DISCUSSION

1. ANNUAL TESTICULAR CYCLE

In amphibians annual testicular cycle exhibits several distinct spermatogenic patterns. In Caecilians spermatogenesis continues throughout the year (Wake, 1980), in anurans four distinct spermatogenic patterns identified and such patterns coincides with the geographical distributions and constrains of environmental factors (Basu, 1969). Spermatogenic patterns in some anurans with references to their geographical distribution is given in the Table 3.

Table 3

Species	Geographical Distribution
Continuous Type	
Bufo parachemis	S. America
Bufo melanostictus	India , Java
Rana cancrivora	Java
Rana hexadactyla	India
Pseudis mandidactyla	India
Discontinuous type	
Rana temporaria	Europe
Rana pipiens	USA
Pleurodema bufonia	S. America
Continuo – discontinuous type	
Rana esculenta	Europe
Rana gracea	S. America
Rana tigrina	India
Rana nigromaculata	Japan
Variable Type	
Rana esculenta ridibunda	Europe, Mediterranean region
Discoglossus pictus	Europe, Mediterranean region

Among the urodeles two distinct spermatogenic patterns are recognized :

a) Classical annual Pattern : In aquatic breeders such as members of Hynobiids, Cryptobranchids, Sirenids, Amphumids and most Salamandrids, spermatogenesis begins in the spring.

b) Biennial Pattern : The biennial gametogenesis recorded in the species living under similar climatic condition and is characteristics of Plethodontid salamanders.

In earlier studies of testicular cycle in *Tylototriton verrucosus*, suggest that onset of spermatogenetic activity is palpable in the April testes and meiotic upsurge observed in June to July followed by spermiogenesis commences on and from June and continues during the months from July to December. Fully formed spermatozoa are found during the months from July to February and spermeation start in the month of August and continues up to December. The author suggested that the spermatogenesis in *Tylototriton* follows an Annual pattern like *Trituroides hongkongensis* (Tso and Lofts, 1977) and *Cynop pyrrhogaster pyrrhogaster* (Tanaka and Iwasawa, 1979). However, in the present study suggest that spermatogenesis in *Tylototriton* exhibit throughout the year, during the month from April to July spermatogenesis exhibit the maximum speed while spermiogenesis remain in low wave, therefore, it may be suggested that spermatogenesis is not a synchronous event in all lobules throughout the testis and occurs in a wave like fashion. It can also be suggested that in *Tylototriton* spermeation takes place twice in a year. In first year specimens spermatogenesis starts in the month of April when environmental conditions are favorable for the spermatogenetic activity. These activity considers with the resorption of the Yellow zone which starts in April. Vellano, 1969; reported that, the yellow zone composed of residual Sertoli cell, controls the spermatogenic activity. Similar reports are available from works of Tso and Lofts, 1977; Tanaka and Iwasawa, 1979; and Verrell et al., 1986.

Aron (1924), reported that "yellow zone" might be associated with the secretion of testicular hormone and involve in the final development of secondary sexual characters. Steroidogenic activity of "yellow zone", have been evidenced from variety of species - e.g. *Pleurodeles waltlii* (Certain et al., 1964; Collenot, 1965; Picheral, 1970), *Salamandra salamandra* (Joly, 1966) *Trituroides hongkongensis* (Tso and Lofts, 1977), *Cynop pyrrhogaster pyrrhogaster* (Tanaka and Iwasawa, 1979) *Trituroides vulgaris* ((Verrell et al. 1986).

When annual testicular patterns of *Tylototriton verrucosus* is compared with that of *Trituroides hongkongensis* and *Cynop pyrrhogaster pyrrhogaster* is apparently evened. However, testicular composition and events of spermatogenesis differ among these three species (Figure 2).

Biannual spermiation in *Tylototriton verrucosus* can be suggested as an activity which coincides with reproductive strategies. During winter months adult males migrate down hill as much as 500 to 800 meters where the ambient temperature remain around 20°C, which is supportive for spermatogenic activity at low wave, the kinetics of spermatogenesis reaches at maximum level when the specimens return to their breeding sites which starts from March to April.

2. SPERMATOGENESIS

Spermatogenesis is a dynamic process and conventionally studied under three events, viz. spermatocytogenesis, meiotic phase and spermiogenesis. Although in recent years new event has been added to it under the heading 'Pre spermatogenesis'.

Each event in spermatogenesis is cytologically, genetically, and biologically *important and thus spermatogenesis in general has been attended by various* researchers of different field of biology. George (1876) first described various stages of spermatogenesis in five species of amphibians from observations on testicular tissues, fresh or fixed in osmium tetroxide and laid the ground work for modern terminology in this field, viz. 'spermatogonium', 'spermatocyst' and 'spermatocyte'. Fleming (1887) used the spermatocyte of *Salamandra* in his study of meiotic division. Witschi (1924) provided the first detailed description of spermatogenesis in *Rana tempororia* and the electron microscopic studies were made by Brokelman (1964) in frog, Fawcett et al., (1961) in *Triturus*, Picheral (1967,1977) in *Pleurodèles* and others (Dym and Fawcett, 1971; Lin, et al., 1990; Lin and Jones, 1992; Asa et al., 1998; Baccetti et al., 1991; Phllips et al., 1989; Soley et al., 1994; Clark,1967; Dehlawi et al., 1992; Jamieson, 1993,1995; Jamieson et al., 1996; Healy et al., 1992; Teixeira et al., 1999, 2002; Scheltinga, et al., 2000; Griger, 1981; Billard, 1986; Meura, et al., 1991; Romagosa, et al., 1999; Gusmao et al., 1999; Medina et al., 2000; Garda et al., 2002).

At histological level spermatogenic activity is made by principle as laid down by Van Oordt (1956) and subsequently modified by Saidapur (1989).

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* spermatogonia. At histological level spermatogenic activity vis a vis changes are assessed or made following the principal led 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

The Stage 0 includes the primary spermatogonia stage and their mitosis. The primary spermatogonia are the largest germ cells located adjacent to the basement membrane of the seminiferus tubules. Like mammals, in amphibians two types of primary spermatogonia are known (Rastogi, 1985, Ray, 1978, Ray et al., 1980) as pale and dark type of spermatogonia. In the present investigation similarly two distinct types are recognized. Even their ultrastructural differences are well convincing. The pale type exhibits large voluminous with two to three nucleoli and with numerous chromatin dots, cytoplasm rich with oval to club shaped mitochondria, microtubules and many lysosomes. The dark type is with prominent nucleolus, chromatin dots and the cytoplasm is with prominent vacuoles. Similar observations are available in *Triturus* (Fawcett, 1961) and *Pleurodeles* (Picheral, 1967).

Primary spermatogonia divides mitotically to form secondary spermatogonia which are smaller in size and share more or less similar ultrastructural features as found in primary spermatogonia, except the nucleus which is more compact and smaller in size. The number of spermatogonial mitosis is variable, viz - 8 in *Rana temporaria* (Witschi,1924), 6 in *Triturus vulgaris* (Callan and Taylor, 1968), 6 in *Xenopus laevis* (Kalt, 1976), and *526 in Rana esculanta* (Rastogi et al., 1983). In *Tylototriton verrucosus* gonial mitosis continues for 8 repeated cycles where the pale type spermatogonia represents the stem cells which

maintain their own number by self-renewal and gave rise to committed or differentiated dark side cells (Ray, 1978). The primary spermatogonia divide mitotically and give rise either two daughter primary spermatogonia or through repeated divisions form a cyst composed of secondary spermatogonium. Only a randomly chosen part of the total primary spermatogonial divides at a given time (Saidapur, 1989).

At the end of this growth phase, the secondary spermatogonia give rise to primarary spermatocytes whish are generally situated in the central part of the cross-sected testis tubules or found attached with the wall of testis tubules. In the cross-section of primary spermatocyte cysts various stages of meiosis can be visualized and when divides after first meiotic division produced secondary spermatocytes which have smaller size, dark nuclear chromatin and vacuolated cytoplasm. After second meiotic division spermatids are formed. Spermatids are small globular cells, smaller than the secondary spermatocytes, strongly basophilic and compact chromatin material with the initiation of spermiogenesis, spermatids become more and more elongated as a result of compactation of chromatin material and ultimately give rise to sickle shaped spermatozoa. Events therefore, more or less similar to other amphibian species.

3. SPERM ULTRASTRUCTURE

The present study indicates that the ultrastructural features of the spermatozoa of ***Tylototriton verrucosus*** strongly resemble with earlier studies made by Picheral (1967) on ***Pleulodeles waltlii*** and with ***Triturus viridescens*** (Fawcett, 1961). However, sperm organization is very much different with ***Notophthalmus viridescens*** (Fawcett, 1970).

Organization of the spermatozoa both at light and electron microscopy level has demonstrated that important morphological differences can be found between different species and can be used for taxonomic purposes (Jamieson,

1991, 1992, 1995,1999; Lee et al., 1992,1993; Selmi et al., 1997; Scheltinga et al., 2001; Garda et al., 2002).

On the basis of the available ultrastructural details of the sperms of *Triturus, Pleurodeles, Notophthalmus* and *Tylototriton* a comparative phylogenetic relationship among the four species can be constructed as follow:

Elongated compact head with distinct barb and perforatorium, distinct middle piece and long tail with undulating membrane are the characteristics of all members of Salamandridae.

(a) In *Notophthalmus,* a long slender head with a long pointed perforatorium (harpoon-like) and a long tail with a short wavy undulating membrane may be considered as species-specific feature of the spermatozoon.

(b) Whereas in *Triturus* curve perforatorium may be considered as the main unique feature.

(c) In *Pleurodeles,* the head and the perforatorium is long straight and blunt, the tail is long, with a finlike undulating membrane can be considered as species specific feature.

(d) In *Tylototriton*, sperm ultrastructure is uniquely similar with that of *Pleulodeles waltlii* as described by Picheral (1967,1977). Scanning electron microscope and transmission electron microscope observations on sperm morphology of *Tylototriton verrucosus* have revealed three unique forms of mature spermatozoa, of which the spermatozoa with slightly curve, sharp pointed perforatorium can be comparable with that of *Triturus* while the spermatozoa with long elongated head and blunt perforatorium is similar to those of *Pleulodeles waltlii* and the spermatozoa with bulb like blunt, short perforatorium have not observed in any other species studied so far.

On the basis of spermatozoal ultrastructural details, the author intend to

suggest that ***Tylototriton verrucosus*** is phylogenetically more close to *Pleulodeles waltlii* and supports renomenclature of ***Tylototriton verrucosus*** as ***Pleulodeles verrucosus*** Andersen as suggested by recent taxonomists.

4. SPERM POLYMORPHISM

Sièbold (1836) recorded the existence of two types of spermatozoa in the pond snail *Paludina* , Since that time many cases of conspicuous polymorphism have been found in invertebrates and in some vertebrates (Ankel, 1924, 1930, 1933, 1958; Gupta, 1964; Heberer; 1932; Koehler and Birky, 1966; Macleod; 1970; Ray; 1978; Ray and Roy, 1989; Roy, 1989; Ray et al., 1999; Patra et al., 2002; Chatterjee et al., 2002).

The polymorphism of spermatozoon may manifest not only gross morphological deviation of nuclear and cytoplasmic characteristics, simple size differences (Tuzet, 1930), but also in their genetic and the biological functions (Koehlelr and Birky, 1966; Gupta 1964; Macleod, 1970; Ray et al., 1999, Mukhopadhyay et al., 2002).

The biological origin of different sperm types within a species as well as in an individual has been reviewed in light of genetic and evolutionary consequences. Ankel (1924) was able to show that in many species the atypical series of spermatozoa is recognizable already in spermatogonial stage. In *Bythinia* and closely related species the atypical spermatozoa are morphologically very similar to typical ones, but they are oligo or hyperpyrene and all of them arise in meiosis. Furrow (1935) described in *Valvata* three different kinds of atypical spermatozoa, which departed from normal development at various stages, all post spermatogonial. The first resulting in on umbrella-shaped head, can be traced back to an atypical anaphase of second meiotic division. The second, an oligopyrene or apyrene 'micro-sperm' about one quarter the length of a normal sperm develops early in

spermiogenesis and the third originates later and becomes 'macro-sperm' with sickle-shaped hyperpyrene heads. The last occurs with approximately one tenth the frequency of typical spermatozoa. While the umbrella type degenerates quickly, the microsperm survives until spermiation , but does not reach the efferent duct. The macrosperm is ejaculated together with typical sperm. Ankel (1930) also noticed that in the marine snail *Janthina* atypical spermatozoa also has distinct cytological differences and such atypical spermatozoa follow an entirely different pathway of development. Such spermatozoa develop from atypical spermatogonia which recognizable early. In contrast to typical spermatogonia which develop in or near the lumen, the atypical ones remain attached to the wall of the follicle by a cytoplasmic stalk until they are almost as big as fully-grown oocyte. They result atypical very big sized spermatocytes and do not enter meiotic division, the chromatin becomes fragmented and appears to be lost and thus becomes apyrene. Ankel (1935) also recognized that in *Fusitriton oregonesis*, (a prosobranch) the atypical spermatozoa follow a different pathway of development. Ankel suggested that such atypical spermatozoa develop from the same primordial cell along with typical ones but a set of complex enzyme system operates differently to differentiate the typical and atypical spermatozoa.

Existence of atypical spermatozoa within a species apart from typical ones is not the monopoly of the invertebrates. Existence of sperm polymorphism has been recorded in a number of vertebrates (Macleod et al., 1970; Ray, 1978, Ray et al., 1989; Mukhopadhyay et al., 1999, Patra et al., 2002; Chatterjee et al., 2002).

Roy and Ray (1984) recognized that in a number of amphibians there is a possible existence of different clones of spermatogonial mother cells and a concept of hypo-hyper diploidy was suggested. They recognized that in *Bufo himalayanus* three such clones exist, having normal diploid Chromosome set (2n = 24), hyper diploid chromosome set (2n = 26/28) and hypo-diploid

chromosomal set (2n = 22/20). Roy (1989) also recognized such clones in Himalayan Newt, *Tylototriton verrucosus*. Roy and Ray (1984) and Roy (1989) have also reported that such variation in chromosomal Constituent is not an aberrant or random one, but follows a discrete pattern of Chromosomal 'loss' or 'duplication'.

In many cases of polymorphism the aberrant types are morphologically very characteristic and sometimes show a behavior which suggests that they have become functional components of the reproductive process although they have never been to accomplish normal fertilization. In the rotifer *Asplanchna* atypical spermatids produce immotile 'rod spermatozoa' which are not cells but cellular products of abnormal spermatids, filled with macrotubules (Koehler and Birrky, 1966). The function of these 'rods' is unknown. It may be related to the peculiar intradermal fertilization of these animals. In the spermatophores of fresh water calanoid shrimps two types of spermatozoa are seen, the typical spindle shaped sperm and large polygonal cells which swell when the spermatophore gets in contact with the water. Their increase in volume exerts a pushing action on the typical sperm during evacuation (Heberer, 1932). Gupta (1964) reported that in *Diaptomus* similar atypical spermatozoa without a chromatoid body acts a 'swelling' spermatozoa . Ankel (1930) reported that atypical spermatozoa of *Janthina* (a marine snail) with highly yolky material serves as a carrier for hundred of typical ones. In *Fusitriton* atypical spermatozoa also act as carrier spermatozoa and believed to act as nutrient cells when the typical sperm are on journey for fertilization. Similarly in man about 3%, 9%, 1% sperm are atypical macrosperm, microsperm and bicephalic, respectively and their biological function is not known and they do not participate in fertilization, (Macleod, 1970) .

In recent years, sperm morphology as well as spermatogenesis have been evaluated in the context of serial and co-ordinated action of sperm specific genes and a number of mutations, artificial or normal, have been found to be

related with various morphological as well as functional forms of spermatozoa. Gene expression in the sperm is stagespecific. The initiation of spermatogenesis during puberty in mammals is probably regulated by a gene called BMP8B which enhances the synthesis of their protein by the spermatogonia. When the product of BMP8B reaches a critical concentration the spermatogonia differentiate in spermatids (Zhao et al., 1996). Mice lacking BMP8B gene do not initiate spermatogenesis. Similarly in man DAZ gene located on the long arm of the Y chromosome, when deleted, causes infertility. DAZ gene is exclusively expressed in germ cell line specially in the spermatogonia and it appears to encode an RNA binding protein (Reijo, et al., 1995; Menke, et al., 1997). In *Drosophila*, similarly , two genes RB97D and 'Boule' are essential for spermatogonial kinetics and when mutated cause degeneration of spermatogonia and the cells do not enter meiosis (Karsch et al., 1993, Eberhart et al., 1996). Some genes are also specific for male meiosis (Hoyle and Raff, 1990; Nishioka et al., 1990). In addition to gene transcription in diploid cells during meiotic prophase certain genes are transcribed in spermatids (Palmites et al., 1984; Peschon et al., 1997; Ray et al., 1991). Therefore, existence of reported different clones in amphibians with different chromosomal complement may be a plausible answer of the existence of polymorphism in sperm.

In the present investigation, 4 distinct forms of spermatozoa have been reported in *Tylototriton verrucosus*, they are, typical slender headed sperm with pointed perforatorium, atypical spermatozoa with hook-shaped head with perforatorium, microcephalic and macrocephalic sperm without perforatorium. Their ultrastructural differences as described in the earlier chapter (Sperm polymorphism) are significant and well convincing. FACS analysis of spermatozoa has also supported that polymorphism of morphology is related with DNA values, as 91.56% sperm 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.

Till date, the sperm polymorphism has been reviewd in terms of morphological variations and their functional role in fertilization. But the phenomenon needs a separate attention to address. The author suggests that the existence of the different forms of spermatozoa has an evolutionary significance. Each form of spermatozoa with hyper or hypohaploid DNA value reflects the evolutionary status of the species. The hypohaploid form represent the class which is lower in the evolutionary status of the species (viz. *Triton* in this case) while hyperdiploid form specifies the next phase of evolution the species which give rise or already gave result.