


Sex steroid receptors in the ovarian follicles of the lizard *Sceloporus torquatus*

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Research Article

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Summary

Estradiol and progesterone have been recognized as important mediators of reproductive events in the female mainly via binding to their receptors. This study aimed to characterize the immunolocalization of the estrogen receptor alpha (ER α), estrogen receptor beta (ER β) and progesterone receptor (PR) in the ovarian follicles of the lizard *Sceloporus torquatus*. The localization of steroid receptors has a spatio-temporal pattern that depends on the stage of follicular development. The immunostaining intensity of the three receptors was high in the pyriform cells and the cortex of the oocyte of previtellogenic follicles. During the vitellogenic phase, the granulosa and theca immunostaining was intense even with the modification of the follicular layer. In the preovulatory follicles, the receptors were found in yolk and additionally, ER α was also located in the theca. These observations suggest a role for sex steroids in regulating follicular development in lizards, like other vertebrates.

Introduction

The hypothalamus–hypophysis–gonad axis orchestrates the seasonality of reproduction via intracrine, paracrine, and endocrine signalling (Norris, 2018). In female lizards, as in other vertebrates, sex steroids are considered the major hormones involved in the reproductive events (development, gametogenesis, mating behaviour, and courtship among others; Edwards, 2005; Sánchez and Smitz, 2012; Ramírez-Pinilla *et al.*, 2015). Generally, estradiol (E₂) and progesterone (P₄) have a dynamic behaviour depending on the stage of the reproductive cycle in vertebrate females (Amey and Whittier, 2000; Rhen *et al.*, 2000; Edwards and Jones, 2001; Wack *et al.*, 2008; Kummrow *et al.*, 2010).

Although genomic (receptor-mediated) and non-genomic signalling is recognized for sex steroid actions in other vertebrates, the second has been scantily studied in Squamata (Contrò *et al.*, 2015; Yatsu *et al.*, 2016; Yaşar *et al.*, 2017). In genomic signal transduction, sex steroids exert their effects by activating nuclear receptors that bind to their promoter regions and modulate gene expression (Sato *et al.*, 2016; Garg *et al.*, 2017). In general, sex steroid receptors are conformed by a N-terminal region that stimulates gene transcription (domain A/B), a DNA binding domain (C), a nuclear localization signal (D), and a ligand-binding region (E) (Medina-Laver *et al.*, 2021). These receptors have shown high-functional plasticity due to their number of combinations in modulators, levels of activity, and responses (Jacobsen and Horwitz, 2012).

In female lizards, E₂ promotes follicular development and the synthesis of vitellogenin (VTG) in the liver. This hormone increases with the onset of vitellogenesis and ovulation in various species (Jones, 2011; Ramírez-Pinilla *et al.*, 2015; Barbosa-Moyano *et al.*, 2020). The ovary is the principal source of E₂ to exert its multiple effects via receptors in different tissues (Contrò *et al.*, 2015). The estrogen receptor (ER) has two isoforms: the alpha (ER α) and beta (ER β). These receptors were reported in many lizard species such as *Bradypodion pumilum*, *Plestiodon finitimus*, *Gekko japonicus* (Yatsu *et al.*, 2016), and *Hemodactylus flaviviridis* (Tripathy and Rai, 2017). At least one receptor has been detected in *Podarcis sicula* (Verderame and Limatola, 2010), *Anolis carolinensis* (Beck and Wade, 2009), and *Calotes versicolor* (Inamdar *et al.*, 2015). In *P. sicula*, ER α is involved in vitellogenesis (Verderame and Limatola, 2010), while in *H. flaviviridis*, it enhances aromatase activity via gonadotropin hormones (Tripathy and Rai, 2017).

P₄ is another hormone with pleiotropic effects in females (Cox, 2020). This hormone also modulates vitellogenesis and ovulation (Crews, 2005; Kabelik *et al.*, 2008; Al-Amri *et al.*, 2012). However, its main roles are pregnancy maintenance and parturition in viviparous species due to

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its influence on uterine epithelium proliferation, uterine contractility inhibition, and downregulation of follicular development by modification of gonadotropin secretion and antiestrogenic capacity (Bonnet *et al.*, 2001; Girling *et al.*, 2002; Martínez-Torres *et al.*, 2003; Custodia-Lora *et al.*, 2004; Holmes and Cree, 2006; Adams *et al.*, 2007). The principal source of P_4 is the corpus luteum (Hosie *et al.*, 2003; Martínez-Torres *et al.*, 2003, 2010, 2014). This progestogen shows low concentrations during follicular development, a peak before ovulation and the highest concentrations near oviposition or during embryo development (Jones, 2011; Ramírez-Pinilla *et al.*, 2015; Barbosa-Moyano *et al.*, 2020).

Two forms of progesterone receptor (PRA and PRB) have been reported and are associated with the stage of the reproductive cycle in reptiles (Custodia-Lora and Callard, 2002; Biazik *et al.*, 2012; Hammouche *et al.*, 2012; Motta *et al.*, 2020). The difference between PRA and PRB resides in an extra-domain with activating function (AF) in the PRB (Garg *et al.*, 2017). In the lizard *Uromastix acanthinura*, PRA and PRB are positively influenced by E_2 , which denotes heterogeneity in responses among species (Hammouche *et al.*, 2012). In the turtle *Chrysemys picta*, RPA expression inhibits vitellogenin synthesis (Duggan and Callard, 2003), whereas RPB promotes it (Giannoukos and Callard, 1995). In Squamata, the diminution of uterine contractions may be regulated via RPA by limiting the activity of proteins involved in cellular adhesion (Custodia-Lora and Callard, 2002; Lovern, 2011; Hoss *et al.*, 2014; Blackburn, 2018; Refsnider *et al.*, 2019).

Recent research on lizard reproductive biology has evaluated changes in sex steroid concentration and the molecular components involved in these processes (Motta *et al.*, 2004, 2020; Tamaro *et al.*, 2008; Tripathy and Rai, 2017). However, most of the studies involve oviparous species. Here we propose the spiny collared lizard (*Sceloporus torquatus*), a viviparous lizard with distribution in the central region of México (Guillette Jr. and Méndez-de la Cruz, 1993), to provide more information on the reproductive biology of lizards. The females begin vitellogenesis in summer, ovulate in autumn, are pregnant until the following spring, and give birth in April–June (Guillette and Méndez-de la Cruz, 1993; Ortiz *et al.*, 2001; Cruz-Cano *et al.*, 2021). This work aimed to characterize the presence of estrogen receptors (α and β) and PR in the ovarian follicles (previtellogenic, vitellogenic and preovulatory) of the lizard *S. torquatus* and relate them to the concentration of these sex steroids.

Materials and methods

Animals

Fifteen sexually mature females (snout–vent length > 70 mm) of *S. torquatus* were collected in the Sierra de Guadalupe State Park (19° 37'N, 99°12'W), State of México. The collecting scientific license was granted by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT; SGPA/DGVS/00579/17 and SGPA/DGVS/02921/19). The collection was carried out during follicular development (June to November).

Sample collection

Immediately after the capture, we collected a 100- μ l blood sample by cardiac puncture with a heparinized syringe to measure sex steroid concentrations. The samples were taken to the Reproductive Biology Laboratory at 4°C and were centrifuged to

store the plasma until hormonal quantification. We anaesthetized females and performed a paramedian celiotomy to extract the ovarian follicles (Cruz-Cano *et al.*, 2021). Then, the tissues were fixed with Bouin solution, and processed for routine histological technique and paraffin embedded.

As soon as the lizards fully recovered, we returned them to the site where they were collected.

E_2 and P_4 quantification

Hormone quantification was performed according to Cruz-Cano *et al.* (2021). We used ASCENT software to obtain the values of the sex steroids. E_2 was measured using a commercial immunoenzyme assay kit (Abcam ab108667, USA). The sensitivity was 8.68 pg/ml, according to the manufacturer's protocol. The inter-assay and intra-assay coefficients of variation were 5.9% and 7.2%, respectively. P_4 was quantified with a commercial radioimmunoassay kit (PROGEST-RIA, CISBIO). We used 125 I-labelled P_4 as a reactive tracer. The sensitivity of the assay was 0.05 ng/ml. The inter-assay and intra-assay coefficients of variation were 4.6% and 7.5%, respectively.

Immunohistochemistry (IHC)

We obtained 5- μ m sections from follicles of at least five lizards per stage (previtellogenesis, vitellogenesis, and preovulatory). The slides were deparaffinized in xylol, rehydrated through graded alcohols, and rinsed with distilled water. Immunohistochemistry was performed using the Vectastain Universal Quick Kit PK-8800 (Vector Laboratories Inc., Burlingame, CA, USA). Antigen retrieval was carried out with 0.1 M citrate buffer (pH 6.0) using heat treatment in a water bath set at 100°C for 20 min. Quenching of endogenous peroxidase was made with 0.3% H_2O_2 for 30 min and washed in phosphate-buffered saline (PBS) 0.1 M (pH 7.4). Background-staining was blocked with preincubation with a solution containing horse serum (1 drop) and bovine serum albumin (BSA) 5% in 2 ml of PBS for 30 min and rinsed with PBS. Sections were incubated overnight in a humidified chamber at room temperature with the polyclonal primary antibodies (rabbit anti-ER α , Thermo Fisher PA5-16440; anti-ER β , Thermo Fisher 51-7700; or anti-PR, Thermo Fisher PA5-94983, diluted 1:100). We incubated tissues with a biotinylated Pan-Specific Universal antibody for 15 min and washed them twice with PBS. Then, the slides were incubated with streptavidin/peroxidase complex for 10 min. We used 0.03%, diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) in PBS and 0.1% H_2O_2 to reveal the peroxidase activity. The sections were mounted in ClearVue (Thermo Fisher). The specificity and effectiveness of the IHC were assessed with two controls, a negative by omitting the primary antibody and a positive with mouse ovaries.

Statistical analysis

The values of E_2 and P_4 were assessed for normality with the Shapiro–Wilk test on raw data. We checked the homogeneity of the variances with Levene's test. To determine the changes in the sex steroid concentrations among stages, we performed an analysis of variance (ANOVA) followed by a Holms–Sidak multiple comparison method. All the statistical analyses were performed with the significance level $\alpha = 0.05$ using the SPSS program for macOS (Version 22).



Figure 1. Morphology of ovarian follicles in the lizard *S. torquatus*. Previtellogensis: (A) The follicles have a homogeneous ooplasm (o) with a cortex in the oocyte periphery (c); the granulosa is a multilayered structure with polymorphic cells small (s), intermediate (i) and pyriform (p). The zona pellucida (z) and a fibrous theca (t) with blood vessels (v) are clearly distinguishable. Vitellogensis: (B) The oocyte contains yolk granules (y), and the granulosa is comprised by a monolayer of small cells (s), the zona pellucida (z) is thicker, the theca (t) has blood vessels (v) and mast cells (mc), and the small cells (s) begin to flatten. Preovulatory: (C) The zona pellucida (z) decreases in thickness and the small cells (s) are flattened in a single layer, also there is a decrease in the blood vessels (v). (A–C Masson's Trichrome stained.)

Results

Ovarian follicles morphology

The morphology of the ovarian follicles presented changes associated with the stage of the reproductive cycle. The granulosa layer in the previtellogenic phase was composed of small, intermediate, and pyriform cells (Figure 1A). When the vitellogenesis began, the regression of the pyriform and intermediate cells reduced the height of the granulosa layer (Figure 1B). When the follicles reached the preovulatory stage, the small cells flattened, and the zona pellucida thickened (Figure 1C).

Sex steroid concentrations

Sex steroid concentrations changed depending on the stage of follicular development (Table 1). E_2 concentrations were low in the previtellogenic (254.61 ± 26.88 pg/ml) and vitellogenic stage (241.84 ± 40.71) and significantly higher in the preovulatory phase (625.95 ± 54.80) (ANOVA $F_{(2,12)} = 26.522$, $P < 0.001$). For P_4 , there were no statistical differences among the stages studied ($P > 0.05$ in all cases) and ranged from 1464 to 1790 pg/ml.

Sex steroid receptors

The previtellogenic follicles from June to September and November showed an intense mark of $ER\alpha$ in pyriform cells (Figure 2A) and granular structures in the cytoplasm of pyriform cells and the cortex (Figure 2B). However, these follicles showed signs of atresia, such as thickening of the theca, abundant small cells, and absence of a clearly defined zona pellucida.

Therefore, we studied the previtellogenic follicles obtained in October that showed a weak mark in the cytoplasm of the granulosa and ooplasm with a strong localization in some granules close to the cortex and some pyriform cells granules (Figure 3A). In addition, the $ER\beta$ was strong in the oocyte cortex, erythrocytes, and moderate in granulosa and theca (Figure 3D). For PR, the stain was intense in the pyriform cells and moderate in the oocyte cortex and some small cells (Figure 3G). With the beginning of vitellogenesis, the $ER\alpha$ staining was strong in the erythrocytes located in the blood vessels and weak in the oocyte cortex (Figure 3B). Conversely, $ER\beta$ was moderate in theca, granulosa cells, and oocyte's cortex (Figure 3E). The PR mark was strong during pyriform cells regression, and moderate in the oocyte cortex and cytoplasm of small cells (Figure 3H).

In the preovulatory stage, there is an evident reduction in the immunostaining of the three receptors. The $ER\alpha$ was intense in the cortex and some regions of the theca (Figure 3C). There was no $ER\beta$ expression in this stage (Figure 3F). Finally, the presence of PR was moderate in yolk (Figure 3I).

Discussion

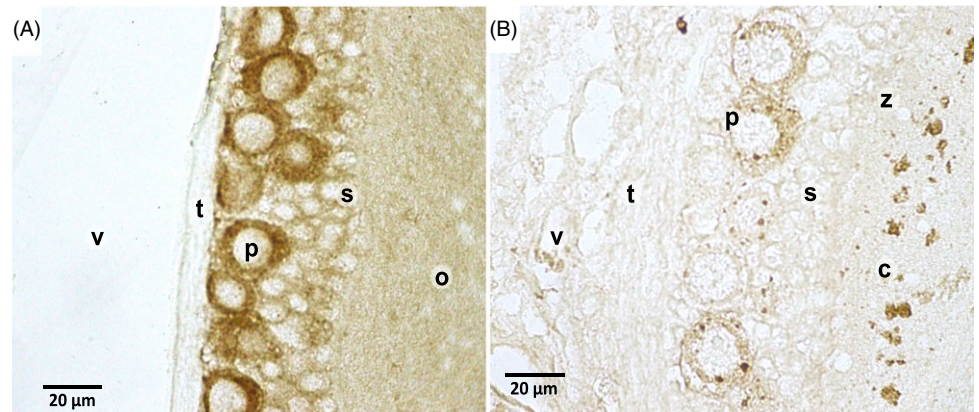
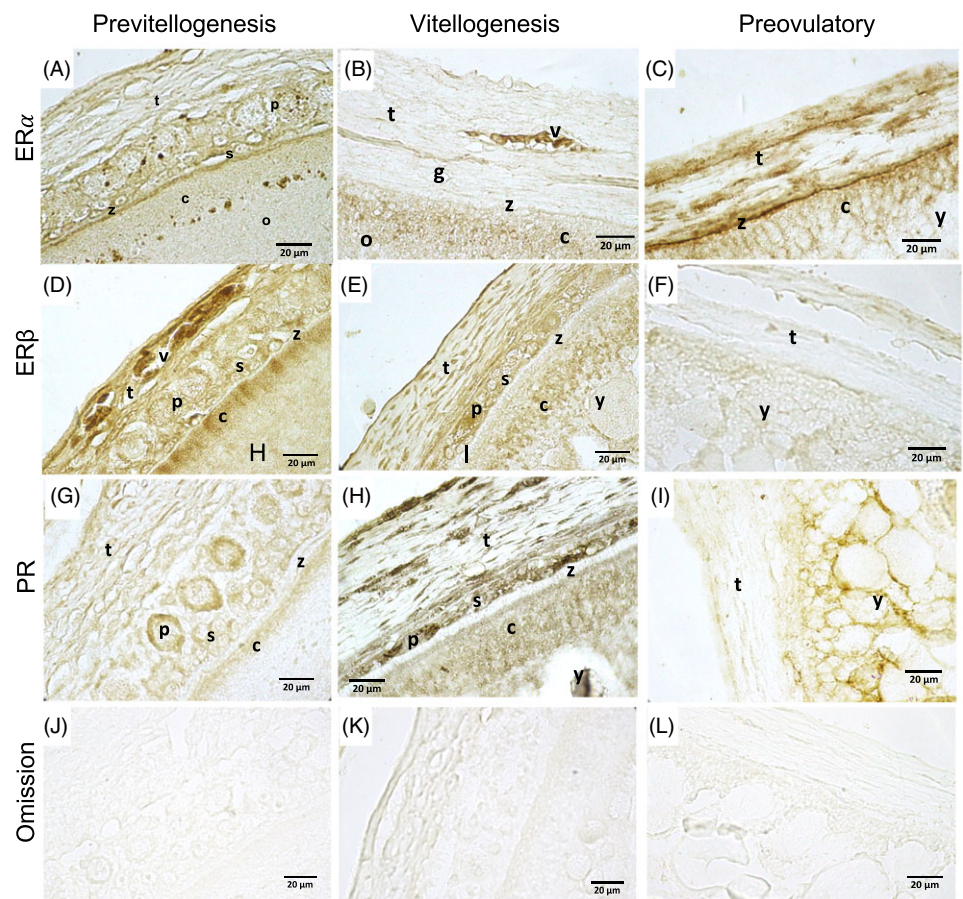
Sex steroid receptors are involved in multiple events in the reproductive biology of female vertebrates. They have different patterns of expression depending on the tissue and the reproductive status of the organism (Hammes and Levin, 2007; Jacobsen and Horwitz, 2012; Baker, 2019; Fuentes and Silveyra, 2019). The interaction of these hormones with their receptors is necessary to activate the expression of one or multiple genes that act on the phenotype (Cox, 2020). As they have cytoplasmic and nuclear localization in the cells of many tissues (Dressing *et al.*, 2011), they show high-functional plasticity due to their large number of combinations in modulators, levels of activity, and responses (Jacobsen and Horwitz, 2012). Numerous studies have reported their participation in the reproductive biology of female vertebrates (Hamilton *et al.*, 2017; Baker, 2019; Fuentes and Silveyra, 2019). However, there is limited information concerning their presence in lizards. In this study, we observed immunolocalization of $ER\alpha$, $ER\beta$, and PR in previtellogenic, vitellogenic, and preovulatory follicles of *S. torquatus*.

The ovarian follicles showed changes in their morphology as reported for other lizards (Uribe *et al.*, 1995; Raucci and di Fiore, 2010; Motta *et al.*, 2020). The more evident changes were the modification of the granulosa layer and the deposit of yolk inside the oocyte. The expression of steroid receptors changed according to the stage of follicular development and the cellular types registered. E_2 concentrations were significantly higher in the preovulatory stage. In addition, P_4 showed similar levels among the different phases of follicular development. The same behaviour is reported for *Sceloporus virgatus* (Weiss *et al.*, 2002), *Phrynosoma cornutum* (Wack *et al.*, 2008), *Chameleo calypttratus* (Kummrow *et al.*, 2010), *Salvator merianae* (Zena *et al.*, 2019) and *Mabuya* sp. (Barbosa-Moyano *et al.*, 2020). ER, and PR presence was not related to the sex steroid concentrations, as reported in the lizard *U. acanthinura* (Hammouche *et al.*, 2017).

We found that the ER labelling was mainly expressed in the granulosa layer and oocyte cortex. In the previtellogenic phase, $ER\alpha$ in the small cells could be associated with the proliferative

Table 1. Plasma sex steroid concentrations in different phases of the follicular development in the lizard *S. torquatus*

Follicular development stage	Sex steroid concentrations	
	17 β -Estradiol (pg/ml)	Progesterone (pg/ml)
Previtellogenic (<i>n</i> = 5)	254.61 \pm 26.88	1790.80 \pm 368.14
Vitellogenic (<i>n</i> = 5)	241.84 \pm 40.71	1592 \pm 214.35
Preovulatory (<i>n</i> = 5)	625.95 \pm 54.80*	1464.60 \pm 181.32

P* < 0.01 vs previtellogenic and vitellogenic phase.Figure 2.** Immunolocalization of ER α in atretic previtellogenic ovarian follicles. Previtellogenesis: (A) Immunostaining is moderate in the small (s) and intense in pyriform cells (p), a weak staining is detected in the ooplasm (o). (B) Intense ER α immunostaining in granules on the pyriform cells (p) and cortex (c).**Figure 3.** Immunolocalization of ER α (A, B, C), ER β (D, E, F) and PR (G, H, I) in the ovarian follicles. Previtellogenesis: (A) Intense ER α immunostaining in granules on the pyriform cells (p) and cortex (c). (D) Strong ER β immunostaining in cortex (c), erythrocytes located in the blood vessels (v) and moderate in granulosa (p, s) and theca (t). (G) Strong immunolabelling of PR in pyriform cells (p), moderate in oocyte cortex (c) and small cells (s). Vitellogenesi: (B) ER α intense immunostaining in erythrocytes located in the blood vessels (v) and weak in cortex (c). (E) ER β was moderate in theca (t), granulosa cells (s, p) and oocyte cortex (c). (H) Strong PR immunolabelling in regressed pyriform cells (p) and moderate in the oocyte cortex (c) and small cells (s). Preovulatory: (C) Intense ER α immunoreactivity in the cortex (c) and theca (t). (F) No evident ER β expression. (I) Moderate PR in the yolk (y). (J, K, L) omission controls.

activity to differentiate in a polymorphic granulosa (Kocanova *et al.*, 2010; Hamilton *et al.*, 2017). Also, its presence in the cytoplasm of the pyriform cells may indicate their role in the estrogen stimulation synthesis and secretion in previtellogenic follicles (Hammouche *et al.*, 2017; Fuentes and Silveyra, 2019), inducing aromatase activity at the beginning of the reproductive season (Tripathy and Rai, 2017). This steroidogenic capacity has been observed in the granulosa cells of previtellogenic and early vitellogenic follicles in *S. torquatus* (unpublished data).

For ER β , its hepatic expression remained constant in the reproductive cycle of the lizard *P. sicula* (Verderame and Limatola, 2010) and changed in the ovaries of *H. flaviviridis* (Tripathy and Rai, 2017). Both receptors' presence may be involved with the regression of pyriform cells in *S. torquatus* during vitellogenesis, as we observed an increased expression in atretic follicles.

Although previous studies have reported the presence of sex steroid receptors in diverse tissues on lizards (Young *et al.*, 1994; Beck and Wade, 2009; Verderame and Limatola, 2010; Hammouche *et al.*, 2012; Inamdar *et al.*, 2015; Yatsu *et al.*, 2016), there is limited information available on this group, as their expression was mainly cytosolic, further studies must consider signalling via non-genomic mechanisms. ER presence in the cytosol is associated with an unbound state that allows the translocation between the nucleus and cytoplasm (Kocanova *et al.*, 2010).

The expression of PR in *S. torquatus* showed a different spatial-temporal pattern expression. However, identifying the behaviour of each isoform could shed light on its role in the phases of follicular development. In reptiles, both isoforms have been reported and are associated with the stage of the reproductive cycle (Custodia-Lora and Callard, 2002; Biazik *et al.*, 2012; Hammouche *et al.*, 2012; Motta *et al.*, 2020). During follicular development, PR could stimulate the proliferation in the germinal beds, recruitment, and transition to the primary oocyte (Custodia-Lora and Callard, 2002; Jones, 2011; Holding *et al.*, 2014; Ramírez-Pinilla *et al.*, 2015; Duarte-Méndez *et al.*, 2018; Motta *et al.*, 2020). As the follicular development progressed, we observed an increase in immunolabelling intensity. A similar effect was observed in *Uromastix acanthinura*, where E₂ positively influenced PRA and PRB (Shao *et al.*, 2003; Hammouche *et al.*, 2012). In the turtle *Chrysemys picta*, PRA expression inhibits VTG synthesis (Duggan and Callard, 2003) whereas PRB promotes it (Giannoukos and Callard, 1995). In the preovulatory stage, PR presence can regulate ovulation and caspase 3 activity (Shao *et al.*, 2003; Hammouche *et al.*, 2017). Therefore, the differences in the events may be mediated by each receptor in the different species.

Our observations indicate that granulosa cells can have different roles in oocyte maturation, depending on the sensitivity to diverse intraovarian factors. The immunolabelling of the sex steroid receptors studied in the granulosa cells and, particularly the pyriform cells, have similar behaviour as other receptors influencing gonadotropin secretion of bradykinin, c-kit, and stem cell factor (Singh *et al.*, 2008; Raucci and di Fiore, 2011). The above confirms their support role in transferring diverse components to the oocyte after their breakdown (Neaves, 1971; Taddei, 1972; Filosa and Taddei, 1976; Andreuccetti, 1992; Motta *et al.*, 1995; Aldokhi *et al.*, 2019).

In conclusion, our study revealed that the immunolabelling of ER and PR was more pronounced in the pyriform cells and oocyte cortex. These observations suggest that in females of *S. torquatus*, steroid receptors participate in the regulation of follicular

development as in other vertebrates. Those receptors have a spatio-temporal expression depending on the stage of the reproductive cycle.

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Competing interest. None

Ethical standard. The UNAM Bioethical Committee approved all experimental procedures.

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