Scanning Microscopy

Volume 2 | Number 2 Article 42

10-19-1987

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canning Microscopy, Vol. 2, No. 2, 1988 (Pages 1089-1095) 0891-7035/88$3.00+.00
canning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

ULTRASTRUCTURE AND HISTOCHEMISTRY OF THE VAS DEFERENS OF THE SALAMANDER RHYACOTRITON OLYMPICUS: ADAPTATIONS FOR SPERM STORAGE

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(Received for publication February 27, 1987, and in revised form October 19, 1987)

Abstract

The vas deferens of the salamander Rhyacotriton olympicus is composed of (1) a peritoneal epithelium, (2) connective tissue with fibroblasts, melanophores, circular smooth muscle, capillaries, and unmyelinated nerves within a collagenous matrix, and (3) an inner layer of cuboidal epithelium partially covered by ciliated squamous cells at the lumen. The lumen and apical cytoplasm of both epithelial cell types contain strongly PAS-positive granules. The cuboidal cells contained numerous swollen rough endoplasmic reticulum cisternae, mitochondria, and apical dense granules suggesting a high degree of secretory activity possibly involved in sperm maintenance. Fewer mitochondria, rough endoplasmic reticula, and granules in squamous cells suggest less secretory activity. Squamous cells may protect the cuboidal cells from possible abrasion by sperm masses and/or their cilia may aid in distributing secretory products in the lumen.

Key words: Amphibian, salamander, caudata, Wolffian Duct, histochemistry, Rhyacotriton olympicus, vas deferens, reproductive tract, ultrastructure.

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Introduction

In salamanders, spermatozoa are produced seasonally in the testes and then transferred through a series of ducts to the vas deferens, where they are stored up to 12 months (Blanchard, 1928; Bruce, 1975, 1978; Jones, 1986; Nussbaum and Tate, 1977). Although general descriptions of male reproductive systems have been reported for salamander species from the families Ambystomatidae, Cryptobranchidae, Proteidae, Plethodontidae, Salamandridae, and Sirenidae (Baker, 1965; Baker and Taylor, 1964; Chase, 1923; Francis, 1934; Ratcliff, 1965; Rogers and Risley, 1938; Rosenquist and Baker, 1967; Spengel, 1876; Strickland, 1966; Weichert, 1945; Williams et al., 1984; Yamagiwa, 1924), there have been few reports on histology and histochemistry (Chase, 1923; Weichert, 1945; Williams et al., 1984) and none on ultrastructure. In addition, Norris et al. (1985), Weichert (1945), and Williams et al. (1984) have characterized seasonal changes in the histology of the vas deferens.

In the family Ambystomatidae, there have been few gross anatomical investigations (Rogers and Risley, 1938; Baker and Taylor, 1964) and only one histological study (Norris et al., 1985) of the vas deferens, and only a brief anatomical note for the dicamptodontid, Dicamptodon (De Marco, 1952). Descriptions of the male reproductive tract in the only other dicamptodontid genus, Rhyacotriton, are limited to a single life-history report of sperm volume based upon smears and general morphology (Nussbaum and Tait, 1977). In the current study of the R. olympicus vas deferens, light and electron microscopy were employed to (1) describe basic histology, (2) determine if ultrastructural changes are seasonal, (3) correlate structure with function, and (4) compare our results with those of previous investigations on other salamanders and mammals.

Materials and Methods

Seventy-one male R. olympicus were collected from February 1985 through November 1986 in southeast Skamania county, Washington (Table 1). Snout-vent lengths (SVL) were measured on anesthetized animals from the tip of the snout to the posterior edge of the vent. Within 48 h after collection, specimens were anesthetized in 2% Tricaine Methane sulfonate (Crescent Research Chemical, Inc.) and their reproductive tracts excised and fixed. For electron microscopy, either the right or left vas deferens was placed in 3% glutaraldehyde buffered with 0.1 M phosphate (pH 7.2); segments from the cranial, middle, and caudal regions were removed for further processing. The samples were then postfixed in 3% OsO_4, dehydrated through an ethanolic series, and embedded in Epon 812 (Luft, 1961) for transmission electron microscopy (TEM), or critical point dried in a Bomar SPC (Cohen, 1974; Cohen et al., 1968) for scanning electron microscopy (SEM). After gold coating (ca. 30 nm) the SEM samples in a Hummer 1 D.C. sputter coater apparatus, they were examined with an ETEC U1 Autoscan scanning electron microscope. Thin sections (70-90 nm) were cut with glass or diamond knives on a Reichert OMU2 ultramicrotome, picked up on Formvar-coated copper grids, and stained sequentially with half-saturated aqueous uranyl acetate for 15 minutes and alkaline lead citrate (Reynolds, 1963) for 5 minutes at room temperature. These sections were examined with a Hitachi HS-8 transmission electron microscope at 50 kV.

For light microscopy, 0.5 µm plastic sections and 10 µm paraffin sections (fixed in 10% buffered formalin) of vasa deferentia were examined. All histochemical tests (Table 2) were performed on 10 µm paraffin sections, except those for lipids in which plastic sections were used. Luminal surface cell area was determined using a Bioquant II image analysis program with an Apple IIe computer.

Table 1. Number of Rhyacotriton olympicus vasa deferentia examined.

Month	Ja	Fe	Mr	Ap	My	Jn	Jl	Au	Se	Oc	No	De
Year(s)	86	85/6	85	85	85/6	85	85/6	85	85	85	86	85
Number of vasa deferentia	12	22	22	16	6	8	6	2	10	12	14	12

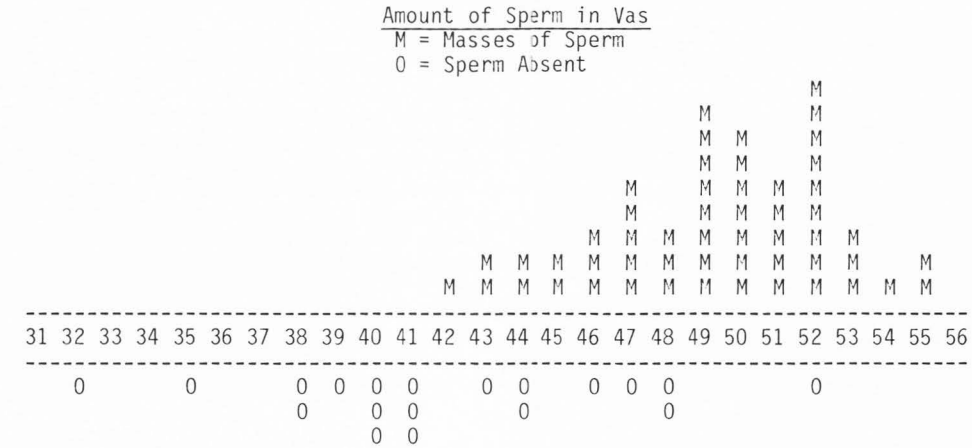
Table 2. Staining techniques and histochemical methods used on paraffin sections of Rhyacotriton olympicus vasa deferentia.

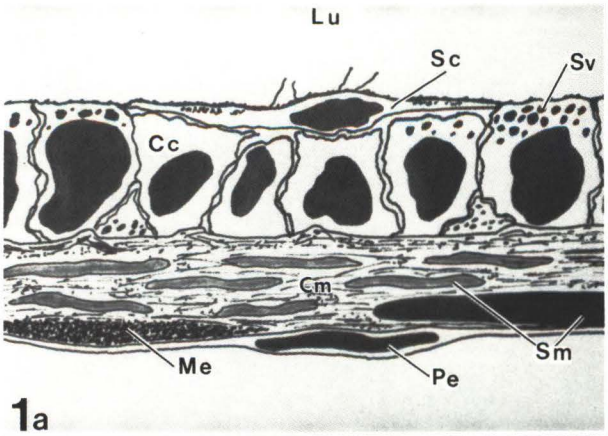
Specificity	Technique	Reference
General staining	Hematoxylin-Eosin	Humason (1967)
	Picro-Gomori	Menzies (1959)
Lipids	Sudan Black	Chiffelle & Putt (1951)
	Osmium tetroxide	Pearse (1968)
Carbohydrates	Periodic acid Schiff	Lillie (1965)
	Alcian Blue	Humason (1967)
DNA	Feulgen	Humason (1967)

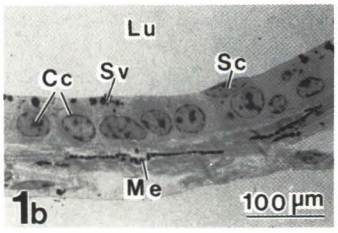
Results

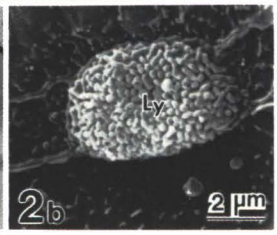
The vas deferens of breeding males greater than 48 mm SVL showed only minor seasonal or individual differences in epithelial height and/or secretory activity. Males of this size or larger were collected for all months except August. Vasa deferentia from the R. olympicus males greater than 48 mm SVL, with the exception of a 52 mm SVL male collected in July, were full of spermatozoa (Table 3). The descriptions presented below represent the typical condition of the breeding male's vasa deferentia from September through May.

Table 3. Snout-vent lengths in mm for male Rhyacotriton olympicus from all months of the year. Snout-vent length is from tip of snout to posterior side of vent for animals preserved in 70% ethanol and represents an average 7% reduction in length due to dehydration (based on the average difference between fresh and preserved specimens for all males used in this study).









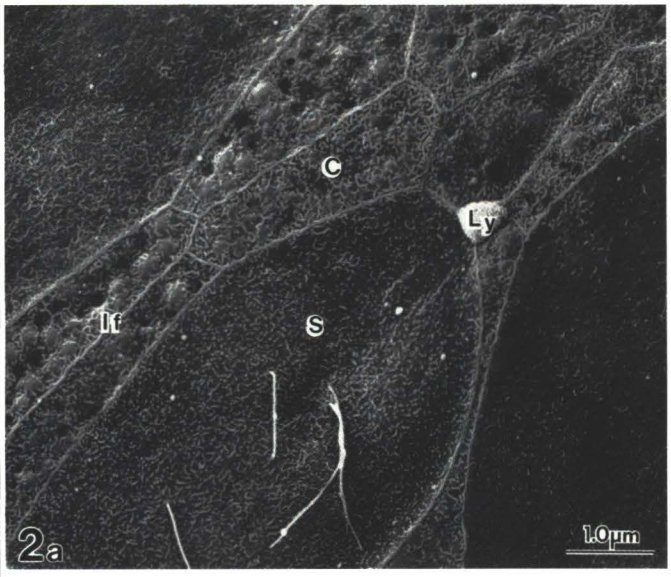


Figure 1a. Diagram of a cross-section of a Rhyacotriton olympicus vas deferens to show smooth muscle (Sm), squamous cells (Sc), peritoneal epithelium (Pe), melanophores (Me), cuboidal cells (Cc), collagenous matrix (Cm), and secretory vesicles (Sv). Lumen (Lu).

Figure 1b. Light microscopy cross-section with structures indicated as in Figure 1a.

Figure 2a. Scanning electron micrograph of the luminal surface of the vas deferens to show ciliated squamous cells (S), a possible lymphocyte (Ly), and non-ciliated cuboidal cells (C) meeting at distinct intercellular surface folds (If).

Figure 2b. High magnification scanning electron micrograph of a possible lymphocyte (Ly).

A diagrammatic representation of the major tissue layers of the vas deferens is presented in Figure 1. From the outer to the luminal surface, the vas deferens was composed of a peritoneal epithelium, a connective tissue sheath, and an inner layer of cuboidal cells partially covered with ciliated squamous cells at the lumen (Fig. 1). Lymphocyte-like cells were occasionally seen protruding between cuboidal and squamous cells at the luminal surface (Fig. 2).

The peritoneal epithelium enclosed a connective tissue layer (15 µm thick) of fibroblasts, melanophores, circular smooth muscle, capillaries, and unmyelinated nerve fibers within a collagenous matrix (Figs. 1,3,4). Melanophores and nerves were located near the outer boundary of this layer and smooth muscle near its inner boundary. Unmyelinated nerve fibers surrounded by Schwann cells and capillaries were rarely seen.

The epithelium lining the lumen was composed of non-ciliated cuboidal and ciliated squamous cells (Fig. 1). In 7 randomly chosen low magnification SEMs taken of the luminal surface of vasa deferentia from 2 males (Fig. 5), 619 cells were identified. The ratio of cuboidal to squamous cells was 17:1. However, because the squamous cells have a greater surface area (mean squamous = 2560 sq µm, sd = 1074; mean cuboidal = 230 sq µm, sd = 111; N = 30 for each cell type) they covered about 40% of the luminal sheath. Cuboidal cell nuclei had little heterochromatin associated with the inner side of the nuclear envelope (Fig. 3). The cytoplasm contained mitochondria with lamellar cristae, occasional Golgi membranes near the nuclei, a few microtubules and filaments, and large quantities of rough endoplasmic cisternae swollen with product. Numerous ribosomes were scattered among the RER. Apically located electron-dense granules, appearing ellipsoidal in cross-section (Fig. 6), averaged 2.9 µm in width and 3.7 µm in length (N = 20). Occasional dense granules were seen basally in apparent extensions of the cuboidal cells (Fig. 3). Although cuboidal cells lack cilia, they have short (1 - 2 µm long) and thin (0.5 - 1.0 µm wide) microplicae (Fig. 6).

While cuboidal cells were separated laterally by a 0.5 µm space, they were in contact with each other through an interdigitating system of 1 µm processes (Fig. 3). Along their bases these cells were associated with a basal lamina by hemidesmosomes. Apically, the cuboidal cells are attached to one another and with squamous cells by typical junctional complexes (zonula adherens, macula adherens, and zonula occludens).

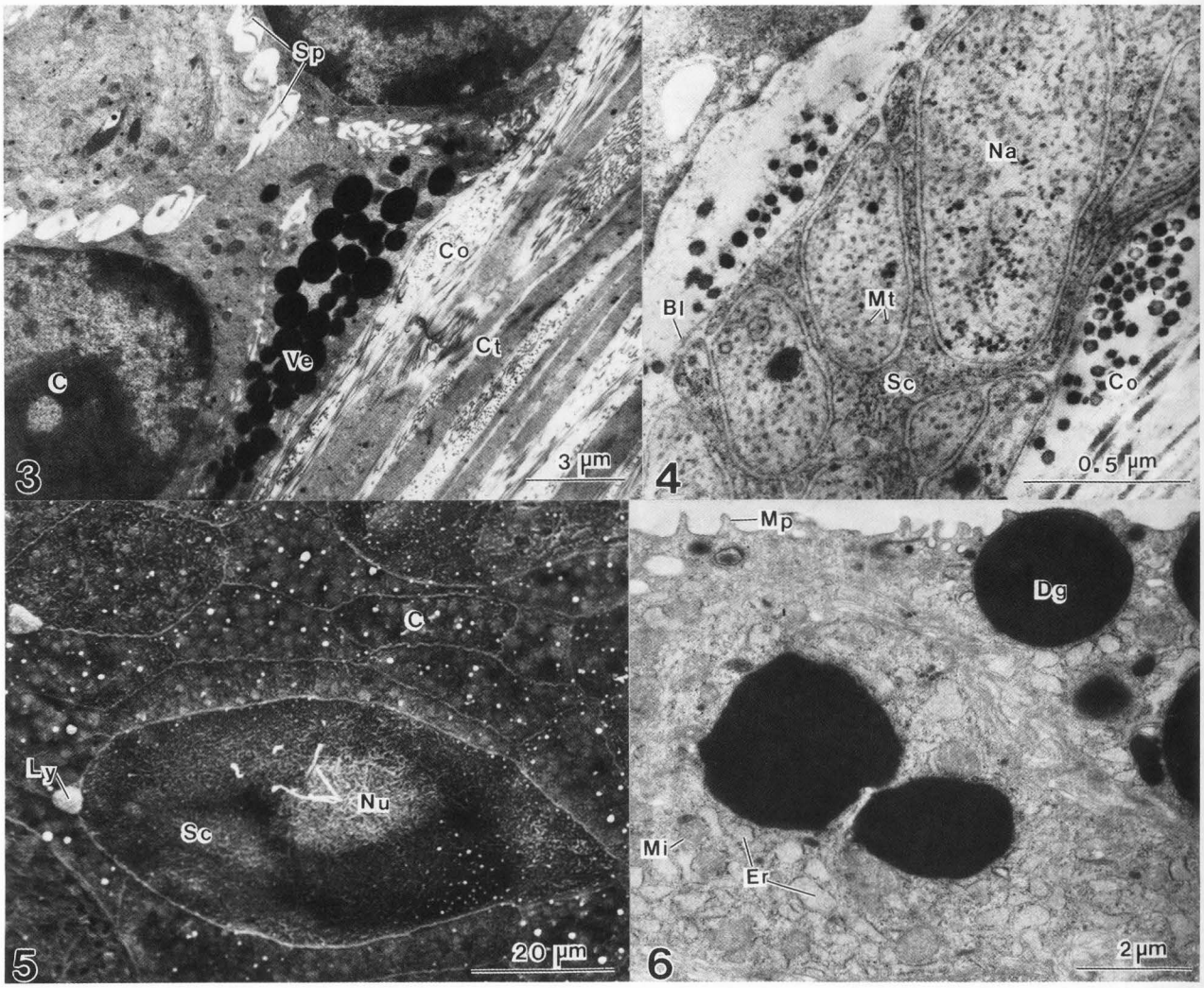


Figure 3. Basal vesicles (Ve) probably in a cuboidal cell process. Also present are cuboidal cell nuclei (C), small processes (Sp) extending into the intercellular space, and a connective tissue layer (Ct) with collagen (Co).

Figure 4. Unmyelinated nerve axons (Na) with microtubules (Mt). Note Schwann cell cytoplasm (Sc), basal lamina (Bl), and collagen (Co).

Figure 5. Scanning electron micrograph of the luminal epithelial surface to show cuboidal cells (C), a possible lymphocyte (Ly), and squamous cells (Sc) with their nuclei (Nu) and cilia.

Figure 6. Apical region of a cuboidal cell with mitochondria (Mi), distended rough endoplasmic reticulum (Er), microplicae (Mp), and dense granules (Dg).

Although ciliated squamous cells with elongate nuclei (ca. 30 µm wide) and little heterochromatin formed a net over the cuboidal cells, they were rarely in contact with one another (Figs. 1,2,5). In a randomly selected sample of 30 squamous cells, 73% had cilia (Fig. 7) with an average of 4 and a range of 0-17 cilia per cell (sd = 4.7). Ciliogenesis within these cells was suggested by the wide range in the number of cilia per cell, and by cytoplasmic structures normally involved in the production of cilia (fibrous granules, cylindrical structures, and basal bodies) located among numerous mitochondria (Fig. 8). Microplicae similar in size to those of the cuboidal cells covered the apical surface (Fig. 7).

Less production of secretory product by squamous cells than cuboidal cells was suggested by less RER and Golgi, fewer mitochondria and single ribosomes. However, numerous dense, biconcave discs were located apically and may be vesicles filled with a secretory product (Fig. 9).

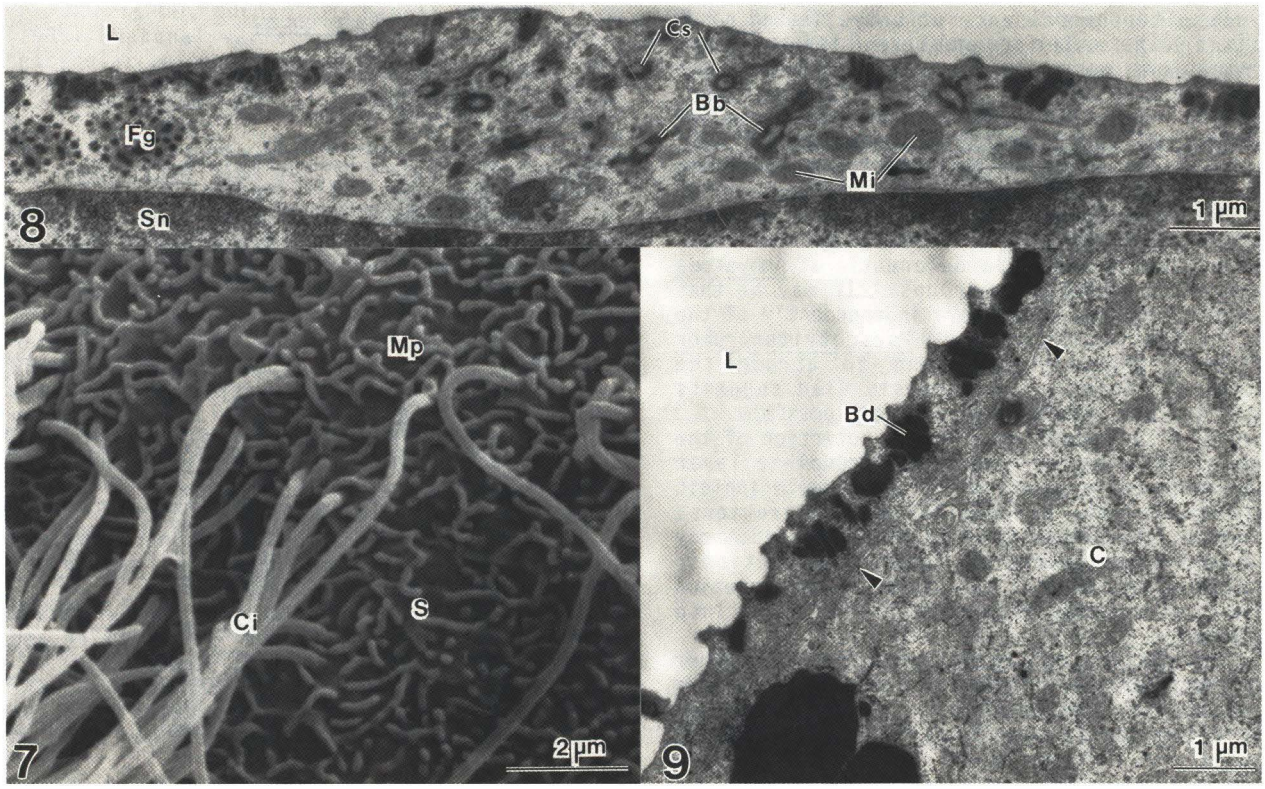


Figure 7. Scanning electron micrograph of squamous cell surface (S) to show cilia (Ci) and microplicae (Mp).

Figure 8. A squamous cell exhibiting ciliogenesis. Note nucleus (Sn), fibrous granules (Fg), cylindrical structures (Cs), basal bodies (Bb), mitochondria (Mi), lumen (L).

Figure 9. Apex of a squamous cell to show dense, biconcave discs (Bd). Below is a cuboidal cell (C). Arrows indicate a cuboidal-squamous cell junction. Lumen (L).

Cytoplasmic microtubules (250 nm) and microfilaments (80 - 100 nm) were more abundant than in cuboidal cells and were primarily oriented parallel to the apical plasmalemmae.

Squamous cell processes were about 0.5 - 2.0 µm in width (Fig. 9). Because the squamous cells were essentially flat, their nuclei appeared as distinct mounds in scanning electron micrographs of the luminal surface (Fig. 5).

Nuclei of the squamous cells were strongly Alcian blue-positive and PAS-negative. Cuboidal cell nuclei were lightly PAS and Alcian blue-positive. The lumen and apical cytoplasm of both cell types contained strongly PAS-positive granules but there was no positive reaction to Alcian blue. Tests for lipids were negative.

Discussion

Our study (Table 3) supports the report by Nussbaum and Tait (1977) that sperm-packed vasa deferentia occur in most sexually mature R. olympicus males (>48 mm SVL) from September to May. Because the spermatozoa of many salamander species are apparently stored in the female's spermatheca for many months, sperm transfer from male to female can occur just prior to or long before oviposition. The ability for both sexes to store spermatozoa for extended periods of time increases the opportunities for successful mating, possibly to whenever sexually mature males and females meet except during the summer.

While the length of time individual spermatozoa can survive in the vas deferens of male salamanders is unknown, the types, numbers, and organization of cytoplasmic organelles (numerous Golgi membranes and vesicles, swollen RER, and secretory granules) within the cuboidal cells, suggests that they are actively providing nutrients for sperm support. PAS-positive reactions in the apical regions of the cuboidal and squamous cells and within the lumen also give credence to this suggestion. Secretions from the cuboidal cells appear to be released through the apical surface between squamous cells. Hafez and Sherman (1978) suggested that in the human oviduct, the ciliated cells help distribute the product throughout the lumen. Possibly the ciliated squamous and cuboidal cells of the vas deferens of R. olympicus function similarly.

Despite the presence of unmyelinated nerve axons in the outer connective tissue layer, terminations were not observed. The smooth muscle located in the connective tissue layer, possibly controlled by such nerves, may cause the expulsion of sperm from the vas deferens.

In addition to having cilia for distributing secretory products, squamous cells may protect the cuboidal cells from possible abrasion by the sperm masses. Russell et al. (1981) reported that spermatozoa in the vasa deferentia of Ambystoma texanum are clumped in groups. It may be that clumps of sperm, which move independently of the wall of the vas deferens in Pseudotriton ruber (Zalisko, unpublished), tend to abrade the epithelial lining. Ciliogenesis also suggests that cilia are continually replaced; possibly because of such abrasion. While protection of the cuboidal cells may be important, a complete layer of squamous cells covering the lumen could inhibit efficient release of cuboidal cell secretions. Therefore, the organization of this epithelium may be a compromise between two important functions; partial protection of the vas wall and secretion via a direct pathway from the cuboidal cells into the lumen.

Information on the vasa deferentia of other salamander families, indicates some general trends in epithelial composition. Species having an epithelium similar to R. olympicus include D. ensatus (Dicamptodontidae), Ambystoma macrodactylum (Ambystomatidae), and Notophthalmus viridescens and Cynops pyrrhogaster (Salamandridae) (Zalisko, unpublished). The plethodontids, Eurycea longicauda, E. lucifuga (Williams et al., 1984), E. bislineata (Weichert, 1945), Plethodon jordani (Zalisko, unpublished) and the proteid Necturus maculosus (Chase, 1923) have a non-ciliated simple cuboidal epithelium. Until more information is available on the vasa deferentia of a variety of salamander species, the functional significance of this structure's variation among the families will remain unresolved.

Although structurally different, the vas deferens of R. olympicus resembles the epididymis of mammals in its function. The mammalian epididymis is a sperm storage organ which provides supportive secretions (Dadoune, 1981) and functions in sperm maturation (Courot, 1981). In his work on in vitro fertilization of ambystomatid salamanders, Brandon (1977, personal comm.) has found that spermatozoa from the cranial end of the vas deferens are less capable of fertilizing ova than those from the middle or caudal regions. While this suggests a physiological maturation of the sperm from anterior to posterior, further study is required to see if concomitant changes occur in their structure.

Acknowledgements

We would like to thank R. Herrington, J. Monda, P. Smith, and A. Zalisko for assistance in the field. The comments of T. Aire, M. Bakst, R. Brandon, J. Mallatt, P. Schroeder, R. Wallace, and an anonymous reviewer were helpful and are greatly appreciated.

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Discussion with Reviewers

T.A. Aire: Why did the authors limit their studies to the vas deferens? Why were the testes not examined to determine their phase(s) in the reproductive cycle of the animals?
Authors: The present study is part of a larger survey on the ultrastructure of the salamander reproductive system. The testes will be analyzed later.

M.R. Bakst: Any evidence of phagocytosis of sperm by cells lining the vas deferens?
Authors: Although looked for in all samples, no signs of phagocytosis of spermatozoa were observed.

M.R. Bakst: Is there any direct interaction of sperm and the luminal epithelial cells of the vas during prolonged storage which would suggest a "supportive" function?
Authors: If direct interaction means a physical relationship involving the enveloping of spermatozoa by epithelial cells, perhaps similar to the Sertoli cell-spermatid relationship, none was ever seen. Any support of spermatozoa by the epithelial cells is apparently via secretion.