DOSE–RESPONSE AND TIME COURSE RELATIONSHIPS FOR VITELLOGENIN
INDUCTION IN MALE WESTERN FENCE LIZARDS (SCELOPORUS OCCIDENTALIS)
EXPOSED TO ETHINYLESTRADIOL

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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 (Sceloporus occidentalis) given intraperitoneal injections of 17α-ethinylestradiol (EE2). Plasma Vtg was quantified directly with an antibody-capture enzyme-linked immunosorbent assay (ELISA) and indirectly using plasma alkaline-labile phosphate (ALP) in order to compare these two methods. Both ELISA and ALP predicted similar median effective dose (ED50 [dose causing a 50% maximal response]) values for plasma Vtg induction (0.167 mg/kg for ELISA and 0.095 mg/kg for ALP). In addition, both ELISA and ALP detected significant Vtg induction at a dose of 0.003 mg/kg of EE2, which was the lowest dose used in our study. 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 EE2-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 with developing a Vtg ELISA in fence lizards exposed to estrogenic compounds.

Keywords—Vitellogenin  Alkaline-labile phosphate  Reptiles  Estrogenic  Ethinylestradiol

INTRODUCTION

In recent years, it has become increasingly clear that chemicals in the environment from both natural and anthropogenic sources may interfere with endocrine physiology [1–3]. However, data are often insufficient to resolve the ecological 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 [4]. There is a recognized need to develop animal models for all vertebrate classes [1,5]. In particular, existing methods suitable for identifying chemicals with endocrine-disrupting mechanisms in reptiles are limited [1,5,6]. We hypothesize that fence lizards (Sceloporus spp.) are good candidates for laboratory reptile models for assessing endocrine-mediated toxicity in vivo.

Reptiles are considered to be suitable as contaminant biomonitor due to their persistence in a variety of habitats, widespread geographic distribution, longevity, and site fidelity [3]. However, with the exception of a few species, this class of vertebrates has been relatively understudied [6]. Currently, the red-eared slider turtle (Trachemys scripta elegans) [7] and American alligator (Alligator mississippiensis) [3,8] are being evaluated for use in assessment of endocrine-mediated toxicity.

In comparison with other reptile models, fence lizards may 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 (Sceloporus undulatus) and western (Sceloporus occidentalis) fence lizards inhabit most of the continental United States and are common throughout this range [9]. Although the natural history characteristics of eastern and western fence lizard populations may vary under natural conditions, many populations mature under laboratory conditions in as few as four to eight months after hatching [10], and mature females typically lay 2 to 6 clutches of between 8 and 15 eggs in a breeding season [11]. Individuals are easily sexed after hatching and offer a variety of endpoints that can be evaluated relative to endocrine-mediated toxicity.

The induction of Vtg in male oviparous vertebrates represents a sensitive biochemical endpoint indicating exposure to estrogenic compounds [1,12]. Vitellogenin 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 [13]. 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. However, males do have the capacity to express this protein if exposed to exogenous estrogens. Thus, the presence of Vtg in the plasma of male oviparous vertebrates indicates a physiological response to exogenous estrogens [1,12]. This is an ideal endpoint to measure in males because the majority of Vtg remains in systemic circulation rather than being sequestered into oocytes, as would occur in females [14].

Vitellogenin is a highly phosphorylated protein [13], and a measure of the phosphate associated with this protein has been used as an indicator of plasma Vtg concentration [15–18]. Plasma phosphoproteins can be separated from other phosphorylated plasma molecules (e.g., phospholipids) with a series of extractions. Because Vtg is the major plasma phos-
phosphoprotein in an estrogen-exposed oviparous animal [15], simple colorimetric measurement of alkaline-labile phosphate (ALP) released from extracted plasma phosphoproteins has been used as an indirect measure of circulating Vtg in fishes [16–19]. In rainbow trout (*Oncorhynchus mykiss*), increasing levels of plasma Vtg were correlated with increases in plasma ALP [19]. However, the relationship between plasma vitellogenin and ALP has not been characterized in reptiles.

As an initial step toward evaluating fence lizards as a laboratory reptile model for endocrine-mediated toxicity, the present study determined dose–response and time course relationships of Vtg induction in males exposed to EE2. A main objective of this study was to compare two methods of measuring this response, a direct measure of Vtg using an enzyme-linked immunosorbent assay (ELISA) and an indirect measure of the ALP associated with this protein.

**MATERIALS AND METHODS**

**Animals**

Male western fence lizards (*S. occidentalis*) from Reno (NV, USA) and San Joaquin Valley (CA, USA) were acclimated to laboratory conditions prior to use. Animals were housed in glass aquaria with a constant photoperiod of 14:10 h light:dark. Food (crickets) and water were provided ad libitum.

**Chemicals**

17α-Ethynylestradiol (EE2, 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 EE2 in male fence lizards (*n* = 4 lizards) and analyzed plasma Vtg both directly by ELISA and indirectly using plasma ALP. The EE2 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 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 and 0.001, 0.01, 0.1, 1, and 10 mg EE2/kg body weight. An additional experiment was also conducted to examine the effect of vehicle (corn oil and propylene glycol) on Vtg induction. Animals were given a single injection of vehicle at a volume of 5 μl stock solution/g body weight or sham injection.

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 and 5 μl of 1.36 mg/ml heparin in normal saline to inhibit proteolysis and clotting, respectively. Plasma was collected following centrifugation at 5,000 rpm for 10 min 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.

**Dose–response experiment**

Single i.p. injections of a wider range of EE2 doses were administered to male lizards from Reno in order to describe the dose–response curve for Vtg induction and generate ED50 values. Stock solutions of EE2 were prepared as described above using propylene glycol as a carrier. Based on data from the preliminary experiments, the experimental groups (*n* = 8 lizards per dose) chosen were sham control, propylene glycol vehicle control, and 0.0003, 0.003, 0.001, 0.01, 0.1, 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 were calculated using the formula (liver wt/(body wt − liver wt)) × 100.

**Comparison of EE2 and 17β-estradiol (E2)**

Stock solutions of EE2 and E2 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. Lizards from San Joaquin Valley (*n* = 8 per group) received an i.p. dose at an injection volume of 5 μl/g body weight. Blood was collected at day 15 and plasma was stored at −80°C until time of analysis.

**Vitellogenin ELISA**

Vitellogenin was isolated from plasma collected from male fence lizards injected i.p. with EE2 by precipitation and purified using diethylaminoethyl Sephadex protein chromatography [20]. Highly specific polyclonal antisera against purified fence lizard Vtg were produced in rabbits (L.P. Weber et al., unpublished data). Microtiter plates (Greiner America, Lake Mary, FL, USA) were coated with purified Vtg (200 ng/well) using sodium carbonate buffer (0.1 M NaCO3, pH 9.6) overnight at 4°C. Anti-Vtg antiserum (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 to 16 h at 4°C. Plates were washed three times with PBS-T between each ELISA step. Coated plates were blocked with 1% bovine serum albumin 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 antirabbit secondary antibody (1:1,000 in PBS-T) was added to plates, incubated at room temperature for 1 h, and detected with p-nitrophenylphosphate 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 min (Bmax = 0.9–1.0) and absorbance values measured at 405 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Nonspecific binding was determined in uncoated wells without primary antibody, and maximum binding of anti-Vtg (Bmax or zero standard) was determined in coated wells in every assay.

Intraassay coefficient of variation for the ELISA was 4.9% (*n* = 6 determinations). Interassay variability was 13.3% (*n* = 12). The detection limit of the Vtg ELISA was 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 ALP were performed as described by Wallace...
and Jared [15] with minor modifications. Briefly, 5 μl of plasma and 30 μl of 1% bovine serum albumin 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 7,000 rpm for 10 min with ice-cold 5% (w/v) TCA (30 min at 50°C), 100% ethanol (80°C for 1 min), chloroform:ether:ethanol (1:2:2), acetone, and ether. Following the ether wash, pellets were allowed to dry and then were reconstituted in 250 μl of 2 N sodium hydroxide and incubated for 15 min at 100°C. The samples were then neutralized by adding 250 μl of 2 N 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 2 N sodium hydroxide and 2 N HCl, were assayed in duplicate, and inorganic phosphate was determined by colorimetric assay using a commercially available kit (Sigma 670). To correct for bovine serum albumin added prior to extraction, 30 μl of 1% bovine serum albumin was extracted, determined for every assay, and all samples were corrected based on this determination.

Intraassay coefficient of variation for plasma ALP was 5.6% (n = 6). Interassay variability was 10.7% (n = 12). The detection limit of the plasma ALP assay was 0.006 μM phosphate. 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 analysis of variance (ANOVA) followed by Fisher’s protected least significant difference posteriori tests as appropriate. All other data were analyzed using Student’s t test or one-way ANOVA, followed by Dunnett’s posteriori tests as appropriate. The ED50 values were calculated using nonlinear, four-parameter logistic regression. A value of p < 0.05 was considered statistically significant. Data are expressed as mean ± standard error of the mean (SEM). Sample size (n) indicates the number of lizards used.

RESULTS

In preliminary experiments, plasma Vtg concentrations increased dose dependently in male lizards (n = 4) receiving a single i.p. dose of EE2 (Fig. 1A). For all doses, plasma Vtg was increased on day 3 of blood collection and remained elevated over the 27-d course of the experiment. Experimental groups (n = 4 per group) receiving multiple i.p. doses of EE2 displayed high levels of plasma Vtg on day 9 of blood collection (Fig. 1B). Unlike the single injection groups, the levels of plasma Vtg consistently decreased over the 27-d course of the experiment at the two highest doses.

Before initiation of the 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 with propylene glycol (Fig. 2, p = 0.03, repeated measures ANOVA across all time points) with peak Vtg levels observed 21 d 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 (Fig. 2).

Based on our preliminary time course experiment, day 15 following a single i.p. injection was chosen for the dose–response experiment because it appeared to be a consistent point of maximum Vtg induction. In this experiment, plasma Vtg followed a sigmoidal dose–response relationship, and from this
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Fig. 3. Plasma vitellogenin (Vtg) levels in untreated (sham injection and propylene glycol vehicle [PPG] controls) and ethinylestradiol-treated male western fence lizards determined using enzyme-linked immunosorbent assay (ELISA). Plasma samples were collected 15 d after a single intraperitoneal injection. Values are mean ± standard error of the mean ([SEM] [n = 7–8]). From this curve, a median effective dose (ED50) value of 0.167 mg/kg was calculated for Vtg induction.

Fig. 5. Time course and preliminary dose–response relationships of plasma alkaline-labile phosphate (ALP) induction in male western fence lizards (n = 4) treated with ethinylestradiol either as a single intraperitoneal injection (A) or five additive i.p. doses administered every second day (B).

Gross effects were also seen at the organ and organism levels. The EE2 caused a significant reduction of body weight at the highest dose (Fig. 4A; p < 0.05 Dunnett’s a posteriori test after one-way ANOVA). Liver size, measured as hepatosomatic index, was significantly elevated in the four highest doses (p < 0.05, Fig. 4B). At the two highest doses, hepatosomatic indices were almost twofold greater compared with control lizards (p < 0.01).

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-injection groups, plasma ALP followed a similar trend as plasma Vtg, increasing with dose and time (Fig. 5A). In the multiple-injection treatment groups, plasma ALP was elevated at the time of first blood collection (Fig. 5B). However, unlike the plasma Vtg measurements in the same samples, ALP levels remained elevated over the duration of the 27-d time course at all doses tested.

In the dose–response experiment, plasma ALP followed a similar trend as plasma Vtg, with an ED50 of 0.095 mg/kg.
gested that the sensitivity of ALP did not extend to low levels of Vtg measured in our study. 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 substitute for Vtg ELISA, as both assays predicted similar ED50 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, at least at the levels of Vtg seen in nonreproductive females and juvenile animals. However, the detection limit for ALP in the present study was 0.006 μM phosphate (equivalent to 3.6 ng Vtg/ml plasma), 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 EE2-exposed male lizard were analyzed using both ELISA and ALP.

### 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 for both plasma Vtg and ALP induction in adult male fence lizards exposed to EE2. Relatively little research has been conducted with fence lizards under laboratory conditions, and it is important to establish baseline physiological responses in model species [5,8].

Two responses were measured following treatments with EE2, i.e., 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 [15].

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 [19] suggested that the sensitivity of ALP did not extend to low levels of Vtg seen in nonreproductive females and juvenile animals. However, the detection limit for ALP in the present study was 0.006 μM phosphate (equivalent to 3.6 ng Vtg/ml plasma), and ALP was significantly elevated at the lowest dose administered (0.003 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 EE2-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 substitute for Vtg ELISA, as both assays predicted similar ED50 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, at least at the levels of Vtg measured in our study.

Surprisingly, few studies have determined dose–response relationships between treatment with EE2 or other potent estrogens and Vtg induction in oviparous vertebrates. To our knowledge, there are no studies that have reported an ED50 for Vtg induction in reptiles. In male Japanese quail (Coturnix coturnix japonica), EE2 did induce Vtg, measured as protein-bound phosphorus (PBP) [21], similar to the measure of ALP utilized in our study. Although PBP induction in quail did not appear to reach a maximum response, it appears that this avian species has a similar sensitivity to EE2 as fence lizards. In fish, the relationship between EE2 exposure and Vtg induction has been established; however, few dose–response studies have been conducted. In a recent study in rainbow trout [22], animals were given intravascular EE2 injections and exhibited a similar dose–response relationship for Vtg induction when compared with fence lizards given i.p. injection. These studies suggest that fence lizards exhibit a similar sensitivity to EE2
exposure compared with 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 EE2. In contrast, plasma ALP measurements were 40-fold different between plasma samples collected from control and high-dose EE2-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 chromogenic phosphomolybdate complex is formed per phosphate in the ALP assay. Despite this apparent difference in magnitude of response, ALP was comparable with Vtg ELISA in other assay parameters such as intra- and interassay 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 trend to decrease over the 27-d period at the highest EE2 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 proteolyzed 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 with the propylene glycol vehicle. The presence of phytoestrogens in the corn oil may have been responsible for the observed estrogenicity [23,24]. Although this result was statistically significant, the level of induction may not be biologically significant since it was one order of magnitude less than the plasma Vtg measured at the lowest dose of EE2. 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.

In addition to induction of Vtg, it has been shown in oviparous vertebrates that exposure to estrogenic chemicals can result in adverse physiological effects. At the highest dose of 10 mg/kg, we saw a significant decrease in body weight during the 15-d experimental period. It was qualitatively noted in our dose–response experiments that lizards exposed to higher doses of EE2 reduced their food intake in addition to regurgitation of food. This observation is consistent with a study by Heck et al. [25] that reported decreased feeding in turtles treated with estradiol extending for a period of 50 d.

At the organ level, the four highest doses of EE2 caused a significant increase in hepatosomatic index. This stimulatory effect on hepatocyte hyperplasia and/or hypertrophy in response to estrogenic compounds has also been observed in fishes. Histopathological changes were associated with hypertrophy in the liver and kidneys of summer flounder [26] and rainbow trout [27]. Although it is not clear if the presence of Vtg in plasma is related to 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 EE2 and 17β-estradiol (E2). The EE2 caused a much greater induction of plasma Vtg (100-fold higher) and ALP (10-fold higher) than E2 15 d after a single i.p. injection. This difference may be a result of differences in toxicokinetics due to the longer half-life of EE2 and faster clearance of E2. This is consistent with other studies in which EE2 was found to be 10 times more potent than E2 as a Vtg inducer, presumably because EE2 is synthesized to withstand biotransformation [28]. Furthermore, it has also been suggested that xenooestrogens may not be subject to the same homeostatic mechanisms (e.g., steroid binding proteins) as E2 based on structural differences [29], making these compounds more bioavailable.

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

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