Testicular structure in three viviparous species of teleosts in the genus *Jenynsia* (Anablepidae)

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Histological structure of the testes and development of spermatozoa in *Jenynsia* species is described using light, scanning and transmission electron microscopy. The testis type is restricted spermatogonial, wherein spermatogonia are restricted to the distal ends of lobules, typical of the Atherinomorpha, and spermatogenesis is continuous throughout the year in wild-caught fish. Within the testicular lobes there are lobular germinal compartments wherein the functional units are spermatocysts, whose borders are formed by Sertoli cells. Spermatocysts may contain meiotic primary spermatocytes, secondary spermatocytes, spermatids, undergoing spermiogenesis, or spermatozoa. Spermatocysts with later stages of developing sperm are located proximal to the testicular ducts. During spermiogenesis, spermatid nuclei become elongated. As this occurs, the nucleus develops a deep, central fossa that contains the centriolar complex. As the flagellum grows, enlarging spermatid mitochondria migrate posteriorly alongside the flagellum but remain separated from it by the cytoplasmic canal, an indentation of the plasma membrane. Between the enlarged mitochondria and plasma membrane, a sub-mitochondrial net develops. In longitudinal sections, the enlarged mitochondria are stacked in a zig-zag fashion, and in transverse sections they appear as a ring surrounding the flagellum, but separated from it by the cytoplasmic canal. Spermatozoa of the 3 jenynsiid species have an introsperm complex composed of a long mid-piece whose flagellum has a single "wing." Within the efferent ducts and the tubular gonopodium, sperm are lightly packed in a side by side fashion which facilitates their transfer into the female reproductive tract. This study presents detailed descriptions of testicular organization and cytological characterization of the stages of spermatozoa differentiation in 3 species of *Jenynsia* from northwestern Argentina (*J. alternimaculata*, *J. multidentata* and *J. maculata*), in order to contribute to the understanding of testicular structure and development of spermatozoa in the context of evolution of viviparity in this fish lineage.

Introduction

Fishes of the subfamily Jenynsiinae are endemic to rivers in South America. They compose a group of small, viviparous teleosts that exhibit sexual dimorphism. Both the nomenclature and the historical systematics of the genus *Jenynsia* have been reviewed.¹ Twenty 3 species of *Jenynsia* have been described.¹⁻⁶ In Neotropical Argentina, species of *Jenynsia* are broadly distributed in the Río de La Plata basin, in habiting the central and north-western regions, as well as Atlantic coast drainages of the south and the south-east of the country.

Studies regarding viviparity have been carried out on *J. lineata* females related to maternal–fetal relationships.⁷⁻¹² In males, testis structure¹³ and spermatozoon ultrastructure¹⁴ have been described. Testicular organization, spermatogenesis and spermatozoon formation are not known. Defined 2 types of teleost testicular morphology,¹⁵⁻²⁰ basal teleosts, such as salmonids and silurids, have anastomosing tubular testes whereas derived teleosts have lobular testes. Lobular testes have 2 patterns of organization based on the distribution and

arrangement of spermatogonia. The unrestricted spermatogonial testis type, where spermatogonia are arranged along the lengths of lobules, is found in the majority of teleosts.¹⁷ The restricted spermatogonial testis type, where spermatogonia are restricted to the distal termini of lobules that extend from the testis ducts and end blindly beneath the testis periphery, is typical of atherinomorphs.¹⁷⁻²¹

Spermatogenesis has a characteristic pattern among fishes, but structural variations on this pattern occur between fish with external fertilization compared to those with internal fertilization.^{17,22} These variations have warranted classification of fish spermatozoa as plesiomorphic aquasperm, a simple type of spermatozoon typical of externally fertilizing teleosts, or introsperm, found in many internally fertilizing teleosts.²³ Introsperm morphology is more complex than that of aquasperm. The nucleus and midpiece of the latter are modified, generally being elongated. Introsperm are characteristic of viviparous fishes, such as the cyprinodont poeciliids *Poecilia reticulata*,²⁴ *P. latipinna*,²⁵ *Xiphophorus helleri*, *X. maculatus*,²³ *Gambusia affinis*,^{23,26} anablepids, *Jenynsia lineata*¹⁴ and

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Anableps anableps,²⁷ Hemirhamphidae, *Normorhamphus*, *Dermogenys* and *Hemirhamphodon*,^{28,29} perciform *Cymatogaster aggregate*.³⁰ Moreover is present in species from the internally-fertilizing subfamilies Glandulocaudinae and Cheirodontinae, order Characiformes,^{22,31} and the internally-fertilizing siluriform, *Trachelyopterus lucenai*, *T. galeatus* (family Auchenipteridae) and *Scoloplax dicra* (family Scoloplacidae).^{22,23,32}

Internal fertilization is essential to viviparity, and it requires sperm transfer mechanisms.^{18,22,24,33,34} In *Jenynsia* species, a tubular gonopodium transfers sperm to the female gonopore.^{35,13} Observing fishes in natural populations, combined with the high diversity of mating systems, make them excellent subjects for studying sperm competition and conducting intraspecific and interspecific tests of sperm competition theory. This study presents detailed descriptions of testicular organization and cytological characterization of the stages of spermatozoa differentiation in 3 species of *Jenynsia* from northwestern Argentina (*J. alternimaculata*, *J. multidentata* and *J. maculata*), in order to contribute to the understanding of testicular structure and development of spermatozoa in the context of evolution of viviparity in this fish lineage.

Results

Testis structure

The 3 *Jenynsia* species had similar testicular structure. The testes were located in the abdominal cavity, backward the gut and lateral to the swimming bladder. Usually, one testis was smaller than the other. Mature testes were white to pale yellow, whereas immature testes were small, elongated and white to translucent. From the inner side of the testis, the spermatid ducts were joined and extended to the gonopore, connecting with the gonopodium (Fig. 1).

Light microscopy

Testes were attached to the abdominal cavity by a thin layer of connective tissue, the mesorchium. The testis wall was formed by the enveloping tunica albuginea (fibrous connective tissue) that was rich in blood vessels (Figs. 1A, 2A).

The 3 *Jenynsia* species presented the same testis type lobular, restricted spermatogonial type, with primordial germ cells limited to the termini of the testicular lobules that ended blindly at the testis periphery (Figs. 2A-C, 3A). The lobules were extended to short efferent ducts that opened into a common intratesticular duct running longitudinally in the testes (Fig. 2D). The lobules consisted of the germinal compartment, between which was the interstitial compartment (Fig. 3C-D). A basement membrane separated these 2 compartments. The germinal compartment contained 2 types of cells, somatic Sertoli cells and germ cells (Figs. 2C, 3A). The interstitial compartment contained vascularized connective tissue, Leydig cells and boundary cells. Germ cell stages, spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa, were observed within testis lobules (Figs. 2A-C, 3A). The most developed germ cells occurred near the efferent ducts (Fig. 3B, D). At the testis periphery, spermatogonia were observed within the lobules bordered by Sertoli cells

Figure 1. Testis and gonopodium of *Jenynsia maculata*. Testicles of *Jenynsia* are paired organs, each testicle (T) there emerges a spermatid duct (SD) that carries sperm to the gonopodium (G). Scale bar = 4 mm.

(Figs. 2B, C, 3A). The stages of spermatogenesis were confined to spermatocysts, defined by the Sertoli cells surrounding the germ cells. There were spermatocysts containing primary spermatocytes (Figs. 2A-C, 3A), secondary spermatocytes (Fig. 2A), spermatids (Figs. 2A-C, 3B) and spermatozoa (Figs. 2A, 3B-E). The germ cells inside a spermatocyst had synchronous development (Figs. 2, 3).

Spermatogonia were arranged singly or in small groups at the periphery of the testicular lobules. They were spherical in shape, attaining 8–10 μm in diameter with hyaline cytoplasm and having a centrally located, prominent nucleus containing a nucleolus (Figs. 2A, B, 3A). They were the largest germinal cells. Spermatogonia were associated with Sertoli cells, surrounded individually by Sertoli cell processes. This association of both cell types forms a cluster, a spermatocyst, whose border was the Sertoli cell processes. Within the spermatocysts, the spermatogonia enter in meiotic process forming the primary spermatocytes. These cells were spherical in shape, 4–4.5 μm in diameter (Figs. 2A-C, 3A), and contained filamentous chromatin in the different phases of prophase I of meiosis. Secondary spermatocytes were spherical in shape, 3–3.5 μm in diameter with strongly basophilic nuclei and reduced cytoplasm.

The spermatocysts, close to the efferent ducts, contained the later stages of spermatocytogenesis (Figs. 2D, 3B, D). Spermatid nuclei were 2.8–3 μm in diameter. During spermiogenesis, the nuclei of spermatids changed from rounded to elongated as chromatin condenses, (3C, E). A mature

Figure 2. Testis structure of *Jenynsia alternimaculata*. (A, B): Lobules terminating at the periphery of the testis. All the stages of spermatogenesis are observed. The spermatogonia (Sg) are restricted to the distal termini of the lobules, at the testis periphery, whereas primary (S1) and secondary spermatocytes (S2), spermatids (Sm) and spermatozoa (S) are distributed along the lobules, basically, in that order. The synchronously developing stages of spermatogenesis of the germinal cells, inside spermatocysts, are well defined. H-E. Scale bars = 40 μ m. (C): Distal termini of lobules of the testis. Spermatogonia (Sg) are observed at the periphery of the testis, they are surrounded by Sertoli cells (SC). A blood vessel (BV) is seen adjacent to the spermatogonia. Spermatocysts contain synchronous germinal cells, primary spermatocytes (S1) and spermatids (Sm). Nuclei of Sertoli cells (SC) are seen at the border of the spermatocysts. H-E. Scale bar = 60 μ m. (D): Spermatocysts (Scy) containing germinal cells at the periphery of the testis and masses of numerous mature spermatozoa (S) flow within the efferent ducts (ED), at the center of the testis in the common intratesticular duct (CID). H-E. Scale bar = 400 μ m.

Figure 3. Testis structure of *Jenynsia maculata* (A, B, C) and *Jenynsia multidentata* (D, E). (A): Distal termini of lobules of the testis. Spermatogonia (Sg) are observed at the periphery of the testis, they are surrounded by Sertoli cells (SC) having a triangular nucleus. Spermatocysts containing primary spermatocytes (S1), are seen. A blood vessel (BV) is observed near the spermatocytes. H-E. Scale bar = 10 μ m. (B): Spermatocytes containing spermatids (Sm) and abundant mature spermatozoa (S) near the efferent ducts (ED) at the center of the testis. Masson's trichrome. Scale bar = 90 μ m. (C): Transition stages from late spermatid to spermatozoa. Some spermatocytes contain developing spermatids (Sm), others contain mature spermatozoa (S). At the end of spermiogenesis the heads of spermatozoa are elongated and strongly basophilic and face toward the Sertoli cells. H-E. Scale bar = 30 μ m. (D): Spermatocytes containing abundant mature spermatozoa (S) near the efferent ducts (ED) at the center of the testis. Masson's trichrome. Scale bar = 100 μ m. (E): Similar disposition in this species to that seen in **Figure 2C**, of mature spermatozoa (S) with the heads toward the Sertoli cells. H-E. Scale bar = 30 μ m.

spermatozoon had its head toward the periphery of a spermatocyst while its flagellum extended into the lumen (Fig. 3C, E). Spermiation occurred close to the efferent ducts when the spermatocyst opened and mature spermatozoa were released. In the efferent ducts, and then, in the common intratesticular duct, spermatozoa were stored (Figs. 2D, 3B, D). Between the lobules, fibroblasts, blood vessels and Leydig cells (Fig. 3E) were observed.

The efferent ducts were lined by cuboidal epithelium while the common intratesticular duct was lined by columnar epithelium, which stained positively with both PAS and alcian blue reactions, characteristic for glycoproteins (Figs. 2D and 3D). Masses of spermatozoa occupy the lumen of the main testis ducts (Fig. 2D). The

main ducts emerged and fused forming a spermatic duct, which extended inside the tubular gonopodium (Fig. 1).

The length of the *Jenynsia* sexually mature male (SL) and testis length are presented in **Table 1**.

Ultrastructure

The ultrastructure analysis defines the synchrony of the germ cells inside a spermatocyst (Fig. 4A). Spermatogonia (Fig. 4B, C), primary spermatocytes (Fig. 4A, B, D) and early spermatids (Fig. 4E) were spherical cells. The spermatozoa had an elongated nucleus, mid-piece containing the flagellum surrounded by mitochondria and the flagellum (Fig. 4F). The spermatogonia divide by mitosis with incomplete cytokinesis remaining a

Table 1. Length of the sexually maturity male (SL) and testis length, in the studied male *Jenynsiids*.

Species	Sexually Mature Fish (SL-mm)	Testis Length mm
<i>Jenynsia alternimaculata</i>	24–42,8	5–10
<i>J. maculata</i>	21–42,5	5–11
<i>J. multidentata</i>	19,5–27,3	5–9

communicating both cells. Connecting canals remain in meiotic phases. Primary spermatocytes had a central nucleus whose chromatin changed during meiosis. During the zygotene phase, the synaptonemal complex was evident, joining the homologous chromosomes (Fig. 5A) that are paired in the pachytene phase. During the diplotene phase, they began to separate remaining united at some points by chiasmata. The cytoplasm contained numerous, small vesicles, the polysomes and mitochondria which were distributed homogeneously (Fig. 5A). Connecting canals are observed between primary spermatocytes (Fig. 5B, C). The secondary spermatocytes (Fig. 5C, D) had a large spherical nucleus with remnant-condensed chromosomes. They underwent the second meiotic division. In the spermatocyte cytoplasm, numerous elongated or rounded mitochondria and vesicular structures, similar to Golgi, were observed (Fig. 5C, D). The centriolar complex, formed by 2 centrioles, was close to the nucleus (Fig. 5C). Connecting canals between secondary spermatocytes (Fig. 5B, C) were also observed (Fig. 5C). Division of the secondary spermatocytes formed haploid spermatids.

Spermiogenesis

The spermatids contained a spherical nucleus in which granular chromatin was homogeneously distributed (Fig. 6A-C). The centriolar complex is close to the nucleus (Fig. 6A-D) and a deep, central nuclear fossa was formed in which the centriolar complex resides (Fig. 6F-H). Later, the distal centriole moves to the base of the nucleus as the flagellum forms. During spermiogenesis, the spermatid cytoplasm moved to encompass the forming flagellum (Fig. 6A).

Progressively occurs a chromatin condensation (Fig. 6F-I). As the cytoplasm migrated around the developing flagellum, an indentation of the plasma membrane occurred forming the cytoplasmic canal (Fig. 6A). Numerous mitochondria were accumulated around the cytoplasmic canal and the proximal portion of the flagellum as the midpiece of the future sperm developed (Fig. 6B-D). The axis of the spermatid flagellum was shifted to a perpendicular position at the base of the nucleus. The flagellum had the typical pattern of 9+2 arrangement of microtubule doublets that were surrounded by groups of 5 to 8 elongated or rounded mitochondria, that became larger than those observed previously and having numerous cristae and electron-dense matrix (Fig. 6C-E). In this area, an endo-membranous system was formed by numerous cisternae (Fig. 6C). Spermatids exhibit lateral structures with numerous vesicles that occurred in the region of the spermatid that will form the midpiece (Fig. 6H). These vesicles were eliminated along with the residual body.

As spermiogenesis progresses, the nuclear fossa is formed, and chromatin condensation became electron dense at the periphery of the nucleus (Fig. 6G-I). The proximal centriole was then located within the nuclear fossa (Fig. 6F-H). The distal centriole was associated with the plasma membrane and will form the cytoplasmic canal (Fig. 6F) as spermatid morphogenesis ensues and the cytoplasm migrates around the developing flagellum. Later, the centriolar complex shifted deeply into the nuclear fossa. The cytoplasmic mass continued moving around the flagellum, more distinctly forming the midpiece of the spermatozoon (Fig. 6F-H). Surrounding the flagellum and beneath the mitochondria, a zig-zag pattern of electron-dense fibrillae formed the sub-mitochondrial net. This was observed after 5 to 8 mitochondria surrounded the flagellum membrane (Fig. 6E, F, H). Enlarged mitochondria became located around the cytoplasmic canal for the length of the mid-piece (Fig. 7F, H).

Spermatozoon

The spermatozoon of the 3 *Jenynsia* species was of the intro-sperm, anacrosomal complex type (Fig. 7A). The elongated head measures 3–3.2 μm in length and 1.5 μm in diameter, and was surrounded by a thin cytoplasmic layer (Fig. 7A, F). The nucleus had condensed chromatin (Fig. 7A, B, F). A deep nuclear fossa housed the centriolar complex. The elongated mid-piece, approximately of 10 μm length came to contain a large, ring-shaped mitochondrion surrounding the flagellum (Fig. 7F-I). The mitochondria formed a single, cylindrical and narrow mitochondrion, which contained longitudinal cristae (Fig. 7B-F). The sub-mitochondrial net was observed surrounding the flagellum, but separated from it by the cytoplasmic canal (Fig. 7F-H). There were some morphological differences observed among the 3 *Jenynsia* species. In *J. alternimaculata* (Fig. 7A, B), the mid-piece displayed small electron-dense spots and was connected to the flagella membrane, and in *J. maculata* and *J. multidentata*, the sub-mitochondrial net formed a ring over the plasma membrane, bordering the cytoplasmic canal (Fig. 7A, F). In the 3 species, the proximal flagellum had thin lateral expansions, approximately 18 μm in length. These were absent from the final flagellum (Fig. 7F-I).

Sertoli cells formed the spermatocyst walls (Fig. 8A-C). They have a typical triangular nucleus in spermatogonias cyst and spermatocyst with primary spermatocytes. In spermatocyst with secondary spermatocytes and spermatids, they have a flat nucleus; the cytoplasm contained numerous mitochondria and a well-developed endoplasmic reticulum (Fig. 8B). By the time spermatocysts contained spermatozoa, the Sertoli cells became hypertrophied due to an increase in cytoplasm including number and size of mitochondria, endoplasmic reticulum, and especially the occurrence of numerous electron-dense secretory granules (Fig. 8C). Mature sperm heads were contained in cytoplasmic recesses of Sertoli cells, which bordered the efferent ducts (Fig. 8C). Leydig cell were situated in the interstitial tissue around lobules and efferent ducts. The nucleus was large and contained abundant mitochondria and dense secretory granules (Fig. 8D).

Figure 4. Scanning electron micrographs (SEM) of spermatogenesis in *J. alternimaculata*. (A): Longitudinal and transversal limits of a spermatocyst and their size are indicated (gray arrows). Spermatocyst containing primary spermatocytes (S1). Scale bar = 20 μm . (B): Peripheral region of the testis containing spermatocysts in several stages of spermatogenesis: spermatogonia (Sg), primary spermatocytes (S1) and secondary spermatocytes (S2). Sertoli cells (SC), border the spermatocysts. Scale bar = 10 μm . (C): Spermatogonia (Sg). Scale bar = 2.5 μm . (D): Primary spermatocytes (S1). Scale bar = 2.5 μm . (E): Spermatids (Sm) with developing flagella. Scale bar = 5 μm . (F): Spermatozoa showing the head (H), mid-piece (Mp) and flagellum (F). Scale bar = 2.5 μm .

Figure 5. Transmission electron micrographs (TEM) illustrating development of spermatocytes within spermatocysts. (A): Primary spermatocytes of *J. maculata* with pachytene chromosomes in the nucleus (n) and mitochondria (m) distributed in the cytoplasm. Scale bar = 10 μm . (B): Detail of organelles in the cytoplasm of primary spermatocytes of *J. maculata*. Mitochondria (m), ribosomes (r), vesicles (v) of the smooth endoplasmic reticulum. Ring canal (asterisk) connecting 2 spermatocytes. Scale bar = 5 μm . (C): Secondary spermatocytes of *J. alternimaculata*. Ring canal (asterisk) connecting 2 spermatocytes. Nucleus (n) and cytoplasmic organelles as centrioles (c), mitochondria (m) and vesicles (v) of the smooth endoplasmic reticulum are seen. Scale bar = 1 μm . (D): Secondary spermatocyte of *J. alternimaculata*. Nucleus (n), mitochondria (m) and vesicles (v) of the smooth endoplasmic reticulum are evident. Scale bar = 0.5 μm .

Discussion

The analysis of the testicular structure and spermatogenesis of *Jenynsia alternimaculata*, *J. multidentata* and *J. maculata* confirms

that they have lobular testes with spermatogonia being restricted to the distal termini of lobules, this being an “atherinomorph” testis type.¹⁶ These results lend additional support to monophyly of the Atherinomorpha.¹⁷ In all atheriniforms studied,

Figure 6. Transmission electron micrographs (TEM). Spermiogenesis in *Jenynsia* spp. (A): Early spermatid of *J. alternimaculata* showing the round nucleus (n), the centrioles (c), numerous, large vesicles (v) and mitochondria (m). Scale bar = 0.5 μm. (B): Spermatid of *J. alternimaculata*, with evident nucleus (n), abundant mitochondria (m), and flagellum (F) disposed near the nucleus. Sertoli cell (SC) at the periphery of the spermatocyst. Scale bar = 1 μm. (C): Spermatid of *J. alternimaculata*. Near the nucleus (n), mitochondria (m) are located around the flagellum (F), displacing numerous vesicles (v). Scale bar = 0.5 μm. (D): Spermatid of *J. maculata*. Near the nucleus (n), mitochondria (m) are located around the flagellum (F). Scale bar = 0.5 μm. (E): Spermatid of *J. maculata*. Mitochondria (m) surround the flagellum (F). Sub-mitochondrial net (smn) disposed between the mitochondria and flagellum. Scale bar = 0.5 μm. (F): Spermatid of *J. alternimaculata*. Sub-mitochondrial net (smn) and mitochondria (m) surround the flagellum (F). White asterisk indicates the beginning of chromatin condensation. Scale bar = 0.5 μm. (G): Spermatid of *J. alternimaculata*. Longitudinal and transverse sections are displayed. Chromatin is strongly condensed (white asterisk); a cytoplasmic canal around the flagellum (F) is evident (asterisk); large vesicles (v) are seen. Scale bar = 1 μm. (H-I): Spermatids of *J. alternimaculata*. The chromatin is strongly condensed (white asterisk). The proximal centriole has inserted deeply into the nuclear fossa (nf). The mid-piece (Mp) and the flagellum (F) are seen. Scale bars = 2 μm and 1 μm.

Figure 7. Transmission electron micrographs (TEM). *Jenynsia* sp. spermatozoa. (A): Head (H) and mitochondria around the flagellum (F) of spermatozoa of *J. multidentata*. Scale bar = 0.5 μ m. (B): Head (H) and mid-piece (Mp) of spermatozoa of *J. maculata*. Scale bar = 1 μ m. (C-D): Mid-piece (Mp) and sub-mitochondrial net (smn) of spermatozoa of *J. alternimaculata*. Scale bar = 0.5 μ m. (E): Mid-piece (Mp) and sub-mitochondrial net (smn) of a spermatozoon of *J. maculata*. Scale bar = 0.5 μ m. (F): Head (H), mid-piece (Mp) and flagella (F) of spermatozoa of *J. maculata*. Scale bar = 0.3 μ m. (G): Mid-piece (Mp), sub-mitochondrial net (smn) and flagella (F) of spermatozoa of *J. maculata*. Scale bar = 0.2 μ m. (H): Mid-piece with mitochondria (m) and sub-mitochondrial net (smn) around the flagellum (F) of a spermatozoon of *J. multidentata*. Scale bar = 0.3 μ m. (I): Flagellum (F) of a spermatozoon of *J. multidentata*. Lateral, membranous expansions (e) of the flagellum are indicated. Scale bar = 0.2 μ m.

spermatogonia, are situated singly or in small groups that are located at the distal ends of the testicular lobules.^{16,18,20,36} By mitotic division, these stem cells produce new generations of spermatogonia.

The periphery of the testis is the spermatogenic zone, whereas the mid testis, in which the efferent ducts are located, is a sperm storage zone. In *Jenynsia* species, continuous spermatogenesis was observed, as occurs in the most studied atheriniforms^{13,15,24,33,29,37} and, in

Figure 8. Light photomicrographs and Transmission electron micrographs (TEM) of Sertoli Cells. (A): Sertoli cells around spermatocysts and efferent ducts of *J. alternimaculata*. The Sertoli cells (SC) around the spermatocysts (Scy) form a squamous epithelium; whereas around the efferent ducts (ED) form a cuboidal epithelium. H-E. Scale bar = 300 μm . (B): Sertoli cells of spermatocyst surrounding spermatids (Sm) in *J. alternimaculata*. There are seen 2 Sertoli cells (SC) of adjacent spermatocysts. The nuclei (n) of both cells are seen. Mitochondria (m) are also observed. Scale bar = 0.5 μm . (C): Sertoli cells of an efferent duct (ED) with spermatozoa (S) in the *J. maculata*. The nucleus (n) of Sertoli cell (SC) is observed. Scale bar = 5 μm . (D): Leydig cell (LC) in the interstitial tissue around an efferent duct of *J. maculata*. The nucleus (n) and abundant mitochondria (m) and dense secretory granules (g) are seen. Scale bar = 0.5 μm .

particular, in viviparous teleosts.¹⁸ Mature spermatozoa were observed in the testes throughout the year in wild specimens. Concomitantly, male specimens observed in aquaria also display mating behavior throughout the year (pers. obs.). Sperm storage in *Jenynsia* in the intratesticular duct was observed in other atheriniforms, such as *Anableps dowi*,³⁸ Hemiramphidae,^{31,29} Embiotocidae³⁰ and in characiforms, specifically the inseminating glandulocaudine fishes.²²

The Sertoli cells surround one to 8 spermatogonia forming the spermatocyst walls. Sertoli cells form a simple, epithelial border around the cysts. Each cyst, within a lobule, is separated from the interstitium by a thin basement membrane coinciding with a similar characteristic that occurs in the labrid, *Thalassoma bifasciatum*.³⁶ Inside the cysts, the spermatogonia enter meiosis, becoming primary spermatocytes. The formation of spermatocytes begins spermatogenesis, coinciding with the gradual growth of spermatocysts and subsequent development of Sertoli cells. Within each spermatocyst germ cells develop quite synchronously.

A testicular feature observed in *Jenynsia* species occurred at the end of spermatogenesis when Sertoli cells hypertrophied and formed the wall of the efferent ducts. A system of efferent ducts has been reported for other freshwater teleosts, as species in the families Salmonidae, Cyprinidae, Esocidae.³⁹ The Sertoli cells develop a secretory function within the efferent ducts. Their secretions stain with both PAS and alcian blue, indicating the presence of neutral and acid glycoproteins, respectively. Our results are similar to those reported in species of the families Poeciliidae,^{15,16,18} Cyprinodontidae and Oryziatidae.²⁴

Spermatogonia and spermatocysts are morphologically similar to those described in other fishes.^{18,19,40-42} Although, differences regarding specific aspects in spermiogenesis do exist. Ultrastructure of earlier stages of spermatogenesis in the 3 *Jenynsia* species analyzed in this study corresponds to the teleostean pattern. As the spermatid nucleus rotates, a deep central nuclear fossa forms that contains the centriolar complex. The depth of the nuclear fossa is similar to that reported in other cyprinodontiforms, such as *Gambusia affinis*, *Poecilia reticulata* and *Poecilia latipinna*.²³ However, the 3 *Jenynsia* species studied here do not exhibit elongated microtubules extending from the proximal centriole to the apex of the nuclear fossa, as observed in *P. latipinna*,²⁵ *P. reticulata*⁴³ and *G. affinis*.²⁶

Spermatozoa with elongated heads and mid-pieces are related to internal fertilization.²³ Because the mature spermatozoa of *Jenynsia* sp. possess an elongated nucleus, with highly condensed chromatin surrounded by a fine cytoplasmic layer, they belong to the introsperm type. An elongated nucleus is considered to be a common sperm modification in inseminating teleosts.^{22,23,28} The narrow and hydrodynamic nucleus shape improves sperm transfer to the female,²² improves sperm movements while reducing friction within viscous female secretions, permits sperm to form clusters improving movement, and reduces the loss of sperm cells. The sperm midpiece is short in species of fish with external fertilization, but has increased size in inseminating species.^{23,28,42} Introsperm, with elongated nuclei and mid-pieces, were described in viviparous fishes of the atherinomorph families Poeciliidae, Jenynsiidae and Anablepidae.²³ In the *Jenynsia* species examined herein, a very long sperm mid-piece, with large mitochondria surrounding

the flagellum, was observed. The sperm were classified as belonging to an anacrosomal, introsperm complex. During spermiogenesis, the position of a centriolar complex becomes located inside a deep nuclear fossa. Along the mid-piece, a long cytoplasmic canal develops which is surrounded by a sub-mitochondrial net, between the plasma membrane and large mitochondria. A sub-mitochondrial net was reported for *J. lineata*¹⁴ and *G. affinis*.²⁶ Although, in *G. affinis* it is less defined than in *Jenynsia* species. These results suggest that a well-developed, sub-mitochondrial net is a characteristic of the genus *Jenynsia*. Its function could be the stiffening of the mid-piece, making it more resistant to lateral forces that are developed during active movements of sperm through viscous fluids of the ovarian lumen. Those functions are addressed by accessory microtubules located in the head and mid-piece of some species of ostariophysan glandulocaudines, such as *Mimagoniates inequalis* and *Macropsobrycon uruguayanae*.^{22,32,44} But, in *Jenynsia* species, the intermediate filaments in the sub-mitochondrial net perform this function.

In *Jenynsia* species the single flagellum shows a pair of short lateral membranous expansions. These expansions do not reach the distal end of the flagellum. A similar sperm structure was also reported for in *J. lineata*,¹⁴ *P. latipinna*,^{23,25} *G. affinis*, *Xiphophorus helleri* and *X. maculatus*.²³

Our results define a pattern of sperm flow inside the spermatid ducts. Within the efferent ducts and inside the gonopodium, spermatozoa are frequently disposed parallel to each other. This is dissimilar to the viviparous Poeciliidae, a family of fishes that form spermatozeugmata with outwardly-projecting sperm nuclei, and is also dissimilar to the Goodeidae, another family of viviparous fishes of the order Cyprinodontiformes,¹⁷ in which sperm flagella course over the surfaces of spermatozeugmata.^{19,20,25} Each family is characterized by having evolved separate mechanisms for sperm transfer within an aquatic environment (i. e., they do not cast free spermatozoa into the aqueous environment). Our results agree with other authors,²² who stated that the absence of packed spermatozoa (spermatozeugmata), does not define their transfer as free spermatozoa. Sperm mass has been found in the ovary of 3 *Jenynsia*.⁴⁵ The flow of spermatozoa within the reproductive system of male *Jenynsia* species is aided by sperm being loosely associated with each other by macromolecules, which results in "clumped" spermatozoa for transfer into female reproductive tract via a tubular gonopodium. We did not find differences in testicular structure between the 3 species, the dimensions of the different types of germ cells are very similar. Males produce sperm throughout the year, but females of each species have mature oocytes at different times (*J. alternimaculata* in August with births between November and March, *J. maculata* in October with births between December and April, while *J. multidentata* in June with births between October and February) (unpublished data). In laboratory conditions, multiple, polyan-drogenic and coercive matches are observed throughout the year, *Jenynsia* females stored sperm in ovarian folds and within the germinal epithelia of the lamella.⁴⁵ Sperm storage and multiple insemination in *Jenynsia* surely play an important role in internal sperm competition, increasing heterogeneity, mixing parenting and adjustment of offspring in unfavorable environments.

Materials and Methods

A total of 24 sexually mature males of each species of *Jenynsia* were included in this study. The species studied are located in different environments and elevations in Salta province, northwestern Argentina. *J. maculata* is located to the west, at 2324 m altitude, in the Calchaquí river (25°07'26.59"S–66°09'42.55"W) in *monte* environments, *J. alternimaculata*, is registered at 1200 m altitude in streams and rivers from the Lerma Valley (25°23'36.58"S–64°09'36.58"W), in *Chaco Serrano* environments. *J. multidentata* is located to the east at 518 m altitude, in effluents of Medina River (24°48'12.50"S–65°26'56.03"W), in environments of dry Chaco plain, in the Sierra de Metán. Species of *Jenynsia* were collected monthly, the specimens were anesthetized and euthanized by immersion in a 5% solution of benzocaine. For light microscopy, small pieces of gonads were fixed in 10% formalin in phosphate buffer (0.1 M, pH7.3) for 24 h. The samples were dehydrated along an ascending series of ethanol, embedded in histoplast, serially sectioned (4–6 µm thick) and stained with 4 techniques: hematoxylin-eosin, Masson's trichrome, PAS–alcyan blue and PAS–hematoxylin. The samples were observed with an Olympus BX40 microscope and photographed with a digital camera Olympus DP 25. For electron microscopy, small pieces of gonads were fixed for 24 h in 1, 66% glutaraldehyde, and sodium phosphate buffer 0.1 M (pH 7, 4) and incubated overnight at 4°C, postfixed with a 1/1 solution of sodium phosphate buffer/2% osmium tetroxide (OsO₄). The samples were washed with distilled water, and a 1/1 solution of

phosphate buffer/uranil acetate was added. After incubation in the dark, the samples washed with ascending series of ethanol and acetone. Then the samples were incubated in a 1/1 solution of acetone/Spurr resin. The specimens were included in Spurr resin. Ultra-thin sections were made with an ultramicrotome, mounted on copper grids and contrasted with uranyl acetate and lead citrate.⁴⁶ The samples were observed with a Zeiss EM109 (Carl Zeiss NTS GmbH, Oberkochen, Germany) transmission electron microscope. For scanning electron microscopy (Jeol 6480 LV, Japan), samples of fixed testes were critical point dried and gold plated. Voucher specimens were stored in the ichthyological collection of the Museo de Ciencias Naturales, Universidad Nacional de Salta, Argentina.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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