

Testicular structure and germ cells morphology in salamanders

Mari Carmen Uribe* and Víctor Mejía-Roa

Laboratorio de Biología de la Reproducción; Departamento de Biología Comparada; Facultad de Ciencias; Universidad Nacional Autónoma de México; Ciudad Universitaria; México DF, México

Keywords: salamanders, lobular testis, cyst, cephalo-caudal spermatogenesis, spermatophores

Testes of salamanders or urodeles are paired elongated organs that are attached to the dorsal wall of the body by a mesorchium. The testes are composed of one or several lobes. Each lobe is morphologically and functionally a similar testicular unit. The lobes of the testis are joined by cords covered by a single peritoneal epithelium and subjacent connective tissue. The cords contain spermatogonia. Spermatogonia associate with Sertoli cells to form spermatocysts or cysts. The spermatogenic cells in a cyst undergo their development through spermatogenesis synchronously. The distribution of cysts displays the cephalo-caudal gradient in respect to the stage of spermatogenesis. The formation of cysts at cephalic end of the testis causes their migration along the lobules to the caudal end. Consequently, the disposition in cephalo-caudal regions of spermatogenesis can be observed in longitudinal sections of the testis. The germ cells are spermatogonia, diploid cells with mitotic activity; primary and second spermatocytes characterized by meiotic divisions that develop haploid spermatids; during spermiogenesis the spermatids differentiate to spermatozoa. During spermiation the cysts open and spermatozoa leave the testicular lobules. After spermiation occurs the development of Leydig cells into glandular tissue. This glandular tissue regressed at the end of the reproductive cycle.

Introduction

The basic progression of sperm development in urodeles proceeds similarly to the rest of vertebrates. Spermatogenesis occurs in the testes and is maintained by a stem cell population, the spermatogonia, which permit constant supplies of abundant male gametes. Spermatogonial stem cells divide through mitosis to produce generations of spermatogonia which enter the spermatogenic cycle. Spermatogonia develop into spermatocytes that undergo meiosis through primary and secondary spermatocytes to produce haploid cells, the spermatids. Spermatids differentiate into mature sperm through morphological and functional changes during spermiogenesis, which results in the mature spermatozoa.

*Correspondence to: Mari Carmen Uribe; Email: mari3uribe3@gmail.com
Submitted: 09/03/2014; Revised: 11/04/2014; Accepted: 11/10/2014
<http://dx.doi.org/10.4161/21565562.2014.988090>

Testicular Structure

The testes are paired and elongated organs, attached to the dorsal side of the body cavity by the mesorchium. The testes are aligned in parallel position to the long axis of the body, and consequently, they are parallel to the mesonephros, Wolffian or mesonephric ducts, and rudimentary Müllerian or paramesonephric ducts.¹⁻¹⁰

The testes of urodeles are composed of one or several lobes. Each lobe is morphologically and functionally a similar testicular unit. In some species, sexually immature males have a testis containing only one lobe, whereas mature males have a testis containing multiple lobes.¹¹ The number of testicular lobes depends on the age of the animal.^{3,12} Testis with multiple lobes were described in various species: *Desmognathus fuscus*, *Diemyctylus viridescens*, *Diemyctylus torosus*, and *Salamandra atra*¹¹; *Triturus viridescens*¹; *Taricha granulosa*²; *Trituroides hongkongensis*¹³; *Triturus cristatus*¹⁴; *Diemyctylus viridescens*, *Cynops pyrrhogaster*⁴; *Triturus marmoratus*¹⁵; *Salamandra salamandra*^{3,16,12,17}, *Bolitoglossa occidentalis*, *B. rostrata*, *Dendrotriton bromeliacia*, *Pseudoeurycea goebeli*¹⁸, *Pleurodeles waltl*.¹⁹ Other species have simple testes, formed by one lobe, regardless of their age, as *Eurycea quadridigitata*,²⁰ *Salamandrina terdigitata*⁵ and *Lissotriton italicus*.²¹

The lobes of the testis are joined by narrow interlobar cords covered by a single peritoneal epithelium and subjacent vascularized connective tissue. These cords contain spermatogonia among the connective tissue.^{4,11,12,22} The lobes and interlobar cords are illustrated in *Taricha granulosa* (Fig. 1A).

Several authors have described the structure of testicular lobes in urodeles.^{4,7-10,14,23-26,27-30} The testicular lobes are surrounded by fibrous connective tissue which forms the tunica albuginea. The internal structure of the testicular lobes consists of abundant longitudinal lobules, separated by trabeculae of thin and vascularized connective tissue, that is a continuation of the tunica albuginea. Among the trabeculae of connective tissue and near the capillaries there are Leydig cells. Each lobule contains several cysts with spermatogenic cells in different stages of development. The spermatogenic cells in a cyst undergo their development through spermatogenesis synchronously.

The distribution of cysts displays the cephalo-caudal gradient in respect to the stage of spermatogenesis (Figs. 1A, 2A,B). This type of spermatogenesis process has been termed the spermatogenesis wave.³¹ The cephalic edge of the testis contains spermatogonia, either as individual cells or in small groups as hollow chambers lined by the spermatogonia. Progressively, there are lobules within

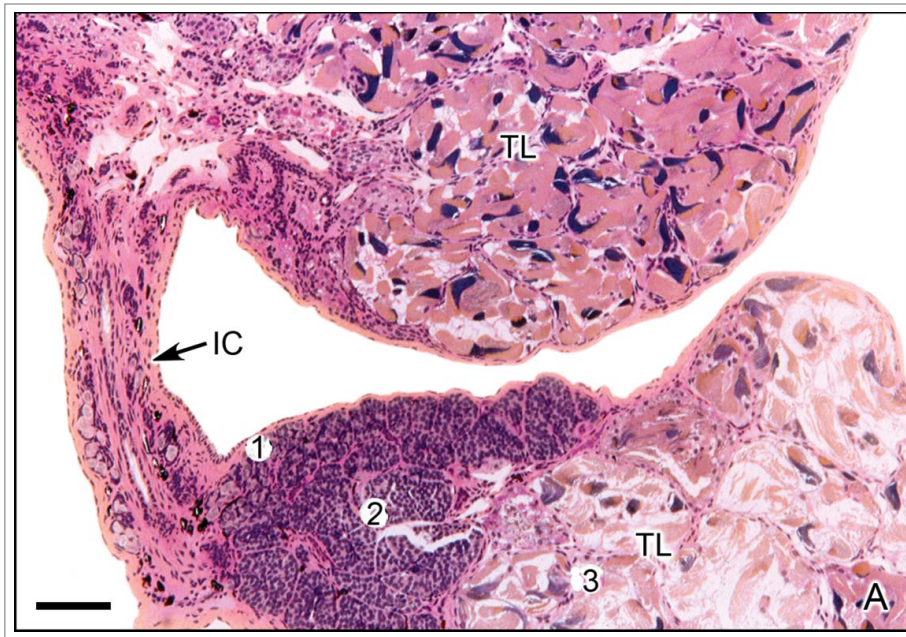


Figure 1. A. Testicular lobes of *Taricha granulosa*. The testicular lobes (TL) are joined by an interlobar cord (IC). The cephalic region of the lobe contains spermatogonia (1) and spermatocytes (2) and the caudal region contain spermatozoa (3). Hematoxylin-eosin. Bar = 200 μ m. Courtesy of Dr. Harry J. Grier.

which primary and secondary spermatocytes, spermatids, and spermatozoa are formed.^{19,20,29,30,32,33} The continuous formation of cysts at cephalic end of the testis causes their migration along the lobules to the caudal end. Consequently, the disposition in cephalo-caudal regions of spermatogenesis can be observed in longitudinal sections of the testis (Fig. 2A), meanwhile, in transversal sections, all cysts are approximately at the same stage, resulting in a spatial and temporal segregation of differentiating germ cells.

During the reproductive cycle the type of spermatogenic cells and the number of cysts differ.^{2,4,12,34,35} For instance, the endemic urodeles of Mexico, *Ambystoma dumerilii* from Patzcuaro Lake, and *A. mexicanum* from Xochimilco Lake, have a spring breeding season. In this annual cycle sperm mature in summer and autumn and occupy the most of testicular lobe and only a small area, at the cephalic end of the lobe, contains spermatogonia and some cysts with spermatocytes and spermatids. Spermatozoa are stored during several months in the testis and in the ducts system. When females spawn in the next spring, then, the males release spermatozoa and the fertilization occurs. In contrast to this cycle, *Cryptobranchus alleghaniensis* and *Necturus maculosus*, spermatozoa are produced only shortly before spawning by a very rapid phase of spermiogenesis.^{3,4} We illustrate the testis and spermatogenesis in urodeles here with the species *Ambystoma dumerilii* and *A. mexicanum*.

A cyst is formed when a primary spermatogonium becomes surrounded by a Sertoli cell (Figs. 2C,D). Thus, the Sertoli cell forms the wall of the cyst.^{3,12,15,36,37,38} The synchronous development of spermatogenic cells inside a cyst, during spermatogenesis is a result of persistent intercellular cytoplasmic bridges between germ cells, and these bridges are due to incomplete cytokinesis during the mitotic divisions of germ cells. The Sertoli cells have complex and

essential functional relationships with the spermatogenic cells. Sertoli cells maintain a permeability barrier to spermatogenic cells during spermatogenesis, determine the endocrine activity that controls spermatogenesis, phagocytose degenerating spermatogenic cells and residual bodies during the spermiogenesis, and form specific antigens.^{25,31,37,39,40} During spermiation, when the cysts open and spermatozoa leave the testis, Sertoli cells (Fig. 2D) remain inside the lobule (Fig. 2D) and undergo morphological changes (Figs. 3C,D) consistent with the acquisition of steroid secreting behavior. Sertoli cells then degenerate and disappear. According to the cell cycle of Sertoli cells, they are not a permanent cell type in the testis; consequently, their cyclic supply is of great importance for the development of cysts and maintaining spermatogenesis in the males.³⁸

The Leydig cells are in the testicular interstitial tissue, between the lobules. These cells present cyclic morpho-physiological changes according to the stage of spermatogenesis. The Leydig cells are

small and irregular in shape during spermatogenesis, but initiate proliferation, hypertrophy and maturation, developing a glandular tissue around the lobules, during and after spermiation.^{39,41,42-44} The cephalo-caudal disposition of spermatogenesis in the testis defines the spermiation at the caudal region; consequently, the development of Leydig cells at this region involves a complex interaction that demonstrates local regulatory control of testicular function.¹⁰ Leydig cells attain 35–55 μ m in diameter^{7,9} and contain lipids, becoming sites of androgen synthesis (testosterone and 5 α -dihydrotestosterone). During the regression period, Leydig cells suffer rapid involution.²⁸ The morpho-physiological changes of Leydig cells are described in several species: *Ambystoma tigrinum*^{26,28}; *Trituroides hongkongensis*¹³; *Cynops pyrrhogaster pyrrhogaster*⁴⁵; *A. mexicanum*^{32,39}; and *Necturus maculosus*^{31,43}; *Triturus marmoratus*.^{36,44}

Spermatogenesis

In most urodeles, spermatogenesis has cyclic changes under the control of the neuroendocrine system and is influenced by environmental factors, such as temperature, rainy season and photoperiod.^{27,28,29,46,47} Spermatogenesis is regulated by follicle-stimulated hormone (FSH) released by the adenohypophysis. Sertoli cells express FSH receptors from early spermatogenesis through the spermatid stage. FSH, through its actions on Sertoli cells, is involved in the mitotic activity of the spermatogonia, the meiotic divisions of primary and secondary spermatocytes during the formation of spermatids and spermiogenesis throughout the development of the spermatozoa.^{32,38} Luteinizing hormone (LH)

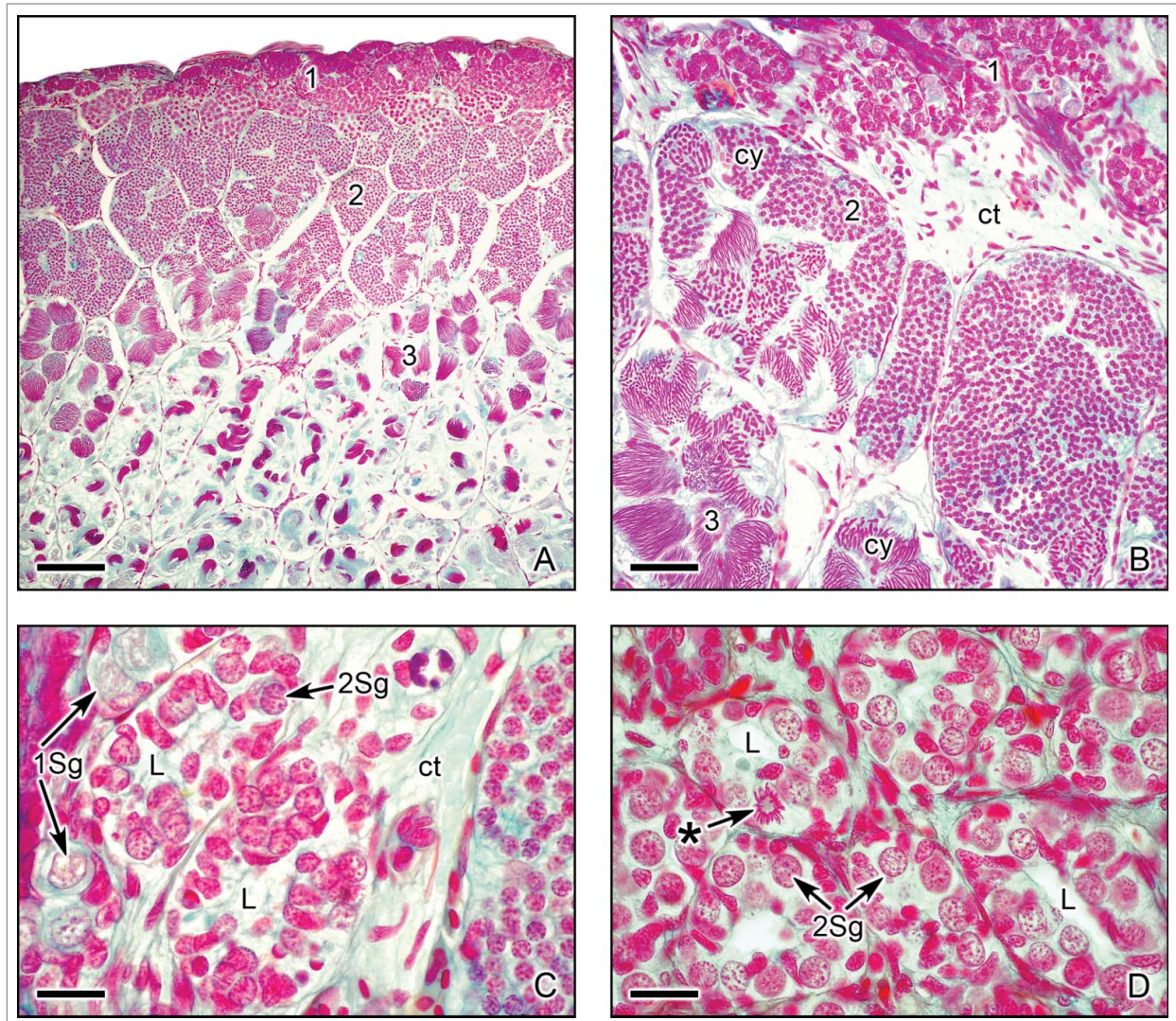


Figure 2. Cephalo-caudal disposition of the spermatogenic stages in longitudinal sections of the testes of *Ambystoma dumerilii*. (**A,B**). Three testicular regions of spermatogenesis advance progressively along the cephalo-caudal axis. (1) Starting at the cephalic region, the testis contains numerous lobules with early stages of spermatogenesis, which include spermatogonia, primary and secondary spermatocytes. (2) Subsequently, middle stages of spermatogenesis with spermatids. (3) Followed by late stages of spermatogenesis with spermatozoa. The testicular lobules enclose several cysts (cy) with germinal cells in synchronous stages of spermatogenesis. Alcian blue. Bar = 200 μm . Bar = 50 μm . (**C,D**). Sections of the peripheral cephalic region of the testis with cysts of proliferating spermatogonia. Primary spermatogonia (1Sg) and secondary spermatogonia (Sg2) are seen. The secondary spermatogonia form clusters surrounding a central lumen (L), structures that originate the testicular lobules. The clusters of secondary spermatogonia are delimited by loose connective tissue (ct). One secondary spermatogonium shows mitotic activity (*). Alcian blue. Bar = 20 μm .

plays a role in spermiation and testosterone secretion.^{28,31} Additionally, spermatogonia and spermatocytes of *Triturus marmoratus marmoratus* showed a positive reaction to androgen, estrogen α , and estrogen β receptors during the annual reproductive cycle, suggesting that there is an androgen-estrogen regulation of the function and development of the new testis.⁴⁸

As discussed previously, during the cyclic changes a longitudinal wave of spermatogenesis occurs along the length of the testis.^{11,31} Spermatozoa are formed by mitotic multiplication of spermatogonia followed by meiosis in spermatocytes and spermiogenesis in spermatids. According to these observations, the testicular cycle of urodeles advances from a period of spermatogenesis to a period of maturation, spermiation and regression.

During spermiation, spermatozoa are progressively released from the testes to the efferent ducts.^{13,28,39,34,49,44} The urodele testicular cycle is synthesized in several phases: proliferative phase, characterized by mitotic divisions, when proliferation of spermatogonia forms new cysts where spermatocytes advance in spermatogenesis; meiotic phase, characterized by meiotic divisions, when primary and second spermatocytes are formed and develop spermatids; spermiogenesis phase when spermatids differentiate to spermatozoa; and, spermiation when cysts of spermatozoa open and spermatozoa leave the testicular lobules. After spermiation occurs the development of Leydig cells into glandular tissue. This glandular tissue regressed at the end of the reproductive cycle.¹⁰

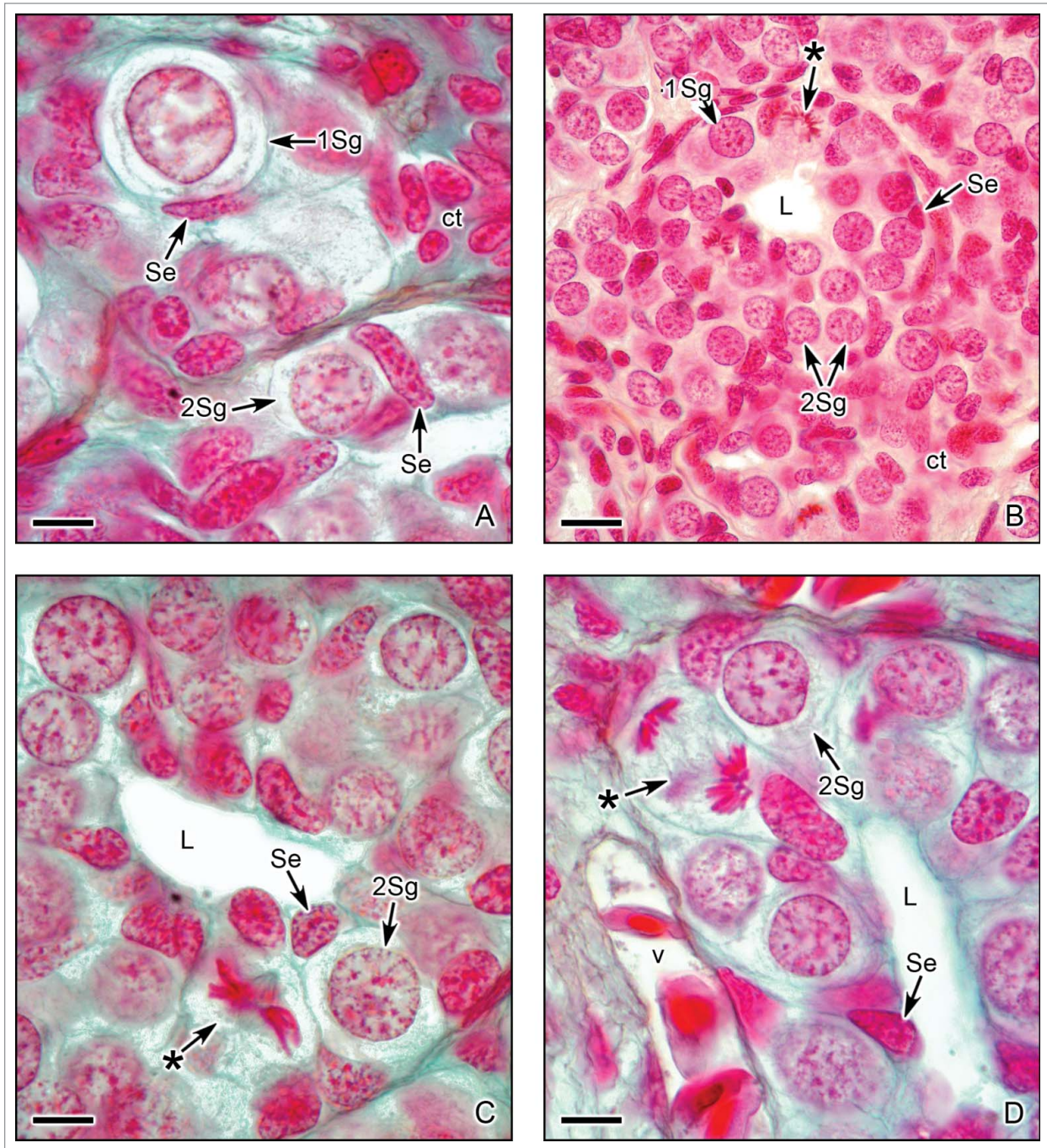


Figure 3. Cephalic region of the testis of *Ambystoma dumerilii* with primary and secondary spermatogonia. (A) A primary spermatogonium (1Sg) and several secondary spermatogonia (Sg2) surrounded by Sertoli cells (Se). Sertoli cells have elongated nuclei with granular chromatin and bordered by connective tissue (ct). Alcian blue. Bar = 10 μ m. (B,C,D) The proliferation of spermatogonia is evident by chromosomes during mitotic phases, as metaphase (*) in Figs. B and C and anaphase (*) in Fig. D. The fibers of the spindle are observed in mitotic phases in Figs. C and D. The clusters of secondary spermatogonia (2Sg) have a central lumen (L). The spermatogonia are surrounded by Sertoli cells (Se). The connective tissue contains blood vessels (v). Hematoxylin-eosin. Bar = 20 μ m. Alcian blue. Bar = 10 μ m.

Morphology of germ cells during spermatogenesis

The stages of spermatogenic cell maturation, in a variety of species of urodeles, was described by several authors: in *Desmognathus fusca*²³; in *Necturus maculosus*^{24,31}; in *Ambystoma tigrinum*^{25,26,28}; in *Pleurodeles waltlii*⁵⁰; in *Tritutoides*

*hongkongensis*¹³; in *A. mexicanum*^{27,49}; in *Salamandrina terdigitata*⁵; in *Triturus marmoratus*^{44,47}; in *Salamandra salamandra*^{12,16,17}; in *A. dumerilii*²⁹; in *Batrachuperus pinchonii*⁵¹; Uribe in *A. mexicanum* and *A. dumerilii*^{8,9}; in *Lissotriton italicus*.²¹ A cinematographic study of meiosis in salamander

spermatocytes in vivo was done.⁵² This study put in relevance the movements of bivalents homologous chromosomes during the first meiotic division.

Primary and secondary spermatogonia

The primary spermatogonia are situated in the cephalic region of the testis as individual cells surrounded by connective tissue (Fig. 3A), and in multilobular testis, also between lobes, in the interlobar cords among the connective tissue (Fig. 1A). Some spermatogonia are also observed between the connective tissue that surrounds lobules in other regions of the testis, not only in the cephalic region but in the direction of the middle part of the testis. The spermatogonia are the biggest germinal cells (40–55 μm in diameter). Primary spermatogonia are spherical cells with light granular cytoplasm and spherical or irregular nuclei containing diploid granular chromatin and one or 2 nucleoli (Figs. 3A,B). Primary spermatogonia proliferate mitotically (Figs. 3B–D), thereby renewing the pool of germ cells and giving rise to secondary spermatogonia.

Secondary spermatogonia attain 35–45 μm in diameter and the nucleus is similar to those of the primary spermatogonia (Figs. 3A–D). They form clusters during several

mitosis. Clusters are interconnected by cytoplasmic bridges. Sertoli cells, progressively, surround the secondary spermatogonia (Figs. 3A–D); in consequence, secondary spermatogonia become enclosed within a cyst. During the development of a cyst, the number of both spermatogonia and Sertoli cells increases dividing mitotically several times before the initiation of meiosis. A central lumen appears in the center of the clusters of secondary spermatogonia (Figs. 3B–D). Into the cyst, secondary spermatogonia enter meiosis and transform into primary spermatocytes.

Primary and secondary spermatocytes

Primary spermatocytes are spherical cells, similar in size to secondary spermatogonia (35–45 μm in diameter). Primary spermatocytes contain duplicated chromosomes that present a gradual thickening along with prophase I of meiosis. Different stages of prophase I can be easily observed within primary spermatocytes: leptonema (fine reticular chromatin with irregular distribution of deep-stained granules of varying size), zygonema (fine fibrillar pattern of duplicated homologous chromosomes in synapsis) (Fig. 4A), pachynema (duplicated chromosomes in crossing-over) (Figs. 4A,B, 5A–C), diplonema (separation of

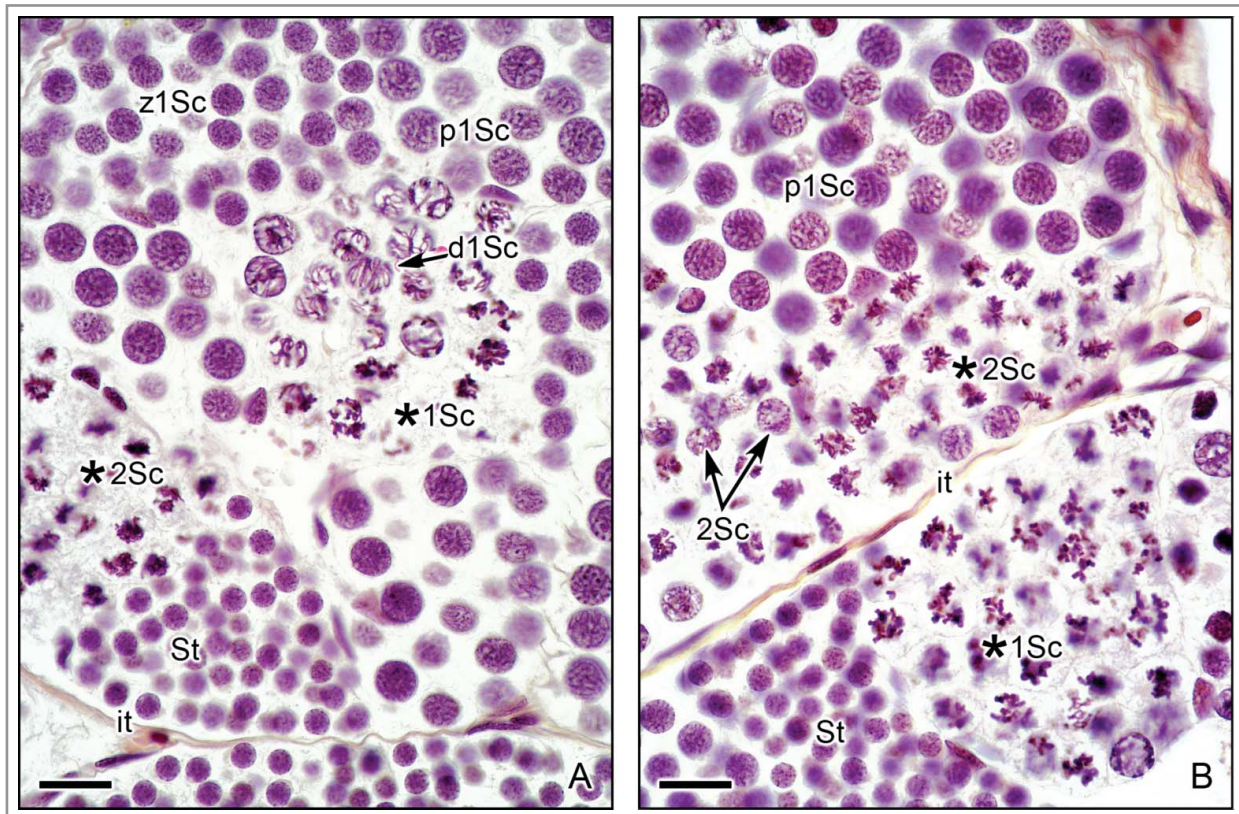


Figure 4. Spermatogenesis in the testis of *Ambystoma dumerilii* with cysts of primary and secondary spermatocytes. (A,B). Primary spermatocytes in several stages of the prophase I of meiosis are seen, as: zygotene (z1Sc) with fibrillar chromosomes; pachyene (p1Sc) also with fibrillar chromosomes and the cell diameter increases; diplotene (d1Sc), the chromosomes have characteristic chiasmata; first meiotic division (*1Sc) with dense chromosomes in the spindle. Secondary spermatocytes (*2Sc) in the second meiotic division also contain dense chromosomes in the spindle. Compare the different size diameter of the primary and secondary spermatocytes in division, being smaller the secondary spermatocytes. Cysts with spermatids (St), near the secondary spermatocytes. Elongated nuclei of Sertoli cells (Se) surround the different cysts. Hematoxylin-eosin. Bar = 20 μm .

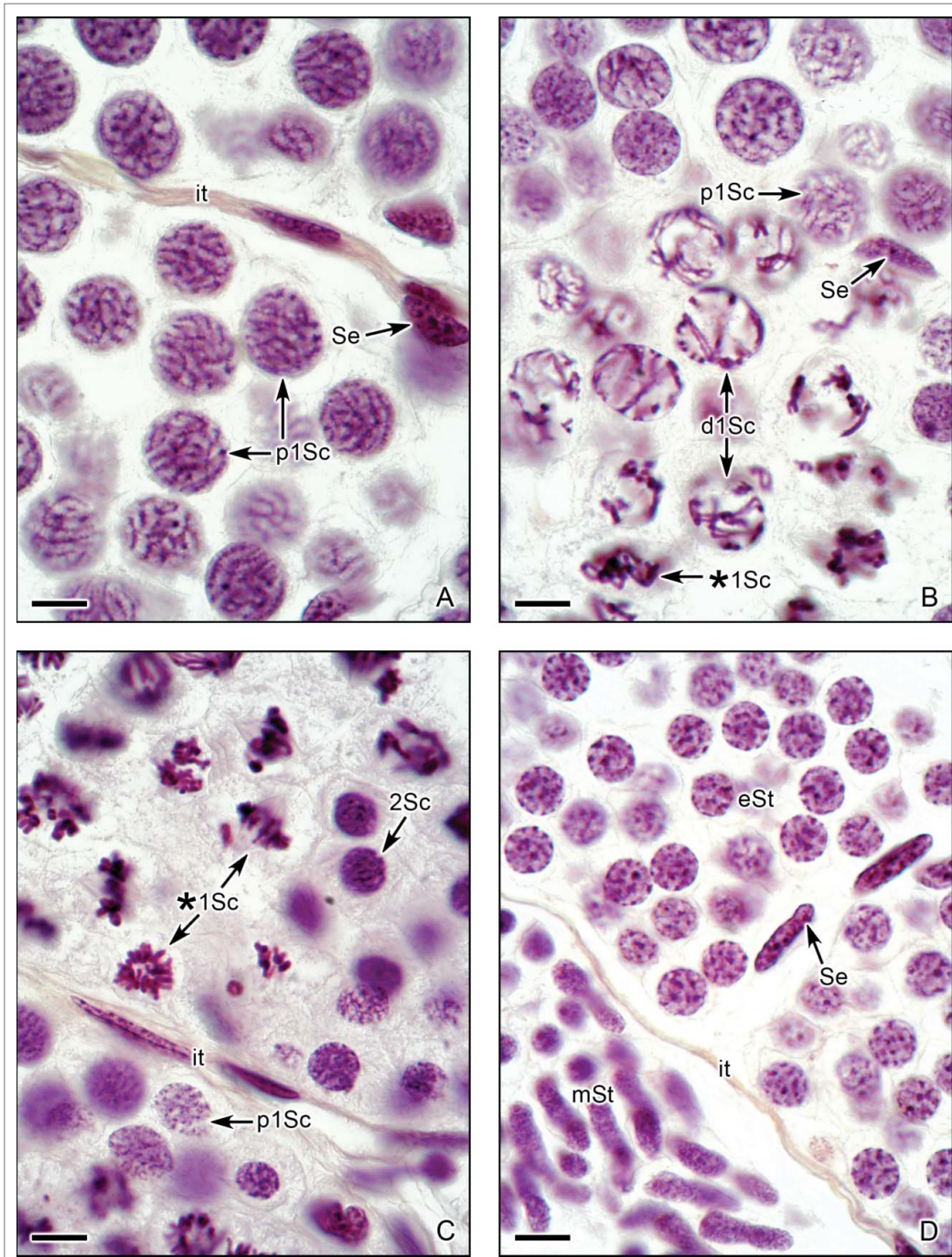


Figure 5. Spermatogenesis in *Ambystoma dumerilii* with morphological details of primary spermatocytes and spermatids. (A). Primary spermatocytes during pachytene (p1Sc). The nuclei contain dense paired chromosomes. Sertoli cell nucleus (Se) and the interstitial tissue (it) are seen. (B). Primary spermatocytes during diplotene (d1Sc). The separation of the paired chromosomes is evident, remaining united in the chiasmata. Other spermatocyte is in metaphase of the first meiotic division (*1Sc). A Sertoli cell nucleus (Se) is seen. (C). Primary spermatocytes during metaphase of the first meiotic division (*1Sc). The division of the primary spermatocytes originates the secondary spermatocytes (2Sc). The secondary spermatocytes contain filamentous chromosomes previous to the second division of meiosis. Compare the smaller size of the secondary spermatocytes with the primary spermatocytes. A primary spermatocyte during pachytene (p1Sc) and the limit of the lobule with interstitial tissue (it) are also seen. (D). Early spermatids (eSt) with round nucleus, and middle spermatids (mSt) with elongation of the nucleus are evident. Sertoli cell nuclei (Se) and interstitial tissue (it). (A-D): Hematoxylin-eosin. Bar = 10 μ m.

homologous duplicated chromosomes, that remain attached in some regions which form chiasmata) (Figs. 4A, 5B) and diakinesis (preparation for cell division). The cells during these meiotic stages of prophase I are named as leptotene, zygotene, pachytene and diplotene spermatocytes. The primary spermatocytes at pachynema stage are relatively abundant, whereas those in leptotema, zygonema, and especially in diplonema are rarely seen. This reflects the duration of meiotic stages, among which pachynema is the longest, leptonema and zygonema are shorter, and diplonema is the shortest stage.⁵³ The primary spermatocytes enter the first division of meiosis (Figs. 4B, 5B,C) through metaphase I, anaphase I, and telophase I. Two secondary spermatocytes are the result of the first meiotic.

Secondary spermatocytes are spherical cells and have an average diameter of 18–20 μ m. Their nuclei contain haploid chromosomes, but duplicated. Their chromosomes have fibrillar shape due to their immediate entrance to the second division of meiosis (Figs. 4B, 5C). They are seen less frequent, as they enter the second meiotic division (Figs. 4A,B) after a short interphase, rapidly giving rise to 2 spermatids. Therefore, secondary spermatocytes are often observed in the same lobule along with dividing primary spermatocytes (Fig. 5C).

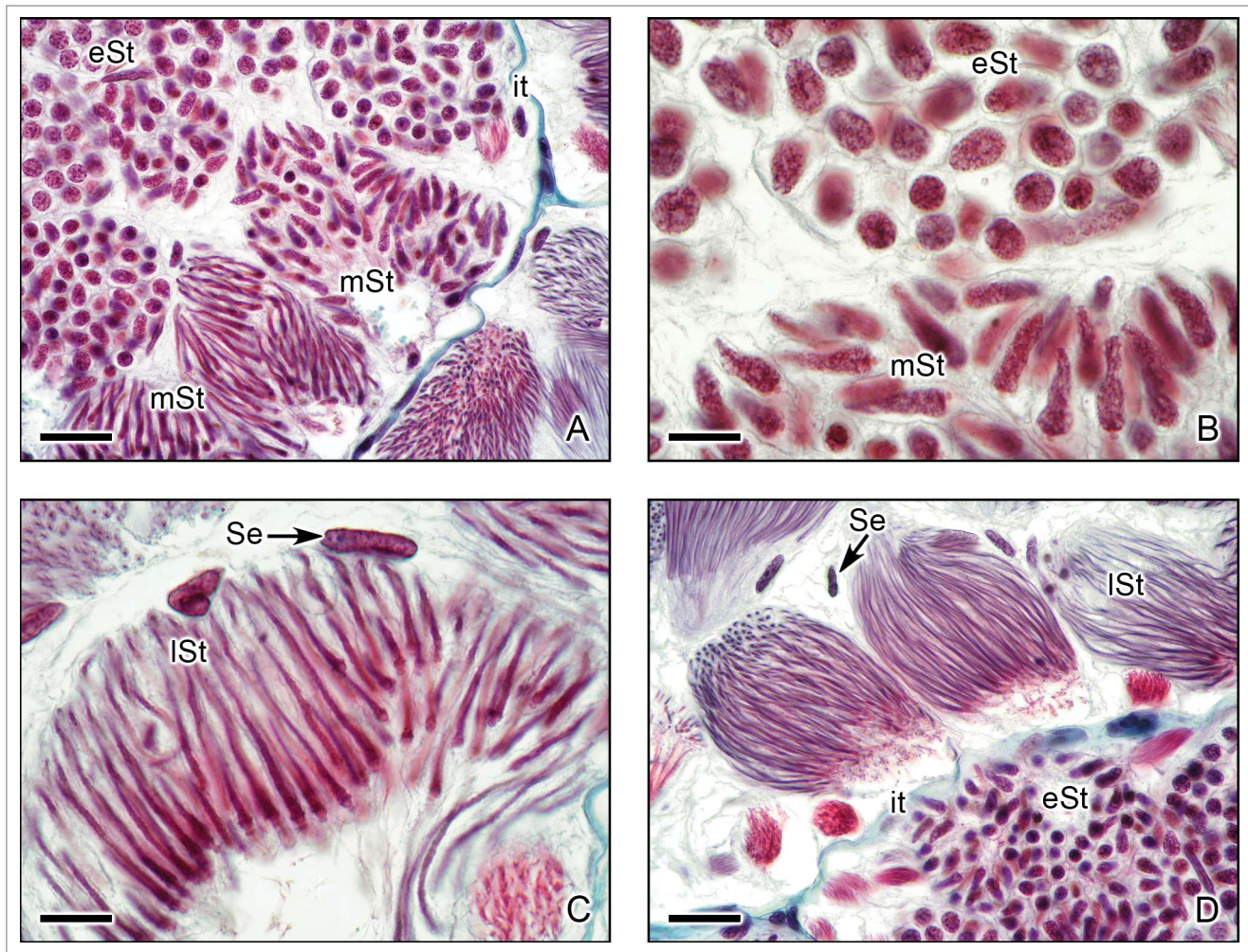


Figure 6. Caudal region of the testis of *Ambystoma dumerilii* (A,C,D) and *A. mexicanum* (B) with details of morphological changes of the spermatids during spermiogenesis. (A,B,C,D). Lobules with cysts that contain early spermatids (eSt) with round nucleus, and middle spermatids (mSt) with different levels of elongation of the nucleus and late spermatids (lSt) with evident and progressive elongation. Sertoli cell nuclei (Se) surround the cysts, interstitial tissue (it). (A): Masson's trichrome. Bar = 10 µm. (B): Hematoxylin-eosin. Bar = 10 µm. (C,D): Masson's trichrome. Bar = 10 µm.

Spermatids and spermatozoa

The result of meiosis is 4 haploid spermatids. Early spermatids are spherical in shape, and attain a diameter of 14–17 µm (Figs. 6A,B). Their nuclei stain denser than the nuclei of secondary spermatocytes. The spermatids transform into spermatozoa through spermiogenesis and become progressively elongated cells (Figs. 6A–D). As spermiogenesis proceeds, the nuclei of spermatids become very long and the chromatin shows increasing degree of condensation (Figs. 6D, 7A–D), defining the head and a large flagellum (Figs. 7B,D).

The morphology of spermatozoa is relatively uniform. A detailed review of the structure of mature spermatozoa of urodeles was done.⁵⁴ The spermatozoa consist of an elongated large head, a neck and a flagellum (Figs. 7B,D, 8A,B). The head of the spermatozoa contains the acrosome and nucleus. The nucleus has an elongated shape, with narrower cephalic part. The proximal centriole lies close to the nucleus, and the distal one is a part of the basal body of the axonema.

Throughout the long middle piece of the spermatozoa, mitochondria, that provide the energy for locomotion, are closely applied. The basal body is formed by a dense ring around the distal centriole.^{29,30,55} The spermatozoa of salamanders possess undulating membrane.^{51,54,55,56,57,58,}

Spermatozoa have a swirl arrangement inside the cyst, with their heads oriented in the same direction as observed in *Lissotriton italicus*²¹ and *Ambystoma dumerilii* illustrated here (Figs. 8A,B). The total length of spermatozoa of urodeles is usually longer than those of other amphibians, and other vertebrates (for review see⁵⁴). The shortest spermatozoa were reported for *Hynobius nebulosus* with a length of 156 µm, whereas the longest with about a length of 1mm was observed in *Necturus maculosus*.⁵⁹ The lengths of spermatozoa were also measured in several species: *Lissotriton italicus* (360 µm),²¹ *Ambystoma mexicanum* (444 µm), *A. dumerilii* (451 µm),⁵⁸ *E. bislineata* (459 µm), *E. lucifuga* (523 µm), *Desmognathus wrighti* (504 µm), *D. aeneus* (388 µm),

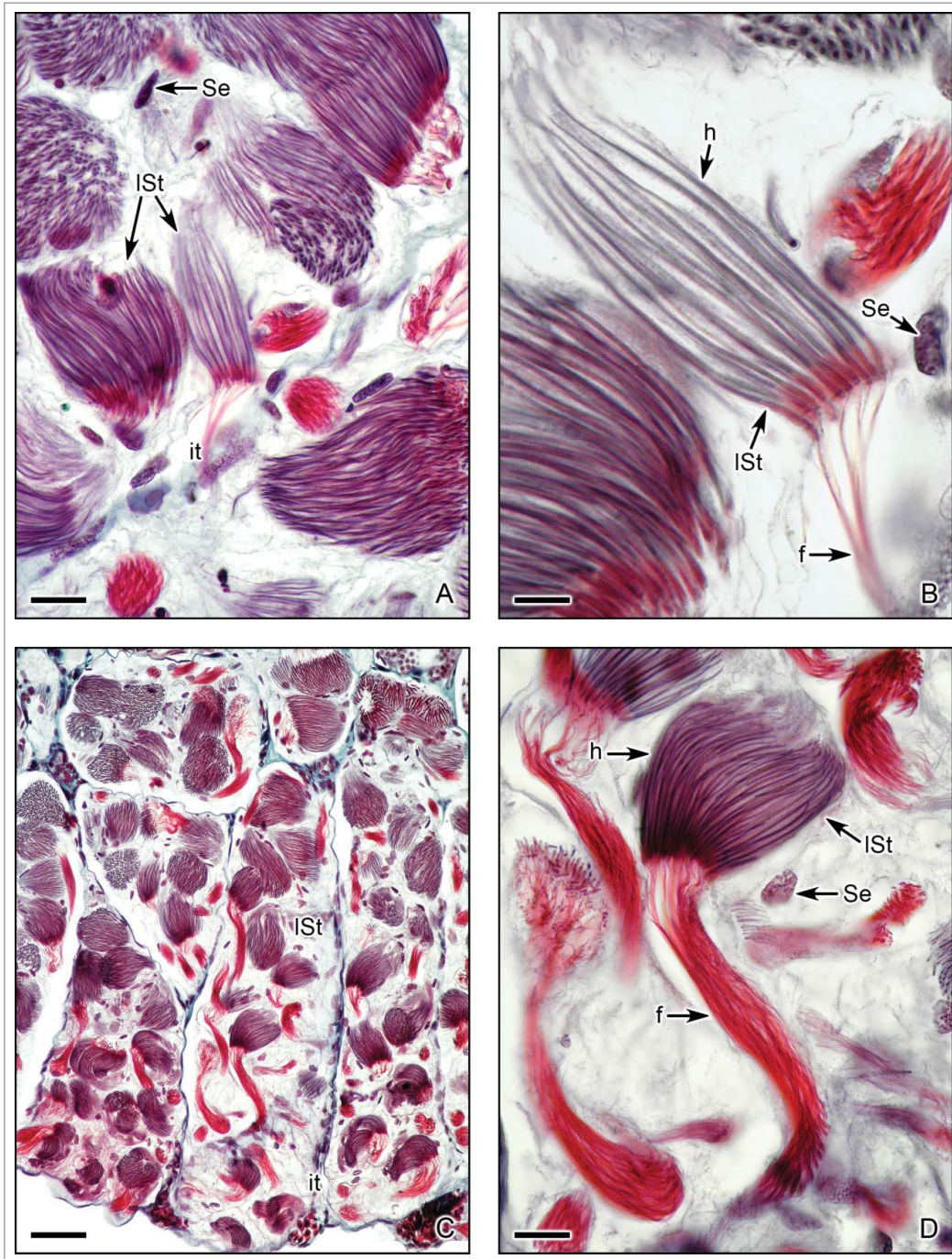


Figure 7. Caudal region of the testis of *Ambystoma dumerilii* with details of morphological changes of the late spermatids during spermiogenesis. (A,B,C,D). Lobules with cysts that contain late spermatids (Ist) with final elongation of the nucleus on the head (h) and development of a thin and large flagella (f). Nuclei of Sertoli cells (Se) and interstitial tissue (it) are seen. Masson's trichrome. (A,B,D): Bar = 10 μ m. (C): Bar = 50 μ m.

spermiation. In several cysts remain some abnormally sized and shaped spermatozoa (Fig. 8C). These spermatozoa eventually are phagocytized by Sertoli cells.¹⁷ The Sertoli cells remain in the lobules, gradually degenerate and are absorbed.³¹ Among the Sertoli cells some spermatogonia may be seen. Around the lobules, Leydig cells hypertrophy, attaining columnar or polyhedral shape (Figs. 9A,B). At the end of spermiation, this tissue regresses constantly and consists of small and irregular cells with pyknotic nuclei and amorphous and lightly stained cytoplasm.^{9,31,36}

Reproductive ducts

Down stream of the intratesticular ducts, the spermatozoa continue to a ducts system which provides secretions that form a favorable condition for the spermatozoa and transport the spermatozoa to the cloaca.^{6,8,34} Sperm transport from the testicular lobules to the Wolffian ducts occurs through the nephrons of the genital kidney, which form transversal ducts. Analysis of the genital kidney of the salamanders *Ambystoma maculatum*⁶⁰ and *Notophthalmus viridescens*⁶¹ described that these nephrons have reduced reabsorptive capacity and aid in the transportation of sperm. The transversal ducts run to the anterior region of the kidney (Figs.,

Plethodon cinereus (507 μ m), *P. dorsalis* (535 μ m), and *P. dunni* (626 μ m).⁵⁴ The biological significance of differences in the lengths of spermatozoa is unknown.

After completion of spermiogenesis, the cysts open and spermatozoa are released from Sertoli cell in a process termed

10A,B). They have a cuboidal epithelium with long stereocilia at the apical end, surrounded by connective tissue, thin smooth muscle and squamous peritoneal epithelium (Figs. 10C,D). Wolffian ducts have a wide lumen that may contain abundant spermatozoa after spermiation. The

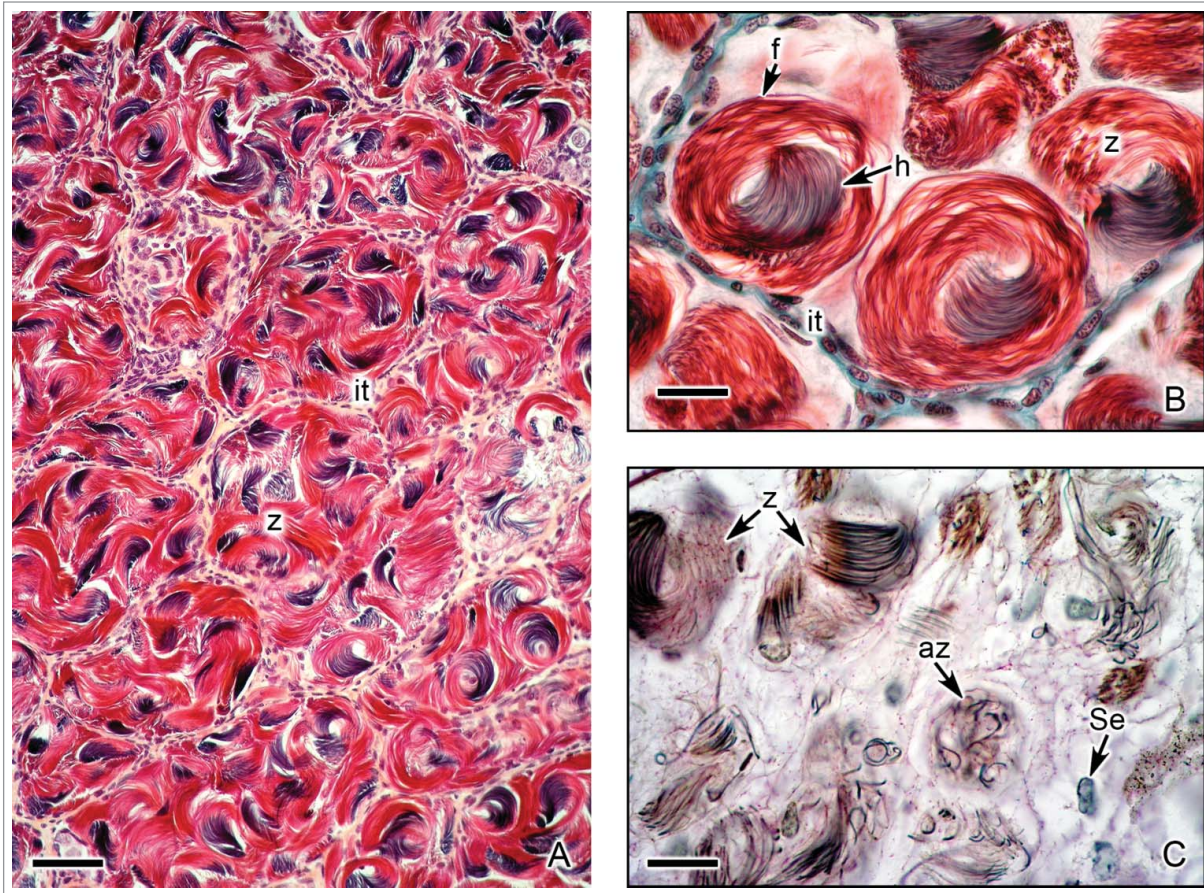


Figure 8. Caudal region of the testis of *Ambystoma mexicanum* with spermatozoa. (A). Lobules containing abundant cysts with spermatozoa (z). Interstitial tissue (it) surrounds the lobules. Hematoxylin-eosin. Bar = 50 μ m. (B). Detail of cysts with spermatozoa (z) formed by the head (h) and flagellum (f). At the end of spermiogenesis the spermatozoa are swirled into the cysts. Masson's trichrome. Bar = 10 μ m. (C). During spermiation, abnormal spermatozoa (az), showing irregular morphology, may remain into the cysts. Nuclei of Sertoli cells (Se) are seen. Alcian blue. Bar = 10 μ m.

epithelium has irregular cuboidal or low columnar cells, with adjacent connective tissue which may have some melanocytes, and surrounding, there are some smooth muscle fibers and a squamous peritoneal epithelium (Figs. 10C,D).

Spermatophores

The spermatophore is a characteristic structure of most male urodeles which have internal fertilization, more than 90%, with the exception of species belonging to the families

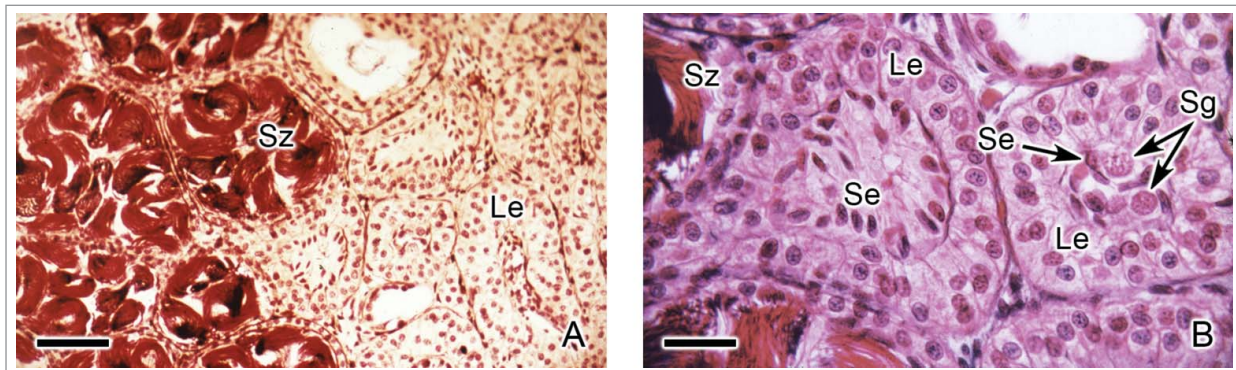


Figure 9. Caudal region of the testis of *Ambystoma mexicanum* during spermiation. (A). There are lobules with spermatozoa (Sz) and, more caudally, there are lobules after spermiation without spermatozoa. Leydig cells (Le) hypertrophy around the lobules. Hematoxylin-eosin. Bar = 50 μ m. (B). Detail of the Fig. 9A. During hypertrophy, Leydig cells (Le) become cuboidal, columnar or polyhedral in shape. The Leydig cells surround the lobules, where residual Sertoli cells (Se) and some spermatogonia (Sg) may be seen. Hematoxylin-eosin. Bar = 10 μ m.

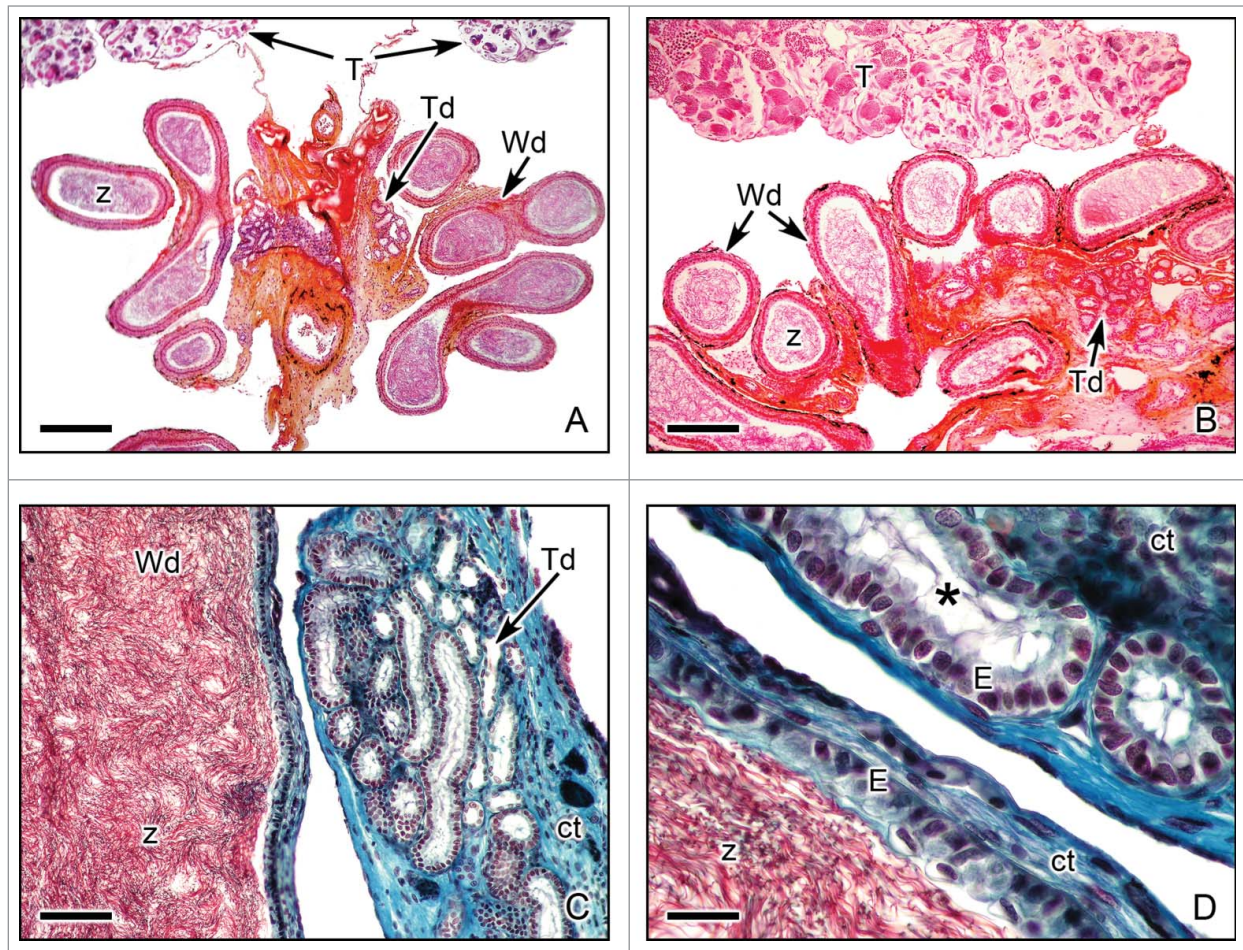


Figure 10. Efferent ducts of *Ambystoma dumerilii* after spermiation. (A,B). Panoramic view of sections with 2 types of efferent ducts: transversal ducts (Td) and Wolffian ducts (Wd). The lumen of the Wolffian ducts contains abundant spermatozoa (z). Near the ducts, the periphery of both testes (T) is seen. Hematoxylin-eosin. Bar = 50 μ m. Bar = 40 μ m. (C,D). Details of the efferent ducts of *Ambystoma dumerilii*. Both types of ducts, transversal ducts (Td) and Wolffian ducts (Wd) have columnar epithelium (E). The epithelium of the transversal ducts presents long stereocilia (*) in the apical end. Wolffian ducts contain abundant spermatozoa (z). Connective tissue (ct) surrounds the ducts. Masson's trichrome. Bar = 20 μ m. Bar = 10 μ m.

Hynobiidae and Cryptobranchidae, which have external fertilization.³ Spermatophores are formed by the cloacal glands and consist of a gelatinous base and a stalk surmounted by a sperm-containing gelatinous cap. Spermatophores contain a package of spermatozoa released from the Wolffian duct and the gelatinous capsule formed by secretions of the cloacal gland complex. Sever described and compared the anatomy and evolution of the cloacal glands in several species.^{46,62-64} The cloacal region exhibits a glandular complex structure. The types of cloacal glands are dorsal, pelvic, ventral and Kingsbury glands.^{46,62-64,65} The spermatophores may present differences in size and shape.⁶⁶ The spermatophores are formed during courtship, deposited by a male in the exterior and picked up by cloacal labia of the female.³ The structure and histochemistry of spermatophores of several species of the families Ambystomatidae, Salamandridae, and

Plethodontidae are described.^{66,67} In *Necturus*, the spermatophores are deposited by a male to a female directly by cloacal apposition and the stalk may be absent in these species.³

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Gerardo Gómez-Ríos for processing some histological specimens and valuable discussions during the early progress of this work; we are grateful to Adriana García-Alarcón and Gabino De la Rosa-Cruz for helpful technical assistance with digital photography; and José Antonio Hernández Gómez who kindly assisted with the digital preparation of figures.

References

- Adams EA. Sexual conditions in *Triturus viridescens*. III. The reproductive cycle of the adult aquatic form of both sexes. *Amer J Anat* 1940;66:235-276; <http://dx.doi.org/10.1002/aja.1000660205>
- Baker CL. The male urogenital system of the Salamandridae. *J Tennessee Acad Sci* 1965;40:1-5.
- Lofts B. Amphibians. In G.E. Lamming (ed.). *Marshall's Physiology of Reproduction*. Vol. One. Reproductive Cycles of Vertebrates. Churchill Livingstone, Edinburgh 1984. pp:127-05.
- Lofts B. Testicular function. In D.O. Norris and R.E. Jones (eds.), *Hormones and Reproduction in Fishes, Amphibians and Reptiles*. Plenum Press, New York 1987. pp:288-98.
- Brizzi R, Calloni C, Vanni S. Spermatogenic cycle in *Salamandrina terdigitata* (Lacépède, 1788) (Amphibia: Salamandridae). *Z Mikrosk Anat Forsh Leipzig* 1985;99:271-92; PMID:4024700
- Norris DO. *Vertebrate Endocrinology*. Academic Press, New York 1997. pp:634.
- Uribe MC. Reproductive systems of caudata amphibians. In H.M. Dutta and J.S. Datta Munshi (eds.). *Vertebrate Functional Morphology*. Horizon of Research in the 21st. Century. Science Publishers, Enfield, USA. New Hampshire 2001. pp:267-293.
- Uribe MC. The testes, spermatogenesis and male reproductive ducts. In D. Sever (volume ed.), and B.G.M. Jamieson (Series ed.). *Reproductive Biology and Physiology of Urodela*. Science Publishers, Enfield (NH), USA. Plymouth, UK 2003. pp:183-202.
- Uribe MC. Chapter 3. Spermatogenesis and male reproductive system. Urodela. In Ogielska M. (ed.). "Reproduction of Amphibians." Science Publishers, Inc. Enfield, NH, USA; Plymouth, UK 2009. pp:100-124.
- Propper CR. Chapter 3. Testicular structure and control of sperm development in Amphibians. In: Norris David O. and Lopez Kristin H. (Editors). Volume 2: Amphibians, "Hormones and Reproduction in Vertebrates." Ac Press, Elsevier 2011. pp:39-53.
- Humphrey RR. The multiple testis in urodeles. *Biol Bull* 1922;43:45-67; <http://dx.doi.org/10.2307/1536690>
- Bergmann M. The morphology of the testis in *Salamandra salamandra* (L.). In: Greven HB. Thiesmeier (eds.). *Mertensiella*. Supplement to *Salamandra*. Proceedings of the Symposium "Biology of *Salamandra* and *Mertensiella*" Nummer 4. Bonn 1994. pp:75-82.
- Tso ECF, LOFTS B. Seasonal changes in the newt, *Trituroides hongkongensis* testis. I. A histological and histochemical study. *Acta Zool-Stockholm* 1977;58:1-8; <http://dx.doi.org/10.1111/j.1463-6395.1977.tb00230.x>
- Franchi E, Camatini M, de Curtis I. Morphological evidence of a permeability barrier in urodele testis. *J Ultra Mol Struct R* 1982;80:253-63; [http://dx.doi.org/10.1016/S0022-5320\(82\)80038-5](http://dx.doi.org/10.1016/S0022-5320(82)80038-5)
- Fraile B, Paniagua R, Rodríguez MC, Saez FJ Jiménez A. Annual changes in the number, testosterone content and ultrastructure of glandular tissue cells of the testis in the marbled newt, *Triturus marmoratus*. *J Anat* 1989;167:85-94; PMID:2630543
- Schindelmeiser J, Greven H, Bergmann M. The immature part of the testis in *Salamandra salamandra*, (L.) (Amphibia, Urodela). *Arch Histol Jap* 1983;46(2):159-72; PMID:6882150
- Schindelmeiser J, Bergmann M, Greven H. Cellular differentiation in the urodele testis. In Duncker HR, Fleischer G. (eds.). *Functional Morphology in Vertebrates*. Proceedings of the 1st International Symposium on Vertebrate Morphology Giessen, 1983 Fortschritte der Zoologie, Band 30. Gustav Fischer Verlag, Stuttgart, New York 1985. pp: 445-7.
- Chan LM. Seasonality, microhabitat and cryptic variation in tropical salamander reproductive cycles. *Biol J Linn Soc* 2003;78:489-496; <http://dx.doi.org/10.1046/j.0024-4066.2002.00157.x>
- Flament S, Dumond H, Chardard D, Chesnel A. Life-long testicular differentiation in *Pleurodeles waltli* (Amphibia, Caudata). *Reprod Biol Endocrinol* 2009. 7:21; PMID:19265523; <http://dx.doi.org/10.1186/1477-7827-7-21>
- Trauth SE. Reproductive biology and spermathecal anatomy of the dwarf salamander (*Eurycea quadridigitata*), in Alabama. *Herpetologica* 1983;39:9-15.
- Sperone E, Bonacci A, Brunelli E, Tripepi S, Jamieson BGM. Male reproductive system in the Italian newt *Lissotriton italicus* (Peracca 1898) (Amphibia, Urodela): ultrastructural and morphological study with description of spermiogenesis, spermatozoon and spermatozophore. *Zoomorphology* 2009;128:183-195; <http://dx.doi.org/10.1007/s00435-009-0087-7>
- Takahashi H, Hanaoka KI. Cultivation in vitro of the testis cord of the newt, *Triturus pyrrhogaster*. *Dev Growth Differ* 1969;11:186-202; PMID:5392793; <http://dx.doi.org/10.1111/j.1440-169X.1969.00186.x>
- Kingsbury BF. The spermatogenesis of *Desmognathus fusca*. *Am J Anat* 1901;1:99-135; <http://dx.doi.org/10.1002/aja.1000010202>
- Humphrey RR. The interstitial cells of the urodele testis. *Am J Anat* 1921;29:213-79; <http://dx.doi.org/10.1002/aja.1000290204>
- Carrick R. The spermatogenesis of the axolotl (*Ambystoma tigrinum*). *Trans Royal Society Edinburgh* 1934;58:63-76; <http://dx.doi.org/10.1017/S0080456800023048>
- Moore FL. Spermatogenesis in larval *Ambystoma tigrinum*: Positive and negative interactions of FSH and testosterone. *Gen Comp Endocrinol* 1975;26:25-33; PMID:1181244; [http://dx.doi.org/10.1016/0016-6480\(75\)90175-6](http://dx.doi.org/10.1016/0016-6480(75)90175-6)
- Miltner MJ, Armstrong JB. Spermatogenesis in the Mexican axolotl, *Ambystoma mexicanum*. *J Exp Zool* 1983;227:255-63; <http://dx.doi.org/10.1002/jez.1402270209>
- Norris DO, Norman MF, Pankak MK, Duvall D. Seasonal variation in spermatogenesis, testicular weights, vasa deferentia and androgen levels in neotenic tiger salamander, *Ambystoma tigrinum*. *Gen Comp Endocrinol* 1985;60:51-7; PMID:4054587; [http://dx.doi.org/10.1016/0016-6480\(85\)90291-6](http://dx.doi.org/10.1016/0016-6480(85)90291-6)
- Uribe MC, Gómez ríos G, Brandon RA. Spermatogenesis in the urodele *Ambystoma dumerilii*. *J Morphol* 1994;222:287-99; <http://dx.doi.org/10.1002/jmor.1052220306>
- Podney J. Spermatogenesis in nonmammalian vertebrates. *Microsc Res Tech* 1995;32:459-97; PMID:8605396; <http://dx.doi.org/10.1002/jemt.1070320602>
- Podney J, Canick JA, Mak P, Callard GV. The differentiation of Leydig cells, steroidogenesis, and the spermatogenic wave in the testis of *Necturus maculosus*. *Gen Comp Endocrinol* 1983;50:43-66; PMID:6852522; [http://dx.doi.org/10.1016/0016-6480\(83\)90241-1](http://dx.doi.org/10.1016/0016-6480(83)90241-1)
- Lecouteux A, Garnier DH, Bassez T, Joly J. Seasonal variations of androgens, estrogens, and progesterone in the different lobules of the testis and in the plasma of *Salamandra salamandra*. *Gen Comp Endocrinol* 1985;58:211-221; PMID:3996890; [http://dx.doi.org/10.1016/0016-6480\(85\)90337-5](http://dx.doi.org/10.1016/0016-6480(85)90337-5)
- Uribe MCA, GÓMEZ RÍOS G, LÓPEZ ARRIAGA C. Cambios morfológicos del testículo de *Ambystoma dumerilii* durante un ciclo anual. *Bol Soc Herpetol Mex* 1991;3:13-8.
- Williams AA, Brandon RA, Martan J. Male genital ducts in the salamanders *Eurycea lucifuga* and *Eurycea longicauda*. *Herpetologica* 1984;40(3):322-30.
- Eisthen HL, Krause BC. Ambiguities in the relationship between gonadal steroids and reproduction in axolotls (*Ambystoma mexicanum*). *Gen Comp Endocrinol* 2012;176(3):472-80; PMID:22245262; <http://dx.doi.org/10.1016/j.ygcen.2011.12.034>
- Fraile B, Paniagua R, Saez FJ, PANIAGUA R. The cycle of follicular and interstitial cells (Leydig cells) in the testis of the marbled newt, *Triturus marmoratus*. *J Morphol* 1990;204:89-101; PMID:2338719; <http://dx.doi.org/10.1002/jmor.1052040110>
- Grier HJ. Comparative organization of Sertoli cells including the Sertoli cell barrier. In Russell LD, Griswold MD. (eds.). *The Sertoli Cell*. Cache River Press, Clearwater, Florida. 1993. pp:704-39.
- Bouma J, Cloud JG. Chapter 5. Sertoli cell biology in fishes and amphibians. In: Skinner MK, Griswold MD (Eds.). *Sertoli Cell Biology*. Elsevier, Ac Press, San Diego, CA, USA 2005. pp:71-9.
- Lazard L. Steroidogenesis in axolotl testis. Histochemistry of two major enzymes related to cell type, spermatogenesis, and substrate. *Gen Comp Endocrinol* 1979;39:381-87; PMID:499762; [http://dx.doi.org/10.1016/0016-6480\(79\)90135-7](http://dx.doi.org/10.1016/0016-6480(79)90135-7)
- Jin Y, Uchida I, Eto K, Kitano T, Abe S. Size-selective junctional barrier and Ca²⁺-independent cell adhesion in the testis of *Cynops pyrrhogaster*: Expression and Function of Occludin. *Mol Reprod Dev* 2008;75:202-16; PMID:17342736; <http://dx.doi.org/10.1002/mrd.20662>
- Lazard L. Spermatogenesis and 3b-HSDH activity in the testis of the axolotl. *Nature* 1976;264:796-97; PMID:1012324; <http://dx.doi.org/10.1038/264796a0>
- Callard GV, Canick JA, Pudney J. Estrogen synthesis in Leydig cells: structural-functional correlations in *Necturus* testis. *Biol Reprod* 1980;23:461-79; PMID:6968228; <http://dx.doi.org/10.1095/biolreprod23.2.461>
- Podney J, Callard GV. Organization of interstitial tissue in the testis of the salamander *Necturus maculosus* (Caudata: Proteidae). *J Morphol* 1984;181:87-95; PMID:6471107; <http://dx.doi.org/10.1002/jmor.1051810108>
- Fraile BR, Paniagua MC, Rodríguez Saez FJ. Effect of photoperiod and temperature on spermiogenesis in the marbled newt, *Triturus marmoratus marmoratus*. *Copeia* 1989;357-63; <http://dx.doi.org/10.2307/1445432>
- Imai K, Tanaka S. Histochemical and electron microscopic observations on the steroid hormone-secreting cells in the testis of the Japanese red-bellied newt, *Cynops pyrrhogaster pyrrhogaster*. *Dev Growth Differ* 1978;20 N°2:151-67; <http://dx.doi.org/10.1111/j.1440-169X.1978.00151.x>
- Sever DM. Morphology and seasonal variation of the mental hedonic glands of the dwarf salamander (*Eurycea quadridigitata*), (Holbrook). *Herpetologica* 1975;31:241-51
- Ricote M, Alfaro JM, García-Tuñón I, Arenas MI, Fraile B, Paniagua R, Royuela M. Control of the annual testicular cycle of the marbled-newt by p53, p21, and Rb gene products. *Mol Reprod Dev* 2002;63:202-9; PMID:12203830; <http://dx.doi.org/10.1002/mrd.10167>
- Arenas MI, Royuela M, Lobo MVT, Alfaro JM, Fraile B, Paniagua R. Androgen receptor (AR), estrogen receptor- α (ER- α) and estrogen receptor- β (ER- β) expression in the testis of the newt, *Triturus marmoratus marmoratus* during the annual cycle. *J Anat* 2001;199:465-72; PMID:11693307; <http://dx.doi.org/10.1046/j.1469-7580.2001.19940465.x>
- Armstrong JB. Spermatogenesis. In Armstrong JB, Malacinski GM. (eds.), *Developmental Biology of the Axolotl*. Columbia University Press, New York 1989. pp:36-41.
- Picheral B. Les elements cytoplasmiques au cours de la spermiogenese du Triton *Pleurodeles waltlii* Michah. III. L'évolution des formations caudales. *Z Zellforsch* 1972;131:399-416; PMID:5074143; <http://dx.doi.org/10.1007/BF00582858>
- Wang Hong Y. Studies on microstructure and infrastructure of testis of little salamander, *Batrachuperus pinchonii*. Master's thesis. Shaanxi Normal University. Xi'an, China 2002.
- Seto T, Kezer J, Pomerat CM. A cinematographic study of meiosis in salamander spermatocytes in vivo. *Z Zellforsch* 1969;94:407-24; PMID:5791454; <http://dx.doi.org/10.1007/BF00319185>

53. Cobb J, Handel MA. Dynamics of meiotic prophase I during spermatogenesis: from pairing to division. *Semin Cell Dev Biol* 1998;9:445-50; PMID:9813191; <http://dx.doi.org/10.1006/scdb.1998.0202>
54. Scheltinga DM, Jamieson BGM. The mature spermatozoa, In Sever D (volume ed.), Jamieson BGM. (Series ed.). *Reproductive Biology and Phylogeny of Urodela*. Science Publishers, Enfield (NH), USA. Plymouth, UK 2003. pp:203-74.
55. Wortham JWE Jr, Brandon RA, Martan J. Comparative morphology of some Plethodontid salamander spermatozoa. *Copeia*. 1977(4):666-80; <http://dx.doi.org/10.2307/1443165>
56. Fawcett DW. A comparative view of sperm ultrastructure. *Biol Reprod Suppl* 1970;2:90-127; PMID:12254595; http://dx.doi.org/10.1095/biolreprod2.Supplement_2.90
57. Bedford JM, Calvin HI. Changes in -S-S- linked structures of the sperm tail during epididymal maturation, with comparative observations in sub-mammalian species. *J Exp Zool* 1974. 187(2):181-203; PMID:4205051; <http://dx.doi.org/10.1002/jez.1401870202>
58. Brandon RA, Martan J, Wortham JWE, Englert DC. The influence of interspecific hybridization on the morphology of the spermatozoa of *Ambystoma* (Caudata, Ambystomatidae). *J Reprod Fert* 1974;41:275-84; <http://dx.doi.org/10.1530/jrf.0.0410275>
59. Wortham JWE Jr, Murphy JA, Martan J, Brandon RA. Scanning electron microscopy of some salamander spermatozoa. *Copeia* 1982(1):52-60; <http://dx.doi.org/10.2307/1444267>
60. Siegel DS, Aldridge RD, Rheubert JL, Gribbins KM, Sever DM, Trauth SE. The testicular sperm ducts and genital kidney of male *Ambystoma maculatum* (Amphibia, Urodela, Ambystomatidae). *J Morphol* 2013;274(3):344-60; PMID:23192852; <http://dx.doi.org/10.1002/jmor.20100>
61. Nicholson AE, Siegel DS. Modifications of the genital kidney proximal and distal tubules for sperm transport in *Notophthalmus viridescens* (Amphibia, Urodela, Salamandridae). *J Morphol* 2014;275(8):914-22; PMID:24643856; <http://dx.doi.org/10.1002/jmor.20268>
62. Sever DM. Cloacal anatomy of male salamanders in the families Ambystomatidae, Salamandridae and Plethodontidae. *Herpetologica* 1981;37:142-55.
63. Sever DM. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). VII. Plethodontidae, *Herpetological Monographs*. 1994. 8 pp:276-337.
64. Sever DM. Courtship and mating glands, In Sever D (volume ed.) Jamieson BGM. (Series ed.). *Reproductive Biology and Phylogeny of Urodela*. Science Publishers, Enfield (NH), USA. Plymouth, UK 2003. pp:323-81.
65. Brizzi R, Calloni C. Male cloacal region of the spotted salamander, *Salamandra salamandra giglioli* (Amphibia, Salamandridae). *Boll Zool* 1992;59:377-85; <http://dx.doi.org/10.1080/11250009209386697>
66. Zalisko EJ, Brandon RA, Martan J. Microstructure and histochemistry of salamander spermatophores (Ambystomatidae, Salamandridae and Plethodontidae). *Copeia*. 1984(3):739-47; <http://dx.doi.org/10.2307/1445158>
67. Russell LD, Brandon RA, Zalisko EJ, Martan J. Spermatophores of the salamander *Ambystoma texanum*. *Tissue Cell* 1981;13(3):609-21; PMID:6172883; [http://dx.doi.org/10.1016/0040-8166\(81\)90031-8](http://dx.doi.org/10.1016/0040-8166(81)90031-8)