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Assessment of Vitellogenin Synthesis in *Salvator merianae* as a Biomarker of Exposure to Xenoestrogens in Terrestrial Ecosystems

Evaluación de la síntesis de vitelogenina en *Salvator merianae* como biomarcador de exposición a xenoestrógenos en ecosistemas terrestres

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ABSTRACT

Agricultural and livestock expansion in Argentina has significantly increased the use of pesticides, adversely impacting animal and human health. These chemical compounds act as xenoestrogens and alter the reproductive physiology of wildlife. In this context, vitellogenin, a hepatic protein typically elevated in oviparous females during reproduction, can also be induced in males and immature females by exposure to xenoestrogens. The objective of this study was to investigate the vitellogenin production in response to exogenous estrogens in male *Salvator merianae* lizards, a species widely distributed in Argentina. Eighteen adult specimens from the Universidad Nacional de Tucumán were used, distributed in three groups: males treated with 17 β-estradiol, females in vitellogenesis phase (positive control), and untreated males (negative control). The treated males received doses of 17 β-estradiol every three days for two weeks, and blood samples were taken before and during treatment for analysis by SDS-PAGE, spec-

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trophotometry, and immunohistochemistry. The results revealed a significant increase in triglyceride levels and the synthesis of a high molecular weight lipoprotein in treated males, a pattern similar to that observed in females during vitellogenesis and absent in negative controls. This pioneering study demonstrates the induction of hepatic vitellogenin in *Salvator merianae* males treated with estradiol, highlighting the potential utility of this species as a biomarker of environmental contamination by xenoestrogens.

Keywords: Black and with tegus, bioindicator, environmental monitoring, conservation biology, vitellogenesis.

RESUMEN

La expansión agrícola y ganadera en Argentina ha incrementado significativamente el uso de plaguicidas, impactando adversamente en la salud animal y humana. Estos compuestos químicos, actúan como xenoestrógenos y alteran la fisiología reproductiva de la fauna silvestre. En este contexto, la vitelogenina, una proteína hepática típicamente elevada en hembras ovíparas durante la reproducción, también puede ser inducida en machos y hembras inmaduras por exposición a xenoestrógenos. El objetivo de este trabajo fue estudiar la producción de vitelogenina en respuesta a estrógenos exógenos en machos del lagarto *Salvator merianae*, especie con amplia distribución en Argentina. Se utilizaron 18 ejemplares adultos de la Universidad Nacional de Tucumán, distribuidos en tres grupos: machos tratados con 17 β-estradiol, hembras en fase de vitelogénesis (control positivo), y machos sin tratamiento (control negativo). Los machos tratados recibieron dosis de 17 β-estradiol cada tres días por dos semanas, y se tomaron muestras de sangre antes y durante el tratamiento para análisis mediante SDS-PAGE, espectrofotometría e inmunohistoquímica. Los resultados revelaron un incremento significativo en los niveles de triglicéridos y la síntesis de una lipoproteína de alto peso molecular en los machos tratados, patrón similar al observado en hembras durante la vitelogénesis y ausente en los controles negativos. Este estudio pionero demuestra la inducción de vitelogenina hepática en machos de *Salvator merianae* tratados con estradiol, destacando la utilidad potencial de esta especie como bioindicador de contaminación ambiental por xenoestrógenos.

Palabras clave: Tegu blanco y negro, bioindicador, monitoreo ambiental, biología de la conservación, vitelogénesis.

INTRODUCTION

In recent decades, a significant percentage of the natural forests and rangelands in Argentina have been transformed into areas designated for agricultural and livestock production (Viglizzo et al., 2011). This development has been accompanied by an intensification in the adoption of advanced technologies and a significant increase in pesticide use. These pesticides have shown adverse effects on the health of wild, domestic animals , and human (Guillette et al., 1994; Jones, Coen, Tremblay, Giesy, 2000; Rey et al., 2006; Soto and Sonnenschein, 2010; Verderame and Scudiero, 2019). Specifically, glyphosate-based herbicides have been implicated in causing teratogenic effects and developmental failures in amphibians, both in larval and adult stages (Bach, Marino, Natale, Somoza, 2018; Wagner, Reichenbecher, Teichmann, Tappeser, Lötters, 2013), as well as in fish (Lopes, Moraes, Martins, 2022). In reptiles, exposure to glyphosate has resulted in genotoxicity, immunological alterations, and increases in body temperature (Carpenter, Monks, Nelson, 2016; Schaumburg, Siroski, Poletta, Mudry, 2016).

These pesticides act as xenoestrogens, either mimicking or blocking natural hormones by binding to estrogenic receptors, thereby generating effects similar to those of endogenous estrogens. This activity can trigger significant reproductive disorders in wildlife (Guillette et al., 1994; Jones et al., 2000; Rey et al., 2006; Verderame and Scudiero, 2019). It has been documented that these contaminants disrupt the functionality of the hypothalamic-pituitary axis and affect hormone biotransformation in the liver, causing alterations in the plasma concentrations of sexual steroids (Guillette and Gunderson, 2001). These endocrine disruptors interfere with animals endocrine systems by mimicking or antagonizing natural hormones, altering their synthesis, degradation, and transport in the body. Moreover, these chemical agents can induce changes in gene expression through epigenetic effects. Such hormonal alterations can cause problems in the development and functionality of reproductive organs, resulting in malformations, reduced viability of eggs, and decreased fertility, negatively impacting the health and survival of animal populations (Milnes and Guillette, 2008).

In recent decades, vitellogenin (Vtg) has been widely recognized as a biomarker of environmental contamination. This lipoglycophosphoprotein, synthesized in the liver of oviparous females in response to estradiol, acts as the precursor protein of vitellus, the main nutritional source for the developing embryo (Finn, 2007; Jones, 2011). Normally, circulating levels of Vtg significantly increase in adult females during their reproductive period and remain virtually undetectable outside this phase (Finn, 2007; Van Dyke and Griffith, 2018). However, in males and sexually immature females, where the Vtg gene normally is not expressed, it can be activated by exposure to exogenous estrogens or xenoestrogens (Huang, Ying, Liang, Liu Y-S, Liu S-S, 2013; Lei et al., 2013; Thomas-Jones et al., 2003).

To date, the majority of studies employing Vtg as a biomarker of endocrine disruption have focused on aquatic systems, mainly using fish as research models (Arukwe and Goksøyr, 2003; Del Giudice et al., 2012; Harries et al., 1999; Jones et al., 2000; Mills and Chichester, 2005; Yamamoto, Garcia, Kupsco, Oliveira Ribeiro, 2017). Research has also been conducted on marine and freshwater turtles (Irwin, Gray, Oberdörster, 2001; Sifuentes-Romero, Vázquez-Boucard, Sierra-Beltrán, Gardner, 2006) and caimans (Rey et al., 2006). However, research on terrestrial reptiles remains sparse, underscoring the need for further studies in this area.

The induction and detection of Vtg have been documented in turtles (Tada et al., 2004) and in lizards exposed to estrogenic compounds in laboratory settings (Verderame, Limatola, Scudiero, 2016). Studies with juvenile alligators have reported alterations in plasma testosterone concentrations and in the activity of essential liver enzymes, suggesting altered hormonal biotransformation with potential adverse repercussions on reproduction and development (Guillette and Gunderson, 2001). Verderame and colleagues (2016) identified the synthesis of Vtg in both hepatic and extrahepatic tissues in males of the terrestrial lizard *Podarcis sicula* treated with estradiol. Further research has shown that the herbicide glyphosate can also induce the synthesis of Vtg in lizards, highlighting their potential as a successful model for the ecotoxicological analysis of soil contamination (Verderame and Scudiero, 2019).

This study was carried out on the lizard *Salvator merianae*. This group of large South American lizards inhabits temperate, subtropical, and tropical zones of South America (Jarnevich et al., 2018). Recently, *Salvator merianae* has been proposed as a sentinel animal for detecting environmental contamination by pesticides, due to its intrinsic characteristics. These include its wide geographic distribution, remarkable plasticity to thrive in both natural and anthropized habitats, and strong fidelity to these environments, which make it an ideal biological model for pollution studies (Schaumburg et al., 2016). Additionally, its biological traits, such as the ability to attain a large body size, a broad diet that includes fruits, invertebrates, small vertebrates, eggs, and carrion, and its adaptability to a variety of habitats, along with its ability to hibernate in underground refugia during adverse conditions, make it particularly valuable for monitoring the long-term effects of environmental pollutants (Goetz et al., 2021; Mestre, 2021; Mestre et al., 2023; Valdez, 2021). Moreover, *S. merianae* is considered a sentinel species due to its high representativeness in agri-environments (Schaumburg et al., 2016; Mestre, 2021). Among probable exposure routes, maternal transfer, direct contamination from soil, water, air, and nest material, as well as feeding, could pose significant risks to this species. Given that agrochemical applications in Argentina coincide with the reproductive season of *S. merianae*, embryos may be exposed to contaminants through the absorption of xenobiotics from contaminated nest material or maternal transfer. This is particularly concerning because the flexible eggshells of

S. merianae require water absorption from the substrate during incubation, which can also introduce contaminants into the developing eggs, potentially affecting the health and development of embryos, hatchlings, and juveniles (Schaumburg et al., 2016).

The purpose of this work was to induce the synthesis of Vtg in male *Salvator merianae* through the administration of exogenous estrogens and to propose its plasma determination as a potential biomarker of contamination by xenoestrogens in agricultural areas of Argentina.

MATERIALS AND METHODS

Animals and Conditions for the Study

Adult individuals from the *Salvator merianae* lizard breeding facility at the Facultad de Agronomía, Zootecnia y Veterinaria, Universidad Nacional de Tucumán, located in the province of Tucumán, northwest Argentina (26º51´S and 65º17´W), were used in this study. The lizards were housed outdoors in enclosures bordered by masonry walls 1.2 meters high. Each enclosure was equipped with shelters of dry grass and shade, providing a surface area of 2 m^2 per individual, in accordance with the recommendations of Manes (2016). Water troughs with potable water and ad libitum food were provided, consisting of a specially formulated diet of chicken heads and feet (Vega Parry and Manes, 2000).

Each animal was individually tracked using a subcutaneous radio frequency identification device with a transponder (ID-100A) and electronic reader (Trovan, Ltd., Electronic Identification Systems, East Yorkshire, UK).

Experimental Design

A study was conducted with 18 adult lizards divided into three groups: six males treated with 17 β-estradiol (treated males' group), six females in vitellogenesis confirmed by ultrasound (positive control), and six untreated males (negative control). Female specimens had a snout-vent length of 35 cm or more, while male specimens were 39 cm or more.

The males in the treated group were administered an intramuscular injection of 17 β-estradiol at a dose of 1 mg/kg body weight every three days for two weeks, following the protocol of Sifuentes-Romero (2006). In all groups, weekly blood samples (1.5–2.0 ml) were collected from the caudal vein using a heparinized syringe. Aprotinin (Sigma, A1153-5 MG) was added at a concentration of 0.5 mg/ml to the samples to prevent proteolysis. The samples were centrifuged at 2500 rpm for 15 minutes, and the plasma was stored at -20°C until further processing.

All experiments have been examined and approved by the Ethics Committe of Consejo de Investigaciones de Universidad Nacional de Tucumán (CIUNT). Approved protocol number 086-2023.

Determination of Triglycerides and Plasma Vitellogenin

To determine triglyceride values, plasma samples were processed using the glycerol phosphate oxidase technique (GTLab790220) and read using a BECKMAN UV/Visible spectrophotometer, model DU 530.

Protein separation by electrophoresis on sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) was performed according to the method of Laemmli (1970). Known amounts of protein per lane were seeded together with a wide-range molecular weight marker (Thermo Scientific, 26630). Samples were treated with solubilising solution (1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 0.1% bromophenol blue). Electrophoretic runs were performed at 200 V with a constant amperage of 0.02 A at a temperature of 4ºC. Gels were stained with Coomassie Brilliant Blue R-250.

Saline Precipitation of Vitellogenin

A partial purification protocol for Vtg via saline precipitation was employed, based on the method by Wiley, Opresko, Wallace (1979), with subsequent modifications by Rey et al. (2006). Aliquots of 200 μl of the sample were mixed with 800 μl of 20 mM EDTA-Na2 (Ethylenediaminetetraacetic acid) (pH 7.7) and 46 μl of 0.5 M $MgCl_2$. The mixture was centrifuged at 2500 rpm for 15 minutes at 4ºC. The supernatant was discarded, and the precipitate, containing the presumptive Vtg, was redissolved in 80 μl of a buffer consisting of 50 mM Tris-HCl and 1 M NaCl (pH 7.5). This solution was centrifuged again at 2500 rpm for 30 minutes at 4ºC to remove impurities. The supernatant, containing the dissolved Vtg, was precipitated by reducing ionic strength with the addition of 1 ml of distilled water, followed by centrifugation for 15 minutes at 4ºC. The supernatant (A) was separated, and the precipitate was dissolved in 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.5). It was then centrifuged at 13000 g for 10 minutes at 4ºC to discard the lipid fraction at the surface. Finally, the resultant solution was mixed with the previously obtained supernatant (A). The resulting precipitates were analyzed by SDS-PAGE.

Immunological Identification of Vitellogenin

For the immunohistochemical identification of Vtg, liver samples were obtained from an estrogenized male 12 days after the last administration

of estradiol. For the animal's anesthesia, a combination of diazepam (2.5 mg/kg) and ketamine (25 mg/kg) was administered intramuscularly. Subsequently, euthanasia was performed via intracardiac injection of sodium pentobarbital at a dose of 100 mg/kg, following the protocols established by Baer (2006) and HSUS (2013). The liver samples were fixed in a 4% formaldehyde solution and processed according to standard histological technique procedures. The obtained histological block was sent to the Instituto de Salud y Ambiente del Litoral (ISAL), where the immunohistochemistry was performed using a specific anti-vitellogenin antibody from caiman.

RESULTS

Plasma Triglyceride Levels

Drastic increases in plasma triglyceride levels were observed in individuals treated with 17 β-estradiol during the first week of treatment, reaching extremely high average values of 2905 ± 572.76 mg/dl. These levels decreased during the second week of treatment to an average of 1255 ± 502 mg/dl, but remained significantly elevated compared to the baseline values observed in the control males, where triglyceride levels ranged from 28 to 135 mg/dl. In reproductive females (positive control), a notable increase in plasma triglyceride levels associated with the vitellogenic process was recorded. The lowest values were observed during the previtellogenic stage, at 93.21 ± 18.4 mg/dl, similar to those of the control males. The maximum values were recorded during full vitellogenesis, averaging 402.05 ± 18.4 mg/dl, although they were notably lower than the levels observed in the treated males. In all cases, the increase in plasma triglycerides coincided with a change in the macroscopic characteristics of the plasma. In treated individuals, the collected plasma changed from a clear amber and translucent appearance before treatment to a whitish color, opaque appearance, and viscous consistency at 12 days post-treatment.

Determination Plasma Vitellogenin

The administration of 17 β-estradiol induced the expression of a monomeric protein with a molecular weight of 250 kDa, identical to the molecular weight of the protein observed in the plasma of females during vitellogenesis. This protein remained present in the plasma throughout the entire treatment period. In contrast, this protein was undetectable in untreated males (Figure 1).

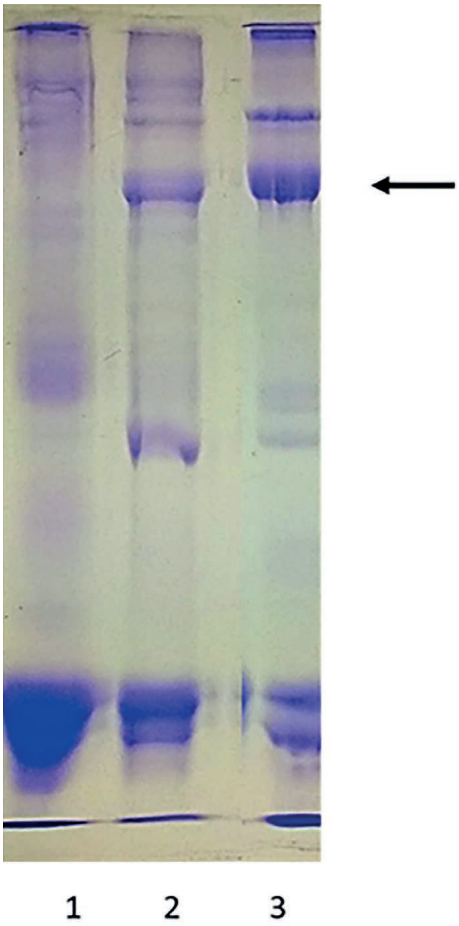

Figure 1. SDS-PAGE (6%) of plasma samples. Lane 1: plasma sample from an untreated male Salvator merianae; Lane 2: plasma sample from a male treated with 17 β-estradiol after the second week of treatment; Lane 3: positive control (plasma from vitellogenic females). The arrow indicates the presence of vitellogenin (Vtg). Coomassie Blue staining.

Saline Precipitation of Vitellogenin

Salt precipitation using EDTA-Na_2/$MgCl_2$ proved effective for the selective separation of Vtg in *S. merianae*, both in females during vitellogenesis and in treated males. As illustrated in Figure 2, this methodology facilitated the release of most co-associated plasma proteins, leaving the sample enriched with Vtg after precipitation.

Immunological Identification of Vitellogenin

The application of immunohistochemical techniques to the liver of treated males confirmed the presence of cytoplasmic aggregates of Vtg in the hepatic tissue, observed 12 days after induction with 17 β-estradiol (Figure 3).

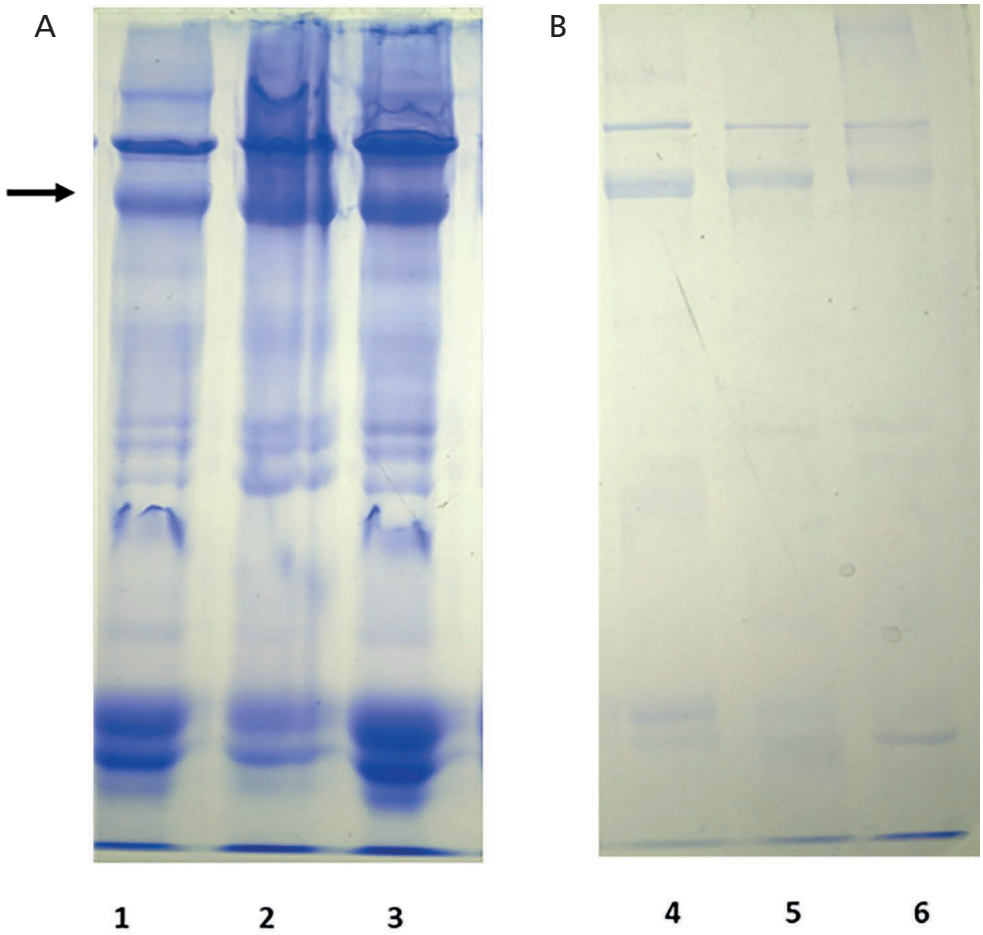

Figure 2. SDS-PAGE (6%) of plasma samples. A) Electrophoretic separation before salt precipitation: Lanes 1 and 2 contain plasma from males Salvator merianae treated with 17 β-estradiol; Lane 3 contains plasma from a vitellogenic female. The arrow indicates vitellogenin (Vtg). B) Salt precipitation of the same plasma samples: Lanes 4, 5, and 6 show the results post-precipitation. Coomassie Blue staining.

DISCUSSION

This study has effectively demonstrated the induction of Vtg synthesis in male *Salvator merianae* through the administration of 17 β-estradiol, setting a significant precedent in research on environmental contamination and its impact on terrestrial fauna. The presence of Vtg, a biomarker for xenoestrogen contamination, in the treated males not only validates the use of this marker in terrestrial reptiles but also highlights the sensitivity of these animals to endocrine disruptors (Arukwe and Goksøyr, 2003; Guillette and Gunderson, 2001).

Some plasma metabolites, such as calcium, phosphorus, and triglycerides, are associated with Vtg and can be considered indirect markers of vitellogenesis. Increases in the plasma levels of these metabolites have been documented during ovarian maturation in various species, such as the snakes *Sistrurus catenatus* (Allender, Mitchell, Phillips, Gruszynski, Beasley, 2006; Slater, Faust, Hileman, Lavin, 2017), the tuatara *Sphenodon punctatus*

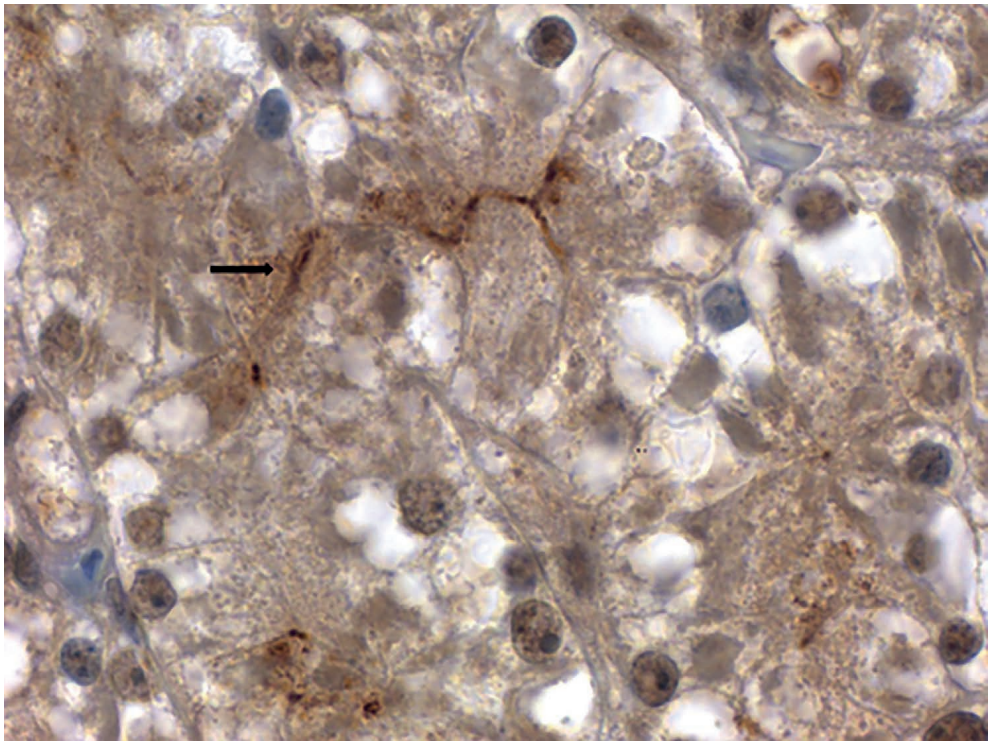

Figure 3. Expression of vitellogenin in the liver of Salvator merianae. Immunohistochemical detection was performed using the LETH-VTG antibody, visualized with 3,3′-diaminobenzidine (DAB) and counterstained with Mayer's Hematoxylin. The image shows a liver section from a male Salvator merianae treated with 17-β-estradiol. Arrows indicate cytoplasmic aggregates of hepatic vitellogenin (Vtg). Magnification: 100X. Immunohistochemistry conducted at the Instituto de Salud y Ambiente del Litoral (ISAL) following the methodology described by Rey et al. (2006).

(Brown, Cree, Chambers, Newton, Cockrem, 1991), and the turtles *Caretta caretta* (Deem et al., 2009; Kawazu, Maeda, Kino, Oka, 2013) and *Chelonia mydas* (Hamann, Limpus, Whittier 2002). Specifically in *Salvator merianae*, previous studies have demonstrated a significant difference in plasma triglyceride levels between females that experienced vitellogenesis and those that did not (Sánchez-Loria, García-Valdez, Arce, Chamut, 2021). The results of the present study highlight a considerable alteration in plasma triglyceride levels in treated males, suggesting a profound metabolic and physiological disruption induced by exposure to exogenous estrogens. This response is notably more pronounced in induced males than in vitellogenic females, which would indicate an adverse impact on the animal's homeostasis and health due to hormonal imbalance (Finn, 2007).

Research on xenoestrogenic biomarkers in terrestrial vertebrates is still limited, exacerbated by the scarcity of specific commercial antibodies and insufficient cross-reactivity among different species with the available antibodies (Selcer et al., 2001, 2006, and Sánchez-Loria Olga Luz pers. comm., 2024) . In this study, the immunological identification of Vtg in the hepatic tissue of treated males provides clear evidence of induced protein expression, supporting the known mechanisms of action of estrogens

and xenoestrogens in vertebrates (Thomas-Jones et al., 2003). Although additional studies on hepatic architecture and the effects of commercial pesticides on Vtg are still required, similar findings can be expected in the South American lizard *Salvator merianae*. This projection underscores the need to expand research to better understand the influence of environmental contaminants on the biology of this species.

Adapting techniques such as salt precipitation and immunohistochemistry to detect Vtg in reptiles introduces an innovative methodological approach for future research in ecotoxicology (Rey et al., 2006; Verderame et al., 2016). The salt precipitation protocol with $MgCl_2$/EDTA, initially designed by Wiley and collaborators (1979), has proven to be highly effective for the selective precipitation of Vtg in vertebrates, yielding outstanding results in species such as *Xenopus laevis* (Palmer, Huth, Pieto, Selcer, 1998), *Trachemys scripta* (Selcer and Palmer, 1995), *Sphenodon punctatus* (Brown, Carne, Chambers, 1997), *Chelonia mydas* (Sifuentes-Romero et al., 2006), and *Atractosteus tropicus* (Martínez García, Hernández Vidal, Hernández Franyutti, Contreras Sánchez, Álvarez González, 2013), highlighting in all cases a high degree of Vtg purity. Although many researchers currently choose to combine salt precipitation with gel filtration or ion-exchange chromatography techniques for a more refined purification of Vtg (Rey et al., 2006; Sifuentes-Romero et al., 2006).Tada et al. (2004) argue, in their study with turtles *Chinemys reevesii*, *Mauremys japonica*, and *Trachemys scripta*, that Vtg purified using $MgCl_2$-EDTA precipitation achieved results similar to those obtained by additional purification methods and consider that, for turtles, $MgCl_2$-EDTA precipitation without complementary techniques is sufficient to purify Vtg. Based on our evidence, we propose that $MgCl_2$-EDTA precipitation, with minor modifications, constitutes an economical and effective technique for the partial purification of this protein in *Salvator merianae*.

This study has demonstrated that the administration of 17 β-estradiol in male *Salvator merianae* induces the synthesis of Vtg, leading us to propose the potential utility of this species as a biomarker for xenoestrogen contamination. Increases in plasma metabolites underscore a significant metabolic and physiological disruption in these reptiles, reflecting their high sensitivity to endocrine disruptors. This advancement not only enhances our understanding of the environmental impact on *S. merianae*, but also underscores the need to integrate innovative approaches to protect biodiversity from emerging contaminants.

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