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ORIGINAL PAPER

Development and gonadal sex differentiation in the neotenic urodele: *Ambystoma mexicanum*

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Abstract *Ambystoma mexicanum* is an endemic neotenic urodele amphibian from Mexico; although it belongs to the Salamandridae family, it is characterized by retaining larval structures and hence has been widely used as an experimental model. In the present study, we describe the main events of gonadal morphogenesis in *A. mexicanum* and correlate these with stages in embryonic and larval development. In this way, it was established that during stage 41 (St41), the gonadal primordium is formed, consisting of primordial germ cells (PGC) and somatic cells. During St45, the undifferentiated gonad is formed from a larger number of PGC interacting with somatic cells. During St53, the germ and somatic cells arrange into the cortical and medullary region. As development proceeds between St55 and 57, morphological differentiation of the gonadal sex takes place, primarily manifested in ovarian differentiation. Our observations and the way these correlate with other urodeles suggest that gonadal morphogenesis in *A. mexicanum* does not depend on larval age. Besides, onset of gonadal sexual differentiation takes place from St53 onward, evidenced by ovarian structural changes, thus neotenic condition does not influence gonadal differentiation events. Finally, it has been established that gonadal development is controlled by chronological regulation that differs from that of somatic development which in the case of *A. mexicanum* suggests that gonadal development is completely independent of metamorphosis, thus implying a process of heterochrony.

Introduction

Amphibians have been used as a model for studying different aspects of vertebrate development. Under laboratory conditions, these organisms are suitable for experiments due to their easy manipulation, the large number of eggs laid in one batch and because larvae are aquatic and easily absorb chemical substances and hormones that are added to the medium, facilitating their study (Hayes 1998; Jamil et al. 2008).

With regard to processes of gonadal sexual determination and differentiation, anurans from the amphibian group have been most studied. Contrastingly, only few species of urodela have been studied and these include *Pleurodeles waltl*, *P. poireti*, *Cynops cristatus*, *C. pyrrhogaster*, *Salamandra salamandra*, *Ambystoma tigrinum* and *A. mexicanum*. Studies on these species include the way hormones influence sexual differentiation (Chardard et al. 1995), temperature-related sex reversal (Chardard et al. 2004; Flament et al. 2011), the effect of androgens and P450 aromatase on sexual differentiation (Kuntz et al. 2003). *P. waltl* is the most studied urodela in terms of the biology of sexual differentiation (Dumond et al. 2008) including

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expression of genes that regulate these processes (Dumond et al. 2011).

Generally, the mechanism for gonadal sexual differentiation follows a similar pattern among anura and urodela. Both groups are gonochoristic (Dumond et al. 2008); i.e. that they develop a single functional sex throughout their lifetime. During gonad morphogenesis, processes of migration, colonization and cell differentiation take place that will lead to the establishment of an undifferentiated gonad consisting of cells of from both the somatic and germ line. In this regard, it has been observed that the formation of the undifferentiated gonad in amphibians is related to the packing of epithelial cells located in the ventral region of the mesonephros, which during very early stages of development are known as genital ridges. Later, these genital ridges are colonized by primordial germ cells (PGCs) of extra-gonadal origin, while somatic cells continue to proliferate (Hayes 1998; Kuntz et al. 2003; Dumond et al. 2008; Flament et al. 2011). Therefore, the undifferentiated gonad is established by the migration and proliferation of PGCs and the proliferation of somatic cells that together form two regions, one external corresponding to the cortical region and one internal that forms the medullary region (Wallace et al. 1999; Kuntz et al. 2003). In this way, the undifferentiated gonad acquires the morphological characteristics which differentiate either into an ovary or a testis; meaning that this is a bipotential organ, whose differentiation will depend on physiological and molecular factors.

Once the undifferentiated gonad is established, morphological differentiation initiates. In the case of ovarian differentiation, this is characterized by the development of the cortex due both to the proliferation of somatic cells and gonocytes or primary oogonia. Some of the primary oogonia form groups of secondary oogonia. These groups are separated from other groups and primary oogonia by a layer of somatic prefollicular cells. When this layer of somatic prefollicular cells proliferate and separate surrounding the individual secondary oogonia which enter meiosis, the first diplotene oocytes appear forming the oocyte follicles (Ogielska and Kotusz 2004). The ovary acquires an ovisac structure because the medullary region undergoes regression resulting in formation of the ovarian cavities (Flament et al. 2009). In contrast, during testicular differentiation, a germinal compartment forms in the medullary region made from lobules that contain the GC and the Sertoli cells of somatic origin. Within the lobules, germline cells are found at different stages of development, grouped within cysts and associated with Sertoli cells. Surrounding the lobules is the cortex region, which is devoid of germ line cells. This gives rise to the tunica albuginea formed mainly from loose connective tissue surrounding the testicle (Kuntz et al. 2003; Flament et al. 2009).

Salamanders from the *Ambystoma* genus have provided important models for biological studies focusing on development, ecology and evolution (Voss and Smith 2005; Smith and Voss 2009; Eisthen and Krause 2012). The axolotl, *Ambystoma mexicanum* is an amphibian from the Urodela order, pertaining to the Ambystomatidae family (Servín 2011). It is a Mexican endemic species that inhabits the lakes of Xochimilco and Chalco in Mexico City (Zambrano et al. 2004). Currently, it is in critical danger of extinction as the censuses from 1998, 2004, 2006 and 2009 have shown a drastic decrease (Wrigth and Whitaker 2001; CITES 2009; Contreras et al. 2009; CONABIO 2011). *A. mexicanum* is a neotenic salamander; meaning that it reaches sexual maturity even though it retains morphological characteristics from its larval stage (Eisthen and Krause 2012).

The axolotl has become an excellent model for medical and biological research. Studies have considered the distribution and conservation of the species (Contreras et al. 2009; Recuero et al. 2010), its regeneration (Song et al. 2010) and the mechanism of neoteny (Thompson et al. 2014). Concerning mechanisms of sexual determination and differentiation, studies on the genetic basis of sexual determination (Smith and Voss 2009), ultrastructure, localization (Ikenishi and Nieuwkoop 1978) and germ line specification (Johnson et al. 2003; Chatfield et al. 2014) have been undertaken, as well as the expression of some genes specific to germ cells, such as *Axdazl*, *Axvh*, *Axoct-4* and *AxKi*t (Bachvarova et al. 2004).

Although *A. mexicanum* has been bred and maintained under laboratory conditions for several years, surprisingly, its reproductive patterns including its physiology during this process have not been documented in detail (Eisthen and Krause 2012). Likewise, a correlation between sexual differentiation and the different stages of embryonic development has not been established in *A. mexicanum*. The objective of this research was thus to investigate the main morphological events leading to gonadal sexual differentiation and their correlation with stages of embryonic and larval somatic development; in order to assess whether the neoteny has any influence at the time of the gonadal morphogenesis in urodeles, independently of the somatic development of the organism as proposed in anurans (Ogielska and Kotusz 2004).

Materials and methods

Animals

Four batches of *A. mexicanum* eggs were donated by the Laboratory for Ecological Restoration at the Institute of Biology, UNAM, FAUT-0112. The batches were

transferred to the Aquarium at the Faculty of Sciences, UNAM, where they were maintained under proper development conditions, with a photoperiod of 12 h light:12 h dark, at a temperature of 16 ± 2 °C and individuals at the larval stage were fed larvae from *Artemia franciscana*, *Culex stigmatosoma* mosquitoes and *Tubifex tubifex*.

Embryos and larvae were collected from these egg batches at different stages of development, and categorized according to the tables previously drawn up by Bordzilovskaya et al. 1989 and Nye et al. 2003. Days of development were counted from the moment of fertilization (days post-fertilization: dpf), and correlated with stages of development (St). According to previous analyses of gonadal development among urodela (Ikenishi and Nieuwkoop 1978; Dumond et al. 2008), the decision was taken to collect and analyze organisms between St41 and St57 and 120-day larvae ($n = 10$ per stage). For purposes of killing, we complied with all procedures described in the Guide to use of Laboratory Animals from the Ethics Committee of the Biomedical Research Institute (UNAM). Organisms were placed in a solution of 2% NaHCO$_3$ at 4 °C, in order to anesthetize them, proceeding immediately to their killing by decapitation (Guerrero and Moreno 2012). A cross-sectional cut was taken to obtain the mid-caudal region of the embryos between St41 and St54; whereas for St55 to St57, the gonads were isolated by micro-dissection. Each caudal region was placed in phosphate buffer (PBS) together with the isolated gonads, to be subsequently fixed applying the appropriate process.

Transmission electron microscopy analysis

Each mid-caudal region obtained from embryos at St41, larvae between St42 and St54 and gonads isolated from larvae between St55 and St57, were processed by light microscopy and transmission electron microscopy (TEM). For this, tissues were fixed in Karnovsky's solution (Karnovsky 1965) for 24 h at 4 °C and then incubated in cacodylate buffer (0.1 M, pH 7.4) and stored at 4 °C for 24 h. Subsequently, the tissues were post-fixed with 1% osmium tetroxide (OsO4) (Sigma-Aldrich, St Louis, MO, USA) for 1 h, dehydrated in a gradated series of ethanol solutions (70–100%, JT Baker), placed in acetonitrile (JT Baker) and infiltrated with solutions of Epon/acetonitrile (EMS/JT Baker) at 1:1 and the at 2:1. Finally, the tissues were incubated in pure Epon for 24 h and polymerized in plastic blocks at 60 °C for 24 h. Semi-thin sections (1 μm), obtained using an ultramicrotome (NOVA, Leica/LKB, Wetzlar, Germany) were stained with toluidine blue (0.1% in water) and observed under light microscope (E200, Nilon, Melville, NY, USA). For electron microscopic analysis, ultrathin sections (25- to 100-nm-thick sections covering a total thickness of 0.5 nm) were mounted on copper grids and contrasted using 2.5% uranyl acetate and 0.3% lead citrate for observation under transmission electron microscopy (JEM-1010, JEOL, Tokyo, Japan).

Morphometrics

For each development stage of *A. mexicanum* St41, St45, St50, St53, St55 and St57, the length and width of both gonads ($n = 6$) were measured, as well as the width and length of germ cells detected. Measurements were made using a micrometric eyepiece with 40× magnification in a CX-31 model Olympus microscope. The data obtained indicated average and standard deviation, using the Statistical program, version 10.

Results

Ambystoma mexicanum larvae at St57 (Fig. 1a) the gonads are paired, whitish and elongated structures, located in the posterior mid-dorsal region of the body and closely related to the mesonephros (Fig. 1b). The mesonephros is distinguished by a yellowish opaque color, located to one side of the gonad (Fig. 1b). In semi-thin cross sections from the mid-caudal region of the larvae, gonads appear to be attached to the ventral surface of the mesonephros and to adjacent the intestinal mesentery. There is evidently a close relationship between the gonads and other structures such as the Wolffian duct, dorsal aorta and intestinal mesentery located within the coelomic cavity (Fig. 1d).

Once we had anatomically and structurally located the gonads, we performed a sequential analysis for gonadal development initiating at St41 that corresponded to embryos of 11 dpf and larvae between St42 (13 dpf) and St57 (90 dpf), as well as 120 dpf.

The undifferentiated gonad

In the case of *A. mexicanum* embryos at St41 (11 dpf), the gonad appears to be morphologically undifferentiated. In transverse sections of embryos taken from the mid-posterior caudal region, gonadal primordia or urogenital ridges are observed within the coelomic cavity, on both sides of the mesentery that contains the intestine, close to the Wolffian duct and in close proximity to the mesonephros. The gonadal primordium is a compact oval-shaped structure with an average diameter of $22.6 \pm 3.6 \times 11 \pm 1.8$ μm, where some relatively large cells ($9.2 \pm 2.3 \times 5.3 \pm 1.0$ μm in diameter) with oval shape, prominent nucleus, lipid droplets and electron-dense bodies can be observed in its cytoplasm; morphological characteristics which suggest they consist of primordial germ cells (PGCs) (Fig. 2a). Ultra-structural analysis confirms the presence of PGCs that appear as large
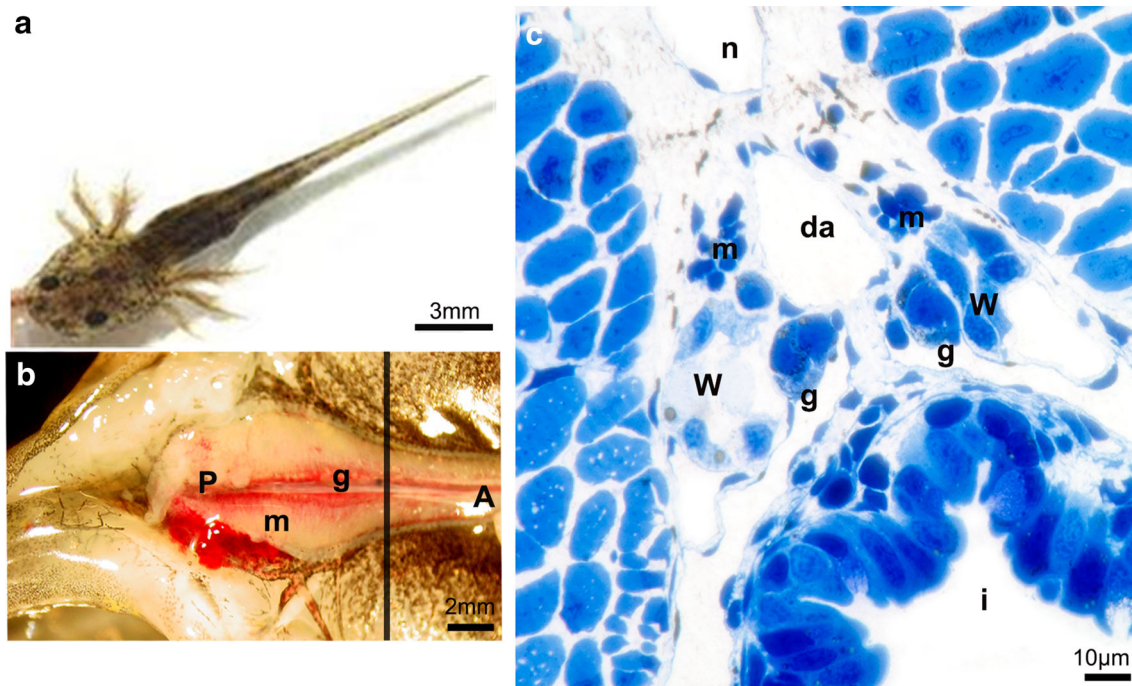
Fig. 1 Larva and location of the gonad in *A. mexicanum*. **a** *A. mexicanum* larvae during St50. **b** Larva caudal region at St57, where the gonad (g)-mesonephros (m) complex is attached to the body wall of the dorsal region. *P* posterior, *A* anterior region. **c** Transverse section at level marked in B of an embryo at St46 where the gonads (g) are located in the coelomic cavity on both sides of the mesentery holding the intestine (i), closely related to the mesonephros (m), the Wolffian duct (W) and the dorsal aorta (da); the notochord (n) is also visible

cells with prominent nuclei and finely granular chromatin; in its cytoplasm lipid droplets, electron-dense bodies and abundant mitochondria are visible (Fig. 2b).

As development progressed, apparently there were no significant changes in morphogenesis of the gonadal primordium. At St45 (15 dpf), the average size of the gonad was $24.2 \pm 6.9 \times 12 \pm 1$ μm; whereas that of germ cells (primary oogonia) was $9.6 \pm 2.91 \times 5.6 \pm 1.3$ μm. The primary oogonia are more clearly defined, presenting a more rounded shape and they have a prominent and oval nucleus with an evident nucleolus, and in the cytoplasm, an increase in yolk droplets and electron-dense bodies can be observed (Fig. 2c). At this stage, there is a close relationship between GCs and somatic cells; the latter can be identified by their irregular morphology and their location both in the periphery of the gonad as well as among the GCs (Fig. 2c, d).

At St50 (28 dpf), larvae still manifest the morphology of an undifferentiated gonad; however, this is larger than at previous stages and has an average diameter of $38.5 \pm 8.5 \times 17.8 \pm 1.8$ μm. Anatomically, gonads are suspended in the coelomic cavity attached to the dorsal wall of larvae by a mesentery. Gonads have now acquired an elongated and irregular morphology and consist of a greater number of somatic cells in close contact with a greater number of primary oogonia (Fig. 3a). Surrounding

the gonad, somatic cells can be observed to form a cubic epithelium (Fig. 3a). Primary oogonia have an average diameter of $10.0 \pm 0.9 \times 6.0 \pm 1.5$ μm and apparently consist of a large nucleus and evident nucleolus, and have a smaller number of electron-dense bodies and lipid droplets in their cytoplasm (Fig. 3a). Ultra-structurally, primary oogonia manifest a prominent nucleus with homogeneously distributed chromatin, a well-defined nucleolus, mitochondria, and a few electron-dense bodies in their cytoplasm (Fig. 3b, c). No significant morphological changes were observed in the development of the gonad during St51 and St52 (data not shown).

Gonadal sex differentiation

During St53 (42 dpf), the gonad continues to increase in size ($54.2 \pm 10.2 \times 20.8 \pm 4.4$ μm in diameter), and has taken on a saccular and pedunculated shape. There is an evident increase in the number of somatic cells in the hilar region that bind it to the body wall. The limiting epithelium is composed of irregular somatic cells and there are a greater number of GCs, which are located contiguous to one another in the cortical region of the gonad. In some individuals, the structure of the medullary region of the gonad appears compact, but notably certain spaces or
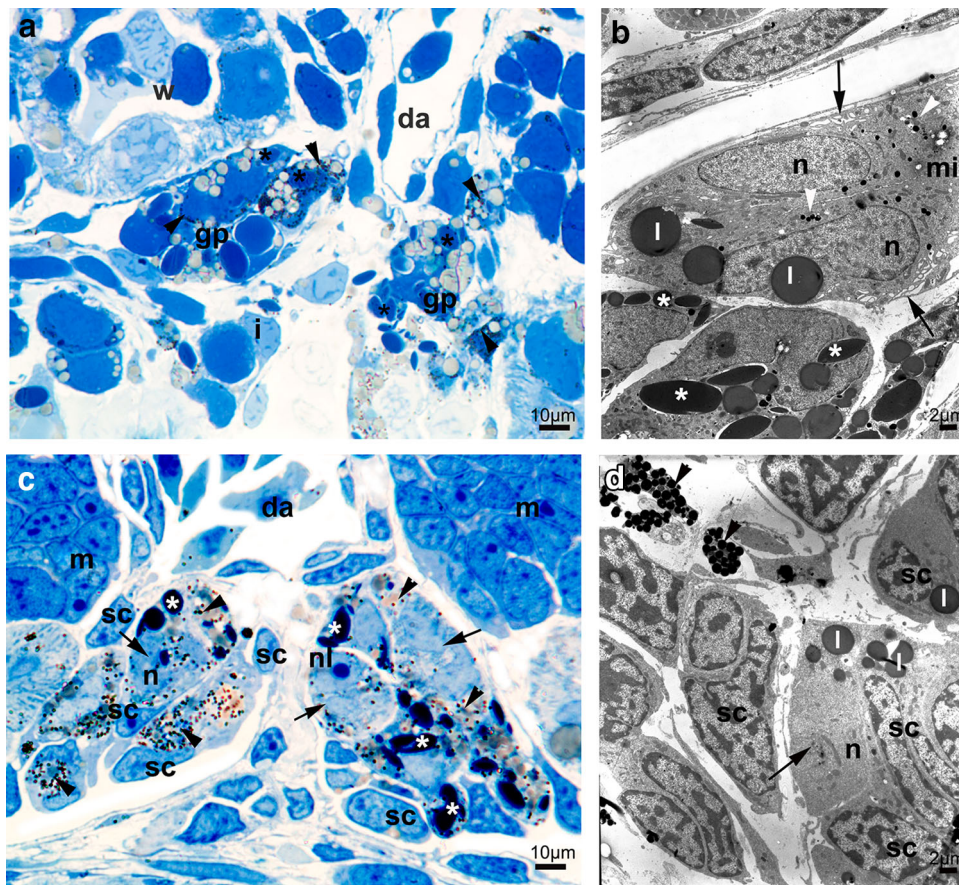
Fig. 2 Undifferentiated gonad of *A. mexicanum*. **a** Transverse section of the mid-caudal region of an embryo during St41 (11 dpf) where gonadal primordium (gp) is visible and strictly related to the Wolffian duct (W), the intestinal mesentery (i) and the dorsal aorta (da). Large germ cells with yolk droplets (*asterisk*) and electro-dense bodies (*arrowhead*) immersed in their cytoplasm are visible. **b** Electron micrograph of the gonadal primordium, where the prominent nucleus (n) of each germ cell (*arrow*), lipid drops (l), yolk drops (*asterisk*), mitochondria (mi) and electro-dense bodies (*arrowhead*) in their cytoplasm are visible. **c** Undifferentiated gonads of a larva during St45 (15 dpf) located adjacent to the mesonephros (m) and the dorsal aorta (da). Note the evident nucleus (n) and nucleolus (nl) of the germ cells (*arrows*), yolk drops (*asterisk*) and electro-dense bodies (*arrowhead*) in their cytoplasm, as well as the close relationship with somatic cells that have irregular morphology (sc). **d** Electron micrograph of a germ cell (*arrow*) with a prominent round nucleus (n), lipid drops (l) in their cytoplasm and their close relationship to somatic cells (sc). Note the presence of electro-dense bodies (*arrowhead*) of a germ cell located at the *upper end*

cavities begin to appear between both somatic cells and in the middle of the medullary region, making them appear loose (Fig. 3d). GCs are more rounded and have an average diameter of $10.8 \pm 2.1 \times 7.2 \pm 1.1$ µm, the electron-dense bodies in their cytoplasm have decreased considerably and yolk droplets are no longer visible. Interaction between somatic cells and GCs is closer (Fig. 3e, f). These morphological changes suggest the onset of ovarian rather than testicular differentiation.

Ovary differentiation

Clear morphological regionalization is apparent during ovarian differentiation, defined by the presence of a cortical region and a medullary region (Fig. 4a). These structural changes are clearest during St55 (52 dpf), when the

forming ovary has an average size of $88.7 \pm 17.2 \times 45.7 \pm 23.3$ µm in terms of diameter and is delimited by an epithelium of cubic somatic cells. In cross sections of the anterior region of the gonad, there is evidently a structure adjacent to the hilar region, known as the progonad that will subsequently give rise to fatty bodies (Fig. 4a). In the medullary region of the gonad, somatic cells still appear to be irregular, limiting the central cavity, although there are still some spaces between the cells. In the cortical region, a greater number of closely packed primary and secondary oogonia form two or three layers of cells. The mean diameter of the all oogonia at this stage was $12.7 \pm 3.5 \times 12.3 \pm 2.4$ µm; and apparently some of these are dividing (Fig. 4a). Ultra-structural analysis shows that primary oogonia have clear cytoplasm with abundant mitochondria, electron-dense bodies and a prominent
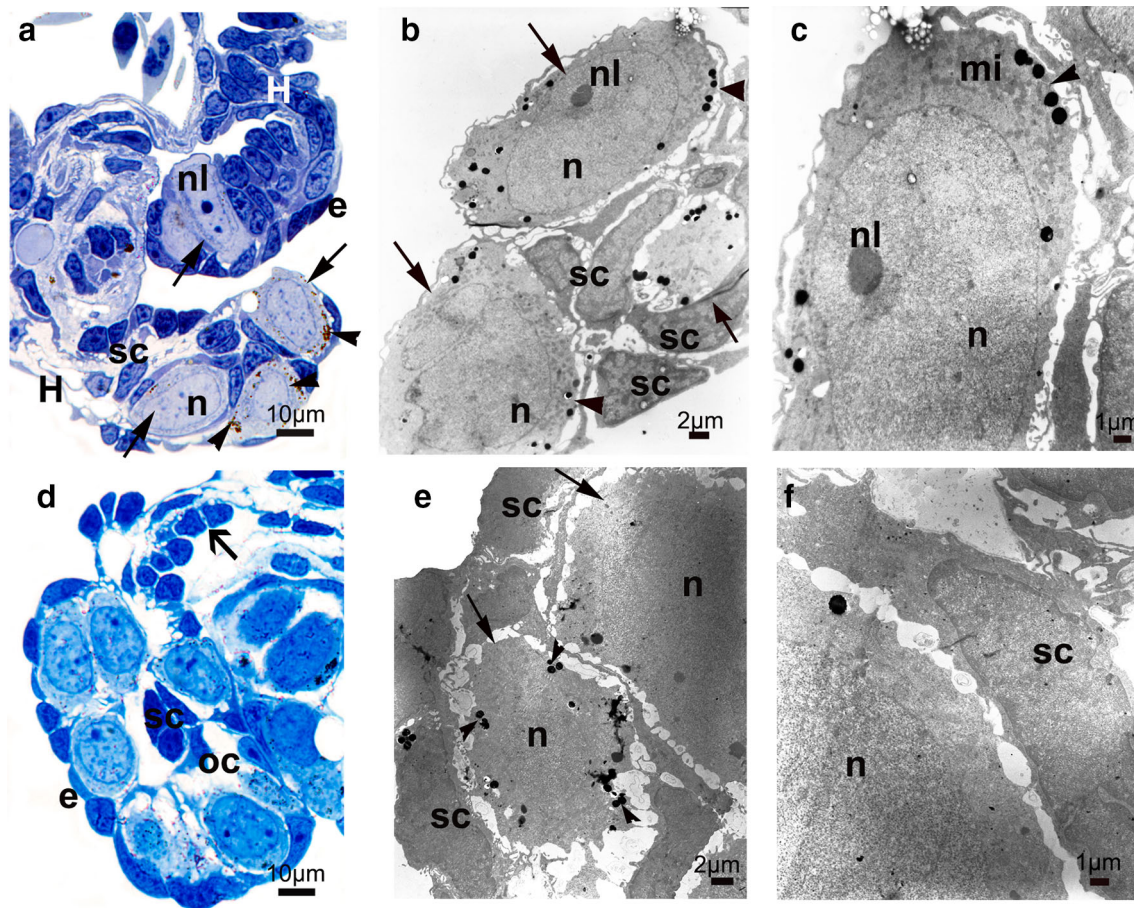
Fig. 3 Undifferentiated gonad of *A. mexicanum*. **a** Undifferentiated gonad of *A. mexicanum* larvae at St50 (28 dpf). The gonads were observed suspended by the hilar region (H). They consist of germ cells (*arrow*) notable for their prominent nucleus (n), nucleolus (nl) and electro-dense bodies (*arrowhead*) in their cytoplasm. Somatic cells (sc) are located among the germ cells, as well as peripheral epithelial cells (e). **b** Electron micrograph showing germ cells (*arrow*) with evident nucleus (n), nucleolus (nl) and electron-dense bodies (*arrowhead*) in their cytoplasm. Somatic cells (sc) are also visible between them. **c** At higher resolution, the nucleus (n) and nucleolus (nl), as well as mitochondria (mi) and electron-dense bodies (*arrowhead*) in its cytoplasm are visible in germ cells. **d** Beginning of gonadal differentiation at St53 (42 dpf). Gonad suspended from the hilar region (*thick arrow*) where two regions are visible: a cortical region where germ cells are located, and a medullary region with many somatic cells (sc), as in the periphery of the gonad forming the epithelium (e). Note the onset of ovarian cavity (oc) formation in the medullary region. **e** Electron micrograph of the gonad, where two germ cells are visible, with a round nucleus (n) and electro-dense bodies (*arrowhead*) and closely related to somatic cells (sc). **f** Detail of a germ cell, with its prominent nucleus (n) and a closely related somatic cell (sc)

spherical nucleus. Secondary oogonia, were observed with structures similar to synaptonemic complexes, characteristic of cells in meiotic division (Fig. 4b).

During St57 (90 dpf), the developing ovary had a larger diameter (96 ± 6.6 × 61.3 ± 11.3 µm), as a result of the increase in GCs (oogonias) and somatic numbers. The ovary at this age, which is located adjacent to the progonad appears as a compartmentalized structure, where we find a cortical region formed by two or three layers of GCs with an average diameter of 14.8 ± 2.3 × 10.8 ± 0.9 µm; and a medullary region, which has been reduced considerably and where few cells apparently form a simple epithelial layer, delimiting the central cavity of the ovary (Fig. 4c). The ultrastructure reveals the prominent nuclei of primary oogonia with homogeneously distributed chromatin, mitochondria in their cytoplasm and prefollicular cells that are closely related to primary oogonia (Fig. 4d).

After 3 months of larval development (120 dpf), ovaries are anatomically located in the abdominal cavity and suspended dorsally from the body wall by the mesovary. They lie symmetrically on either side of the midline of the body, parallel to the kidneys. Morphologically, the ovary appears to be a saccular structure with a large central cavity (Fig. 4e); the limit to the ovary is defined by epithelial cells that are cubic or flattened in shape and in some places on this delimiting epithelium, groups of oogonias are observed to structure the germinal epithelium or niches (Fig. 4e). At the periphery of the ovary, meaning in the cortical region,

Fig. 4 Ovarian differentiation in *A. mexicanum*. **a** Location of the gonad of a larva during St55 (52 dpf), cortical region (cr) with peripheral germ cells and medullary region (mr) consisting of somatic cells (sc) in retreat leading to formation of the ovarian cavity (oc). Note the progonad (p) closely related to the back region of the gonad. **b** Electronic micrography of a germ cell with indications of meiosis, evidenced by the presence of synaptonemic complexes (s). Mitochondria are also apparent (mi) and electro-dense bodies (*arrowhead*) in its cytoplasm, and the relationship with somatic cells is evident (sc). **c** Gonad of a larva at St57 (90 dpf), where germs cells in the cortical region (cr) are visible and regression of the medullary region (mr) forming a central ovarian cavity (oc). **d** At greater resolution the nucleus (n) of a germ cell is visible, and in its cytoplasm mitochondria (mi) can be seen. **e** 3-month-old larva ovary (120 dpf), surrounded by an epithelium (e), with an evident ovarian cavity (oc). Attached to the epithelium are ovarian follicles structured by the oocyte (*asterisk*) (f). Note a niche of oogonias (no) in the wall of the ovary. **f** Electron micrograph of the wall of an oocyte with peripheral vitelline vesicles (vv) and mitochondria (mi) in their cytoplasm and surrounded by follicular cells (f), of the theca (t) and ovarian epithelium (e)
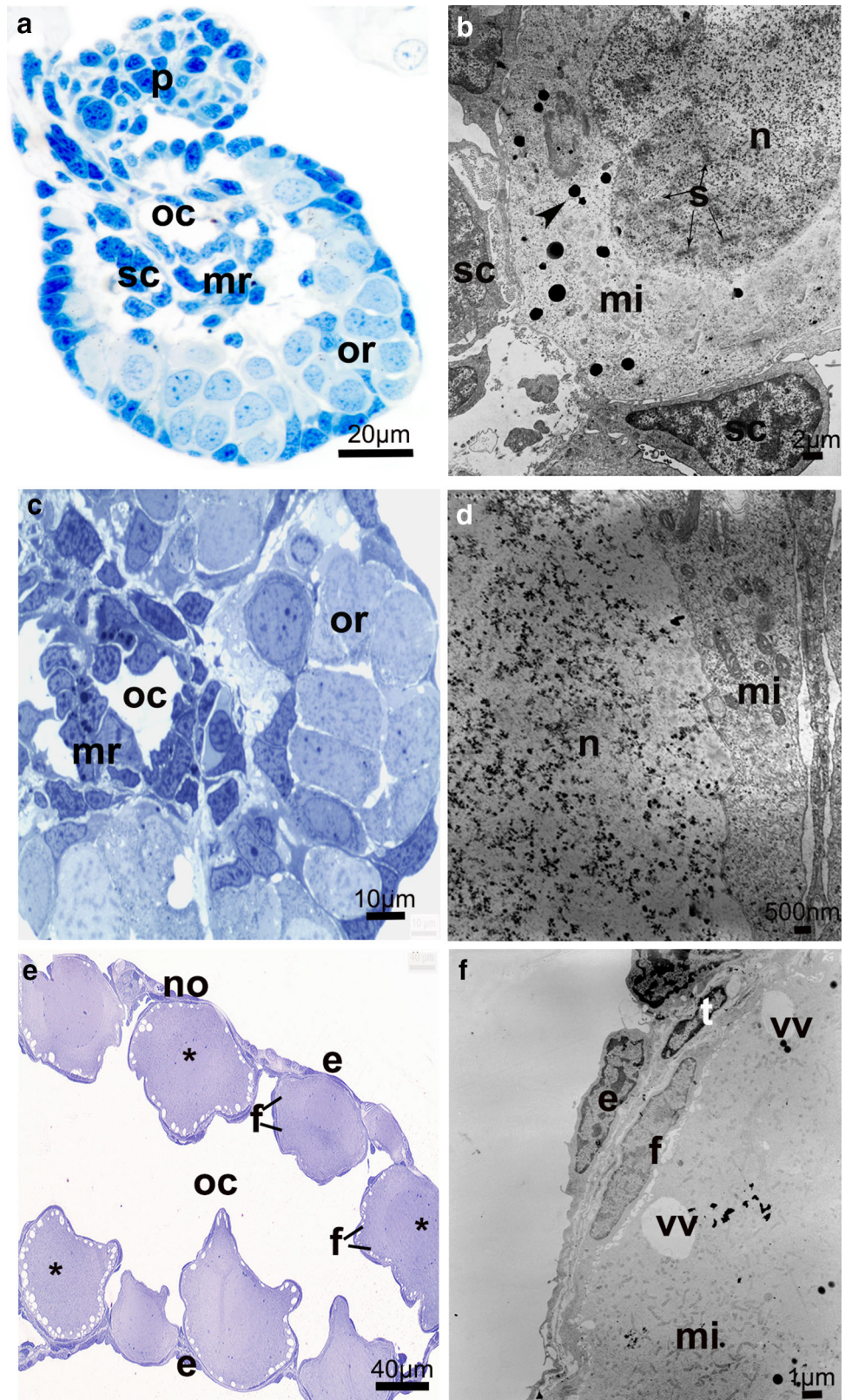
follicles attached to the ovarian epithelium can be observed. These are formed during the diplotene oocyte, presenting a prominent nucleus with several peripheral nucleoli. The cytoplasm is homogeneous with peripheral vitelline vesicles surrounded by a layer of flattened follicular cells and a thin theca also formed from flattened cells (Fig. 4f).

Testis differentiation

During St55 (52 dpf), a notable increase in the size of the differentiating gonad occurs, in the testicular direction (78.5 ± 14.1 × 63.0 ± 5.3 μm in diameter). The two regions that structure the gonad manifest changes; the cortical region is now restricted to a layer of flattened epithelial cells that surround the gonad and these continue with the mesentery to suspend this from the wall of the body. In the medullary region, a large amount of primary spermatogonia are observed to form groups in close relation to somatic cells, organized in such a way that they structure groups delimited by other internal somatic cells, thus forming the testicular cords (Fig. 5a). Primary spermatogonia are apparently larger in size than somatic cells, with an average diameter of 11.3 ± 0.8 × 8.3 ± 0.8 μm. These primary spermatogonia consist of a large nucleus with an eccentric nucleolus and it is evident that some primary spermatogonia groups are undergoing cell division (Fig. 5a). Ultra-structurally, it is notable that in the primary spermatogonia nucleus, chromatin is finely granular and this nucleus is surrounded by a large amount of peripheral mitochondria. In the primary spermatogonia cytoplasm, the presence of some dense granules is still apparent (Fig. 5b). At this stage of testicular differentiation, two types of somatic cells are evident, some of irregular shape intimately attached to the primary spermatogonia, which are pre-Sertoli cells, and others more peripheral that are possibly part of the stromal tissue and therefore future peritubular or myoid cells (Fig. 5b). At this stage, as well as during ovarian morphogenesis the progonad appears, which will later form fatty bodies (Fig. 5a).

At St57 (90 dpf), the testes are apparently larger (80 ± 7.2 × 44.3 ± 4.0 μm). In the medullary region, testicular cords are becoming organized; this is where the majority of primary spermatogonia are located, identifiable by their size (with an average diameter of 14.0 ± 0.8 × 9.3 ± 1.8 μm). These are electron-light in appearance with a prominent nucleus, with one or two nucleoli. The germ line (spermatogonias) is closely related to somatic cells (future Sertoli cells) and structures the testicular cords (Fig. 5c, d). Among the testicular cords, somatic cells present different morphologies, some are fusiform and located at the periphery of the cords, structuring future myoid or peritubular cells, and others are irregular in shape, appearing as part of the interstitial tissue and finally there are others with lipids included in their cytoplasm that are located near blood vessels; possibly future Leydig cells (Fig. 5c, d). In the GCs, the presence of a granular chromatin in the nuclei and a large amount of mitochondria in the cytoplasm close to the periphery of the nucleus is ultra-structurally evident (Fig. 5d).

At 3 months of development (120 dpf), testicular differentiation is apparent. The testes appear as same described in before stage. The testes are parallel to the axis of the body, and closely related to the mesonephros. They are surrounded by fibrous connective tissue that forms the tunica albuginea with an interior consisting of testicular lobes. Each testicular lobe consists of several cysts with primary spermatogonias and each cyst consists of one or more Sertoli cells which make up the cyst wall (Fig. 5e). Externally, surrounding each lobe, it is possible to observe the myoid or peritubular cells. Between the testicular lobules, the interstitial tissue is loose and is formed out of connective tissue, where the Leydig cells are located close to blood vessels (Fig. 5e). Ultra-structurally, the primary spermatogonias can be identified by the presence of a large round nucleus with finely granular and homogeneous chromatin (Fig. 5f). In the interstitial region, cells with accumulations of lipid droplets are apparent, suggesting the differentiation of Leydig cells (Fig. 5f).

Notably, the size of the gonad as well as the size of the GCs increases as larval development progresses, with a tendency to be bigger at the beginning of gonadal differentiation, especially in the female gonad (Table 1).

Discussion

The Mexican axolotl (A. mexicanum) is a pedomorphic salamander; meaning that it becomes sexually mature while preserving morphological characteristics from the larval stage. Some morphological and molecular aspects related to germline specification and germ cell migration have been described for some stages of embryonic and larval development (Ikenishi and Nieuwkoop 1978; Dournon et al. 1989; Johnson et al. 2001; Bachvarova et al. 2004); but morphological events involved in the processes of gonadal sexual determination and differentiation have been little explored. Similarly, neither has the correlation between these morphogenetic events and the establishment of the germ line been reported. As this is a neotenic organism, it is of great interest to identify and correlate processes of gonadal differentiation with stages in embryonic development, as well as considering that observed among other non-neotenic urodeles, such as P. waltl. Anatomically in A. mexicanum, as in anurans and P. waltl, the gonads develop as paired organs that are located in the posterior mid-dorsal region of the body of the organism, closely related to the mesonephros and suspended in the coelomic cavity (Ogielska 2009; Flament et al. 2011).

Gonad sexual development in amphibians is a process that has principally been described among anurans (Ogielska 2009; Piprek et al. 2010; Flament et al. 2011), whereas little is known about urodeles (Dournon et al.

Fig. 5 Testicular gonadal differentiation in *A. mexicanum*. **a** Larval gonad at St55 (52 dpf), where testicular cords (*box*) are visible in the medullary region, structured by germ cells and surrounded by somatic cells (sc). Notice the epithelial cells (e) that surround the gonad and the progonad (p) at *upper right*. **b** Electron micrograph of a germ cell with a large nucleus (n) with homogeneously distributed chromatin, mitochondria (mi) and electro-dense bodies (*arrowhead*) in their cytoplasm. Note the close relationship between two somatic cells, one of them practically attached; pre-Sertoli future Sertoli cell (S) and the other more peripheral; future peritubular cell (P). **c** Larval gonad during St57 (90 dpf), where testicular cords comprising primary spermatogonia (Sp) are visible in close relation to pre-Sertoli (S) cells, peritubular cells (P) and in the interstitium near to the blood vessels (bv) are future Leydig cells (L). **d** Electron micrograph of a primary spermatogonia (Sp) with a large nucleus (n) and mitochondria (mi) in its cytoplasm, note the close relationship with somatic cells: future pre-Sertoli (S), peritubular (P), and Leydig (L). **e** Testes of a 3-month-old larva (120 dpf) with cysts of primary spermatogonia (Sp) inside, consisting of one or more sertoli (S) cells. Peritubular cells (P) are visible around the lobules and in the interstice there is a Leydig cell (L) near to a blood vessel (bv). **f** Electron micrograph showing the detail of a primary spermatogonia (Sp) cyst, closely related to sertoli (S) cells; in the interstitial tissue, a leydig (L) cell with lipid droplets in its cytoplasm is visible
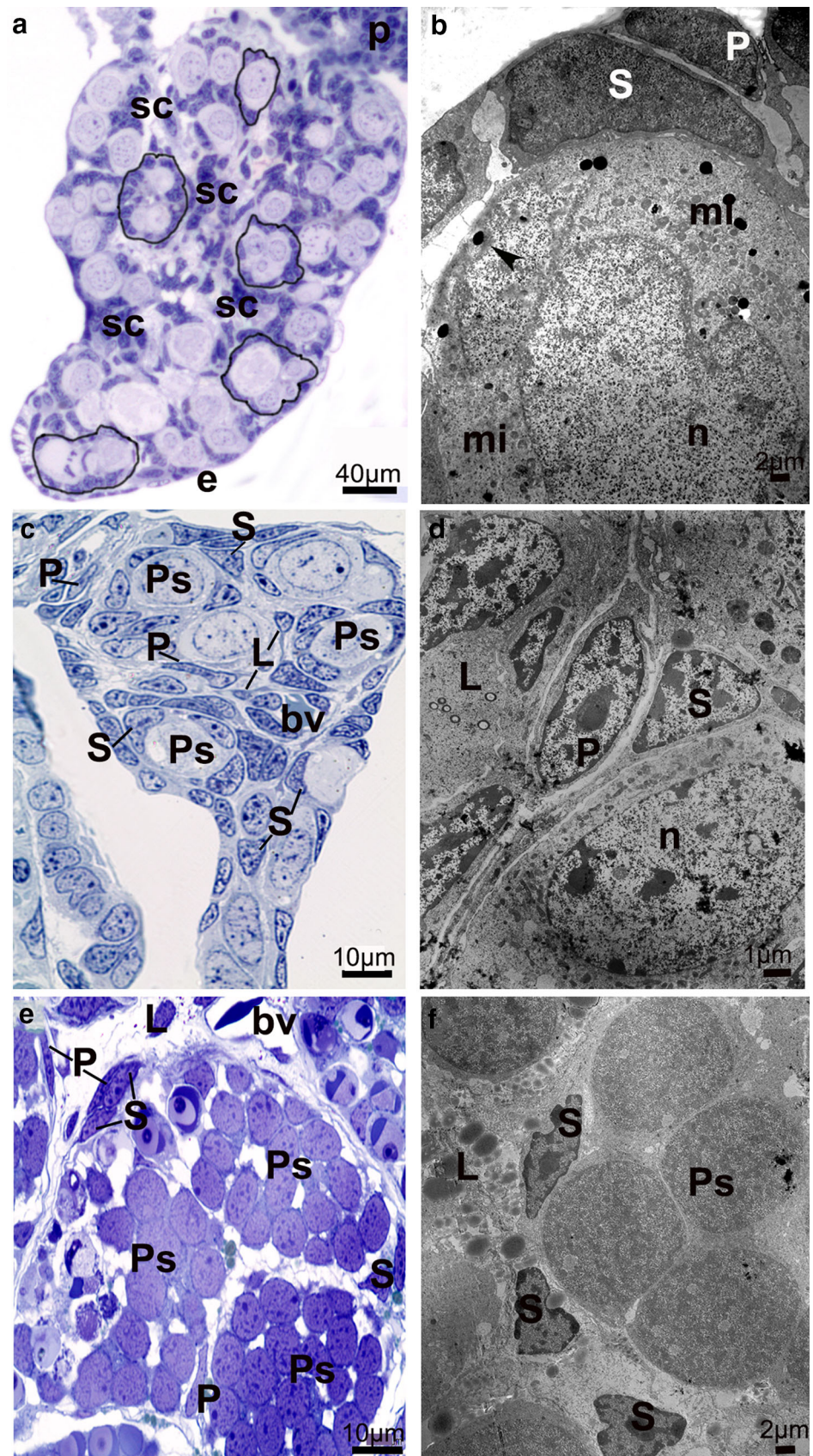
Table 1 Average diameters (length and width) of the gonad and the germ cells (GC) and area of germ cells of *A. mexicanum* at different stages of development

Development stage	Gonad length (μm)	Gonad width (μm)	GC length (μm)	GC width (μm)	GC area (μm^2)
41	22.6 ± 3.6	11 ± 1.8	9.2 ± 2.3	5.3 ± 1.0	488 ± 153.6
45	24.2 ± 6.9	12.0 ± 1.0	9.6 ± 2.9	5.6 ± 1.3	620 ± 97.2
50	38.5 ± 8.5	17.8 ± 1.8	10.0 ± 0.9	6.0 ± 1.5	576 ± 101
53	54.2 ± 10.2	20.8 ± 4.4	10.8 ± 2.1	7.2 ± 1.1	706 ± 96
55 O	88.7 ± 17.2	45.7 ± 23.3	12.7 ± 3.5	12.3 ± 2.4	709 ± 110
57 O	96.0 ± 6.6	61.3 ± 11.3	14.8 ± 2.3	10.8 ± 0.9	1053 ± 180
57 T	80.0 ± 7.25	44.3 ± 4.0	14.0 ± 0.8	9.3 ± 1.8	638 ± 74

1990). In this study, the main morphological events leading to the establishment of the undifferentiated gonad and its morphological differentiation in *A. mexicanum* were similar to those described for anurans and the *P. walt* urodela (Dournon et al. 1990). The formation of a genital ridge is apparent during St41, when a group of somatic cells are found suspended below the mesonephros and adjacent to the Wolffian duct. This stage coincides with the location of PGCs close to the Wolffian duct, concurring with previous studies (Ikenishi and Nieuwkoop 1978; Dournon et al. 1990; Bachvarova et al. 2004; Dumond et al. 2008; Flament et al. 2011). Based on our observations, the genital ridge seems to retain, at least morphologically, the potential to differentiate into a testis or an ovary, depending on the chromosomal complement of the organism (ZZ-ZW). Between St42 and 44, no obvious morphological changes were observed, only an increase in the size of the gonad probably due to the invasion of PGCs, apparently similar to that reported among *Xenopus laevis* and *Rana pipiens*, as the gonad during the larval stage contains few somatic cells (Falconi et al. 2004; Ogielska 2009). Likewise, it has been reported that during these stages, PGCs may be in a period of mitotic rest, as apparently in *P. waltl* there is a period between St35 and St41, characterized by zero mitotic index (Po), during which no proliferation occurs (Dournon et al. 1989, 1990).

In *A. mexicanum* from St45 until St52, it is already possible to observe the developing gonadal primordium as a more compact structure where the germ line becomes clearly distinguished from the somatic line. In this respect, in our observations, as in other studies, it is evident that the number of germ cells appears to increase as does their size (Ikenishi and Nieuwkoop 1978; Dournon et al. 1990; Dumond et al. 2008). Therefore, we suggest that during these stages of larval development in *A. mexicanum* urodela, independent of the chromosomal sex of the organism, the undifferentiated gonad consists mainly of germ cells and somatic cells. Thus, the morphological events leading

to the development and establishment of the undifferentiated gonad in *A. mexicanum* occur between St41 and St53, similar to that reported for the *P. waltl* salamander (Dournon et al. 1990; Dumond et al. 2008; Flament et al. 2011). As in most vertebrates and owing to its morphological characteristics, the undifferentiated gonad in *A. mexicanum* can be considered as a bipotential structure, meaning that morphologically it has the capacity to differentiate into an ovary or testis.

During St45, it has been reported that the *P. waltl* salamander has a structure formed out of somatic cells which is attached to the gonad, known as the peduncle or hilar region. In the case of *A. mexicanum,* this structure appears during St50, coinciding more with that described for other amphibians (Jamil et al. 2008).

Structural changes in the undifferentiated gonad during St53 of *A. mexicanum*, such as the rearrangement of germ cells in the cortical region, an increase in their proliferation and the beginning of the formation of a cavity in the internal zone by regression of the medullary region, suggest that at the end of this stage, sexual differentiation of the gonad initiates. Similar to that reported in *P. waltl* and contrary to that found in other vertebrates, in *A. mexicanum*, it seems that the first morphological manifestation of gonadal sexual differentiation occurs in the female gonads, as in the male gonad no morphological changes were detected with respect to the structure of the undifferentiated gonad; only an evident increase in size, coinciding with previous observations (Dournon et al. 1990). Testicular morphogenesis is evident during St55, when germ cells located in the medular region of the gonad interact with somatic cells, whereas the cortical region is restricted to a layer of flattened epithelial cells that surround the gonad. All these cellular processes of gonadal differentiation coincide with patterns reported for anuran amphibians and urodeles (Dournon et al. 1990; Kuntz et al. 2003; Dumond et al. 2008; Flament et al. 2011; Al-Assad et al. 2013; Haczkiewicz and Ogielska 2013).

The electro-dense bodies that are contained in germ cells that make up the *A. mexicanum* gonad decrease as gonadal development progresses during St53. Ikenishi and Nieuwkoop (1978) observed the presence of mitochondrial arrangements in the cytoplasm of germ cells of *A. mexicanum* during St40, which they called "nuage", also known as electro-dense material or "cloud" in other amphibians. In other urodeles such as *Triturus pyrrhogaster*, *Hynobius* and *P. waltl*, these groups of mitochondria have also been observed in germ cells (Ogielska 2009), indicating that these structures appear with characteristic of the germline, as they have also been found to be closely related to mitochondria, which are part of the nuclear material and contain ribonucleoproteins (Guerrero and Moreno 2012; Voronina et al. 2011). In mammals, "dense nuclei" are equivalent to "nuage" material and it has been proposed that these are organelles specializing in the reception of signals from the extracellular environment that may modify the behavior and differentiation status of PGCs (Merchant and Álvarez 1981; Voronina et al. 2011). Therefore, the decrease in electro-dense material in germ cells at the moment when morphological differentiation initiates in the gonad in *A. mexicanum* (St53), may indicate a role in maintaining the bipotential germline state.

The structural arrangement of the developing ovaries and testes was similar to that described for other species of anuran amphibians such as *X. laevis* (Merchant and Villalpando 1981), *X. tropicalis*, *R. pipiens*, *Bombina orientalis* among others (Hayes 1998), as well as for the *P. walt* urodela (Dournon et al. 1990; Hayes 1998; Kuntz et al. 2003; Dumond et al. 2008). In *A. mexicanum*, during St55, a structure appears for the first time in the anterior region and adjacent to the gonad known as an anterior progonad, which will give rise to fatty bodies, as observed in other urodeles (Ogielska 2009).

Morphogenetic changes undergone by undifferentiated gonads of both sexes in order to differentiate themselves, as well as the time involved, seem to be independent of the neotenic condition of *A. mexicanum*. As in the *P. waltl* salamander, the beginning of the organization of the cortical and medullary regions for the formation of a testis or ovary begins during St53, when both species are at the larval stage (Dournon et al. 1990; Kuntz et al. 2003; Dumond et al. 2008). In other amphibians, the differentiation process occurs only during the larval lifespan and in some cases occurs near to metamorphosis (Dournon et al. 1990; Hayes 1998). During processes of gonadal differentiation, some of these occur before, and others after metamorphosis, as is the case on entering germ line meiosis. In the ovary of *P. waltl*, oogonias enter meiosis during metamorphosis, that is approximately during St56 (Dournon et al. 1990; Dumond et al. 2008), whereas in the testicle, PGCs do this during the juvenile stage (Dumond

et al. 2008; Flament et al. 2011; Al-Assad et al. 2013). In the case of *A. mexicanum*, although they do not undergo complete metamorphosis, something similar to that described for *P. waltl* occurs. In *A. mexicanum*, ovary germ cells initiate meiosis during St55, evidenced by the presence of synaptonemic complexes.

At 3 months of age, the gonad has completed its morphological differentiation, becoming an ovary or a testicle. The ovary of *A. mexicanum* is a saccular structure, coinciding with that observed in *Necturus maculosus* (Kessel and Panje 1968), *P. waltl* (Dumond et al. 2008; Wallacides et al. 2009; Flament et al. 2011) and *A. dumerilli* (Uribe 2009, 2011). In comparison with the testicle, the presented structure is compact, similar to that observed in other urodeles such as *P. waltl* (Dumond et al. 2008; Flament et al. 2011) and *A. dumerilli* (Uribe et al. 1994).

In conclusion, our observations suggest that the time period for embryonic development in *A. mexicanum* is similar to that observed in *P. waltl*. However, larval development in *A. mexicanum* is faster than the development of *P. waltl*; this may be due to the neotenic condition of *A. mexicanum*; implying that when they are not undergoing metamorphosis, they can follow a pattern of constitutive growth independent of the temperature at which larvae develop. The different events of gonadal development observed in *A. mexicanum* coincide with those described for *P. waltl* in terms of stages; however, fewer days are required for *A. mexicanum* to advance from one stage to another. This change in development speed coincides with the stage at which the thermo-sensitive period proposed for *P. waltl* begins, suggesting that in *A. mexicanum* urodela, temperature does not influence somatic development. However, in both urodela species, the onset of gonadal development occurs from St35 (11–12 dpf) and the establishment of the undifferentiated gonad is observed from St50, independent of the days post-fertilization. This suggests that gonadal morphogenesis is independent of days of larval development. Likewise, the onset of gonadal sexual differentiation in *A. mexicanum* takes place from St53, evidenced by ovarian structural changes. Likewise in *P. waltl*, it is known that gonadal sexual differentiation takes place in stages prior to metamorphosis (St53–St56); stages that coincide with differentiation in *A. mexicanum*, meaning that the neotenic condition does not influence events related to gonadal morphogenesis.

According to Wakahara (1996) gonadal development is controlled by a chronological regulation that differs from somatic development; this suggests that gonadal development is completely independent of metamorphosis, implying a process of heterochrony.

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