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Epididymis of the Lizard *Eutropis carinata*: A Light Microscopic and Ultrastructural Seasonal Study

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Abstract

The epididymis of the lizard *Eutropis carinata* can be divided into four regions viz., an initial segment (extra testicular) Efferent ductules (Eds) and three regions, the anterior region, a broad middle, and a narrow posterior, comparable to the caput, corpus, and cauda epididymidis of mammals. The anterior region of the epididymis is closely associated with a whitish initial segment (extra testicular), the Efferent ductules (Eds). The epididymal wall consists of an epithelial layer lining the lumen resting on a basement membrane surrounded by four to five layers of smooth circular muscles, a layer of connective tissue, and a layer of serosa. The ultrastructure study of the initial segment of epididymis the Eds during breeding season discloses four cell types in its epithelium viz., ciliated, non-ciliated, Apical-Mitochondria Rich Cells (AMRC), and basal cells. The anterior, middle, and posterior regions of the epididymis show five different cell types namely principal, basal, AMRC, narrow, and clear cells. Ciliated and non-ciliated cells are limited to the initial segment of the epididymis while, principal, narrow, and clear cells are found in the rest of the three regions of the epididymis. Basal and AMRC are found in all four regions. AMRCs are the most abundant cell type in the initial segment while principal cells are the major components of the epithelial lining of the remaining regions of the epididymis. During the non-breeding season, all the cell types are present but with regression and altered cytology of the cells without any sign of cellular activity in the different regions of the epididymis. During the breeding season, even the circulating testosterone levels are significantly higher compared to the non-breeding season. This is the first report describing different cell types in the initial segment and three different regions of the epididymis with ultrastructural seasonal variations in the Keeled Indian Mabuya, *Eutropis carinata* (Scincidae).

Keywords: Electron Microscopy, Epididymis, Epithelial Cells, Light Microscopy, Lizard, Seasonal Study, Ultrastructure

1. Introduction

In mammals, testicular spermatozoa, which are neither motile nor fertile, require discrete post-gonadal maturation to be able to fertilize the ova[1,2]. The final stages of spermatozoa differentiation occur outside the gonad and are not under the genomic control of germ cells. Thus, the epididymis is the main site for sperm maturation and sperm storage. The epididymis is a long tube with very active secretory and reabsorption functions and can create a sequential change in the composition of luminal fluid along its length. In reptiles, the genital region of the Wolffian duct differentiates into epididymis[3,4]. Albeit the epididymis evolved for the first time in reptiles among vertebrates, it is the least investigated compared to the other amniotes. Reptiles were the first vertebrates that successfully adapted to life on land due to the evolution of internal fertilization and cleidoic eggs as major adaptations to terrestrial life[3,5-7]. The advent of internal fertilization in vertebrates was associated with spermatozoa maturation and storage in accessory reproductive organs, of which the epididymis is a major organ. Studies on reptilian epididymis are important as they might lead to understanding the evolution of the spermatozoa maturation process in vertebrates as reptiles are a pivotal group in vertebrate evolution. Light microscopic studies

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on the epididymis have been carried out in many species of reptiles[8-14]. The epididymis in lizards *Calotes versicolor*, *Lacerta vivipara*, and *Hemidactylus flaviviridis*is is divided into three regions, *viz.,* anterior, middle, and posterior regions based on the tubule diameter and comparable with caput, corpus, and cauda epididymidis of mammals[8,10,14]. In the lizard, *Psammophilus dorsalis* the epididymis is divided into three regions based on the localization of steroid-metabolizing enzymes[15]. Further, in *C. versicolor* the epididymal epithelium is differentiated along its length into five regions[10]. Unlike the lizards, the epididymis of the crocodile, *Caiman crocodilus,* and turtle *Chrysemys picta* is divisible into only two regions *viz.,* proximal and distal[16,17]. However, Olukole, *et al.,* observed three regions across epididymis in the African side neck turtles *Pelusios castaneus* with no significant difference in the duct diameter[18].

Ultrastructural study of the epididymis has been carried out only in a few species of reptiles. Studies in the lizard *L. vivipara*[19] and the crocodile *C. crocodilus*[16] disclose the presence of only two types of cells *viz.,* columnar secretory cells and basal cells. A preliminary study in the lizard *M. carinata* (now renamed *Eutropis carinata*) described the presence of a single cell type in the epididymal epithelium[9]. Studies in the turtle *C. picta* record four kinds of cells as identified by Holmes and Gist in the epididymis and they are vesicular and basal cells which are major types followed by narrow and AMRC in the epididymal epithelium[17]. Meeran, *et al.,* based on light microscopic observations, have identified seven cell types in the epididymis of the lizard *C. versicolor viz.,* principal, narrow, apical, clear, dark, basal, and halo cells[13]. Pagliarini, *et al.,* studied the morphological changes of the epididymis of *Phrynops geoffroanus* with light microscopy, and the cellular types of the epididymal epithelium corresponded to only principal, basal, and narrow cells[20]. An ultrastructural study of the epididymis in the fan-throated lizard *Sitana ponticeriana* reveals that the ductus epididymidis is differentiated into four histologically distinct zones, namely the initial segment, caput, corpus, and cauda[21]. The initial segment, caput, and corpus appear to be associated with secretion and absorption/endocytosis, while the cauda is concerned with absorption/endocytosis and storage[21]. Further, in this study, Akbarsha, *et al.,* report the presence of six cell types in the epithelium based on ultrastructural studies[21]. Light microscopy histology of efferent ductules (initial segment) and the ultrastructural organization of the epithelium have been studied in the fan-throated lizard *S. ponticeriana*. The epithelium is formed of four different types of cells. Transmission electron microscopy confirms that two cell types, non-ciliated cells, and ciliated cells are predominant in the epithelial lining of the efferent ductules, but occasionally a few dark cells and flat cuboidal basal cells are also present[22].

Ultrastructural studies conducted hitherto in reptiles have not revealed differences if any, in the distribution of different cell types in different regions of the epididymis except for one report in *S. ponticeriana*[21]. Further, the nature of ultrastructural changes in the epididymis during the breeding season, compared to the non-breeding season, is not reported in reptiles. In addition, there is no information as to whether multiple cell types are present throughout the year, or appear only during the breeding season. It is also evident that reports are not explicit with reference to cell types in the epididymis epithelium of reptiles. Since mammals and modern reptiles have evolved from the same ancestral reptilian stock, it would be interesting to know whether modern reptiles failed to achieve multiple cell types in the epididymis similar to mammals or whether the occurrence of a lesser number of cell types is merely a species difference. Hence, more studies on different species of modern reptiles and a thorough analysis of cell types are required to generalize the concept. Accordingly, the present study was carried out to identify different cell types in the epididymal epithelium based on their ultrastructural features and regional variation in their occurrence during the breeding and non-breeding seasons in the lizard *Eutropis carinata*.

2. Materials and Methods

Sexually mature males of *Eutropis carinata* were collected from in and around the Mysore University Campus, spread over 739 acres of woody habitat (76.6° longitude, 12.3° latitude) in Mysuru district, Karnataka, India. The collection of lizards (5 lizards per season) was carried out during breeding (October-December) and non-breeding (January–July) seasons of the reproductive cycle[23]. The lizards were sacrificed immediately after their capture by euthanizing them through exposure to ether, a procedure approved by the Institutional Animal Ethics Committee (IAEC) of the University of Mysore and CPCSEA for

handling and sacrifice of animals (No.MGZ/455/2005-06 dated March 31, 2006). Blood was drawn and cold centrifuged (−4°C) at 4,000 xg for 10 min and serum was separated for the determination of testosterone levels by ELISA using a testosterone kit (DRG International, Germany, Product No.: EIA-1559). The epididymis was dissected out and used for both light and electron microscopic examinations.

The epididymis of one side was used for Electron Microscopy (EM), and the other for light microscopy. For EM studies tissues were fixed in Karnovosky's reagent for 24 hours and then in 1% osmium tetroxide at 4°C for 2 hours (post-fixation). After rinsing in phosphate buffer (0.1 M, pH 7.4) for 15 minutes, tissues were dehydrated through a graded series of ethanol. The epididymis was then en-bloc stained in uranyl acetate (2% in 95% ethanol) for 90 minutes at 2-3 °C. Staining was performed after passing the sections through 90% ethanol and before reaching absolute ethanol during the dehydration process. Propylene oxide was used as a clearing agent. The tissues were then transferred to a mixture of propylene oxide and araldite and placed in a rotator overnight. The materials were subsequently placed in epoxy resin at room temperature for 4 hours and then embedded in Araldite. Araldite was allowed to polymerize for about 48 hours at about 60°C. Semithin (1µm) and ultra-thin (600-700 A°) sections were cut using glass knives. Semithin sections were stained with toluidine blue whereas ultrathin sections were picked up on a copper grid, contrasted with uranyl acetate and lead citrate, and viewed in a transmission electron microscope (JEOL-JEM-100CX II) at 60 kV.

For the light microscopic study, the epididymis was fixed in Bouin's fluid, embedded in paraffin wax, and 5 µm thick serial sections were cut and stained with hematoxylin and eosin. Serial sections were used to measure the tubule diameter and epithelial cell height using an ocular micrometer calibrated with a stage micrometer.

Morphometric data were analyzed using a two-way analysis of variance to test the influence of seasons and regions on tubule diameter and epithelial cell height. One-way ANOVA was conducted for those data, where there was a significant interaction between seasons and regions as judged by two-way ANOVA. Further, ANOVA was followed by Duncan's Multiple Range Test (DMRT). Significant differences between the mean values of breeding and non-breeding seasons of each parameter were compared by Student's *t*-test.

3. Results

3.1 Anaomical and Histological Differentiation of Epididymis

The epididymis of *Eutropis carinata* is a highly convoluted duct surrounded by connective tissue about four centimeters in length during the breeding season. The ductus epididymidis of this lizard is differentiated along its length into four distinct regions viz., a transparent initial segment (Eds), an anterior epididymis, a broad middle epididymis, and a narrow posterior epididymis are identified (Figure 1), based on gross morphology, morphometry, and histology. The initial segment, Efferent ductules (Eds), and luminal content show sparse sperm and secretory granules (Figure 1A). The ductus epididymidis has a central lumen immediately surrounded by a single layer of columnar epithelium resting on a basement membrane followed by four to five layers of circular smooth muscles and a layer of connective tissue. The tubules of the anterior epididymis are small and lined with high columnar epithelium and their lumen contains abundant spermatozoa (Figure 1A). The tubules of the

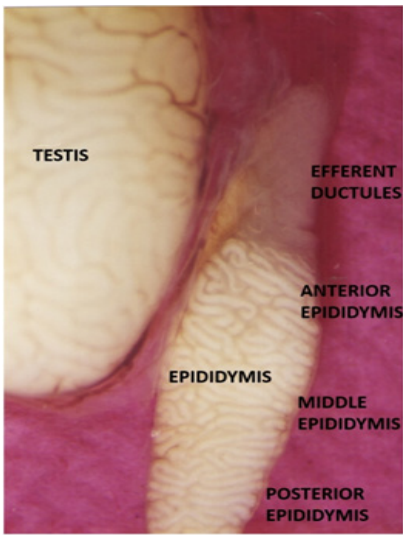


Figure 1. Photomicrograph of the testis and epididymis of the lizard *Eutropis carinata* during the breeding season. Note the anterior whitish efferent ductules, and the three regions of the epididymis the anterior, middle, and posterior.

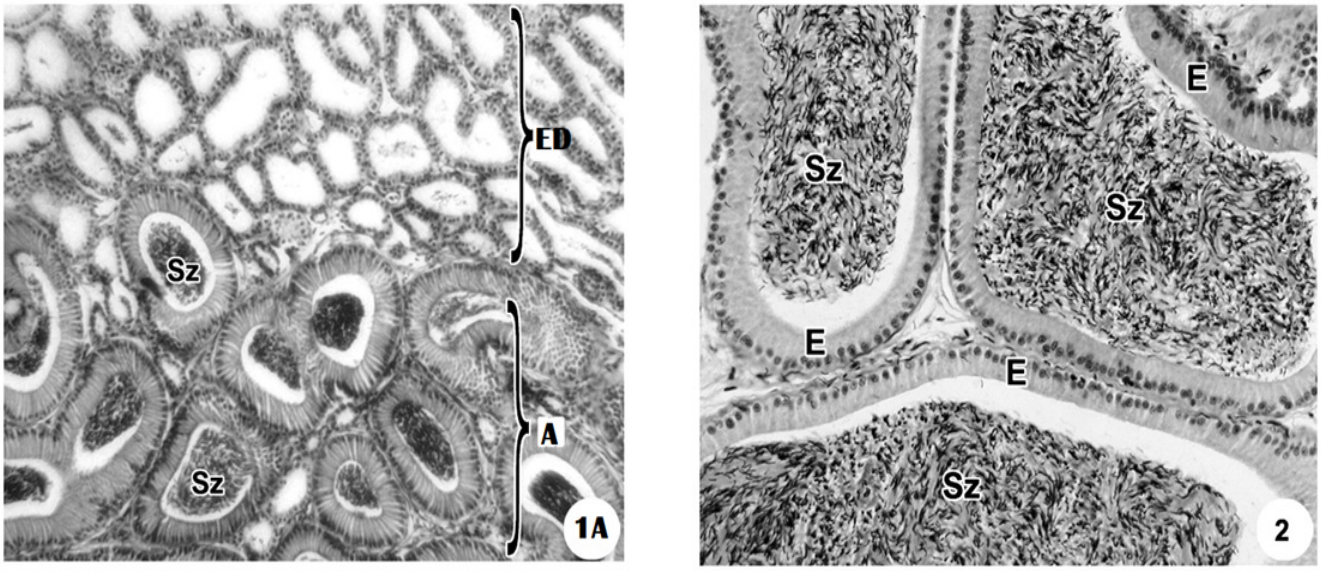


Figure 2. Photomicrographs of histological sections of the epididymis during the breeding season in *Eutropis carinata*. **1A.** Paraffin section of epididymis showing the initial segment with the extra-testicular Efferent Ductules (ED) followed by the caput, the anterior (A) region, during the breeding season. Copious spermatozoa (Sz) and tall columnar epithelium are seen in the anterior region of the epididymis (x200). **2.** Cross section of the posterior region of the epididymis showing large tubules with low columnar Epithelium (E), and densely packed spermatozoa (Sz) in the lumen (x400).

middle epididymis are larger compared to the anterior epididymis, lined with high columnar epithelium and their lumen consists dense spermatozoa. Although the posterior epididymis is narrow compared to the middle epididymis in its gross morphology, the tubules are larger than those of the middle epididymis and are lined with low columnar cells. Their lumen is filled with dense spermatozoa fully packed without any space between the epithelium and the lumen (Figure 2).

During the nonbreeding phase of the reproductive cycle in the lizard *Eutropis carinata*, there is a decrease in reproductive activity, which is reflected in the morphology of the reproductive organs. Specifically, there is quiescence in the reproductive organs. In the epididymis, which is a structure where sperm mature and are stored, there is an overall reduction in the size of all three regions (Figures 2A to 2C). Additionally, the secretory epithelium, which produces the material that nourishes and protects the sperm, is also absent. The epithelium is pseudostratified and the lumen, which is the central space within the epididymal tubules, is empty. There are some degenerating spermatozoa from the previous breeding season present in some of the tubules. There is also a significant reduction in the diameter of the tubules in all three regions of the epididymis when compared to the breeding season, and most of the space is occupied by connective tissues (Figures 2A to 2C).

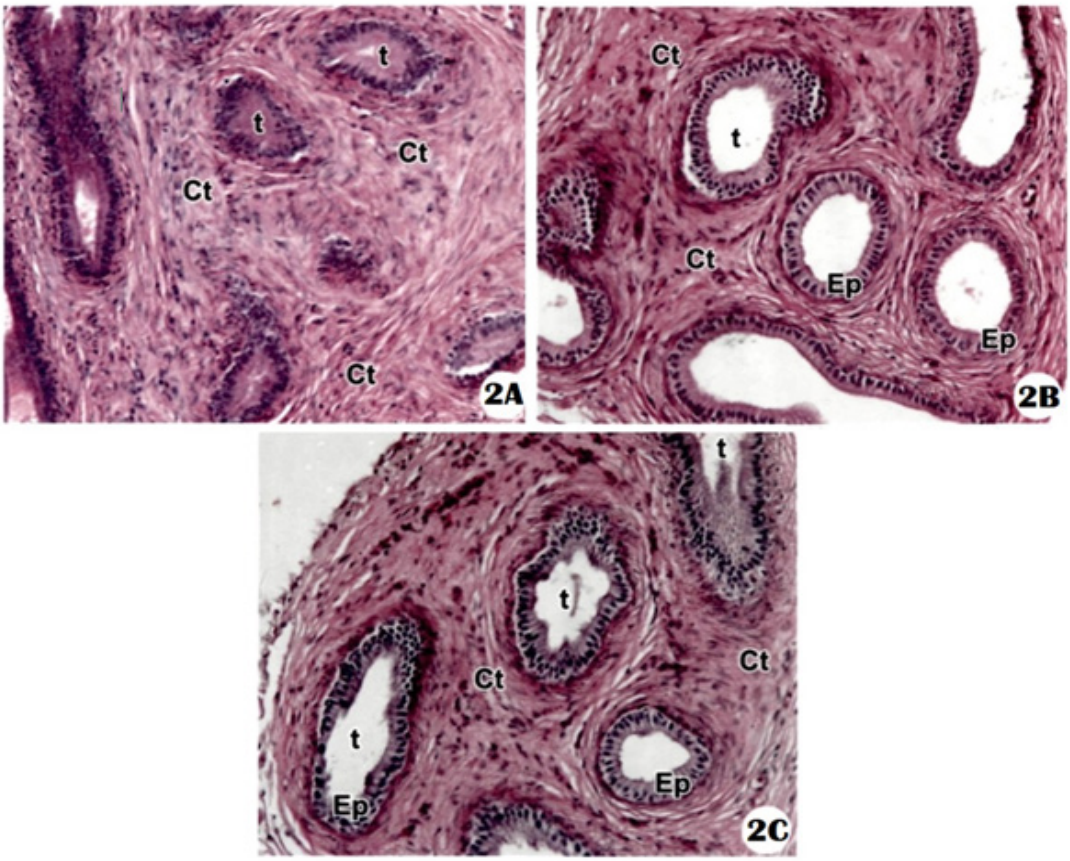
3.2 Tubule Diameter and Epithelial Cell Height (Morphometry)

Tubule diameter and epithelial cell height in different regions of the epididymis during the breeding and non-breeding seasons are shown in Table 1. Three different regions are identified in the epididymis based on gross morphology, histomorphology, and morphometry. During the breeding season, the anterior epididymis showed minimum tubule diameter followed by an increase in the middle epididymis and a maximum in the posterior epididymis (anterior < middle < posterior). A similar pattern was seen during the non-breeding season too wherein there was a significant reduction in the tubule diameter of all three regions when compared to that of respective regions during the breeding season (Figure 2A to 2C). Epithelial cell height also showed significant variation in three different regions. During the breeding season, the epithelial cell height in the

Table 1. Tubule diameter and epithelial cell height of the epididymis of the lizard *Eutropis carinata* during breeding and non-breeding seasons of the reproductive cycle

	Mean tubule diameter (µm ± SE)		**Mean epithelial cell height (µm ± SE)**	
	Breeding season	**Non-breeding season**	**Breeding season**	**Non-breeding season**
Anterior epididymis	79.14 ± 1.4[a]	30.26 ± 1.5* [a]	18.20 ± 0.2[a]	9.7 ± 0.9* [a]
Middle epididymis	177.32 ± 3.9[b]	88.12 ± 2.2* [b]	29.12 ± 0.1[b]	11.52 ± 0.9* [b]
Posterior epididymis	200.22 ± 2.6[c]	117.01 ± 4.5* [c]	12.08 ± 1.1[c]	5.22 ± 0.8* [c]
One way ANOVA F-Value	595504.32 (p<0.01)	486473.19 (p<0.01)	21067.704 (p<0.01)	1171.178 (p<0.01)

Note: Significance is shown by an asterisk for the Student's t-test, and by superscript letters for DMRT. *Significantly different (p<0.01) compared to the breeding season as judged by Student's t-test (rows). DMRT: Mean values with different superscript letters in a column are significantly different (p<0.01).



Figures 2A, 2B, and 2C. Photomicrographs of hematoxylin and eosin-stained histological sections of the epididymis of the lizard *E. carinata* during the non-breeding season. **2A.** Transverse section of the anterior region of the epididymis showing regressed tubules (t) and increased inter-tubular connective tissue (Ct). x200. **2B.** Transverse section of the middle region showing regressed tubules (t), increased intertubular connective tissue (Ct), and regressed epithelium (Ep). x200. **2C.** Transverse section of the posterior region showing regressed tubules (t), increased inter-tubular connective tissue (Ct), and regressed epithelium (Ep). x200.

posterior epididymis is the least followed by the anterior region, and maximum in the middle region (middle < anterior < posterior). There is a significant reduction in epithelial cell height of each region in the non-breeding season compared to that of respective regions of the breeding season. Two-way ANOVA revealed a significant interaction between the regions and seasons on the tubule diameter ($F = 72.941$, $P<0.01$) and epithelial cell height ($F = 954$, $p<0.01$).

3.3 Serum Testosterone Levels

The levels of serum testosterone during different seasons of the reproductive cycle of *E. carinata* were significantly different. The serum testosterone level was high during the breeding season compared to the non-breeding season. During the breeding season, the serum testosterone concentration was 18.0 ± **1.7** ng/mL and during the non-breeding season, the circulating serum testosterone concentration was 3.0 ± **0.2** ng/mL.

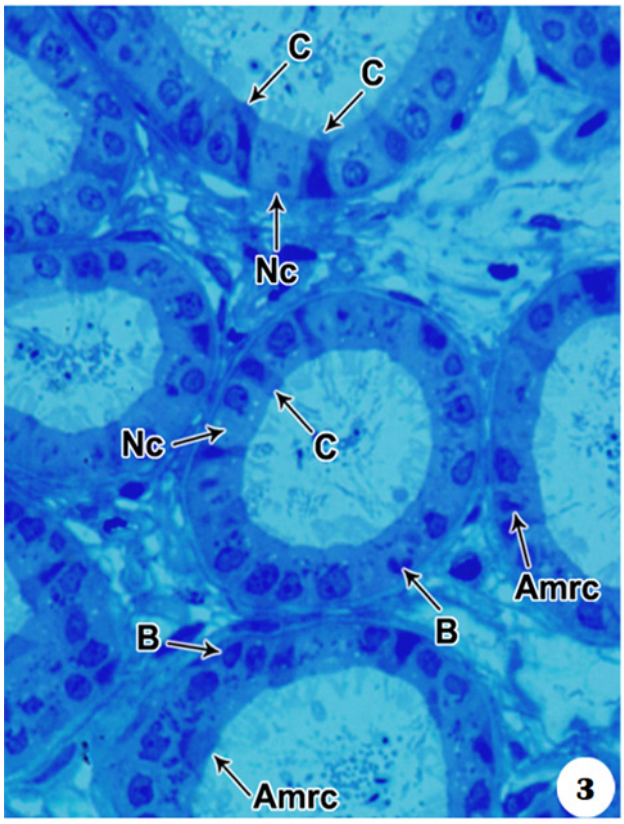


Figure 3. Toluidine blue-stained semithin section of the initial segment (efferent ductules) of the epididymis during breeding season showing ciliated cells (CCs), Non-Ciliated Cells (NCCs), basal cells (B), and Apical Mitochondria-Rich Cell (AMRC), (x100).

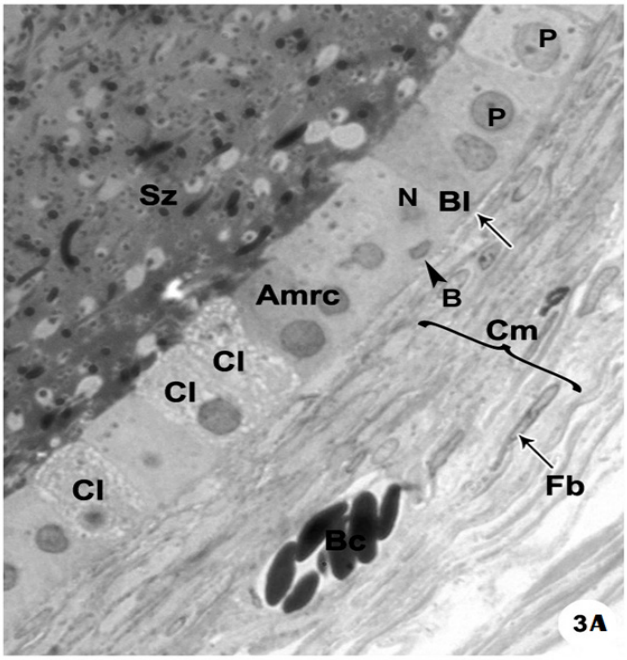


Figure 3A. Semithin section of the posterior region of the epididymis during breeding season showing highly vacuolated clear cells (Cl), principal cells (P), basal cell (B), narrow cell (N), Apical Mitochondria-Rrich Cell (AMRC), basal lamina (Bl), blood capillaries showing blood cells (Bc), smooth circular muscles (Cm), fibroblast (Fb) and lumen containing densely packed spermatozoa (Sz) (x1000).

3.4 Ultrastructural Organization of Epithelium of Epididymis

The epithelial lining of the epididymal tubule is pseudostratified. Epithelial cells rest on the basal lamina. There is no epithelial folding projecting into the lumen. The epithelium is surrounded by four to five layers of smooth circular muscles (Figure 3A). The muscularis is in turn surrounded by a connective tissue layer made up of collagen and fibroblasts. Numerous blood vessels are seen lying between the muscle and connective tissue. Based on cell and nuclear morphology, and ultrastructural features, four different cell types *viz.*, ciliated, non-ciliated, basal, and apical mitochondria-rich cells are identified in the initial segment, the efferent ductules (Eds) of the epididymis (Figure 3). In the anterior, middle, and

posterior regions of the epithelium five different types of cells *viz.,* principal, basal, AMRC, clear, and narrow cells are identified (Figure 3A).

3.5 Cell Types Lining the Initial Segment (The Efferent Ductules)

Four different cell types viz., ciliated, non-ciliated, basal, and apical mitochondria-rich cells are identified in the initial segment, the Efferent ductules (Eds) of the epididymis (Figure 3).

3.5.1 Ciliated Cells (Figure 4)

These cells have long cilia in their apical plasma membrane. The nucleus is horizontally elongated without a smooth surface and is present in the basal region of the cell. These cells are dark and narrow. Spherical-shaped mitochondria are present in the apical region of the cell. Numerous electron-dense bodies are distributed throughout the cytoplasm. Well-developed Golgi bodies, rough endoplasmic reticulum (rER), and secretory granules are also seen in the cytoplasm. Lateral membranes are interdigitated with adjacent cells to form intercellular gaps. During the non-breeding season, these cells appeared dark like in the breeding season (Figure 5). A few long cilia were present in the apical membrane facing the lumen. The cytoplasm contained elongated and indented nuclei and sparse cell organelles. Numerous electron-dense bodies were distributed throughout the cytoplasm. Lateral membranes did not interdigitate between adjacent cells.

3.5.2 Non-ciliated Cells (Figure 4)

These cells are broader than the ciliated cells and have numerous long microvilli in the apical plasma membrane facing the lumen. The apical membrane possesses coated pits. A spherical nucleus and numerous elongated mitochondria are present in the basal region of the cell. The apical region of the cell contains many multivesicular bodies. Secretory granules and electron-dense bodies are

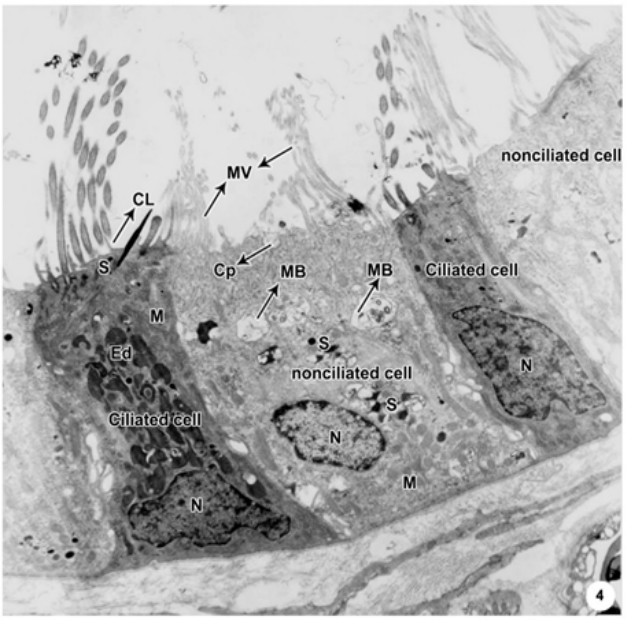


Figure 4. Transmission Electron Micrographs (TEM) through the initial segment during breeding season showing ciliated and non-ciliated cells. The darkly staining ciliated cells show long cilia (CL), flattened nuclei (N), mitochondria (M), secretory granules (S), and electron-dense bodies (Ed). The lightly stained non-ciliated cells show microvilli (MV) in the apical region, round nucleus (N), Multivesicular Bodies (MB), mitochondria (M), and secretory granules (S) (x2700).

Figure 5. TEM through the initial segment during the non-breeding season showing ciliated and non-ciliated cells. Cells show regression with highly indented narrow nucleus (N), ciliated cells with fewer cilia (Cl), and non-ciliated cells with a few apical microvilli (Mv), and cytoplasm lacking cell organelles are noted (x5000).

present in the cytoplasm. These cells are larger than the other cell types in the initial segment. Some non-ciliated cells possess large vacuoles at the apical cytoplasm (Figure 6). During the non-breeding season, these cells appeared broad and pale. They have numerous long microvilli in the apical plasma membrane facing the lumen. The basally located nucleus is irregular in shape and slightly elongated when compared to the breeding season. Mitochondria are small, round, and reduced in number. Multivesicular bodies are absent but a few large vesicles are still present. Secretory granules are completely absent (Figure 5).

3.5.3 Apical Mitochondria-rich Cells (AMRC) (Figure 6)

These cells are not confined to the apical region of the epithelium but their cytoplasm extended from the basal lamina. The apical region is broad while the middle and basal regions are narrow. They are more in number in the initial segment of the epididymis and their number declines towards the posterior region. The cytoplasm is electron-dense; hence, cells appear dark. The apical plasma membrane shows sparse microvilli and cilia. Numerous mitochondria are present throughout the cytoplasm of the cell. The nucleus is irregular in shape with a highly indented surface and is present at the base of the cell. Coated vesicles are present in the ad-luminal cytoplasm and other cell organelles are sparse. The lateral plasma membrane is smooth while the basal membrane is highly folded. During the non-breeding season, there is no drastic change in the shape of the cells (Figure 7). A few very short cilia are present facing the lumen whereas microvilli are absent. The nucleus appears elongated and occupies three fourth of the entire cytoplasm of the cell from the basal to the apical region. The ad-luminal cytoplasm contains a few electron-dense bodies and the entire cytoplasm is electron-dense. The coated vesicles are absent and the basal membrane is folded like that seen during the breeding season.

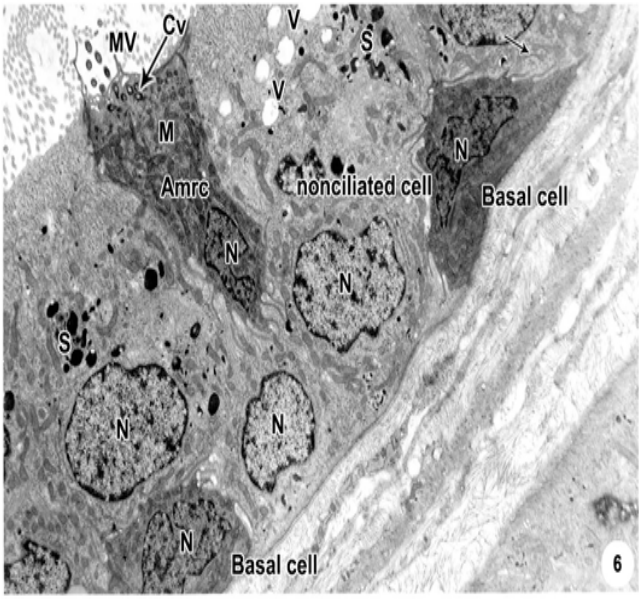


Figure 6. Apical Mitochondria-Rich Cells (AMRC) and basal cells during the breeding season. TEM through the initial segment of the epididymis during breeding season shows apical mitochondria-rich cells (Amrc) with abundant mitochondria (M), indented nucleus (N), coated vesicles (Cv), and basal cell showing pyramidal nucleus (N) and apical membrane with inter-digitating folds. Note vacuoles (V) and secretory granules (S) in non-ciliated cells (x2700).

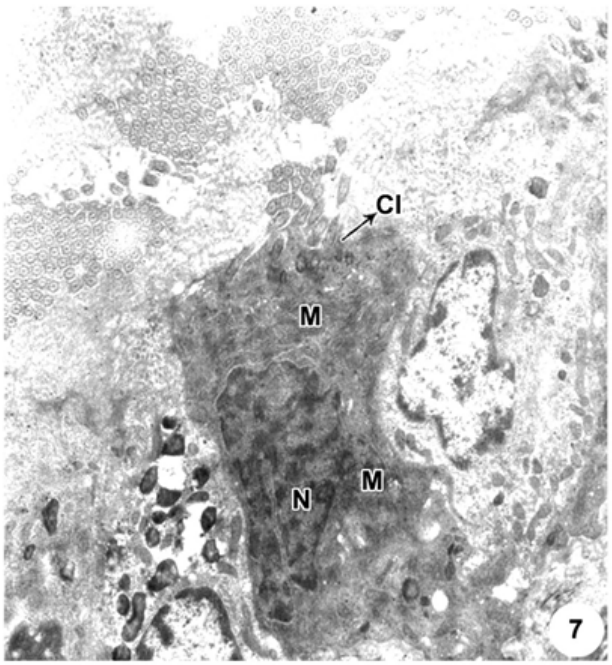


Figure 7. TEM through the initial segment of the epididymis during the non-breeding season shows a regressed apical mitochondria-rich cell with a small mitochondrion (M), highly indented nucleus (N), and apical portion showing a few cilia (Cl) (x6000).

3.5.4 Basal Cells (Figure 6)

These cells are the next abundant cell type found in the basal region of the epithelium. Basal cells are small and dark, pyramidal with indented and triangular-shaped nuclei. The nucleus is surrounded by numerous mitochondria and with small Golgi apparatus and a few rough endoplasmic reticulum (rER) strands. The apical membrane of these cells interdigitated with the basal membrane of the other overlying cells. During the non-breeding season, the cells are found attached to the basal lamina with a highly folded basal plasma membrane (Figure 8). The nucleus of the cells is highly indented forming an irregular shape. The cytoplasm is scanty and major cell organelles are absent.

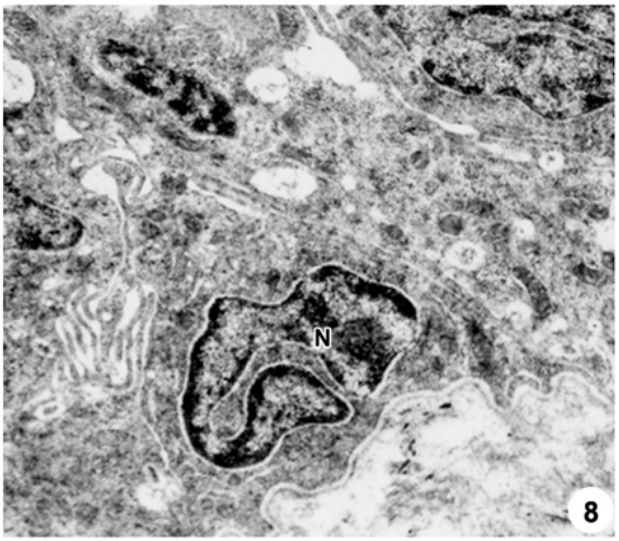


Figure 8. TEM through the initial segment of the epididymis during the non-breeding season showing regressed basal cells with a highly indented nucleus (N), cytoplasm lacking cell organelles and wavy basal plasma membrane (x6000).

3.6 Epithelial Cell Types of the Anterior, Middle, and Posterior Regions of the Epididymis

There are five different cell types in the anterior, middle, and posterior regions of the epididymis of which basal cells and AMRC are common with the initial segment whereas, principal, clear, and narrow cells are new to these regions (Figure 3A).

3.6.1 Principal Cells

These cells (Figure 9) are the major cell type found in the epithelium. They are tall columnar in the anterior and middle regions and low columnar in the posterior region of the epididymis extending from the basal lamina to the lumen. They have basally situated round or oval-shaped nuclei with smooth surfaces and prominent nucleolus. The adjacent cells are anchored by tight junctions at the apical region. In the supranuclear region, principal cells have numerous well-developed Golgi complexes, several mitochondria, and predominantly rER. Numerous small and large secretory granules are distributed in the entire cytoplasm of the cell. Two types of granules are found; some are surrounded by vesicles and others are without vesicles. Congregations of secretory granules are also found below the nucleus and apical cytoplasm. Small and large vacuoles/vesicles are present throughout the cytoplasm.

During the non-breeding season, these cells are reduced in height and their nuclei are deeply indented (Figure 10). Junctional complexes are absent, and sparse and very short microvilli are present at the apical membrane facing the lumen. Numerous vesicles are present in the entire cytoplasm. A few elongated and round mitochondria are seen in the basal region of the cells and other cell organelles are not prominent. Noticeably, cells lack secretory granules. The lateral plasma membranes of the adjacent cells interdigitate forming numerous intercellular lacunae. The basal region of the cells shows a wavy plasma membrane.

3.6.2 Basal Cells and Mitochondria-rich Cells (AMRC)

These cells are like those found in the initial segment of the epididymis during the breeding and non-breeding seasons of the reproductive cycle. They show similar ultrastructural cellular characteristics. However, the number of mitochondria-rich cells declined in the three regions of the epididymis compared to the initial segment. The basal cells of the initial segment have darkly stained cytoplasm (Figure 6) whereas, in the anterior, middle, and posterior epididymis, they are pale-stained. They are confined to the region closer to the basal lamina. The principal cells, narrow cells, AMRC, and clear cells spread to lie on top of the basal cells.

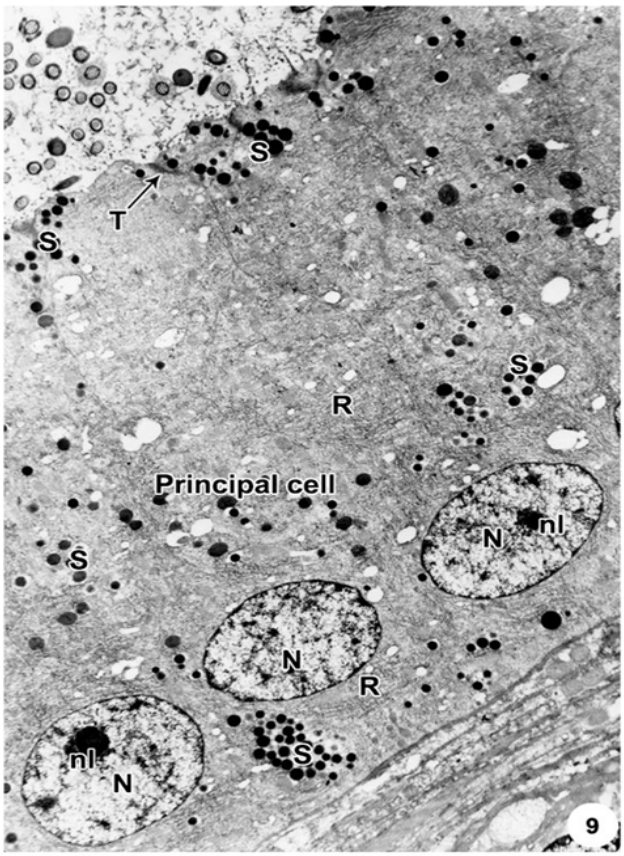


Figure 9. TEM of principal cells of the middle region of the epididymis during the breeding season showing an oval nucleus (N) with evenly distributed karyoplasm and a prominent nucleolus (nl), rough endoplasmic reticulum (R), and abundant secretory granules congregations (S). Note the tight junctions (T) at the apical portion (x2000).

3.6.3 Clear Cells

The clear cells are found in the anterior, middle, and posterior regions and are absent in the initial segment (Figure 11). They are generally found in between the principal cells with distinct ovoid nuclei placed slightly above the basal position. The cellular features of these cells include extensive supranuclear vacuoles and endocytosed secretory granules at the apical portion (inset figure) and basal region of the cell. Large clear vacuoles with distinct membranes are present in the basal region. Their frequency increases in the posterior region of the epididymis. During the non-breeding season, these cells show a basally situated small nucleus with deep

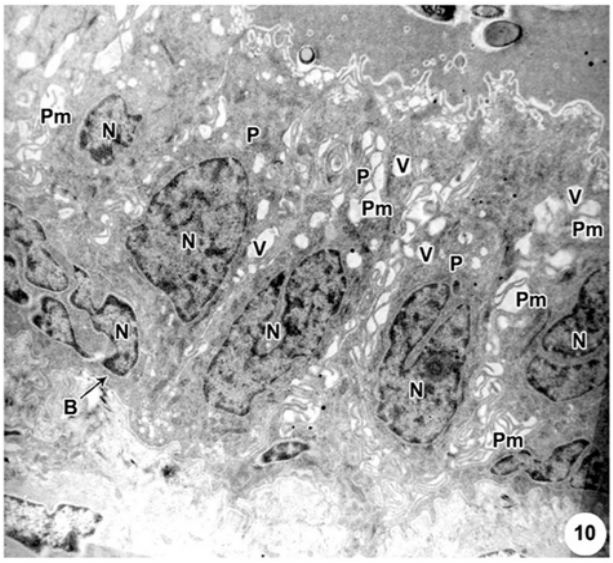
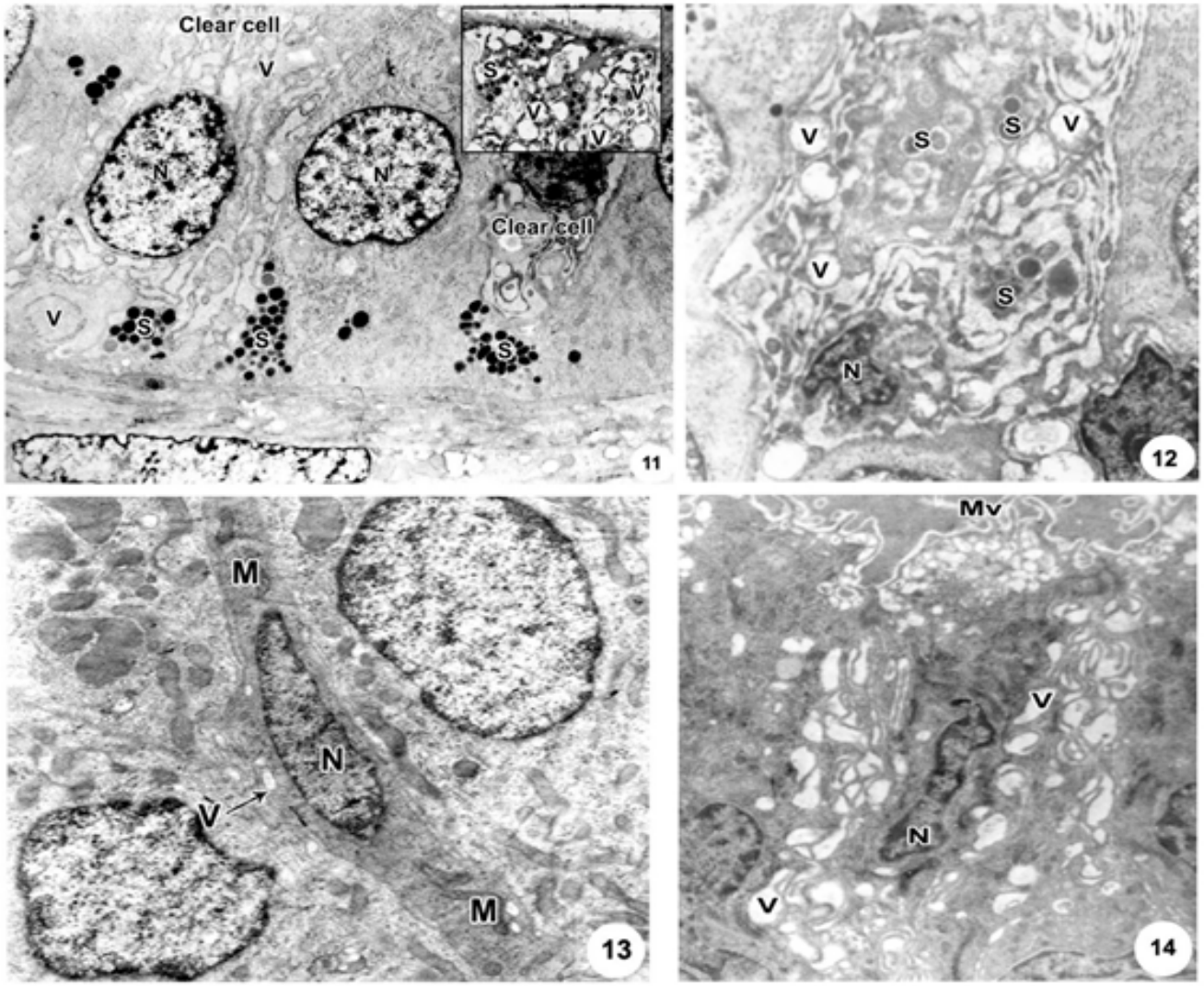


Figure 10. TEM of the middle region of the epididymis during the non-breeding season showing principal cells and basal cells. Regression of cells is noted; plasma membranes interdigitate (Pm); nuclei (N) are highly indented, and cytoplasm lacks major cell organelles in principal cell (P) and basal cell (B). Note the numerous vacuoles (V) in the cytoplasm and cytoplasm of the cells devoid of any secretory granules (x4000).

indentations. The cytoplasm shows numerous empty vesicles and a few granules. The entire cytoplasm shows many membrane-bound vesicles of different sizes and shapes (Figure 12).

3.6.4 Narrow Cells

Narrow cells are the fifth type of cells that are found with less incidence (Figure 13). These cells are columnar and thin and possess a small elongated or oval nucleus with evenly distributed karyoplasm in contrast to the larger spherical nuclei of other cell types. The cytoplasm of the narrow cells contains a few cell organelles like elongated mitochondria, vesicles, and rER without any secretory granules in the cytoplasm. At the apical portion plasma membrane of these cells shows a few microvilli extending into the lumen. During the non-breeding season, narrow cells are columnar and thin (Figure 14). They possess a few microvilli on the apical plasma membrane and at the ad-luminal cytoplasm, numerous vesicles are



Figures 11 to 14. Clear cell and narrow cell during breeding and non-breeding seasons. **11.** TEM of clear cell showing basally located large round nucleus (N), cytoplasm with many vacuoles (V) (x3000), and in the apical and basal portions ingested granules (S) (inset picture) (x700). **12.** TEM through the posterior region of the epididymis during the non-breeding season showing regressed clear cells with a small regressed nucleus (N). Note many vacuoles (V) and a few granules (S) in the cytoplasm (x6000). **13.** TEM through the posterior region of the epididymis showing narrow cells during the breeding season. Cells show basally situated elongated nucleus (N) and cytoplasm with sparsely distributed elongated mitochondria (M) and vacuoles (V) (x3000). **14.** TEM of the posterior region of the epididymis during the non-breeding season showing regressed narrow cells with a highly indented nucleus (N) and many vacuoles (V) in the cytoplasm and a few small microvilli (Mv) in the apical portion of the cell (x6000).

present. The nucleus is elongated and indented with a few elongated mitochondria, and the lateral and basal plasma membranes are highly interdigitated, forming numerous intercellular lacunae.

4. Discussion

Unlike mammals, in the reptilian epididymis, there is no clear-cut demarcation dividing the epididymis into anatomical segments such as head, body, and tail as

noted in primitive mammals[24]. However, the reptilian epididymis is not simply a spermatozoa storage organ but it is a highly dynamic organ secreting constituents of the seminal plasma[9,10,13,17,19,21,25-30]. Major changes that occur during epididymal maturation in mammalian spermatozoa are the ability to move, recognize and bind to the zona pellucida, and to fuse with the plasma membrane of the oocyte[31]. Although epididymis has appeared for the first time in reptiles among vertebrates, there is not much understanding of spermatozoa maturation and the role of accessory gland secretions in this process in reptiles except for a few reports[21,25,27,29]. The first fine-structure analysis of reptilian epididymis was that of Mesure, *et al.,* in the lizard *Lacerta vivipara*, in which four regions, caput, corpus, proximal cauda, and distal cauda, with only small differences from the head to the distal corpus were distinguished[19]. Later Akbarsha, *et al.,* reported a detailed study on histological variation along and ultrastructural organization of the epithelium of the ductus epididymidis of the fan-throated lizard *Sitana ponticeriana*[21]. Spermatozoa from the epididymis of the Chinese soft-shelled turtle *Pelodiscus sinensis* were studied to understand the mechanism of sperm storage and was found that epididymis does play a role in the storage of spermatozoa[32,33]. Comparative studies on epididymis of amniotes reveal similarities and differences in its structure and functions and its role in spermatozoa maturation which is necessary to understand the evolution of the spermatozoa maturation process in the vertebrates.

In the present investigation, in the lizard *Eutropis carinata*is the epididymis is divided into four regions viz., an initial segment Efferent ductules (Eds) (extra testicular) and three regions, the anterior region, broad middle, and a narrow posterior regions comparable to caput, corpus, and cauda epididymidis of mammals based on tubule diameter, epithelial cell height, and histology of the epididymis. These regions are identified as similar to observations carried out in the lizards *Sitana ponticeriana*[21]. The presence of three regions comparable to caput, corpus, and cauda is also reported in many species of reptiles *H. flaviviridis, C. versicolor*, *L. vivipara*, and *Phrynops geoffroanus* (Testudines: Chelidae)[8,10,13,14,20]. The epididymal tubules of *H. flaviviridis* and *C. versicolor* in the posterior region show the largest diameter and are filled with spermatozoa during breeding season[4,10]. However, in the turtle, *C. picta* spermatozoa are found in the epididymis throughout the year[17]. The epididymis in *P. geoffroanus* is a simple, long, and highly convoluted tubule that receives efferent ductules throughout its extension. It is covered by a pseudostratified columnar epithelium with three cellular types: the principal cells, which are the most abundant followed by basal cells, and a small narrow cell[20]. In the present investigation also like the overall reported trend in the tubule diameter and epithelial cell height of the three regions of epididymis was seen[8,10,15,21]. There is an increase in the diameter of the tubules from the anterior region to the posterior region and a decrease in the height of the epithelium of the posterior region compared to the middle (maximum height) and anterior region. During the non-breeding season also a similar pattern is observed with a significant reduction in tubule diameter and epithelial cell height.

An earlier preliminary study on the lizard *E. carinata* described the epididymis as a simple structure without demarcation into different regions, composed of contorted tubules lined with a single layer of epithelial cells and the lumen filled with spermatozoa during the breeding season[23,34]. Further, the observation of the ultrastructural cellular features of the anterior and posterior parts of the epididymis of *E. carinata* by Sarkar and Shivanandappa[23] showed secretory granules and stereocilia. However, the present study in *E. carinata* reveals different regions in the epididymis based on gross morphology and histological organization. These are the initial segment, the efferent ductules (Eds), and the anterior, middle, and posterior regions of the epididymis based on their position as well as statistically significant differences in tubule diameter (posterior > middle > anterior) and epithelial cell height (middle > anterior > posterior). In addition, the epithelium is columnar in the anterior region and high columnar in the middle region with abundant spermatozoa and low columnar with densely packed spermatozoa in the posterior region of the epididymis during the breeding season. The presence of histologically defined regions in the epididymis of *E. carinata* is similar to that reported in the lizard *H. flaviviridis*, *C. versicolor*, *L. vivipara*, *P. dorsalis* and *S. ponticeriana*[8,10,14-15,21]. In the lizard *E. carinata*, the anterior portion of the epididymis is occupied by the efferent ductules (Eds), and the ductus epididymidis originates only at a distance posteriorly where the ductules are found among the coiled ductus epididymidis, which is the case in the crocodile[16], turtle[17], fan-throated lizard[21], birds[35,36] and human[37].

The terminology used for the cell types of the epididymal epithelium in *E. carinata* is based on a comparison of the ultrastructural features of these cells with those of the mammalian epididymis. In the initial segment of the epididymis, four different cell types are identified based on the ultrastructural features of the cells *viz.*, ciliated, non-ciliated, basal, and apical mitochondria-rich cells (AMRC). Cells with long cilia extending into the lumen of the tubules with the basally situated elongated nucleus are identified as ciliated cells in *E. carinata*. The presence of mitochondria, well-developed Golgi bodies, rER, and secretory granules indicate the secretory activity of these cells. These cells with the help of prominent long cilia might aid in the movement of luminal fluid and spermatozoa, which are pushed from the initial segment EDs to the next region by the ciliary action of these cells. Cells with similar ultrastructural features are found in the lizard *S. ponticeriana*[21] and the epididymis of mammals too[38].

Cells with numerous long microvilli in the apical plasma membrane projecting into the lumen and appearing light are identified as Non-Ciliated Cells (NCCs). Similar cells in mammals are thought to play a role in the absorption of water and ions which is the main function of the epithelial cells of the initial segment of the epididymis, where the concentration of spermatozoa is increased by fluid absorption[39]. The presence of microvilli, coated pits, vesicles, multivesicular bodies, and electron-dense bodies in the cytoplasm of these cells in *E. carinata* further supports the ingestion activity of these cells. The secretory role of NCCs is indicated by the presence of secretory granules in the cytoplasm. Ciliated and NCCs are the major types of cells found in the initial segment of the epididymis of *E. carinata* and similar outcomes are reported in the lizard *S. ponticeriana*[21], the crocodile *C. crocodilus*[16] and the turtle *C. picta*[17] and many mammals such as guinea pigs, rabbits, and rats[40-43]. The major role of the NCCs of the ductules of the homeotherms, in general, as inferred from ultrastructural analyses supported by tracer studies is endocytosis of fluid and particulate material from the lumen through fluid-phase and adsorptive processes. Since the NCCs of the lizard (this study), other reptile studies of EDs match in ultrastructural organization those in birds and mammals, particularly with reference to the endocytic apparatus, it could be inferred that the NCCs of the epithelium of the EDs of this lizard would participate in endocytosis. And so, provide for the reabsorption of fluid, proteins, etc., and uptake of particulate material, to modify the concentration and composition of the fluid arriving at the ductus epididymidis.

Pyramidal cells with triangular and indented nuclei found in the basal region lying flat on the basal lamina are identified as basal cells. These are also the next abundant cell type after principal cells in the anterior, middle, and posterior regions of epididymis. Basal cells, a constant feature of the mammalian epididymis, is also found in the present report and previous reports with respect to lizards *L. vivipara*[19], *P. sicula*, and *S. ponticeriana*[21], and turtle *C. picta*[17]. The basal cells are confined to the basal aspect of the epithelium and never reach the lumen. These cells contain a few organelles, like mitochondria, Golgi complex, and a few rER, and are electron-dense. These ultrastructural characters reveal that these cells may not be actively involved in secretory activity. Because of their basal location, one would expect them to be mitotically active (previously thought to be stem cells) but in the present study as well as in other studies mitotic figures are rarely encountered in these cells[21,43-46]. It is reported that basal cells participate in the detoxification process or act as scavenger cells in the mammalian epididymis as well as in the epithelium of the vas deferens of the lizard *S. ponticeriana*[45,47-50]. Basal cells are attributed with a supportive role and immune surveillance of sperm and other testicular antigens[51,52].

Apical mitochondria-rich cells are more in number in the initial segment and anterior region of the epididymis and their number declines in the posterior epididymis region. This observation is comparable to that seen in mammals where they are numerous in the caput region and their number declines in the cauda region[43,53-54]. The presence of numerous mitochondria is an important characteristic of these cells. Similar cells are also found in the middle and posterior regions of the epididymis in *E. carinata* and other lizards in between the principal cells[21]. Even though the functional significance of these cells is still unclear, it has been suggested that they are involved in the reabsorption and acidification of the epididymal luminal fluid in mammals[54]. This view is supported by the presence of coated vesicles and microvilli in these cells in the present study and other studies. The aggregation of mitochondria in the apical cytoplasm suggests that they may play a role in the generation of ATP, which is

required for the transport of H^+ and Cl^- ions across the cell membrane[46].

The anterior, middle, and posterior regions of the epididymis in *E. carinata,* in contrast to the initial segment, shows five different types of cells. Principal cells are the major cell types found here, with numerous well-developed Golgi complexes, several mitochondria, and predominantly rER indicating active protein synthesis. Numerous small and large secretory granules distributed in the entire cytoplasm of the cell reveal the secretory nature of these cells. The study of the epididymis of *S. ponticeriana* reveals ultrastructural features of the principal cells of the ductus epididymis as responsible for the secretion of electron-dense biphasic granules[21]. However, in the present study two types of granules are found, some are surrounded by vesicles and others are without vesicles but not biphasic. These differences in secretory granules might indicate species differences that occur. The principal cells possess a prominent Golgi apparatus and all versions of endoplasmic reticulum, including rough, smooth, and sparsely granulated. Principal cells with similar ultrastructural secretory features are also noted in birds and mammals[35,44-46]. The presence of many membrane-bound vesicles containing a homogeneous electron-dense material in the basal cytoplasm of the principal cells is lipid droplets, and such lipid droplets have been reported in other reptiles and mammals[21,45]. The ultrastructural organization of the principal cells of mammals and reptiles is similar. The present study adds one more species of lizard, *E. carinata*, to the list of reptilian species whose epididymal secretion is in the form of discrete granules.

Cells with light staining properties and the presence of numerous large membrane-bound vesicles in the cytoplasm are identified as clear cells. The occurrence of clear cells in the reptilian epididymis was reported for the first time in the lizard *S. ponticeriana*[21] and in the present study they are noticed. Clear cells are well described in mammals[38,45]. Clear cells are also referred to as light cells, holocrine cells, pale cells, or foamy cells. These cells are absent in the initial segment of the epididymis and are present in the anterior, middle, and posterior regions of the epididymis of *E. carinata*. Their occurrence is most abundant in the posterior region. Extensive cytoplasmic vacuoles and endocytosed secretory granules in the cytoplasm suggest that they are involved in the absorption of substances from the epididymal lumen. The ultrastructural organization of the clear cells of the lizard epididymis with the presence of microvilli, the occurrence of endocytic vesicles, and the abundance of lysosomes provide evidence for a role for clear cells in endocytosis.

Cells with a small elongated nucleus in contrast to the larger spherical nucleus of other cell types and occurring less frequently are identified as narrow cells. Narrow cells with similar cellular features are found in turtles, lizards, rats, and other mammals[17,21,38,55]. Like the mammalian narrow cell, these cells in the present study are found distinguished from other cells by their ovoid nucleus, the presence of a few elongated mitochondria, and a few vesicles. However, they lack the C-shaped vesicles characteristic of mammalian narrow cells. These cells are thought to be involved in endocytosis. A significant reduction in the epididymal tubule diameter and epithelial cell height and shrunken tubules without spermatozoa was noted during the non-breeding season indicating idle epididymis. Parallel with these changes was quiescence in testicular activity and decreased serum testosterone level (3 ± 0.2 ng/mL). These observations indirectly indicate the dependence of the epididymis on the testis as found in other reptiles[8,10,12,23]. The serum hormone level study supports this observation where circulating serum testosterone level is significantly reduced during the non-breeding season compared to the breeding season, which shows high levels of circulating serum testosterone (18 ± 1.7 ng/mL).

It is interesting to note the presence of all the cell types in the regressed epididymis during the nonbreeding season as revealed by the ultrastructural observations in *E. carinata*. Further, all the cells showed regressive changes *viz.,* irregularly shaped nuclei with deeply indented surfaces, intense folding of lateral and basal plasma membranes forming intercellular lacunae, cytoplasm lacking all the major cell organelles of protein synthesis, and absence of secretory granules, rather than the absence of one or more cell types. Hence, seasonal changes in epididymal epithelial cells are changes in their secretory activity, rather than their absence from the epithelium during the nonbreeding season and re-appearance during the breeding season.

Ultrastructural studies on the epididymis in the lizard, *L. vivipara*[19], and the crocodile, *C. crocodilus*[16], revealed two cell types *viz.*, columnar secretory cells and basal cells in epididymal epithelium whereas, a similar study in the turtle, *C. picta*, revealed four kinds of cells *viz.,*

vesicular cells, basal cells, narrow cells, and mitochondria rich cells[17]. A preliminary ultrastructural study in the lizard *E. carinata* although it reported one cell type in the epididymal epithelium did not name the cell type and further noted secretory granules and stereocilia[23]. However, the light microscopic study by Meeran, *et al.,* identified seven cell types in the epididymal epithelium of *C. versicolor*[13]. The epithelium of the epididymis of the fan-throated lizard *S. ponticeriana* shows six different cell types, principal, narrow, apical, clear, basal, and intraepithelial leucocytes[21]. These earlier studies in reptiles, except the study in the lizard *S. ponticeriana,* neither focused on the regional distribution of different cell types nor are unequivocal regarding the number of cell types in the epididymal epithelium. The present study in *E. carinata* focuses on these aspects and reveals the regional difference in the distribution of cell types. It is evident from the present study that the initial segment of the epididymis of *E. carinata* contains ciliated, non-ciliated, basal, and apical mitochondria-rich cells, in contrast to the presence of the principal, basal, mitochondria-rich, clear, and narrow cells in the anterior, middle, and posterior regions of the epididymis. Basal cells and mitochondria-rich cells are common in all three epididymal regions, whereas others are specific to the initial segment or anterior, middle, and posterior regions. The presence of multiple cell types in the epithelium of the epididymis and the region-specific distribution of cell types is comparable to mammalian epididymis.

Earlier studies in *E. carinata* reported that non-motile testicular spermatozoa from the testis exhibited different patterns of motility when incubated with the luminal contents of different regions of the epididymis[29]. The epididymal luminal fluid of *E. carinata* from the reproductively active phase when subjected to size exclusion chromatography gel electrophoresis separation and purification methods combined with LC-MS/MS identified nine proteins including three enzymes and three heat shock proteins, and most of them were localized in the cytoplasm[48]. During the annual reproductive cycle, the epididymis of the lizard *E. carinata* undergoes dramatic changes, both morphologically and biochemically, that occur in a well-organized sequence. Enzymes, such as acid phosphatase, alkaline phosphatase, and α-glucosidase, are highly sensitive to seasonal changes and their activity parallels the production of epididymal proteins[29,56-58]. Beta-hexosaminidase (Hex) is the major lysosomal enzyme associated with the event of fertilization. The presence of Hex in the epididymis of the lizard *E. carinata* has been demonstrated[58]. The studies further indicated that Hex is released from the epididymal epithelium and binds to the spermatozoa and, in the lumen, it gradually increases from the anterior to the posterior region of the epididymis. The latter report also suggested that Hex A which binds to the epididymal spermatozoa originates from the epididymis[58]. The above studies on the epididymis of *E. carinata* implicate that the biochemical complexity of sperm activation observed is consistent with those reported in mammals. Hence, based on the present study and previous studies in *E. carinata,* it is suggested that modern reptiles have also achieved structural complexity in the epididymal epithelium comparable to mammals. However, the occurrence of different numbers of cell types in other reptiles might be due to species differences[16-17,19-21]. In conclusion, it can be said that post-testicular physiological maturation of spermatozoa is crucial for attaining the morphological and functional capabilities needed for successful fertilization and epididymal epithelium offers a favourable milieu for the same in all amniotes.

5. Conclusion

This light and transmission electron microscopic seasonal study in the lizard *E. carinata* (1) establishes histologically distinct zones in lizard ductus epididymidis comparable to the initial segment, caput, corpus, and cauda of the mammalian ductus epididymidis, (2) reports the occurrence in the epithelium of five different cell types during both breeding and non-breeding seasons, (3) provides ultrastructural evidence for secretion of two types of granules, some surrounded by vesicles and others without vesicles, from the principal cell, (4) shows evidence for well-developed endocytic activity in the posterior region, and (5) all the cell types are present throughout the breeding and non-breeding season of the reproductive cycle.

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