased circulating thyroxine causes a decrease in oxygen consumption in lizards (Chandola-Saklani and Kar, 1990) and this could have caused the dose dependent decrease in total oxygen consumption throughout incubation.

CONCLUSION

Similar to results found by Brasfield et al. (2004) with passive Cd exposure, perchlorate also appeared to cross the eggshell and affect western fence lizard embryos. Embryos were exposed via spiked substrate because this exposure route is ecologically relevant to fence lizard eggs. More common experimental exposure routes include painting and injecting techniques. When reptile eggs are painted with an environmental contaminant approximately 2% (Willingham and Crews, 2000) to 8% (Podreka et al.,

1998) of the amount applied is incorporated into the embryonic tissue. Although injecting eggs may be a more reliable exposure method in that an exact dose is delivered to the egg yolk, this is not an ecologically relevant technique and general mortality increases, presumably due to egg trauma (personal observations).

In the present study, perchlorate appeared to penetrate eggs during incubation and affected oxygen consumption after four weeks of exposure, which was the fifth week of incubation. Therefore, we feel the unique egg properties that enable eggs to uptake environmental contaminants in an ecologically relevant manner and the ease of measuring metabolic rate via oxygen consumption are properties that will make this model useful for ecotoxicological studies that involve soil contaminants.

LITERATURE CITED

Amdur MO, Doull J, Klaassen CD. 1991. *Casarett and Doull's Toxicolog; the Basic Science of Poisons, Fourth Edition.* Pergamon press Elmsford, NY, USA.

Angilletta MJ, Winters RS, Dunham AE. 2000. Thermal effects on the energetics of lizard embryos: implications for hatchling phenotypes. *Ecology* 81:2957-2968.

Brasfield SM, Weber LP, Talent LG, Janz, DM. 2002. Dose-response and time course relationships for vitellogenin induction in male western fence lizards (*Sceloporus occidentalis*) exposed to ethinylestradiol. *Environmental Toxicology and Chemistry* 21:1410-1416.

Brasfield SM, Bradham K, Wells JB, Talent LG, Lanno RP, Janz DM. 2004. Development of a terrestrial vertebrate model for assessing bioavailability of

cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. *Chemosphere* 54:1643-1651.

Burnham DK, Lackey A, Manering M, Jaensson E, Pearson J, Tyler DO, Melson D, Talent LG. 2003. Effects of 17 alpha-ethinylestradiol on immune parameters in the lizard *Sceloporus occidentalis*. *Environmental Toxicology* 18:211-218.

Chandola-Saklani A, Kar A. 1990. Evidence for the role of thyroxine as a hormone in the physiology of a lizard. *General and Comparative Endocrinology* 78:173-179.

Goleman WL, Carr JA, Anderson TA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environmental Toxicology and Chemistry* 2:590-597.

Hopkins WA, Rowe CL, Congdon JD. 1999. Elevated trace element concentrations and standard metabolic rates in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. *Environmental Toxicology and Chemistry* 19:2391-1263.

Kyung OY, Narayanan L, Mattie DR, Godrey RJ, Todd PN, Sterner TR, Mahle DA, Lumpkin MH, Fisher JW. 2002. The pharmacokinetics of perchlorate and its effect on the hypothalamus-pituitary-thyroid axis in the male rat. *Toxicology and Applied Pharmacology* 182:148-159.

McNabb FMA, Larsen CT, Pooler PS. 2004. Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. *Environmental Toxicology and Chemistry* 23:997-1003.

Moeller LA. 2004. Effects of metal contamination on developing red-eared slider turtles (*Trachemys scripta)* and implications for the species as a biomonitor. MS Thesis. Oklahoma State University Stillwater, OK.

Motzer WE. 2001. Perchlorate: problems, detection, and solutions. *Environmental Forensics* 2:301-311.

Nagle RD, Rowe CL, Congdon JD. 2001. Accumulation and selective maternal transfer of contaminants in the turtle *Trachemys scripta* associated with coal ahs deposition, *Archives of Environmental Contamination and Toxicology* 40:531-536.

Packard MJ, Packard GC, Boardman TJ. 1982. Structure of eggshells and water relations of reptilian eggs. *Herpetologica* 38:136-155.

Podreka S, Georges A, Maher B, Limpus CJ. 1998. The environmental contaminant DDE fails to influence the outcome of sexual differentiation in the marine turtle *Chelonia mydas. Environmental Health Perspectives* 106:185-188.

Roe JH, Hopkins WA, Talent LG. 2005. Effect of body mass, feeding, and circadian cycles on metabolism in the lizard *Sceloporus occidentalis*. *Journal of Herpetology* 39:595-603.

Stanbury JB, Wyngaarden JB. 1952. Effect of perchlorate on the human thyroid gland. *Metabolism-Clinical and experimental* 1:533-539.

Sylber CK. 1988. Feeding-habits of the lizards *Sauromalus varius* and S*auromalus hispidus* in the Gulf of California. *Journal of Herpetology* 22:413-424.

Talent LG, Kumont JN, Bantle JA, Janz DM, Talent SG. 2002. Evaluation of western fence lizards (*Sceloporus occidentalis*) and Eastern fence lizards (*Sceloporus undulatus*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 22:899-905.

Thompson MB, Russell KJ. 1998. Metabolic cost of development in one of the world's smallest lizard eggs: Implications for physiological advantages of the amniote egg. *Copeia* 1998:1016-1020.

Tracy CR. 1980. Water relations of parchment shelled lizard (*Sceloporus undulatus*) eggs. *Copeia* 1980:478-482.

Urbansky ET. 1998. Perchlorate chemistry: Implications for analysis and remediation. *Bioremediation Journal* 2:81-95.

Willingham E, Crews D. 2000. The red-eared slider turtle: an animal model for the study of low doses and mixtures. *American Zoologist* 40:421-428.

Table 1. Nominal sodium perchlorate ($NaClO_4$) and perchlorate (ClO_4^-) concentrations in spiked pore-water and substrate of exposure chambers.

Treatment Groups	Concentration			
	Pore Water (mg/L)		Substrate (µg/g)	
	Sodium Perchlorate	Perchlorate	Sodium Perchlorate	Perchlorate
Control	0	0	0	0
1	0.867	0.702	1.95	1.58
2	8.67	7.02	19.5	15.8
3	86.7	70.2	195	158
4	867	702	1,950	1,585
5	8,670	7,020	19,500	15,852

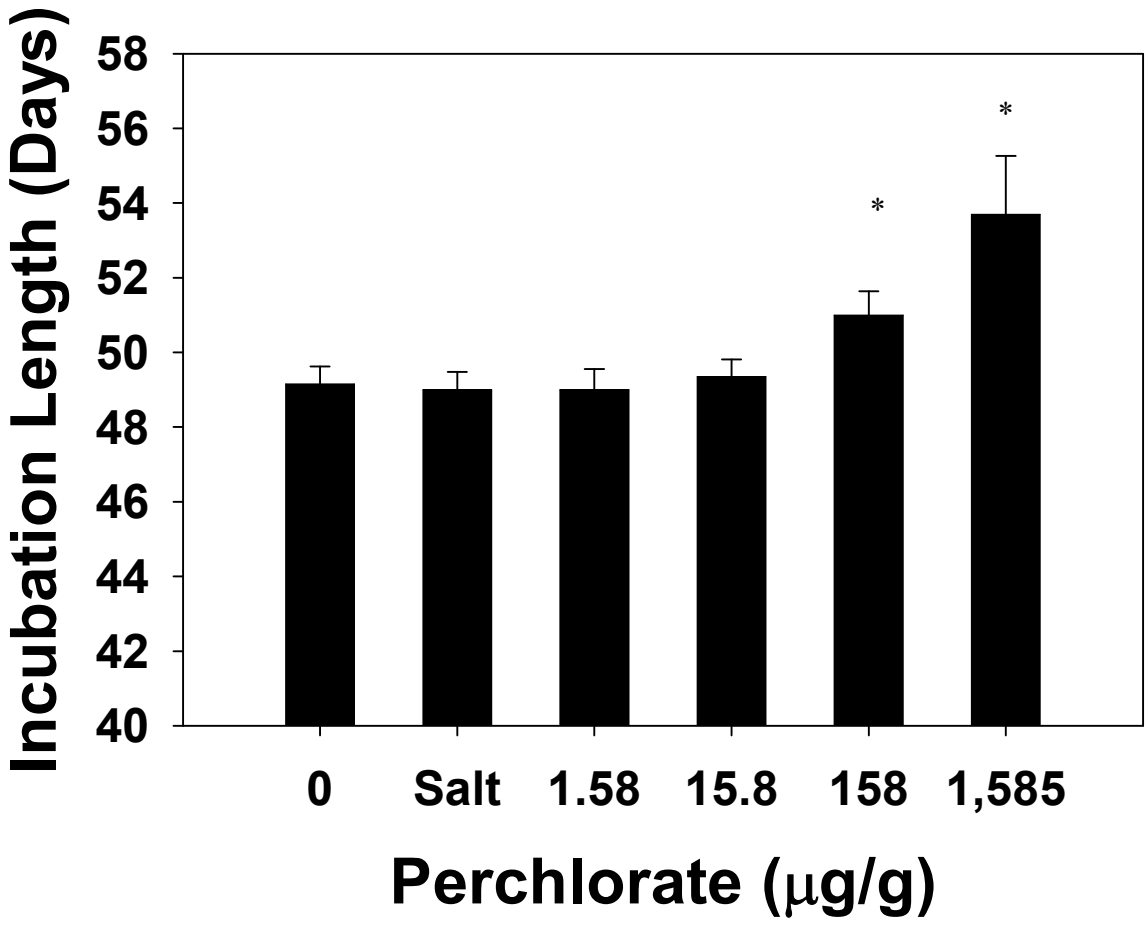


Figure 1. Length of incubation of western fence lizard eggs that hatched after *in ovo* exposure to sodium perchlorate spiked perlite. Exposure began at day 7. Data points are presented as mean ± SEM for a sample size of 8-20 eggs. Asterisks indicate significant differences between lizards in the perchlorate-concentration group and the control.

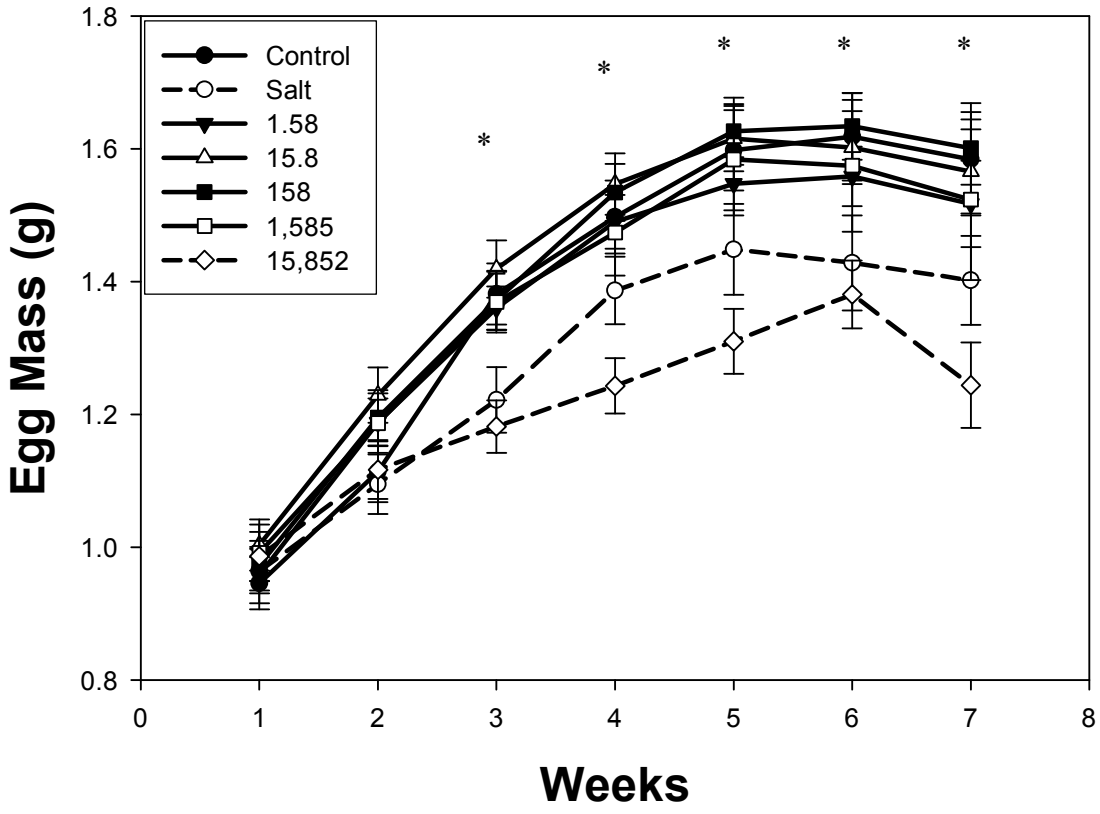


Figure 2. Weekly egg mass for eggs incubated on perlite spiked with perchlorate solutions, reagent grade water or a salt control. Exposure to perchlorate-spiked substrate (µg $NaClO_4$/g perlite) began on day 7. Data points are presented as mean ± SEM for a sample size of 8-20 eggs. Dashed lines indicate concentrations that differ from control eggs. Asterisks denote location of significant differences.

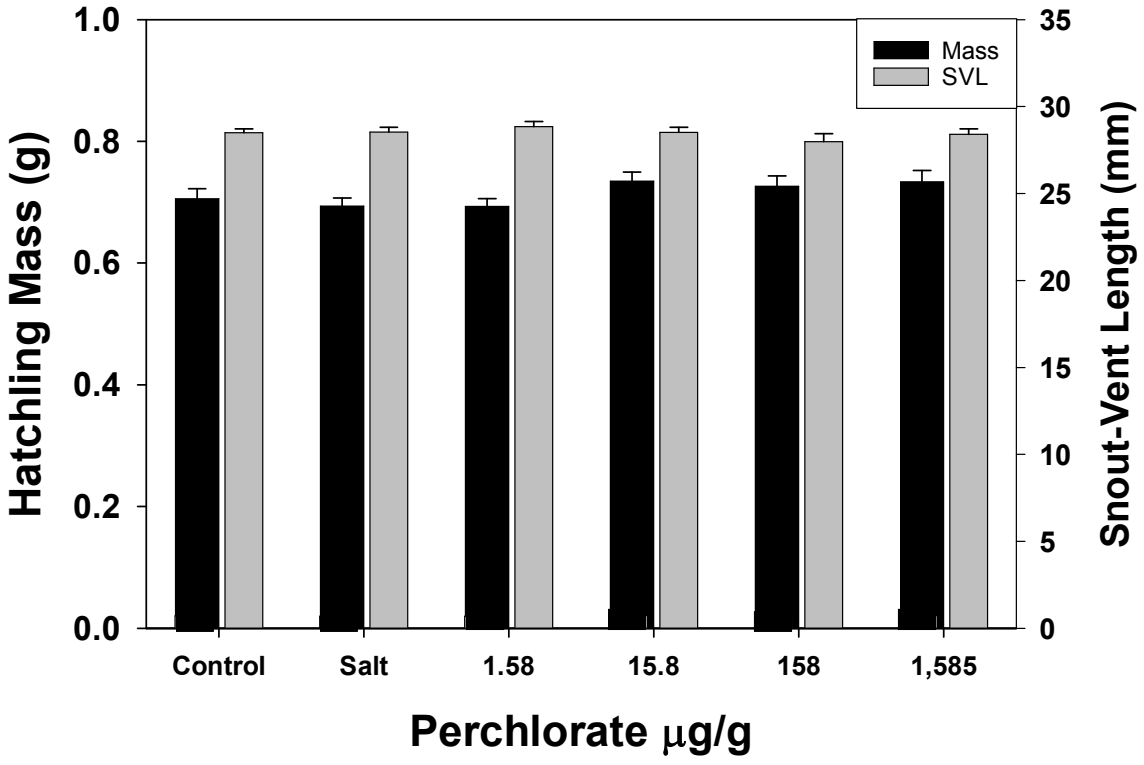


Figure 3. Hatchling mass and snout vent length after *in ovo* exposure to perchlorate throughout incubation. The 1,585 non-hatching (NH) and 15,852 (NH) groups were two weeks older than controls when mass measurements were taken. Data are presented as mean ± SEM for a sample size of 8-20.

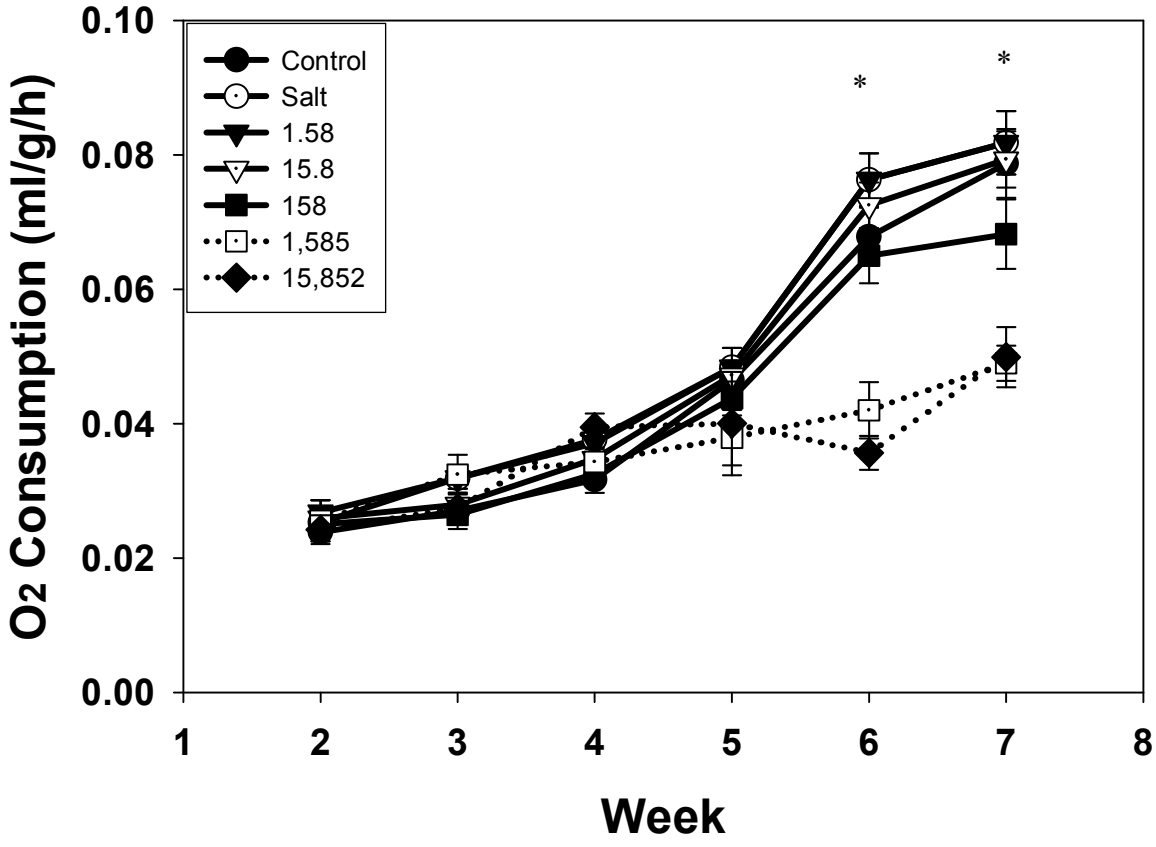


Figure 4. Weekly oxygen consumption rates (ml oxygen/egg mass/hour) of western fence lizard embryos during incubation on perchlorate-spiked substrate. Oxygen consumption measurements began seven days after exposure began, which was 14 days after oviposition. Data are represented as mean ± SEM. Sample size is 20 for each exposure group. Dashed lines indicate oxygen consumption rates of exposure groups that are different than control embryos. Asterisks denote locations where differences in oxygen consumption rates occur.

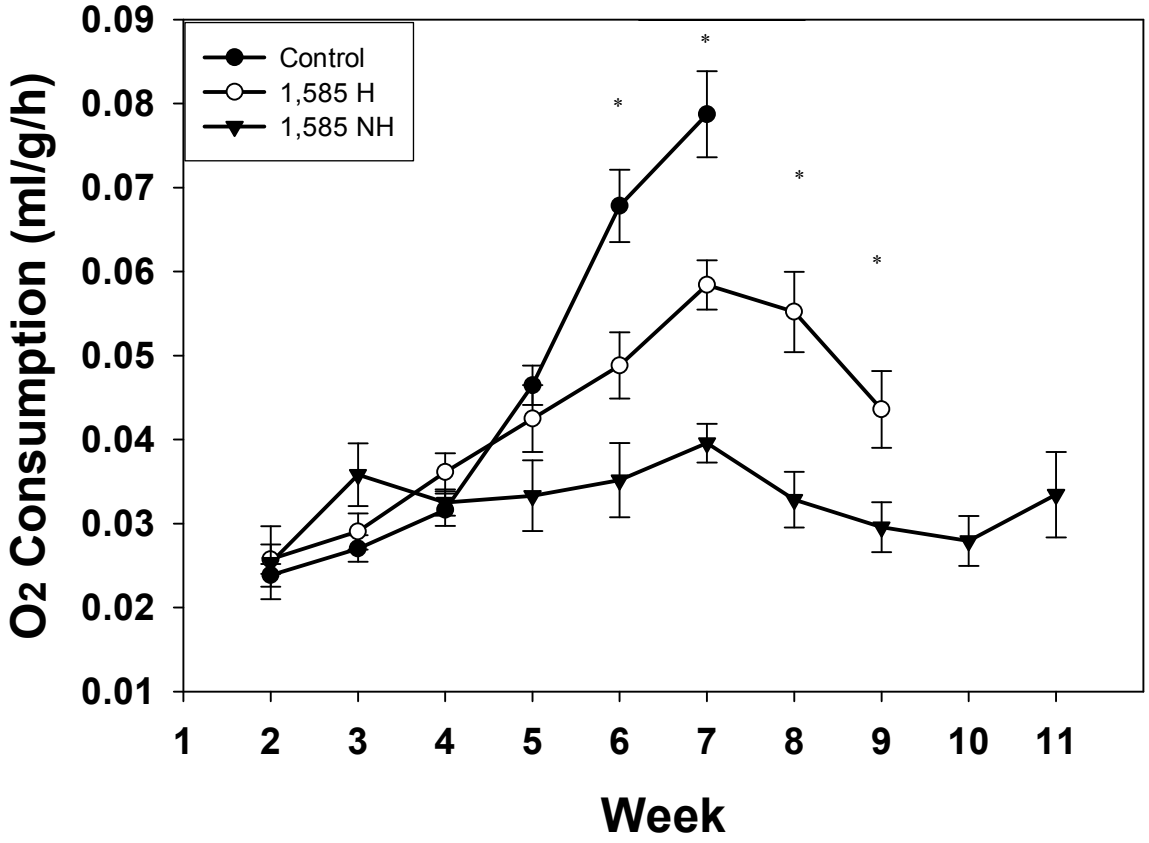


Figure 5. Comparison of weekly oxygen consumption rates (ml oxygen/egg mass/hour) of western fence lizard control eggs, those that were exposed to 1,585 μg ClO_4^-/g that hatched (H) and eggs exposed to 1,585 μg ClO_4^-/g that failed to hatch (NH) after *in ovo* exposure to perlite. Oxygen consumption measurements began seven days after exposure began, which was 14 days after oviposition. Data are represented as mean ± SEM. Sample size is 8-20 for each exposure group. Asterisks denote oxygen consumption rates in exposure groups that are different from control embryos and each other

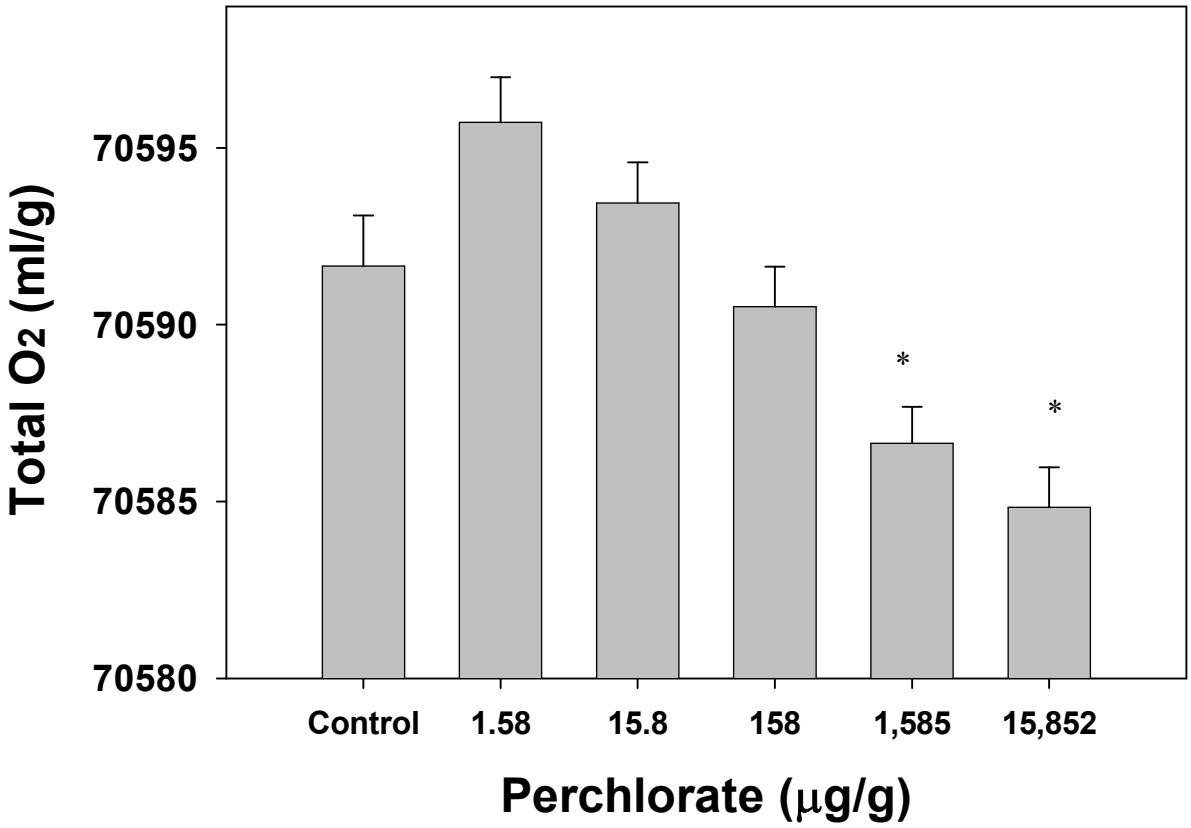


Figure 6. Total oxygen consumption levels of western fence lizard embryos throughout incubation on perchlorate-spiked substrate. Data are represented as mean ± SEM of n = 18-20 lizards. Asterisks indicate significant differences between lizards in the perchlorate exposure group and the control.

CHAPTER IV

IN OVO PERCHLORATE EXPOSURE AFFECTS INCUBATION, GROWTH, AND REPRODUCTION OF FENCE LIZARDS (*SCELOPORUS SP*.)

ABSTRACT

Perchlorate is known to prevent iodide uptake by the thyroid gland, which interferes with synthesis of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). When fence lizard eggs are incubated on perchlorate spiked substrate, perchlorate crosses the eggshell and decrease whole body thyroid hormones in hatchling lizards. Decreases in circulating embryonic thyroid hormones may interfere with growth and development of embryos and could have permanent effects. This research was designed to determine the effects of long term *in-ovo* perchlorate exposure on incubation length, growth and breeding capabilities of fence lizards. Eggs for this study were collected from a laboratory population of eastern (*Sceloporus undulatus*) and western (*S. occidentalis*) fence lizards on the day of oviposition and incubated on perchlorate-spiked perlite at a constant temperature of 28˚C. Concentrations greater than 243 μg perchlorate/g incubation substrate delayed or inhibited the pipping response in fence lizards. After hatch, female eastern fence lizards exposed to the highest concentration of perchlorate (972 μg perchlorate/g incubation substrate) grew larger than control lizards and displayed a significantly higher relative clutch mass due to increased egg and clutch

size compared to control pairs. However, there were no detectible differences in adult serum hormones measured.

INTRODUCTION

In the realm of ecotoxicology, reptiles have been largely ignored (Hopkins et al., 2002) for a number of reasons including the perception that information on reptile toxicology was not urgently needed (Urban and Cook, 1986). Regulatory agencies presumed that regulations based on toxicological data extrapolated from birds and mammals also provided adequate protection for reptiles (Urban and Cook, 1986). Furthermore, toxicologists who conduct laboratory studies have historically worked with species that were easy to maintain under laboratory conditions. Until recently, self-sustaining breeding reptile colonies have been difficult to maintain in captivity and it is understandable why they were avoided as laboratory models. However, because reptiles are ectothermic, their physiology is different from endothermic vertebrates and they may not be protected by regulations designed to protect birds and mammals. Reptile metabolic rates are lower and they may eliminate and detoxify environmental contaminants slower than endotherms. In addition, the enzymes produced by reptiles may not be as efficient at degrading toxicants as those produced by birds and mammals (Walker and Ronis, 1989). Also, any contaminant that is more toxic at lower body temperatures may be more toxic to reptiles than to birds and mammals (Talent, 2005).

Reptiles occur in many areas where environmental contamination occurs, including both aquatic and terrestrial habitats. Nevertheless, most toxicological studies on reptiles have been concerned with aquatic species such as turtles and alligators

(Sparling et al., 2000). As a result, ecotoxicological studies on terrestrial reptiles have been relatively neglected. Terrestrial reptiles may be susceptible to soil contamination because contaminants can enter reptiles by several routes including ingesting contaminated material, percutaneous absorption, maternal transfer of contaminants into eggs, and direct uptake of contaminants from soil by incubating eggs. Although a number of studies have examined the trophic transfer of soil contaminants into reptiles (Hopkins et al., 2004; Hopkins et al., 2005), few studies exists on the effects of direct uptake of contaminants from soil by incubating eggs (Brasfield et al., 2004; Moeller, 2004).

The purpose of this research was to evaluate the effects of *in ovo* exposure to a soil contaminant on all life stages of eastern and western fence lizards (*Sceloporus undulatus* and *S. occidentalis, respectively*). Talent et al. (2002) and Brasfield (2004) suggested that fence lizards had potential as laboratory reptile models and suggested that fence lizard eggs would be useful for ecotoxicological studies specifically focusing on soil contaminants. Fence lizards are small in size, easy to handle, and breed well in captivity. Furthermore, they bury their eggs in moist soil where they incubate for two to three months (Zug et al., 2001). *Sceloporus sp.* may be especially sensitive to soil contamination because they, like most oviparous lizards, lay parchment-shelled eggs that readily absorb environmental pore water from the substrate (Packard et al., 1982; Tracy, 1982; Ackerman et al. 1985) which provides a unique route for embryonic exposure to water soluble soil contaminants. Their eggs are porous and absorb environmental pore water from soil throughout incubation, at times doubling in size (Packard et al., 1982). Not only does pore water move into the eggs, water vapor also moves out of the eggs

(Ackerman et al., 1985). Because contaminated pore water continues to enter the eggs and water vapor exits, bioaccumulation of soil contaminants are likely.

Perchlorate (ClO_4^-) was selected as the soil contaminant to use in our research because it is common in areas inhabited by oviparous lizards and their eggs may be exposed to perchlorate at nest sites. Perchlorate contamination is often associated with military bases where heavy munitions training occurred because ammonium perchlorate is the primary fuel oxidizer in solid fuel propellants used in missiles and rockets. Sodium perchlorate ($NaClO_4$) was used as the experimental soil contaminant instead of ammonium perchlorate because once in the environment, ammonia is usually replaced with a sodium cation (Urbansky, 1998). Therefore, using sodium perchlorate as the soil contaminant is environmentally relevant as it is the perchlorate compound to which terrestrial lizards would likely be exposed.

Perchlorate is a highly water soluble, persistent contaminant of soil and water that affects thyroid hormone synthesis and may adversely affect reptiles. Perchlorate contamination in parts of the western United States has been measured in concentrations up to 3,700 ppm in surface water (Urbansky, 1998). Because the perchlorate anion itself is approximately the same size and charge as iodide, it is preferentially taken up by the thyroid gland reducing the amount of iodide available for hormone synthesis and toxic levels of perchlorate can produce hypothyroidism (Wolff, 1998). Hypothyroidism can prevent normal metabolism, growth, and reproduction in vertebrates (Stanbury and Wyngaarden, 1952). Furthermore, inadequate thyroid hormones during critical stages of organogenesis can result in permanent changes in some organs, including gonads of both

males and females (Zertashia et al., 2002; Cooke et al., 2004; Mendis-Handgama and Ariyaratne, 2004).

Exposure of lizard eggs to perchlorate during incubation is known to result in hatchlings that have abnormally low levels of thyroid hormones (Chapter II). However, nothing is known about the effects of hypothyroidism during embryonic development on subsequent growth and reproduction of lizards. Therefore, the specific objectives of our research were to determine: 1) The effects of *in ovo* exposure to perchlorate on incubation length, embryo survival, and post-hatch growth of eastern and western fence lizards, and 2) to determine the dose-related effects of *in ovo* exposure on reproduction relative to steroid and thyroid hormones, clutch size, egg size, and relative clutch mass.

MATERIALS AND METHODS

Research Animals, Rearing and Breeding Cage Setup

Eastern fence lizard eggs used for this study were obtained from a laboratory population of eastern fence lizards that was established from lizards collected in Clark County, Arkansas (Talent et al., 2002). Western fence lizard eggs were obtained from a laboratory population of fence lizards that was established from lizards collected in San Joaquin Valley, CA (Talent et al., 2002). All lizards were housed on corncob substrate in 74.2-L glass aquaria that were covered with a 3-mm steel mesh lid. A 60-W incandescent light bulb was positioned over one end of each cage to permit thermoregulation across a temperature gradient of approximately 26-40 ºC. Ambient room temperature was maintained at approximately 22 ºC and a 14:10 h light:dark light cycle was provided. Lizards were provided with a water source and fed daily with house

crickets (*Acheta domestica*) of the appropriate size that had been dusted with a Herptivite® and Rep-Cal® mixture (1:1 v/v) (Rep-Cal Research Labs, Los Gatos, CA, USA). Reproductive lizards were also provided with an oviposition site that consisted of a container of moist sand.

Perchlorate Exposure

We exposed developing lizard embryos to perchlorate by incubating eggs on a sodium perchlorate – spiked substrate, i.e. perlite. Prior to conducting the definitive study, a range finding test was conducted to determine the highest sublethal concentration of sodium perchlorate that could be used to spike the egg incubation substrate. Subsequently, exposure chambers were prepared for each egg by placing 4 g of oven dried, size-separated perlite (<4 >0.85 mm) into acid-washed, oven-dried borosilicate cylinders (70 mm deep). Sodium perchorate solutions were prepared by dissolving sodium perchlorate (> 99%, Sigma) in reagent grade water (18 MΩ). Nominal concentrations in the solutions were 1, 10, 100, 200, 400, and 800 mg $NaClO_4$/L. Exposure chambers were spiked with perchlorate by adding 6 ml of the perchlorate solutions to the chambers. These concentrations of $NaClO_4$ solutions resulted in nominal ClO_4^- concentrations of 1.21, 12.15, 121.5, 243, 486, and 972 µg ClO_4^-/g of perlite in the exposure chambers (Table 1). The controls were prepared by adding an equivalent volume of reagent grade water to chambers.

For each species, eggs were collected and distributed into the control and six treatment groups within 24 hours after oviposition. Initial sample size for the control and each treatment group was 35 eggs. LC_{50}s were calculated using a sample size of 35 eggs/

exposure concentration. Additional eggs were added to the 486 and 972 µg ClO_4^-/g exposure groups increasing the sample size to 70 and 80 eggs, respectively. We added extra eggs to the 486 and 972 µg ClO_4^-/g exposure groups to insure that we had a sufficient number of lizards survive in the higher exposure groups for conducting the reproductive study when they matured.

All control and experimental eggs were placed on the perlite substrate of the exposure chambers and chambers were sealed with a plastic cap. A 1-mm hole was drilled in the center of each plastic cap for gas exchange. Chambers were placed into a Precision incubator at 28ºC ± 0.5 ºC and each egg was checked daily for mortality. Chamber water balance was maintained weekly by adding reagent grade water to replace any water lost due to evaporation. Embryos were monitored until they hatched or died.

Rearing and Breeding Experimental Lizards

At hatching, mass, snout-vent length (SVL), sex, and length of incubation of hatchlings were recorded. Thereafter, mass and SVL were recorded monthly for four months. Hatchlings were reared 10 per cage under conditions previously described. At the age of four months, fence lizards were acclimated for artificial hibernation. Lizards were placed into a temperature-controlled room set at 10 °C in cages similar to those they were reared in. A 60-W incandescent light bulb was positioned over one end of each cage and provided a temperature gradient of approximately 10 to 34 °C. For the first month, a 4:20 h light:dark light cycle was provided daily. Lizards were provided water but no food. During the second month, the lights were turned on for four hours once per

week and water continued to be provided. After the two-month acclimation period, the lights were turned off and lizards were maintained at 10 °C for two additional months.

Due to lack of space, only eastern fence lizard pairs from the control and two highest exposure concentrations were evaluated in the reproduction phase of this study. After hibernation, 9, 11, and 10 heterosexual pairs of from the control, 486, and 972 μg ClO_4^- /g exposure groups, respectively were returned to pre-hibernation conditions with regard to cage setup, food, water, temperature, and photoperiod. Male and female eastern fence lizards from the same perchlorate treatment group were paired and a single pair of lizards was placed in each cage. Lizards were monitored daily for signs of sexual behavior and readiness to lay a clutch. Restless and digging gravid females were placed individually in a laying chamber that contained moist sand. If eggs were not deposited within 24 hours, the female was placed back in her cage for 24 hours before being returned to the laying chamber. Usually eggs were laid the first or second time that females were placed in the laying chambers. Eggs were removed from the laying chamber within 12 hours of oviposition. The female was weighed and measured and then returned to her cage. The number of eggs in each clutch was determined and mass of each egg was determined to the nearest 0.01g. In addition, the relative clutch mass (clutch mass/post-oviposition female mass) of each female was determined. Fourteen days after a female successfully laid her first clutch of eggs, both the female and her mate were sacrificed by decapitation and blood was collected from carotid arteries for hormone analysis.

Hormone Analysis

Enzyme linked immunosorbant assay (ELISA) kits (MP Biomedicals, Orangeburg, NY) were used to determine serum levels of thyroxine in males and females and testosterone in males only. Serum was diluted by a dilution factor of 2 with a phosphate buffer solution for the thyroxine assay. For the testosterone assay, serum was diluted by a dilution factor of 100. Inter-assay variation was below 15% and intra-assay variation was less than 10%. Parallelism with the standard curve was determined by the serial dilution of spiked samples for all hormones.

Data Analysis

Experiments were constructed in a statistical blocked design to account for variation within lizard clutches. Normality within each exposure group and homoscedasticity among exposure groups were determined by Kolmogorov Smirnov and Bartlett tests, respectively. Analysis of variance (ANOVA) followed by a Dunnett's post hoc test was then performed. A level of $p < 0.05$ was considered to be statistically significant. LC_{50}s were calculated using a Trimmed Spearman Karber analysis.

RESULTS

Egg Mortality and Incubation Length

Egg mortality during incubation was less than 6% in the control and all perchlorate exposure groups except for the highest concentration for both eastern and western fence lizards. 54% of eastern fence lizard embryos and 45% of western fence lizard embryos exposed to 972 μg ClO_4^-/g died before they hatched. The calculated LC_{50}

for eastern and western fence lizards was 789.51 and 846.18 μg ClO_4^-/g, respectively (Table 2).

At 28˚C, the mean incubation period for the control group of eastern and western fence lizards was 49.77 and 50.57 days, respectively. However, the variability in incubation length increased at higher perchlorate exposure levels for both species. Furthermore, the mean incubation length was significantly longer ($p < 0.0001$) for eastern and western hatchlings in the 486 and 972 μg ClO_4^-/g exposure groups. Eastern and western fence lizards exposed to 972 μg ClO_4^-/g exposure group had an incubation length that averaged 6.09 and 11.35 days, respectively, longer than the controls (Fig. 1).

Lizard Survival and Growth

Survival of the eastern fence lizards for the four-month growth period was greater than 88% for controls and all perchlorate exposure groups except for the 486 and 972 μg ClO_4^-/g exposure groups where survival was only 77.1 and 69.7%, respectively. Most mortality during the growth phase occurred within the first week after hatching. For example, 25% percent of the hatchlings exposed to 972 μg ClO_4^-/g died during the first week after hatching and only an additional 6.25% died during the remainder of the four-month growth period. Western fence lizard survival was above 90% for all exposure groups.

At the time of hatching, there were no significant differences among the control and any perchlorate exposure group relative to hatching mass or SVL for either fence lizard species. During the four-month growth period, there were no significant differences among the control and exposure groups in growth in mass for eastern or

western fence lizard males (Fig. 2 A and B) or females (Fig. 3 A and B). In addition, there were no significant differences in growth in SVL among controls and exposure groups for eastern or western fence lizard males (Fig. 4 A and B) or western fence lizard females (Fig 5 B). However, eastern fence lizard females in the 972 μg ClO_4^-/g exposure group grew significantly ($p = 0.009$) more in SVL than controls and at four months old, their mean SVL was 64 mm whereas the mean SVL of controls was 54 mm (Fig. 5 A).

Reproduction

There were dose responses relative to clutch size, egg size, and relative clutch mass. Eastern fence lizards in the 972 μg ClO_4^- /g exposure group lay significantly ($p=0.0257$) more eggs ($\bar{x} = 11$) per clutch than controls ($\bar{x} = 8.4$) (Fig. 6). However, clutch size was proportional to body size. Females exposed to 972 μg ClO_4^- /g were considerably larger than lizards in the control and the 486 μg ClO_4^-/g exposure group. The mean SVL of post-ovipositional females exposed to 972 μg ClO_4^-/g was 65.67 mm whereas the mean SVLs of females in the 486 μg ClO_4^- /g exposure group and the control were 61.36 and 62.17 mm, respectively.

The mean mass of individual eggs laid also varied in a dose dependent manner. The mean mass of individual eggs laid by control and the 486 μg ClO_4^-/g exposure group females were similar in mass at 0.33 and 0.34 g, respectively. However, the mean mass of eggs laid by females exposed to 972 μg ClO_4^-/g was 0.37 g which was significantly ($p=0.0001$) more than the mass of control eggs (Fig. 7). Furthermore, the relative clutch mass of females also varied in a dose dependent manner. The relative clutch mass of the control, 486, and 972 μg ClO_4^-/g exposure groups were 0.33, 0.40, and 0.45, respectively.

However, only the relative clutch mass of the 972 μg ClO_4^-/g treatment group was significantly ($p = 0.03$) larger than the control (Fig. 8).

Two weeks after females laid their clutches, the pair was sacrificed and plasma samples were examined for hormones. No significant differences in circulating levels of testosterone in males, or T_4 in both sexes were detected among any of the perchlorate exposure groups (Table 3).

DISCUSSION

The objective of this study was to explore the effects of embryonic perchlorate exposure on the life cycle of eastern and western fence lizards. Previous studies indicate perchlorate crosses the eggshell and depresses oxygen consumption of the embryo (Chapter III). Furthermore, lizards incubated on perchlorate spiked substrates have an increased incubation length and are hypothyroid upon hatching (Chapter II).

Rather than begin exposure on day 7 of incubation as done previously, exposure in the current study began on the day eggs were laid. Due presumably to the increased exposure duration, the highest concentration in which eastern or western fence lizard eggs would hatch at was 972 μg ClO_4^-/g, which is 61% of the highest perchlorate concentration in previous studies (Chapter II).

Spiking the incubation substrate one day after oviposition with perchlorate produced an apparent dose response in eastern and western fence lizards at several levels. However, no differences between the two species *S. occidentalis* and *S. undulatus* exposed to the same concentrations of perchlorate were detected. Embryonic mortality for both species increased with dose primarily because they continued to live in the eggs

long past the hatch date of controls and died presumably when they had used up yolk reserves. It is unknown why the embryos failed to hatch. However, prior research with western fence lizards demonstrated that the growth and development of embryos was decreased by exposure to perchlorate. Nevertheless, fence lizard embryos should have hatched after they reached normal hatching size if reduced growth was the only effect of perchlorate. Apparently perchlorate produced multiple developmental problems because although some embryos did hatch after an extended incubation period, many did not. Therefore, we suspect that hypothyroidism caused reduced growth rates which in turn caused the length of incubation to significantly increase in the 486 and 972 μg ClO_4^-/g treatment groups. However, 54% and 45% of the eastern and western fence lizard embryos respectively, exposed to 972 μg ClO_4^-/g failed to hatch even after most reached the typical size of hatchlings.

The pipping response involves several steps that can be observed. The first external indication that an embryo is going to hatch within the next 24 hours is beads of water that start to appear on the shell, i.e. the egg appears to sweat. Backlighting the egg will show that the blood vessels that typically adhere to the shell membranes are being pulled away as the yolk sac is retracted into the abdominal area of the embryo (Zug et al., 2001). During this period, the embryo will slit the egg with an egg tooth and protrude its nose through the slit and start breathing. After the yolk sac is fully retracted into the abdomen, the hatchling emerges fully from the egg. The embryos that failed to hatch in the higher perchlorate exposure groups never initiated the yolk sac retraction process, possibly due to a hormone deficiency.

Although exposure to perchlorate increased the period required for incubation, there were no significant differences in hatching mass or SVL among the different treatment groups. However, for eastern fence lizards, there was significantly higher post-hatching mortality in the 972 μg ClO_4^-/g exposure group compared to the control and other treatment groups. Most of the post-hatch mortality occurred within the first week after hatching and appeared to be due to weak hatchlings with poor coordination that did not start feeding.

If the hatchlings were vigorous enough to feed, they had a tendency to survive and grow during the four-month juvenile phase. However, eastern fence lizard females exposed to 972 μg ClO_4^-/g grew significantly more in SVL during the four-month growth period than the controls and other exposure groups. The explanation for this difference in growth is unclear. Juvenile growth rates of lizards are affected by thyroid hormones (Gerwien and John-Alder, 1992). However, comparing our data with other published studies is difficult because other studies that have used growth rate as an endpoint to determine effects of perchlorate exposure on oviparous vertebrates measured growth during exposure (McNabb et al., 2004; Goleman, et al., 2002). This study was different in that embryos were exposed to a thyroid inhibiting chemical throughout incubation rather than during the juvenile growth stage. Although western fence lizard embryos exposed *in ovo* are hypothyroid at hatching (Chapter II), once the lizard hatched, exposure to sodium perchlorate ceased. Therefore, it is unclear how long it took after hatching for the thyroid gland to recover and produce normal amounts of thyroid hormones. We suspect that the thyroid recovered quickly because growth rates of hatchlings from the perchlorate exposure groups were as high as or higher than controls.

It is questionable whether or not growth is a sensitive endpoint for evaluating the effects of perchlorate, especially when studies are conducted after the thyroid gland has developed and is functioning normally. McNabb et al. (2004) concluded that growth was an insensitive endpoint for evaluating the effects of perchlorate exposure on growing bobwhite quail (*Colinus virginianus*). Because the thyroid gland stores synthesized hormone and circulating thyroxine acts as a source for the more biologically active triiodothyronine, it may take chronic exposure to a thyroid inhibiting chemical to deplete tissue hormone levels to a point where growth would be reduced (Hadley, 2000).

In our study, hatchling mass was unaffected by perchlorate exposure, despite probable hypothyroidism. However, after they hatched, the female eastern fence lizards in the highest perchlorate exposure group (972μg ClO_4^-/g) grew faster and larger in SVL than the controls. It is tempting to speculate that the increased growth rate of these females was due to a hormesis. Support for this contention may be provided by Goleman et al. (2002) who exposed *Xenopus laevis in ovo* to ammonium perchlorate concentrations and continued the exposure for 70 days post hatch. Ten days after hatching, the frogs exposed to concentrations of 425 ppm ammonium perchlorate had significantly shorter snout-vent lengths than control frogs. However, they were able to recover and catch up to control frogs within 40 days while still submerged in ammonium perchlorate solution. Nevertheless, we are reluctant to suggest that hormesis caused the rapid growth and large adult snout-vent lengths of female fence lizards exposed to 972 μg ClO_4^-/g because we did not see a significant increase in female western fence lizard growth. Also, 54 % of eastern fence lizard embryos exposed to 972 μg ClO_4^-/g failed to hatch and 31% of those that hatched died before they matured. The embryonic and

subsequent survival rate for western fence lizards was higher. Therefore, eastern fence lizard females that survived may have been especially hardy resulting in rapid growth rate.

It is probable that the explanation for the larger clutch size of eastern fence lizards exposed to 972μg ClO_4^-/g also is related to their larger body size because there is a direct correlation between female SVL and clutch size in eastern fence lizards (Zug et al., 2001). However, body size does not explain why the eggs of females in the 972 μg ClO_4^- /g exposure group were significantly heavier than the eggs of control lizards. Egg mass typically does not vary with body size in eastern fence lizards that are reared under laboratory conditions (Ferguson and Talent, 1993). Moreover, body size also does not explain why the relative clutch mass of females exposed to 972 μg ClO_4^-/g was significantly greater than the relative clutch mass of females in the control. However, the key difference between the 972 μg ClO_4^-/g exposure group and controls is egg mass which would produce the difference in relative clutch mass. The mass of an egg can be due to at least three factors: 1) the ovarian follicles may have produced ova with higher water content, 2) yolks may have been larger, and 3) the eggshells may have been denser (Fernie et al., 2000). We have no way of knowing which of these processes took place because we did not determine the dry mass of eggs or weigh eggshells. Nevertheless, our data suggests that *in ovo* exposure to perchlorate caused some developmental change in ovarian tissue or the liver that resulted in heavier eggs.

It is known that hypothyroidism during rat development can affect the structure of reproductive organs (Doufas and Mastorakos, 2000; Zertashia et al., 2002). Prenatal hypothyroidism causes an increase in ovarian weight and graafian follicle diameter

(Zertashia et al., 2002) and abnormal folliculogenesis (Dijkstra et al., 1996) in mature female rats although the mechanism behind the perturbations is not known. The sertoli and leydig cells of males that are transiently hypothyroid during development continue to multiply resulting in a much larger testicle at maturity (Kirby et al., 1996; Cooke et al., 2004; Mendis-Handagama and Ariyaratne, 2004). Transient neonatal hypothyroidism can also lead to an increase in sperm production in rats after they mature (Cooke et al., 2004). However, nothing is known about how perchlorate affects these processes in reptiles. Nevertheless, our results suggest that *in ovo* perchlorate exposure may enhance reproductive output of female fence lizards. However, additional research is needed to determine whether the increase in egg mass and relative clutch mass was due to differential survival of females or to developmental changes due to perchlorate exposure.

In conclusion, our research and the work of Brasfield et al. (2004) suggest that exposing fence lizard eggs to contaminated substrate is a practical method for evaluating the effects of embryonic soil contaminant exposure on reptiles. Furthermore, incubating eggs on contaminated substrate is a more environmentally relevant method of exposing eggs to contaminants compared to other methods of exposure. Moreover, fence lizards appear sensitive enough to indicate perturbations caused by contaminant exposure yet hardy enough to survive and breed in an artificial environment.

LITERATURE CITED

Ackerman RA, Dmi'el R. Ar A. 1985. Energy and water vapor exchange by parchment-shelled reptile eggs. *Physiological Zoology* 58:129-137.

Brasfield SM, Bradham K, Wells JB, Talent LG, Lanno RP, and Janz DM. 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and *in ovo* effects on hatching size and thyroid function. *Chemosphere* 54:1643-1651.

Cooke PS, Holsberger DR, Witorsch RJ, Sylvester PW, Meredith JM, Treinen KA, Chapin RE. 2004. Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. *Toxicology and Applied Pharmacology* 194:309-335

Dijkstra G, De Rooij DG, Jong FH, Van Den Hurk R. 1996. Effect of hypothyroidism on ovarian follicular development, granulose cell proliferation and peripheral hormone levels in the prepubertal rat. *European Journal of Endocrinology* 134:649-654.

Douofas A.G. Mastorakos G. 2000 The hypothalamic-pituitary-thyroid axis and the female reproductive system. *Annals of the NY Academy of Science* 900:65-76.

Ferguson GW, Talent LG. 1993. Life-history traits of the lizard *Sceloporus undulatus* from two populations raised in a common laboratory environment. *Oecologia* 93:88-94.

Fernie KJ, Bortolotti GR, Smits JE, Wilson J, Drouillard KG, Bird DM. 2000. Changes in egg composition of American kestrels exposed to dietary polychlorinated biphenyls. *Journal of Toxicology and Environmental Health Part A* 60:291-303.

Gerwien RW, John-Alder HB. 1992. Growth and Behavior of thyroid deficient lizards (*Sceloporus undulatus*). General and Comparative Endocrinology 87:312-324.

Goleman WL, Urquidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit

development and metamorphosis in *Xenopus laevis*. *Environmental Toxicology and Chemistry* 21:424-430.

Goleman WL, Carr JA, Anderson TA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environmental Toxicology and Chemistry* 21:590-597.

Hopkins WA, Roe JH, Snodgrass JW, Staub BP, Congdon JD. 2002. Effects of chronic dietary exposure to trace elements on banded water snakes (*Nerodia faciata*). Environmental Toxicology and Chemistry 21:906-913.

Hopkins WA, Staub BP, Baionno JA, Jackson BP, Roe JH, Ford NB. 2004. Trophic and maternal transfer of selenium in brown house snakes (*Lamprophis fuliginosus*). *Ecotoxicology and Environmental Safety* 58:285-293.

Hopkins WA, Staub BP, Baionno JA, Talent LG. 2005. Transfer of selenium from prey to predators in a simulated terrestrial food chain. *Environmental Pollution* 134:447-456.

Kirby JD, Manakar MV, Hardesty D, Kreider DL. 1996. Effects f transient prepubertal 6-N-Propyl-2-Thiouracil treatment on testis development and function in the domestic fowl. *Biology of Reproduction* 55:910-916.

McNabb FMA, Larsen CT, Pooler PS. 2004. Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. *Environmental Toxicology and Chemistry* 23:997-1003.

Mendis-Handagama SMLC, Ariyaratne HBS. 2004. Effects of thyroid hormones on leydig cells in the postnatal testis. *Histology and Histopathology* 19:985-997.

Packard MJ, Packard GC, Biardman TJ. 1982. Structure of eggshells and water relations of reptilian eggs. *Herpetologica* 38:136-155.

Sparling DW, Linder G, Bishop CA, editors. 2000. *Ecotoxicology of Amphibians and Reptiles*. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC) 904 p.

Stanbury JB, Wyngaarden JB. 1952. Effect of perchlorate on the human thyroid gland. *Metabolism-Clinical and experimental* 1:533-539.

Talent LG, Kumont JN, Bantle JA, Janz DM, Talent SG. 2002. Evaluation of western fence lizards (*Sceloporus occidentalis*) and Eastern fence lizards (*Sceloporus undulatus*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 22:899-905.

Talent LG. 2005. Effect of temperature on toxicity of a natural pyrethrin pesticide to green anole lizards (*Anolis carolinensis*). *Environmental Toxicology and Chemistry* 24:3113-3116.

Tracy CR. 1982. Biophysical modeling in reptilian physiology and ecology, Pages 275-321 in C.Gas and H. Pough, eds. *Biology of the Reptilian Vol. 12.* Academic Press, New York.

Urban DJ, Cook NJ. 1986. Hazard evaluation division-Standard evaluation procedure-Ecological risk assessment. EPA 540/9-85-001. Technical report. U.S. Environmental Protection Agency, Washington, DC.

Urbansky ET. 1998. Perchlorate chemistry: implications for analysis and remediation. *Bioremediation Journal* 2:81-95.

Walker CH, Ronis MJJ. 1989. The monooxygenases of birds, reptiles, and amphibians. *Xenobiotica* 19:1111-1121.

Wolff J. 1998. Perchlorate and the thyroid gland. *Pharmacology Review* 50:89-105.

Zertashia A, Jalali S, Ahmad L, Mirza A. 2002. Effect of hypothyroidism induced by propylthiouracil on ovarian function and structure in offspring from treated mothers (rats). *Journal of Experimental Zoology* 293:407-413.

Zug GR, Vitt LJ, Caldwell JP, (Eds). 2001. *Herpetology.* Academic Press. London.

Table 1. Nominal sodium perchlorate ($NaClO_4$) and perchlorate (ClO_4^-) concentrations in the spiked pore-water and substrate of the exposure chambers.

Treatment	Concentration			
Groups	Pore Water		Substrate	
	Sodium Perchlorate	Perchlorate	Sodium Perchlorate	Perchlorate
Control	0 mg/L	0 mg/L	0 µg/g	0 µg/g
1	1 mg/L	0.81 mg/L	1.5 µg/g	1.215 µg/g
2	10 mg/L	8.1 mg/L	15 µg/g	12.15 µg/g
3	100 mg/L	81 mg/L	150 µg/g	121.5 µg/g
4	200 mg/L	162 mg/L	300 µg/g	243 µg/g
5	400 mg/L	324 mg/L	600 µg/g	486 µg/g
12	800 mg/L	648 mg/L	1200 µg/g	972 µg/g

Table 2. LC_{50} and upper and lower 95% confidence intervals for eastern and western fence lizards after incubation on perchlorate spiked substrate.

Fence Lizard	Eastern	Western
LC50	789.51	846.18
Upper 95%	1018.95	1404.07
Lower 95%	611.73	509.95

Table 3. Serum concentrations of thyroxine (T_4) and testosterone in males, and thyroxine in females two weeks after the first clutch of eggs was laid.

Treatment	Males		Females
µg/g ClO4-/g	Testosterone (ng/ml)	T_4 (ng/ml)	T_4 (ng/ml)
Control	29.97 ± 6.77	385.62 ± 38.77	285.32 ± 55.57
486	37.70 ± 4.92	437.83 ± 33.74	375.79 ± 30.91
972	27.78 ± 5.44	384.78 ± 26.94	333.55 ± 47.52

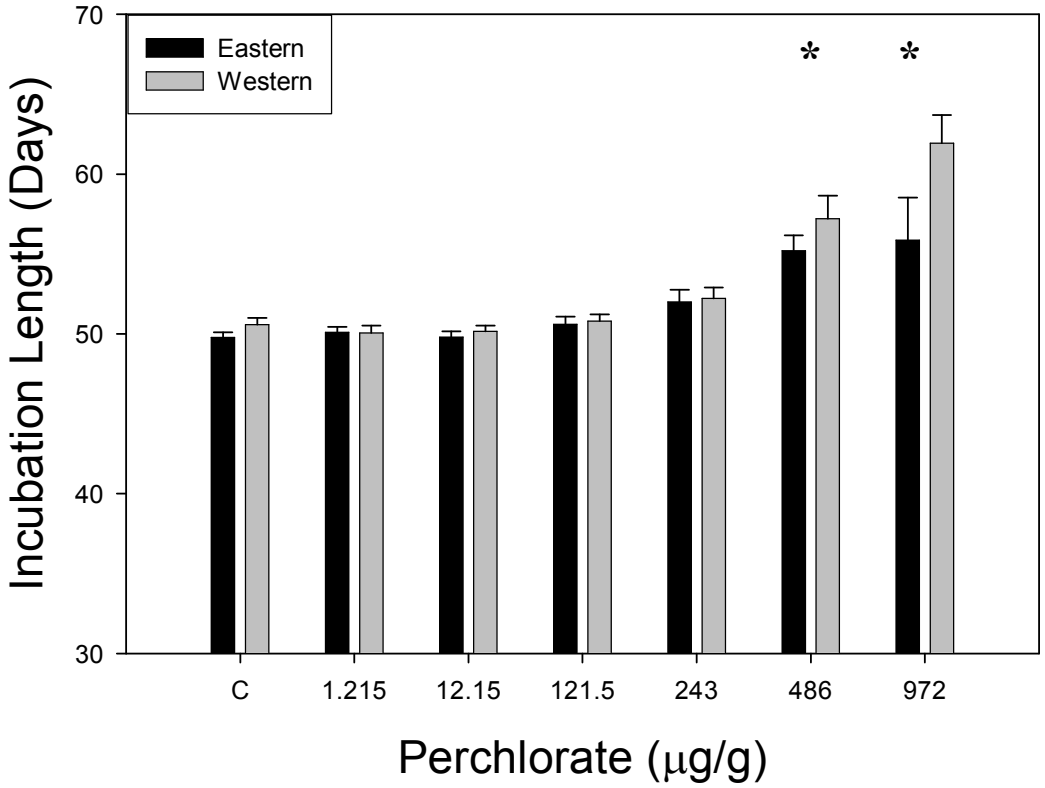


Figure 1. The effects of perchlorate concentration in spiked incubation substrate on the length of the incubation period at 28˚C. Asterisk indicates a significant difference between lizards in the perchlorate-concentration group and the control.

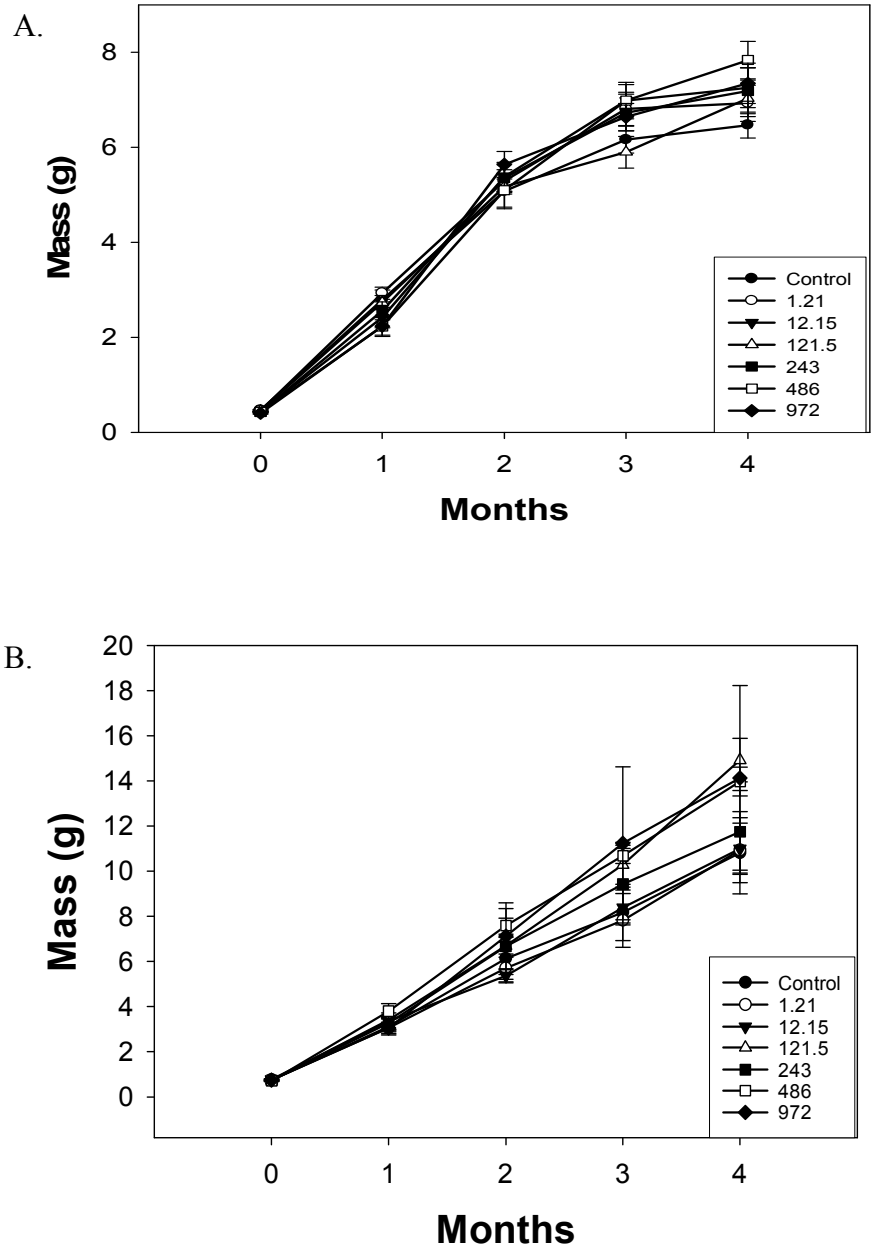
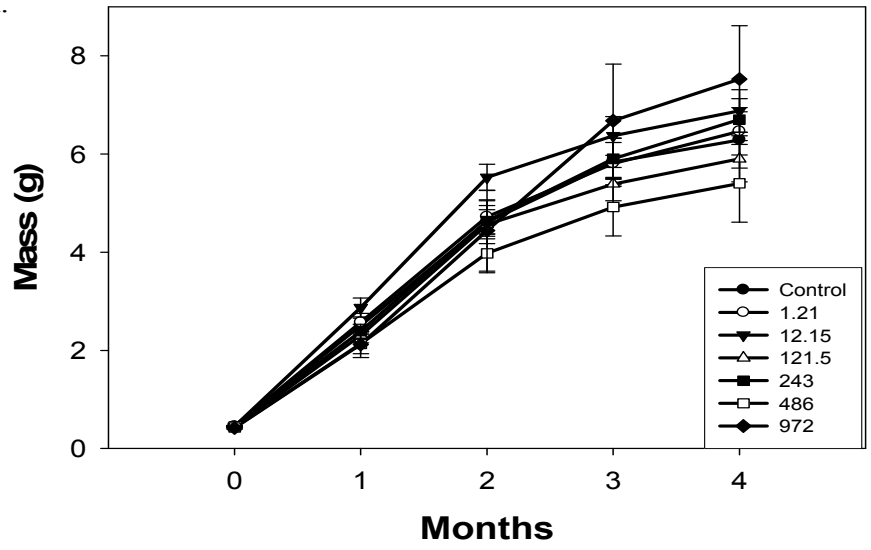


Figure 2. Effects of *in ovo* exposure to perchlorate concentrations in spiked incubation substrate on postnatal growth in mass of male eastern (A.) and western (B.) fence lizards. Concentration units were μg ClO_4^-/g perlite. Sample size is >10 for each concentration.

A.



B.

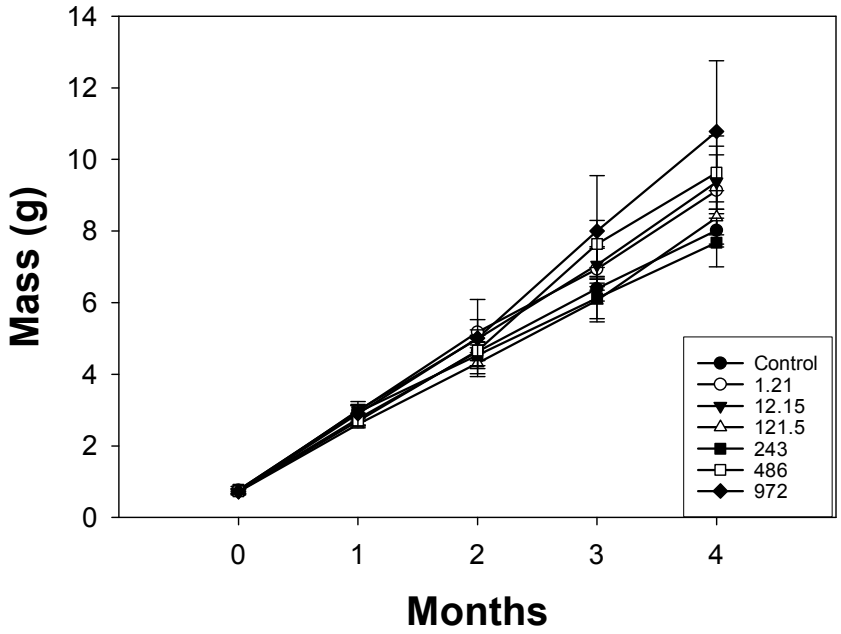
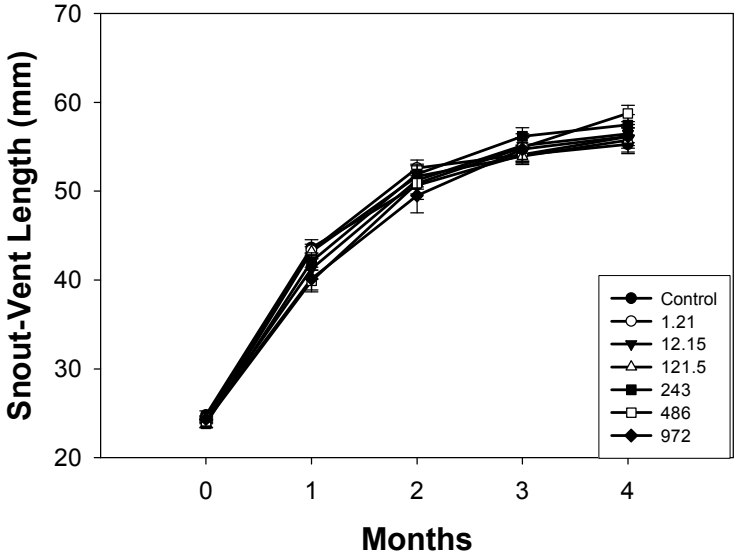


Figure 3. Effects of *in ovo* exposure to perchlorate concentrations in spiked incubation substrate on postnatal growth in mass of female eastern (A.) and western (B.) fence lizards. Concentration units were μg ClO_4^-/g perlite. Sample size is > 10 for each concentration.

A.



B.

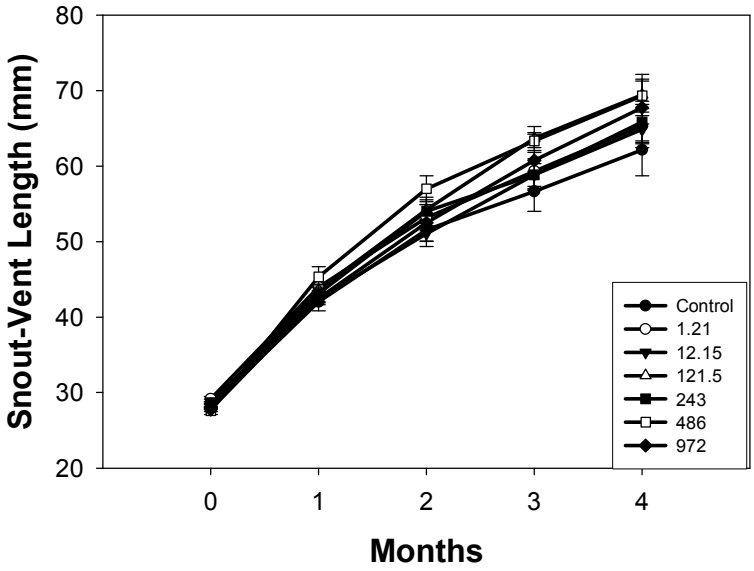
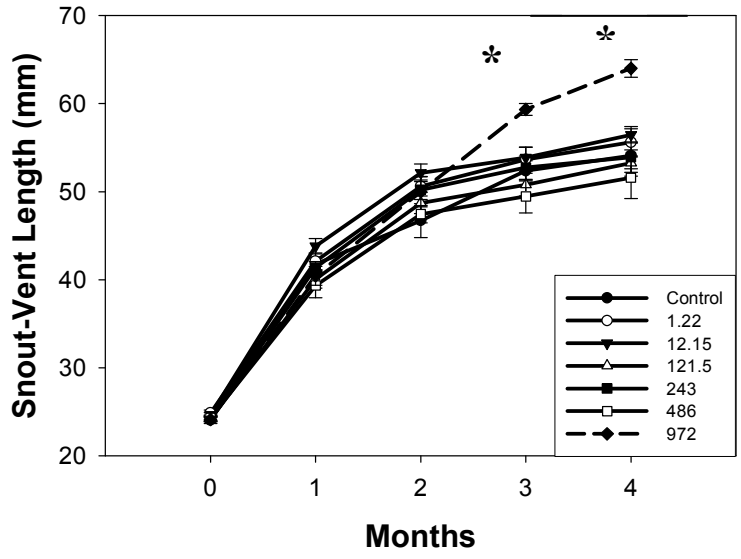


Figure 4. Effects of *in ovo* exposure to perchlorate concentrations in spiked incubation substrate on postnatal growth in snout-vent length of male eastern (A.) and western (B.) fence lizards. Concentration units were μg ClO_4^-/g perlite. Sample size is > 10 for each concentration.

A.



B.

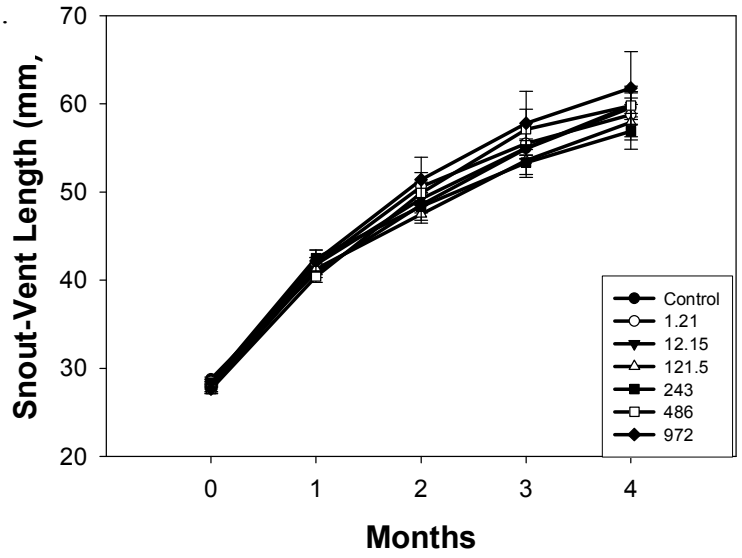


Figure 5. Effects of *in ovo* exposure to perchlorate concentrations in spiked incubation substrate on postnatal growth in snout-vent length of female eastern (A.) and western (B.) fence lizards. Concentration units were μg ClO_4^-/g perlite. Dashed line indicates significant differences in snout vent length between lizards in the perchlorate-concentration group and the control. Asterisk denotes the location of significant differences. Sample size is > 10 for each concentration.

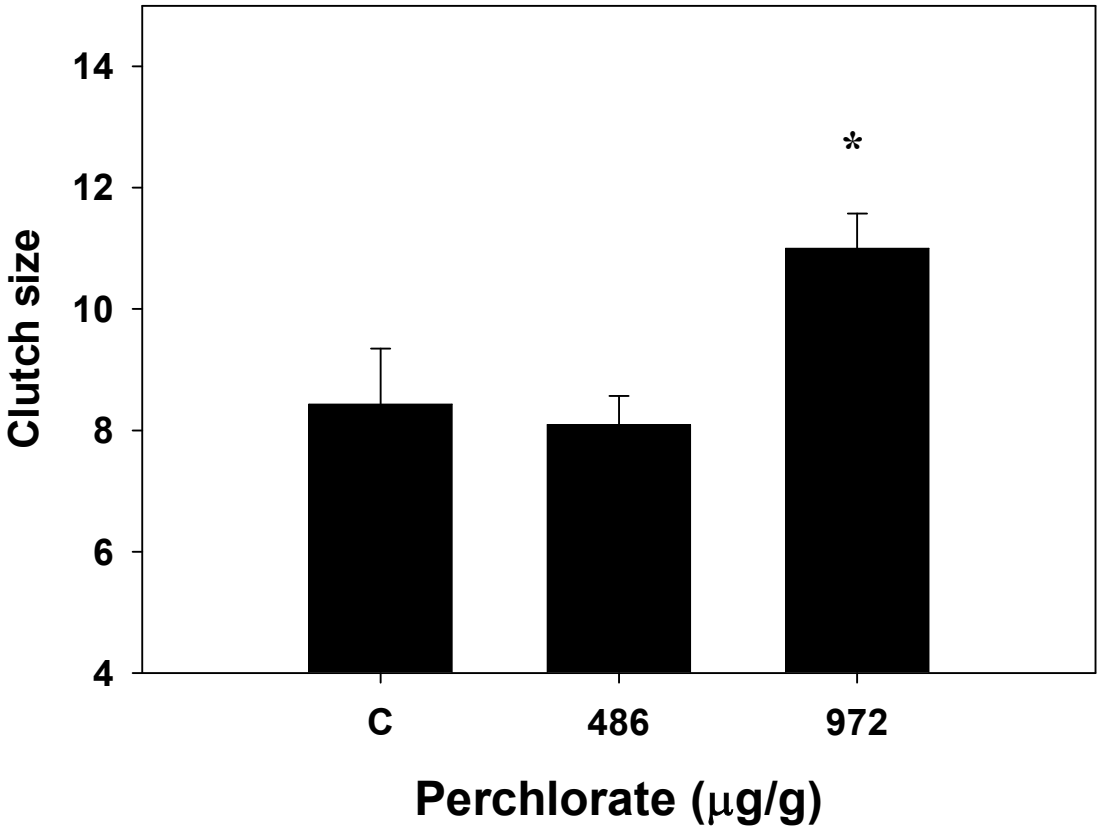


Figure 6. Average number of eggs laid by eastern fence lizards exposed *in ovo* to perchlorate in spiked incubation substrate. Sample size ranges from 8-11 clutches. Error bars represent standard error. Asterisk indicates a significant difference between lizards in the perchlorate-concentration group and the control.

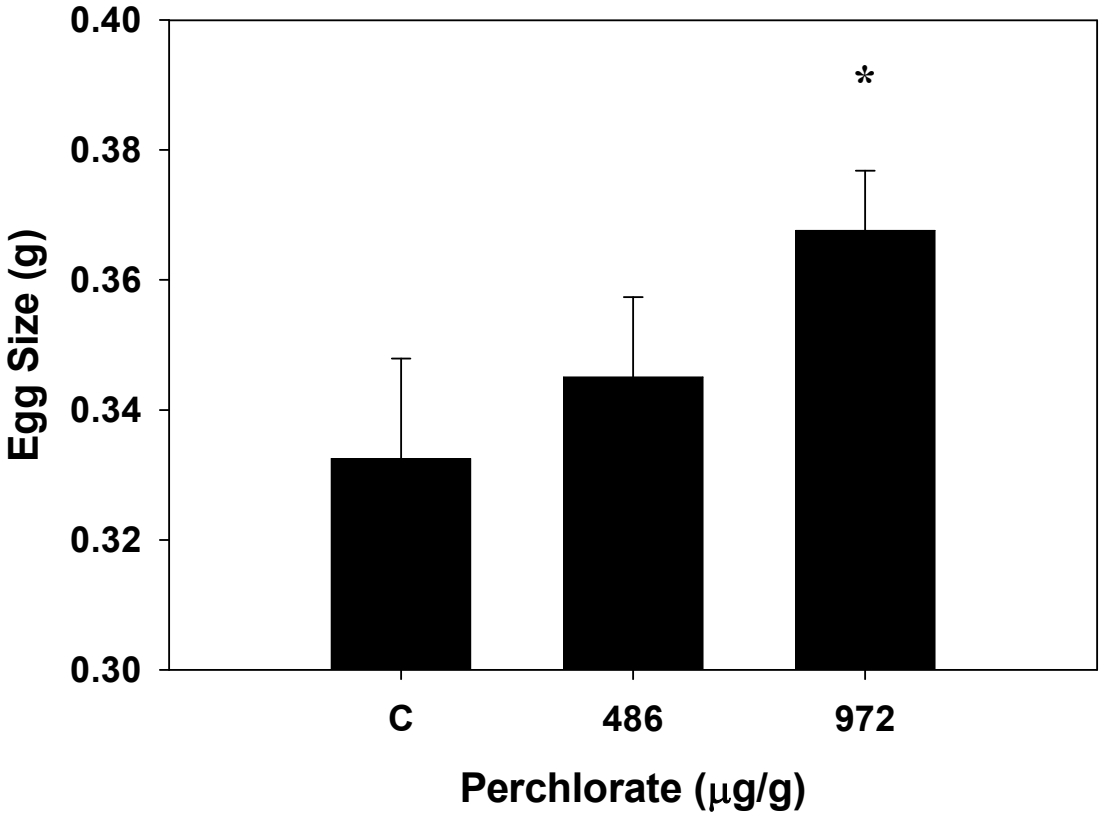


Figure 7. Mean mass of eggs laid by eastern fence lizards exposed *in ovo* to perchlorate in spiked incubation substrate. Sample size ranges 47-83 eggs in each concentration. Error bars represent standard error. Asterisk indicates a significant difference between lizards in the perchlorate-concentration group and the control.

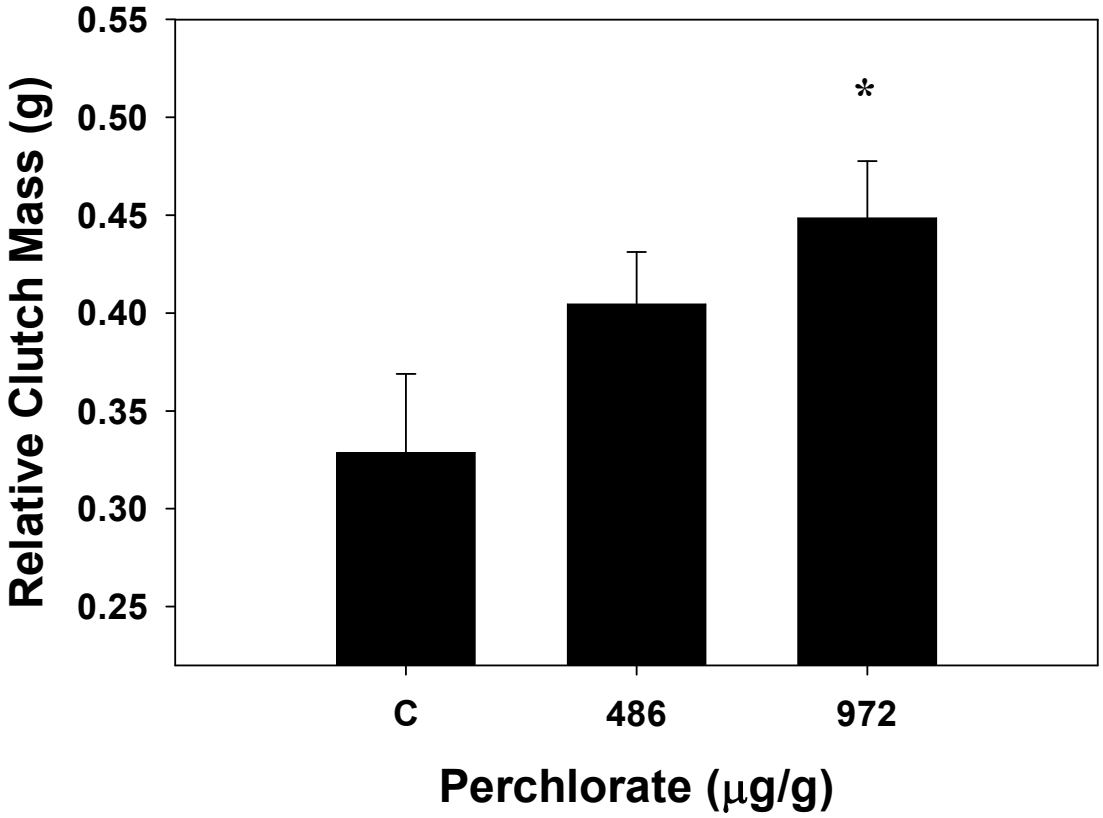


Figure 8. Mean relative clutch mass of eastern fence lizards exposed *in ovo* to perchlorate in spiked incubation substrate. Sample size ranges from 8-11 females. Error bars represent standard error and the asterisk indicates a significant difference between lizards in the perchlorate-concentration group and the control.

CHAPTER V

SUMMARY OF CONCLUSIONS

During the parinatal period, whole body corticosterone levels increased the day before pipping. At the same time, thyroxine began to decrease followed by a spike in triiodothyronine on the day of pipping.

Oxygen consumption decreased in lizards exposed in ovo to 1,585 μg ClO_4^-/g perlite and higher. Decreases were more dramatic in eggs that never hatched when compared to those that did hatch at the same level of exposure.

Embryonic perchlorate exposure via spiked incubation substrate suppressed whole body thyroid hormone levels in hatchling western fence lizards.

Whole body corticosterone appeared to be affected by embryonic perchlorate but not in a predictable manner.

Embryonic perchlorate exposure increased the length of incubation as well as post hatch mortality in a dose dependant manner.

Hatchling size does not appear to be sensitive to embryonic perchlorate exposure.

Western fence lizards were more sensitive to perchlorate exposure when exposure began on day one of incubation rather than on day seven.

LC_{50} concentrations do not differ between eastern and western fence lizards when exposed to perchlorate via spiked substrate.

Juvenile growth rates of female eastern but not western fence lizards increased when exposed to high (972 μg/g) perchlorate concentrations.

Adult female eastern fence lizards exposed to perchlorate in the egg outperformed control lizards with regard to total reproductive effort.

Thyroxine and testosterone hormone levels in adult control and exposed lizards were not different after embryonic perchlorate exposure.

Overall, fence lizards appear sensitive enough to detect contaminant toxicity yet hardy enough to successfully breed under artificial laboratory conditions. These characteristics indicate fence lizards are suitable for use as a model in conducting life cycle effects of contaminants in a laboratory.

VITA

Melinda Suzanne Redick-Harris

Candidate for the Degree of

Doctor of Philosophy

Thesis: EFFECTS OF IN OVO EXPOSURE TO SODIUM PERCHLORATE ON DEVELOPMENT, GROWTH AND REPRODUCTION OF FENCE LIZARDS (*SCELOPORUS SP*.)

Major Field: Zoology Date of Degree: July 2006

Education: PhD in Zoology, Oklahoma State University, Stillwater, OK 74078

M.Sc. in Environmental Science, University of North Texas, Denton, TX 76202. 2002

B.Sc. in BioEnvironmental Science, Texas A&M University, College Station, TX 1997

Experience:

01/06 to present	Teaching assistant, Animal Biology, Department of Zology, Oklahoma State University.
08/05 to 12/05	Instructor of record for Animal Biology course, Department of Zoology, Oklahoma State University.
08/04 to 08/05	Teaching Assistant Animal Biology Laboratory Preparation, Department of Zoology, Oklahoma State University.
08/01 to 08/04	Research Assistant, Department of Zoology, Oklahoma State University.
12/00 to 08/01	Laboratory Coordinator, Institute of Applied Science, University of North Texas.
08/98 to 12/00	Teaching Assistant, Institute of Applied Science, University of North Texas.
05/98 to 08/98	Research technician, Army Corps of Engineers (Lewisville Aquatic Ecosystem Research Facility)

Professional Memberships: Society for Environmental Toxicology and Chemistry

Name: Melinda Suzanne Redick-Harris Date of Degree: July, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF IN OVO EXPOSURE TO SODIUM PERCHLORATE ON DEVELOPMENT, GROWTH AND REPRODUCTION OF FENCE LIZARDS (*SCELOPORUS SP*.)

Pages in Study: 110 Candidate for the Degree of Doctor of Philosophy

Major Field: Environmental Toxicology

Scope and Method of Study: This research was designed to determine the effects of long term in ovo perchlorate exposure on eggs, juveniles and adult fence lizards. Reptile eggs are highly extensible and absorb environmental pore water throughout incubation. Perchlorate is a water soluble environmental contaminant that interferes with synthesis of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) by preventing iodide uptake by the thyroid gland. Decreases in circulating embryonic thyroid hormones may interfere with growth and development of embryos and may have permanent effects. Endpoints measured during incubation included: egg viability, size, oxygen consumption rate, incubation length, and hatchability. Initial size (mass and snout/vent length), thyroid and corticosterone hormone levels, and growth rate were measured in hatchling lizards. Clutch and egg size were recorded as indications of breeding success for adult fence lizards. Adult plasma thyroxine levels were measured in all adults exposed to perchlorate *in-ovo* and plasma testosterone levels were recorded in males. Eggs for this study were collected from a laboratory population of fence lizards on the day of oviposition and incubated on perchlorate-spiked perlite at a constant temperature of 28˚C.

Findings and Conclusions: Corticosterone and thyroid hormones appear to play a significant role in the hatching of western fence lizards. Perchlorate absorbed from spiked substrate caused decreased whole body thyroid hormone concentrations at concentrations greater than 15.8 μg ClO_4^-/g. Throughout incubation eggs exposed to 1,585 μg ClO_4^-/g and higher consumed less oxygen than control lizards. Furthermore, incubation length in ovo mortality and post hatch mortality increase in a dose dependant manner when perchlorate was present in the incubation substrate. Eastern and western fence lizards that survived post hatch appeared to completely recover from embryonic perchlorate exposure. Eastern but not western fence lizards grew more rapidly than control lizards and eastern females outperformed control lizards with regard to clutch production. Adult serum hormone levels were not different in control and perchlorate exposed lizards.

ADVISER'S APPROVAL: _____

Larry G. Talent