SUMMARY

Amphibians have an unique place in the evolutionary history of vertebrates. They are the first vertebrates to establish life on land but preferred to settle at the edge of the water, a reminiscent of their aquatic ancestry. The successful perpetuation of a species and it's survival in the new terrestrial environment is dependent on the successful modulation and development of several anatomical features, viz. - limbs, lungs, skin etc. But biologically the most important modification is the evolution of new reproductive strategy.

Conventionally, the reproductive cycles of amphibians have been reviewed in terms of histological and histochemical studies by many scientists. The advent of new tools and techniques in cell study has revolutionized the earlier observations made. It is evident from the recent observations that the reproductive pattern vis -a- vis the reproductive mode not only encounter gross cytological changes but are also reflection of concurrent submicroscopic changes caused due to one or several intrinsic and extrinsic factors.

In India, about hundred species of anurans, over a dozen species of apodan and only one species of urodele are known to exist. However, studies on the reproductive cycles of Indian amphibians are surprisingly limited to only a half a dozen of species (*Rana cyanophlyctis, Rana tigerina, Rana hexadactyla, Bufo marinus, Bufo melanostictus, Bufo stomaticus*).Therefore, the scope of study at the ultrastructural level is highly immense and is of great necessity. The study would be of significance to bridge the gaps in our understanding on various aspects related to the reproductive biology of amphibians. The present text mainly embraces the ultrastructural changes in the reproductive cycles ofthe Himalayan Newt, ***Tylototriton verrucosus*** Anderson (*Pleurodeles verrucosus* Anderson) , the sole representative of urodele in the Indian sub continent.

Therefore, the present investigation has a potential scope for study the

following aspects of urodelan reproductive biology :

- Structural changes of testis during the annual testicular cycle, i.e. Pre-breeding Breeding, Post-breeding and Regression phases by routine histological technique.

- Surface morphological changes of testis and testicular components using scanning electron microscopy (SEM) during testicular cycle.

- Correlation of the changes of testicular cell components as revealed by light and scanning electron microscopy with that of ultrastructural changes by transmission electron microscopy (TEM).

- Flow-cytometric analysis to substantiate a species level genomic variation of spermatogenic cells.

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 specimen which were selected for the present study had single lobed individual testis. Each lobe is some what spherical or pear shaped and bears a groove in the middle . The branched efferent ductile systems arises from the inner surface of the lobe to meet anterior nephric collecting tubules and ultimately empties into Wolffian duct. The Wolffian duct runs along the lateral edge of the Kidney to open into the cloaca.

The size of the lobe varies seasonally. The maximum size has been observe during the breeding month (July to September), The size gradually decreases on the onset of winter and maximum reduction was observed in the January specimens. 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 are sacrificed during the breeding months. 'Yellow Zone' reappears distinctly during the next winter (January to February).

In *Tylototriton verrucosus,* the testis exhibits cystic arrangement. The cells present in a cyst are in the same stage of the development and derived from a single spermatogonium. However, in a cross-section of the 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. The spermatogenesis starts at the onset of Summer season (April to June). Spermatogenesis reaches its maximum velocity in breeding season (July to September) the Post-breeding season onset with regression of spermatogenetic activity and last for few months (October to December). The Regression phase is recognized as the period in which the yellow pigmentation or 'Yellow Zone' formation reaches its maximum development (January to March).

Annul testicular cycle, as revealed from histological observation shows onset of spermatogenesis in the month of April. Reduction division starts in the month of May while spermiogenesis starts in the month of June. Mature spermatozoon appears in July. The specimen shows distinct annul testicular cycle.

Under the scanning electron microscope the cross-section of pre-breeding testis shows different stages of spermatogenesis and accordingly they are called as gonial cyst, spermatocytic cyst and spermatid cyst. The gonial cysts are found at periphery of the anterior part of the testicular lobe. Such nest are filled with two type of cells - one, larger in size with rough surface and other

cells are relatively smaller in size and with relatively smooth in appearance. 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. 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. Sertoli cells are observed in association with the cluster of germs cells. These cells are radiate from the tubule periphery and extend towards the lumen and invest developing germ cells with cytoplasmic processes during the process of gametogenesis. There are some Leydig cells in the interstitial region. Each Leydig cell is elliptical or ovidal in shape showing a lobular appearance.

The breeding testis exhibits all the cellular component as mentioned in pre-breeding testis. In breeding testis there are some additional cysts with full of mature sperm in a characteristic bundle fashion. In higher magnification each sperm bundle shows a typical conical structure. The post-breeding and regression testis shows mainly mature sperm bundles and evacuated lobules.

Under transmission electron microscope, there are two types of germ cell i.e. spermatogonial cells and spermatocytic cells, and are found in the pre-breeding testis. Some interstitial Leydig cells and Sertoli cells are also found. Two types of spermatogonial cells, as observed under light microscope and scanning electron microscope studies are also recognized under transmission electron microscope. The primary spermatogonia, in general exhibits a conspicuous round nucleus with fine distribution of chromatin materials and two to three nucleolus. 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. Primary spermatogonium divides mitotically to give rise to secondary spermatogonia, which are initially very similar to their mother cell,

except their nucleus is slightly smaller.

The spermatocytes cells 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. 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.

Many interstitial Leydig cells are found in the pre-breeding testis at the interstitial region 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 nature. At the peripheral region of the nucleus electron dense chromatin material is arranged in a laminar fashion. The cytoplasm contains mitochondria, small electron dense protein granules, rough endoplasmic reticulum, lysosomes, lipid droplets and different types of polysomes. The Sertoli cells are the somatic elements of the testis. 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 evident nucleolus. At the periphery of the nucleus there are some electron dense chromatin materials are evident.

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. The ultrastructure of spermatogonia and spermatocytes are same as found in pre-breeding testis. The spermatids are seen in different stages of spermiogenesis as indicated by the degree of

chromatin condensation and the presence of flagella. Initially , they contain chromatin in course granules, evenly distributed throughout the nucleus. With the progress of the spermiogenesis process, the chromatin condensed into dense clot and the cell becomes smaller and more condense. 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. The mature sperm is divided into three parts i.e. head piece, connecting piece, and tail piece. 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. 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. 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. 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. That portion of the tail from the end of the sheath of cytoplasm to the termination of the axial fiber is the principal piece. 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. Under transmission electron microscope, the post-breeding and regression testis shows gonial cells, spermatocytes, spermatids and spermatozoa which are very similar in ultrastructure to breeding testis gonial cells. Apart from these gonial cells, some big sized phagocytotic cells and spherical interstitial cells are also found

in regression testis.

Polymorphism in sperm structure has also been recorded in the present investigation. In the present investigation 4 distinct forms of spermatozoa have been observed in *Tylototriton verrucosus* , (i) typical slender headed sperm with pointed perforatorium, (ii) atypical spermatozoa with hook shaped perforatorium, (iii) microcephalic sperm with dot like head and without perforatorium, (iv) mega cephalic head without perforatorium.

FACS analysis of spermatozoa has also supported that polymorphism of morphology is related with DNA values as 91.56% cells with normal haploid value, 7.04% cells have above haploid DNA value, 0.15% cells have hypohaploid DNA value and 0.3% cells have hyperdiploid DNA value.

The present investigation of the above described parameters suggests that in ***Tylototriton*** the testicular morphology, seasonal changes in the testicular components and annual testicular cycle follow an annual testicular mode or pattern as in other subtropical salamanders. However, in this species sperm release takes twice in year, i.e. on the onset of breeding season, sperm harbored in the spermatophores are released to facilitate fertilization. A second spermiation takes place at the climax phase of spermatogenesis. Spermatogenesis follows the ultrastructural changes that are usually encountered in other urodels.

Sperm ultrastructural features of the species when compared with the other members of the Salamandridae shows that ***Tylototriton*** sperm resembles closely with ***Pleurodeles*** than the ***Triturus*** and a remote resembles with ***Notophthalmus.*** On the basis of comparative ultrastructural features, it has been suggested that ***Tylototriton verrucosus*** should be renamed as ***Pleurodeles verrucosus*** as suggested by recent taxonomists.

In this species, sperm morphology exhibits a phenomenon of polymorphism. Three distinct forms have been recognised. Majority of the sperm has elongated head with pointed perforatorium, conspicuous middle piece and a long tail with fin-like undulating membrane. The second type consist of megacephalic sperm without the perforatorium and a very short tail. The DNA analysis also showed that the DNA values of such sperm varies significantly. The present author suggests that such variation has an evolutionary significance. Hypohaploid forms represent the reminiscent of the lower evolutionary status while hyperdiploid forms represents the new form or species that is likely to appear in nature in near future.