

# Dimorphic protein expression for *Sox9* and *Foxl2* genes in the testicles and ovaries of the urodele amphibian: *Ambystoma mexicanum*

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## Abstract

*Ambystoma mexicanum* is an endemic salamander from Mexico. Due to its neotenic characteristics, it is considered an important model for regeneration and development studies. However, there is little research concerning the pattern of gene expression related to testicular and ovarian function. In this study, we analyse expression of the protein of the *Sox9*, *Foxl2* and *Vasa* genes, and  $\Delta^5$ -3 $\beta$ HSD enzyme activity in ovaries and testicles of *A. mexicanum*. Correlating morphology with the expression of *Sox9* and *Foxl2*, we find that these genes manifest sexually dimorphic expression. *Sox9* is expressed at higher levels in the testicle, whereas *Foxl2* is specific to the ovary. *Vasa* was detected in spermatogonia in the anterior region of the testis, and in the ovary, it was located in previtellogenic oocytes.  $\Delta^5$ -3 $\beta$ HSD was located in Leydig cells of the testicles and theca cells of the ovaries. In summary, the dimorphic expression of *Sox9* and *Foxl2*, and that of *Vasa* in the germ line are similar to that described for other vertebrates, suggesting that the molecular mechanisms related to ovarian and testicular function in *A. mexicanum* follow a similar expression pattern that is not altered by neotenic characteristics.

## KEYWORDS

*Ambystoma mexicanum*, *Foxl2*, *Sox9*, *Vasa*,  $\Delta^5$ -3 $\beta$ HSD

## 1 | INTRODUCTION

The *Ambystoma mexicanum* axolotl is a salamander endemic to Mexico that presents pedomorphic characteristics. This condition of retaining larval characteristics up until sexual maturity has made this axolotl a model organism for biological research, with importance for regeneration and embryonic development studies in general (Eisthen & Krause, 2012). However, despite the neotenic condition that this organism presents, little is known about its reproductive characteristics, particularly about the expression pattern of genes related to testicular and ovarian function and their relationship to the neotenic condition, characteristic of *A. mexicanum*.

In this regard, classic studies that deal with sexually reversed organisms have suggested that *A. mexicanum* females are heterozygous in terms of sex, as they have Z and W heteromorphic sex chromosomes, and the Z chromosome usually represents a male-determining chromosome. Thus, these genetic experiments have established a ZW-type sexual determination mechanism for *A. mexicanum* (Humphrey, 1945, 1957; Smith, & Voss, 2009). Based on the proposal that attests to this type of sexual determination mechanism, recently it has tried to identify specific regions in the W chromosome associated with sex, where an ATRX gene that is a paralogue of ATRW and an orthologous gene called MAP2K3 have been linked to sex, and proposed as markers for the

identification of the sex of axolotls (Keinath, Timoshevskaya, Timoshevskiy, Voss, & Smith, 2018).

Despite the fact that a sex gene or sex-determining genes in *A. mexicanum* have not yet been identified with exactitude, somatic genes conserved in vertebrates have also been related to the processes of sexual determination and differentiation, as well as to gonadal morphogenesis. In this way, a large number of genes related to gonadal sexual determination and differentiation, as well as genes involved in testicular and ovarian function, have been described in other vertebrates, mainly in mammals. Genes that play a more relevant role in maintaining the functionality of the adult testis and/or the ovary of vertebrates include the transcription factor *Sox9* (SRY-box 9). This gene is highly conserved throughout the evolution of vertebrates, including amphibians (Nagai, 2001). It is a member of the family of SOX transcription factors, occupies a domain within a group that manifest high mobility (HMG) to DNA and two transactivation domains (Harley, Clarkson, & Argentaro, 2003). Among mice, it has been described as being expressed when embryonic development initiates in the nuclei of Sertoli cells, as it is necessary for their differentiation, as well as for the formation and maintenance of seminiferous tubules in testicular morphogenesis (Quin and Bishop, 2005). In amphibians, *Sox9* has been studied extensively among several anuran species such as *Rana rugosa* (Takase, Noguchi, & Nakamura, 2000), *Xenopus laevis* (Osawa, Oshima, & Nakamura, 2005) and *Xenopus tropicalis* (El Jamil, Kanhoush, Magre, Boizet-Bonhoure, & Penrad-Mobayed, 2008), where it is apparent that during embryonic and larval development, this gene is expressed in both male and female gonads. However, following metamorphosis, when a testicle and an ovary have already been differentiated, its expression is detected only in the testicles (Flament, Chardard, Chesnel, & Dumond, 2011). In urodele amphibians, the expression of *Sox9* has been studied in the caudate amphibian *Pleurodeles waltl*, where it was observed that this gene is expressed in both sexes during larval development and only in the testis at the end of metamorphosis (Dumond et al., 2011). Subsequently, in adult stages it is once again detected in both sexes, so it has been suggested that *Sox9* may play a role during late gonadal differentiation. As it is expressed in the ovary, there is a suggestion that it plays a role in the functionality of the adult ovary of this newt (Dumond et al., 2011).

Another of the relevant genes in ovarian and testicular differentiation of vertebrates is the transcription factor *Foxl2* (forkhead box L2). *Foxl2* is one of the genes expressed in early development with a sexually dimorphic pattern, acting as a marker of ovarian differentiation in mammals and able to play a role in the differentiation of ovarian somatic cells and in the development or maintenance of folliculogenesis (Bertho et al., 2016; Cocquet et al., 2018). In Anuran amphibians such as *R. rugosa* and *X. laevis*, apparently *Foxl2*

transcripts are expressed in undifferentiated gonads of both sexes before the onset of sexual differentiation, its greatest expression being in females. To identify the cell type expressed by *Foxl2* in the *R. rugosa* ovary, protein expression, detected in somatic cells that surround the oocytes in ovaries just after metamorphosis, was analysed (Flament et al., 2011; Oshima, Uno, Matsuda, Kobayashi, & Nakamura, 2008). In the urodele amphibian *Andrias davidianus*, the expression of *adFoxl2* was greater in the ovary than in other tissues. During the development of the gonads, transcription of *adFoxl2* mRNA was significantly greater in the ovary than in the testicles and increased gradually with respect to age; thus, it appears that this gene plays a role in the development of the oocytes. *Foxl2* transcripts were detected in granulosa cells in the ovary and in spermatocytes of the *A. davidianus* salamander (Hu, Meng, Tian, Zhang, & Xiao, 2016).

In virtually all vertebrates, the testicles and ovaries have the same developmental origin: the primordial gonads. Prior to sex determination, the primordial gonads, also known as bipotential gonads, are made up of multipotent somatic progenitor cells that are able to adopt one or other sex-specific cellular outcome. During sex determination, the support cell lineage differs to become either Sertoli cells in the testicles (Sekido, Bar, Narváez, Penny, & Lovell-Badge, 2004) or pre-granular cells in the ovaries (Chassot et al., 2012). After undergoing cell differentiation, the outcome concerning sexual definition spreads to other somatic lineages, including steroidogenic cells (Leydig cells in the testicles and theca cells in the ovaries) that then promote the acquisition of primary and secondary sexual characteristics, by means of hormonal regulation. During the production of steroid hormones, there are two biosynthesis pathways, known as  $\Delta 4$  and  $\Delta 5$ , which use cholesterol as a precursor. This is unfolded by a series of enzymes, until it transforms into pregnenolone, an intermediate hormone that is common in all types of steroid hormones, produced by both the ovaries and the testicles, as well as by other types of steroidogenic organs (Gore-Langton and Armstrong, 1988). Pregnenolone is in turn converted to progesterone by the  $\Delta 5$ - $3\beta$  hydroxysteroid dehydrogenase enzyme complex ( $\Delta 5\beta$ -HSD). Activity on the part of the  $\Delta 5\beta$ -HSD enzyme complex is essential for carrying out steroid hormone production, which is why it is considered an early physiological marker of steroidogenic cells, in turn influenced by age, sex, cyclic phenomena, diet and stress (Goldman, Baker & Stanek, 1972). Pioneer studies on the histoenzymatic activity of  $\Delta 5\beta$ -HSD were described by Levy, Deane, and Rubin (1959), in order to determine the location and abundance of enzymes and other compounds related to steroid metabolism. Indication of enzymatic activity relates to the transfer of hydroxysteroid hydrogens to the tetrazolium salt, via a pyridine nucleotide (NAD<sup>+</sup>); in this way, the salt is reduced

and as in its reduced form it is insoluble and coloured (formazan salt), when deposited at the reaction site it can be localized. In this way, this enzymatic reaction has been used to locate steroidogenic activity related to both testicular Leydig cells and ovarian theca cells (Warttenberg, 1958; Christensen, 1975; Martinez-Juárez et al., 2018).

Due to the importance of the *Sox9* transcription factor in the development and maintenance of Sertoli cells in adult testis, and of *Foxl2* in the regulation of the development and maintenance of the ovary, the objective of our work was to characterize protein expression pattern of the *Sox9* and *Foxl2* genes in the ovaries and testicles of 18-month-old *A. mexicanum*, using as a germ line marker to the RNA helicase Vasa and as the steroidogenic cell line the activity of the  $\Delta^5$ -3 $\beta$ HSD enzyme. Finally, we correlated our results with the ultrastructure of these tissues.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Ovaries from five female and testicles from five male *A. mexicanum* were obtained. 18-month-old adult organisms were donated by the Laboratory of Ecological Restoration of the Institute of Biology, UNAM, FAUT-0112. Subsequently, these were transferred to the Biomedical Research Institute, where they were sacrificed following procedures described in the Laboratory Animal Use Guide of the Ethical Committee in the Biomedical Research Institute (UNAM). Animals were placed in a solution of 2% NaHCO<sub>3</sub> at 4°C, in order to anaesthetize them, and then immediately sacrificed by decapitation. The ovaries and testes were then surgically removed and cut into several pieces. A fragment was fixed in 4% paraformaldehyde (PFA) for immunofluorescence and immunohistochemistry. Finally, other fragments were frozen for total protein extraction and Western blot analysis.

### 2.2 | Histological preparation

The ovaries and testes were fixed in Bouin solution for 24 hr at 4°C. The tissue was dehydrated in ethanol solutions ranging from 30% to 100%, and then, tissue clearance was performed on xylol (JT Baker, Edo. Mex., México), and subsequently infiltrated and embedded in paraffin liquid, permitting the formation of solid blocks. Sections of 7  $\mu$ m were obtained using an 820 Spencer Microtome (American Optical Corporation, New York, USA) and stained with haematoxylin and eosin (Electron Microscopy Sciences (EMS); Hatfield, PA, USA—Amresco, Solon, OH, USA), employing standard techniques. Samples were prepared

using permanent mounting medium (Entellan, J.T. Baker, Xalostoc, Edo. of México, México).

### 2.3 | Light microscopy

Fragments from ovaries and testicles were fixed in Karnovsky's solution (Karnovsky, 1965) for 24 hr at 4°C and then transferred into sodium cacodylate buffer (0.1 M pH 7.4) and stored for 24 hr at 4°C. Subsequently, tissues were post-fixed in osmium tetroxide (OsO<sub>4</sub>; Sigma-Aldrich, St. Louis, MO, USA) for 1 hr and dehydrated in 70%–100% ethanol solutions (J.T. Baker, Xalostoc, Edo. of México, México). They were then placed in acetonitrile (J.T. Baker) twice for 20 min each and incubated in dilutions of Embed 812 resin (Electron Microscopy Science, Hatfield, PA, USA) acetonitrile (J.T. Baker) 1:1 for 1 hr and 2:1 for another hour. Finally, samples were incubated in pure Embed 812 resin for 24 hr and polymerized in plastic blocks at 60°C for 24 hr. For each sample, semi-thin sections of 1  $\mu$ m thickness and 60 nm thickness were obtained using a Leica ultramicrotome (Wetzlar, Germany). The semi-thin sections were stained with toluidine blue, mounted with Cytoseal mounting medium (Electron Microscopy Science, Hatfield, PA, USA) and observed under a light microscope (Carl Zeiss, Jena, Deutschland).

### 2.4 | Immunofluorescence

Immediately after dissection, ovary and testis fragments were fixed in PFA (4% in 1X PBS, pH 7.1) for 30 min, washed with 1X PBS for 5 min and placed in 30% sucrose in PBS (Sigma-Aldrich) at 4°C, overnight. Samples were soaked in optimum mounting medium for frozen sectioning (OCT; Tissue-Tek, Sakura, CA, USA) and frozen in hexane (JT Baker) at –70°C. Subsequently, we obtained 20- $\mu$ m sections in a cryostat (Leica CM 1850). Immunofluorescence technique was performed as previously described by Moreno-Mendoza, Harley, and Merchant-Larios (1999). Briefly, sections were treated with Triton X-100 (1% in 1X PBS) for 10 min, washed with 1X PBS and blocked for 2 hr with 1% bovine serum albumin (BSA, Sigma-Aldrich). Sections were incubated separately with polyclonal antibodies against VASA (1:250; Abcam, ab13840) and SOX9 (1:250; Millipore, AB5535) and diluted in 1% albumin overnight at 4°C. They were then washed four times with 1X PBS for 5 min and incubated with the secondary antibody cyanine 3 (Cy3) (1:200; Life Technology, A10520) diluted in albumin/1X PBS for 1 hr at room temperature. Finally, sections were mounted in medium for permanent fluorescence (Dako™) and stored at 4°C for analysis under a Zeiss confocal microscope (LSM 5 Pascal; Carl Zeiss, Jena, Germany),

equipped with argon–krypton and helium–neon, employing filters for Cy3 and Nomarski interference contrast technique.

## 2.5 | Immunohistochemistry

Ovaries and testes were embedded in paraffin following the aforementioned light microscopy protocol. Sections were placed in a vacuum for 24 hr, at 4°C. Sections were dewaxed using standard techniques. Subsequently, they were placed in 0.01 M hot pH 6 sodium citrate and allowed to cool to room temperature. The samples were washed with 1X PBS for 10 min. Endogenous peroxidase was inactivated with 3% hydrogen peroxide (J.T. Baker) in methanol for 30 min. Sections were then washed with 1X PBS for 5 min, and 1% Triton X-100 (Sigma-Aldrich, Saint Louis, MO) was added for 10 min. Sections were then washed with 1X PBS, blocked with 1% albumin for 2 hr and incubated overnight with FOXL2 primary antibody (sc-68348; Santa Cruz Biotechnology, Dallas, TX) at a 1:50 dilution. The following day, sections were washed four times in 1X PBS for 5 min each. The Vector AB kit was used, and sections were incubated with the anti-rabbit secondary antibody at a 1:100 dilution in 1% albumin (Sigma-Aldrich) for 1 hr at room temperature. Sections were washed four times in 1X PBS for 5 min each and then placed in the AB reagent prepared following the Vector AB kit instructions (Vectastain ABC Kit, Burlingame, CA). Sections were washed again, four times in 1X PBS for 5 min each and finally developed with diaminobenzidine (Sigma-Aldrich) for 4 min. Slides were allowed to dry at room temperature and mounted using Cytoseal mounting medium.

## 2.6 | Detection of $\Delta 5$ -3 $\beta$ HSD activity

Fourteen-micron-thick sections were extracted from the tissues included in Tissue-Tek, and incubated at 37°C in histoenzymatic detection medium of  $\Delta 5$ -3 $\beta$ HSD, composed of 20 mg of  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NAD; N1636-250; Sigma-Aldrich), 20 mg of Nitro Blue Tetrazolium (N5514-10; Sigma-Aldrich) and 2 mg of dehydroisoandrosterone (D-4000; Sigma-Aldrich) for 1 hr. Sections were washed with distilled water and mounted in a permanent aqueous medium (Dako™) for observation under a light field microscope (Levy et al., 1959).

## 2.7 | Western Blot analysis

Snap-frozen testicle and ovary were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate), in the presence of a protease inhibitor mixture

and centrifuged at 16,000 g for 20 min at 4°C. Supernatants were collected and stored at –80°C until use. Total protein content in supernatants was assayed using the Pierce Bicinchoninic Acid Protein assay (Thermo Scientific, IL, USA). Fifty micrograms of protein from testicle and ovary homogenates, diluted in loading buffer (Laemmli 2 x containing 1% de  $\beta$ -mercaptoethanol), was separated by electrophoresis on 12% SDS-PAGE home-made gels at 150 V for 60 min and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA), using semi-dry blot system (Bio-Rad) at 25 V for 50 min. Membranes were blocked in PBS/2% non-fat dry milk, overnight at 4°C and incubated overnight at 4°C with primary antibodies SOX9 (AB5535, Millipore, Burlington, MA), FOXL2 (sc68348, Santa Cruz Biotechnology, Dallas, TX), VASA (Abcam, ab13840) and  $\beta$ -actin (A2066, Sigma-Aldrich, Saint Louis, MO). After washing with PBS/0.2% Tween, membranes were incubated with HRP-conjugated secondary antibodies anti-rabbit IgG (Invitrogen, CA, USA) at RT for 1.5 hr. Chemiluminescence revealed immunoreactivity bands, employing a Super Signal Wets Dura Extended Duration Substrate Kit (Thermo Scientific, IL, USA) according to the manufacturer's protocol. The optical density (intensity/mm<sup>2</sup>) for each band was quantified by densitometry using ImageJ software.

## 2.8 | Densitometric analysis

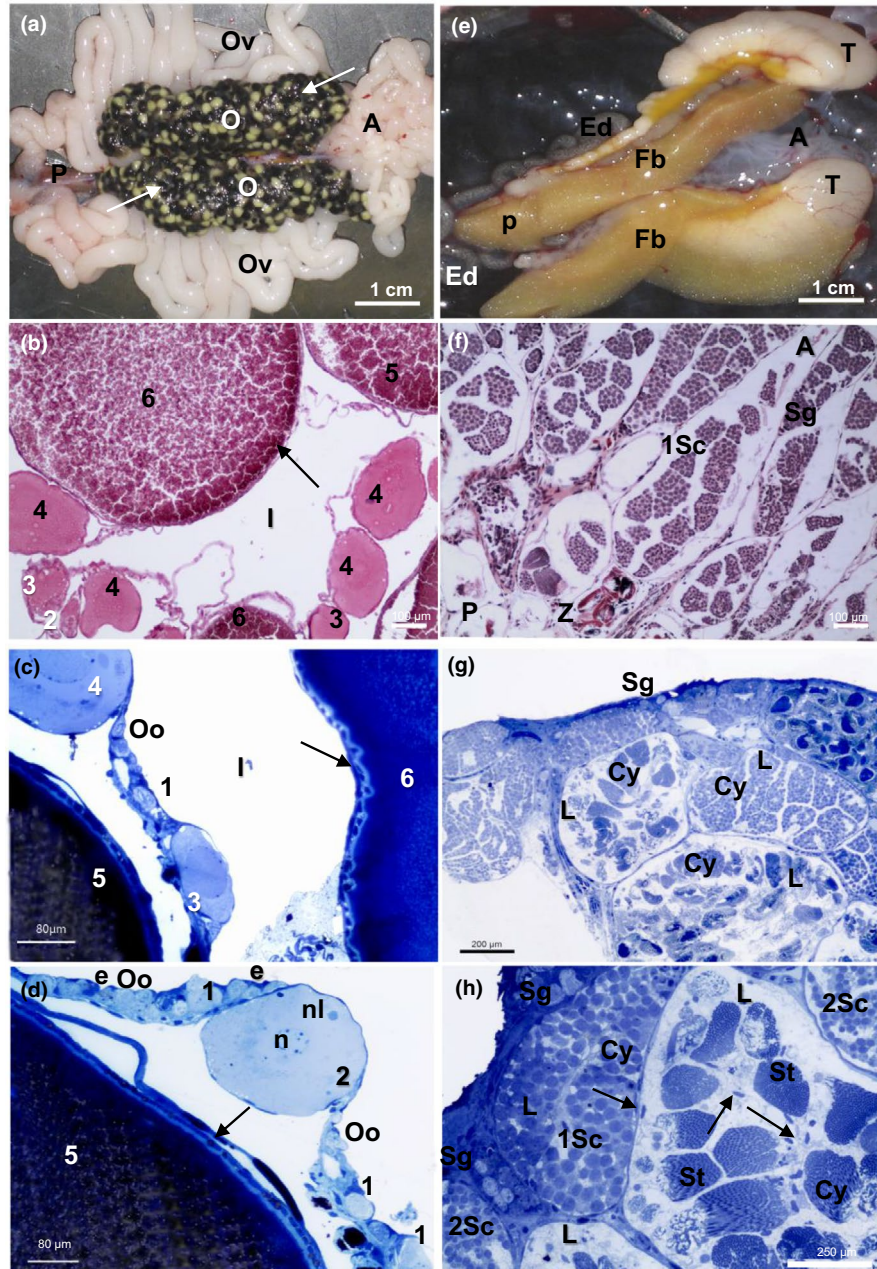
The optical density of each sample was normalized with respect to corresponding actin values. Expression values for each protein are represented as arbitrary relative expression communities, obtained from the ratio of protein interest/actin for each sample. Relative expression values are represented as the mean derived from independent experiments  $\pm$  standard deviation. One-way analysis of variance (ANOVA), followed by a Tukey multiple comparison test with a 95% confidence interval was performed, using the Statgraphics Centurion XV statistical program.

# 3 | RESULTS

## 3.1 | Morphology of *Ambystoma mexicanum* ovary

Ovarian fragments from 18-month-old *A. mexicanum* were processed to provide a structural description; oocytes were classified according to their stage of development based on Uribe (2010) and Erler, Sweeney, and Monaghan (2017). Ovaries were observed as paired, voluminous structures that are located in the posterior mid-body region to either side of the midline, parallel to the kidneys. The wall of the ovary





**FIGURE 1** Morphology of ovaries and testicles of 18-month-old *Ambystoma mexicanum*. (a) Anatomy of the female reproductive system from an anterior (A) and posterior (P) perspective. Ovaries (O), oviducts (Ov), oocytes (arrow). (b) Histological section of an ovarian fragment where oocytes undergoing stages II, III, IV, V and VI are visible in the lumen (l). Pigmentation is evident at the animal pole in stage V and stage VI oocytes. (c) Semi-thin ovary section, where oocytes are visible at stages III and IV and among them an oogonia nest (Oo) and an oocyte at stage I. At the ends, there are two oocytes at stages V and VI surrounded by follicular cells (arrow), and at the centre the lumen (l). (d) Semi-thin ovarian section showing epithelial cells on the ovarian wall (e), oogonia nests (Oo) and oocytes at stages I and II with prominent nucleus (n) and several nucleoli (nl), as well as an oocyte at stage V with follicular and theca cells (arrow). (e) Anatomy of the male reproductive system. Testis (T), efferent ducts (Ed), fatty bodies (Fb), anterior region (A), posterior region (P). (f) Histological section of the testicle, showing different stages of spermatogenesis from the anterior region (A) to the posterior (P). (g) Section of the testicle showing the testicular lobes (Lb) containing cysts (Cy), and showing spermatogonia (Sg) nests at the periphery. (h) Amplification of the anterior region of the testicle, showing cysts (Cy) within the lobes (Lb), note the nuclei of Sertoli cells (arrow) surrounding the cysts. At the limit are isolated or grouped spermatogonia (Sg)

is transparent, making it possible to observe the larger vitellogenic oocytes, which manifest clear regionalization, where the animal pole is darker due to pigmentation and the plant pole, with greater concentration of yolk (Figure 1a), is

lighter. Morphologically, the ovary appears to have a saccular structure; the outer limit of the ovary is made of cubic or flattened epithelial cells and at some points on this delimiting epithelium, groups of oogonia and stage I oocytes can be

observed forming nests (Figure 1c,d). At the periphery of the ovary, attached to the epithelium are the follicles at different stages of development, which hang from the wall, and grow and develop into the ovarian cavity (Figure 1b). The follicles in primary growth at stage I are located in the nests, formed from oocytes that have a spherical nucleus with a nucleolus, and outside a number of flattened follicular cells surround the oocyte (Figure 1b,c,d). In stage II follicles, the nucleus of the oocyte is central and spherical, containing one or more nucleoli and is already completely surrounded by follicular cells. At stage III, the nucleus of the oocyte is large, spherical and has several peripheral nucleoli, and its cytoplasm is homogeneous with peripheral vitelline vesicles, which are surrounded by a layer of flattened follicular cells (Figure 1b,c,d). From stage IV onwards, secondary growth begins, where the presence of yolk is evident at the periphery of the oocyte cytoplasm. During stages V and VI, the size of the oocytes increases markedly by the accumulation of yolk platelets, and at stage V, the animal pole is clearly distinguished by deposition of pigment in the cortical cytoplasm (Figure 1b). At stage VI, the yolk platelets are larger and more abundant and are mostly located at the plant pole. At the periphery of stage V and stage VI oocytes, the follicular wall consisting of thin follicular cells is notable (Figure 1c,d).

### 3.2 | Morphology of the *Ambystoma mexicanum* testicle

Anatomically, *A. mexicanum* testicles were observed as paired organs located parallel to the axis of the body, attached to the dorsal wall of the body by the mesorchium and located prior to or cranial to the kidneys (Figure 1e). These are elongated structures composed of several lobes and are surrounded by fibrous connective tissue that forms the tunica albuginea. Histologically, the testicles consist of testicular lobes, including interstitial connective tissue (Figure 1f,g,h). This morphological conformation indicates that the spermatogenesis process occurs longitudinally from the anterior or cephalic dorsal region towards the caudal or posterior region of the testis; this is a process of gradual and regionalized germ cell maturation, which is also cystic. Each testicular lobe is made up of several cysts and each of these cysts is structured by the relationship between one or several spermatogonia and one or several Sertoli cells, to form the wall of the cyst (Figure 1h). Depending on the testicular region, the lobes present cysts with development that is synchronous with the germ line cells, which mature progressively in a caudal cephalic direction with respect to the testis. In this way, in each testicular region we can observe lobes with cysts that manifest different types of spermatogenic cells, ranging from spermatogonia located at the periphery of the anterior or cephalic region, to cysts of

primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa towards the posterior or caudal region (Figure 1f,g,h). Testicular lobes are surrounded by loose interstitial tissue, where Leydig cells are located.

### 3.3 | Expression of SOX9, FOXL2 and VASA proteins in *Ambystoma mexicanum* testis

Combining Nomarski's microscopy and immunofluorescence techniques, it was apparent that *SOX9* protein expression is located in the nucleus of Sertoli cells. Evidently, within each testicular lobe, spermatogenic cells differentiated to the same degree can be identified, grouped into cysts in the same lobe. Likewise, each cyst is associated with several Sertoli cells (Figure 2a,b,c). Regarding the expression pattern of the *Foxl2* protein, no expression signal was detected in situ in the *A. mexicanum* testis (Figure 3a). As for the expression of the *Vasa* RNA helicase, this was located in the cytoplasm of germ or spermatogonia cells within cysts that form lobes located in the peripheral dorsal and more cephalic region of the testis (Figure 4a,b,c). It was apparent that as sperm cells progress in their differentiation, the cytoplasmic expression of the *VASA* protein declines, which was evident in spermatogonia that are initiating meiosis, that is primary spermatocytes (Figure 4c).

### 3.4 | Expression of SOX9, FOXL2 and VASA proteins in *Ambystoma mexicanum* ovary

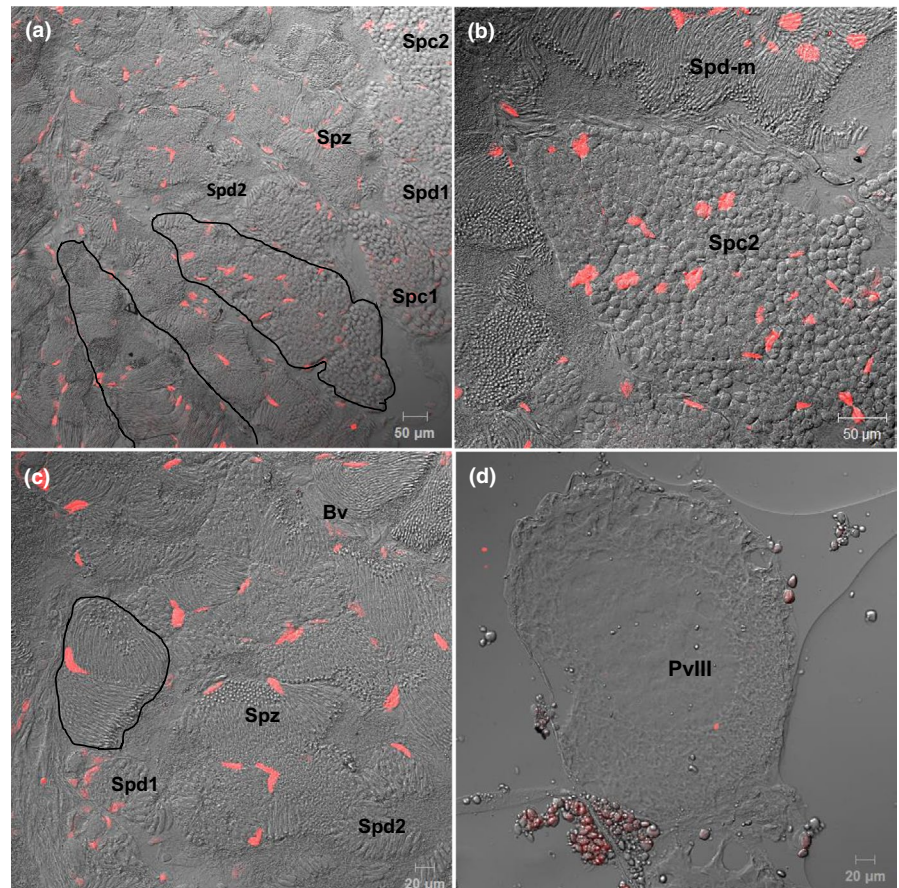
In 18-month-old *A. mexicanum* ovaries, it was not possible to detect the expression of the *Sox9* protein by immunofluorescence (Figure 2d). This may be due to the fact that the ovary is a large and elongated structure, and this technique obtains 20- $\mu$ m sections, so *SOX9* is probably indicated by oogonia nests, as expression of this gene would be required for oogenesis. Contrary to what was found with the *Sox9* transcription factor, *Foxl2* protein expression was observed in the cytoplasm of follicular cells that surround oocytes at different stages of follicular development, being most evident in vitellogenic oocytes, where many yolk platelets have already accumulated (Figure 3). With respect to the expression pattern of the *Vasa* protein, as in most vertebrates, this was observed in the cytoplasm of previtellogenic oocytes or during primary stage III growth (Figure 4d).

### 3.5 | Detection of $\Delta^5$ -3 $\beta$ HSD enzymatic activity in *Ambystoma mexicanum* testes and ovaries

$\Delta^5$ -3 $\beta$ HSD activity was detected by the accumulation of granular formazan deposits, resulting from the transfer of hydroxysteroid hydrogen to the tetrazolium salt, by means of a



**FIGURE 2** Expression of the transcription factor *Sox9* in testis and ovary of *Ambystoma mexicanum*. (a) Expression of the *Sox9* protein in the nuclei of Sertoli cells (red) located between the cysts that make up the testicular lobes (black contour). (b) Amplification of (a) where the nuclei of Sertoli cells (red) are visible, adjacent to cysts containing secondary spermatocytes (Spc2) surrounded by lobes with elongated spermatids (Spd2). (c) Detail of another region of the testicle where lobes with sperm cells are visible at different stages of development grouped in cysts and associated with Sertoli cells, the latter evidenced by the positive expression of *SOX9*. (d) In the ovary, no *SOX9* signal was detected as shown in a stage III previtellogenic oocyte (PvIII)



pyridine nucleotide (NAD<sup>+</sup>). In this way, the salt is reduced, and because its reduced form is insoluble and coloured (formazan salt), this is deposited at the reaction site. Because of this, we identified  $\Delta^5$ -3 $\beta$ HSD activity in *A. mexicanum* testicles, similar to that detected in mouse testicles, used as a positive control for this technique (Figure 5f). Formazan deposits accumulated in cells located in the interstitial compartment that surrounds the testicular lobes (Figure 5a), where Leydig cells are usually found in the testicles of vertebrates. Analysing the accumulation of formazan granule deposits in greater detail, these deposits were detected in cells with steroidogenic characteristics, demonstrated by lipid accumulation (Figure 5b,c). In the ovaries of *A. mexicanum*, deposits of formazan, which are a sign of steroidogenic activity, were detected in some cells located in the theca region of the follicles (Figure 5d). A weak signal was also observed at the periphery of the vitellogenic oocyte cytoplasm (Figure 5e).

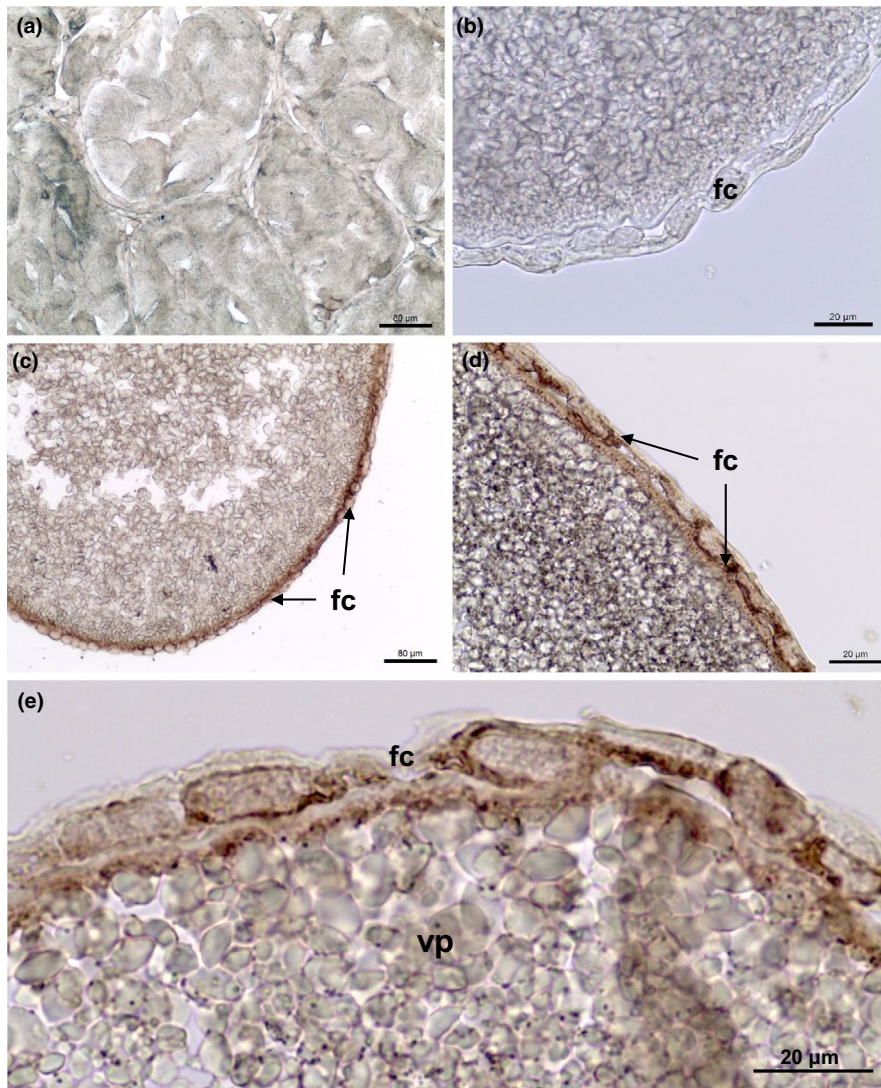
### 3.6 | Quantification of SOX9, FOXL2 and VASA proteins in testes and ovaries of *Ambystoma mexicanum*

Protein quantification was performed by Western blot transfer analysis (Figure 6a). We perceived that similar to the

results obtained for the location of the protein for the transcription factor *Sox9*, this factor was expressed in both sexes; however, the expression of this protein was significantly higher in the testicles than in the ovaries. In the case of the *Foxl2* protein, as shown by the immunohistochemical tests, this was not detected in the testicles; however, in the ovaries, protein expression of this gene was clearly evident. We also discerned that the Vasa protein is expressed both in the ovary and in the testis; however, higher levels were detected in the testis (Figure 6c). The  $\beta$ -actin loading control was used as a positive control, with no apparent differences between ovary and testis (Figure 6b).

## 4 | DISCUSSION

The external morphology and histological structure of the *A. mexicanum* ovary from 18-month-old specimens used in the present investigation confirms that these are sexually mature organisms or reproductive adults. In the ovary, oogonia located in nests were observed at different sites in the ovarian wall with oocytes at different stages of development, that is from primary growth (previtellogenesis) during stages I, II and III to secondary growth (vitellogenesis) during stages IV, V and VI. Likewise, in the male gonads there is typical



**FIGURE 3** Immunohistochemical localization of the *Foxl2* protein in testis (a) and ovary (b-e) of *Ambystoma mexicanum*. (a) In the testicles, no *FOXL2* protein signal was detected in any cell type. (b) Ovarian section used as a specificity control, where the primary antibody was omitted. (c) Ovarian section, where a stage VI vitellogenic oocyte (VgVI) surrounded by follicular cells (fc) is visible. (d) Amplification of (c) where follicular cells (fc) expressing *FOXL2* are visible. (e) Greater amplification of oocyte formed out of yolk platelets (vp), where the positive expression of *FOXL2* in the surrounding follicular cells (fc) is evident

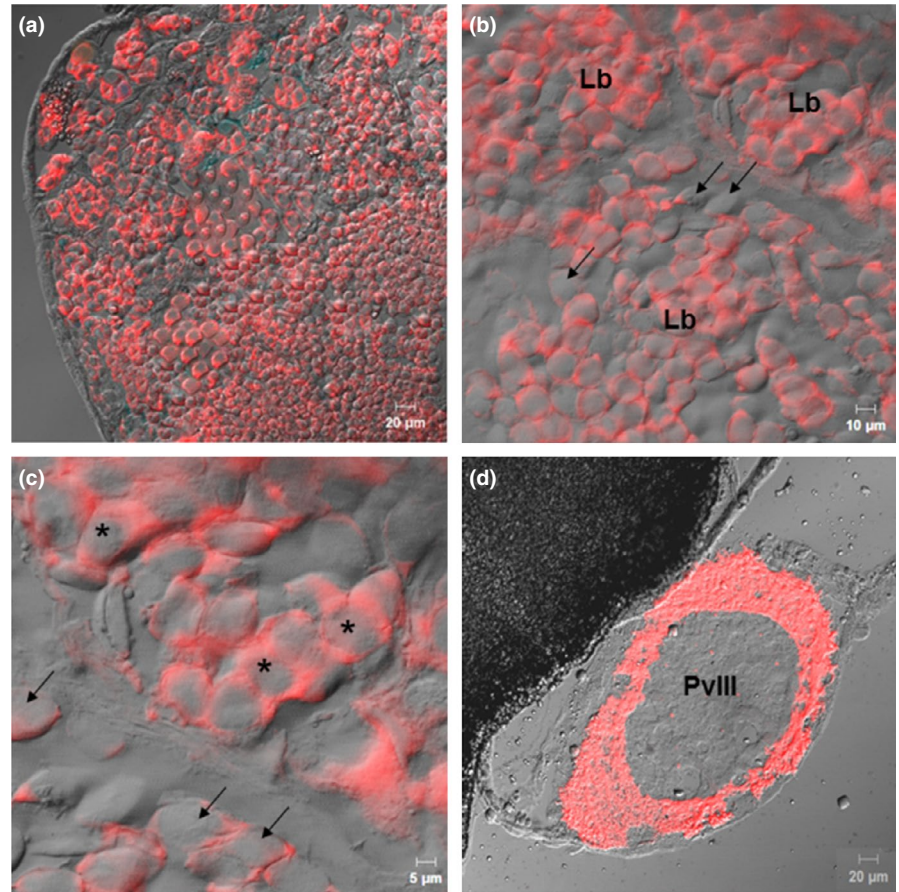
active cystic spermatogenesis with a type of caudal cephalic maturation, coinciding with the characteristics that have been mentioned for other urodeles (Erler et al., 2017; Proper, 2011; Uribe, 2009, 2011; Uribe and Mejía-Roa, 2014). It thus appears that adult organisms of *A. mexicanum* show a typical urodele reproductive pattern, despite continuing to maintain larval characteristics.

In the present study, we analyse *Sox9* gene expression involved in testicular function and *Foxl2* gene involved in the ovarian function of vertebrates, as well as the expression of highly conserved *Vasa* helicase in the development and maintenance of the germ line. Although the morphological changes that occur in the developing testicles and ovaries are well-defined in some amphibian species, especially anurans, the genetic regulation of gonadal morphogenesis in urodele amphibians is poorly understood. However, it is probable that genes involved in testicular and ovarian morphogenesis and their maintenance are expressed in a spatiotemporal manner coinciding with the morphological changes that occur during gonadal differentiation. In the *A. mexicanum*

testicle, the *Sox9* protein was detected in somatic cells, which correspond to Sertoli cells and coincides with their location within the testicular lobes. Therefore, considering what has been observed in other amphibians and vertebrates in general, the *Sox9* gene in *A. mexicanum* may play a role in the development of testicular structures and, therefore, in the differentiation and maintenance of the testis. The expression of *Sox9* in the urodele amphibian *A. mexicanum* suggests an evolutionarily conserved role in the development of vertebrate testicles. The functional importance of *Sox9* in the differentiation of the male phenotype (testicular differentiation) has been widely reported among vertebrates (Kent, Wheatley, Andrews, Sinclair, & Koopman, 1996; Morais da Silva et al., 1996; Moreno-Mendoza et al., 1999; Spotila, Spotila, & Hall, 1998). In particular, among Anuran amphibians, the expression of *Sox9* has been studied in some species of adult amphibians such as *R. rugosa* and *X. laevis*, where the expression of *Sox9* is dimorphic, and only detected in the testis (Flament et al., 2011; Osawa et al., 2005; Takase et al., 2000). However, in other Anuran such as *X. tropicalis* and



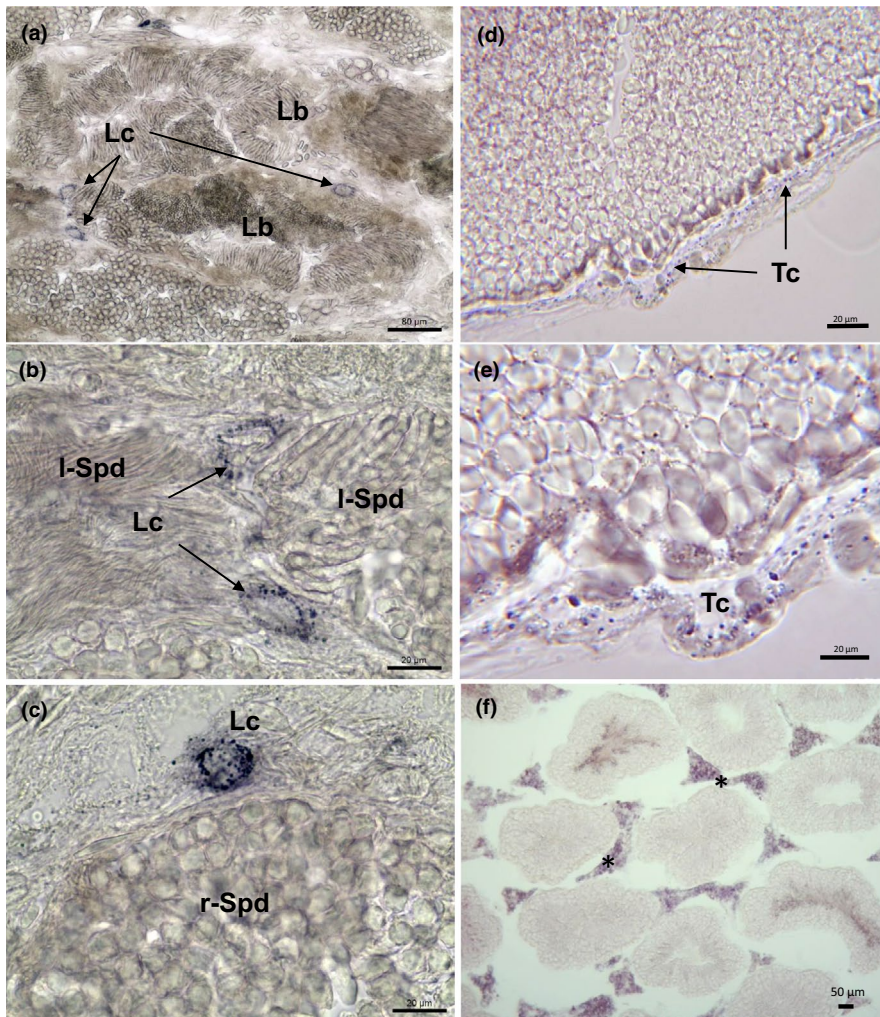
**FIGURE 4** Expression of RNA helicase VASA in the testis and ovary of *Ambystoma mexicanum*. (a) Transverse and anterior section of testis, where VASA protein expression in the cytoplasm of sperm cells is shown in red. (b) Amplification of (a) where three lobes are visible (Lb) containing VASA-positive cells, in which their cytoplasm is completely red and cells marking this appear to decrease (Arrows). (c) Greater magnification shows a lobe with three cysts containing spermatogonia, evidenced by the expression of VASA that completely surrounds the nucleus (\*) of these cells. Adjacent to this lobe, another lobe is found with cells where VASA expression is decreasing, indicating that these are primary spermatocytes (arrows). (d) In the ovary of *Ambystoma mexicanum*, VASA was detected in the previtellogenic oocytes and a stage III oocyte (PvIII) is shown



*Bufo marinus*, in addition to the detection of *Sox9* protein expression in the testicle, it was also observed in the ovary, in the cytoplasm of previtellogenic oocytes (Abramyan, Feng, & Koopman, 2009; El Jamil et al., 2008). Similarly, in the urodele amphibian *P. waltil*, *Sox9* expression was detected as much in ovaries as in testicles (Dumond et al., 2011), findings that corroborate with those of this study. Recently, the Penrad-Mobayed group investigated the nuclear localization of the *SOX9* protein in adult oocytes of the Anuran amphibian species *X. laevis*, *X. tropicalis* and the urodele *P. waltil*. They observed that there is indeed a nuclear localization of the *Sox9* protein in nuclear extracts from adult oocytes of these amphibians and they suggest a role in post-transcriptional processes and possibly a different biological role on the part of this protein, in the later stages of oogenesis (El Jamil et al., 2008; Penrad-Mobayed et al., 2018). Evidently, the expression of the *SOX9* protein in adult ovaries, specifically in oocytes, is not restricted to species of Anuran amphibians or urodeles, but also occurs in fish such as *Oryzias latipes* (Yokoi et al., 2002), *Danio rerio* (Rodríguez-Mari et al., 2005), *Chapalichthys encaustus* (Guerrero-Estévez et al., 2012) and even reptiles such as *Crocodylus moreletii* (Martínez-Juárez, López-Luna, Porrás-Gómez, & Moreno-Mendoza, 2018), so this may in fact be a general characteristic of anamniotes and amniotes (Dumond et al., 2011; Penrad-Mobayed et al., 2018).

Among genes that maintain the morphology and function of the ovary, we analyse the *Foxl2* transcription factor, which has been described as being responsible for the transformation of androgens to oestrogens and granulosa cell differentiation (Nef & Vassalli, 2009; Villareal & Aguilar, 2014) and for maintaining the morphology of the adult ovary in several vertebrate species (Uhlenhaut et al., 2009). In the *A. mexicanum* ovary, we found that *Foxl2* protein expression is located in the cytoplasm of follicular cells, surrounding oocytes undergoing secondary growth during stages V and VI (vitellogenic), during which the large accumulation of yolk platelets is already evident, conforming with the idea that the expression pattern for the *Foxl2* gene is highly conserved throughout evolution (Bertho et al., 2016). In the testicle, *Foxl2* protein expression was barely detectable by Western blot or by immunohistochemistry, which suggests a dimorphic role for this gene during ovarian and testicular differentiation in *A. mexicanum*. It has been proposed that *Foxl2* expression is essential for the differentiation of granulosa or follicular cells and for the maintenance of the ovary, by negatively regulating the expression of *Sox9*. In the absence of *Foxl2*, there is no differentiation of granulosa cells and, therefore no folliculogenesis, as primary follicles die by apoptosis in the absence of functional granulosa cells. As mentioned, in our observations of *A. mexicanum*, *Foxl2* presents a sexually dimorphic expression pattern, so it can be considered an indicator of female





**FIGURE 5** Detection of the  $\Delta^5$ -3 $\beta$ HSD enzyme in testis (a, b and c) and ovary (d and e) of *Ambystoma mexicanum*. (a) Expression of  $\Delta^5$ -3 $\beta$ HSD in Leydig (Lc) cells located in the interstitium surrounding the testicular lobes (Lb). (b) Detail of  $\Delta^5$ -3 $\beta$ HSD expression in the cytoplasm of Leydig cells (Lc) located in the interstitium and adjacent to the membrane of two testicular lobes (Lb) containing elongated spermatids (Spd2). (c) Detection of a Leydig cell with formazan deposits located adjacent to a testicular lobe (Lb) made up of round spermatids (Spd1). (d) Detection of  $\Delta^5$ -3 $\beta$ HSD activity in theca cells (Tc) of a follicle containing a vitellogenic oocyte. (e) Detection of increased  $\Delta^5$ -3 $\beta$ HSD enzyme expression in theca cells (Tc). (f)  $\Delta^5$ -3 $\beta$ HSD activity in Leydig cells (\*) in mouse testis used as a positive control

functional heterogamy (Schmidt et al., 2004; Shoemaker-Daly et al., 2010; Cocquet et al., 2018). *Foxl2* has not been widely studied in amphibians, but as in mammals and other vertebrates, it is also linked to the feminization and maintenance of the gonad (Bertho et al., 2016). In some Anuran species such as *R. rugosa*, *X. laevis* and *Lithobates sylvaticus*, *Foxl2* is expressed in the gonads of both sexes, although subsequently clear dimorphic expression is observed, where expression levels in the ovaries are more evident (Flament et al., 2011 and Bertho et al., 2016; Piprek, Damulewicz, Kloc, & Kubiak, 2018). In the giant salamander *Andrias davidianus*, which has a close phylogenetic relationship with *P. walt*, the expression of *adFoxl2* was characterized by observing that transcript levels gradually increase in the ovary during the first 5 years of life, being detected at significantly higher levels in the ovary than in the testicle. In situ expression is detected in ovarian granulosa cells and weakly in spermatocytes in the testis (Hu et al., 2016). Based on our observations for *A. mexicanum*, we can assume that at some time *Sox9* and *Foxl2* may coexpress during gonadal morphogenesis, in order to carry out some function possibly related to the mechanisms of gonadal sexual determination and differentiation.

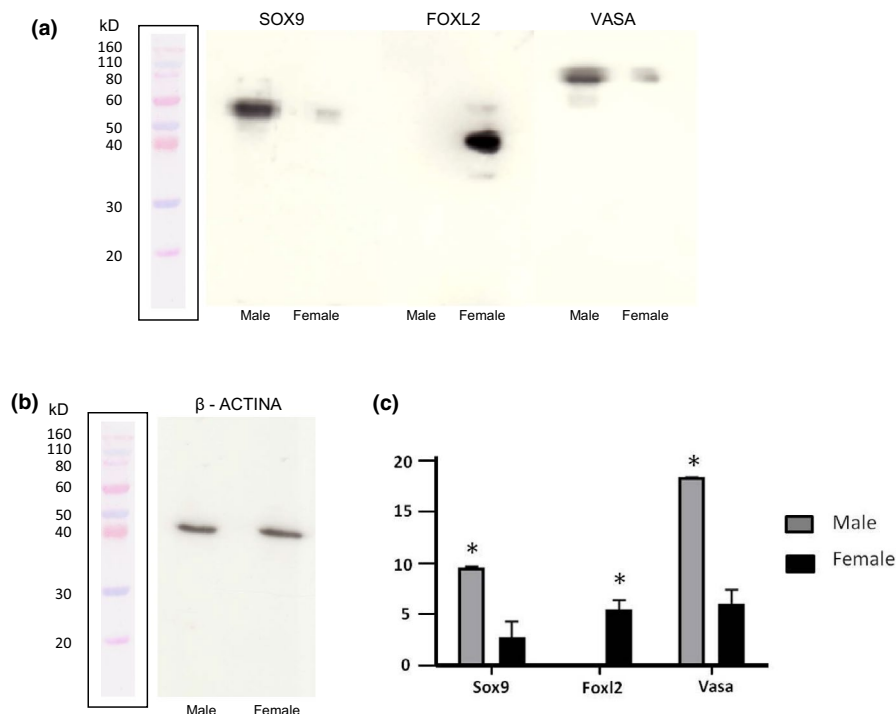
Subsequently, the dimorphic expression of these genes would influence the establishment of ovarian and testicular functionality in sexually mature organisms. In this way, *Foxl2* gene function remains in the granulosa cells, while continuing to regulate and maintain ovarian functionality.

*Vasa* is a gene that has been used as a germ cell marker in vertebrate and invertebrate species (Molyneaux & Wylie, 2004). The *Vasa* gene codes for an RNA-dependent helicase that belongs to the DEAD box protein family, is germ line-specific and is necessary not only for germ cell specification during embryogenesis, but also for gametogenesis (Soatome, Isomura, Seki, Nakamura, & Nakamura, 2010). In this investigation, *Vasa* was located in germ line cells of ovaries and testicles of *A. mexicanum*, specifically in oocytes and spermatogonia, coinciding with that observed in a number of Anuran species (Erler et al., 2017; Komiya, Itoh, Ikenishi, & Furusawa, 1994; Marracci et al., 2007). In the *A. mexicanum* ovary, *Vasa* was expressed in the cytoplasm of oocytes undergoing primary growth (previtellogenic) stage III, which is similar to the pattern of expression observed in previtellogenic oocytes of *Rana ridibunda*, *Rana esculenta* and *Rana lessonae* (Marracci et al., 2007), as well as in the adult testis

**FIGURE 6** Expression of protein from genes *Sox9*, *Foxl2* and *Vasa* in the testis and ovary of *Ambystoma mexicanum*.

(a) Immunoblot for SOX9 (56kDa), FOXL2 (38kDa) and VASA (76kDa). (b) Smooth muscle  $\beta$ -actin (42kDa). (c) The relative expression levels obtained from the densitometric analysis of the bands obtained for each protein are visible; these were normalized with respect to actin protein. Significant differences between sexes

\*( $p < .05$ )



of *X. laevis* and *R. rugosa* (Soatome et al., 2010). *Vasa* protein expression levels were higher in the testis, which may be due to the fact that in the ovary, VASA is detected only in cell groups similar to those detected in nests and to the fact that as the follicular stage progresses and follicles become previtellogenic, VASA expression decreases considerably. Contrarily, in the case of the testicles, VASA was expressed in all the spermatogonia that make up the testicular lobes, probably representing the majority of cells that express this RNA helicase.

The transformation of  $\Delta^5$ - $3\beta$ HSD is essential for the biosynthesis of all kinds of active steroids such as progesterone, glucocorticoids, androgens and oestrogens. These steroid hormones play a crucial role in the differentiation, development, growth and physiology of most human tissues (Morel et al., 1997). The detection of  $\Delta^5$ - $3\beta$ HSD enzymatic activity in the *A. mexicanum* testis was evident in Leydig cells, owing to the accumulation of formazan deposits in the lipid droplets contained in the cytoplasm of these steroidogenic cells. In the ovaries,  $\Delta^5$ - $3\beta$ HSD was observed in cells located in the theca region surrounding the follicles. These positive cells mostly correspond to oocytes undergoing previtellogenesis III, suggesting that steroidogenic cells are active in 18-month-old *A. mexicanum* females. In this regard, in some amphibians such as *Rana catesbeiana*, masculinization has been seen to occur, when  $\Delta^5$ - $3\beta$ HSD activity decreases (Hsu, Hsu, & Liang, 1979; Hsu, Yu, Ku, Chang, & Liu, 1991). Studies of larvae and adults of *X. laevis* revealed  $\Delta^5$ - $3\beta$ HSD enzyme activity in the interrenal, kidney and gonad tissue. Steroidogenic activity is weak but positive during early and advanced stages, similar to that observed in *R. catesbeiana*

larvae. Similarly, after ovarian and testicular sexual differentiation, little hormonal activity is apparent, and no differences concerning this activity between sexes (Kang, Marin, & Kelley, 1995). In *P. waltil*, this enzyme has been detected by histochemistry in testis and ovary (Collenot & Collenot, 1977). In other vertebrates such as the *Mabuya carinata* lizard, during their annual spermatogenic cycle, there is a well-defined seasonal lipid cycle and steroidogenic activity in the testicle. In spermatogenically active testicles, the lipids are scattered and  $\Delta^5$ - $3\beta$ HSD activity is evident in Leydig cells (Shivanandappa & Devaraj-Sarkar, 1979). In the interstitium of *A. mexicanum* testicles, we find what we anticipated considering other reptiles and vertebrates,  $\Delta^5$ - $3\beta$ HSD activity in Leydig cells (which produce steroid hormones), which corroborates the fact that 18-month-old male *A. mexicanum* are already sexually mature and that steroidogenic Leydig cells are located in the interstitial region of the testicles as in all vertebrates.

In conclusion, the 18-month-old *A. mexicanum* females and males are sexually mature. Also at this age, we found novel results that corroborate the dimorphic expression of the *Sox9* and *Foxl2* genes in males and females, respectively, providing evidence that some ovarian and testicular differentiation events are conserved among vertebrates. Similarly, the expression of the *Vasa* gene in the oocytes and spermatogonia of this urodele also maintains a conserved role. Likewise, in this study, for the first time, the steroidogenic thecal and Leydig cells were identified by their physiological activity, indicating hormonal activity in these organisms. In summary, our results suggest that the molecular mechanisms related to ovarian and testicular function in *A. mexicanum*



follow a similar pattern of expression to that described in most vertebrates that are not altered by their neotenic condition. Therefore, these results are expected to promote future research on the reproductive biology of *A. mexicanum* and among urodele amphibians in general.

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## COMPETING INTERESTS

The authors declare that they have no conflicts of interest associated with the contents of this manuscript.

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