OBSERVATIONS

A. TESTICULAR MORPHOLOGY

In *Tylototriton verrucosus* the testes are multilobed. The lobes of the either side are in the form of linear chain and each lobe is connected with each other by slender cord of tissue. However, number of lobes differ with the age of the specimen. The first year specimens which were selected for the present study had single lobed individual testes. The older specimens (2nd year or 3rd year specimens) have more testicular lobes than in the first year specimens.

Each lobe is some what spherical or pear shaped and bears a groove in the middle. The branched efferent ductile system arises from the inner surface of the lobe to meet anterior nephric collecting tubules and ultimately empty into the Wolffian duct. The Wolffian duct runs along the lateral edge of the kidney to open into the cloaca. (Diagram - 2).

Each lobe is light cream in colour and fat bodies are present in the form of a yellow cord attached with the inner surface of the testis lobe along the antero lateral direction. (Plate -2, Fig. - A)

The size of the lobe varies seasonally. The maximum size has been observed during the breeding month (July to September), The size gradually decreases on the onset of winter and maximum reduction is observed in the January specimen. (Figure-1). Each lobe becomes compact and Yellow pigmentation appears within it. The rest of the lobe remains creamy in colour. These yellow pigmentation gradually recognized as a 'Yellow Zone' which gradually increases in the succeeding months and exhibit its maximum development during the month of March and April. The 'Yellow Zone' get resorbed on and from the month of May and only a trace is found in the specimens which were sacrificed during the breeding months. 'Yellow Zone' reappears distinctly during the next winter (January to February). (Plate - 2).

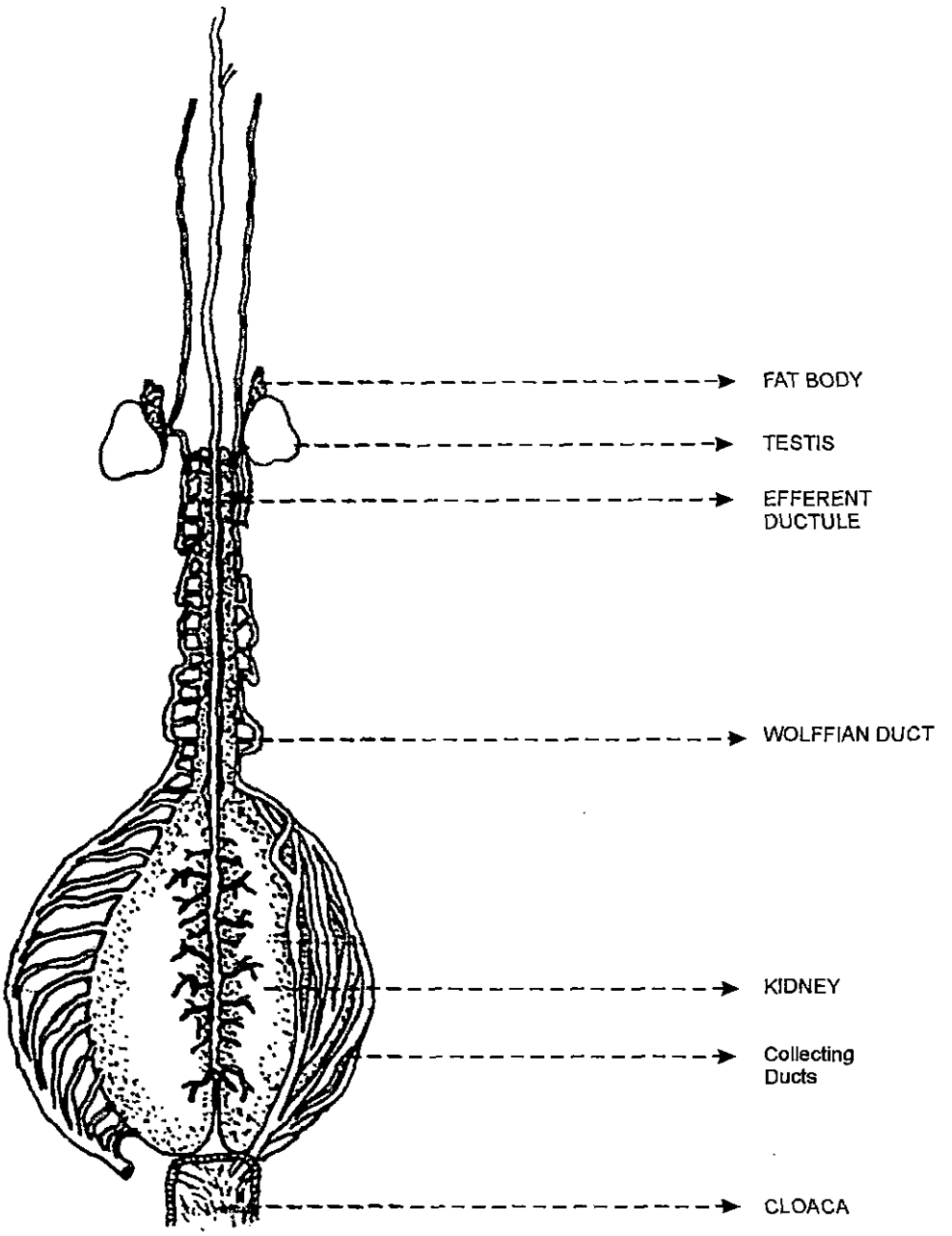
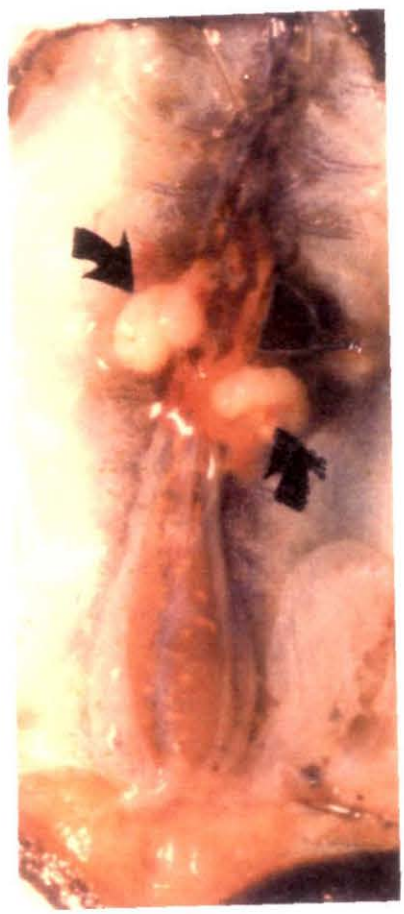


DIAGRAM - 2

Urinogenital organs of ***Tylototriton Verrucosus.***
The collecting ducts on the right side are shown detached from cloaca and spread out for the sake of clarity.

PLATE - 2



Gross morphology of testicular lobes of *Tylototriton verrucosus*, arrow indicates testis, fat bodies and yellow zone.

B. TESTICULAR ANATOMY

The two principal elements of the testis are the seminiferus tubules and interstitial tissue, consisting of connective tissue, blood capillary and closely packed Leydig cells. The testicular elements are covered externally by a thick fiber sheath.

In *Tylototriton verrucosus*, the testis exhibits cystic arrangement. The cells present in a cyst are in the same stage of the development and are derived from a single spermatogonium. However, in a cross-section of seminiferus tubules, cyst in different stages of spermatogenesis are usually encountered. In the present text, such cysts have been designated as spermatogonial cysts, spermatocyte cysts, spermatid cysts and spermatozoal cysts (for a detail description see annual testicular cycle).

The histological structure of each testis has been studied for three consecutive seasons (1999 - 2000, 2000 - 2001, 2001 - 2002) and for the sake of convenience it has been described as Pre-breeding, Breeding, Post breeding and Regression phases.

The spermatogenesis starts at the onset of Summer season (April to June). Spermatogenesis reaches its maximum velocity in breeding season (July to September).The onset of Post-breeding season starts with regression of the spermatogenetic activity and lasts for few months (October to December). The Regression phase is recognized as the period when the yellow pigmentation or 'Yellow Zone' formation reaches its maximum development (January to March).

In the text, therefore testicular histology would be represented in four distinct months i.e. pre-breeding (May), breeding (August), post-breeding (November) and regression (February).

i) Pre-breeding Testis

The testicular lobe contains cysts harboring with spermatogonial cells and primary spermatocytes. Interstitial cells are very few. The 'Yellow Zone' is totally absent.

Gonial cells are present in the peripheral lobules / cysts and are arranged in clusters. The cells are lightly stained, chromatin material condensed and finely distributed throughout the cellular space in a reticular fashion. The inner lobules / cysts contains the primary spermatocytes. Such lobules are compactly arranged and hardly with any interlobular space. The cells are more darkly stained than the spermatogonial cells. Chromatin materials are also darkly stained and condensed than the spermatogonial cells.

Interstitial cells are found distally in some lobules which are elongated and very darkly stained. (Plate - 4 , Figs . A, B, C, D)

ii) Breeding Testis

The testicular lobe contains lobules with different cells types and accordingly are called gonial cyst, spermatocytic cyst, spermatid cyst and spermatozoal cyst. The gonial cysts / lobules are found in the peripheral position of the testis followed by spermatocytic cyst containing primary spermatocytes. The middle part of the lobe contains lobules or cysts with secondary spermatocytes. The secondary spermatocytes are smaller primary spermatocytes with more condensed chromatin material.

The spermatid lobules or cysts are easily recognizable with darkly stained, oval or ovoid and elongated cells which are with highly condensed chromatin material. Spermatozoa are found in the lobules which are distally placed and such cells are arranged in clusters. Some cysts in the distal region exhibit vaculations suggesting the onset of spermiation or sperm release. Interstitial

mass is distinct in a restricted part at the distal end of the lobe. (Plate - 8, Figs A, B, C, D, E, F)

iii) Post-breeding testis

The testicular lobe contains lobules with gonia, spermatocytes, spermatids and spermatozoa. Spermatogonia and spermatocyte cysts are restricted to the peripheral margin of the lobe while spermatid and spermatozoal cysts are present in the middle part. However, some spermatozoal cysts have been located also in the proximal part of the lobe. Sperm evacuation have been noted in some lobules or cyst leaving residual body within them at the distal part of the lobe (Plate 12, Figs A, B, C, D). Interstitial cells are found to be attached to the walls of evacuated lobules or cysts.

iv) Regression Testis

Each lobe has distinct regions according to cellular components of lobules, viz. proximal, middle and distal parts.

The proximal part contains lobules or cysts pact with spermatogonial cells. The middle part contains lobules with spermatocytes, spermatid spermatozoa, and some evacuated lobules or cysts. However, the distal part mainly contains evacuated lobules or cyst. The walls of such cyst are hypertrophid and form a compact mass of tissue called the 'yellow Zone'. The yellow zone is very much pronounced in the testis of this month. (Plate- 16, Fig A, B, C, D)

C. ANNUAL TESTICULAR CYCLE

Annual testicular cycle has earlier been studied by Ray (1978), and Roy (1989), using light microscopic study. The above studies indicate that the testicular cycle can be summarized into four distinct phases on the basis of spermatogenetic activity vis-à-vis the steroidogenic activity of the interstitial cells.

Therefore, in the present work the description of the testicular morphoanatomy is restricted in the months which have been identified as the critical point of each phase or stage. Accordingly the testicular cycle in this text will be grouped as -

(a) Pre-breeding phase

This stage commences shortly after culmination of the winter and onset of the summer season at high altitude and lasts for about three months (April, May and June). At the initial stage, i.e., in the month of April, the mean ambient temperature recorded is 15°C. The ambient temperature rises about 20°C in mid May to June and is followed by a prolonged rainy season which last for three to four months (June, July, August, September)

(b) Breeding phase

The breeding season starts with the advent of the monsoon season and nuptial activity of the specimens are easily noticeable in the breeding spots. Larvae emergence in these spots and the appearance of the secondary sexual characters become more prominent.

(c) Post breeding phase

The post breeding season is restricted in the month of November when the spermatogenetic activity still remain active but at a low eve.

(d) Regression phase

The regression phase is a dramatically concise one, with reduction of testicular size and hyperactive development of the interstitial tissue as 'yellow zone' (Figure - 1).

Therefore, the present study as well as the earlier observations tend to suggest that in *Tylototriton verrucosus* spermatogenetic activity onsets in the month of April but remains in a quiescent phase till mid May. The onset of monsoon as well as rise of ambient temperature (around 20°C) upsurge the spermatogenetic activity and gonial population increase in number. Meiotic upsurge is observed in the June and July specimens and testes of such specimens are found to be studded with spermatocytes at different divisional stages. Spermiogenesis commences in June specimens and continues in the months of July to December. However, spermiogenesis is not a synchronous event in all lobules / cysts and occurs in a wave like fashion. Fully formed spermatozoa are found during the months from July to February.

Spermiation starts in the month of August and continue up to December. However, in the older specimens spermiation is found to occur at early pre breeding stage as a result of which testicular lobules/cysts of older specimens exhibit vaculation in early April to May months.

In the present text, the above mentioned annual testicular cycle pattern, as revealed from the histological studies has been reconfirmed using surface morphological investigation using Scanning Electron Microscopic (SEM) technique and fine structural changes with the aid of Transmission Electron Microscopic technique (TEM).

SCANNING ELECTRON MICROSCOPE OBSERVATIONS

Pre-breeding testis

At the light microscopic level, the cross-section of pre-breeding testis (April to June) shows different stages of spermatogenesis and accordingly they are called as gonial cysts, spermatocytic cysts and spermatid cysts. Spermatogonial cells are easily recognisable, usually they are distributed adjacent to the basement membrane of the seminiferus lobule or cyst. They are bigger in size, lightly stained and chromatin material is finely distributed throughout the nucleus in a reticular fashion (plate 4; Figure. A, C). Spermatocytic cells are very large in size and such cells exhibit various type of meiotic stages. Secondary spermatocytes are smaller in size and found near the primary spermatocytes (Plate 4; Fig. B, D). Spermatids are found in the spermatid nest. These type of spermatid nests are often found attached with the wall of the seminiferus tubules. The interstitial cells are found in between the seminiferus lobules / cysts. They are large ovoid or elliptical in shape. The nucleus is large and filled with dense chromatin granules (Plate 4; Fig. D).

Under the scanning electron microscope (SEM), the pre-breeding testis exhibits all the cellular component as mentioned above.

The whole-mount testis preparation exhibits the following characteristics:

i. The testis is ovoid to elliptical in shape. The anterior or proximal portion is broader than the posterior or distal end. The testis is bordered by a thick fibrous coat. At the surface level such coat forms small crypts with low protuberance giving a honey - comb like appearance of the outer surface.

ii. The gonial cysts are found at the periphery of the anterior part of the testicular lobe. Such nests are filled with two type of cells - one type that is larger in size and with rough surface. The rough appearance is due to the

presence of various projections of variable shape and size . With the help of these cytoplasmic projections gonial cells form a cluster of germ cells. Other cells are relatively smaller in size and with relatively smooth in appearance due to the absence of similar projections (Plate 5 ; Fig. A).

iii. The spermatocytic cysts / lobules are found in the inner side of the middle part of the testicular lobe, such lobules are filled with cells either with large electron-dense smooth wall cells or with small-electron dense cells having smooth appearance. (Plate 5; Fig. D)

iv. Spermatid cysts or lobules are found at periphery of the lobes. These cells are electron-dense, very small and ovoid in appearance. Some spermatids are elongated in shape, forming a head region and a tail region, the latter consisting of a middle piece and principle piece (Plate 5; Fig. B).

v. Sertoli cells are observed in association with the cluster of germs cells, as shown in plate 5; Figure F. These cells radiate from the tubule periphery and extend towards the lumen and invest developing germ cells with cytoplasmic processes during the process of gametogenesis (Plate 5; Fig. F).

vi. There are some Leydig cells in the interstitial region. Each Leydig cell is elliptical or ovidal in shape, showing a lobular appearance. The apical small lobe is demarked from the posterior large lobe by a cleft at the lateral margin. Moreover, the apical lobe exhibits medial cleft on its surface. Both apical and posterior lobe exhibit electron-dense region in the central part suggesting presence of a conspicuous prominent nucleus in the middle (Plate 5; Fig. C).

Breeding Testis

At the light microscopic level, the cross-section of breeding testis (July to September) shows different stages of spermatogenesis and accordingly they are called as gonial cyst, spermatocytic cyst, spermatid cyst and spermatozoal cyst. The periphery of the proximal or anterior part of testis shows spermatogonial cysts (Plate 8; Fig. E). The gonial cysts / lobules are followed by spermatocytic cysts containing primary and secondary spermatocytes. Primary spermatocytic cysts show different stages of meiotic prophase. Secondary spermatocytes show condensed chromatin material and the cytoplasmic layer of the cells is very thin (Plate 8; Fig. C, D, F).The middle part of the lobe contains lobules and secondary spermatocytes. The spermatid lobules or cysts are easily recognisable with darkly stained, oval, ovoid and elongated cells with highly condensed chromatin material. In spermatozoal cysts, spermatozoa are found and arranged in clusters (Plate 8; Fig. A)

Under the scanning electron microscope (SEM) the breeding testis exhibit all the cellular component as mentioned in the above.

The whole mount testis preparation exhibits following characteristics:

i. The testis is ovoid to elliptical in shape. The upper half is broader than the posterior or distal half. The testis is bordered by a relatively thin fibrous coat. At the distal end of the testis there is a small constricted portion which have three lobules, two with cluster of sperm bundles and other one is vacuolated, (Plate- 7).

ii. The anterior part of the testis shows lobules with gonial cells. In higher magnification, these lobules show rough surface gonial cells and relatively smooth surface electron-dense spermatids (Plate 9; Fig. E. F). The periphery of the anterior end is studded with lobules with gonial cells while inner lobules

show spermatocytic and spermatid cells. A few evacuated lobules are also seen (Plate 7).

iii. The middle part of the testis shows lobules with spermatids and late spermatids with forming elongated head region, mid piece, principal piece and tail with a flagellum (Plate 9; Fig. C, D). In the periphery of the middle portion, there are some lobules with round shaped sperm bundles (Plate 9; Fig. C). In higher magnification, these round shaped sperm bundles show both mature and maturing sperm (Plate 9; Fig. D) A few evacuated lobules are also noticed in the middle portion of the testicular lobe (Plate 7).

iv. The distal part of the testis lobe shows lobules with mature sperm bundles (Plate 7; Plate 9; Fig. A). Some lobules are evacuated , suggesting release of sperm bundles (Plate 7). In higher magnification each sperm bundle shows a typical conical structure (Plate 9; Fig. B). In a bundle, the heads are pointed at one end and the tail with characteristics undulating membranes in an elliptical fashion at the other end (Plate 9; Fig. B). At the tip of the distal part of the testis lobe, three lobules are found with in a characteristic constricted portion. Of the three lobules two are studded with the sperm bundles and other one is vacuolated (Plate 7).

Post-breeding Testis

At the light microscopic level, the cross-section of post-breeding testis (October to December) shows lobules with different stages of gonial cells, spermatocytes and mature spermatozoa . Spormatogonial and spermatocyte cysts are restricted to the peripheral margin of the lobe while spermatid and spermatozoal cysts are present in the middle part of the testicular lobe. However, some spermatozoal cysts are located also in the proximal part of the lobe. Sperm evacuation has been noted in some lobules or cysts leaving residual body within them at the distal part of the lobe. Interstitial cells are found to be attached to the walls of evacuated lobules or cysts (Plate 12; Fig.

A, B, C, D).

Under the scanning electron microscope the post-breeding testis exhibits all the cellular components as mentioned above.

The whole mount testis preparation exhibits following characteristics:

i. The testis is kidney or bean shaped. It bears a medial groove. The upper half is slightly narrower than the posterior or distal half. The distal part of the testis shows begging of the yellow zone formation. At the middle portion of the testis there is a cluster of lobules bearing mature sperm. The testis is bordered by a relatively thick fibrous coat (Plate 11).

ii. The anterior part of the testis shows some lobules with a few mature sperms in a disperse form. Some evacuated lobules are also seen at that part (Plate 13; Fig. A). In higher magnification the anterior lobules also show some spermatids and gonial cells and also a batch of mature sperm (Plate 13; Fig. A, B).

iii. The middle portion of the lobe shows much more round-shaped sperm bundle (Plate 13; Fig. C). In higher magnification, round sperm bundles show jagged fashion of orientation (Plate 13; Fig. D) Some interstitial cells are also seen in these lobules (Plate 13; Fig. C, D).

iv. The distal part of the testis shows mature sperm bundles as well as evacuated lobules. Higher magnification shows a characteristic in which the sperm heads are embedded inside the lobule for their nutrition from Sertoli cells. Such Sertoli cells are very big in size with irregular shape cytoplasm radiated from the basement membrane of the seminiferus tubules (Plate 13; Fig. F).

Regression Testis

At the light microscopic level, the cross-section of egression testis (January to March) shows distinct regions according to cellular components of lobules, viz. Proximal or anterior, middle and distal. The proximal or anterior part contents lobules or cysts pact with spermatogonial cells, (Plate 16; Fig. D). The middle part contains lobules or cysts with spermatocytes, spermatids, and spermatozoa and some evacuated lobules or cysts. However, the distal part of the testis mainly contains evacuated lobules or cysts. The wall of such cysts are hypertrophid and form a compact mass of tissue called the 'yellow Zone' The yellow zone is very much pronounced in the testis of this month. Some lobules contains residual spermatozoa (RSPZ) for the next breeding reason (Plate 16; Fig. B, C).

Under the scanning electron microscope, the regression testis exhibit all the cellular components as mentioned above.

The whole mount testis preparation exhibits the following characteristics:

i. The testis is ovoid to elliptical in shape. The proximal and distal half is more or less similar in shape. The testis is bordered by a very thick fibrous coat. At the distal end of the testis there is evidences for 'Yellow zone' formation (Plate 15).

ii. The anterior part of the testis shows several lobules/cysts filled with different types of gonial cells. At the higher magnification the gonial cells show several projections at its surface which helps to form cytoplasmic connections between the gonial cells.

iii. In the middle portion of the testis lobes there are some lobules at the peripheral regions fully loaded with spermatocytes and spermatids. At the inner portion there are some lobules which filled with sperm bundles (Plate 17; Fig. C). In higher magnification some spermatocytic cells are found at dividing

PLATE - 3

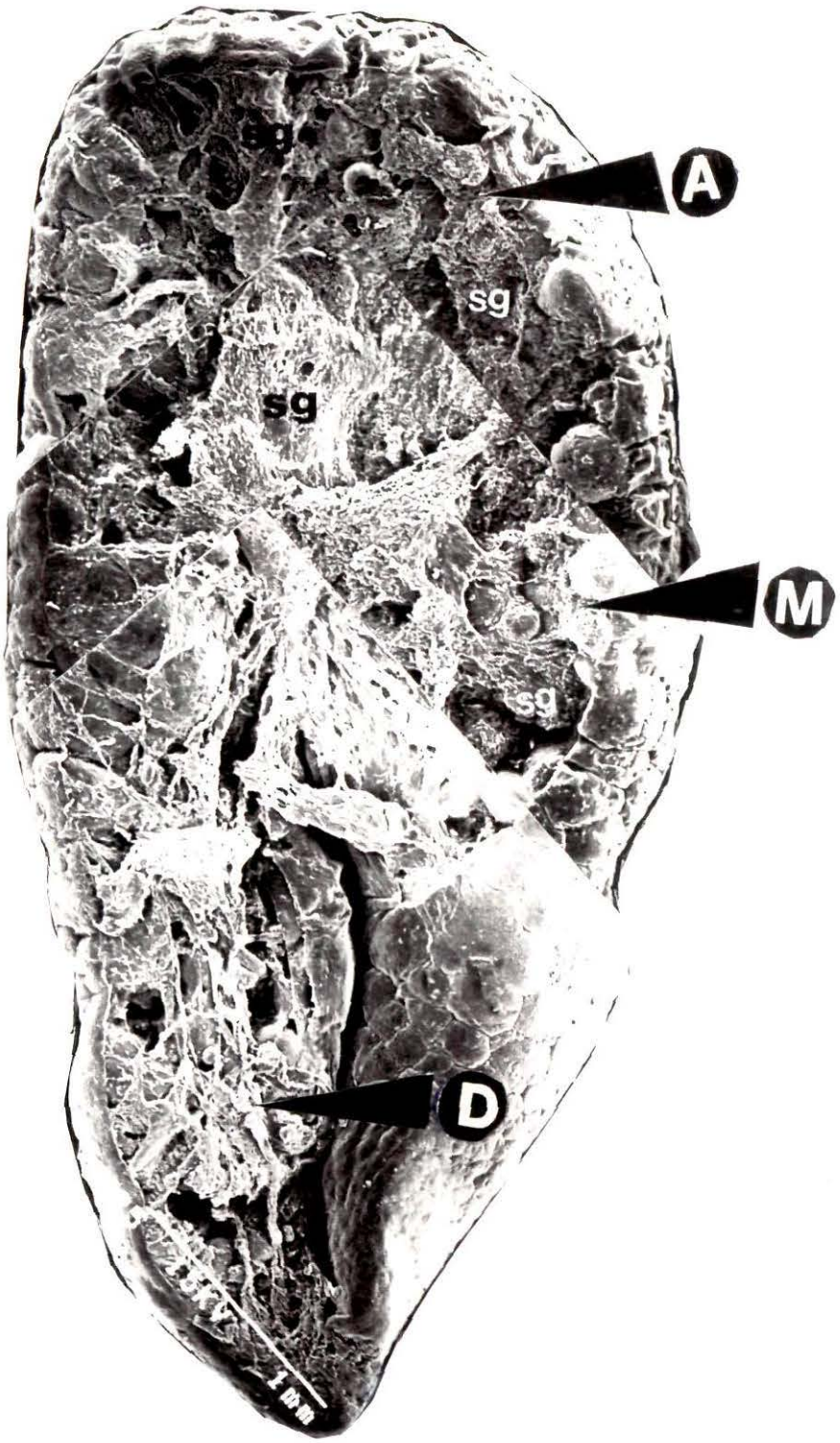


PLATE 3. Scanning Electron Microscope view of pre-breeding testis cross-section. **A** anterior part, **M** middle part, **D** distal part. **sg** indicates spermatogonial cells.

PLATE - 4

Photomicrographs of the histological section (5 μ) of pre-breeding testis of *Tylototriton verrucosus*.

Fixative : Bouins (aqueous)

Stain : Haematoxyline - eosin

Figure A : Peripheral lobules/Cysts at anterior part of the lobe showing spermatogonial cell (SG) and inner lobules showing primary spermatocytes (PSC), ISC indicates interstitial cell.

Figure B : Lobules / Cysts of distal part of the testis lobe showing spermatogonial (SG) cells oriented towards periphery and Primary spermatocytes (PSC) and secondary spermatocytes towards the lumen of the lobe.

Figure C : Spermatogonial cells (SG) displaying the reticular fashion of chromatin material.

Figure D : Primary spermatocytes (PSC) showing condensed chromatin. Arrow indicates interstitial cell (ISC).

PLATE - 4

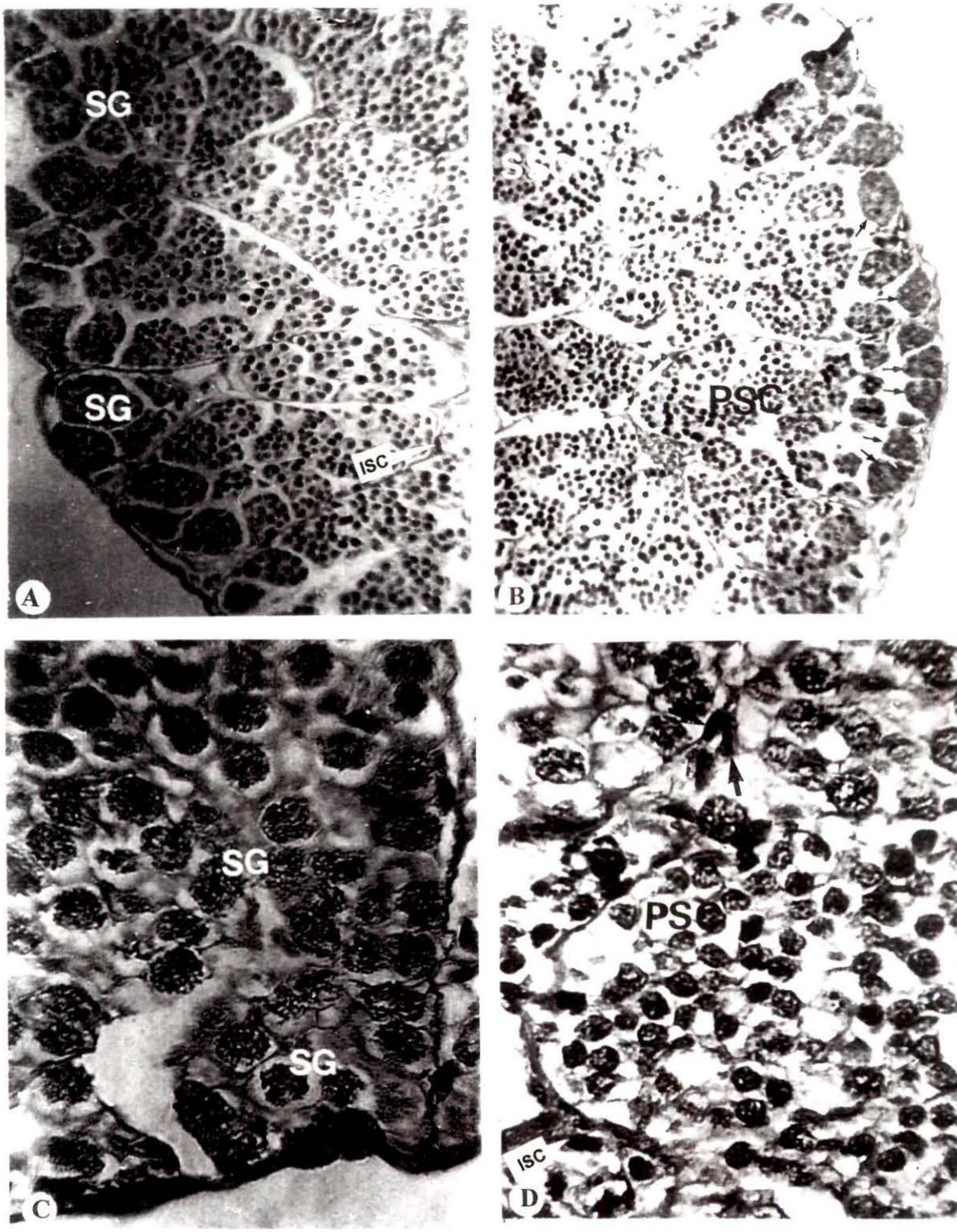


PLATE 5. Scanning Electron Microscope view (magnified) of pre-breeding testis cross-section. **Fig. A.** Cluster of rough surface spermatogonial cells (sg) showing cytoplasmic projections (arrows) and its attachment sites (small arrows). **Fig. B.** Spermatid cyst showing small ovoid electrondense spermatid (arrows) and elongated spermatid (↑) with forming head and tail region. **Fig. C.** Leydig cell. **Fig. D.** Spermatocyte cyst showing spermatocytes (sc). **Fig E. F.** Sertoli cell (S) in association with the cluster of germ cells.

PLATE - 5

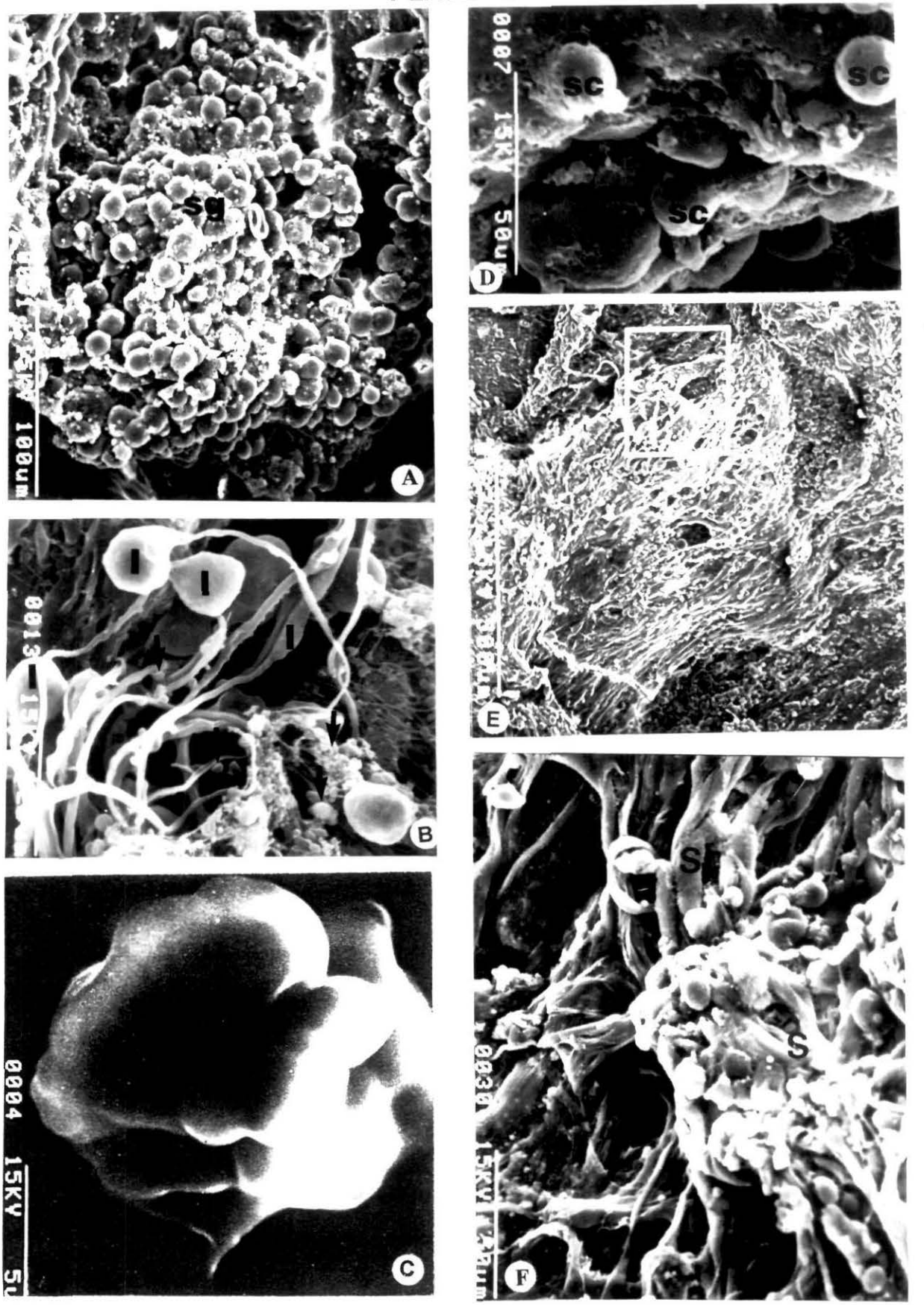


PLATE 6. Transmission electron micrographs of pre-breeding testis of *Tylototriton verrucosus*. **Fig. A.** Primary spermatogonia (PSG) and secondary spermatogonia (SSG). x3450. Cytoplasm shows mitochondria (m), lysosomes (ly) and lipid droplets(li) **Fig. B.** Primary spermatocytes (PSC) and secondary spermatocytes (SSC), arrows indicate chromatin materials. x2170. **Fig. C.** Cytoplasm of gonial cell shows mitochondria (m), lysosomes (ly) and lipid droplets (li). x16585. **Fig. D.** In higher magnification the cytoplasm of spermatocytes shows mitochondria (m), Golgi bodies (g) and lysosomes (ly). x16400. **Fig. E.** Leydig cell (L) shows wavy nuclear membrane (nm) and laminar distribution of chromatin materials (arrows).x14466. **Fig. F.** Sertoli cells show irregular shape nucleus (n), nucleolus (nu) and chromatin materials (arrows).x4246.

PLATE - 6

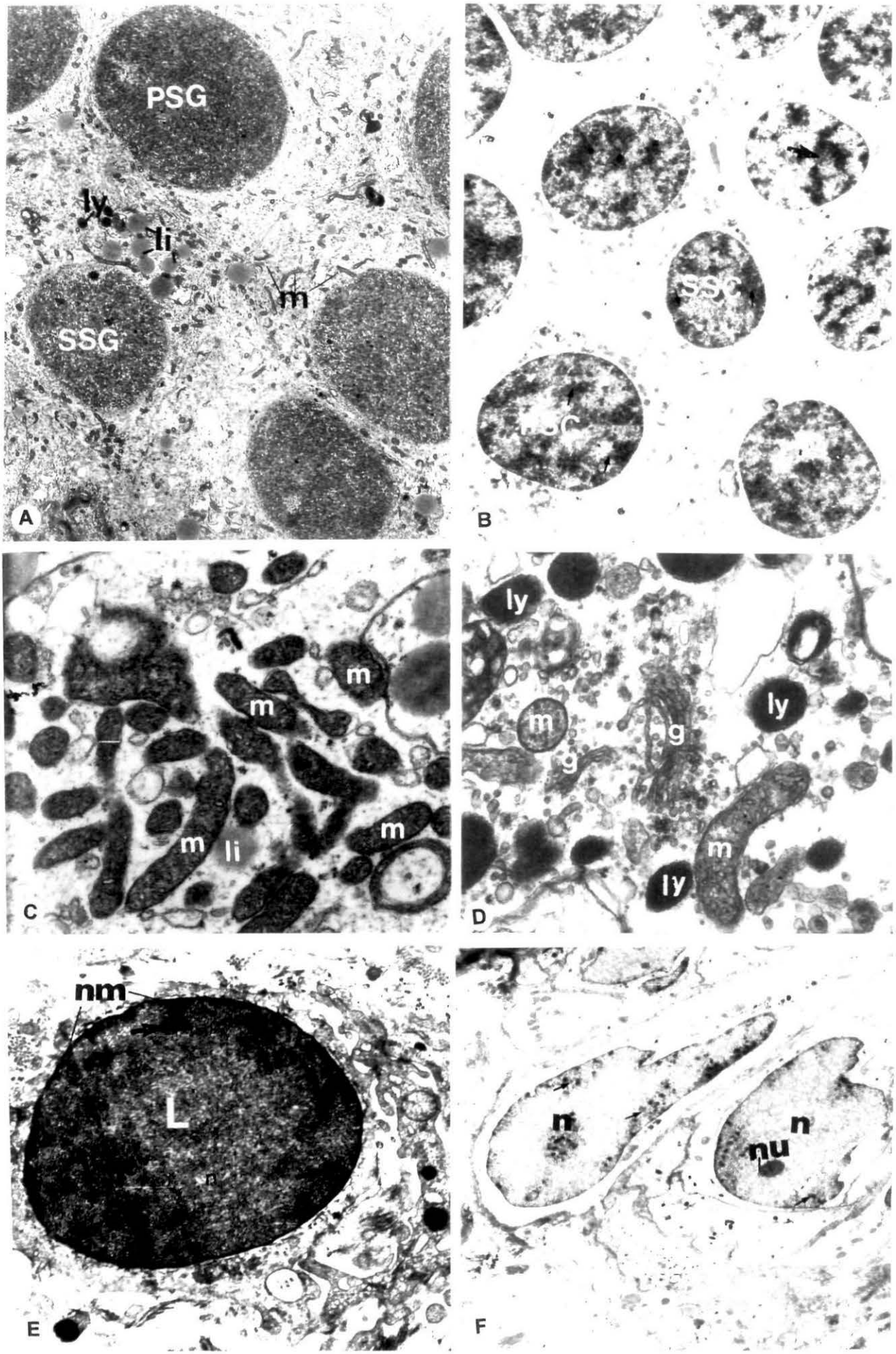


PLATE - 7

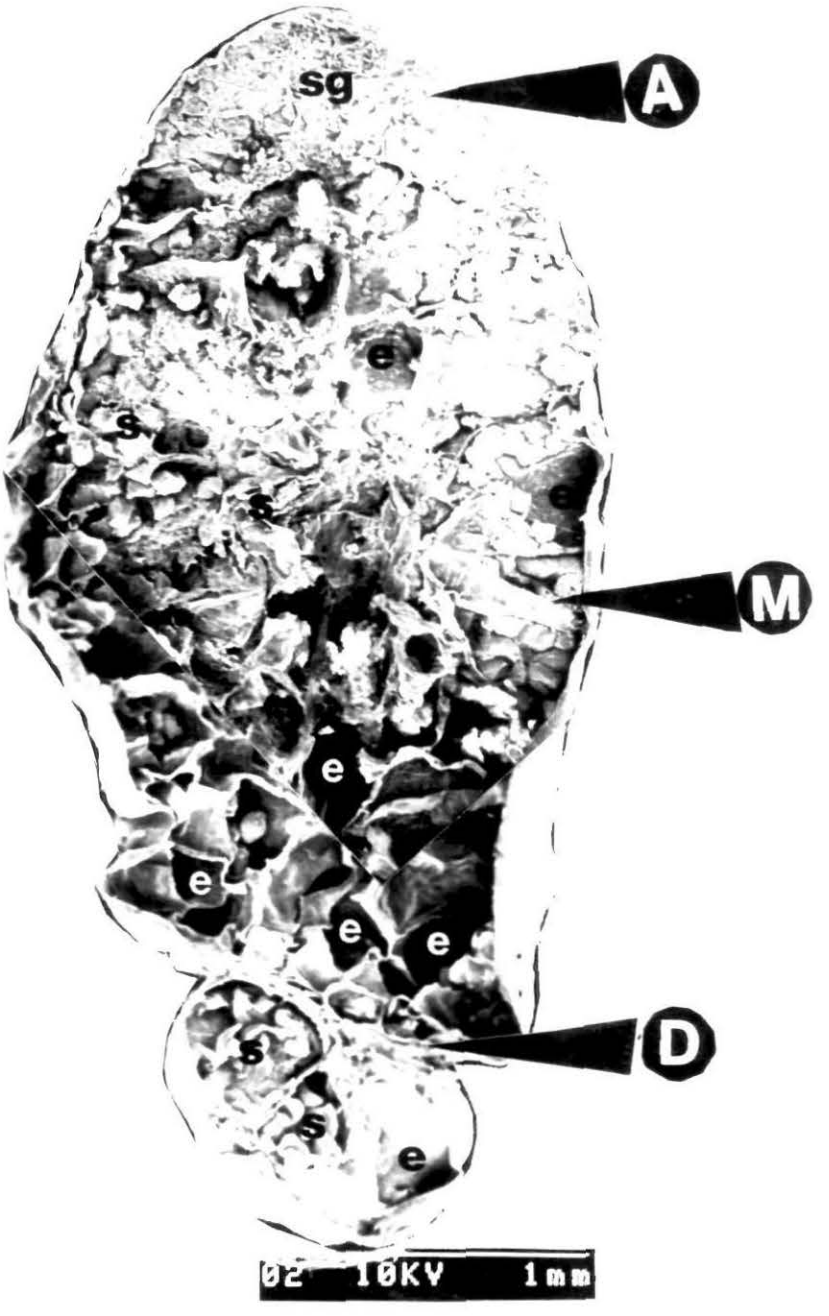


PLATE 7. Scanning Electron Microscope view of breeding testis cross-section. **A** anterior part, **M** middle part, **D** distal part.
sg indicates spermatogonial cells, **e** indicates evacuated lobules, **s** indicates sperm bundles.

PLATE - 8

Photomicrographs of the histological section (5 μ) of breeding testis of *Tylototriton verrucosus.*

Fixative : Bouins (aqueous)

Stain : Haematoxyline - eosin

Figure A : Lobules / Cysts of the distal part of the testicular lobe showing clusters of spermatozoa (SPZ).

Figure B : Lobules / Cysts of the distal part of the testicular lobe showing some spermatid in different maturing stage (STD).

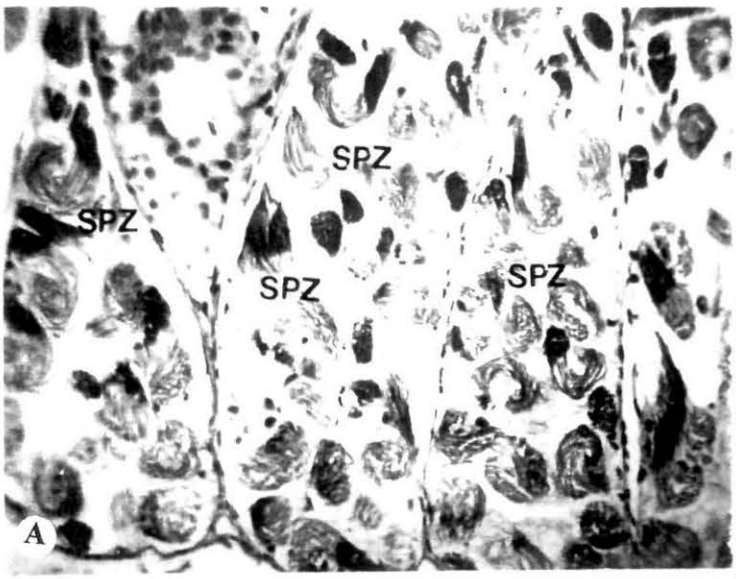
Figure C : Middle part of the testicular lobe contains lobules / cysts, showing secondary spermatocytes (SSC).

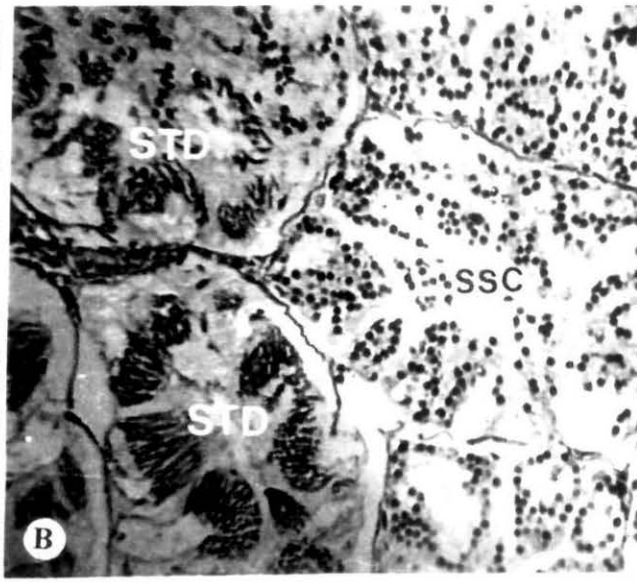
Figure D : Divisional stages of primary spermatocytes (PSC) showing in the spermatocyte lobules / cysts.

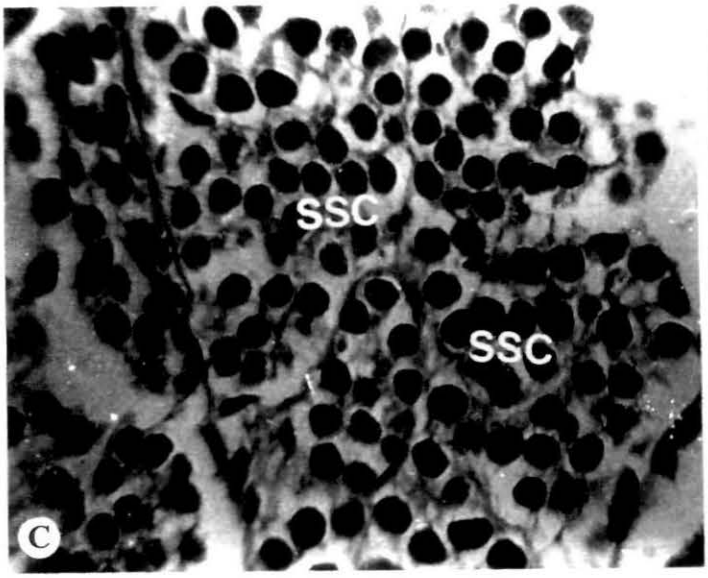
Figure E : Lobules / Cysts of the anterior part of the testicular lobe showing spermatogonial cell (SG) at the peripheral region and spermatocytes (PSC) at the inner region.

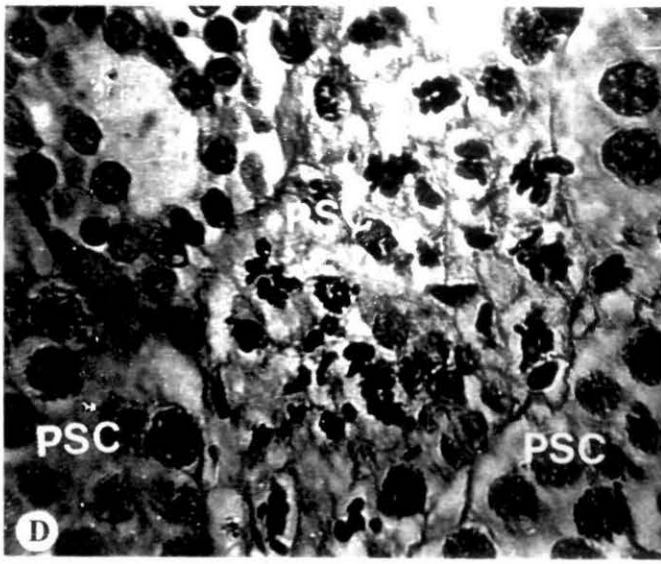
Figure F : Lobules / Cysts showing primary spermatocytes (PSC) and secondary spermatocytes . (SSC).

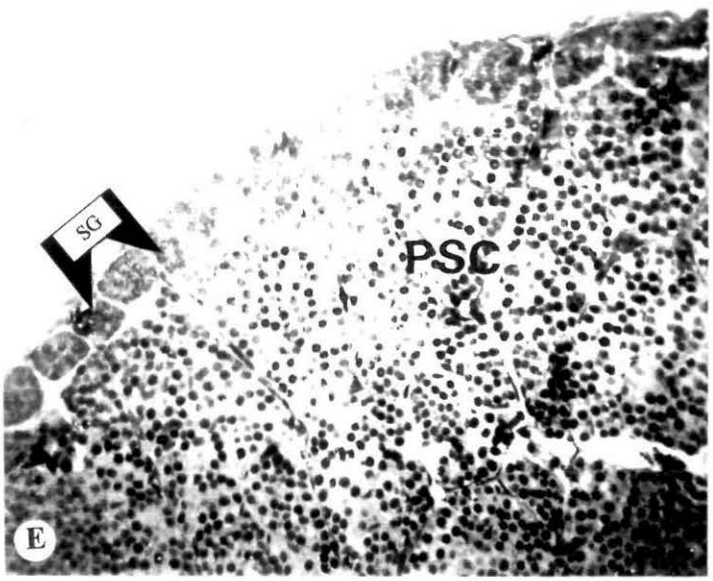
PLATE - 8


SPZ
SPZ
SPZ
SPZ
A


STD
SSC
STD
B


SSC
SSC
C


PSC
PSC
D


SG
PSC
E

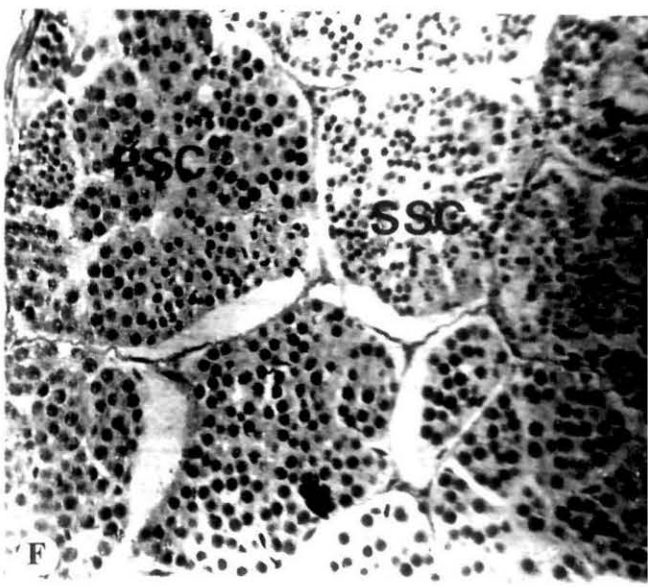
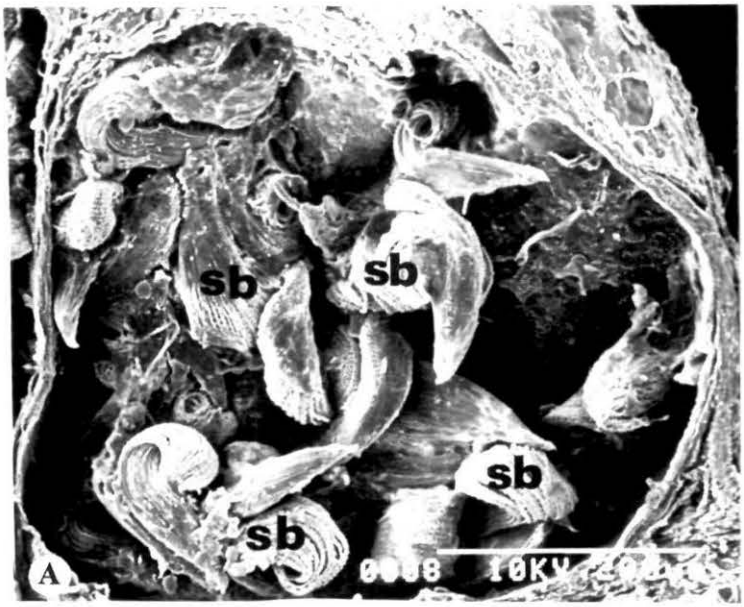
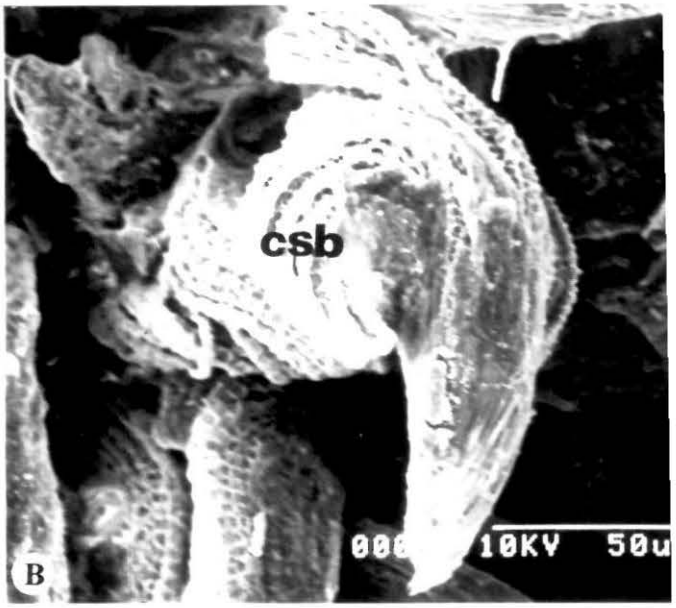
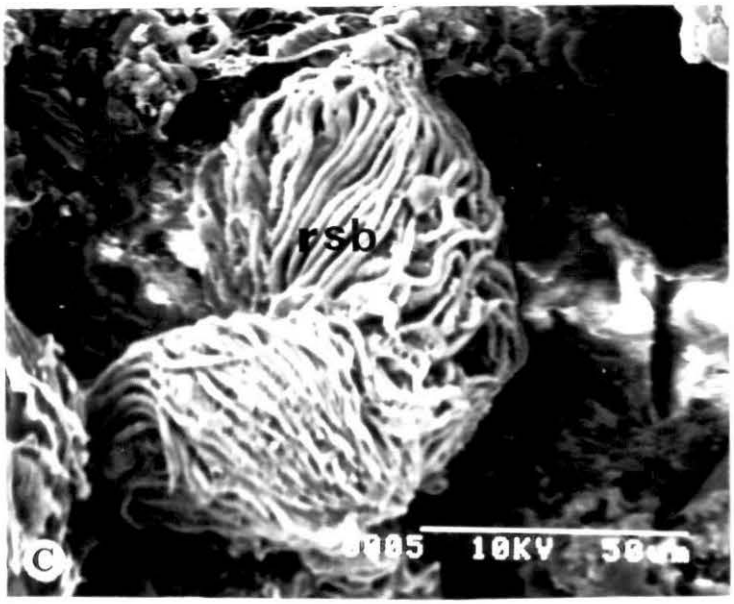
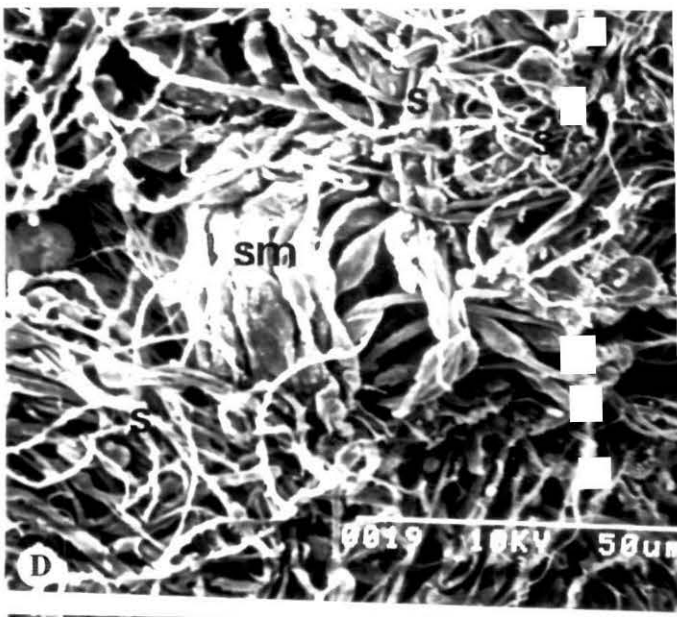

PSC
SSC
F

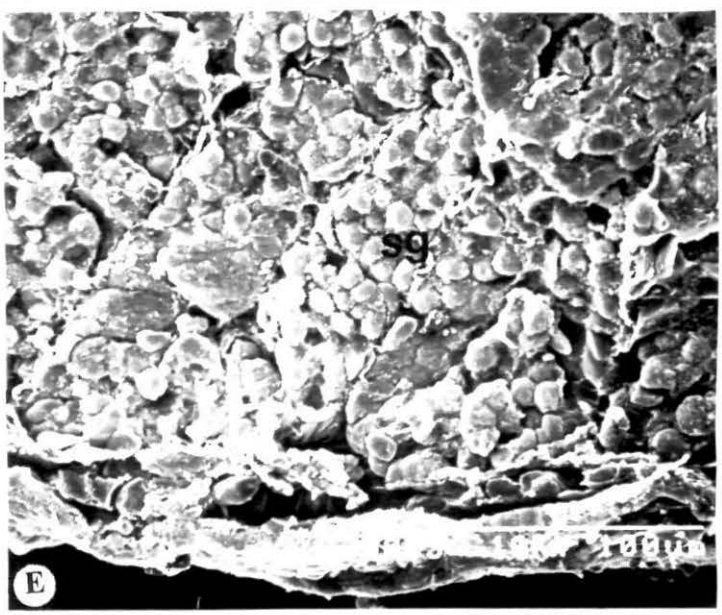
PLATE 9. Scanning Electron Microscope view of breeding testis cross-section. **Fig. A.** Distal part of the testis lobe shows cyst with mature sperm bundles (sb). **Fig. B.** In higher magnification each sperm bundle (csb) shows a typical conical structure. **Fig. C.** Round shaped sperm bundles (rsb). **Fig. D.** Magnified view of round shaped sperm bundle shows both mature (s) and maturing sperm (sm).**Fig. E. F.** Anterior part of the testis shows lobules with different type gonial cells (sg).


sb
sb
sb
sb
A


csb
10KV 50u
B


rsb
10KV
C


s
sm
10KV 50u
D


sg
E

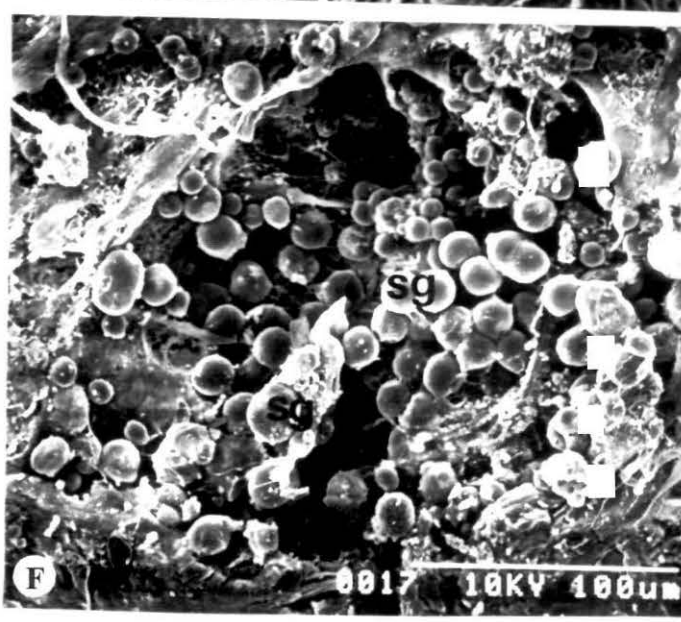

sg
sg
10KV 100u
F

PLATE 10. Transmission electron micrographs of breeding testis of *Tylototriton verrucosus*. **Fig. A.** Spermatogonial cell (SG), shows two nucleolus (arrows). x2628. **Fig. B.** Primary spermatocytes (PSC),arrows indicate chromatin materials. x1980. **Fig. C.** A group of spermatids in different stages of spermiogenesis, note the forming acrosomal region (A), nucleus (N), accumulation of mitochondria at mid-piece region (M). x5467. **Fig. D.** Transverse sections through the middle of the sperm head, note the condensed nucleus (N), thin mantle of cytoplasm free of organelles (C), the cross-section of slender 'Côte' is visible as a small oval profile on one side of the nucleus (arrows). x15428. **Fig. E.** Elliptical spermatids showing acrosome formation (A), uniformly condensed nucleus (N), arrangement of mitochondria (M) behind the nucleus. x16392. **Fig. F.** Transverse sections through the middle piece of the sperm showing horseshoe-shaped profile of the axial fiber (AX) surrounded by a sheath of cytoplasm rich in mitochondria (M), note the undulating membrane (U) and axonemal complex. (arrow).x1740.

PLATE - 10

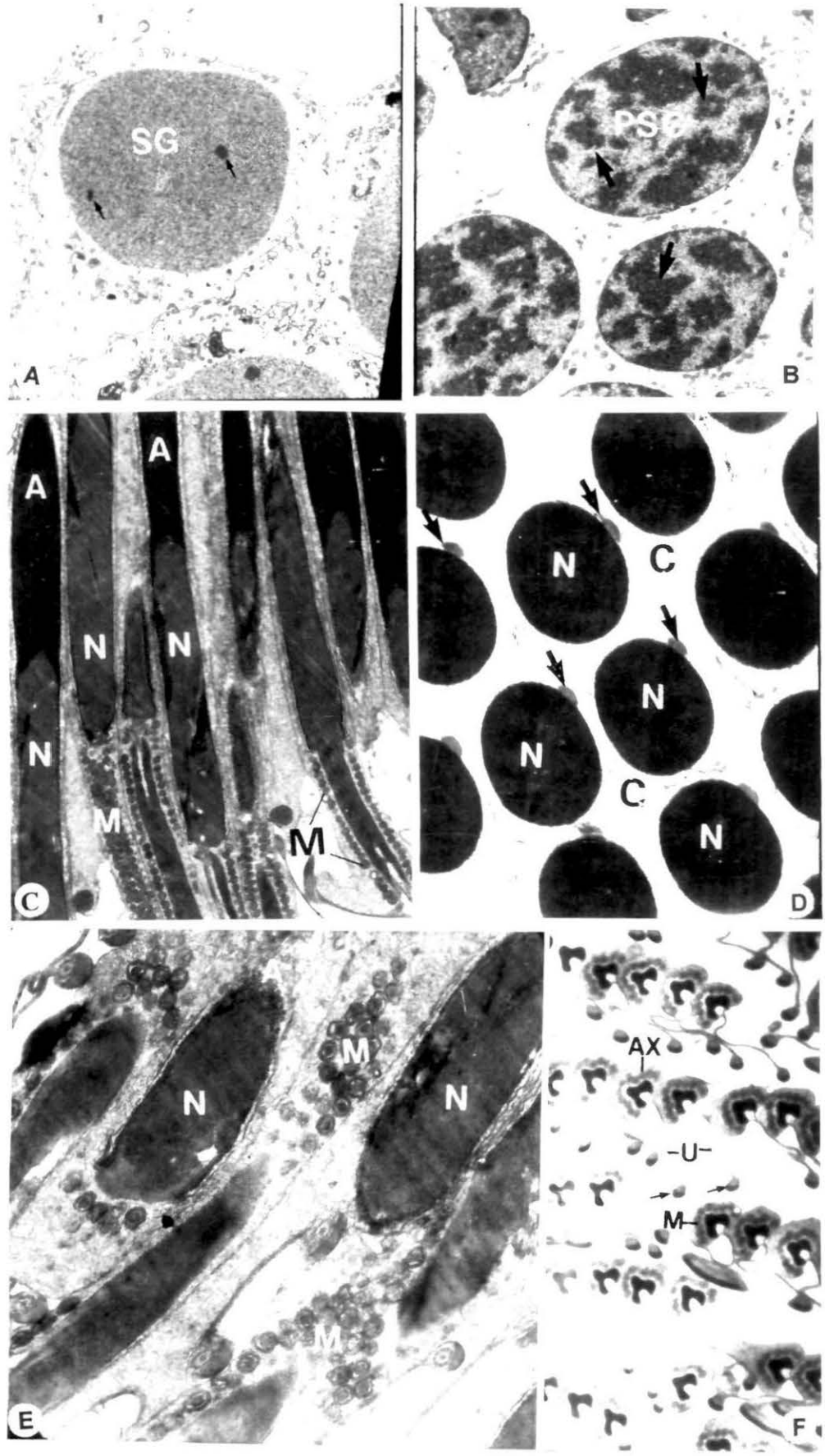


PLATE - 11

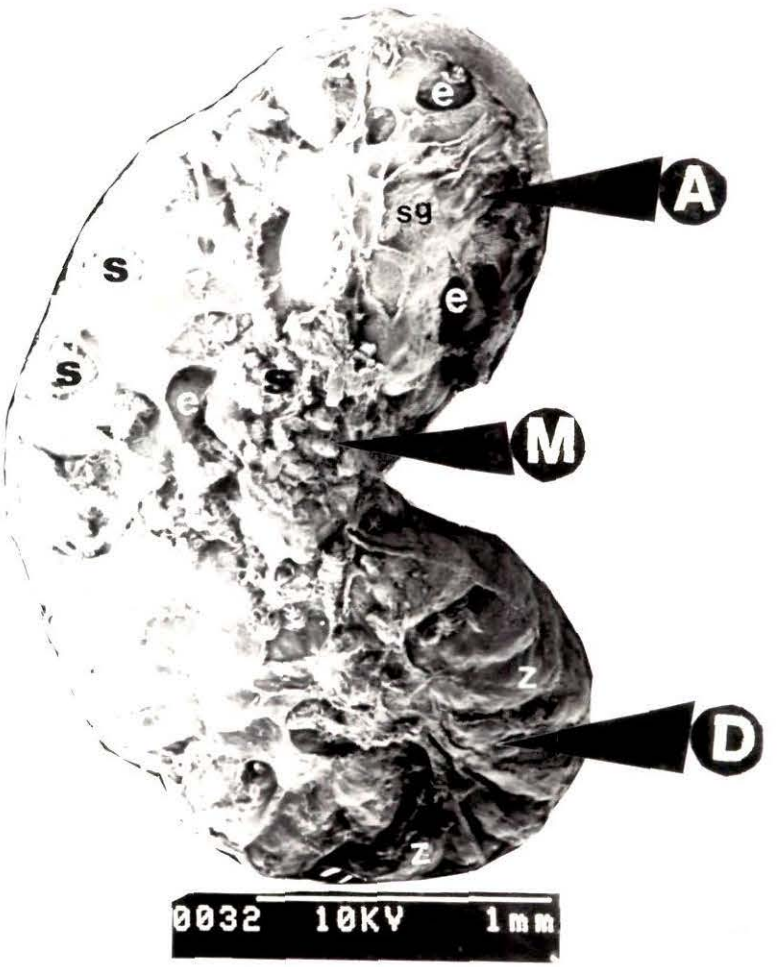


PLATE 11. Scanning Electron Microscope view of post-breeding testis cross-section. **A** anterior part, **M** middle part, **D** distal part. **sg** indicates spermatogonial cells, **e** indicates evacuated lobules, **s** indicates sperm bundles. **z** indicates beginning of yellow zone formation.

PLATE - 12

Photomicrographs of the histological section (5 μ) of post-breeding testis of *Tylototriton verrucosus.*

Fixative : Bouins (aqueous)

Stain : Haematoxyline - eosin

Figure A : Lobules / Cysts of the proximal part of the testis lobe showing spermatids (STD) and spermatozoa (SPZ).

Figure B : Lobules/Cysts showing spermatogonial cells (SG) at the proximal part of the testis.

Figure C : Lobules / Cysts showing very thin lobular wall with interstitial cells merge in it. These lobules / cysts are situated at the middle portion of the testis lobe. Arrow indicates the lobules / cysts showing beginning of evacuation.

Figure D : Evacuated Lobules / Cysts with residual bodies (RB) in the center at the distal part of the testicular lobe.

PLATE - 12

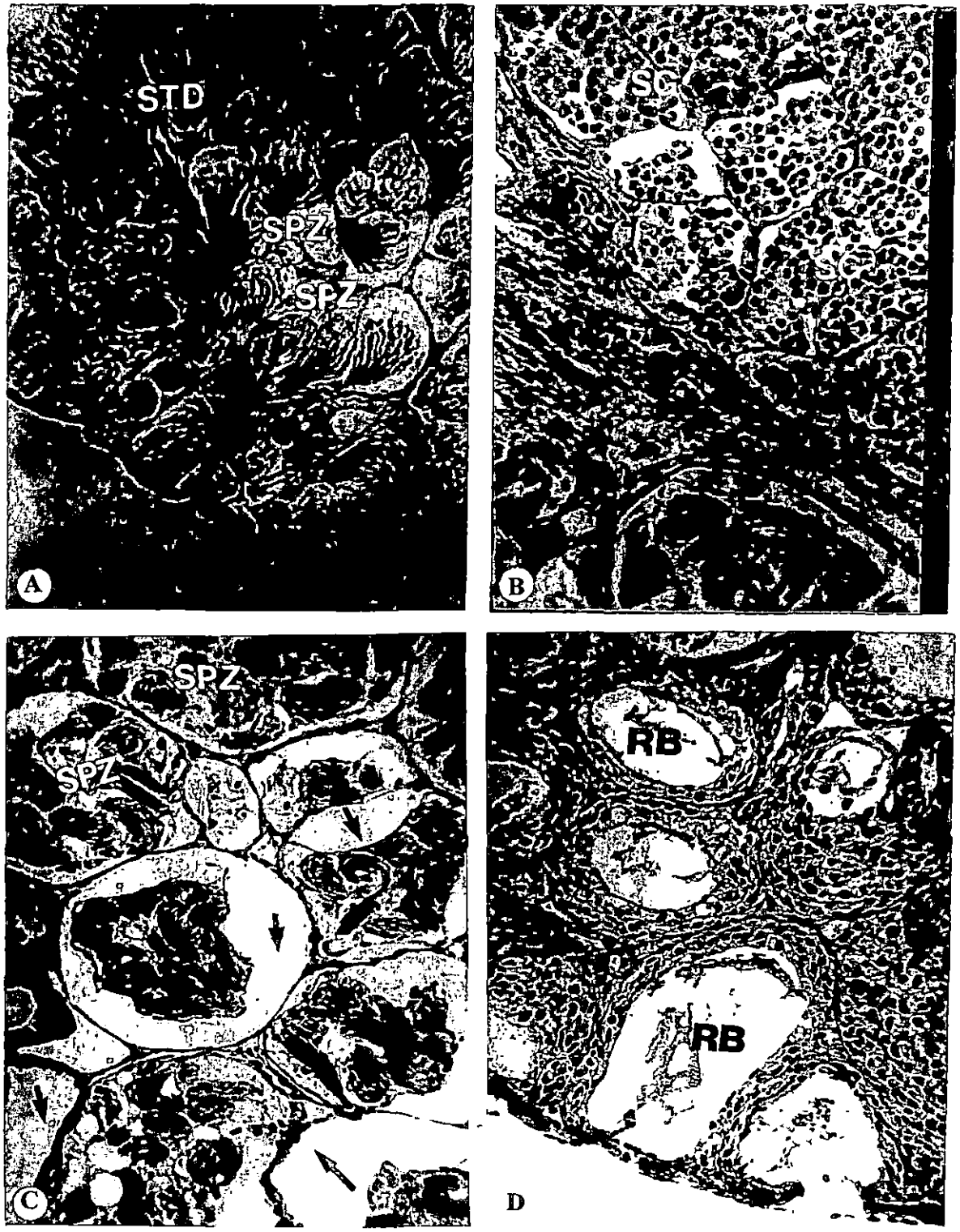


PLATE 13. Scanning Electron Microscope view of post-breeding testis cross-section. **Fig. A.** Anterior part of the testis lobe shows cyst with small amount of mature spermatozoa (sz) and some evacuated lobules (e). **Fig. B.** Different types of gonial cells at the anterior cyst of testis. **Fig. C.** Round shaped sperm bundles (rsb) at the middle portion of the testis. **Fig. D.** Magnified view of round shaped sperm bundle shows jagged fashion of orientation (rj), some interstitial cells (ic) are also found. **Fig. E.** Distal part of the testis shows mature sperm bundles (sb) as well as evacuated lobules (e) **Fig. F.** Higher magnification shows some characteristic of sperm bundle (csz) in which the sperm heads are embedded inside the lobule for their nutrition from Sertoli cell (s).

PLATE - 13

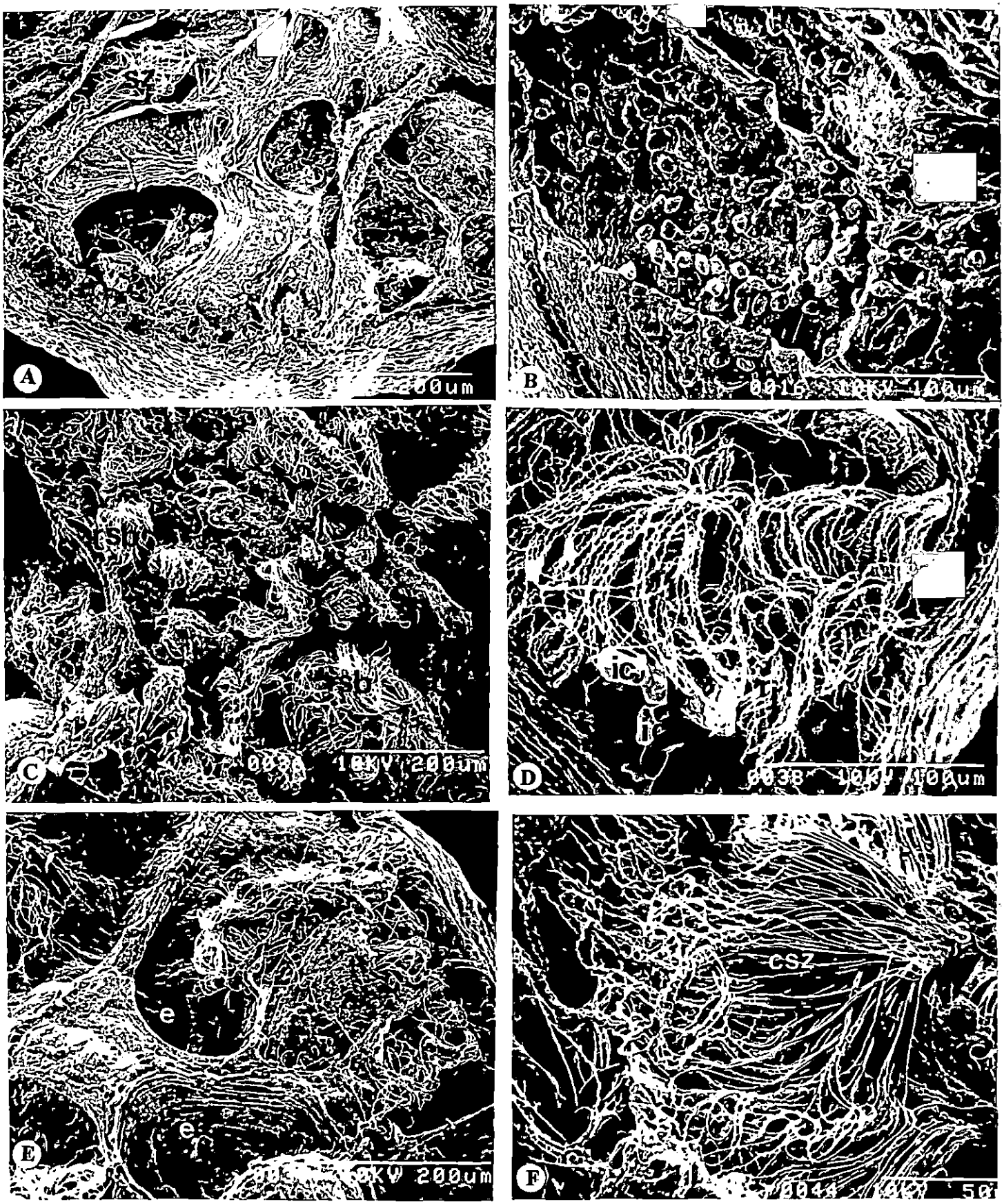


PLATE 14. Transmission electron micrographs of post-breeding testis of *Tylototriton verrucosus*. **Fig. A.** Longitudinal sections of spermatid shows elongated head and forming tail. Note condensed nucleus (n), proximal centriole (pc), axial fiber (ax) and mitochondrial sheath (m). x4783. **Fig. B.** Magnified view of connecting-piece region of elongated spermatid showing proximal centriole (pc), origin of axial fiber (ax) and arrangement of mitochondria (m).x23235. **Fig. C.** Longitudinal sections of spermatids showing accumulation of mitochondria at the mid-piece region (am), more advanced spermatid shows two rows of mitochondria (m) with forming axial filament (ax). x22550. **Fig. D.** Longitudinal sections of forming spermatid tail showing two and three rows of mitochondria. x12600. **Fig. E.** Longitudinal sections of principal-piece region of elliptical spermatid tail showing rows of mitochondria (m) and axial filaments (ax). **Fig. F.** Transverse sections through the tail piece of the sperm showing axial fiber (ax), undulating membrane (u) and axonemal complex with marginal fibers (arrows). x11250.

PLATE - 14

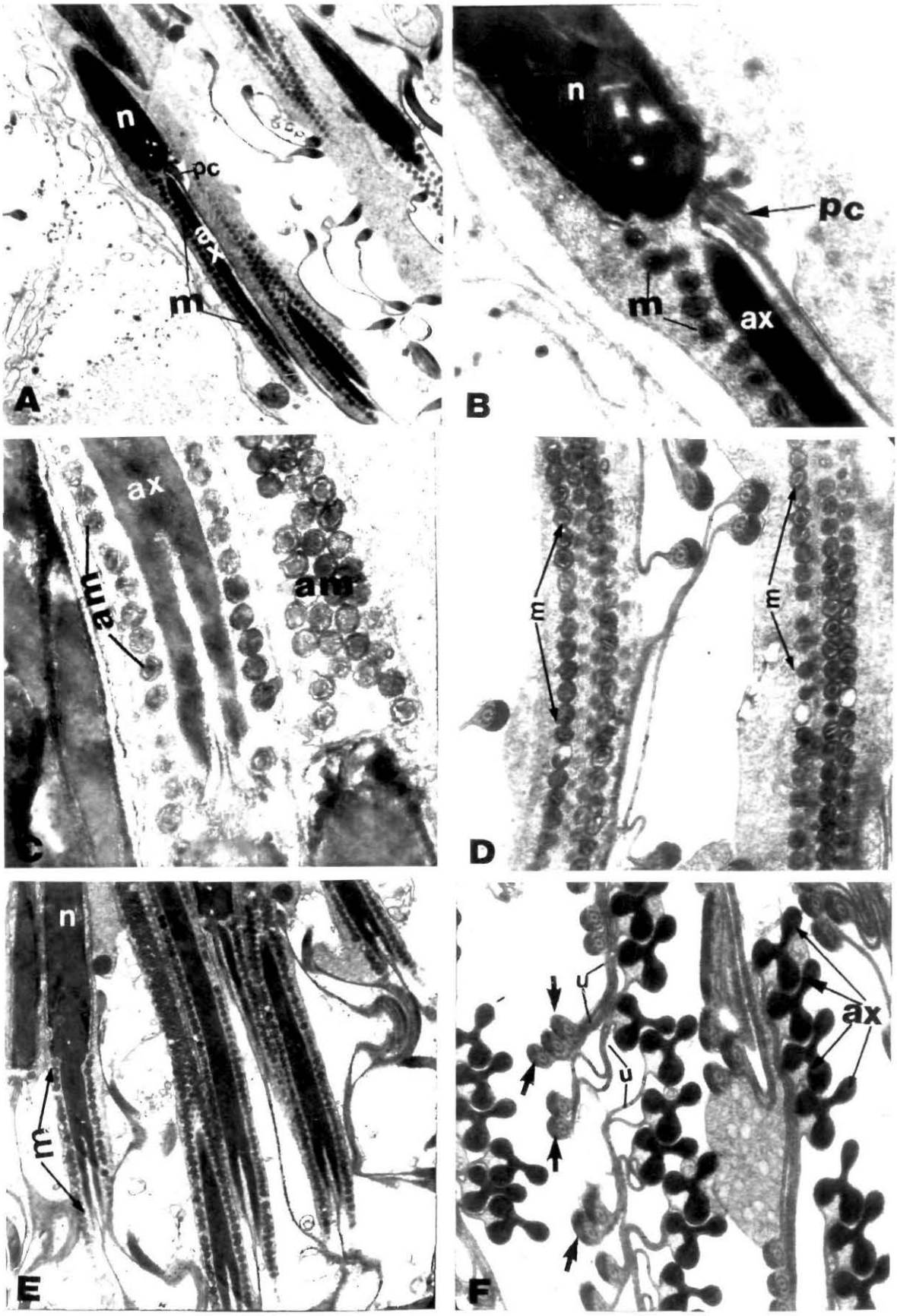


PLATE - 15

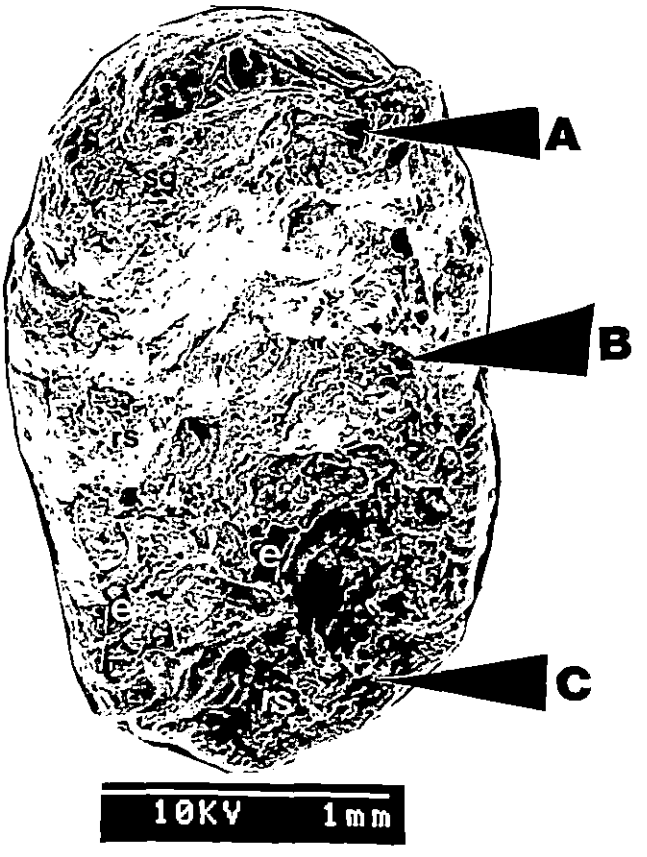


PLATE 15. Scanning Electron Microscope view of regression testis cross-section. **A** anterior part, **B** middle part, **C** distal part. **sg** indicates spermatogonial cells. **rs** indicates residual spermatozoa.

PLATE - 16

Photomicrographs of the histological section (5 μ) of regression testis of *Tylototriton verrucosus.*

Fixative : Bouins (aqueous)

Stain : Haematoxyline - eosin

Figure A : Lobules / Cysts at the middle part of the lobe showing residual spermatozoa (RSPZ).

Figure B : Distal part of the testis lobe showing evacuated lobules / cysts with residual body (R.B.)

Figure C : Distal part of the testis lobe showing hypertrophied lobules / cysts and 'Yellow Zone'.

Figure D : Lobules / Cysts at the proximal part of the testis lobe showing spermatogonial (SG) cells.

PLATE - 16

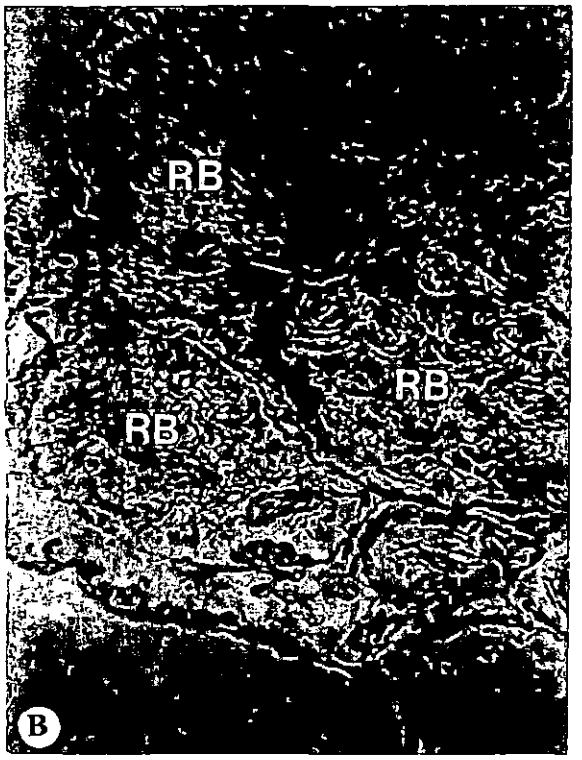


PLATE 17. Scanning Electron Microscope view of regression testis cross-section. **Fig. A.** Anterior part of the testis lobe shows cyst with small amount of gonial cell (SG). **Fig. B.** Magnified view of gonial cells (SG) at the anterior cyst of testis. **Fig. C.** At the middle portion of the testis there are some spermatocytes (SC) and spermatozoa (SPZ). **Fig. D.** Magnified view of spermatocytes show cell division (dc). **Fig. E.** Distal part of the testis shows evacuated lobules (E) **Fig. F.** In higher magnification one lobule shows residual spermatozoa (RSPZ), interstitial cells (IC) and yellow zone (YZ).

PLATE - 17

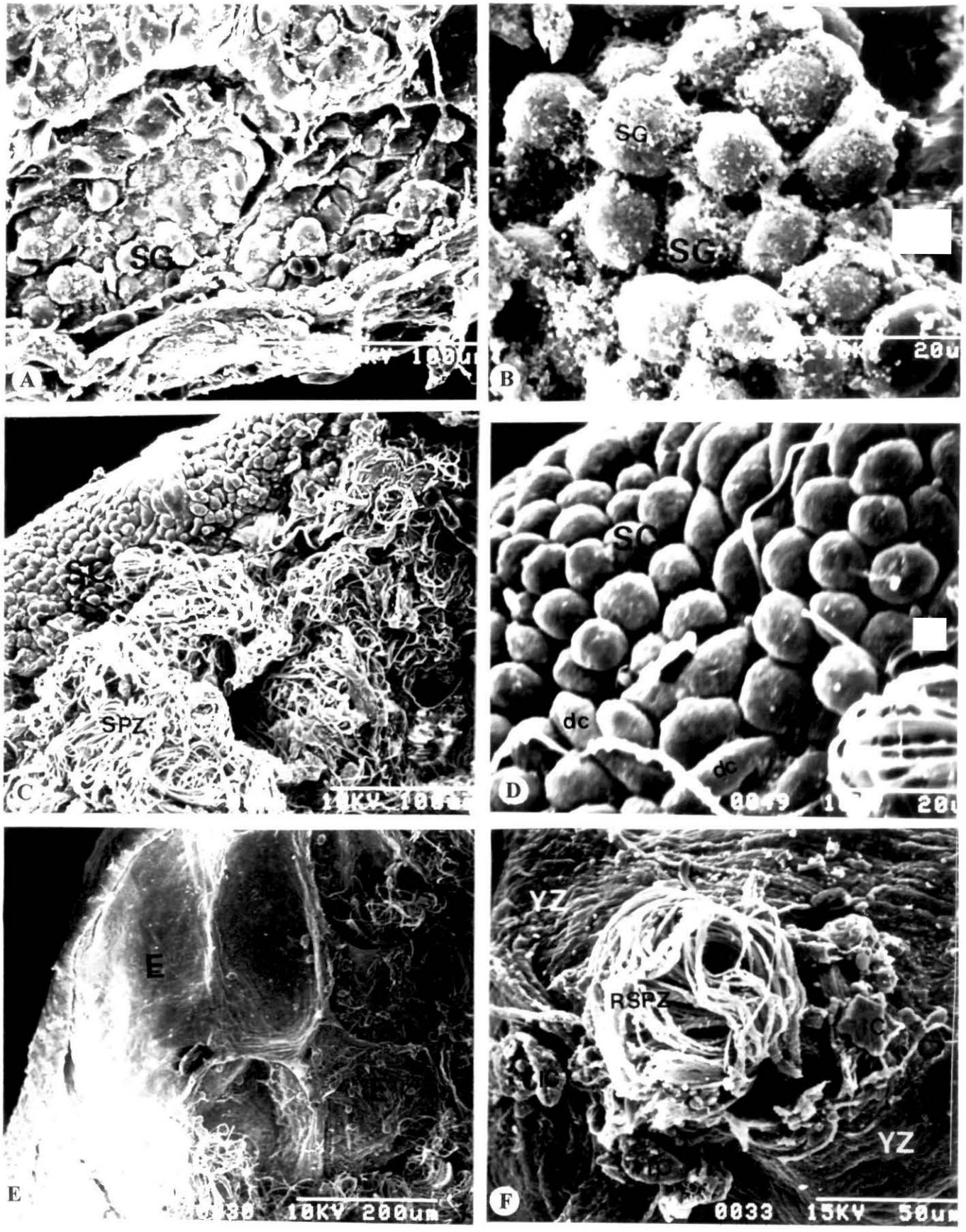
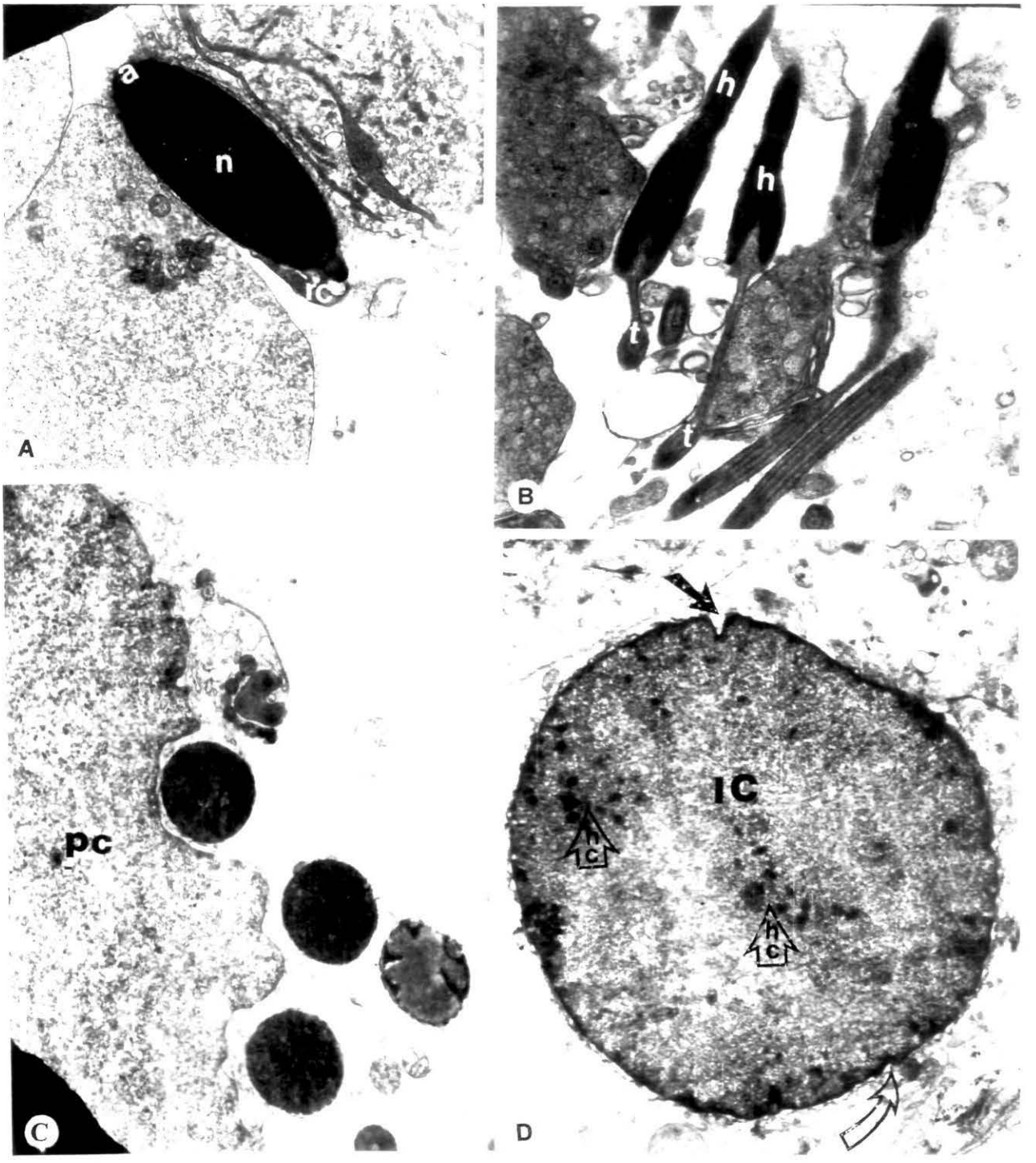

SG
A
SG
SG
B
SPZ
C
SG
dc
dc
D
E
E
10KV 200um
YZ
RSPZ
YZ
F
0033 15KV 50u

PLATE 18. Transmission electron micrographs of regression testis of *Tylototriton verrucosus*. **Fig. A.** Longitudinal section of spermatid showing condensed uniformly dark nucleus (n) with beginning of acrosome (a) and neck region, note residual cytoplasm (rc). x13885. **Fig. B.** More advanced spermatid with elongated head (h) and tail region (t).x12600. **Fig. C.** Phagocytotic cell (pc) engulf discarded cytoplasm of gonial cell and spermatozoa. x10850. **Fig. D.** Interstitial cell shows spherical nucleus with pinocytotic and phagocytotic grooves (arrows) and electron-dense hetero chromatin (hc). x14900.

PLATE - 18



stage (Plate 17; Fig. D).

iv. At the distal end of the testis lobes the lobules/cysts are mainly evacuated (Plate 17; Fig. E). But there are some lobules/cysts which are filled with some residual spermatozoa in a characteristic circular fashion (Plate 17; Fig. F). In higher magnification, one lobule/cyst shows some residual spermatozoa in a characteristic fashion with some interstitial cells. The walls of the lobule/cyst are hypertrophied and form a compact mass of tissue called the 'Yellow Zone'(Plate 17; Fig. F).

TRANSMISSION ELECTRON MICROSCOPE OBSERVATIONS

Pre-breeding Testis

The ultrastructural observations of the pre-breeding testis is confirmatory to that of light and scanning electron microscopic observations. Under transmission electron microscope (TEM) there are two types of germ cells i.e. spermatogonial cells and spermatocytic cells are found in the pre- breeding testis. Some interstitial Leydig cells and Sertoli cells are also found (Plate 15)

1. Spermatogonia

Two types of spermatogonial cells, as observed under light microscope and scanning electron microscope studies, are also recognised under transmission electron microscope. The primary spermatogonia in general exhibit a conspicuous round nucleus with fine distribution of chromatin materials and two to three nucleolus (Plate 6; Fig. A). The nuclear membrane is indistinct. The cytoplasm of the gonial cell contains a rich distribution of spherical and tubular mitochondria. A fair amount lysosomes and smooth endoplasmic reticulum are also present (Plate 6; Fig. C). Primary spermatogonia divides mitotically to give rise to secondary spermatogonia, which are initially very

similar to their mother cell, except that their nucleus is slightly smaller (Plate 6; Fig. A).

2. *Spermatocytes*

The spermatocytes are of two types, primary spermatocytes and secondary spermatocytes. Primary spermatocytes are larger than the secondary spermatocytes and chromatin material are more conspicuously distributed in the nucleus than in the secondary spermatocytes. Primary spermatocytes show various stages of meiotic prophase. The nucleolus is not visible and chromatin masses are irregularly arranged (Plate 6; Fig. B).

Secondary spermatocytes arise from the division of primary spermatocytes and show large nucleus with irregularly distributed chromatin mass. The chromatin masses are concentrated at one pole of the nucleus (Plate 6; Fig. B).

In higher magnification the cytoplasm of the spermatocytes shows large spherical and tubular mitochondria, free ribosomes and well-developed Golgi bodies. A large number of vesicles are found at the middle of the trans face of the Golgi apparatus. Such vesicles contain electron-dense materials (Plate 6; Fig. D).

3. Interstitial Cells

Many interstitial Leydig cells are found in the pre-breeding testis at the interstitial region in between lobules or cysts. They are somewhat elliptical or ovoid in shape. The nucleus of the interstitial cells are ovoid, spherical, elongated or irregular in shape. The nuclear membrane is more or less wavy in outline. At the peripheral region of the nucleus electron-dense chromatin material is arranged in a laminar fashion (Plate 6; Fig. E).

The cytoplasm contains mitochondria, small electron dense protein granules, rough endoplasmic reticulum, lysosomes, lipid droplets and different types of polysomes (Plate 6; Fig. E).

4. Sertoli Cells

The Sertoli cells are the somatic element of the germ line cells. They are distributed in the periphery of the cysts/lobules or around the spermatogonia. The Sertoli cells show irregular shaped nucleus, decondensed chromatin and with an evidence of nucleolus. At the periphery of the nucleus, some electron-dense chromatin materials are evident (Plate 6; Fig. F).

BREEDING TESTIS

The ultrastructural observations of the breeding testis is confirmatory to that of light and scanning electron microscopic observations. Under transmission electron microscope there are four types of germ cells, i.e. spermatogonial cells, spermatocytic cells, spermatids and spermatozoa are found in the breeding testis.

1. Spermatogonia

The number of spermatogonial cells found in breeding testis are relatively less than that found in pre-breeding testis. There are two types of spermatogonial cells as observed under light microscope and scanning electron microscope studies, they are also recognised under transmission electron microscope. The primary spermatogonia exhibit a conspicuous round nucleus with fine distribution of chromatin materials and two to three nucleolus (Plate 10; Fig. A). The nuclear membrane is indistinct. The cytoplasm of the gonial cell contains a rich distribution of spherical and tubular mitochondria. A fair amount of lysosomes and smooth endoplasmic reticulum are also present (Plate 10; Fig. A). The primary spermatogonia divides mitotically to give rise to secondary spermatogonia, which are initially very similar to their mother cell, except their

nucleus which is slightly smaller. These primary and secondary spermatogonial cells are very much similar to the gonial cells which are found in pre-breeding testis, discussed earlier.

2. Spermatocytes

The spermatocytes which are found in the breeding testis are very much similar to that of spermatocytes found in pre-breeding testis. They are of two types, primary spermatocytes and secondary spermatocytes. Primary spermatocytes are larger than the secondary spermatocytes and the chromatin material are more conspicuously distributed in the nucleus than secondary spermatocytes. Primary spermatocytes show various stages of meiotic prophase. The nucleolus is not visible and chromatin masses are irregularly arranged (Plate 10; Fig. B).

Secondary spermatocytes arise from the division of primary spermatocytes and show large nucleus with irregularly distributed chromatin mass. The chromatin masses are concentrated at one pole of the nucleus.

In higher magnification the cytoplasm of spermatocyte shows large spherical and tubular mitochondria, free ribosomes and well-developed Golgi bodies (Plate 10; Fig. B).

3. Spermatids

The spermatids are seen in different stages of spermiogenesis as indicated by the degree of chromatin condensation and the presence of flagellum. (Plate 10; Fig. C). Initially, they contain chromatin in course granules, evenly distributed throughout the nucleus (Plate 10; Fig. E). With the progress of the spermiogenesis process, the chromatin materials condensed into dense clot and the cell becomes smaller and more condense (Plate 24; Fig. A). Simultaneously, the volume of cytoplasm decreases considerably. Some mitochondria increase in volume and encompass the initial part of the

flagellum, forming the mitochondrial sheath, the future middle piece of the spermatozoa (Plate 16; Fig. C). More detailed study of the different forms of spermatids is given in the chapter, dealing with spermatogenesis.

4. Spermatozoa

The head of the sperm is very long and cylindrical, tapering anteriorly to slender point bearing a complex acrosomal cap which is provided with a sharp point and recurving hook-like barb (Plate 25; Fig. E, F). The caudal fourth of what appears under the light microscope to be the sperm head consists of a cylindrical structure which has the same diameter as the nucleus but differs from it in staining affinity and in its density in the electron micrographs. This has been called the connecting piece or intermediate piece. At its caudal end, it is continuous with the axial rod which is the principle supporting structure of the tail. The undulating membrane attaches to the margin of a groove that runs along one side of the axial fiber (Plate 26; Fig. B, Plate 10; Fig. B). In the edge of the membrane is a dense marginal filament – a flexible supporting element that courses longitudinally along one side of a typical 9+2 axonemal complex (Plate 28; Fig. C).

Throughout the greater part of the length of the tail the axial fiber is partially surrounded by mitochondria in the proximal two thirds of the tail, consistency demands that this long segment be called the middle piece – the term in general use for the segment of a sperm containing the mitochondria. The portion of the tail from the end of the sheath of cytoplasm to the termination of the axial fiber is the principal piece (Plate 26; Fig. D, Plate 27; Fig C). The end piece is the short tapering segment of the tail extending beyond the end of the undulating membrane. It consists of the axoneme and a short terminal segment of the marginal filament enclosed only by the plasma membrane. More detailed study of different forms of spermatozoa is given in the chapter

dealing with sperm ultrastructure.

Post-breeding testis

Under transmission electron microscope the post-breeding testis shows gonial cells, spermatocytes, spermatids, mature and maturing spermatozoa.

Different stages of spermatids are seen, viz. spermatids with elongated head and forming tail (Plate 14; Fig. A), spermatids with forming mid piece showing accumulation of mitochondria (Plate 14; Fig. B, C), forming principal piece with rows of mitochondria are also seen (Plate 14; Fig. D). The gonial cells and the spermatocytes show characteristics as found in breeding and pre breeding testes. Their individuals is greatly reduced than that in the pre-breeding testis. The mature spermatozoa looks like same as it is found in the breeding testis (Plate 25; Fig E, F). The number of spermatozoal cysts are very much greater than that of the breeding and pre-breeding testis.

Regression Testis

Under transmission electron microscope, the regression testis shows gonial cells, spermatocytes, spermatids and spermatozoa. Some large phagocytotic cells and spherical interstitial cells are also found (Plate 18; Fig. A, B, C, D).

The gonial cells, spermatocytes, spermatids and spermatozoa are showing the same characteristics as found in the breeding and pre-breeding testes. The phagocytic cells are bigger in size and engulf dead and discarded portion of the gonial cells and spermatozoa (Plate 18; Fig. C). The interstitial cells are spherical in shape. The nuclear membrane is more or less wavy in nature. Some pinocytotic and phagocytotic grooves are also seen. At the peripheral region of the nucleus electron dense chromatin material is arranged in a laminar fashion and patch of dense chromatin materials are also seen in center of the nucleus (Plate 18; Fig. D).

SPERMATOGENESIS

In amphibians, spermatogenesis is of cystic type, i.e., the cells present in a cyst are in the same stage of development and are derived from a single spermatogonium. At the histological level spermatogenic activity vis a vis changes are assessed or made following the principle laid down by Van Oordt (1956) and subsequently modified by Saidapur (1989). Accordingly, the quantitative assessment of spermatogenic activity in histological profile is adopted with the identification of following cell types in a mature testis:

Stage 0	Primary spermatogonia
Stage I	Secondary spermatogonia
Stage II	Primary spermatocyte
Stage III	Secondary spermatocyte
Stage IV	Spermatid
Stage V	Sperm bundle attached to Sertoli cell

In the present text quantative aspect of spermatogenesis has been assessed employing three different methods, Light microscopy, Scanning electron microscopy and Transmission electron microscopy.

A. LIGHT MICROSCOPE OBSERVATIONS

1. Primary spermatogonia

Primary spermatogonial cells are round in shape and moderately stained. The nucleus of primary spermatogonia was round shaped and chromatin materials are uniformly distributed. The nucleus bears one to three nucleolus. A thin layer of cytoplasm is seen around the nucleus. The cytoplasm of the gonial cells is well connected with each other (Plate 19; Fig. A).

2. Secondary spermatogonia

Secondary spermatogonial cells are round in shape and moderately stained. These cells are very much similar to primary spermatogonial cells in context of staining intensity but they are smaller in size. Secondary and primary gonial cells are found in same cysts or lobules (Plate 19; Fig. A).

3. Primary spermatocytes

Primary spermatocytes are the largest cells in the cysts or lobules. They have a large nucleus and very thin cytoplasmic rim around it. The nucleus bears different stages of meiotic prophase. Heterochromatin materials are found all over the nucleus (Plate 19; Fig. B).

4. Secondary spermatocytes

Secondary spermatocyte cells are round in shape and deeply stained. These cells are very much similar to primary spermatocyte cells in context of staining intensity but they are smaller in size. Secondary and primary spermatocytes are found in same cysts or lobules (Plate 19; Fig. B).

5. Spermatids

The spermatids are classified according to their size, shape and nature of staining. An early spermatid has a round shape with compact round shape nucleus which possesses a thin rim of cytoplasm around it (Plate 19; Fig. C). A mid spermatid has an oblong nucleus with a concentrated cytoplasm at the posterior end, representing the neck forming region (Plate 19; Fig. D). Elongated spermatid shows an ovoid nucleus and a distinct neck region (Plate 19; Fig. E). Further condensation of the apical region along with the elaboration of neck region occurred at later stage (Plate 19; Fig. F).

6. Mature Spermatozoon

A mature spermatozoon is long with somewhat corrugated sickle shaped head about 90 μm in length, a short neck region and a tail of about 129 μm in length (Plate 29; Fig. A). The sperm tail is represented by a long axial fiber and a thin undulating membrane (Plate 29; Fig. A). A polymorphism in head morphology of the sperm is highly characteristic. Sickle shaped, club shaped and round shaped heads are observed in the air dry preparation (Plate 29; Fig. A, B, C).

B. SCANNING ELECTRON MICROSCOPE OBSERVATIONS

Mature gonial cell are somewhat spherical in shape and showing rough surface and irregular protubation. The structures suggest connections between gonial cells (Plate 20; Fig. A, E). Spermatocytes are large spherical or ovoid in shape. These cells are electron dense and relatively smooth (Plate 21; Fig. F). Round shape spermatids exhibit thin cytoplasmic rim and electron-dense nuclear region with a protuberance for future neck region (Plate 20; Fig. B). Mid spermatids with spiral neck region and enlarged nuclear region shows somewhat coarse surface (Plate 20; Fig. C). Late spermatids show long tail and elongated head. Elongated head shows beginning of acrosome formation (Plate 20; Fig. G). Pre mature sperm shows broad mid-piece and mature spermatozoa shows a long perforatorium, sickle shaped head, short slender and course mid piece, and a long tail with undulating membrane (Plate 20; Fig. H; Plate 21; Fig. A, B, C, D, E, F).

Mature sperms are lodged in cysts/lobules in a characteristic fashion, where the head of sperm tie up into one end and embedded in to Sertoli cell (Plate 2O; Fig. D). Polymorphism in sperm structure is observed and two type head structure is very common, one with slender pointed perforatorium and the other with hook like perforatorium (Plate 21; Fig. A, B C).

C. TRANSMISSION ELECTRON MICROSCOPE OBSERVATIONS

1. Spermatogonia

Two types of spermatogonial cells, as observed under light microscope and scanning electron microscope are also recognized under transmission electron microscope. The primary spermatogonia exhibit a conspicuous round nucleus with fine distribution of chromatin materials and one to three nucleolus (Plate 22; Fig. B, C). The nuclear membrane is indistinct. The cytoplasm of the gonial cell contains a rich distribution of spherical and tubular mitochondria. A fair amount lysosomes and smooth endoplasmic reticulum are also present (Plate 22; Fig. D). Primary spermatogonium divides mitotically to give rise secondary spermatogonia, which are initially very similar to their mother cell, except that their nucleus is slightly smaller (Plate 22; Fig. A).

2. Spermatocytes

The spermatocytes are of two types, primary spermatocytes and secondary spermatocytes. Primary spermatocytes are larger than the secondary spermatocytes and chromatin materials are more conspicuously distributed in the nucleus than secondary spermatocytes. Primary spermatocytes show various stages of meiotic prophase. The nucleolus is not visible and chromatin masses are irregularly arranged (Plate 10; Fig. B, Plate 23; Fig. A).

Secondary spermatocytes arise from the division of primary spermatocytes and show large nucleus with irregularly distributed chromatin mass. The chromatin masses are concentrated at the one pole of the nucleus (Plate 10; Fig. B).

In higher magnification the cytoplasm of spermatocytes shows large spherical and tubular mitochondria, free ribosomes and well-developed Golgi bodies. A large number vesicles are found at the middle of the trans face of the Golgi apparatus. Such vesicles contains electron dense materials (Plate 23; Fig. B).

Dividing primary spermatocytes show nuclear vaculation and unequal distribution of nuclear materials (Plate 23; Fig. C).Higher magnification of primary spermatocyte nucleus shows distribution of chromatin materials concentrated towards the polar region of the nucleus (Plate 23; Fig. D).

3. Spermatids

The spermatids are seen in different stages of spermiogenesis as indicated by the degree of chromatin condensation and the presence of a flagellum (Plate 10; Fig. E, C). Initially, they contain chromatin in course granules, evenly distributed throughout the nucleus. With the progress of the spermiogenesis process, the chromatin condenses into dense clot and the cell becomes smaller and more condense. Simultaneously the volume of cytoplasm decreases considerably (Plate 24; Fig A). Some mitochondria increase in volume and encompass the initial part of the flagellum, forming the mitochondrial sheath, the future middle piece of the spermatozoa (Plate-10; Fig C, E, Plate - 25; Fig. A, B, C). Some spermatids become elongated and show different stages of acrosome and tail formation. (Plate - 24; Fig. C, B, E). Higher magnification shows elliptical forming head with electron dense dark acrosome and comparatively light electron-dense nucleus (Plate - 24; Fig. D). Some advanced spermatids with elliptical head and a short tail is also observed. These spermatids show many mitochondria in a linear fashion along with axonemal complex and a distinct centriole at the connective piece region of the future sperm. (Plate - 24; Fig. F)

4. Spermatozoa

The general structure of the spermatozoa, described under the section ULTRASTRUCTURE OF SPERMATOZOA.

PLATE 19. Light microscope observation of spermatogenesis. **Fig. A.** Primary spermatogonial cells (psg) show moderately stained round shape nucleus (n) and thin cytoplasmic rim (c), secondary spermatogonial cells (ssg) show small nucleus. [Semithin section (1μm), toluidine blue stain]. **Fig. B.** Primary spermatocytes (psc) show deeply stained round shape nucleus (n) with hetero chromatin material, secondary spermatocytes (ssc) show small nucleus (n). [Semithin section (1μm), toluidine blue stain]. **Fig. C.** Early spermatid (std) with round shape compact nucleus (n) [Air-dry preparation, Leishman stain]. **Fig. D.** Spermatid (std) with forming neck region (arrow). **Fig. E.** Elongated spermatid (estd) shows ovoid nucleus (arrow) and a distinct neck region (small arrow), [Air dry preparation, Leishman stain]. **Fig. F.** Late spermatid (lstd) with differentiating head (h) and tail (t) region. [Air-dry preparation, Leishman stain]. **Fig. G.** Mature sperm bundle (sb) attached with Sertolicell. [Histological section (5μm), Haematoxyline - eosin stain]

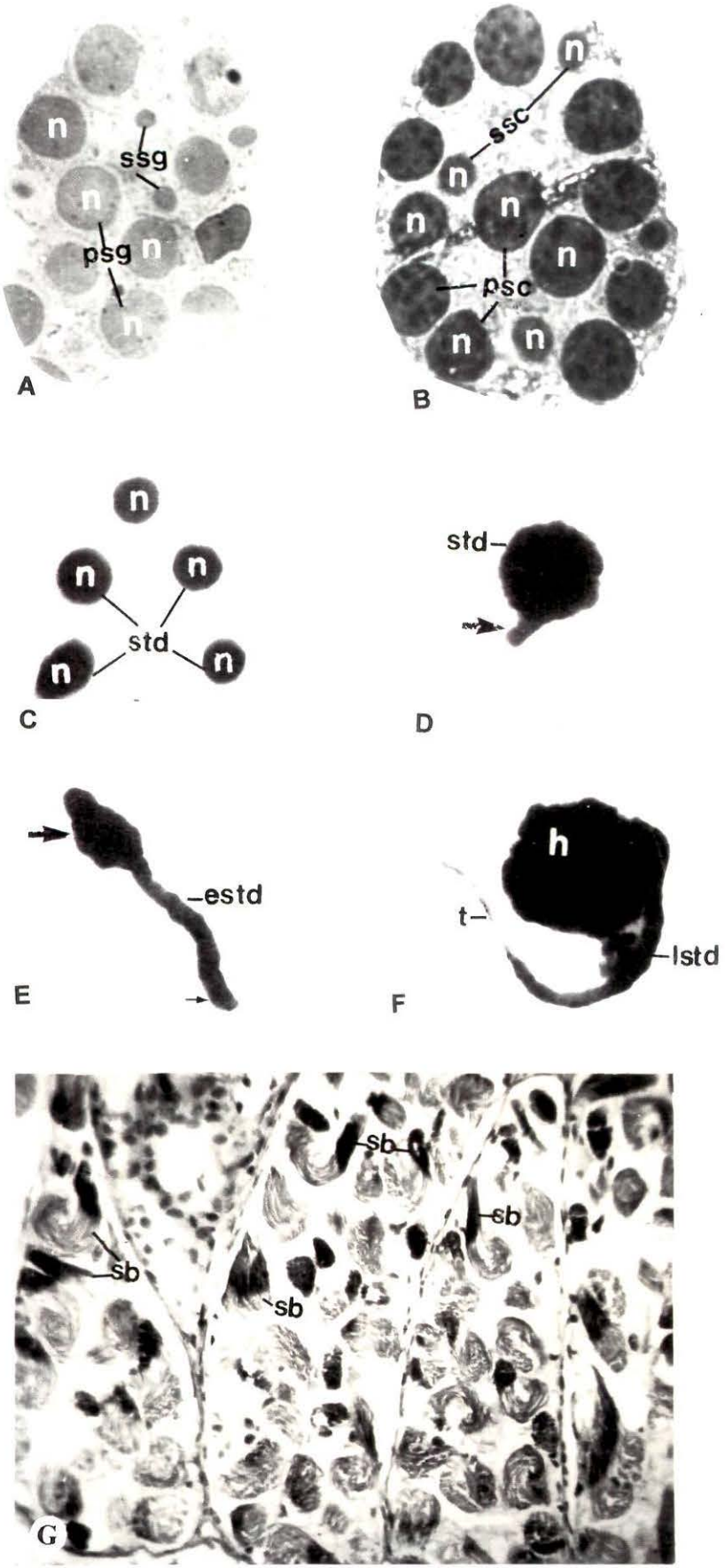

n
ssg
psg
A
ssc
psc
B
std
C
D
estd
E
h
t
lstd
F
sb
G

PLATE 20. Scanning Electron Microscope observation of spermatogenesis. **Fig. A.** Mature gonial cell shows rough surface and irregular protuberance (arrows). **Fig. B.** Round shape spermatid exhibits thin cytoplasmic rim (c) and electron-dense nuclear region (n) with a projection for future neck region (arrow). **Fig. C.** Mid-spermatid with spiral neck region (arrow) and enlarged nuclear region (n). **Fig. D.** Mature sperm bundle (sb) attached with Sertoli cell. **Fig. E.** Dividing gonial cell shows protuberance and protuberance receptor site (arrow). **Fig. F.** Spermatocytes (sc). **Fig. G.** Elongated head of spermatid shows acrosome formation (arrow). **Fig. G.** Mature spermatozoa with long perforatorium (p), sickle shaped head region (h), with mid piece (m) and long tail (t) with undulating membrane (u).

PLATE - 20

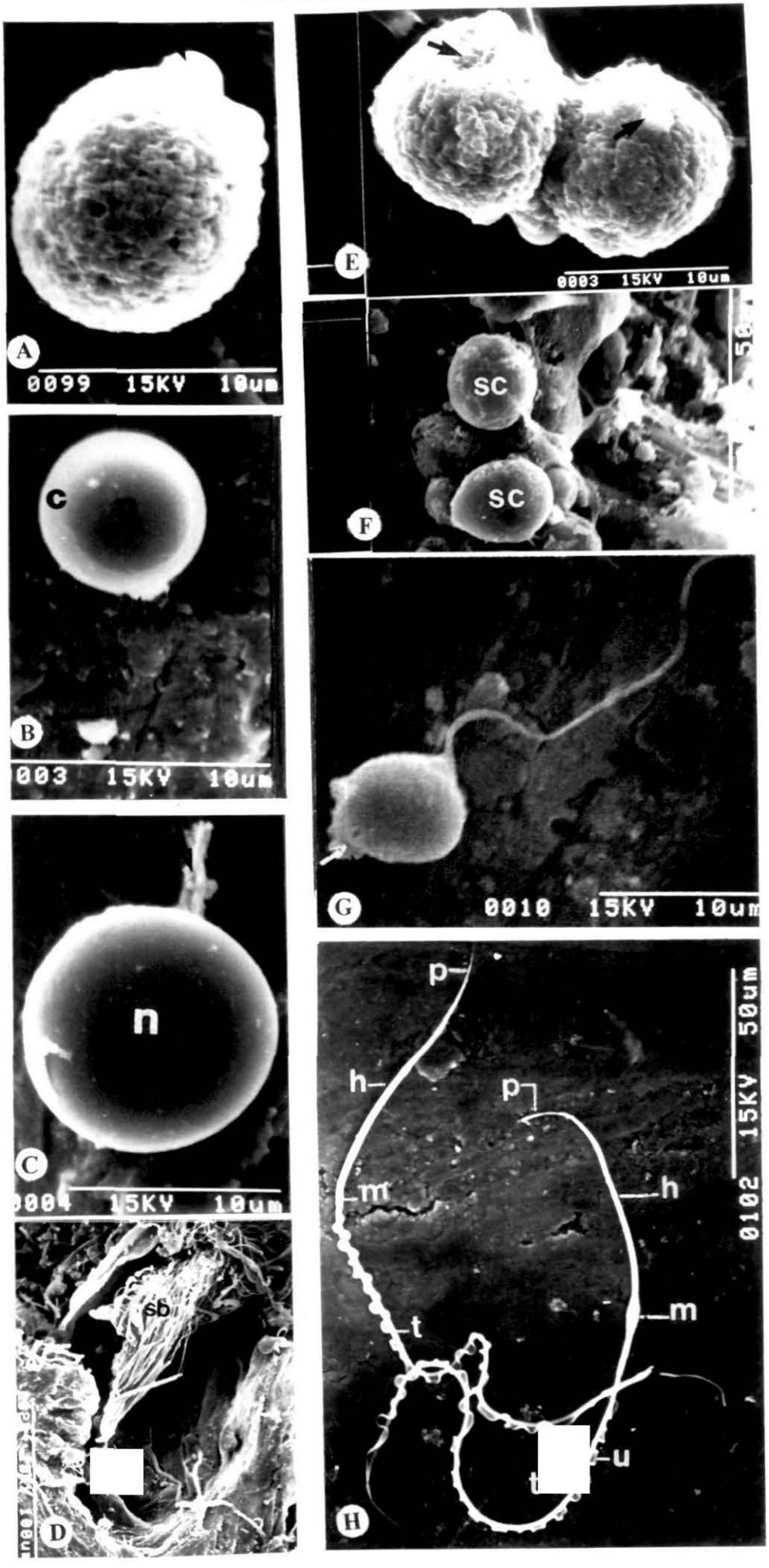
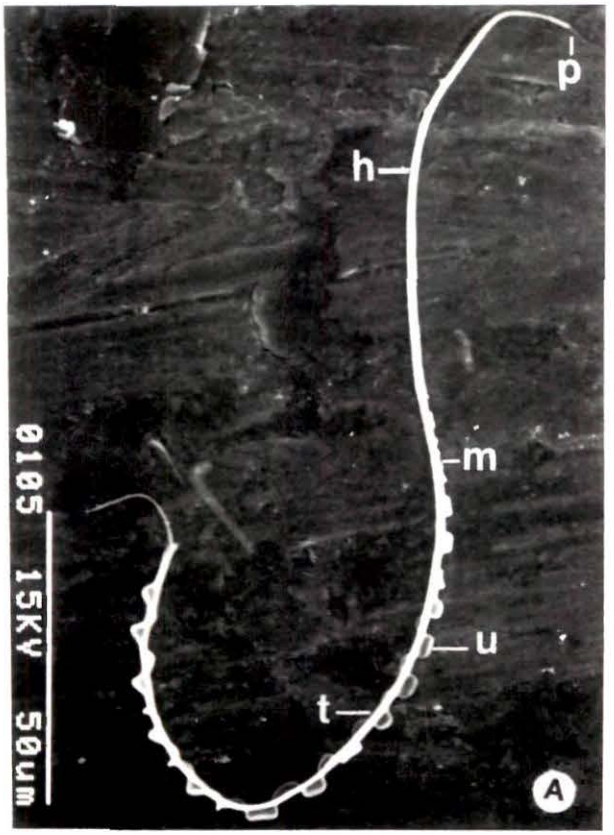
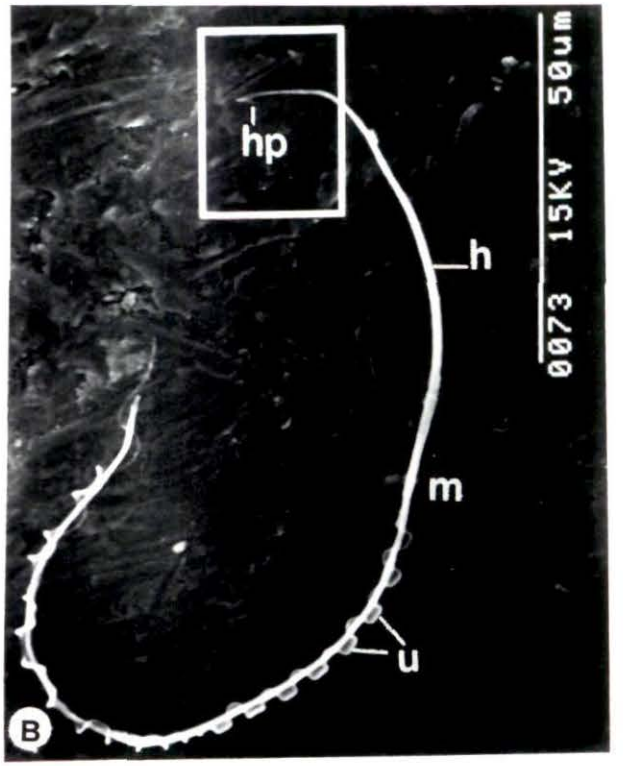
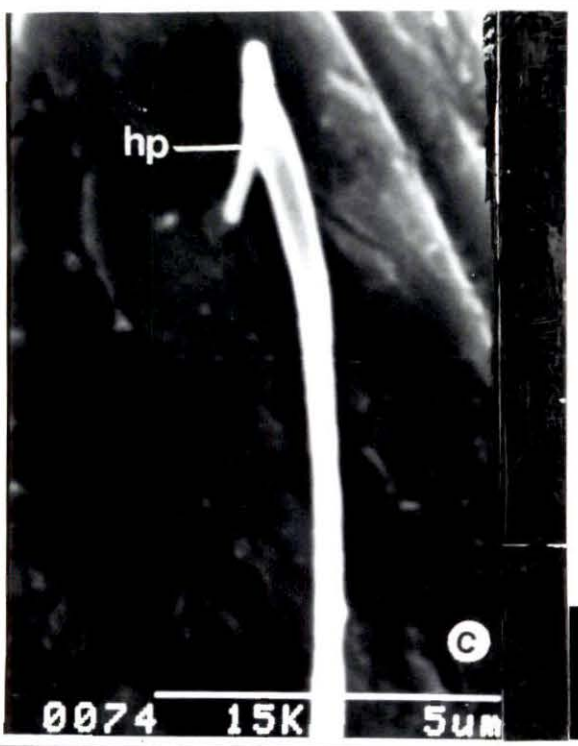

A
0099 15KV 10um
E
0003 15KV 10um
SC
SC
F
c
B
003 15KV 10um
G
0010 15KV 10um
n
C
15KV 10um
p
h
p
m
h
m
t
u
H
0102 15KV 50um
sb
D

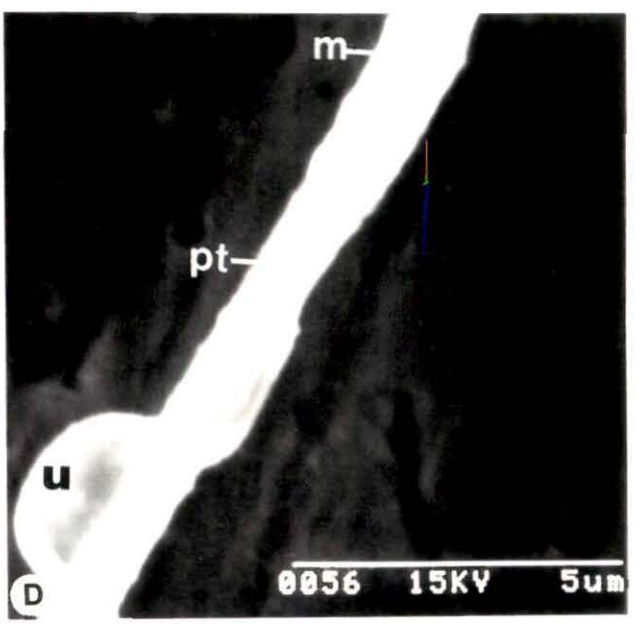
PLATE 21. Scanning electron microscope observation of spermatogenesis. **Fig. A.** Mature spermatozoa showing thin slender perforatorium (p), sickle shaped long head (h), long tail with undulating membrane (u). **Fig. B.** Mature spermatozoa showing hook like perforatorium (hp), sickle shaped long head (h) and long tail with undulating membrane (u).**Fig. C.** Magnified view of hook like perforatorium (hp). **Fig. D.** Magnified view of mid-piece (m) and principal piece of tail (pt). **Fig. E.** Principal piece of sperm showing undulating membrane (u). **Fig. F.** End-piece of tail showing undulating membrane (u).

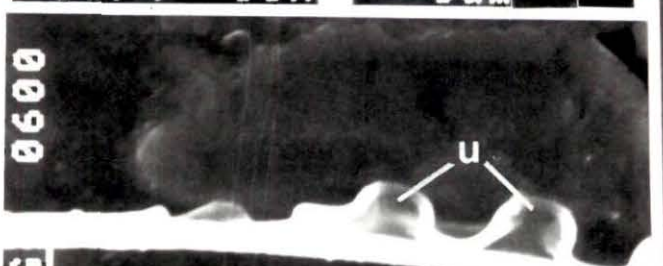
PLATE - 21


p
h
m
u
t
0105 15KV 50um
A

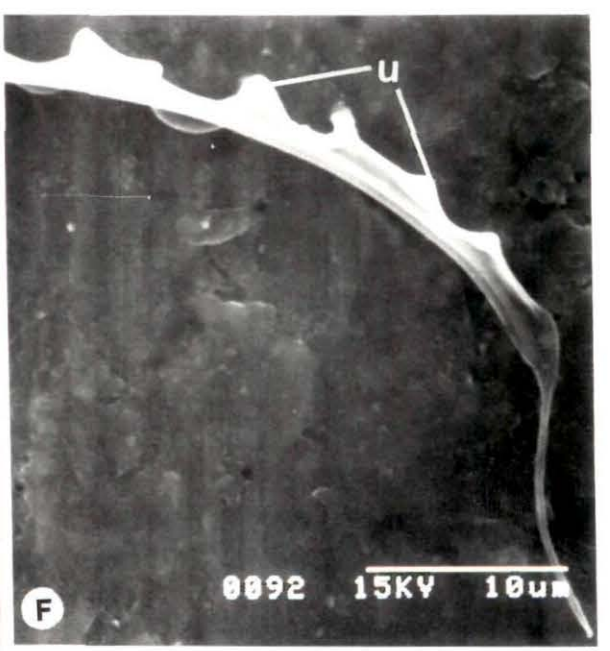

hp
h
m
u
0073 15KV 50um
B


hp
C
0074 15K 5um


m
pt
u
D
0056 15KV 5um


0090
u


5KV 10um
E


u
0092 15KV 10um
F

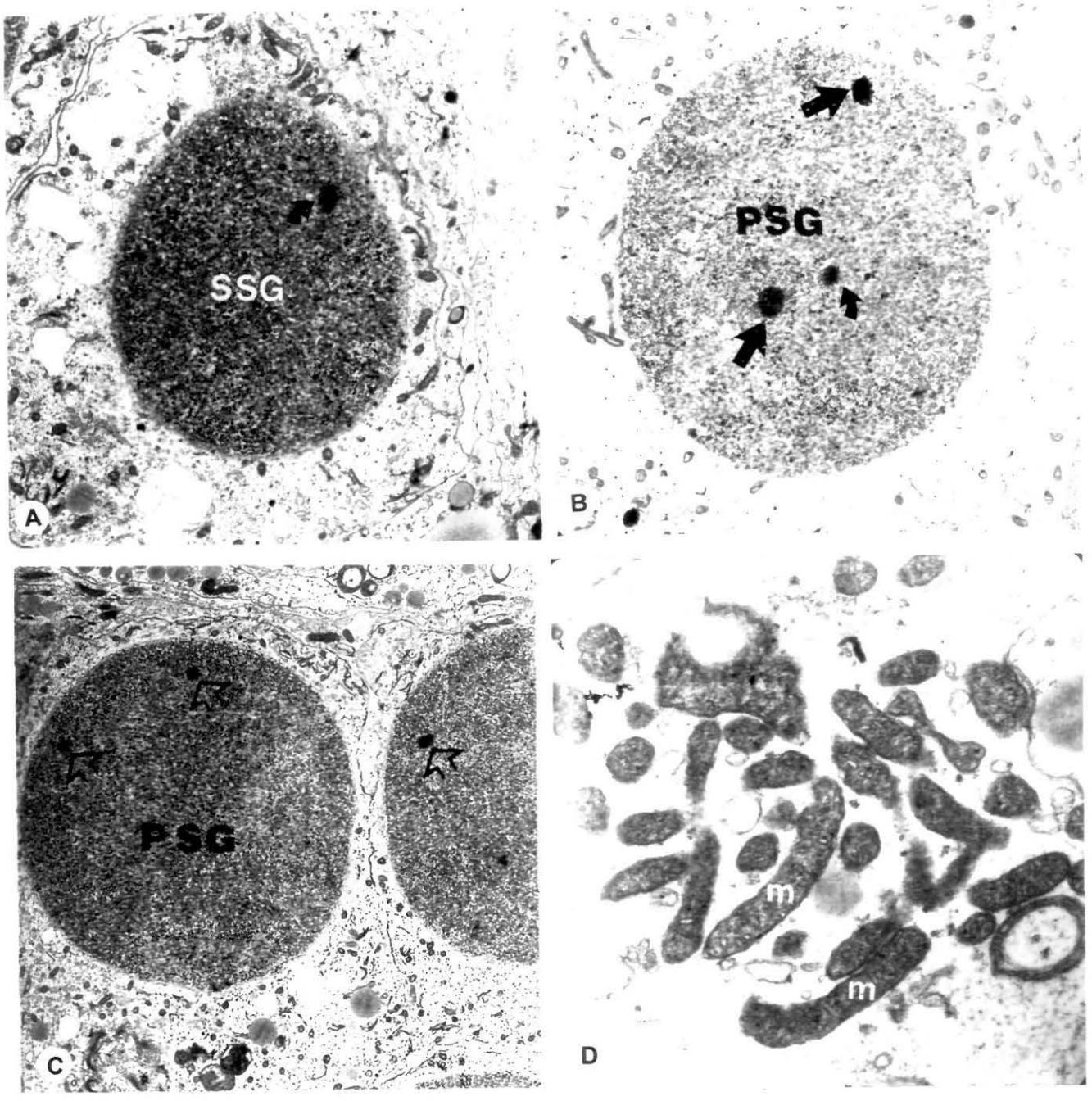


PLATE 22. Transmission electron micrographs of spermatogenesis of *Tylototriton verrucosus*. **Fig. A.** Secondary spermatogonial cell (SSG), note comparatively small nucleus and one nucleolus (arrows). Cytoplasm showing many vacuoles (arrows), mitochondria, ribosomes and lipid droplets. x6815. **Fig. B.** and **C.** Primary spermatogonia cells (PSG),arrows indicate nucleolus. x6150 and x4512. **Fig. D.** Cytoplasm of gonial cell showing rich distributions of mitochondria (m).x16585.

PLATE - 23

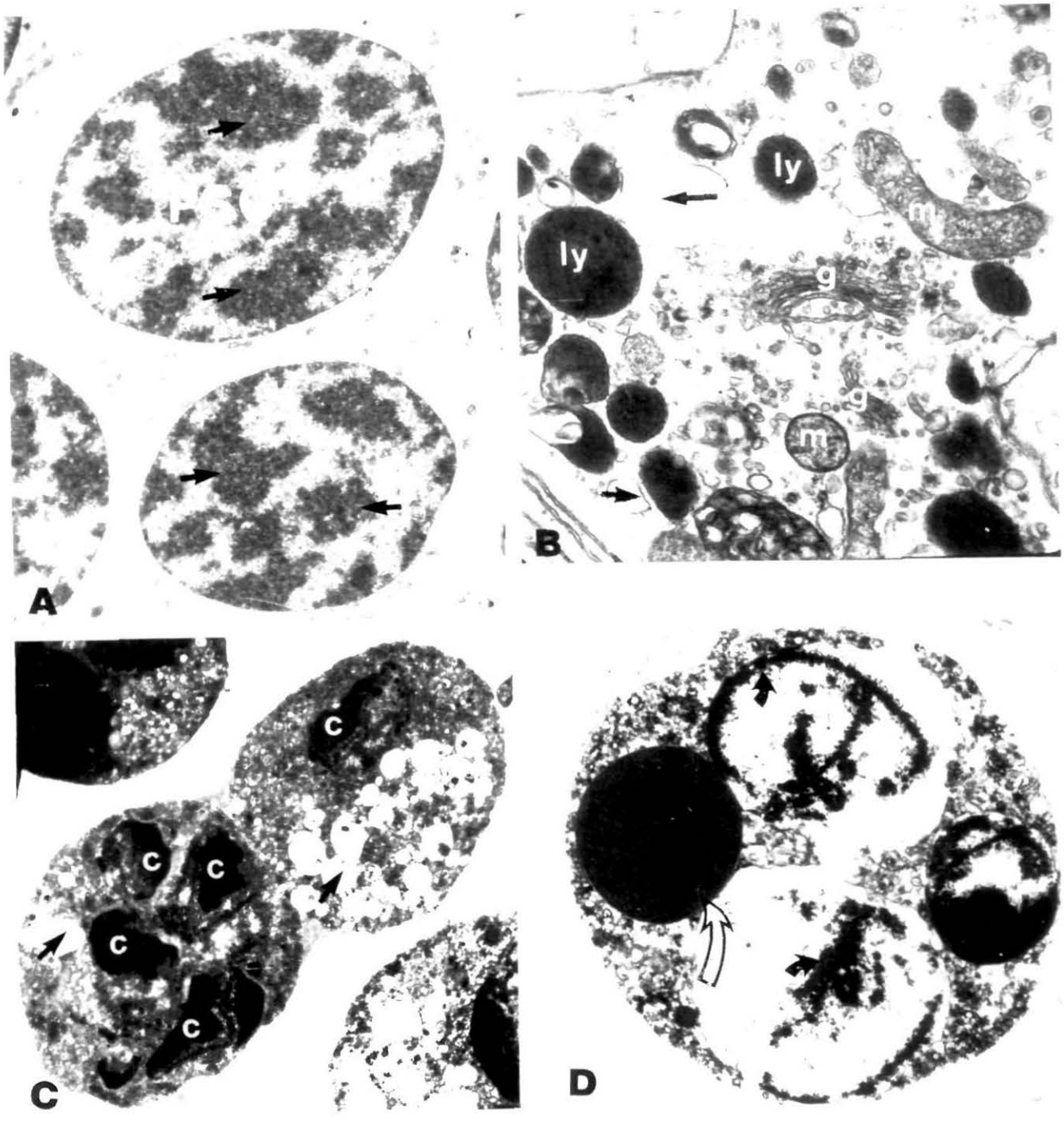
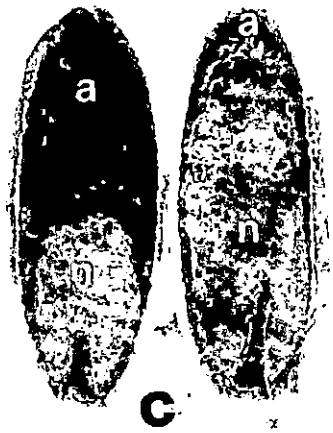


PLATE 23. Transmission electron micrographs of spermatogenesis of *Tylototriton verrucosus*. **Fig. A.** Primary spermatocytes (PSC) showing large nucleus with irregular arrangement of chromatin materials (arrows).x4184. **Fig. B.** Primary spermatocytes cytoplasm showing many mitochondria (m), lysosomes (ly), Golgi-bodies (g), many vacuoles (arrows) and lipid droplets. x21086.. **Fig. C.** Dividing spermatocytes showing condensed chromatin materials (c) and many small vacuoles (arrows).x3524. **Fig. D.** Nucleus of spermatocytes showing characteristics distribution of chromatin materials (arrows). x4420.

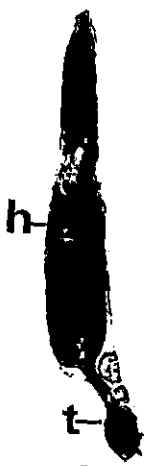
PLATE 24. Transmission electron micrographs of different stages of spermiogenesis **Fig. A.** Longitudinal section of spermatid shows uniformly dark nucleus (n), forming neck (k) and acrosomal (a) region and residual cytoplasm (arrow).x13657. **Fig. B** and **E.** More advanced spermatids with elongated head and tail. x10800. **Fig. C.** Spermatids showing acrosome formation. x19440. **Fig. D.** More advanced stage of acrosome formation showing dark acrosome (a) and gray nucleus (n). **Fig. F.** Premature spermatozoa shows elongated head and forming tail. Note condensed nucleus (n), proximal centriole (pc), axial fiber (ax) and mitochondrial sheath (m). x4783.

PLATE - 24

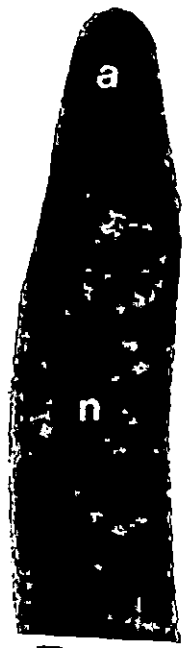
A



C

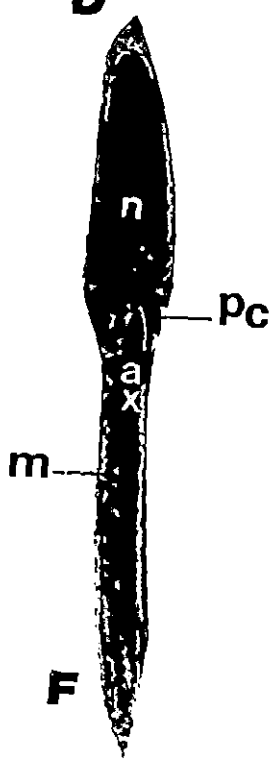


B



D

E



F

PLATE 25. Transmission electron micrographs of different stages of spermiogenesis **Fig. A.** Longitudinal section of spermatid shows uniformly gray nucleus (n), dark acrosome (a) and forming perforatorium (arrow),at the end part of nucleus accumulation of mitochondria is characteristics (m). **Fig. B** and **C.** Shows orientation of mitochondria (m) at the mid-piece region and formation of axial filament (ax).x22550. **Fig. D** Pre-mature sperm head showing perforatorium (pf), acrosome (a) and nucleus. x24600. **Fig. E.** Sperm head shows slender perforatorium (spf), dark acrosome (a) and sub-acrosomal space (sa).x4784. **Fig. F.** Mature sperm head showing hook shaped blunt perforatorium (hpf), dark acrosome (a) with sub-acrosomal space (sa), dark nucleus (n) and plasma membrane (p) with thin layer of cytoplasm. x4784.

PLATE - 25

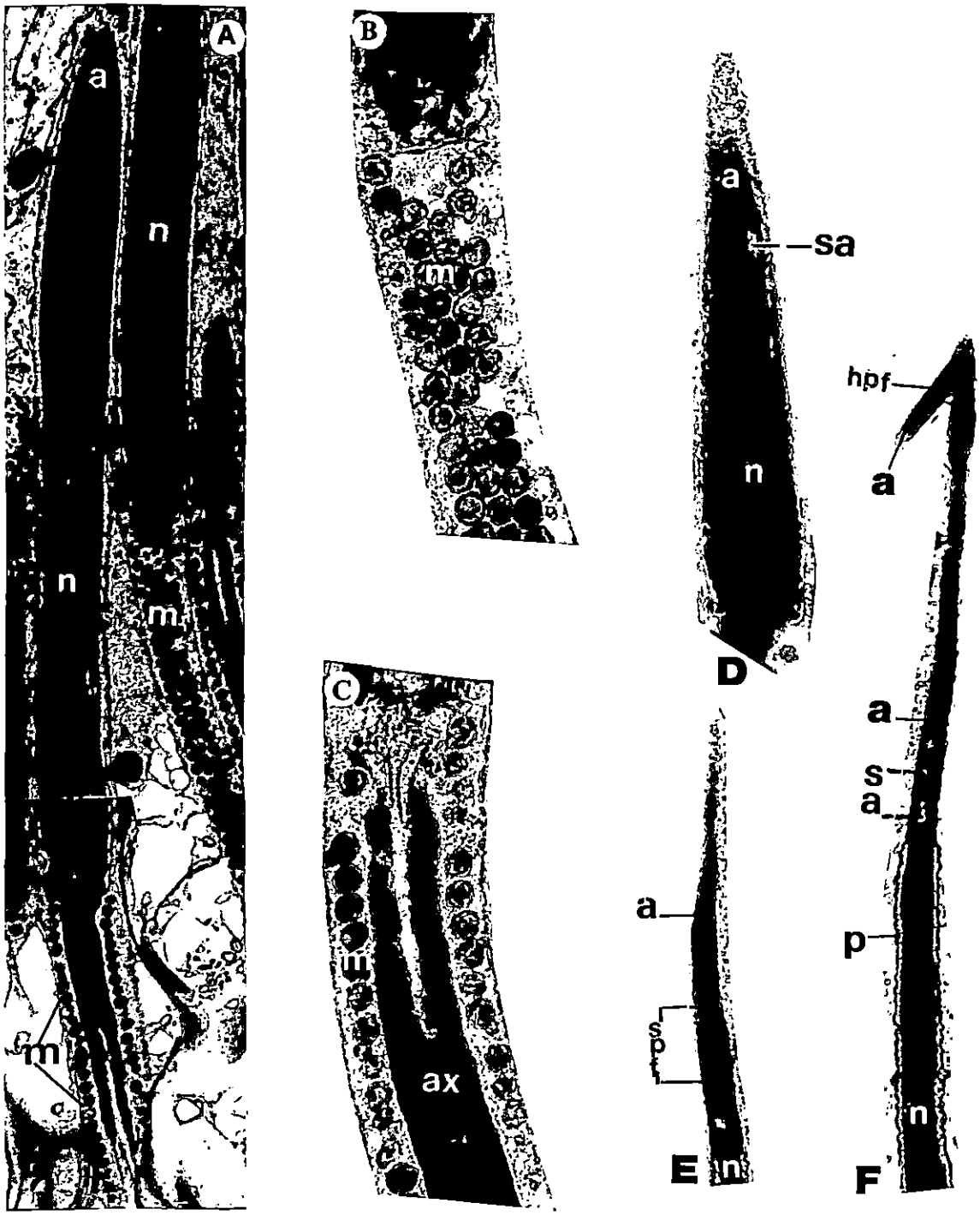


PLATE - 26

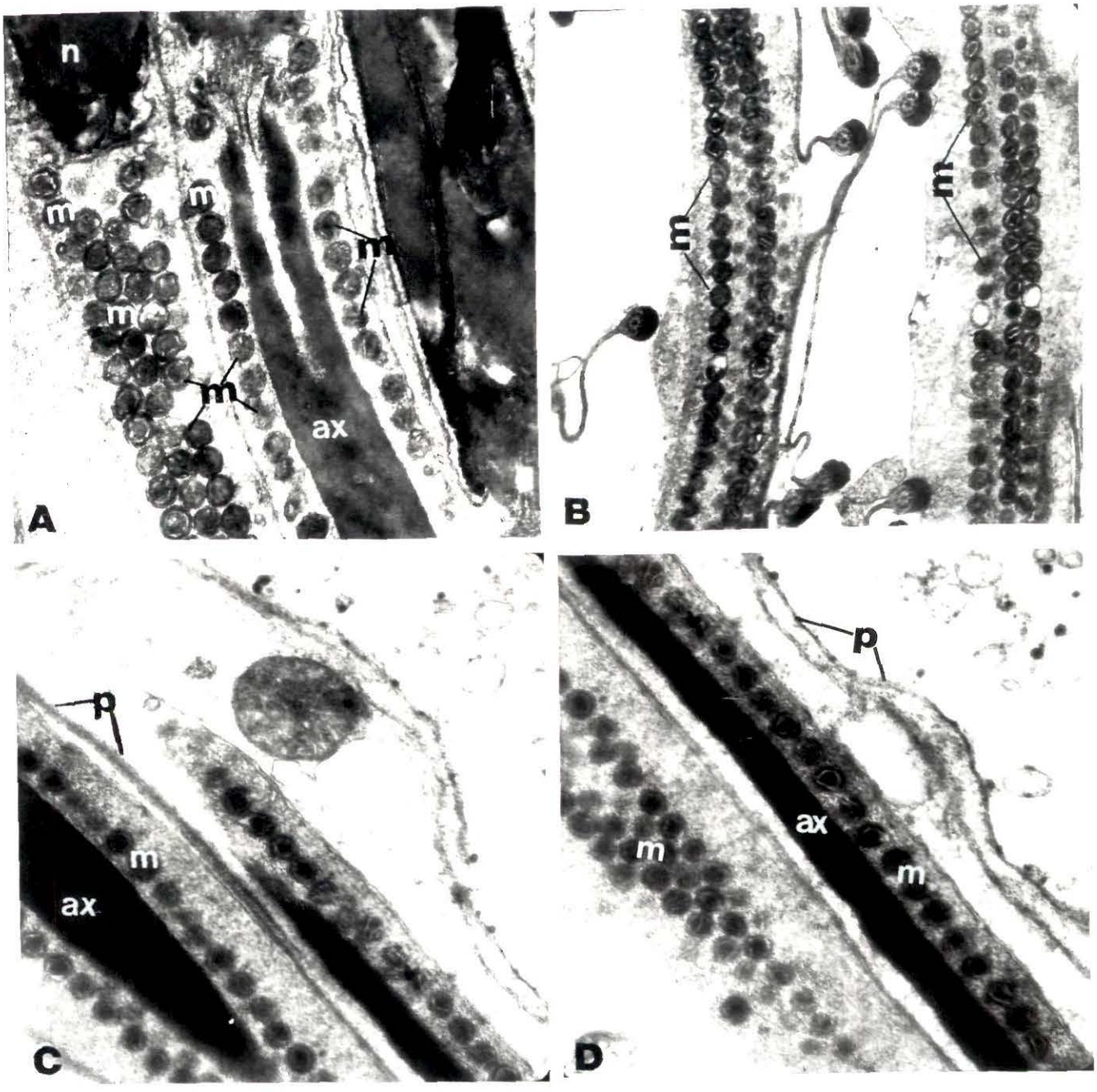


PLATE 26. Longitudinal sections of proximal part of the sperm tail. **Fig. A.** Orientations of mitochondria (m) during formation of axial filament (ax).x22550. **Fig. B.** Two and three rows of mitochondria (m) at the proximal part of the tail. x12000. **Fig. C** and **D.** Longitudinal sections of tail showing mitochondria (m),axial filament (ax) and plasma membrane (p). x24600.

PLATE 27. Micrograph of transverse sections through the sperm head. **Fig. A.** Transverse sections through the anterior portion of sperm head (postacrosomal region), note here the 'côte' (arrow) is a broad crescent nearly encircling the nucleus. The round profile in the center of the cross-section is the perforatorium (pf). Entire structure covered by the plasma membrane (p). **Fig. B.** Transverse sections through the middle portion of sperm head showing condensed nucleus (n), thin mantle of cytoplasm free of organelles (c), the cross-section of slender 'côte' is visible as a small oval profile on one side of the nucleus (arrows). x25457. **Fig. C.** Transverse sections through the caudal portion of sperm showing characteristic arrangement of mitochondria (m), a thin layer of cytoplasm around the nucleus free from organelle (ct) is evident, the cytoplasm containing mitochondria and microtubules is dense in nature and called as 'manchette"(mc).x13672. **Fig. D.** Magnified view of a transverse section through the caudal portion of sperm showing nucleus (n), thin cytoplasmic layer around the nucleus (ct) and manchette (mc). x29520.

PLATE - 27

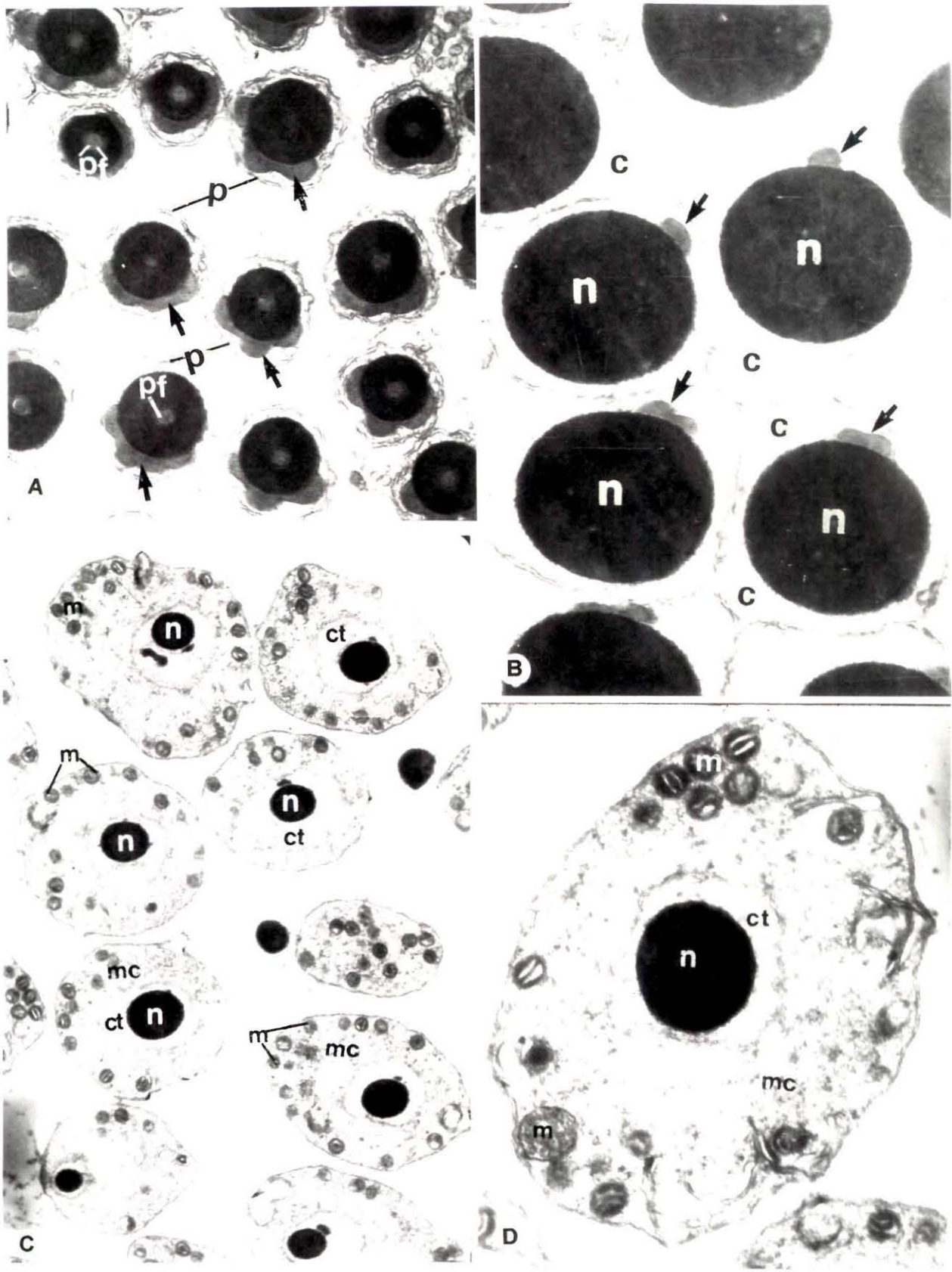


PLATE 28. Fig. A. Transverse sections through the middle of the sperm showing horseshoe-shaped profile of the axial fiber (ax) surrounded by a sheath of cytoplasm rich in mitochondria (m), note the undulating membrane (u) and axonemal complex. (arrow).x10150. **Fig. B.** Magnified view of axial filament shows characteristic arrangement of mitochondria (m) and origin of undulating membrane (arrow). **Fig. C.** Magnified view of axonemal complex shows typical (9+2) structure, undulating membrane (arrow) and marginal fiber (mf). x67500. **Fig. D.** Transverse sections of sperm tail showing trifoliate axial fiber (taf), undulating membrane (u) and axonemalcomplex (ac) with marginal fiber (mf). x21600.

PLATE - 28

ULTRA STRUCTURE OF SPERMATOZOA

General organization of the Spermatozoa

The head of the sperm is very long and cylindrical, tapering anteriorly to slender point bearing a complex acrosomal cap, which is provided with a sharp point and blunt recurving barb (Plate 25; Fig. E, F, Plate 30; Fig. A, B). The caudal fourth of what appears under the light microscope to be the sperm head consists of a cylindrical structure which has the same diameter as the nucleus but differs from it in staining affinity and in its density in electron micrographs. This has been called the connecting piece or intermediate piece (Picheral, 1967). At its caudal end it is continuous with the axial rod, which is the principal supporting structure of the tail. The undulating membrane attaches to the margin of a groove that runs along one side of the axial fiber. In the edge of the membrane is a dense marginal filament – a flexible supporting element that courses longitudinally along one side of a typical (9+2) axonemal complex (Plate 28; A, B, C, D).

Throughout the greater part of the length of the tail the axial fiber is partial surrounded by mitochondria in the proximal two thirds of the tail and demands that this long segment be called the middle piece – the term is in general use for the segment of a sperm containing the mitochondria. The portion of the tail from the end of the sheath of cytoplasm to the termination of the axial fiber is the principal piece (Plate 26; A, B, C, D) The end piece is the short tapering segment of the tail extending beyond the end of the undulating membrane. It consists of the axoneme and a short terminal segment of the marginal filament enclosed only by the plasma membrane.

(a) The acrosome and head piece

The acrosomal apparatus of the mature urodele spermatozoa is very complex and its three dimensional interpretation from electron micrographs of randomly

oriented sections presents considerable difficulty. It has been analyzed in greatest detail by Picheral (1967) in *Pleurodeles* and Fawcett (1961,1970) in *Triturus* and *Notophthalmus*. In *Tylototriton* the head is slender and pointed anteriorly (Plate 30; Fig. B). In some sperms, the perforatorium projecting laterally from the anterior portion of the acrosomal cap, which curves backward and tapers to a sharp point, giving the perforatorium the appearance of the blunt hook (Plate 30; Fig. A).The amorphous substance comprising both the point and the barb is a uniform gray in electron micrographs and somewhat less dense that of more caudal portions of the acrosome. The acrosomal cap is limited, as usual, by a close fitting membrane.

Beneath the cap is a very long, conical subacrosomal space which is eccentrically placed so that in transverse sections at the level of the barb the circular profile of this space has only a thin rim of the cap around one side but a broad oval or rectangular mass on the side toward the barb (Plate 27; Fig. A). The subacrosomal space contains a long, tapered rod or perforatorium of a density similar to that of the acrosome proper and with no resolve substructure comparable to that in the perforatorium of toad sperm (Burgos and Fawcett, 1956). The anterior end of this rod is free in the subacrosomal space, but its posterior portion occupies a cylindrical central cavity in the slender anterior end of the nucleus (Plate 27; Fig A). Thus in cross sections at the level of the barb, the round profile of the rod is surrounded by a narrow clear zone between it and the surrounding acrosomal material. Farther posterior, where the rod is lodged in a deep invagination in the nucleus, its round profile is centrally placed in cross section and is immediately surrounded by a dense rim of condensed chromatin (Plate 27; Fig. A, B). Outside of this is a narrow ring of acrosomal cap. This concentric disposition of the head structures in cross sections continues posteriorly for some distance beyond the caudal margin of the acrosomal cap. The dense ring of chromatin around the central rod gradually increases in thickness at more caudal levels, and in the post acrosomal region a new structure appears around the ring of chromatin. This

was called the nuclear "Côte" by Picheral (Plate 27; Fig. B). At high magnifications, it is seen to be composed of minute tubular sub-units closely packed in hexagonal array and oriented parallel to the long axis of the nucleus. In cross sections in the immediate postacrosmal region, it forms a complete ring peripheral to the chromatin, but at successively more caudal levels, it forms a crescent and finally is reduced to a thin rod that runs along one side of the nucleus for the greater part of its length (Plate 27; Fig A, B, C, D). There is a minor difference of interpretation as to its relationship to the nuclear envelope. Over the "côte" one sees only one membrane, whereas elsewhere around the nucleus the outer and inner membranes are clearly seen. Picheral concluded from this appearance that the "côte" lies between the layers of the nuclear envelope. Fawcett favor the view that it is entirely within the nucleus but that the inner membrane is so intimately fused to the outer surface of the cote that it is not resolved as a separate structure. Hence only one membrane of the nuclear envelope is seen passing over this organelle. No structure comparable to the "côte" is found in sperm of other taxa of vertebrates and its function is unknown.

There have been no detailed studies of the acrosome reaction in urodeles, but there are incidental observations suggesting that the acrosomal cap soon disappears when sperm are removed from their spermatophores and observed in an aqueous environment (Picheral, 1967, 1977). Loss of the acrosome presumably would leave behind the "perforatorium" or subacrosomal rod, but its role, if any, in egg penetration is not established. Despite the present lack of evidence for any acrosomal function other than the widely accepted release of lytic enzymes, it is difficult for a morphologist, confronted by the remarkable complexity of its organizations in *Tylototriton* to dismiss this characteristic elaborate structure.

(b) The connecting piece

This large component of the urodele sperm is presumed to be homologous with the connecting piece of mammalian spermatozoa because it is interposed between the nucleus and middle piece , and it develops around the centrioles and ultimately encloses them in the mature spermatozoon. There the similarity ends, for the connecting piece of urodele sperm shows none of the cross striations characteristic of this structure in mammals. In *Tylototriton,* the connecting piece is a cylinder several microns long with a rounded anterior end that fits into a concavity of confirming shape in the caudal end of the nucleus. Its substance is a homogeneous dark grey in electron micrographs but is distinctly less dense than the black of the condensed chromatin of the nucleus (Plate 27; Fig D). At its caudal end, it appears to be continuous with the axial fiber of the middle piece. Because the latter is of smaller diameter and placed nearer one side of the connecting piece (Plate 27; Fig. C) , their junction is marked by an abrupt, step like offset. Embedded in the substance of the connecting piece in this region are the two centrioles. The one which constitutes the basal body of the flagellum is set in the caudal aspect of the connecting piece at an angle, such that the axonemal complex which originates from it diverges from the axial fiber to run in the edge of the undulating membrane (Plate 26; Fig A, B, C, D, Plate 14; A, B).

(c) The middle piece

In transverse sections of the tail distal to the connecting piece, the axial fiber is horseshoe shaped with a smooth rounded contour on one side and a deep groove on the side toward the undulating membrane (Plate 28; Fig. A, B). When appropriately stained, the axial fiber is found to have a large dense medulla and thinner and slightly less dense cortical layer. Its convex surface is covered with a thick mantle of cytoplasm containing numerous small spherical mitochondria (Plate 28; Fig B). On the opposite side where the mantle of

cytoplasm is lacking, the plasma membrane appears to be firmly attached to the margins of the groove in the axial fiber by two linear accumulations of dense material interposed between the membrane and the underlying fiber. From these two lines of attachment on either side of the groove, the membranes converge to form the two leaves of the undulating membrane which run parallel and enclosing a thin layer of cytoplasmic matrix. Toward the edge of the undulating membrane, the two leaves of membrane diverge again to surround the axoneme and the marginal fiber. The marginal fiber is crescentic in cross sectional profile with its inner surface in very close relation to doublets 8, and 9 of the axonemal complex (Plate 28; Fig C, D). The marginal fiber has a different fine structural texture than the axial fiber and is distinctly less dense. Its crescentic cross section is bisected by a denser line, one end of which is directly opposite doublet number 9, while the other end is marked by a minute ridge centrally located on the convex outer surface of the fiber (Plate 28; Fig. C).

The axial fiber gradually diminishes in thickness toward the distal part of the middle piece and its cross-sectional outline becomes more cordiform. The abrupt termination of the mantle of mitochondria – rich cytoplasm about three quarters of the way along the tail marks the end of the middle piece.

(d) The principal piece

Distal to the termination of the mitochondrial sheath, the plasma membrane is closely adherent to the surface of the axial fiber, which in this region has a trifoliate cross – sectional outline (Plate 28; Fig D). As the axial fiber continues to taper down in thickness, its trifoliate cross-sectional outline gradually gives way to a cresentic shape which then becomes increasingly attenuate and finally ends. In this region of the tail, the axoneme and its associated marginal fiber converge upon the axial fiber so that the undulating membrane rapidly narrows and ends at about the level of rapidly narrows and ends at about the

level of termination of the axial fiber.

(e) The end piece

The axoneme and marginal fiber, continuing beyond the end of the undulating membrane, constitute the end piece. The marginal fiber diminishes in size until it is but a thin line between the axoneme and the flagellar membrane. The central pair of fibers then end, and the peripheral doublets continue for only a short distance beyond this point.

Homologies with tail components of mammalian sperm

In attempting to homologize the various components of the urodele sperm tail with those of the mammal the axial fiber seems to us a correspond to one of the outer dense fibers, probably number 3. Its density its differentiation into cortical and medullary zones, and its relation to the connecting piece all seem to support this interpretation. The marginal fiber, on the other hand, is distinctly different from the axial fiber in density and fine structural texture. Thus it does not seem to correspond to another of the outer fibers of the mammalian sperm. Instead its fine structure and its intimate relationship to doublet number 9 of the axonemal complex suggest the possibility that it corresponds to one of the longitudinal columns of the fibrous sheath of the mammalian spermatozoon. Just as the fibrous sheath enveloping the tensile elements of the mammalian sperm tail, is thought to have a spring-like counteraction to the bending force of the enclosed axoneme it seems likely that the marginal fiber in the case of the urodele undulating membrane may also be an elastic supporting element providing the necessary resistance for the flagellum to work against.

If the current assumptions about the nature of the mammalian outer fibers is correct and if the axial fiber in urodele sperm is the counterpart of one of these, then one might expect that the axial fiber would be capable of contraction. On

the contrary, under the usual conditions of laboratory observation, the axial fiber appears quiescent while the flagellum generates waves in the undulating membrane that move rapidly along the tail from base of tip. The axial fiber has therefore been considered by most investigators to be a stiff supporting structure providing attachment for the base of the undulating membrane. However, a comparable dense fiber associated with the undulating membrane of toad sperm is reported to be capable of executing rapid sinuous movements independent of the beating of the flagellum (Burgos and Fawcett, 1956). It seems not unlikely, therefore, that the axial fiber of *Tylototriton* sperm may be potentially motile but that conditions for its activation have not been achieved in vitro. The association of large numbers of mitochondria with the axial fiber also suggests that the latter is contractile. If the function of the mitochondria were to supply energy for the flagellum, it would be surprising for them to be located at the opposite side of the tail. If the axial fiber is non-motile , it is difficult to imagine what function is served by a long middle piece containing hundreds of mitochondria.

The morphology of spermatozoa is highly variable in anurans and salamanders and too little known about the morphology of caecilian spermatozoa. Among the salamanders various kinds of variation have been noted. For example presence of tail membrane is a characteristics of salamanders under family Salamandridae which is unknown in other salamanders (Marton and Wortham, 1972; Selmi et al., 1997). Similarly, differences in head and neck morphology are also known from several urodele species (Barker and Biesele, 1967; Brandon et al., 1974; Wortham, et al., 1977, 1982; Lee et al., 1996). However, presence of a barb on the acrosome is the characteristics of all salamanders.

SPERM POLYMORPHISM

A. Light microscopic observations

Polymorphism in head morphology of the sperm, both in breeding and post-breeding specimens is highly characteristic. Both club shaped and sickle shaped head frequent in the air dry preparations under the light microscope observation (Plate 29; Fig. A, B). A few microcephalic sperm are also observed under the light microscope. The microcephalic sperm possess 'dot' like spherical head region. (Plate 29; Fig. D). Some macrocephalic sperm are also observed with broad shaped head under light microscope. (Plate 29; Fig. C).

B. Scanning electron microscope observation

Under scanning electron microscope, polymorphism in sperm structure is well pronounced. Four type of sperm heads are found under scanning electron microscope. These are microcephalic sperm with dot like head (Plate 31; Fig. E), sperm with hook like perforatorium (Plate-31; Fig. B, C), sperm without tail (Plate 31; Fig. D) and normal sperm with sickle shaped head are documented (Plate 31; Fig. A).

Differences are also found in the mid piece region of several sperm. Normal mid-piece shows some what slender structure, in higher magnification normal mid-piece shows several folds (Plate-32; Fig. A, C). Some sperm exhibit bulb like mid-piece (Plate 32; Fig. B, C, Plate 33; Fig. A, D, E, F). Sperm with broad mid-piece with a groove are also observed (Plate-33; Fig. B, C).

C. Transmission electron microscope observation

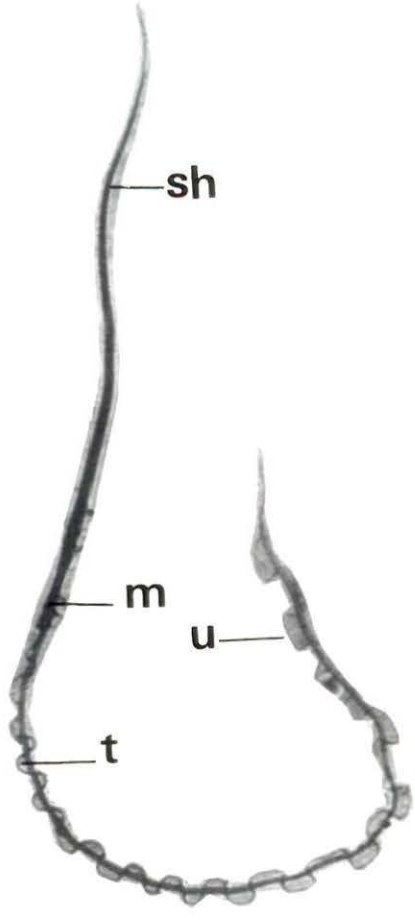
Transmission electron microscope observation substantiate the light microscope and scanning electron microscope observation. Under transmission electron microscope three types of sperm are noticed. Two types sperm are more frequent, one with along pointed slender head perforatorium

other with a recurving head with a hook like perforatorium. (Plate 30; Fig. A, B). The third one with a barb like perforatorium (Plate 30; Fig. C).The plasma membrane at the head region shows a wavy nature and there is hardly any cytoplasm inside the head.

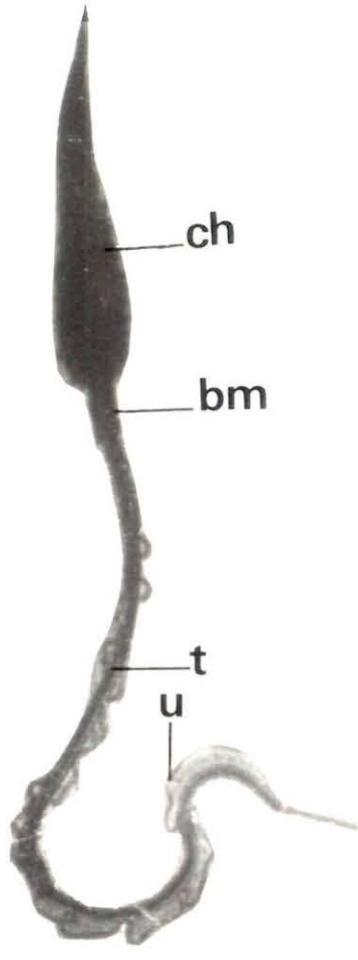
D. FACS study for measuring DNA contains of different sperm

After purification of sperm cells and staining with ethidium bromide the cells are scanned under FACS machine (for the methods see chapter II). The histogram obtained (Figure - 3) after cells scanning show four peaks namely **M1**, **M2**, **M3** and **M4**. Each peak denotes number of cells and their variable DNA values. From this histogram it is known that, 91.56% sperm cells have normal haploid DNA value, 7.04% cells have above haploid DNA value, 0.15% cells have hypohaploid DNA value and 0.3% cells have hyperdiploid DNA value. From this result it is clear that the sperm polymorphism exists not only at morphological level but also at the genetic level.

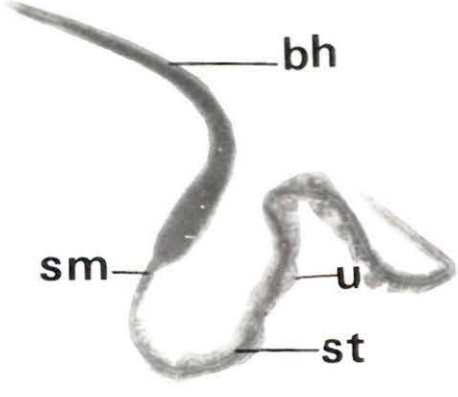
PLATE 29. Light microscopic observation on sperm poly morphism. **Fig. A.** Normal sperm with sickle shape head (sh), short slender mid-piece (m) and long tail (t) with fin like undulating membrane (u). **Fig. B.** Mega-cephalic sperm with club shaped head (ch), short and broad mid-piece (bm) and long tail (t) with undulating membrane (u). **Fig. C.** Mega-cephalic sperm with broad and short head (bh), slender mid-piece (sm) and short tail (st) with undulating membrane (u). **Fig. D.** Micro-cephalic sperm with dot like round shape head (dh), slender mid-piece (sm) and long tail (lt) with reduced undulating membrane.



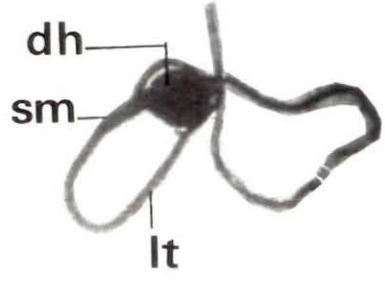
A



B



C



D

PLATE - 30

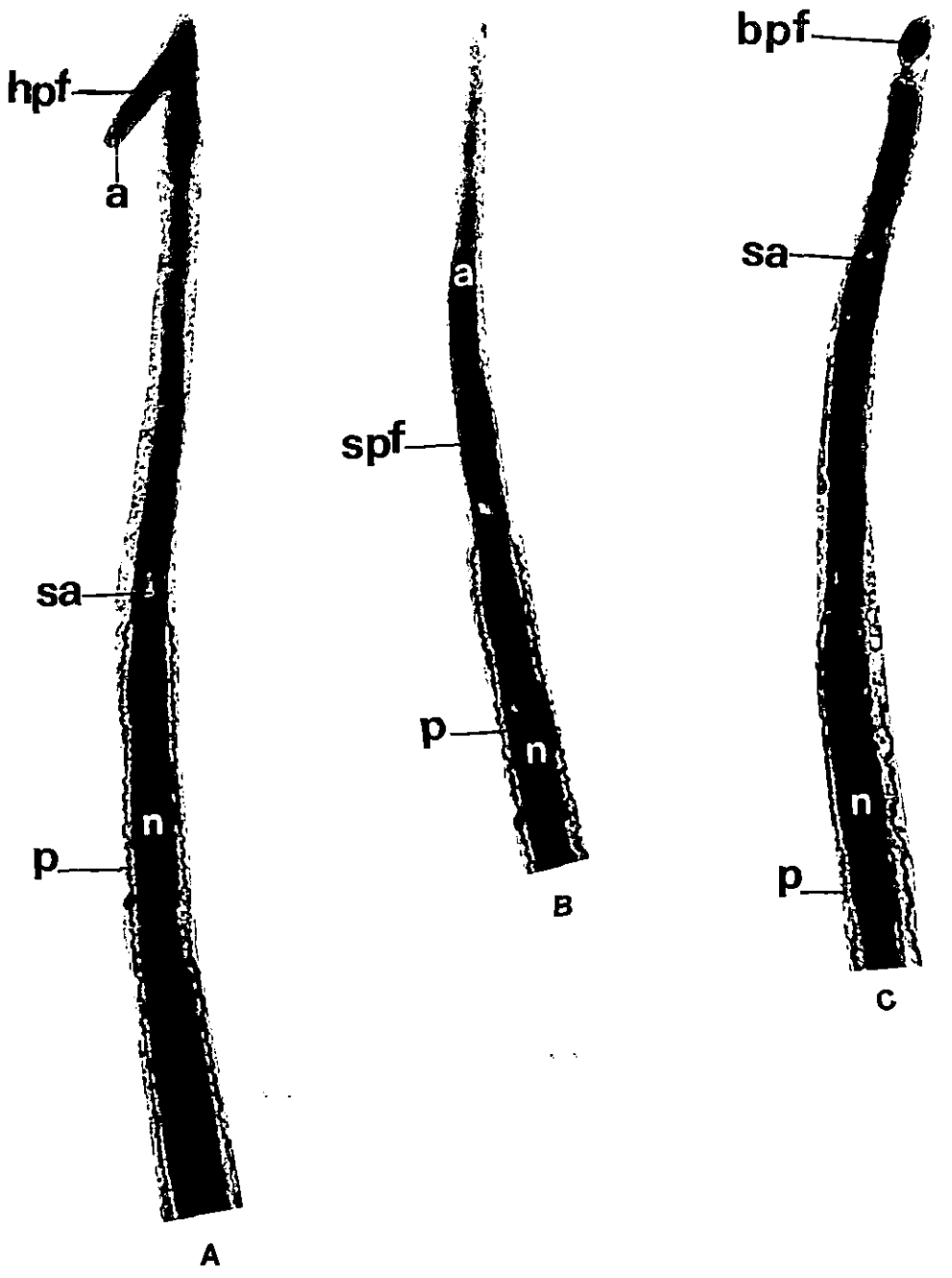
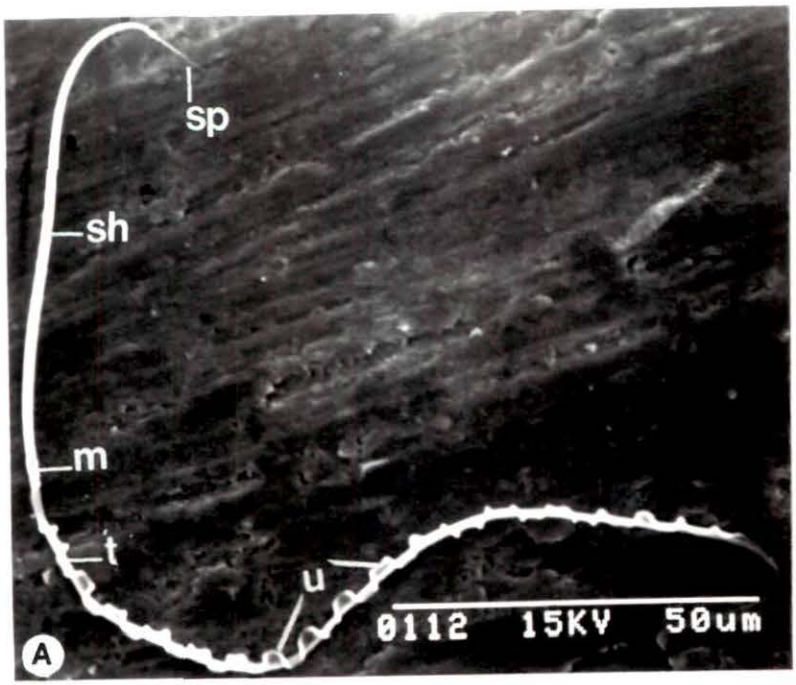
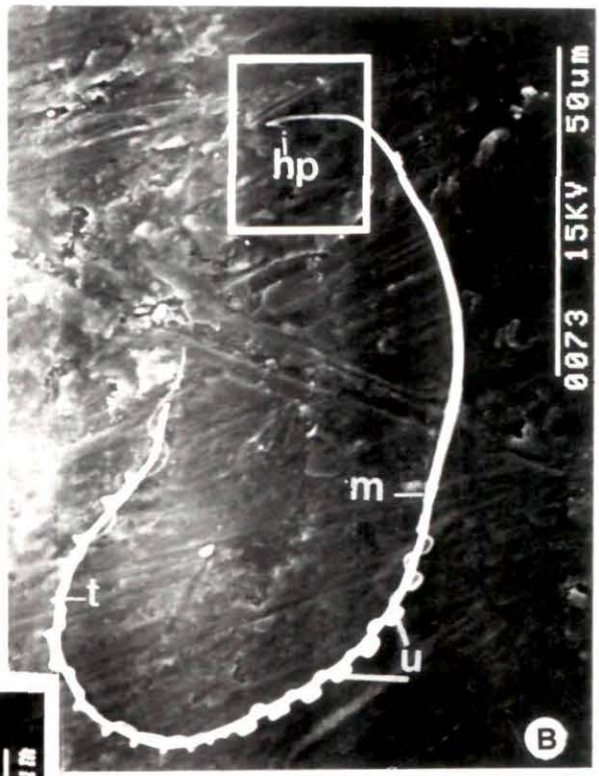


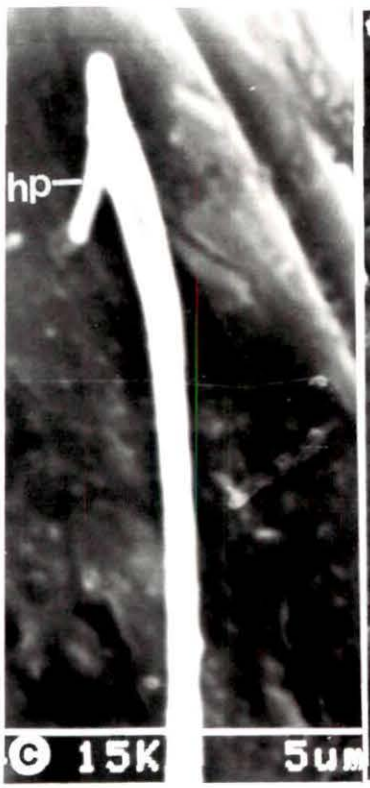
PLATE 30. Transmission electron micrograph of three type sperm heads. **Fig. A.** Longitudinal section of sperm head shows hook shaped blunt perforatorium (hpf), dark acrosome (a), sub-acrosomal space (sa), nucleus (n) and plasma membrane with thin cytoplasm (p). x5467. **Fig. B.** Longitudinal section of sperm head showing slender pointed perforatorium (spf) and acrosome (a) with sub-acrosomal space (sa), dark nucleus (n) and plasma membrane (p) with thin layer of cytoplasm. x5467. **Fig. C.** Longitudinal section of sperm head shows bulb like perforatorium (bpf) with acrosome, long sub-acrosomal space (sa), dark nucleus (n) and plasma membrane. x5467.

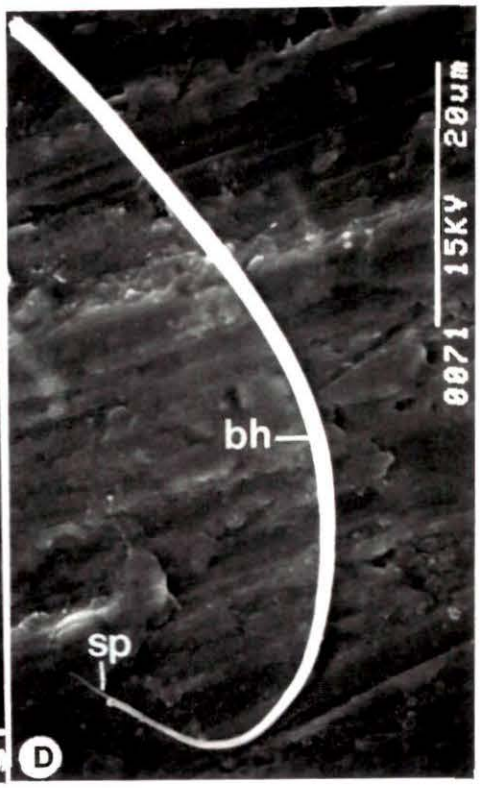
PLATE 31. Scanning electron microscopic observation on sperm polymorphism (head region). **Fig. A.** Normal sperm with sickle shape head (sh) with slender pointed perforatorium (sp), short slender mid-piece (m) and long tail (t) with fin like undulating membrane (u). **Fig. B.** Sperm with hook like perforatorium (hp) short mid-piece (m), long tail (t) with undulating membrane (u). **Fig. C.** Magnified view of hook like perforatorium (hp). **Fig. D.** Sperm with broad head (bh) with slender perforatorium (sp) and without tail. **Fig. E.** Micro-cephalic sperm with dot like round shape head (dh), slender mid-piece (sm) and long tail (lt) with undulating membrane (u).

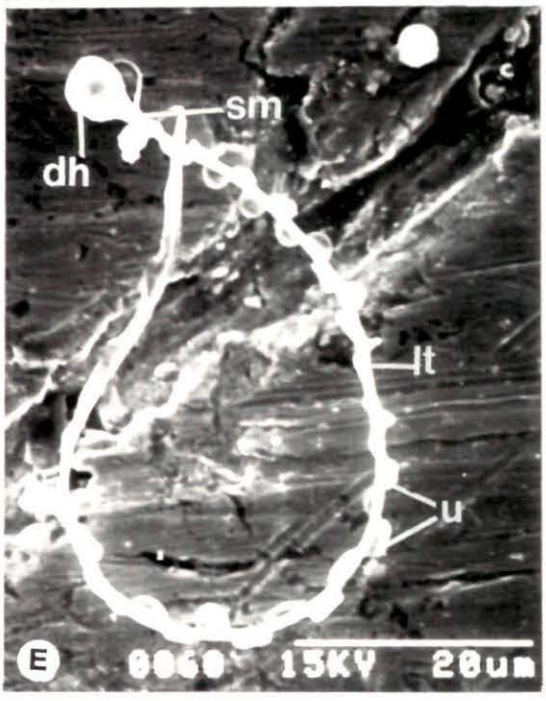
PLATE - 31


sp
sh
m
t
u
0112 15KV 50um
A


hp
m
t
u
0073 15KV 50um
B


hp
C 15K
5um


bh
sp
0071 15KV 20um
D


sm
dh
lt
u
E
15KV 20um

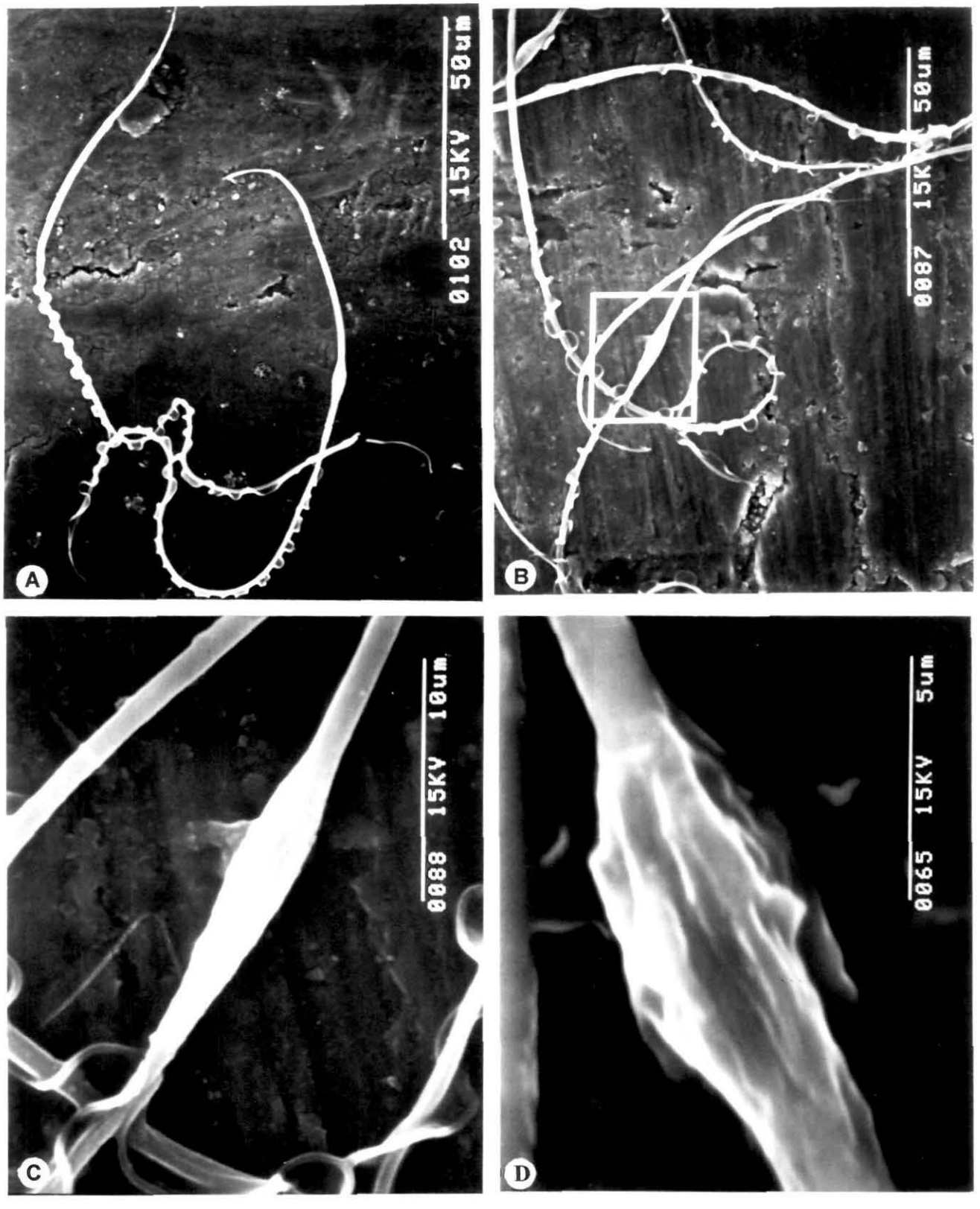
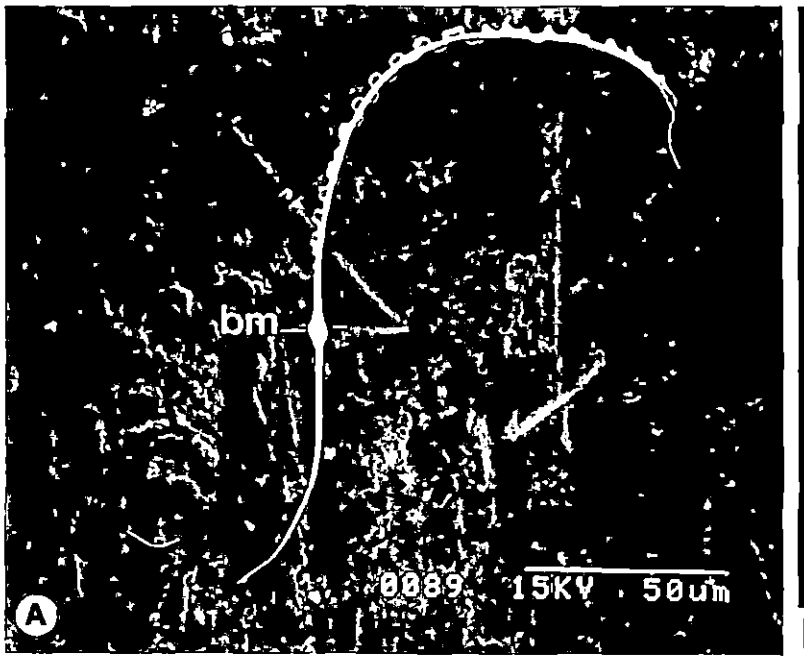
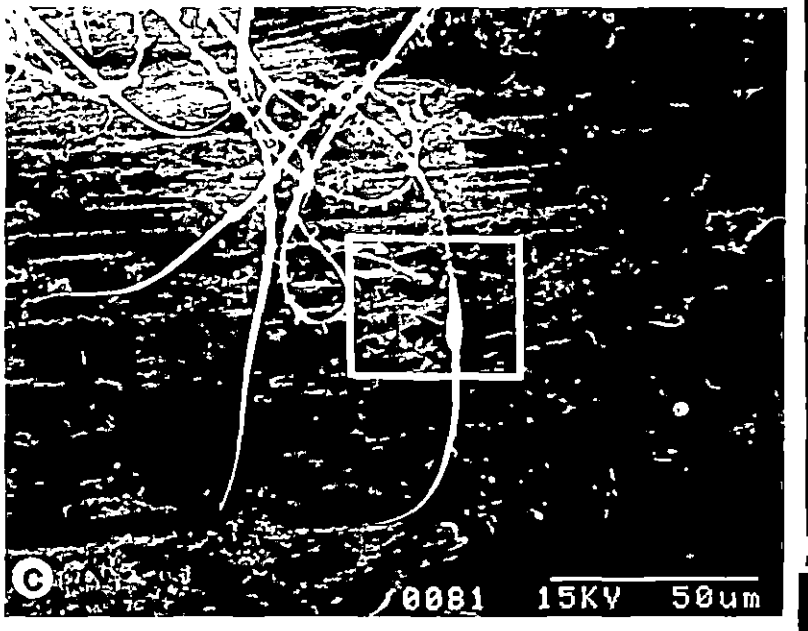


PLATE 32. Scanning electron microscopic observation on sperm polymorphism (mid-piece region). **Fig. A.** Normal sperm with short slender mid-piece (nm) and sperm with broad mid-piece (bm). **Fig. B.** Sperm with broad mid-piece (arrow). **Fig. C.** Magnified view of broad mid-piece (arrow). **Fig. D.** Further magnified view of mid-piece shows several folds (arrows).

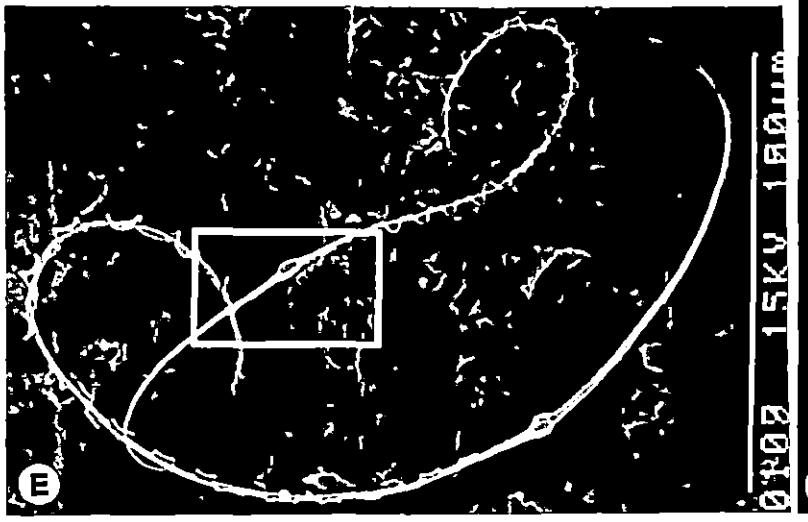
PLATE 33. Scanning electron microscopic observation on sperm polymorphism (mid-piece region). **Fig. A.** Sperm with broad bulb like mid-piece (bm). **Fig. B.** Magnified view of broad mid-piece (highlighted in figure C) with a groove (arrow). **Fig. C.** Sperm with characteristic mid-piece (arrow). **Fig. D.** Magnified view of bulb like mid-piece (figure A) showing several fold (arrow). **Fig. E.** Sperm with characteristics bulb like mid-piece. **Fig. F.** Magnified view of short bulb like mid-piece (highlighted in figure E).

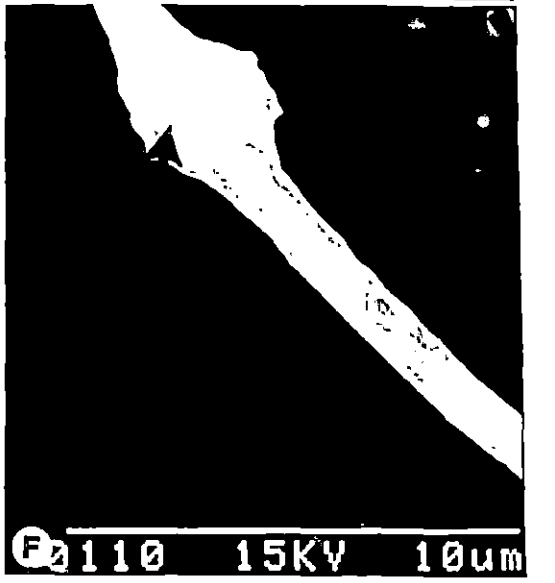

bm
0089 15KV 50um
A


B0082 15KV 10um


C
0081 15KV 50um


D91 15K 10um


E
0108 15KV 100um


F0110 15KV 10um

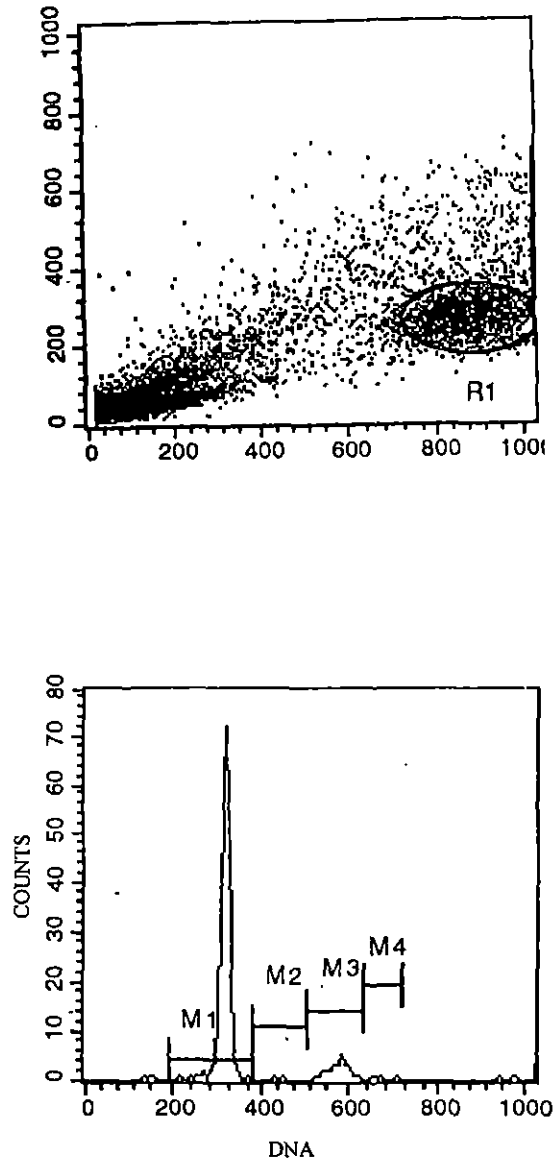


FIGURE - 3

Result of FACS study for measuring DNA content of different sperm