DEVELOPMENT OF A LABORATORY REPTILE MODEL FOR ASSESSMENT OF ENDOCRINE-MEDIATED TOXICITY

By

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NOMENCLATURE

ALP	alkaline labile phosphate
ANOVA	analysis of variance
BSA	bovine serum albumin
E_2	17-β estradiol
ED_{50}	effective dose that causes 50% of the maximal response
EDCs	endocrine disrupting compounds
EE_2	17-α ethinylestradiol
GSD	genotypic sex determination
HCl	hydrochloric acid
HSI	hepatosomatic index
LOEL	lowest observed effects level
NaOH	sodium hydroxide
PBS-T	phosphate buffered saline containing Tween 20
T_3	triiodothyronine
T_4	thyroxine
TSD	temperature-dependent sex determination
Vtg	vitellogenin

Abstract

The overall goal of this research was to develop and validate biological parameters in Western fence lizards (*Sceloporus occidentalis*) for use in assessing the risks of endocrine-mediated toxicity. This work is part of a larger long-term study to evaluate both Eastern and Western fence lizards as laboratory reptile models. The effects of many chemicals considered to be endocrine-disrupting compounds (EDCs) have been studied under laboratory conditions with a variety of vertebrates. However, relatively few laboratory studies have been conducted on the effects of EDCs on reptiles and no standardized tests involving reptile models exist. The induction of the yolk precursor protein, vitellogenin (Vtg) in oviparous vertebrates has been studied extensively in response to both exogenous and endogenous estrogenic compounds. Disruption of natural vitellogenesis in female oviparous vertebrates or the unnecessary induction of vitellogenesis in males and juveniles by exposure to xenobiotics may adversely affect reproductive processes, such as oogenesis and embryonic development, resulting in potential risk for population level impacts and reduced biological productivity. Although inappropriate vitellogenesis in wildlife may not indicate what compound may be causing the effect, it can be used as a rapid, sensitive, and economical initial screen before more costly testing procedures are needed identify the specific contaminating compounds. Dose response and time course relationships between exposure to a synthetic estrogen, ethinylestradiol (EE_2), and plasma Vtg induction were established in male Western fence lizards (*Sceloporus occidentalis*). Plasma Vtg was quantified

directly with an antibody-capture enzyme linked immunosorbent assay (ELISA) and indirectly using plasma alkaline-labile phosphate (ALP), and comparisons were made between these two methods. These methods estimated similar ED_{50} values for Vtg induction by EE_2, 0.167 mg/kg for the ELISA, and 0.095 mg/kg for ALP. Multiple dilutions of EE_2–treated male plasma measured with both methods resulted in a strong correlation. Based on these data in fence lizards, ALP may therefore be a suitable alternative to measuring Vtg by ELISA. Measuring hormones may provide an indication of overall endocrine status, however it is still not clear to what degree circulating levels must be altered before organismal success is hampered. A better definition of baseline conditions for endocrine function is necessary to serve as a basis for assessing effects of endocrine-mediated toxicity. Seasonal hormone profiles in breeding males and females and diel hormone profiles in males were determined for four hormones: testosterone, 17β–estradiol (E_2), triiodothyronine (T_3) and thyroxine (T_4). Seasonally, cycles were seen in both male and female Western fence lizards for the sex steroids, E_2 and testosterone. E_2 levels in male and female lizards were highly elevated during weeks 8-12 following emergence from artificial hibernation. Testosterone was elevated in males during the first 6 weeks of breeding, indicative of active spermatogenesis. Seasonally, thyroid hormones did not fluctuate greatly, but T_3 appeared to increase in weeks 8 and 16. Hormones in males revealed little diel cyclical activity and high levels of variability. In conclusion, these baseline data are useful as an initial step in characterizing this species as a laboratory reptile model for endocrine-mediated toxicity.

Chapter One

Rationale and Literature Review

Introduction

Recently, the issue of endocrine disruption has raised interest in many researchers and has attracted considerable public and political concern. Endocrine-disrupting compounds (EDCs) have been defined as exogenous agents that interfere with the synthesis, storage/release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock et al. 1996). This definition reflects a growing awareness that the issue of EDCs extends considerably beyond that of environmental estrogens and includes anti-androgens and agents that act on other components of the endocrine system such as the thyroid and pituitary glands. Understanding the mechanisms of endocrine function and the general pathways that may be affected by environmental chemicals is essential to providing a better idea of how physiological performance might be affected by EDCs.

The endocrine system consists of a number of brain-hypothalamus-pituitary-target organ feedback pathways involved in the regulation of a multitude of bodily functions and the maintenance of homeostasis. Various hormones are involved in reproduction, growth and development, maintenance of the internal environment, and regulation of energy balance (Van der Kraak et al. 1998). A single hormone can exert various effects in different tissues; conversely, a single function can be regulated by several hormones (Ojeda and Griffin 2000).

Environmental chemicals can affect endocrine physiology through effects on hormone biosynthesis, transport, metabolism, or actions. The majority of our current knowledge comes from information related to the effects of environmental chemicals on sex steroid and thyroid hormone physiology and how these effects influence growth, development, and reproduction (Jobling et al. 1996, Ankley et al. 1998).

There are several sites at which an environmental agent could disrupt endocrine function. The function of virtually all the sex steroid and thyroid hormone receptor-mediated pathways can be disrupted by one or more environmental chemicals (Cooper and Kavlock 1997). Hormones are present in the bloodstream in very low concentrations, thus as a prerequisite for any hormone to exert its actions it must first bind to specific, high affinity cellular receptors (Ojeda and Griffin 2000). The capacity of a cell to respond to a particular hormone depends on the presence of cellular receptors specific for that hormone. After binding, the receptor can become biochemically and structurally altered, resulting in activation. The activated receptor then mediates all of the actions of the hormone on the cell (Mendelson 2000).

Receptors serve the dual functional properties of recognition and transmission of hormone signal to biological response. Receptors are proteins that bind hormones with high affinity, which is appropriate for the low concentrations found in circulation. The receptor must also bind the hormone with high specificity, so that it has a greater affinity for a particular biologically active molecule rather than a related but less biologically active molecule

(Mendelson 2000). The binding of hormone to receptor is a saturable process, as there are a finite number of receptors for a given hormone on a target cell.

Sex steroid and thyroid hormones exert long-term effects on their target cells after binding to specific receptors located within the nucleus. These hormone-receptor complexes regulate the synthesis of specific proteins primarily by altering the rate of transcription of specific genes. Because of their lipophilicity, sex steroids and thyroid hormones travel in the circulation predominantly bound to serum proteins. Only a small proportion of the circulating hormone is free, and it is only the unbound hormone fraction that has the capacity to enter cells by free diffusion.

Disruption of normal endocrine function may occur through several mechanisms. Hormones or analogues that bind to receptors and elicit the same biological response as the naturally occurring hormone are termed agonists (Ojeda and Griffin 2000). Molecules that bind to receptors, but fail to elicit the normal biological response are termed competitive antagonists because they occupy receptors and prevent the binding of biologically active molecules. Molecules that bind to receptors but are less biologically active that the endogenous hormone are termed partial agonists or partial antagonists because these compounds prevent the binding of the native hormone. Most EDCs have been shown to be full or partial agonists of endogenous hormones.

Currently the most studied chemicals are those that interact with the estrogen receptor and pathways. An important factor leading to this bias in the research is related to the promiscuity of the estrogen receptor (Van Der Kraak et

al. 1998). The estrogen receptor can bind with many structurally dissimilar compounds with varying degrees of affinity.

There have been several reports of endocrine-mediated abnormalities in specific wildlife populations of invertebrate, fish, avian, reptilian, and mammalian species (Ankley et al. 1997). In ecological studies, these effects were not recognized until the populations began to decline (Kavlock et al. 1996). However, the indication that a population is stable is not an assurance that EDCs are not affecting reproduction, development, and/or growth of individuals.

Recent legislation enacted by the US Congress has mandated the development, validation, and implementation of screening tests for identifying potential EDCs (Ankley et al. 1997). Although mounting evidence suggests EDCs are causing adverse ecological impacts, it is difficult to assess the extent to which this is occurring because few tests and models for identifying endocrine-specific effects in wildlife have been validated. Accepted test systems and appropriate endpoints are generally not available to assess the significance of the exposure and impacts of many chemicals in the environment (Ternes 1999).

There is a need for better procedures to characterize the potential of environmental agents to disrupt endocrine function in laboratory species, but there is also a need for a more comprehensive understanding of the normal physiological processes associated with reproduction and development in those wildlife species studied (Cooper and Kavlock 1997). There is insufficient science to predict the relative ecological risk associated with environmental contaminants that exert toxicity through alterations in endocrine systems (Ankley et al. 1997). It

is also necessary to have an understanding at the level of the individual of what is normal in terms of baseline endocrinology and physiology.

Relatively few laboratory studies have been conducted on the effects of EDCs on reptiles and no standardized test involving reptiles currently exists (Ankley et al. 1998). Some wild populations of reptiles including turtles and crocodilians have been affected by EDCs (Palmer and Palmer 1995; Guillette et al. 1996; Crain et al. 1998) including altered levels of circulating sex steroids, developmental abnormalities of reproductive structures, and inappropriate vitellogenesis in males. However, with the exception of a few species of turtles and crocodilians, this class of vertebrates has been overlooked in terms of ecotoxicological studies (Hopkins 2000), and reptiles may be at risk following exposure to environmental contaminants.

Ecologically, reptiles are excellent models for the study of contaminant induced endocrine disruption because of their extensive distribution, high trophic status, and site fidelity (Hopkins 2000), and biologically, these animals are unique because they employ two distinct modes of sexual differentiation which can be manipulated in the laboratory (Crain and Guillette 1998; Willingham and Crews 2000). In reptiles, gender is either determined by a gene or genes carried on sex chromosomes, as in the case of genotypic sex determination (GSD), or by the incubation temperature of the eggs during a critical period of embryonic development, as in the case of temperature-dependent sex determination (TSD) (Pieau et al. 1999; Raynaud and Pieau 1985; Bull 1989). Although all the mechanisms underlying this alternative developmental strategy are not

completely resolved (Lance 1997), it is now well established that temperature, whether directly or indirectly, influences the synthesis and activity of aromatase. This enzyme complex that converts androgens to estrogens is very important in ovary differentiation during the thermosensitive period (Pieau et al. 1999). Information available on sex determination in reptiles comes almost entirely from species with TSD, specifically the American alligator (*Alligator mississippiensis*) and one or two turtle species. Both the American alligator and the red-eared slider (*Trachemys scripta elegans*) have been proposed as reptile models for study of EDCs (Crain and Guillette 1998; Willingham and Crews 2000), and both of these species exhibit TSD. Comparisons among reptiles with GSD and TSD may help to better characterize the effects of EDCs on reproduction and development. However, a reptile model with GSD is lacking (Crain and Guillette 1998). Based on a review of sex determining mechanisms in squamate reptiles (Viets et al. 1994), it was suggested that western fence lizards have GSD, and confirmed for eastern fence lizards. We hypothesized that fence lizards (*Sceloporus* spp.) would be an ideal laboratory GSD reptile model for assessing endocrine-mediated toxicity.

Several characteristics of fence lizards make these animals well suited for both laboratory and field study. These lizards are distributed over a wide geographic range in a variety of habitats. Eastern (*Sceloporus undulatus*) and western (*Sceloporus occidentalis*) fence lizards inhabit most of the continental United States and are common throughout this range (Adolph and Porter 1996). Most populations of fence lizards have a relatively small body size, which is

beneficial for laboratory housing and handling. Females have a high fecundity rate in captivity, and both males and females reach sexual maturity in a matter of months (Ferguson and Talent 1993). Following hatching, juveniles can be readily sexed by the early development of secondary sexual characteristics.

Proper characterization of biochemical endpoints in fence lizards will contribute important baseline information in the characterization of a laboratory GSD reptile model of endocrine-mediated toxicity. Thus the overall goal of this thesis research was to determine several endocrinological responses in captive fence lizards.

The presence of vitellogenin in the plasma of male oviparous vertebrates is used as a very sensitive biochemical indicator of exposure to estrogenic environmental contaminants (Ankley et al. 1998, Palmer and Palmer 1995). Vitellogenin is a phosphorylated, glycosylated lipoprotein normally produced in the liver of female oviparous vertebrates in response to the endogenous estrogen, 17β-estradiol (E_2) (Ho 1987). Vitellogenin is released into the bloodstream, carried to the ovary where it is actively sequestered into the developing oocytes, and cleaved into yolk proteins. Vitellogenin is generally a female specific protein, since males have very low circulating levels of endogenous E_2 (Specker and Anderson 1994). However, males have been shown to produce this protein if exposed to exogenous estrogen or estrogenic compounds (Palmer and Palmer 1995; Jobling et al. 1996). This is an effective biochemical marker in males, since vitellogenin remains in the bloodstream until

metabolized and excreted, due to a lack of a natural repository (oocytes) for this protein.

As a high molecular weight protein (170-220 kDa), vitellogenin readily lends itself to analysis by immunological techniques. A highly sensitive method for determination of this protein is the enzyme-linked immunosorbent assay (ELISA) (Figure1) (Specker and Anderson 1994). Several advantages of this approach include high sensitivity, reliability, and avoidance of radioactivity (Bon et al. 1997). For accurate determinations, this immunoassay utilizes a specific antibody made against a purified protein. An indirect ELISA system using a second antibody is ideal because it increases the overall sensitivity by amplifying the signal (Specker and Anderson 1994).

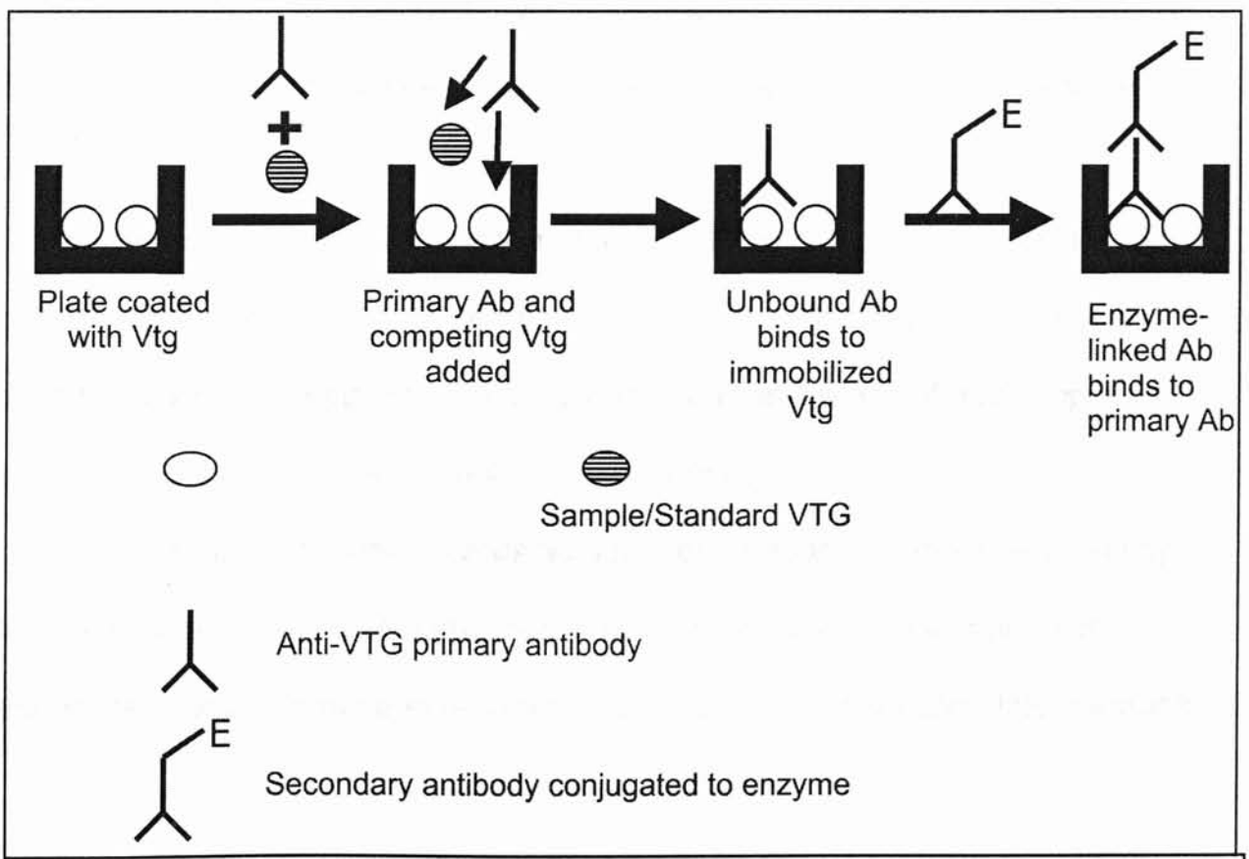

Figure 1 Schematic depicting an indirect Vtg antibody capture ELISA.

Research concerning vitellogenin induction has been primarily focused on amphibians, birds, and fish (Brown et al. 1997). The majority of research on vitellogenin purification and characterization has been conducted in fish, although a few studies have addressed the process in female reptiles such as *Anolis pulchellus* (Morales et al. 1991), the lizard *Lacerta vivipara* (Gavaud 1986), the tuatara (*Sphenodon punctatus)* (Brown et al. 1997), and the male red-eared slider (*Trachemys scripta*) (Palmer and Palmer 1995).

Another method that has been used to indirectly quantify the induction of vitellogenin is determination of alkaline-labile phosphate (ALP) (Parker and McKeown 1987, Kramer et al. 1998). Vitellogenin is a highly phosphorylated protein and release of these phosphate groups from the protein after several extraction steps provides an estimate of vitellogenin concentration in the plasma. Extraction steps are necessary to remove other proteins and free phosphate groups that may interfere with the assay (Figure 2).

However, the overall sensitivity and specificity of ALP as a measure of plasma Vtg is still unknown. A comparison of ALP and the Vtg ELISA should provide information regarding the advantages and limitations of each approach, and to our knowledge this has not yet been conducted.

To characterize fence lizards as a model for endocrine-mediated toxicity, normal circulating levels of hormones must be established. Relatively little endocrine research has been reported in *Sceloporus*, and no complete hormonal

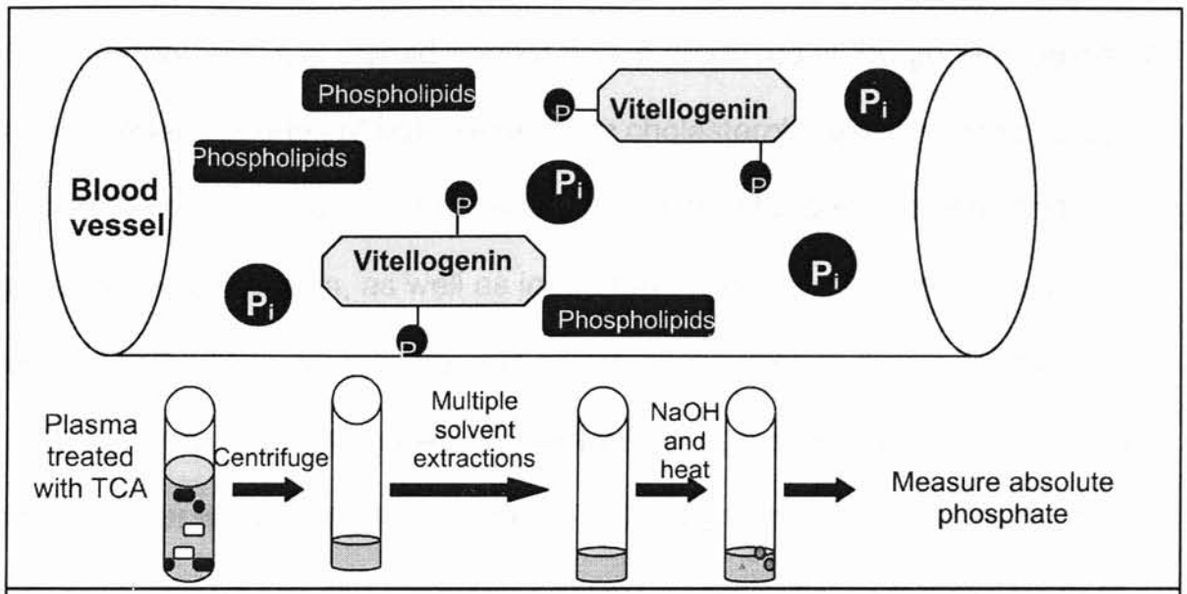

Figure 2 Schematic representing the bloodstream of a male oviparous vertebrate exposed to estrogenic compounds. For the purposes of the ALP assay, free phosphate groups (P_i) and other potentially interfering proteins must be extracted from the plasma sample shown in the lower diagram.

profiles exist. Kavlock et al. (1996) states that the collection of baseline endocrine data is one of the highest priorities for reproductive and developmental parameters associated with assessing the risks of EDC exposure in sentinel species. It is often difficult to make extrapolations of endocrine responses of fish and wildlife species from mammalian studies, and this can be avoided with more baseline hormone information in all vertebrate classes (Van Der Kraak et al. 1998).

The determination of sex steroid and thyroid hormone levels provides essential basic knowledge of endocrine homeostasis of fence lizards maintained under laboratory conditions. This information is important for future studies using *Sceloporus spp.* as bioindicators of adverse endocrine-mediated toxicities.

In vertebrates, sex steroid hormones are produced in the gonads (testis or ovary) and adrenal cortex of both sexes from cholesterol. Testosterone and E_2 are important in both sexes in various aspects of growth, development, and morphologic differentiation, as well as in the development of and regulation of sexual and reproductive behaviors and cycles (Randall et al. 1997). Although many target tissues respond directly to testosterone, this androgen must first be converted either to dihydrotestosterone (DHT) or E_2 to mediate its actions. In both genders, tissue specific expression of the enzymes 5α–reductase for DHT and aromatase for E_2 determines which hormone will be synthesized from testosterone.

Thyroid hormones act on the liver, kidney, heart, nervous system, and skeletal muscle, stimulating cellular respiration, oxygen consumption, and metabolic rate. The acceleration of metabolism stimulated by thyroid hormones leads to a rise in heat production, which is important in thermoregulation of many vertebrates (Griffin 2000). Thyroid hormones are also synergistically involved with growth hormone to promote critical protein synthesis during development (Norris 1997). Recently, it has been demonstrated that thyroid hormones are directly involved with reproductive function, rather than only in a permissive capacity. In rats, thyroid hormones regulate the duration of Sertoli cell proliferation and hence the capacity of the testis to produce sperm (Buzzard et al. 2000).

The two principal thyroid hormones are thyroxine (3,5,3',5'-tetraiodo-L-thyronine), or T_4, and 3,5,3' triiodo-L-thyronine, or T_3. The primary iodothyronine

secreted by the thyroid gland is thyroxine. Most of the circulating T_3 is produced by the monodeiodination, or removal of a single iodide, of T_4 in peripheral tissues. Under physiological conditions most of the activity of T_4 can be accounted for by the T_3 formed from it, and as a result T_4 can be considered a prohormone and T_3 as the physiologically active thyroid hormone (Griffin 2000).

The release of most hormones occurs in a regular and rhythmical manner. These cycles may be as short in duration as 24 hours or encompass the entire reproductive season (Bentley 1998). External influences on the cyclical release of hormones include food availability, light, and temperature (Dunlap 1995). Cycles usually correspond with changes in photoperiod and temperature both throughout the course of a day and season (Randall et al. 1997) As an important aspect of developing *Sceloporus* spp. as a model for endocrine-mediated toxicity, normal diel and seasonal variation in circulating sex steroid and thyroid hormones were determined. To date, the primary focus in the area of endocrine disruption research has been on toxicant interactions with sex steroid receptors to either induce or inhibit endogenous steroid hormone actions (Cooper and Kavlock 1997). The physiological effects of estrogenic chemicals on animals appear to be manifested primarily in the gonads, which may explain the concentration of research in this area (Jobling et al. 1996). However, thyroid hormones are present in many tissues and affect multiple processes, such as metabolism, growth, differentiation, and reproduction. Disruption of the direct and permissive actions of thyroid hormones could be detrimental to overall endocrine homeostasis. Cyclical variations have been reported for thyroid hormones on

both a diel and seasonal basis in many vertebrates (Norris 1997). Environmental factors, such as photoperiod and temperature, have been shown to influence thyroid hormone secretion and release.

Hypotheses

Objective 1: Determine the effects of graded doses of a model estrogenic compound, ethinylestradiol, on vitellogenin induction in adult male fence lizards (*Sceloporus* spp.).

H_o: There is not a dose response relationship between ethinylestradiol on vitellogenin induction in adult male fence lizards.

H_A: There is a dose response relationship between ethinylestradiol on vitellogenin induction in adult male fence lizards.

Objective 2: Develop, optimize, and validate a sensitive and specific enzyme-linked immunosorbent assay (ELISA) to quantify plasma vitellogenin levels in ethinylestradiol treated male fence lizards.

H_o: The ELISA technique will not be sensitive and specific to quantify plasma vitellogenin levels in ethinylestradiol treated male fence lizards.

H_A: The ELISA technique will be sensitive and specific to quantify plasma vitellogenin levels in ethinylestradiol treated male fence lizards.

Objective 3: Compare the reliability and sensitivity of the vitellogenin ELISA with an indirect vitellogenin assay that quantifies plasma alkaline-labile phosphate (ALP).

H_o: There will be no difference in the reliability and sensitivity of the ELISA and ALP assays.

H_A: There will be a difference in the reliability and sensitivity of the ELISA and ALP assays.

Objective 4: Characterize the normal diel and seasonal hormone profiles of male and female fence lizard plasma testosterone, 17β-estradiol (E_2), triiodothyronine (T_3), and thyroxine (T_4).

H_o: Laboratory acclimated fence lizards will not exhibit hormone cycles corresponding to seasonal and diel activities.

H_A: Laboratory acclimated fence lizards will exhibit hormone cycles corresponding to seasonal and diel activities.

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Chapter Two

Dose Response and Time Course Relationships for Vitellogenin Induction in Male Western Fence Lizards (*Sceloporus occidentalis*) Exposed to Ethinylestradiol

Abstract

The long term goal of this research is to develop and validate an *in vivo* reptile model for endocrine-mediated toxicity using fence lizards (*Sceloporus* spp.). One of the best defined estrogenic responses in oviparous vertebrates is induction of the yolk precursor protein, vitellogenin (Vtg). In this study, dose-response and time course relationships for Vtg induction were determined in male Western fence lizards (*S. occidentalis*) given intraperitoneal injections of 17α-ethinylestradiol (EE_2). Plasma Vtg was quantified directly with an antibody-capture ELISA and indirectly using plasma alkaline-labile phosphate (ALP) in order to compare these two methods. Both ELISA and ALP predicted similar ED_{50} values for plasma Vtg induction (0.167 mg/kg for ELISA and 0.095 mg/kg for ALP). A decrease in body weight at the highest dose (10 mg/kg) and an increase in hepatosomatic index at the four highest doses were observed. Serial dilutions of plasma from an EE_2-exposed male revealed a high correlation between plasma Vtg and ALP determinations in this species. In conclusion, our data show that plasma ALP may be a suitable alternative for measuring plasma Vtg compared to developing a Vtg ELISA in fence lizards exposed to estrogenic compounds.

Introduction

In recent years, it has become increasingly clear that chemicals in the environment from both natural and anthropogenic sources may interfere with endocrine systems (Ankley et al. 1998; Van Der Kraak et al. 1998; Crain et al. 1998). However, data are often insufficient to resolve the risk associated with endocrine-disrupting chemicals (EDCs) that exert toxicity through alterations in endocrine systems. Suitable test systems and appropriate endpoints are generally not available to assess the significance of the exposure and impacts of many chemicals in the environment (Ternes 1999). There is a recognized need to develop animal models for all classes of vertebrates (Kavlock et al. 1996).

Reptiles are considered to be suitable as contaminant biomonitors due to their occurrence in a variety of habitats, wide geographic distribution, longevity, and site fidelity (Crain and Guillette, 1998). However, with the exception of a few species, this class of vertebrates has been relatively understudied in this respect (Hopkins 2000). Reptiles are a unique class of vertebrates, with contrasting modes of gender determination and parity (Crain and Guillette 1998). Gender may either be determined chromosomally, as in the case of genotypic sex determination (GSD), or by the temperature at which eggs are incubated, as in the case of temperature-dependent sex determination (TSD) (Bull 1983, Raynaud and Pieau 1985). Comparisons of reptiles with GSD and those with TSD may be important in characterizing the effects of endocrine disrupting chemicals. Reptile models with TSD, including the red-eared slider (*Trachemys scripta elegans*) (Willingham and Crews 2000) and the American alligator

(*Alligator mississippiensis*) (Crain and Guillette 1998, Matter et al. 1998)) are currently being evaluated for use in assessment of endocrine mediated toxicity, however, to our knowledge, there are no existing studies involving a reptile model with GSD (Crain and Guillette 1998).

In comparison with other potential reptile models with GSD, fence lizards (*Sceloporus* spp.) represent ideal candidates due to their wide geographic distribution, small size, high fecundity, short time to sexual maturity, and early development of secondary sexual characteristics. Together, eastern (*S. undulatus*) and western (*S. occidentalis*) fence lizards inhabit most of the continental United States and are common throughout this range (Adolph and Porter 1996). Although the natural history characteristics of each population may vary under natural conditions, many populations, under laboratory conditions, mature in as few as 4-8 months after hatching (Talent unpublished data, Ferguson and Talent 1993), and mature females may lay as many as 4-6 clutches of between 8 and 15 eggs in a breeding season (Talent unpublished data). Individuals are also easily sexed after hatching by presence of much larger post-anal scales in the males.

Existing methods suitable for identifying chemicals with endocrine disrupting effects in reptiles are limited (Ankley et al. 1998). Ankley et al. (1998) also outlines the need for *in vivo* assays in one or more model species in each class of vertebrates. We hypothesize that fence lizards are an ideal GSD laboratory reptile model for assessing endocrine-mediated toxicity *in vivo*. Suitable biochemical markers for use in natural settings remain to be validated

under controlled laboratory conditions (Matter et al. 1998). This study aims to characterize biochemical markers of estrogenic exposure in captive fence lizards.

The induction of vitellogenin (Vtg) in male oviparous vertebrates represents a sensitive biochemical indicator of exposure to estrogenic compounds (Ankley et al. 1997, Palmer and Palmer 1995). Vtg is a high molecular weight phosphoglycolipoprotein produced in the liver of all oviparous vertebrates in response to circulating levels of the endogenous estrogen, 17β-estradiol (Ho 1987). In females, Vtg is then secreted into systemic circulation for transport to the ovary where it is actively incorporated into developing oocytes. Under normal physiological conditions Vtg is a female specific protein since males have very low circulating levels of endogenous estradiol (Specker and Anderson 1994). However, males do have the capacity to express this protein if exposed to exogenous estrogens (Jobling et al. 1996). Thus the presence of Vtg in the plasma of male oviparous vertebrates indicates a physiological response to exogenous estrogens (Palmer and Palmer 1995, Ankley et al. 1998). This is an ideal endpoint in males, since the majority of Vtg remains in systemic circulation rather than being sequestered into oocytes as would occur in females (Specker and Anderson 1994). There is a lack of knowledge regarding the elimination and kinetics of vitellogenin in most species, however in male rainbow trout given intra-arterial doses of ethinylestradiol, the elimination half-life of Vtg was been estimated at 42-49 h (Schultz et al. 2001).

Vtg is a highly phosphorylated protein (Ho et al. 1987), and a measure of the phosphate associated with this protein has been used as an indicator of Vtg

induction in the plasma (Wallace and Jared 1968, Tinsley 1985, Parker and McKeown 1987, Kramer et al. 1995). Alkaline-labile phosphate (ALP) is released from Vtg as *ortho*-phosphate, and following a series of extractions, is easily measured using a colorimetric assay. Plasma ALP has been used previously as an indirect measure of circulating Vtg in several species. In fish, increasing levels of plasma Vtg have been shown to reflect proportional increases in the plasma phosphoprotein (Nagler et al. 1987). Nagler et al. (1987) confirmed this relationship in rainbow trout by correlating plasma Vtg measured by radioimmunoassay with plasma ALP in rainbow trout. However, the relationship between plasma vitellogenin and ALP has not been characterized in reptiles.

As an initial step towards evaluating fence lizards as a laboratory reptile model for endocrine-mediated toxicity, the present study determined dose-response and time course of relationships of Vtg induction in males exposed to ethinylestradiol (EE_2). A main objective of this study was to compare two methods of measuring this response, a direct measure of Vtg by enzyme-linked immunosorbent assay (ELISA) and an indirect measure of the alkaline-labile phosphate associated with this protein.

Methods and Materials

Animals

Male Western fence lizards (*Sceloporus occidentalis*) from Reno, Nevada, and San Joaquin Valley, California, were acclimated to laboratory conditions for no

less than one year prior to use. Animals were housed in glass aquaria with a constant photoperiod of 14L: 10D. Food and water were provided *ad libitum*.

Chemicals

17α-ethinylestradiol (EE_2, purity 98%) and all other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified.

Preliminary dose-response and time course experiments

In preliminary experiments, we examined the effects of single and multiple intraperitoneal (i.p.) injections of EE_2 in male fence lizards (n=4 lizards), and analyzed plasma Vtg both directly by ELISA and indirectly using plasma ALP. EE_2 was dissolved in acetone and added to a carrier solution of corn oil. The acetone was then evaporated under a stream of nitrogen. All experimental groups, including corn oil vehicle controls, received an i.p. injection of 5 μl stock solution/g body weight. Two injection regimens were compared; a single bolus injection, and five additive doses administered every second day. Doses included corn oil vehicle controls, 0.001, 0.01, 0.1, 1, and 10 mg EE_2/kg body weight.

Blood samples were repeatedly collected on days 3, 6, 9, 15, 21, and 27 in the single dose groups and on days 9, 12, 15, 21, and 27 in the multiple injection groups from the same lizards. Blood (~100 μl) was collected from the postorbital sinus using a heparinized microcapillary tube and placed in a tube containing 5 μl of 5.12 mg/ml aprotinin, a protease inhibitor and 5 μl of 1.36 mg/ml heparin in normal saline to inhibit proteolysis and clotting, respectively. Collections could take as long as 1-2 hours, and blood samples were kept on ice

during collection to prevent thermal breakdown of proteins. Plasma was collected following centrifugation at 5000 rpm for 10 minutes at 4° C. Samples were stored at -80° C until analysis. Animals were killed on day 27, body weights were recorded, and livers were excised and weighed.

Definitive dose-response experiment

Single i.p. injections of a wider range of EE_2 doses were administered to male lizards in order to describe the dose-response curve for Vtg induction and generate an ED_{50} value. Stock solutions of EE_2 were prepared as described above using propylene glycol as a carrier. Based on data from the preliminary experiments, the following experimental groups (n=8 lizards per dose) were chosen: sham control, propylene glycol vehicle control, 0.0003, 0.003, 0.001, 0.01, 0.1, 1, and 10 mg/kg. A single i.p. injection (5 μl/g except sham) was administered and lizards were killed after blood was collected on day 15. Body weights were recorded before treatment on day 0 and before blood collection on day 15. Corrected hepatosomatic indices (HSIs) were calculated using the formula (liver weight/(body weight-liver weight)) x 100.

Comparison of EE_2 and 17β-estradiol (E_2)

Stock solutions of EE_2 and E_2 were prepared as described above using propylene glycol as a carrier. Compounds were administered at a dose of 0.167 mg/kg to compare the differential induction of Vtg. This was the ED_{50} dose predicted from the dose response data generated from the ELISA. Animals (n=8

per group) received an intraperitoneal dose at an injection volume of 5μl/g body weight. Blood was collected at day 15 and plasma was stored at -80°C until time of analysis.

SDS-PAGE and Western blotting

Using purified vitellogenin prepared in our laboratory, polyclonal antisera against vitellogenin were produced in rabbits (Weber, Talent, and Janz, unpublished data). Plasma samples, diluted 1:40, were separated under denaturing conditions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Hoefer Model SE600-15-1.5) consisting of 10% stacking gels and 5-10% gradient polyacrylamide separating gels. The proteins were electroblotted from the gel onto a 0.45 μm nitrocellulose membrane for 14-16 hours at 50 constant volts using a Hoefer transfer unit (TE series). The nitrocellulose membrane was allowed to air dry to fix the proteins prior to blocking, or saturation of excess binding sites, with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% blotting grade nonfat dry milk (BioRad). After blocking, the membrane was incubated for 1 hr at room temperature with anti-*Sceloporus* vitellogenin antibody at a concentration of 1:5000 in TBS-T containing 1% dry milk. The membrane was washed 3x (5 min, 10 min, and 15 min washes) in TBS-T. Enzyme-conjugated goat anti-rabbit secondary antibody was diluted 1:2000 in TBS-T containing 1% dry milk, and incubated with the membrane for 1 hr at room temperature. Proteins were visualized using enhanced chemiluminescence (SuperSignal, Pierce Chemical Co.).

Vtg ELISA

Vtg was isolated from plasma collected from male fence lizards injected i.p. with EE_2 by precipitation and purified using DEAE Sephacel protein chromatography (Wiley et al. 1979). Highly specific polyclonal antisera against purified fence lizard Vtg were produced in rabbits (L.P. Weber et al. unpublished data). ELISA microtiter plates (Greiner America, Lake Mary, FL, USA) were coated with purified Vtg (200 ng/well) using sodium carbonate buffer (0.1 M $NaCO_3$, pH 9.6) overnight at 4°C. Anti-Vtg antibody (1:200) was preincubated with standards (2.5-100,000 ng Vtg/ml) or diluted samples (1:10 for control and at least 1:160 for treated) in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T) on a rotating platform for 14-16 h at 4°C. Plates were washed 3x with PBS-T between each ELISA step. Coated plates were blocked with 1% bovine serum albumin (BSA) in PBS-T for 1 h at room temperature. Preincubated standards were added to coated, blocked plates in duplicate and samples were added in triplicate, then incubated for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:1000 in PBS-T) was added to plates, incubated at room temperature for 1 h, and detected with *p*-nitrophenyl phosphate solution (0.91 mg/ml in 10% (v/v) diethanolamine buffer, pH 9.6). Color was allowed to develop in the dark at room temperature for 40 minutes (B_0=0.9-1.0) and absorbance values measured at 405 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Non-specific binding was determined in uncoated wells without primary antibody, and

maximum binding of anti-Vtg (B_o or zero standard) was determined in coated wells in every assay.

Intra-assay coefficient of variation for the ELISA was 4.9% (n=6 determinations). Inter-assay variability was 13.3% (n=12). The sensitivity of the Vtg ELISA was 2.5 ng/ml, with a detection limit of 0.25 ng/ml. Parallelism was observed between the standard curve and serially diluted plasma samples. Internal controls were run in duplicate on every plate and assays were repeated if values deviated >10% from previously determined values.

Plasma alkaline-labile phosphate

Using the same plasma samples assayed in the ELISA, extractions for alkaline-labile phosphate were performed as described by Wallace and Jared (1968) with minor modifications. Briefly, 5 μl of plasma and 30 μl of 1% BSA were added to 1.5 ml of 10% (w/v) trichloroacetic acid (TCA), then allowed to precipitate overnight at 4°C. Pellets were repeatedly washed and centrifuged at 7000 rpm for 10 min with ice-cold 5% (w/v) TCA (30 min at 50°C), 100% EtOH (80°C for 1 min), chloroform:ether:ethanol (1:2:2), acetone, and ether. Following the ether wash, pellets were allowed to dry, reconstituted in 250 μl of 2N NaOH and incubated for 15 min at 100°C. The samples were then neutralized by adding 250 μl of 2N HCl. Extracts were stored at -20°C. Extracts were diluted (1:10 for controls and at least 1:100 for treated) using a 1:1 mixture of 2N NaOH and 2N HCl, assayed in duplicate, and inorganic phosphate was determined by colorimetric assay using a commercially available kit (Sigma Cat. # 670). To

correct for BSA added prior to extraction, 30 μl of 1% BSA was extracted, determined for every assay, and all samples were corrected based on this determination.

Intra-assay coefficient of variation for plasma ALP was 5.6% (n=6). Inter-assay variability was 10.7% (n=12). The sensitivity of the plasma ALP assay was 13.0 μM, with a detection limit of 0.006 μM. Parallelism was observed between the standard curve and serially diluted plasma samples.

Data Analysis

Time course data from the preliminary experiments were analyzed using two-way repeated measures ANOVA followed by Fisher's protected LSD a posteriori tests as appropriate. All other data were analyzed using Students t-test or one-way ANOVA, followed by Dunnett's a posteriori tests as appropriate. ED50 values were calculated using nonlinear, 4-parameter logistic regression. A value of $p<0.05$ was considered statistically significant. Data are expressed as mean ± SEM. Sample size (n) indicates the number of lizards used.

Results

The specificity of polyclonal antisera against Vtg was verified by Western blotting following SDS-PAGE of untreated male plasma, EE_2-treated male plasma, yolk from Western fence lizard eggs, and purified Western fence lizard Vtg (Figure 3). Untreated male plasma (lane 1) showed no evidence of Vtg either in the intact or fragmented forms. Males treated with EE_2 (lane 2), however, expressed proteins that corresponded to the molecular weights of both intact (192 kDa) and

fragmented Vtg (120 kDa). Egg yolk (lane 3) had a large band on both the Coomassie stained gel and the Western blot. Purified Vtg (lane 4) was used for the Vtg ELISA as a standard, and appears to be relatively uncontaminated with other plasma constituents such as albumin, none of which appear to be crossreacting with the anti-VTG antibody (Fig 3).

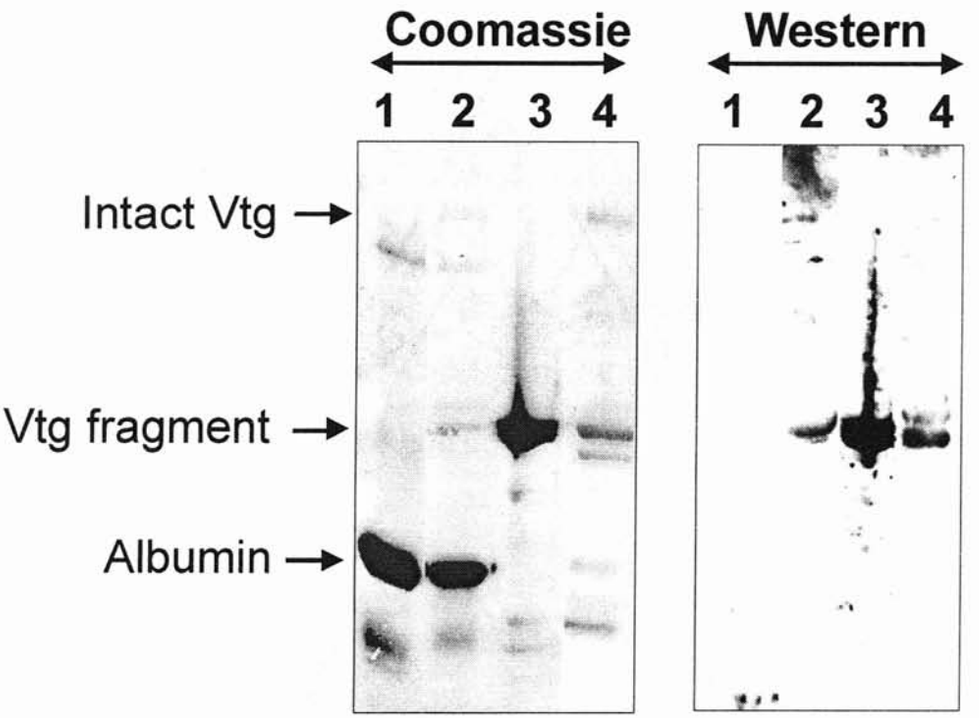

Figure 3 Reactivity of *Sceloporus* vitellogenin antiserum with untreated male (lane 1), EE_2-treated male (lane 2), egg yolk (lane 3), and the purified vitellogenin preparation (lane 4). Proteins were separated using SDS-PAGE (5-10% gradient) followed by either Coomassie staining for all proteins (left) or Western blotting specific for Sceloporus Vtg and related proteins (right). Proteins on the Western blot were visualized using enhanced chemiluminescence.

In preliminary experiments, plasma Vtg concentrations increased dose-dependently in male lizards (n=4) for most groups receiving single i.p. doses of EE_2 (Figure 4A). For all doses, plasma Vtg increased on day 3 of blood

collection, and remained elevated over the 27-day course of the experiment. Experimental groups (n=4 per group) receiving multiple i.p. doses of EE_2 displayed high levels of plasma Vtg on day 9 of blood collection (Figure 4B). Unlike the single injection groups, the levels of plasma Vtg consistently decreased over the 27-day course

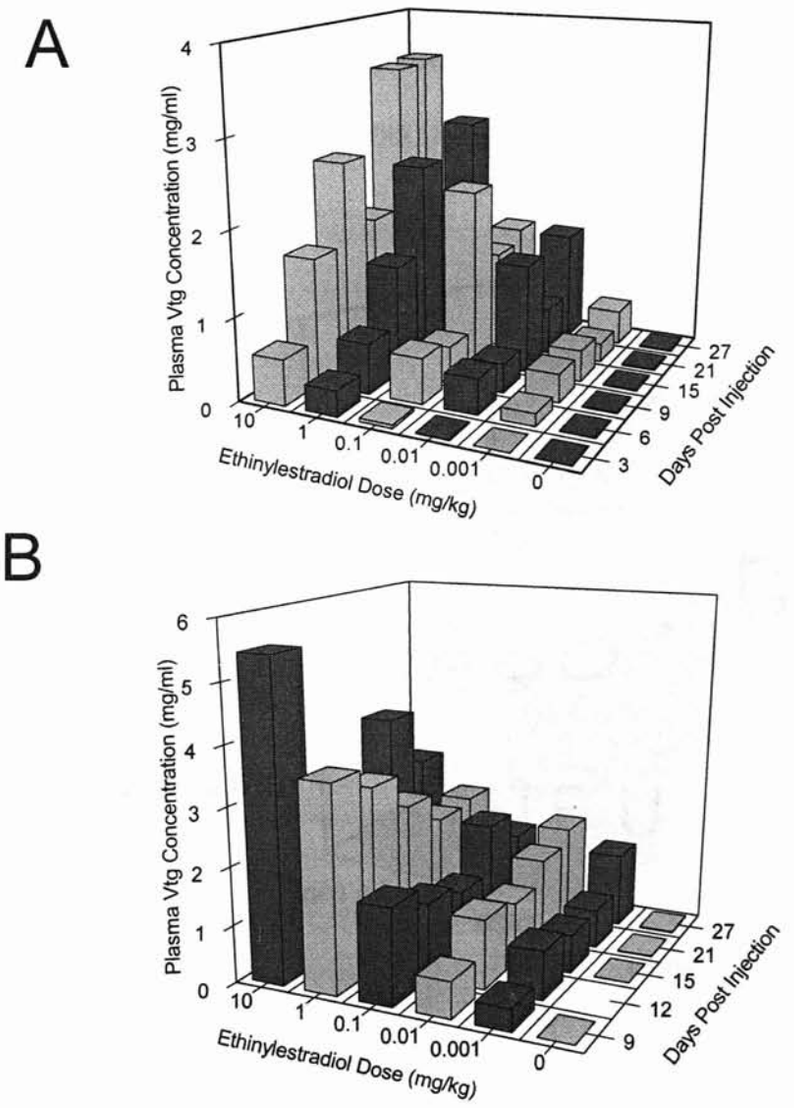

Figure 4 Time course and preliminary dose-response relationships of plasma Vtg in male Western fence lizards treated with EE_2, either as a single i.p. injection (A) or five additive i.p. doses administered every second day (B). Blood was collected from the same animals on the days labeled on the axis.

of the experiment at the two highest doses.

Before initiation of the definitive dose-response experiment, we chose to investigate the effect of injection vehicle on Vtg induction. Corn oil vehicle caused a significantly higher induction of Vtg in comparison to propylene glycol (Fig 5, p=0.03, repeated measures ANOVA across all time points) with peak Vtg levels observed 21 days after a single injection. Plasma Vtg levels in the sham injection group were not significantly different from either corn oil (p=0.07) or propylene glycol (p=0.63) vehicle controls (Figure 5).

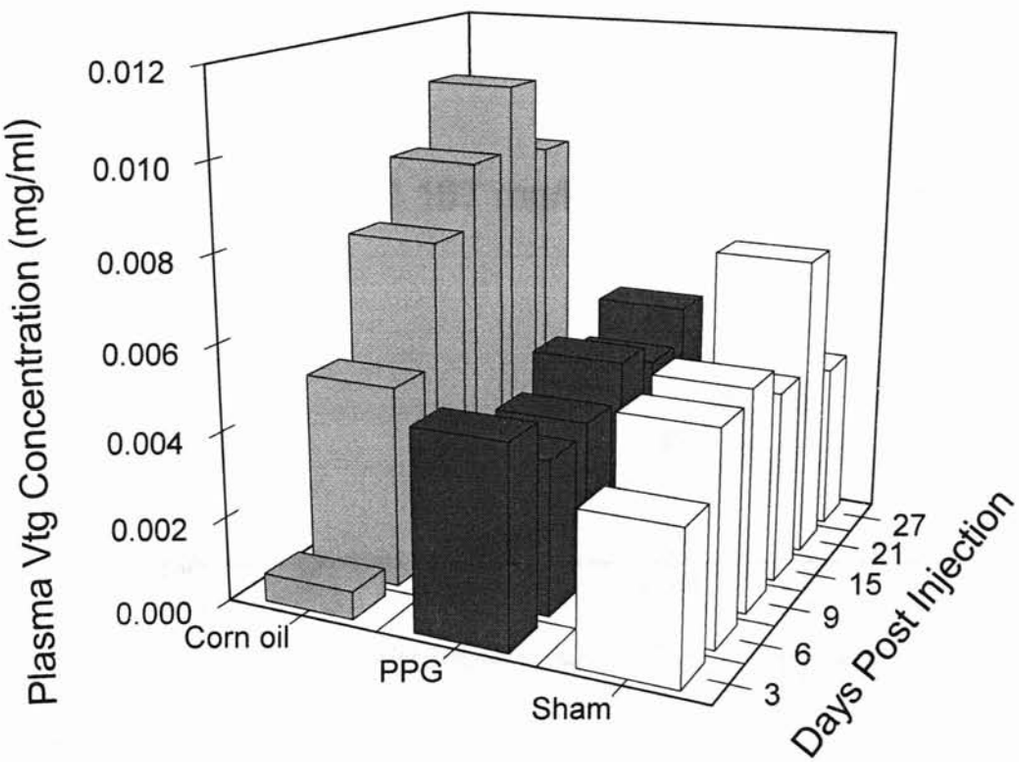

Figure 5 Effect of corn oil and propylene glycol (PPG) vehicles on plasma Vtg induction in male Western fence lizards (n=4). Solutions were administered i.p. at a constant volume of 5 μL/g, and sham controls received a needle stick into the peritoneal cavity. Plasma was collected 15 d following injection and assayed by ELISA. Data were analyzed using a repeated measures two-way ANOVA.

Based on our preliminary time course experiment, day 15 following a single i.p. injection was chosen for the definitive dose-response experiment because it appeared to be a more consistent point of maximum Vtg induction. In the definitive experiment, plasma Vtg followed a sigmoidal dose-response relationship and from this curve an ED_{50} value of 0.167 mg/kg (95% CI: 0.040-0.70 mg/kg) was calculated (Fig 6). Significant induction of Vtg was seen at the lowest dose administered, 0.0003 mg/kg.

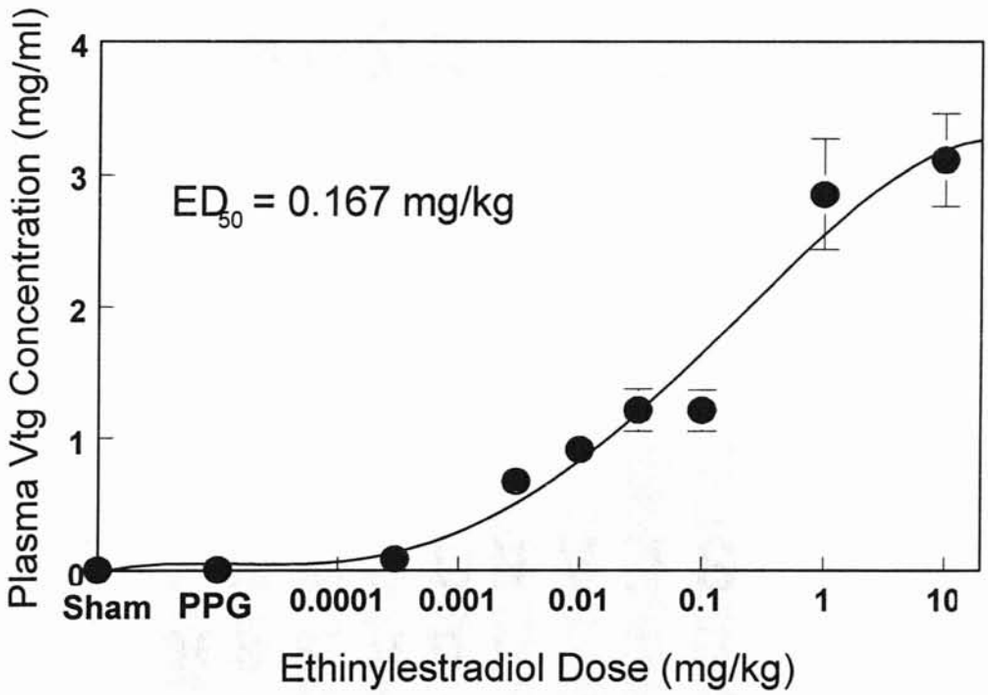

Figure 6 Plasma Vtg levels in untreated (sham and propylene glycol vehicle controls) and EE_2 treated male fence lizards determined using ELISA. Plasma samples were collected fifteen days after a single i.p. injection. Values are mean $\pm$ SEM (n=7-8). From this curve, an ED_{50} value of 0.167 mg/kg was calculated for Vtg induction.

Gross effects were also seen at the organ and organism levels. EE_2 caused a significant reduction of body weight at the highest dose (Figure 7A; $p<0.05$ Dunnett's a posteriori test after one-way ANOVA). Liver size, measured as HSI, was significantly elevated in the four highest doses ($p<0.05$, Figure 7B). At the two highest doses, the HSIs were almost two-fold greater than in the control lizards ($p<0.01$).

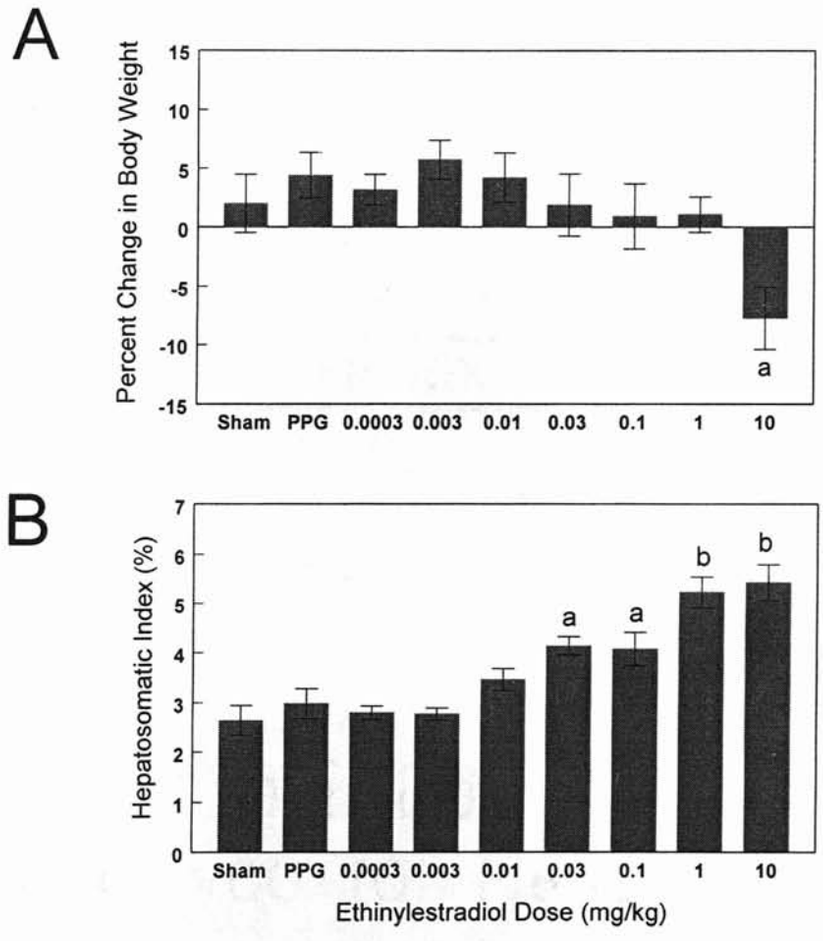

Figure 7 Effects of EE_2 treatment on body weights and corrected hepatosomatic index. Animals received a single i.p. injection and were killed on day 15. Values are mean ± SEM (n=7-8). The letters **a** and **b** denote $p<0.05$ and $p<0.01$, respectively.

Plasma ALP was also determined in the preliminary dose-response and time course experiments using the same samples analyzed for plasma Vtg. In the single injected groups, plasma ALP followed a similar trend as plasma Vtg, increasing with dose and time (Figure 8A). In the multiple injected treatment groups, plasma ALP was elevated at the time of first blood collection (Figure 8B). However, unlike the plasma Vtg measurements in the same samples, ALP levels remained elevated over the duration of the 27-day time course at all doses tested.

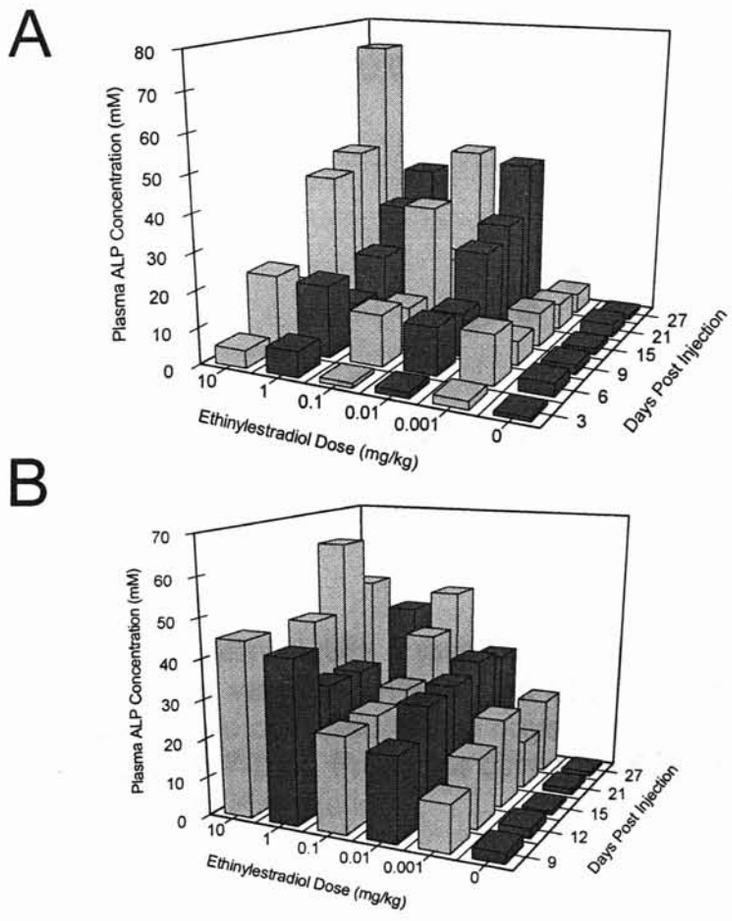

Figure 8 Time course and preliminary dose-response relationships of plasma ALP in male Western fence lizards treated with EE_2, either as a single i.p. injection (A) or five additive i.p. doses administered every second day (B). Blood was collected from the same animals on the days labeled on the axis.

In the definitive dose-response experiment, plasma ALP followed a similar trend as plasma Vtg, with an ED_{50} of 0.095 mg/kg (95 % CI: 0.018-0.68 mg/kg) (Figure 9). Significant increases in plasma ALP were seen in the lowest dose administered, 0.0003 mg/kg.

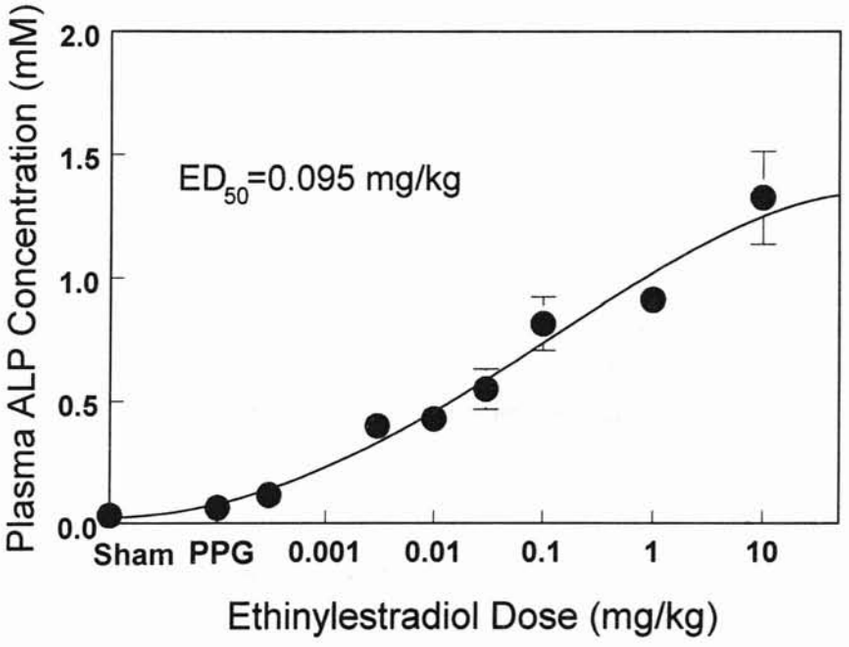

Figure 9 Plasma alkaline-labile phosphate concentrations in untreated (sham and propylene glycol vehicle controls) and EE_2 treated male fence lizards. Plasma samples were collected fifteen days after a single i.p. injection. Values are mean ± SEM (n=7-8).). From this curve, an ED_{50} value of 0.095 mg/kg was calculated for ALP induction.

To further compare the two methods, plasma Vtg and ALP were measured in six sample dilutions to generate a correlation relating these two endpoints

(Figure 10). The line revealed a strong correlation between these two techniques (r=0.99). The equation that describes this line is:

[Vtg] = 0.23 x [ALP] + 0.0022

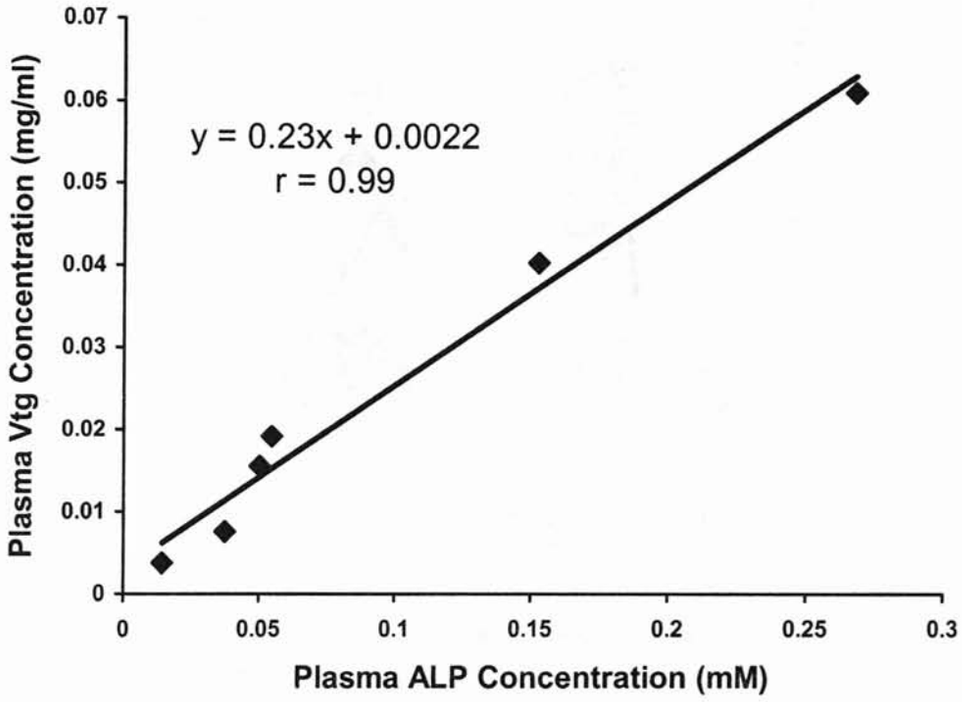

Figure 10 Relationship between plasma Vtg concentration and plasma ALP concentration measured in the same dilutions of an EE_2-induced male plasma sample. Vtg was measured using ELISA. The line generated to fit these data has an equation of y=0.23x + 0.0022, and a correlation coefficient (r) of 0.99.

Comparison of the potency of EE_2 with E_2 in this species was determined by administering both compounds at the EE_2 ED_{50} dose of 0.167 mg/kg. At this dose, EE_2 significantly induced Vtg (p=0.0011, Welch's t-test) and plasma ALP (p=0.011) to a greater extent than E_2 (Figure 11).

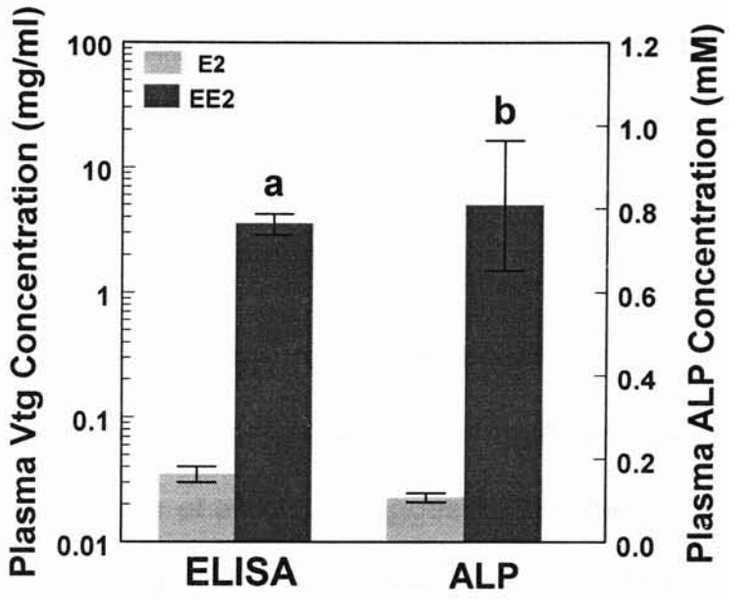

Figure 11 Relative potencies of ethinylestradiol (EE_2) and 17β-estradiol (E_2) measured as plasma Vtg and ALP. Adult male Western fence lizards from the San Joaquin Valley, CA, were given a single intraperitoneal injection of 0.167 mg/kg E_2 or EE_2 and blood was collected on day 15. Values are mean ± SEM (n=8).

Discussion

As an initial step in developing fence lizards as a laboratory reptile model for endocrine mediated toxicity, we characterized dose-response and time course relationships of both plasma Vtg and ALP induction in adult male fence lizards exposed to EE_2. Very little research has been conducted with fence lizards in captivity, and it is important to establish baseline physiological responses in model species (Kavlock et al. 1996, Matter et al. 1998). Two responses were measured following treatments with EE_2: plasma Vtg using an antibody-capture ELISA and plasma ALP using a modified procedure of a commercially available

kit. The antibody-capture ELISA targets the protein using highly specific antibodies for *Sceloporus* Vtg and thus is a direct quantitative measure of Vtg. The plasma ALP method does not measure Vtg directly, but instead quantifies protein-associated phosphate groups. Previous research has shown that when present in the plasma, Vtg represents the majority of the phosphoproteins (Wallace and Jared 1968).

Few studies have examined the relative sensitivity and reliability of ALP as a measure of Vtg expression in reptiles or other oviparous vertebrates. A previous study in fish (Nagler et al. 1987) suggested that the sensitivity of ALP did not extend to low levels of Vtg seen in non-reproductive females and juvenile animals. However the detection limit for ALP in the present study was 0.006 μM (3.5 μg/ml), and ALP was significantly elevated at the lowest dose administered (0.0003 mg/kg). This indicates that ALP might be useful as a biochemical marker at low levels of estrogenic exposure in male oviparous vertebrates. To further compare these techniques, serial dilutions of plasma collected from an EE_2-exposed male lizard were analyzed using both ELISA and ALP. A high concordance between plasma Vtg and ALP determinations was observed that extended to low levels of Vtg. In addition, our dose response data further support the use of ALP as a useful substitute for Vtg ELISA, as both assays predicted similar ED_{50} values, 0.167 mg/kg for ELISA and 0.095 mg/kg for plasma ALP. Thus, we believe the relationship described between ALP and ELISA may be useful in future studies as a relatively rapid and technically less demanding

alternative to direct Vtg measurement with ELISA, both at high and moderately low levels of Vtg.

Surprisingly, few studies have determined dose-response relationships between treatment with EE_2 or other potent estrogens and Vtg induction in oviparous vertebrates. To our knowledge, there are no studies that have reported an ED_{50} for Vtg induction in reptiles. In a previous study of male Japanese quail (*Coturnix coturnix japonica*), EE_2 did induce Vtg, measured as protein bound phosphorus (PBP) (Robinson and Verrinder Gibbins 1984), similar to the measure of ALP utilized in our study. Although PBP induction in quail did not appear to reach maximum response, it appears that this avian species has a similar sensitivity to EE_2 as fence lizards. In fish, the relationship between EE_2 exposure and Vtg induction has been established, however few dose-response studies have been conducted. In a recent study in rainbow trout (*Oncorhynchus mykiss*) (Schultz et al. 2001), animals were given i.v. EE_2 injections and exhibited a similar dose response relationship for Vtg induction when compared to fence lizards given i.p. injection. These studies suggest that fence lizards exhibit a similar sensitivity to EE_2 exposure compared to these commonly used avian and piscine laboratory species.

Differences in the magnitude of response for plasma Vtg and ALP were observed in the present study. The direct measure of Vtg using ELISA revealed a 400-fold increase in the plasma concentration of this protein between controls and lizards exposed to high doses of EE_2. In contrast, plasma ALP measurements were 40-fold different between plasma samples collected from

control and high dose EE_2-treated males. The difference in magnitude could be due to several factors. First, other phosphoproteins in the blood of untreated animals may contribute to higher baseline levels of ALP but have no interference with the Vtg ELISA. Second, our polyclonal antisera may recognize multiple Vtg epitopes which would lead to an amplification of Vtg measured with ELISA. Third, ELISA techniques such as the one used in this study have additional amplification because multiple secondary antibodies bind to each primary antibody and enzyme coupling allows each secondary antibody to produce large amounts of chromagen. In comparison, although Vtg has multiple phosphates per molecule, only one chromagenic phosphomolybdate complex is formed per phosphate in the ALP assay. Despite this apparent difference in magnitude of response, ALP was comparable to Vtg ELISA in other assay parameters such as intra- and inter-assay variability.

In our preliminary experiments, there were similarities in the time courses for induction of plasma Vtg and ALP. However in the multiple injection groups, there was an inconsistency in the relationship between the plasma Vtg and ALP concentrations. When measured in the same samples, plasma Vtg showed a decreasing trend over the 27-day period at the highest EE_2 doses, whereas ALP remained elevated. One possible explanation for this observation might be the route of degradation and excretion of this protein. If Vtg was cleaved into fragments that were not recognizable by the antibody used in the ELISA but were still large enough to be extracted with plasma proteins in the ALP method, this would cause a negative result in the ELISA but a positive result when measuring

the ALP. Further experiments would be necessary to substantiate this uncertainty.

In the experiment that examined effects of injection vehicle, corn oil induced a significantly higher amount of plasma Vtg compared to the propylene glycol vehicle. The presence of phytoestrogens in the corn oil may have been responsible for the observed estrogenicity (MacLatchy and Van Der Kraak 1995; Casanova et al. 1999). Although this result was statistically significant, the level of induction may not be biologically significant, since it was an order of magnitude less than the induction caused by the lowest administered dose of EE_2 determined from the dose-response curve. Many previous studies using injections have utilized vehicles of a botanical origin, but based on our data, we believe that a relatively inert vehicle such as propylene glycol may be a safer choice to avoid any potential additive or synergistic effects on Vtg induction.

It has been shown in several species of oviparous vertebrates that induction of Vtg can be related to adverse physiological effects. In male turtles, Vtg induction resulted in the downregulation of other plasma proteins (Selcer and Palmer 1995) . Studies in male fish have reported atrophy of the testis, impaired spermatogenesis, and reduced gonadosomatic index following treatment with estrogens (Jobling et al. 1996; Panter et al. 1998; Folmar et al. 2001). At the highest dose of 10 mg/kg, we saw a significant decrease in body weight during the 15-day experimental period. It was qualitatively noted in both our preliminary and definitive dose-response experiments that lizards exposed to higher doses of EE_2 reduced their food intake, in addition to regurgitation of food. This

observation is consistent with a study by Heck et al. (1997) that reported decreased feeding in turtles treated with estradiol extending for a period of 50 days.

At the organ level, the four highest doses of EE_2 caused a significant increase in hepatosomatic index (HSI). This stimulatory effect on hepatocyte hyperplasia and/or hypertrophy in response to estrogenic compounds has also been observed in fish (Folmar et al. 2001). Other studies have examined cellular effects resulting from this stimulatory effect on the liver. Histopathological changes were associated with hypertrophy in the liver and kidneys of rainbow trout (Herman and Kincaid 1988) and summer flounder (Folmar et al. 2001). Although it is not clear if the presence of Vtg in plasma causes these deleterious responses or whether estrogenic chemicals directly cause pathology in the liver or kidney, the measurement of Vtg in males can serve as an early indication of whether animals are being physiologically influenced by the estrogenic properties of environmental contaminants.

We conducted an additional experiment to compare the relative potency of EE_2 and 17β-estradiol (E_2). EE_2 caused a much greater induction of plasma Vtg (100-fold higher) and ALP (10-fold higher) than E_2 15 days after a single i.p. injection. This difference may be a result of differences in toxicokinetics due to the longer half-life of EE_2 and faster clearance of E_2. This is consistent with other studies in which EE_2 was found to be ten times more potent than E_2 as a Vtg inducer, presumably because EE_2 is synthesized to withstand biotransformation (Allner et al. 1999). Furthermore, it has also been suggested that xenoestrogens

may not be subject to the same homeostatic mechanisms (e.g. steroid binding proteins) as E_2 based on structural differences (Folmar et al. 2000), making these compounds more bioavailable.

In summary, this study contributes baseline physiological and toxicological information about this species of lizard, and provides a foundation for future experiments involving other estrogenic compounds. The work conducted in this study is part of a ongoing project evaluating several other endpoints in fence lizards in relation to toxicant exposure and we believe that further characterization and development of this reptile laboratory model will be useful in future assessments on the potential impacts of endocrine-modulating environmental contaminants.

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Chapter 3

Seasonal and Diel Sex Steroid and Thyroid Hormone Profiles in Captive Western Fence Lizards (*Sceloporus occidentalis*)

Abstract

Seasonal and diel cycles in serum concentrations of sex steroid (testosterone and 17β-estradiol (E_2)) and thyroid (triiodothyronine (T_3) and thyroxine (T_4)) hormones were determined in captive Western fence lizards (*Sceloporus occidentalis*). Seasonal samples were collected from male and female breeding pairs weekly for a 4-month period following their emergence from artificial hibernation. Diel samples were collected from breeding males at nine time points over a 24-hour period. Seasonal levels of E_2 corresponded with the expected vitellogenic and ovulatory cycles in females, and surprisingly, E_2 in males followed a similar pattern, indicating a possible role in breeding behavior. Serum testosterone was high in male lizards for the first 6 weeks following emergence from artificial hibernation, possibly related to the onset of active spermatogenesis. Thyroid hormones revealed little cyclical activity throughout the breeding cycle, with the exception of small increases of T_3 at weeks 8 and 16, possibly inferring an active role of this hormone with ovulation in females. Diel profiles in male fence lizards exhibited no clear cycles, likely due to high variability in these wild-caught animals related to prolonged stress or parasite loads. Overall, these baseline data are important in developing this animal as a laboratory reptile model for assessment of endocrine mediated toxicity.

Introduction

Circulating levels of sex steroid and thyroid hormones represent an integrated response dependent on multiple factors acting on the brain-hypothalamus-pituitary-gonadal and brain-hypothalamus-pituitary-thyroidal axes, respectively. Most hormones exhibit cyclical fluctuations in serum concentrations, both on a seasonal basis corresponding to breeding cycles (Bentley 1998), and also over a diel period as temperature and light fluctuate (Licht et al. 1985; Cree et al. 1990). Many reptiles display daily variations in activity and body temperature, both of which may be related to circulating levels of sex steroids and thyroid hormones. Sex steroid hormones correspond with breeding and reproduction in *Sceloporus* spp. (Callard et al. 1972) like most other oviparous vertebrates. In female blue spiny lizards (*Sceloporus cyanogenys*) and the freshwater turtle (*Chrysemas picta*) (Callard et al. 1991), oviduct weight changes parallel those of the ovary during the postovulatory period, reflecting changes in ovarian hormone secretion, specifically 17β-estradiol. Testosterone is a gonadal steroid that is necessary in males for both the generation of sperm prior to breeding and the activation of courtship and territorial behaviors in lizards during breeding (McKinney and Marion 1985; Moore and Marler 1987; Dunlap and Schall 1995; Woodley and Moore 1999). Thyroid hormones are directly involved in many physiological processes in fence lizards, including thermoregulation and metabolism, and also indirectly in a permissive capacity in other endocrine systems (Dunlap 1995). For example, altered thyroid hormone secretion in male Western fence lizards in response to a period of food deprivation was related to

an inability to behaviorally thermoregulate in an effort to reduce metabolic rate (Dunlap 1995). However, it is often difficult to document consistent changes in thyroid hormones in oviparous vertebrates because of the influence of several factors including temperature, feeding, and photoperiod (Cyr et al. 1988). Most of our knowledge of physiological actions of sex steroid and thyroid hormones in reptiles is based on the response to exogenous administration rather than on the measurement of the changes in circulating levels of hormones correlated with specific events (Moore et al. 1995).

Descriptive studies of fence lizard hormone levels under laboratory conditions are limited, and few have been reported under natural conditions with respect to season and diel fluctuations. Androgens and their association with the reproductive cycle have been reported in eastern fence lizards (*Sceloporus undulatus*) (McKinney and Marion 1985). However, the majority of hormone research in *Sceloporus* spp. is often related to behavioral and stress-induced alterations in normal circulating levels (Moore 1987, Sinervo and Dunlap 1995, Dunlap and Wingfield 1995, Smith and John-Alder 1999). Although these data contribute to the overall endocrinological knowledge in these species, no complete hormone profiles exist. For the purpose of establishing fence lizards as a laboratory reptile model for endocrine-mediated toxicity, baseline levels of circulating hormones in lizards under laboratory conditions must be established.

Several classes of environmental chemicals have been reported to alter serum concentrations of sex steroid and thyroid hormones in wild reptiles (Guillette et al. 1995,1996; Crain et al. 1998). The mechanisms by which

toxicants disrupt circulating hormone levels are complex and may involve direct effects on metabolic pathways of hormone biosynthesis, secretion, or transport, or indirect effects on feedback loops involved in endocrine homeostasis (Van Der Kraak et al. 1998). An important recommendation made in recent summaries examining endocrine disrupting compounds (EDCs) (Kavlock et al. 1996; Ankley et al. 1998) is that in order to understand endocrine-mediated toxicities in wildlife, more basic knowledge is required regarding normal endocrine processes in such species. Kavlock et al. (1996) states that the collection of baseline endocrine data is one of the highest priorities for reproductive and developmental parameters associated with assessing the risks of EDC exposure in sentinel species. Relatively little endocrine research has been done with captive *Sceloporus*, and no complete hormonal profiles exist. It is often difficult to make extrapolations of endocrine responses to fish and wildlife species from mammalian studies, and more baseline hormone information is needed in other study species (Van Der Kraak et al. 1998). In the majority of nonmammalian species used as models for assessing potential endocrine-mediated toxicities, very little is known regarding normal variation in circulating hormone levels. An important step in the development of *Sceloporus* spp as a model for potential effects of EDCs will be to accurately characterize the extent of diel and seasonal variability in serum sex steroid and thyroid hormone levels of captive individuals. This information will be valuable for future studies investigating endocrine-mediated toxicities in both field and laboratory studies.

The objective of this study was to determine diel and seasonal fluctuations in sex steroid and thyroid hormones in captive Western fence lizards (*Sceloporus occidentalis*). Circulating levels of testosterone and 17-β estradiol (E_2) as well as serum estradiol: testosterone ratios were determined in male and female breeding pairs, providing a measure of *in vivo* reproductive endocrine status. Since thyroid hormones are integral endocrine mediators of growth, development and reproduction and have been proposed to be sensitive targets of environmental toxicants, serum thyroxine (T_4) and triiodothyronine (T_3) were also determined in captive fence lizards. In addition, a ratio of circulating T_3:T_4 was calculated as an indication of thyroid hormone status. Seasonal samples were taken from breeding pairs of male and female Western fence lizards following their emergence from artificial hibernation. Hormones were measured weekly for a period of 16 weeks. Diel samples were taken from breeding male Western fence lizards over a 24-hour period.

Methods and Materials

Animals

Adult male and female Western fence lizards were captured from wild populations near Reno, Nevada. Lizards were acclimated for no less than a year under laboratory conditions including a constant photoperiod (14L: 10D) in glass aquaria prior to use. Animals received water and crickets *ad libitum*.

Blood Collection

For the diel sampling, blood was collected from the post-orbital sinus using heparinized microcapillary and Natelson tubes. At each of the 9 time points, samples were collected from 3-4 male lizards during a single 24-h period. Individually housed males were used in this experiment. Collection times were 0300, 0500, 0700, 0900, 1200, 1500, 1700, 1900, and 2300 h. Serum was collected following clotting overnight at 4°C and centrifugation (Eppendorf Centrifuge 5415C) at 5000 g for 10 min at 4°C. Serum was then stored at -80°C until time of extraction or assay.

Seasonal blood samples were collected from both male and female lizards starting one week after emerging from artificial hibernation. Blood was collected weekly for 16 weeks at 1000 h to avoid possible diel variation in circulating hormone levels. Samples were obtained from one group of 8 breeding pairs each week, and 4 of these groups were staggered to ensure that the animals were bled only once per month. In the event of mortality during experiment, reserve breeding pairs that underwent simultaneous artificial hibernation and conditioning were available to substitute within the group. Blood was allowed to clot overnight at 4°C and was then centrifuged at 5000 g for 10 min at 4°C. Serum was stored at -80°C in multiple aliquots to prevent repeat thawing and refreezing.

Sample Preparation

For sex steroid determinations, serum samples were extracted twice using diethyl ether to separate the lipophilic steroids from the plasma binding proteins using a method modified from McMaster et al. (1992). A volume of 25 μl of serum

was combined with 975 μl sodium phosphate assay buffer containing gelatin (pH 7.6) in a 16x150 mm borosilicate test tube and mixed well. Diethyl ether (5 ml) was added to each sample and vortexed vigorously for 40 s. Phases were allowed to separate for a minimum of 10 min. Tubes were then snap frozen in liquid nitrogen for 20 s, warmed by hand for 10 s, and immersed in liquid nitrogen for another 20 s. The ether phase was carefully transferred into a clean borosilicate test tube (12x75 mm) and evaporated at 50°C. After sufficient thawing, the aqueous phase was re-extracted as before and then combined with the previous ether extract. Extracts were then completely evaporated at 50°C and under a constant stream of N_2 gas. Lipids were reconstituted with 250 μl assay buffer (1:10 dilution), separated into 2 aliquots for each sex steroid (E_2 and testosterone) and stored at -20° C until assayed. Extraction efficiencies were determined by extracting a known amount of sex steroid and calculating recoveries. These values were 92% and 93% for testosterone and E_2, respectively.

Hormone Determinations

Serum testosterone, 17β-estradiol (E_2) , triiodothyronine (T_3), and thyroxine (T_4) levels were determined using enzyme immunoassays (EIAs) (testosterone from Cayman Chemical, Ann Arbor, MI, E_2 from Oxford Biomedical, Oxford, MI, T_3 and T_4 from ICN Biomedical, Costa Mesa, CA) validated for measurement in Western fence lizards by demonstrating parallelism

of serial dilutions of serum with the standard curves of each assay (McMaster et al. 1992).

To establish optimal dilution for each assay, ranges of dilutions were tested for all hormones as well as in both males and females. For testosterone, extracted samples were diluted 1:10 for females, and 1:100 for males. E_2 extracted samples were diluted 1:50 for females and 1:5 for males. T_3 and T_4 were assayed unextracted from serum, and both were determined using 1:2 dilutions in both males and females.

Performance characteristics for hormone assays included intra-assay and inter-assay variability in addition to parallelism. These parameters were estimated by measuring a pooled sample repeatedly. Intra-assay variability (n=6) determined for testosterone and E_2 EIAs was 5.1% and 6.7%, respectively. Inter-assay variation (n=12) for the testosterone and E_2 EIAs was 5.2% and 6.7%, respectively. For the thyroid hormones, intra-assay variation (n=6) for T_3 and T_4 was 10.6% and 7.3%, respectively. Inter-assay variation (n=12) for T_3 and T_4 was 11.6% and 15.3%, respectively. Parallelism between the standard curve and serial dilutions of lizard serum was observed for each of the hormone assays.

Results

The 16-week hormone profiles in males and females coming out of artificial hibernation revealed cycling of the sex steroids, testosterone and E_2. Testosterone levels were very high in males during the first six weeks of breeding activity, reaching levels greater than 1000 ng/ml (Figure 12A). Following this

initial period of testosterone elevation, levels decreased and remained relatively constant (70.7-230 ng/ml) for the remainder of the sampling period. Seasonal levels of testosterone for female lizards ranged from 2.60-14.3 ng/ml and remained relatively constant, with a slight increase at week 10 (Figure 12A).

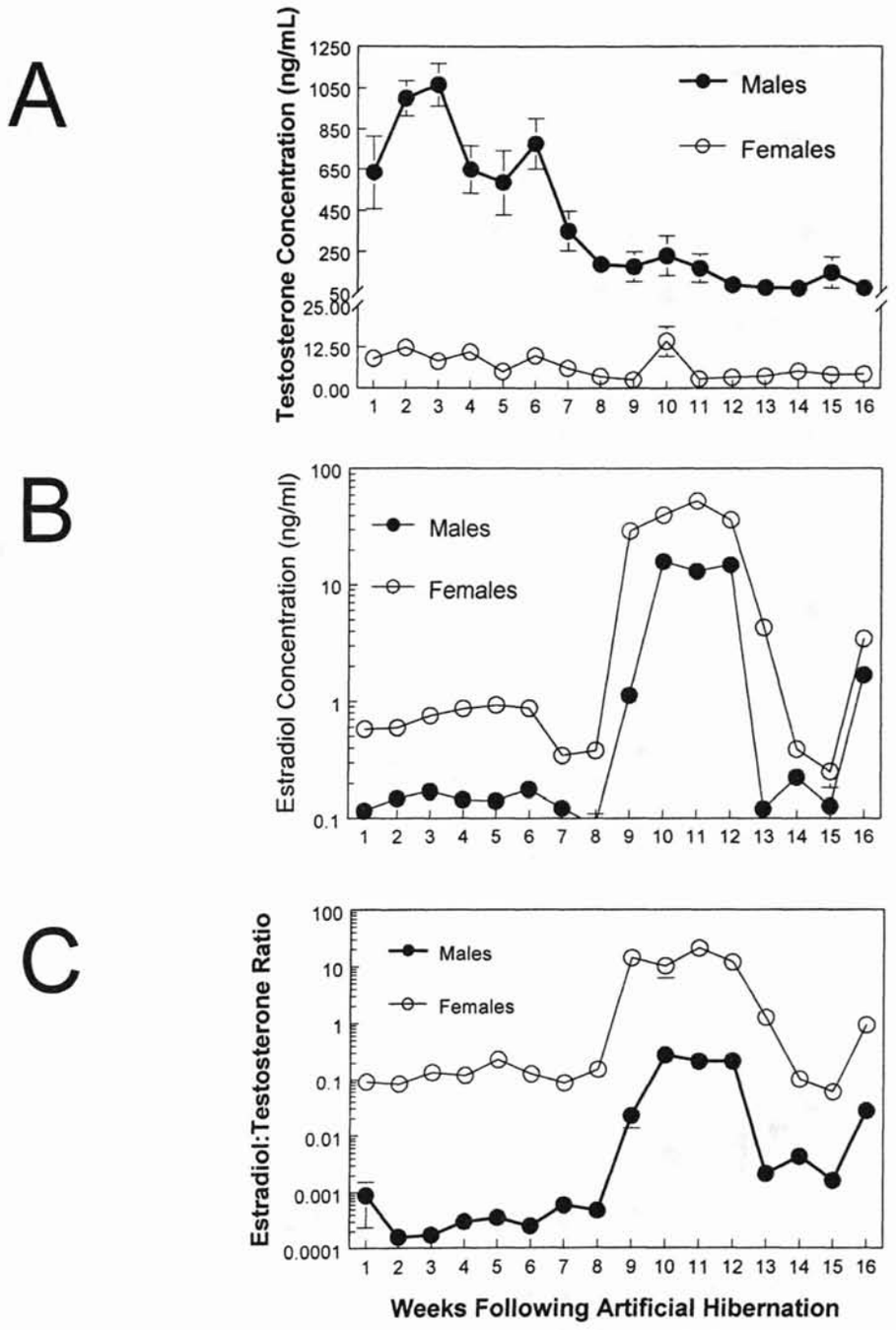

Figure 12 Seasonal profiles of testosterone, estradiol, and estradiol:testosterone ratios measured in male and female Western fence lizards. Blood was collected from the postorbital sinus from each animal only once every four weeks. Hormones were measured using validated EIAs. Values are means ± SEM; n=7-8.

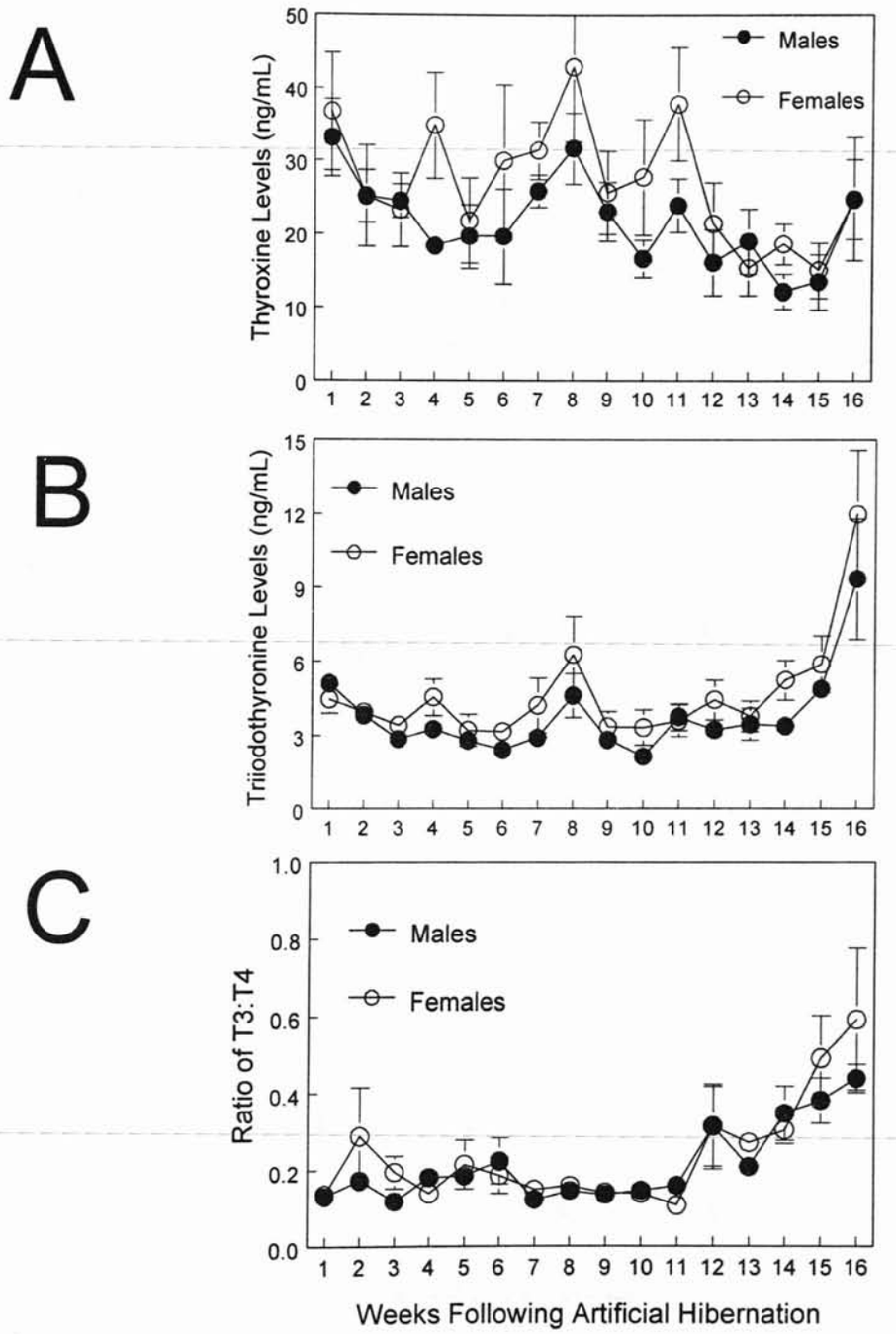

Figure 13 Seasonal profiles of thyroxine (T_4), triiodothyronine (T_3), and T_3:T_4 ratios measured in male and female Western fence lizards. Blood was collected from the postorbital sinus from each animal only once every four weeks. Hormones were measured using validated EIAs. Values are means ± SEM; n=7-8.

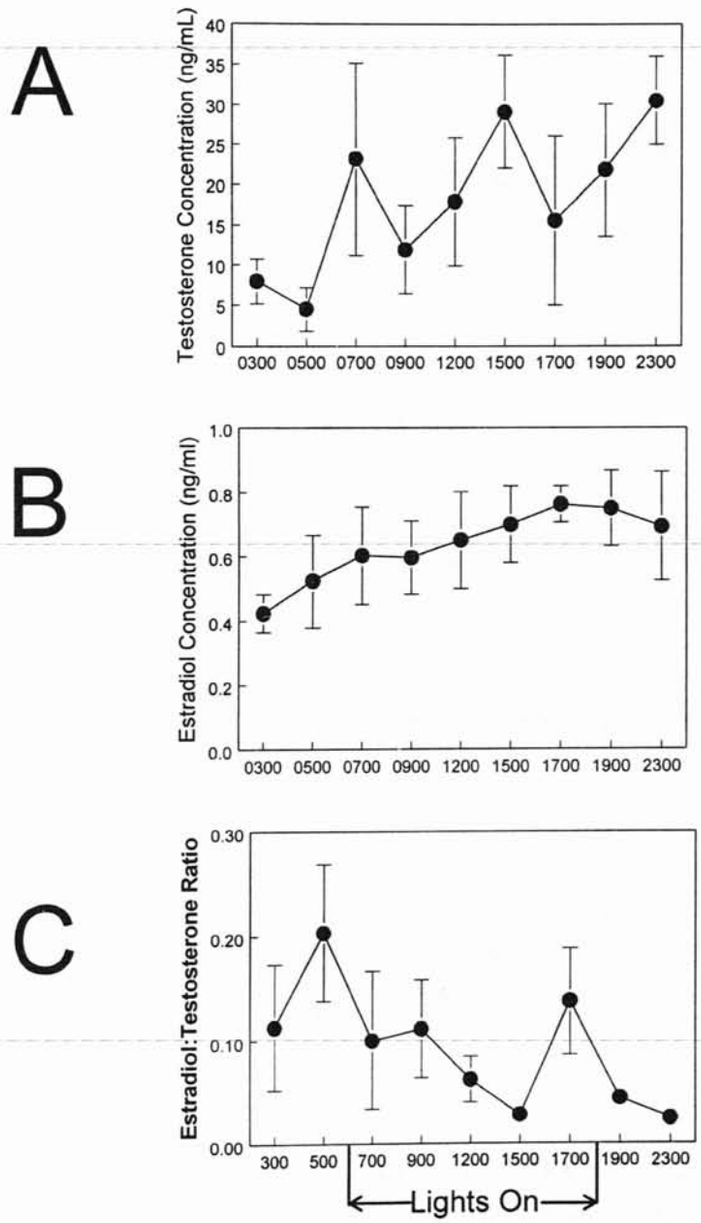

Figure 14 Diel profiles in estradiol (E_2), testosterone, and E_2:T ratios measured in male Western fence lizards. Blood was collected from the postorbital sinus and from each animal only once during the sampling period. Values are mean ± SEM for n=3-4.

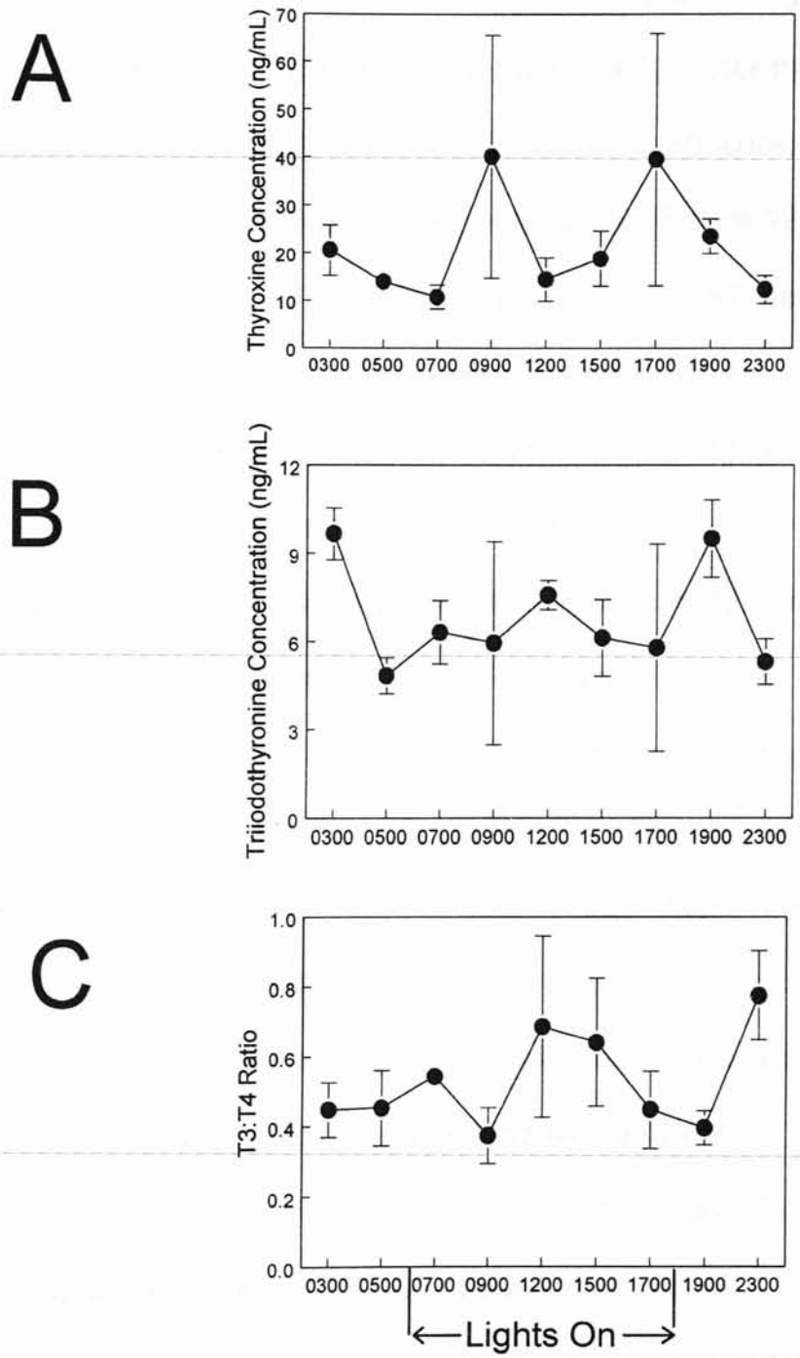

Figure 15 Diel profiles in thyroxine (T_4), triiodothyronine (T_3), and T_3:T_4 ratios measured in male Western fence lizards. Blood was collected from the postorbital sinus and from each animal only once during the sampling period. Values are mean ± SEM for n=3-4.

E_2 levels followed similar cycles in both males and females, with levels remaining constant for the first six weeks of breeding (Figure 12B), before increasing over 180-fold in males and over 140-fold in females during weeks 9-12. After this elevated period, levels decreased in both males and females during weeks 14-15, and appeared to be beginning to increase again at week 16 (Figure 12B). Similar to the E_2 levels, the ratio of E_2 to testosterone over the 16 week period indicated an overall sex steroid hormone level increase in weeks 9-12, a decrease in weeks 13-15, and a subsequent elevation in week 16 (Figure 12C).

Seasonal profiles in thyroid hormones revealed little cyclical activity in either males or females. Thyroxine levels ranged from 12.2-33.1 ng/ml in males and from 15.4-43.0 ng/ml in females (Figure 13A). Triiodothyronine levels appeared to increase during weeks 8 and 16 (Figure 13B), and ranged from 2.10 to 9.30 ng/ml in males and from 3.20 to 12.0 ng/ml in females. The ratio of T_3 to T_4 indicated an increase in overall thyroid hormone activity after week 11 that continued to elevate until week 16 (Figure 13C).

Diel sex steroid hormones in males (Figure 14) revealed no obvious patterns over a 24-hour period due to high levels of variability between individuals. Circulating testosterone levels ranged from 4.50-30.5 ng/ml in males over this 24-hour period, and testosterone levels appeared to increase throughout the day (Figure 14A). Serum E_2 levels remained relatively constant over the sampling period, with a circulating range of 0.42-0.76 ng/ml (Figure 14B). The ratios of E_2: testosterone further revealed no cyclical diel patterns in sex steroid hormone release in these animals (Figure 14C).

Diel thyroid hormones in males (Figure 15) appeared to decrease as the lights came on, but then sampling times of 0900 and 1700 h resulted in high variability for both T_3 and T_4, making any patterns unclear (Figure 15A, 15B). Over the 24-hour sampling period, T_3 in males ranged from 5.30 to 9.67 ng/ml and T_4 levels ranged from 10.7 to 40.1 ng/ml. The ratio of T_3:T_4 (Figure 15C) showed an overall increase in thyroid hormone activity during midday (1200-1500 h) and late night (2300 h).

Discussion

Reproductive hormones in the serum of both male and female fence lizards change seasonally but can also vary among lizards at any given time (Moore and Marler 1987; Saad et al. 1990; Dunlap and Schall 1995). The seasonal profiles of both sex steroid and thyroid hormones in the present study corresponded with the patterns of breeding and ovulation observed in previous research with these lizards (Talent unpublished data). It was expected that the female lizards would lay the first clutch of eggs at approximately weeks 6-8 and a second clutch between weeks 14-16 following artificial hibernation.

Serum testosterone levels in males were high for the first six weeks after artificial hibernation, indicating active spermatogenesis was occurring in preparation for mating. This is in agreement with what has been documented previously in this species, with testosterone levels peaking in the spring at the beginning of the activity period and breeding season (Eisen and DeNardo 2000). In another lizard species, *Podarcis s. sicula,* a similar trend in seasonal

testosterone was seen, however sampling increments were monthly rather than weekly (Ando et al. 1990) and levels peaked at less than 200 ng/ml, much less than the values reported in this study.

In female lizards, there was a large peak in serum E_2 concentrations observed between weeks 8-12 and a second increase beginning at weeks 15-16. These peaks in E_2 indicate the vitellogenic, or growth phase of oogenesis. Palpation of female lizards during this experiment revealed growing cohorts of follicles in preparation for ovulation. Interestingly, male lizards also had a peak of E_2 levels, although much lower than females. The reason for these elevated levels of E_2 in males is uncertain, although it has been suggested that both estrogens and androgens play important roles in sexual behavior in lizards (Tokarz 1986; Denardo and Sinervo 1994; Klukowski and Nelson 1998). In other oviparous vertebrates including the Japanese quail (*Coturnix coturnix japonica*), it has been demonstrated that masculine copulatory behaviors are estrogen dependent, suggesting that central aromatization of androgen may be responsible for the initiation of these behaviors (Schlinger and Callard 1990). Although these data revealed hormonal evidence of oogenesis and spermatogenesis, only a few of the breeding pairs successfully produced fertile eggs.

There were increases in serum thyroid hormones occurring at approximately weeks 8 and 16, corresponding with E_2 levels. However, variability was high in these data throughout the sampling period, making it difficult to determine cycling activity. Thyroid hormones are known to play a role in the

reproductive physiology of oviparous vertebrates, and these increases correspond with the sex steroid hormone cycles and proposed time of ovulation in females. As was seen in these data, concordance in the profiles of thyroid and sex steroid hormones would imply some interplay between reproduction and thyroid hormones (Cyr et al. 1988), indicating that these hormones have more than just an indirect role in this dynamic process. In birds, thyroid hormones are essential for reproduction with regard to stimulating and maintaining egg laying in females, terminating this period, and for inducing molting (Forgo et al. 1996). In fish, E_2 administration can lower both free T_3 and T_4 levels presumably because vitellogenin can act as a thyroid hormone binding protein and thus alter the balance between bound and free thyroid hormones (Flett and Leatherland 1988). This is in disagreement with the thyroid profiles seen in this study, as thyroid hormone activity appeared to increase during weeks 8 and 16 along with E_2 levels. This would allow the conclusion that it is likely that thyroid hormones are involved in the reproduction of these animals.

Diel variation in serum hormones revealed significant variability among male lizards in this study. Sample size was low (n=3-4) and this most certainly contributed to the varied hormone concentrations. Another potential reason for the high variability may be that wild caught lizards were used. These animals may have varied in age, disease status or parasite load. It has been shown previously that male Western fence lizards infected with a malarial parasite displayed fewer courtship and territorial behaviors, have altered sexually dimorphic coloration, and have smaller testes, whereas females have reduced

clutch size when infected (Dunlap and Schall 1995). Despite a period of acclimation, it is likely that these animals were still stressed from capture and change in housing conditions. In this species, it has been shown that malarial parasites can negatively affect several life history traits including testosterone levels and other factors related to reproductive success (Eisen and DeNardo 2000).

It would be useful in future work to repeat this experiment in laboratory-reared lizards of the same age, however these data represent novel basic biological data and will contribute to establishing normal ranges of diel thyroid and sex steroid hormone levels in captive fence lizards.

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Summary and Conclusions

There was a clear dose response relationship between vitellogenin induction and ethinylestradiol exposure in male Western fence lizards.

Vitellogenin ELISA and ALP measurements predicted similar ED_{50} values for each method (0.167 mg/kg for ELISA, 0.095 mg/kg for ALP).

There was a strong correlation (r=0.99) between samples measured with the ELISA and ALP.

The magnitude of response differed between ELISA (400x) and ALP (40x).

Ethinylestradiol exposure caused decreased body weight and increased hepatosomatic index in a dose dependent manner in male lizards.

Ethinylestradiol is much more potent than 17β-estradiol in male lizards, causing a 100 fold higher induction of vitellogenin and a 10 fold higher induction of ALP.

Measuring ALP may be a suitable low cost alternative to developing an ELISA for measuring plasma vitellogenin in male oviparous vertebrates exposed to estrogenic compounds.

Seasonal profiles of estradiol and testosterone in captive lizards revealed cycling that corresponded to reproductive activity.

Serum testosterone in males was elevated to very high levels for 6 weeks following emergence from artificial hibernation, indicating spermatogenic activity.

Thyroid hormones revealed slight cyclical activity seasonally, as T_3 peaked at weeks 8 and 16, corresponding with the sex steroids.

Diel profiles revealed little cyclical activity in males due to high levels of individual variability possibly related to differences in age, weight, and parasite loads.

Overall, this lizard species is a good laboratory reptile model for assessment of endocrine-mediated toxicity, and these data should be useful for future studies and further characterization of this model.

Future Research Considerations

Although this thesis research represents novel biological data and will be useful in the characterization of fence lizards as a laboratory reptile model, much work remains to be done. The Nevada population of Western fence lizards used predominantly in this study did not exhibit optimal characteristics in the laboratory, and it would be beneficial to repeat this study on a laboratory population with better performance. In addition, it will be necessary to evaluate these same responses in a selected population of Eastern fence lizards to observe any differences between species.

The dose response data presented in this research using ethinylestradiol provide a good foundation for the characterization of estrogenic responses in male fence lizards. However, it would also be useful to evaluate other compounds including weak estrogens and also more environmentally relevant concentrations and routes of exposure.

Seasonal and diel hormonal profiles should be established in F2 and F3 generation captive reared lizards from the optimal populations of both Eastern and Western species. The variability seen in these data may be reduced, allowing for more cyclical activity to be observed. Controlling for factors such as age and parasite load by using captive reared animals may also provide more useful information in developing fence lizards as a laboratory model.

Appendix A: Fence Lizard Vitellogenin ELISA Protocol

Day 1: Coating plates, pre-incubating standards and samples.

1. Coating:

A. Prepare 100 mL 0.1M carbonate coating buffer as follows:
   1.06 g Na_2CO_3
   90 mL dH_2O
   pH to 9.6 with 1N HCl
   make up to 100 mL with dH_2O
B. Dilute VTG stock solution (1.271 mg/mL) 1:1271 by adding 15.7 μL stock to 20 mL carbonate buffer to give final concentration of 1000 ng/mL.
C. **Excluding the first 4 wells (A1-D1),** add 200 μL to each well of a 96 well Greiner ELISA plate.
D. To the first four wells (blank and nonspecific binding(NSB)), add 200 μL carbonate coating buffer excluding vitellogenin.
E. Cover plate with acetate plate sealer, and incubate for 16-24 hrs at 4°C.

2. Antibody Capture Incubation:

A. Standard curve:
   To ensure consistent solution preparation, observe the following standard preparations carefully and mix WELL between each dilution:

   Solution A: VTG stock solution (1.271 mg/mL)
1. Solution B: Add 78.7 μL A to 921.3 μL PBS-T (100,000 ng/mL).
2. Solution C: Add 7.87 μL A to 992.1 μL PBS-T (10,000 ng/mL).
3. Solution D: Add 3.93 μL A to 1 mL PBS-T (5,000 ng/mL).
4. Add 25 μL B to 975 μL PBS-T (2500 ng/mL)
5. Add 100 μL C to 900 μL PBS-T (1,000 ng/mL).
6. Add 100 μL D to 900 μL PBS-T (500 ng/mL).
7. Add 50 μL D to 950 μL PBS-T (250 ng/mL).
8. Add 100 μL 250 ng/mL solution to 900 μL PBS-T (25 ng/mL).
9. Add 100 μL 25 ng/mL solution to 900 μL PBS-T (2.5 ng/mL).

B. Prepare the primary antibody solution (1:100) by adding 100 μL anti-VTG rabbit serum to 10 mL PBS-T.
C. In 2 labeled tubes, add 1.5 mL PBS-T for the blank and NSB wells.
D. In another tube labeled B_o, add 750 μL PBS-T and 750 μL primary antibody solution, resulting in a final antibody concentration of 1:200.
E. For each of the prepared standards
   1. Add 750 μL primary antibody solution
   2. Add 750 μL corresponding standard solution

This should result in a final volume of 1.5 mL of each solution.

F. Mix each of these solutions well, and place on a rotator at 4°C for 16-24 hours.

Day 2: Blocking, main incubations, secondary antibody, and detection.

1. Blocking:

A. Dissolve 2 mL10% BSA super stock solution in 18 mL PBS-T to yield 20 mL 1% BSA blocking solution.

B. Shake out coating antigen buffer and knock dry on a paper towel.

C. Wash by adding 300 μL PBS-T to each well for 5 minutes. Shake out wash buffer and knock dry on a paper towel. Repeat this procedure for a total of 3 washes.

D. Add 200 μL of 1% BSA blocking solution to each well, including the NSB wells.

E. Seal and incubate at room temperature for 1 hour, with shaking.

F. Shake out blocking solution and wash plate as described previously.

2. Main Incubations:

A. Add 200 μL of each pre-incubated standard (from the antibody capture step) to the designated wells.

B. Add 200 μL of incubated PBS-T to the 2 blank and 2 NSB wells.

C. Seal and incubate at room temperature, with shaking for 2 hrs.

3. Secondary Antibody:

A. Dilute the alkaline phosphatase-conjugated goat anti-rabbit antibody 1:1000 by adding 22 μL to 22 mL PBS-T.

B. Shake out plate containing main incubations and wash 3x as described previously.

C. **Excluding the first 2 wells (A1-B1),** add 200 L secondary antibody solution to each well.

D. Add 200 μL PBS-T to the first two wells.

E. Seal and incubate at room temperature for 1 hour with shaking.

4. Substrate:

A. Add 20 mg of *p*-nitrophenyl phosphate (PNPP) to 22ml of 10%diethanolamine buffer (v/v pH 9.6). Mix well, ensuring full dissolution. Keep out of light until use.

B. Shake out the plate containing secondary antibody solution and wash 3x as described previously.

C. Add 200 μL of substrate solution to each well.

Detection:

A. Turn on the microplate spectrophotometer and open SoftMax program.

B. Wipe bottom of plate to remove any fingerprints or residues, and place plate in the plate reader.

C. Plate should mix for 40 minutes and then read at 405 nm.

Appendix B: Final Protocol for Plasma Alkaline-Labile Phosphate Assay

A) Extraction

1. Pipette 10 µl of serum/plasma/sample into a 1.65 ml microcentrifuge tube.
2. Add 1.5 ml cold 10% trichloroacetic acid (TCA), vortex and incubate overnight at 4°C.
3. Centrifuge in the Eppendorf microcentrifuge at 7000 rpm for 10 min at 4°C.
4. Discard supernatant (carefully pipette supernatant off pellet - the pellet is sometimes loose). Add 1.5 ml 5% TCA and incubate on block heater for 30 min at 90°C (setting 3.2 on high temp control).
5. Centrifuge at 7000 rpm for 5 min.
6. Discard supernatant. Add 1.5 ml 80°C 100% ethanol.
7. Centrifuge at 7000 rpm for 5 min.
8. Discard supernatant. Add 1.5 ml chloroform:ether:ethanol (1:2:2).
9. Centrifuge at 7000 rpm for 5 min.
10. Discard supernatant. Add 1.5 ml acetone.
11. Centrifuge at 7000 rpm for 5 min.
12. Discard supernatant. Add 1.5 ml ether.
13. Centrifuge at 7000 rpm for 5 min.
14. Let tubes sit in fume hood for several minutes or until the pellets are dry.
15. Add 250 µl of 2 N NaOH and incubate for 15 min at 100°C (setting 4.0 on high temp heating block) or until pellet is dissolved.
16. Neutralize sample by adding 250 µl of 2 N HCl.

B) Phosphate Determination

1. Use Sigma Inorganic Phosphorus Diagnostics kit (Catalogue #670) to determine the alkaline-labile phosphate in samples, but using the following modified procedure.
2. Prepare standards using a 1:1 mixture of 2 N NaOH and 2 N HCl as a blank and diluent. The phosphate standard stock concentration is 0.65 mM.

Standard #	Concentration (µM)	Volume Std. Stock to Add (µl)	Volume Diluent to Add (µl)
1	13.0	10	490
2	32.5	25	475
3	65	50	450
4	130	100	400
5	325	250	250

3. Pipette 200 µl of diluent into each blank well. Pipette 200 µl of standard or sample into appropriate wells. Highly vitellogenic samples may need to be diluted 1/10 before assay to keep on standard curve.

4. Add 40 µl of acid molybdate solution to each well.
5. Add 10 µl of Fiske & SubbaRow Reducer to each well.
6. Mix plate in microplate reader for 5 min, but make sure to read absorbance at 660 nm within 10 min of step #5.

Appendix C: Ether Extractions for Sex Steroid Hormones

Fresh RIA buffer must be prepared each time:

RIA Buffer

Na_2HPO_4	0.575 g
$NaH_2PO_4*H_2O$	0.128 g
Gelatin	0.100 g

Mix ingredients into 95.0 mL distilled H_20. Heat at 45-50°C for 15 minutes to dissolve the gelatin. Let cool. Adjust pH to 7.6 and fill to 100 mL with distilled H_2O. Store at 4°C.

Extraction Efficiencies

Along with the sample preparation, label one large (16x150) and small (13x 75) test tube for each steroid you will be extracting (testosterone and/or estradiol). Using the standard provided in the hormone kit, make a 0.5 mL solution at a concentration of 100 pg/mL. Add 100 μL of this solution to the larger test tube. Freeze the remaining portion of the stock solution at -20°C and label it 'unextracted'. At the end of the extraction procedure, reconstitute the extract in 100 μL RIA buffer. Aliquot the extract in two tubes and label it extracted. After determining the hormone concentration of both the extracted and unextracted, calculate a percentage as follows: (extracted/unextracted)*100. This is the fraction of steroid that was successfully removed from the sample.

Procedure

1. Add 25 μL serum from sample to 16x150 mm test tubes.
2. Adjust volume to 1.0 mL with RIA buffer
3. Add 5 mL of ether and vortex vigorously for 40 seconds.
4. Let stand for 10 minutes so that the ether and aqueous phases separate.
5. Snap freeze aqueous layer by immersing the tube into liquid nitrogen for 20 seconds. Remove the tube and warm by hand for 10 seconds. Refreeze for 20 seconds.
6. Carefully remove upper phase (unfrozen) and transfer to small glass test tube.
7. Repeat steps 3-6 and combine extracts to ensure that all steroid has been recovered from each sample.
8. Evaporate ether at 50°C under a constant stream of nitrogen.
9. Add a small amount of ether to wash steroids down to bottom of test tube. Allow evaporating to occur.
10. Reconstitute sample in 250 μL (1:10 dilution of original sample) RIA buffer. Aliquot equally into 2 tubes and store at -20°C.

Appendix D: Protocol for Testosterone EIA

Kits were purchased from Cayman Chemical Co., Ann Arbor, MI

Samples were extracted as described previously and stored at -20°C. Dilutions may be necessary depending on hormone titer.

Buffer Preparation

1. EIA Buffer-Dilute the contents of one vial of EIA Buffer Concentrate with 90 mL of ultra pure water. Be sure to rinse the vial to remove any excess salts that may have precipitated. Store at 4°C until use.
2. Wash Buffer (1 L)-Add 2.5 mL of the Wash Buffer Concentrate to a total volume of 1 L ultra pure water. Add 0.5 mL Tween 20 and mix well. Store at 4°C until use.
3. For one plate, reconstitute the 100 determination (dtn) Testosterone Tracer with 6 mL EIA Buffer. Store the tracer at 4°C and use **within one week**.
4. For one plate, reconstitute the 100 dtn Testosterone Antiserum with 6 mL EIA buffer. Store the antiserum at 4°C. It will be stable for 4 weeks.
5. For one plate, reconstitute one vial Ellman's reagent with 20 mL Ultra Pure water. Store out of light until use.

Preparation of Standards

This procedure will vary with the manufacturer's instructions for each kit.

1. Label 9 glass test tubes.
2. Transfer 100 µL of the testosterone standard (50 ng/mL) into a tube containing 900 µL ultra pure water. Mix well. This solution (5 ng/mL) will serve as the bulk standard and can be stored at 4°C with an airtight cap for 6 weeks.
3. In the tube labeled Standard #1, aliquot 900 µL EIA buffer. Transfer 100 mL of the bulk standard to this tube and mix thoroughly.
4. Aliquot 500 µL EIA Buffer into each of the remaining tubes (#2-8).
5. Serially dilute the standard by removing 500 µL tube #1 and placing in tube #2. Mix thoroughly.
6. Repeat this process for tubes #3-8.
7. These diluted standards should not be stored for more than 24 hours.

Plate Preparation

Determine how many well will be needed for the assay.

1. The standards will include 2 blank wells (EIA buffer only), 2 non-specific binding (NSB) wells (tracer only), 2 total binding (B_o) wells (receives tracer and antiserum), and an 8-point standard curve.

2. Each sample should be assayed in two dilutions, and each dilution should be assayed in duplicate.
3. Add 150 μL EIA buffer to blank wells.
4. Add 100 μL EIA buffer and 50 μL tracer to NSB wells. Add 50 μL EIA buffer, 50 μL tracer, and 50 μL antiserum to B_o wells.
5. Add 50 μL of standards or samples to the appropriate wells, following the plate template.
6. Add 50 μL tracer to each sample and standard well.
7. Add 50 μL antiserum to each sample and standard well.
8. Cover plate with plate sealer and incubate at room temperature on a rotary shaker for 1 hour.
9. Empty the well and rinse five times with wash buffer. Add 200 mL of Ellman's reagent to each well.
10. Before reading, the bottom of the plate should be wiped to clean any smudges, fingerprints, etc.
11. Place on the orbital shaker or in the microplate reader out of the dark. Typically this plate develops in 40-60 minutes, but absorbance (read at 405 nm) values should be checked every 15 minutes. B_o wells should reach an absorbance of 0.300 or greater.

Appendix E: Protocol for Estradiol EIA

Kits were purchased from Oxford Biochemical

Samples were extracted as described previously and stored at -20°C. Dilutions may be necessary.

Preparation of Standards
This procedure will vary with the manufacturer's instructions for each kit.

Supplied with Estradiol standard of 1μg/mL Solution **A**.

1. Nine glass test tubes should be labeled.
2. Add 20 μL of **A** to 980 μL of EIA buffer (also provided with kit) to yield 1mL of solution **B** at a concentration of 20 ng/mL. Mix well, using vortex.
3. Add 200 μL of **B** to 1.8 mL of EIA buffer to yield 2 mL of solution **C** (2 ng/mL).
4. Add 200 μL of **C** to 1.8 mL of EIA buffer to yield 2 mL of solution **D** (0.2 ng/mL).
5. Prepare standards as follows:

Standards	Ng/mL	EIA buffer (μL added)	C Standard μL	D standard μL
S	0	As is	-	-
S_1	0.02	900		100
S_2	0.04	800		200
S_3	0.10	500		500
S_4	0.20	-	-	As is
S_5	0.40	800	200	-
S_6	1.00	500	500	-
S_7	2.00	-	As is	-

Plate Preparation

1. Determine the number of wells to be used.
2. Dilute the estradiol enzyme conjugate. Add 1 μL of enzyme conjugate into 50 μL total volume of EIA buffer for each well assayed. For the whole plate, add 110 μL of the enzyme conjugate into 5.5 mL total volume of EIA buffer. Mix the solution thoroughly.
3. Add 50 μL of standards or unknowns (samples diluted as necessary) to the appropriate wells in duplicate.
4. Add 50 mL of the diluted enzyme conjugate to each well.
5. Cover plate with plastic film or plate cover and incubate at room temperature for one hour.

6. Dilute concentrated wash buffer with deionized water (10X). Mix thoroughly.
7. After incubation, dump out contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
8. Wash each well with 300 μL of the wash buffer. Repeat for a total of three washings.
9. Add 150 μL of substrate to each well. Mix by shaking plate gently.
10. Allow further mixing on shaking platform for 30 minutes.
11. Wipe bottom of plate with KimWipe to remove any moisture or fingerprints.
12. Read plate in a microplate reader at 650 nm.

Appendix F: Thyroid Hormones EIA Protocols

Both T_3 and Total T_4 kits were purchased from ICN Pharmaceuticals

Extractions were not necessary for these assays and samples were stored at -80°C until use. Samples were diluted 1:2 in each of the two assays using steroid stripped serum also obtained from ICN.

Total Thyroxine (T_4) EIA

Buffer Preparation

1. All reagents should reach room temperature before use.
2. To prepare working T4-HRPO Conjugate Reagent: add 0.1 mL of the T4-HRPO Conjugate Concentrate (11x) to 1.0 mL Conjugate Diluent. Each well assayed will require 100 µL of this solution, and the amount of conjugate diluted depends on the assay size. The working conjugate reagent is stable at 4°C for at least 24 hours.
3. To prepare the H_2O_2/TMB solution (substrate): prepare a 1:1 mixture of color reagent A with color reagent B for up to 1 hour before use. Shake gently to ensure complete mixing. This solution should be made **at least 15 minutes** before use and is stable at room temperature **out of light** for up to three hours.

Standards Preparation
The standards for this assay come premixed, however to ensure that we would have a more complete standard curve, two lower standards were added to the range.

Assay Procedure

1. Secure the desired number of wells in the holder.
2. Pipette 25 µL of standards, specimens, and controls into the appropriate well. All samples and standards should be assayed in duplicate.
3. Add 100 µL of the Working Conjugate Reagent (prepared previously) to each well.
4. Mix thoroughly by placing on a rotary shaker for 1 hour at room temperature.
5. Remove the incubation mixture by flicking the plate contents into a waste container.
6. Rinse the microtiter wells 5 times with distilled H_2O.
7. Strike the wells sharply on paper towels to remove all residual water droplets.
8. Dispense 200 µL of H_2O_2/TMB solution into each well. Mix well.
9. Incubate at room temperature, **in the dark**, for 20 minutes.
10. Stop the reaction by adding 50 µL of Stop Solution to each well.
11. Ensure that all blue color has changed completely to yellow.
12. Read absorbance at 450 nm with a microplate reader within 30 minutes.

Triiodothyronine (T_3) EIA

Buffer Preparation

1. All reagents should reach room temperature before use.
2. To prepare working T3-HRPO Conjugate Reagent: add 0.1 mL of the T3-HRPO Conjugate Concentrate (11x) to 1.0 mL Conjugate Diluent. Each well assayed will require 100 µL of this solution, and the amount of conjugate diluted depends on the assay size. The working conjugate reagent is stable at 4°C for at least 24 hours.
3. To prepare the H_2O_2/TMB solution (substrate): prepare a 1:1 mixture of color reagent A with color reagent B for up to 1 hour before use. Shake gently to ensure complete mixing. This solution should be made **at least 15 minutes** before use and is stable at room temperature **out of light** for up to three hours.

Standards Preparation

The standards for this assay come premixed, however to ensure that we would have a more complete standard curve, two lower standards were added to the range.

Assay Procedure

1. Secure the desired number of wells in the holder.
2. Pipette 50 µL of standards, specimens, and controls into the appropriate well. All samples and standards should be assayed in duplicate.
3. Add 50 µL of the T3 Antibody Reagent into each well. Mix thoroughly.
4. Add 100 µL of the Working Conjugate Reagent (prepared previously) to each well.
5. Mix thoroughly by placing on a rotary shaker for 1 hour at room temperature.
6. Remove the incubation mixture by flicking the plate contents into a waste container.
7. Rinse the microtiter wells 5 times with distilled H_2O.
8. Strike the wells sharply on paper towels to remove all residual water droplets.
9. Dispense 200 µL of H_2O_2/TMB solution into each well. Mix well.
10. Incubate at room temperature, **in the dark**, for 20 minutes.
11. Stop the reaction by adding 50 µL of Stop Solution to each well. Ensure that all blue color has changed completely to yellow.
12. Read absorbance at 450 nm with a microplate reader within 30 minutes.

VITA

Sandra M. Brasfield

Candidate for the Degree of

Master of Science

Thesis: DEVELOPMENT OF A LABORATORY REPTILE MODEL FOR ASSESSMENT OF ENDOCRINE-MEDIATED TOXICITY

Major Field: Zoology

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Education: Graduated from Riverdale High School, Murfreesboro, Tennessee, in May, 1995; received Bachelor of Science degree in Biology with a minor in Chemistry from Middle Tennessee State University, Murfreesboro, Tennessee, in May 1999. Completed the requirements for the Master of Science degree in Zoology at Oklahoma State University in December 2001.

Experience:

Employed by Oklahoma State University, Department of Zoology as a graduate research assistant, January 2001 to present, and as a teaching assistant in the courses of Vertebrate Morphology and Human Anatomy, August 1999-December 2000.

Employed by Middle Tennessee State University, Department of Biology, as an undergraduate research assistant, January 1998 through April 1999 to participate in a population survey of a coastal plains amphibian population.

Employed as an intern in the Research Experience for Undergraduates (REU) by the NSF at the University of Georgia exploring the effects of leaf and wood litter exclusion on headwater stream metabolism in the Southern Appalachian Mountains.

Employed by Middle Tennessee State University, Department of Animal Science, as an undergraduate research assistant.

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