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Martín Martínez-Torres
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Piña-Amado José Juan
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Juana Luis
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Mario Cárdenas León
Laboratorio de Hormonas Proteicas, Departamento de Biología de la Reproducción, Instituto de Ciencias Médicas y de la Nutrición Salvador Subirán, México DF, México

María Maricela Campos-Solórzano
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

María Eugenia Garín Aguilar
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Farmacobiología. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Carmen Álvarez-Rodríguez
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Corresponding Author:
Martín Martínez-Torres
Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala. Laboratorio de Biología de la Reproducción. Av. de los Barrios 1, Los Reyes Iztacala, CP54090, Tlalnepantla Estado de México, México

Effects of deluteinization on gestation and progesterone concentration in the viviparous Mexican lizard *Sceloporus grammicus* (Sauria: phrynosomatidae)

Martín Martínez-Torres, Piña-Amado José Juan, Juana Luis, Mario Cárdenas León, María Maricela Campos-Solórzano, María Eugenia Garín Aguilar and Carmen Álvarez-Rodríguez

Abstract
Aim: The corpus luteum is a transitory endocrine gland present in all vertebrate gravid females. Several authors have assigned a central role to the capacity to produce progesterone in egg retention during gestation and in the evolution of reptilian viviparity. However, its role in maintaining gestation in viviparous reptiles is currently under discussion. Here, we describe the effects of lutectomy on progesterone concentration and the maintenance of gestation in the temperate Mexican viviparous lizard *Sceloporus grammicus*.
Materials and Methods: For this purpose, sixteen pregnant females were assigned to three different groups: lutectomy, sham lutectomy, or non-surgical treatment, the latter used as a control group. Blood samples were obtained before surgery, as well as 24 hours and 8 weeks after the procedure to determine the effects of lutectomy on the concentration of progesterone and the maintenance of gestation.
Results: We found a significant increase in the plasma concentration of progesterone 24 h after surgery (lutectomized and sham lutectomized lizards); however, these progesterone levels dropped significantly after 8 weeks. There were no significant changes in this regard in the control group at equivalent times when compared with the other groups. Normal parturition and no abortion events were observed in any of the study groups.
Conclusion: These observations indicate that the corpus luteum, although capable of progesterone production, is not necessary for the maintenance of gestation nor for parturition. Moreover, these results suggest the existence of a secondary source of progesterone capable of maintaining gestation.

Keywords: Viviparity, pregnancy, ovary, reptiles, hormones

1. Introduction
The goal of this study was to determine the effects of lutectomy on the maintenance of gestation and progesterone plasma concentrations in the viviparous temperate lizard *Sceloporus gammicus.* The corpus luteum (CL) is a transitory endocrine gland present in vertebrate gravid females (Xavier, 1987) [39]. Several authors concur in that embryo retention in gravid reptiles is controlled by the capacity of CL to produce progesterone (P_4) (reviewed in Xavier, 1987 [39], Albergotti and Guillette, 2011) [1]. In oviparous lizards, there is a positive correlation between luteal function and longtime of the egg retention (Jones and Guillette, 1982) [22] since luteal regression occurs just before (*Sceloporus anneus*, Guillette and Jones, 1985) [16], or immediately after oviposition (*Calotes versicolor*, Varma 1970; *Uromastick hardwicki*, Arslan *et al.*, 1978) [37, 4]. Further, the surgical removal of the luteal tissue causes premature oviposition and diminished P_4 levels (*Sceloporus undulatus*, Roth *et al.*, 1973; *Cnemidophorus uniparens*, Cuellar, 1979) [34, 8]. However, viviparity requires that the period of the egg retention *in utero* be extended until the embryonic development is complete (Guillette, 1993) [18]. Moreover, there is no direct correlation between luteolysis and parturition in viviparous lizard's species, since luteal regression can occur at any time during pregnancy and differs among species Jones and Guillette, 1982; Xavier, 1987) [22, 39]. Further, the extirpation of either the ovaries or the CL in gestating viviparous lizards have different effects according

to species and pregnancy stage. In this regard, abortion can be induced as a result of lutectomy or ovariectomy if performed during early pregnancy in *Xantusia vigilis* (Yaron, 1972) [40] and *Sceloporus jarrovi* (Guillette, 1987) [17]. However, the same treatment in *Lacerta vivipara* (Panigel, 1956) [30], *Sceloporus cyanogenys* (Lien and Callard, 1968, Callard *et al.*, 1972) [24, 6], and *Barisia imbricata imbricata* (Martínez-Torres *et al.*, 2010) [28] does not result in abortion, but cause abnormal parturition (stillborn and delayed parturition). In other species, such as *Mabuya carinata* (Sekharappa and Devaraj-Sarkar, 1978) [35] and *Chalcides ocellatus* (Badir, 1968) [5], the extirpation of the luteal tissue or ovariectomy had no effect in gestation length or parturition. These data highlights that, at present, it is unclear if the CL participates in maintaining gestation in viviparous lizards.

Sceloporus grammicus is a viviparous temperate lizard with a wide distribution in México (Arevalo *et al.*, 1991), it is listed as least concern species (UICN, 2018). The populations in the highlands of the central plateau mountain regions present a fall reproductive cycle (Ramirez-Bautista *et al.*, 2004, 2005, Jiménez-Cruz *et al.*, 2005; Hernández-Salinas *et al.*, 2010) [31, 19]. Males begin their gametogenesis in May or June, with a maximal testicular volume reached in July, August, or September (according to the region they inhabit) (Guillette and Casas, 1980; Jiménez-Cruz *et al.*, 2005; Ramirez-Bautista *et al.*, 2005) [14, 32]; whereas in the females the vitellogenesis occurs between May-September, ovulation in October-November and the young birth in April, May or July (Ramirez-Bautista *et al.*, 2005; Jiménez-Cruz *et al.*, 2005; Ramirez-Bautista *et al.*, 2012) [21, 32]. The reproductive ecology of this species has been studied extensively (Guillette y Casas-Andrew, 1980; Ramírez-Bautista *et al.*, 2004; Jiménez Cruz *et al.*, 2005; Ramírez-Bautista; 2012) [21, 14], as well as histology follicular development, CL and placenta the *S. grammicus*; however, their reproductive endocrinology remains unapproached.

2. Materials and Methods

2.1. Experimental design

We collected adult females (snout-vent length: 59.0 ± 6.0 mm; body weight: 7.3 ± 2.1 g) (Fig. 1) of the viviparous lizard S. grammicus in Santa Ana Ixtlauaca (19°60'21" N, 99°90'53" W; 2,750 m altitude), State of México in early November 2014, late December 2016, and early January 2015 (License SGPA/DGVS/09K5-1028/06/14) and were toe-clipped for individual identification. At following day of capture were transported to laboratory and were released in a terrarium (2.5 m x 2.5 m x 5.0 m) located within the greenhouse of the Unidad de Morfología y Función, Facultad de Estudios Superiores Iztacala of Universidad Nacional Autónoma de México (UMF-FES Iztacala UNAM) (19°52'62" N, 99°19'01" W, altitude 2,240 m). The lizards were assigned into three groups at random: A) lutectomy (n = 7), B) sham lutectomy (n = 5) and C) control females (n = 6).

Fig 1: Gestant females of the viviparous lizard *Sceloporus grammicus*. A) dorsal view, B) ventral view.

2.2 Experimental procedure

One week after capture, the specimens assigned to either the lutectomy or sham lutectomy groups were anaesthetized with sodium pentobarbital (16.0 µg/10 g body weight) via IP; afterwards, a ventrolateral incision was performed. In the females subjected to lutectomy, the ovaries were exposed, all CL were surgically removed from each ovary and its total number registered. Additionally, two atretic vitellogenic follicles (AVF) were obtained from 3 females (Fig. 2). One CL and one AVF from each female were fixed in 10% buffered formalin and processed for routine histological methods; the remaining samples were embedded in tissue-teck and immediately frozen in a mixture of dry ice and acetone and stored at -40 ºC until the time for histochemistry analysis of Δ^{5-4}3β-hydroxyl steroid dehydrogenase (Δ^{5-4}3β-HSD) (Levy *et al.*, 1959) [23]. The specimens subjected to sham lutectomy were treated identically; however, the CL and AVF were not removed and only their number was registered. In addition, the embryo development stage was determined according to the development table for *Lacerta vivipara* (Dufaure and Hubert, 1961) [11].

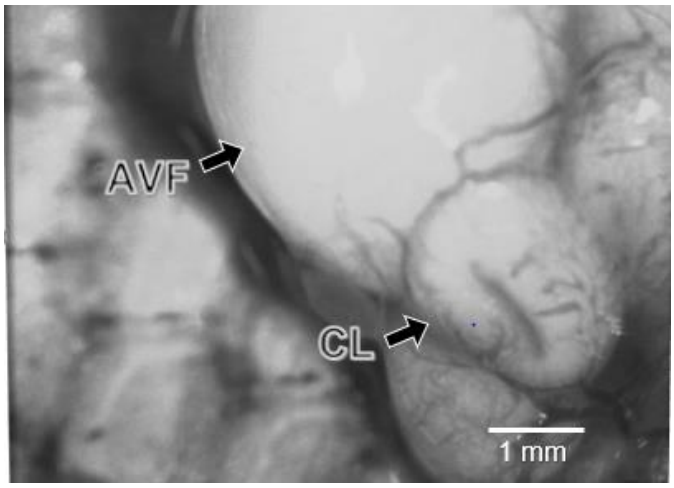

Fig 2: Corpus luteum (CL) and Atretic vitellogenic Follicle (AVF) of a pregnant female of *Sceloporus grammicus* in early gestation. 10x

Once the surgical procedure was completed, the lizards were sutured and each female deposited in individual terrariums within our laboratory, with free access to food and water (the diet included *Tenebrio* mealworms, *Galleria mellonela* wax worms, *Achaeta spp*. domestic crickets, and grasshoppers) during the following three days to allow for their recovery. On the day of surgery, each lizard received an injection of 5,000 IU of penicillin G, and of 500 µg streptomycin sulfate (IM) in the following two days. Lizards of the control group did not receive surgical treatment but were administered the antibiotics treatment regardless. After the recuperation period, all females were housed individually in terrariums (30 cm × 50 cm × 30 cm) and kept in the greenhouse of the UMF-FES Iztacala UNAM throughout the pregnancy period. All lizards had unrestricted access to water and food (mealworms, grasshoppers and crickets) and were kept under natural temperature and photoperiod throughout the experiment. The terrariums were scrutinized daily from the day of surgery until parturition to detect early or late embryo expulsion, or non-viable eggs. The dates of abortion, expulsion of non-viable eggs, and parturition, as well as the number of offspring, including live neonates, stillborn embryos or nonviable eggs, were registered. We also recorded whether parturition was

normal or abnormal and each lizard was dissected the following day after labor for to confirm CL absence in the ovaries, as well as the lack of embryos or eggs in the uterus of lutectomized females. The uteri of sham-lutectomized specimens were analyzed in the same manner; the number of CL was recorded, in addition to the state of the uterus, in the control specimens as well.

2.3. Histology
Previously fixed CL and AVF were processed according to routine histological technique and embedded in paraffin. Serial sections (7 µm thick) were obtained and stained using Harris hematoxylin and eosin method (Luna, 1968) [26]. and observed under the microscope to determine the histological characteristics for CL and AVF and development stage for CL according to Martínez-Torres *et al.*, (2003) [27].

2.4. Histochemistry
Corpora lutea and AVF samples embedded in tissue-tek were sectioned (12 µm thick) in a cryostat and processed according to Levy *et al.*, (1959) [23]. The freshly frozen sections were placed in medium containing 40 mg nicotine adenine dinucleotide and 20 mg nitro blue tetrazolium dissolved in 40 ml phosphate-buffered solution, pH 7.4 and incubated at 37 ºC for 1 h. As a substrate, we used 2 mg dehydroepiandrosterone (3b-hydroxyandrost-5-en-20-one) dissolved in 0.5 ml N, N-dimethylformamide. Control sections of mice ovaries and kidneys were incubated in a medium with or without substrate. All reagents were provided by Sigma Chemical.

2.5. Effect of lutectomy on progesterone concentration
One day before surgery (lutectomy or sham lutectomy), a blood sample of 80 ± 10 µl was obtained from each female by intracardiac puncture with a heparinised syringe. Blood samples were also obtained 24 hours and eight weeks after surgery. Control females were bled in similar manner as the experimental specimens. The blood samples were centrifuged immediately after collection (6000 rpm/1 min), and the plasma decanted and stored at - 40°C until the P_4 radioimmunoassay (RIA) was performed (Martínez-Torres *et al.*, 2003) [27]. All aliquots were obtained between 9:00 a.m. and 11:00 a.m. Plasma P_4 concentration was determined using a commercial kit (Coat-A-Count Progesterone, Diagnostic Products Corporation, Ca 90045). The assay was performed without duplicates, using 50 µl of plasma without prior dilution or extraction. ^{125}I-labelled P_4 was supplied as a reactive tracer. We used a P_4 specific antiserum, finding a steroid cross-reaction (relative to P_4, 100%) with androstenediol (non-detectable), corticosterone (0.9%), cortisol (0.03%), 11-deoxicorticoesterone (2.2%), 20α-dihydroprogesterone (0.2%), oestradiol (non-detectable), 17α hydroxyprogesterone (3.4%), 5b-pregnan-3a-ol-20-one (0.05%), 5a-pregnan-3, 20-dione (3.2%), pregnenolone (0.1%), and testosterone (0.1%). Inter- and intra-assay coefficients of variation were of 7.9 and 7.6%, respectively. The sensitivity of the assay was of 0.02 ng/ml. The values were obtained using the GAMBYT software. All experimental procedures were approved by the FES Iztacala-UNAM Bioethical Committee.

Fig 3: Intracardiac puncture to obtain the blood sample.

2.6. Statistical analysis
To determine the difference in litter size and number of live offspring, as well as to compare the number of CL among the groups, we used a one-way ANOVA. A two-way ANOVA for repeated measures was used to determine any significant changes in P_4 concentrations. Post-hoc tests were carried out using the Holm-Sidak method to determine differences; the significance threshold was set at $p< 0.05$ (Ambrose and Ambrose, 1987). All statistical analyses were performed with the Sigma Stat software (version 3.5 for Windows).

3. Results
The eggs of specimens subjected to lutectomy during early pregnancy (two females collected in early November) presented a “cicatricela” (Fig. 4A), and, according to Dufaure and Huber (1961) [11], these embryos were between stages 4-7. In the remaining lizards, surgery was performed during the second part of gestation, when the embryos were in stages 29 (two lizards) and 32 (one lizard) (Fig. 4B) (according to Dufaure and Hubert, 1961) [11].

Fig 4: Gestant females. A) Early pregnancy (➔: cicatricela). B) Mid gestation (➔ embryo stage 32).

3.1. Effects of lutectomy on the maintenance of gestation
We observed no abortion events in any of the lutectomized lizards; parturition was normal and occurred from the first week in March through April and May. A similar situation was observed in sham-lutectomized and control females. Further, we did not observe any significant difference in the number of neonates among groups (Table 1).

3.2 Histology
The CL samples obtained during late October and early November showed a histological appearance corresponding to

stage I of luteal development (Fig. 5A). This stage is characterized by an irregular cavity filled with erythrocytes and surrounded by the luteal cell mass (LCM) (Fig. 5A). The latter showed oval or rounded cells with acidophilic cytoplasm, a vesicular nucleus, a nucleolus (Fig. 5B), and a few cells with condensed nucleus. The CL obtained from lutectomized specimens during the second half of gestation showed typical histological characteristics of luteal tissue in regression. The luteal cell mass of CL obtained from females with embryos in stage 29 showed cells with slightly shrunken nuclei and without nucleolus, corresponding with stage I of regression (Fig. 5C), whereas CL from females with embryos in stage 32 showed CL in stage II of regression (cells with shrunken nuclei and connective tissue trabeculae). In the AVF, the theca contains many fibroblasts, blood vessels and acidophilic intercellular material. The oocyte lacks zona pellucida, and has been invaded by follicular cells, the cytoplasm presents some blood vessels and scanty vitelline residuals (Fig. 5D)

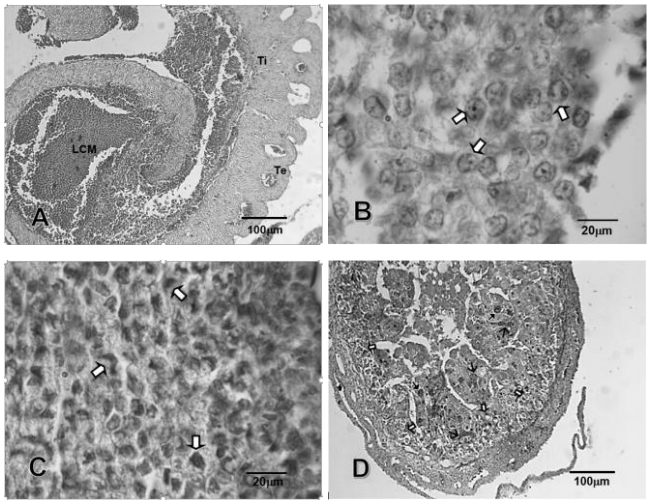

Fig 5: Histology of the corpus luteum and of an atretic vitellogenic follicle of *Sceloporus grammicus*. Histological sections of corpus luteum in stage I of development (A, B) and stage II luteolysis (C). A) Note that cavity surrounded by luteal cells mass (LCM), theca intern (Ti) and theca extern (Te). 10X. B) Note the vesicular nuclei with one or two nucleoli of the luteal cell mass.100X. C) Note the presence of nucleus slightly shrunken and without nucleolus.100X. D) Histological section of atretic vitellogenic follicle. Note the presence of blood vessels (➔), follicular cells and some vitelline residuals (➔)

3.3. Histochemistry

All CL obtained from early and middle pregnancy stages were positive. The highest activity of Δ $^{5-4}$3β-HSD was observed in the luteal cell mass, whereas lower activity was observed in the thecal tissue (Fig. 6A). Atretic vitellogenic follicles also showed low activity in both theca (Fig. 6B) and the oocyte cytoplasm (Fig. 6C), however, the formazan granules were observed mainly in the theca.

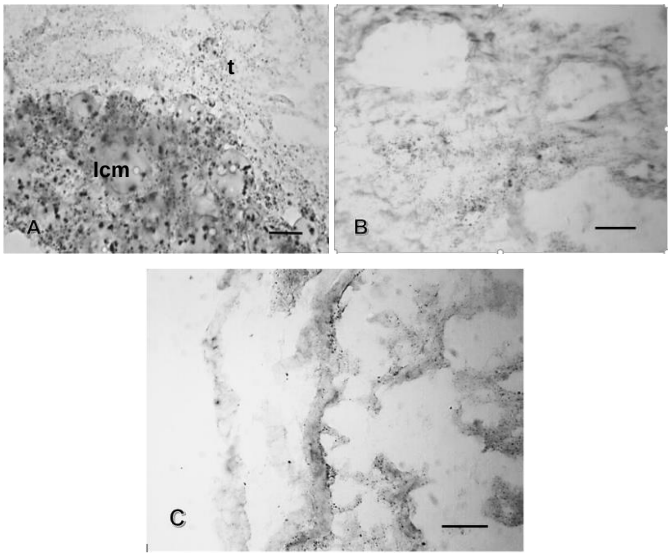

Fig 6: Histochemical activity of Δ $^{5-4}$3β-3β-hydroxyl steroid dehydrogenase. Corpus luteum in stage I of development (A). Observe the abundance and thickness of the formazan granules in the luteal cell mass (l cm), whereas in the thecal tissue the granules are sparse and thin (t). Atretic vitellogenic follicle (B, C). Note that formazan granules are scanty in oocyte (B) as in follicular wall (C). 100X.

3.4. Effects of lutectomy on progesterone concentration

The changes in plasma P_4 concentration in *S. grammicus* females (lutectomized, sham lutectomy, and control lizards) are shown in Table 1. We found a significant increment in plasma P_4 concentration in lutectomized lizards 24 h after surgery [F (4, 28) = 9.43, $p< 0.05$]. The values obtained previous to lutectomy were of 0.438 ± 0.09 ng/ml, whereas after surgery these were of 2.29 ± 0.63 ng/ml. However, the levels of this hormone diminished significantly eight weeks after lutectomy (0.16 ± 0.03 ng/ml) [F (4, 28) = 9.43, $p< 0.05$]. Regarding sham-lutectomized lizards, we also found a significant increment of P_4 concentration in plasma [F (4, 28) = 7.57, $p< 0.05$] (previous to surgery: 0.55 ± 0.18 ng/ml; 24 h after surgery: 1.11 ± 0.32 ng/ml). A significant decrement ($p< 0.05$) in P_4 levels was observed at 8 weeks after surgery as well (0.34 ± 0.12 ng/ml). In contrast, the control specimens did not show any significant changes in P_4 concentration in the evaluated time points (previous to surgery: 0.33 ± 0.13 ng; 24 hr. after surgery: 0.62 ± 0.36 ng/ml).

Table 1: Effects of lutectomy on maintaining of gestation and progesterone plasma concentration in the viviparous lizard *Sceloporus grammicus*

Treatment	Number of CL (means ± SD)	Number young (means ± SD)	Progesterone* (means ± SEM)		
			BS	24h AS	8 wAS
Lutectomy (n = 7)	4.8 ± 2.2	4.8 ± 2.4	0.44 ± 0.09[a]	2.29 ± 0.6[b]	0.16 ± 0.03[c]
Sham Lutectomy (n = 5)	4.5 ± 1.9	4.5. ± 2.8	0.55 ± 0.18[d]	1.11 ± 0.3[e]	0.34± 0.12[f]
Intact (n = 6)	5.2 ± 1.6	5.6 ± 1.9	0.34 ± 0.13[g]	0.62 ± =0.3[h]	0.36± 0.13[i]

*ng/ml
SD = standard deviation
SEM = standard error medium
BS = before surgery
AS = after surgery
wAS = weeks after surgery
[n] = number of females used.
[b] = the means was significantly higher (at $p< 0.05$) with respect to remaining times of pregnancy examined (whiting to the same group and among groups).
[e] = the mean was significantly higher (at $p< 0.05$) with respect to remaining times of pregnancy examined (whiting to the same group).

4. Discussion

Previous studies have evidenced the role of the luteal tissue as a major source of gestational P_4 in all reptiles (reviewed in Yaron, 1985; Xavier, 1987) [41, 39]. Since gestation is supported by this hormone, several authors have assigned a central role to this gland in the maintenance of the gravidity period (Yaron, 1985; Guillette, 1987; Callard *et al.*, 1992) [41, 17]. However, the role of the CL in the maintenance of gestation is yet in dispute since surgical deluteinization causes different effects according to species and gestation stage in which the surgical procedure is performed (Guillette, 1987) [17].

In this study, we found evidence that the CL is not essential for the maintenance of gestation in *S. grammicus* as lutectomy did not result in abortion. In this species, luteal development occurs during the first third of gestation whereas luteolysis occurs during the last two (Villagrán-Santa Cruz, 1989) [38], where luteal regression occurs gradually and ends around the time of parturition. In our study, we found that the luteal tissue of this species is positive for Δ^{5-4}3β-HSD activity in both early and middle stages of gestation, when the CL are in development and regressive stages, respectively. These observations suggest that, same as in other studied lizards (reviewed in (Xavier, 1987) [39], the CL of *S. grammicus* is capable of producing P_4 throughout gestation. Several authors have reported that both in viviparous lizards (*Tiliqua rugosa*, (Fergusson and Bradshaw, 1991; *Barisia imbricata*, Martínez-Torres *et al.*, 2010) [12, 28] and in snakes (*Thamnophis elegans*, (Highfill and Mead, 1975) [20] lutectomy results in a decreased concentration of P_4, either immediately (*e.g.* 24 h after surgery) or in the long term (*T. rugose*, Fergusson and Bradshaw, 1991; *T. elegans*, Highfill and Mead, 1975; *B. i. imbricata*, Martínez-Torres *et al.*, 2010) [12, 20, 28]. In contrast, we observed that in *S. grammicus* there is a significant increment (around five-fold when compared to the observed values prior to CL extirpation) of this hormone in the plasma of all lutectomized lizards 24 h after surgery. However, the plasma concentration of P4 decreased significantly after 8 weeks and fell below the levels observed prior to surgery, although not below the detection limit. In sham lutectomized lizards, we observed that the concentration of P_4 increased significantly as well; however, this increment was below the observed in lutectomized females. In control females, P_4 concentration did not change significantly in any of the evaluated time points. These results make clear that in *S. grammicus*, as previously suggested for other viviparous lizards (Fergusson and Bradshaw, 1991; Martínez-Torres *et al.*, 2010) [12, 28], exist a secondary source of this steroid capable to maintenance of gestation in absence of CL. Some authors have suggested that AVF may be an important ovarian source of P_4 during gestation (Guillette *et al.*, 1981; Villagrán-Santa Cruz, 1989) [15] [38]. Further, Villagrán-Santa Cruz (1989) [38] suggested that the AVF may be capable of P_4 production since it possesses a glandular appearance, proposing that these structures could assist the CL in the synthesis of this hormone. In our study, we found that the AVF obtained from lutectomized lizards showed low Δ^{5-4}3β-HSD activity when compared with the luteal tissue. These observations suggest that in this species the contribution of AVF towards P_4 production during gestation is minimal; therefore, we consider that the AVF does not participate in maintaining gestation in this lizard. On the other hand, Guillette *et al.*, (1981) [15] have proposed that the chorioallantoic placenta (CAP) could be a secondary source of P_4 in *S. jarrovi*, since the presence of this placental tissue coincides with CL regression. Painter and More (2005) [29] demonstrated that the CAP is capable of metabolizing steroids in this lizard. According to Villagrán-Santa Cruz (1989) [38], *S. grammicus* develops the CAP during the last stage of gestation (from stage 30 of embryonic development). In our study, we performed the lutectomy procedure during early and middle gestation stages, when the CAP would not have formed yet. Although it is unknown if the CAP is capable of synthetizing P_4 in this species, we do not discard the possibility that the placenta may participate in the production or metabolism of this hormone, in similar manner to *S. jarrovi* (Painter and More, 2005) [29] and *Chalcides chalcides* (Guarino *et al.*, 1998) [13].

In some species of viviparous lizards, such as *Xantusia vigilis*, (Yaron, 1972) [40] and *Sceloporus jarrovi* (Guillette 1987) [17], lutectomy causes abortion when performed during early pregnancy. However, the same treatment did not provoke abortion in either *Lacerta vivipara* (Panigel, 1956) [30], *Sceloporus cyanogenys* (Lien and Callard, 1968; Callard *et al.*, 1972) [24], or *B. i. imbricata* (Martínez-Torres *et al.*, 2010) [28]; however, it did cause abnormal parturition (stillborn young and delayed parturition). In other species, such as *Mabuya carinata* (Sekharappa and Debaraj-Sarkar, 1978) [35] and *Chalcides ocellatus* (Badir, 1968) [5], neither the extirpation of luteal tissue nor ovariectomy affected the length of gestation or parturition. In *S. grammicus*, we did not observe abortion or abnormal parturition in lutectomized lizards. In a previous paper, Martínez-Torres, *et al.*, (2010) [28] proposed that although the reptilian luteal tissue is capable of producing P_4, their relevance in the maintenance of gestation in the viviparous saurians has been overshadowed by the emergence of other structures capable of P_4 production, such as the adrenal gland (AG). Moreover, there is evidence on the capacity of the AG to produce P_4 in several viviparous squamata species, such as *Lacerta vivipara*, (Dauphin-Villemant and Xavier, 1985 [9] and Dauphin-Villemant *et al.*, 1990), *Thamnophis elegans*, (High fill and Mead, 1975) [20], and that it could be, therefore, participating in maintaining gestation in the absence of CL (*T. rugosa*, Fergusson and Bradshaw, 1991; *T. elegans*, Highfill and Mead, 1975; *B. i. imbricata*, Martínez-Torres *et al.*, 2010) [12, 20, 28], this is supported by the fact that the levels of plasma P_4 concentration never fell below the limit of detection despite being lutectomized. In contrast, adrenalectomy results in decreased plasma P_4 concentration, at non-detectable levels, in non-pregnant *T. elegans* snakes (Highfill and Mead, 1975) [20]. These evidences support the idea that the AG actively participates in P_4 production and maintaining gestation in viviparous lizards. Moreover, we consider that these observations demonstrate the necessity of evaluating the role of the AG in maintaining gestation in viviparous squamata.

5. Conclusion

1. Although the CL of *S. grammicus* is capable of producing P_4, it is not necessary for maintaining gestation or parturition.
2. There is a plausible possibility of a 'secondary' organ capable of producing P_4 and maintaining gestation.
3. It is necessary to re-evaluate the participation of the corpus luteum in maintaining gestation in viviparous saurians.

6. Conflict of interest

The authors declare that there is no conflict of interest that could affect the integrity of the currently reported results. The corresponding author asserts that the co-authors have read the final manuscript, agree with its contents and consent to joining the list of authors of this paper.

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