# INTRODUCTION

Spermatogenesis is a dynamic phenomenon which includes a series of changes and is conventionally studied as three gross events.

(a) Spermatocytogenesis - the multiplications of the spermatogonial mother cells to form spermatogonial population.

(b) Meiotic division - which results in the formation of haploid spermatids from diploid primordial germ cells.

(c) Spermiogenesis - transformation of spermatids into spermatozoa.

The events include a series of changes -

- Development and differentiation of germ cells.
- Multiplication of primordial germ cells.
- Reduction of chromosome numbers.
- Transformation of haploid products (spermatids) into spermatozoa.
- Post metamorphic maturations.

Considerable notes are available on the above parameters in mammals (Roosen-Runge, 1951; Fawcett, 1958, 1965, 1970; Clermont, 1962,1972; Vogl et al., 1985; Russel, 1984, 1993; Dym, 1994; Segatelli et al., 2000; Werner and Moutairou, 2000 ), birds (Asa et al., 1986; Phllips, 1989; Lin et al., 1990; Baccetti et al., 1991; Lin and Jones, 1992; Soley, 1994; Góes et al, 2002), reptiles (Clark, 1967; Dehlawi, 1992; Jamieson, 1993;1995; Jamieson et al., 1996; Teixeira et al., 1999, 2002, Scheltinga et al., 2000) and fishes (Grier, 1981; Baccetti et al., 1984; Billard, 1986; Jamieson, 1991; Tanaka et al., 1995; Hara et al., 1998; Romagosa et al., 1999; Gusmao et al., 1999; Medina et al., 2000).

In urodela information are mostly confined to the European , Japanese and American species viz - *Ambystoma* (Bishop,1949; Baldauf,1952; Hassinger et al., 1970; Brandon et al., 1974), *Pleurodeles* (Janssens, 1901; Thorn, 1968; Steward, 1970; Picheral, 1967,1977), *Amphiuma* (Cagle, 1948; Barker and Biesele, 1967), *Siren* (Hanlin and Mount, 1978), *Triturus* (Fawcett, et al., 1961).

Information on the Indian salamander ***Tylototriton verrucosus***,Anderson (*Pleurodeles verrucosus*) is meager and scanty (Ray, 1978; Roy, 1989;).

The present review is, therefore, confined on the above parameters and reflects a comparative studies , among the urodele species (*Trituroides hongkongensis, Cynop pyrrhogaster pyrrhogaster, Triturus viridescens, Pleurodeles waltlii, Notophthalmus viridescens*).

## A. ORIGIN OF GERM CELLS

Germ cells of gonads are of extragonadal in origin. They set aside early in development to become the stem cells, i.e. primordial germ cells of germ line. Light and Electron microscopic, cytochemical, biochemical, and immunological analyses (Bounoure, 1934; Blackler, 1966; Savage et al., 1993; Robb et al, 1996) have clearly shown easily recognizable differences between primordial germ cells and somatic cells in the blastula and early gastrula stages in many species.

In anurans, viz *Xenopus,* (Whittington and Dixon, 1975), the primordial germ cells are found at the vegetal pole of the egg in the two cell stage. They aggregate in small patches being located several millimeters away from the pole. As the two cell stage divides, they aggregate and move towards the cleavage furrow and coalesce medially (Diagram -1).At the end of gastrulation and during tail bud stages primordial germ cells are found to be located in the deep floor of the archenteron.

## DIAGRAM 1

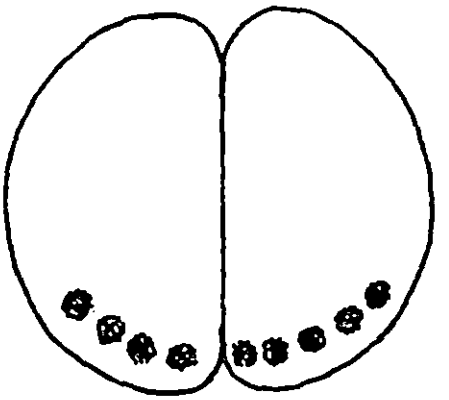
Location and state of aggregation of the germplasm granules during the first two cleavage divisions in *Xenopus.*

A. Two celled stage showing individual aggregates in the vegetal pole formation .

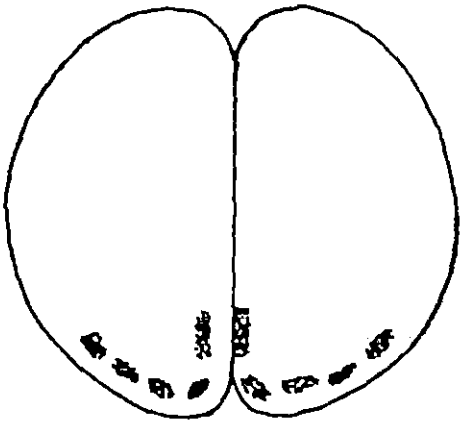
B. Beginning of the second cleavage showing the aggregates moving toward the cleavage furrow and coalescing.

C. Section of four- celled stage showing granules in a single aggregate in each cell along the cleavage furrow.

(Redrawn from Hooper and Hart, 1985)



A



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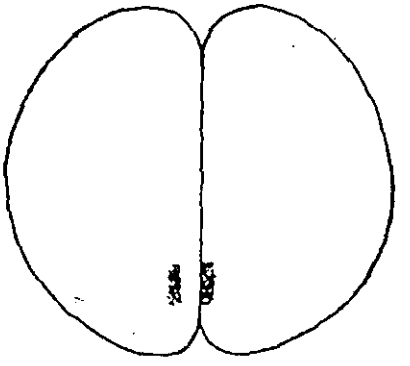


C

DIAGRAM - 1

In urodela primordial germ cells have been shown to be the forerunners of entire germ line (Hopper and Hart, 1985).The germplasm can not be seen at the ventral pole of cleaving egg as found in anuran. The primordial germ cells are not delineated until the gastrula stage, when they may be distinguishable among the cells of lateral mesoderm.

Transplantation experiments of belly endoderm between two species of urodeles have shown that neither the germplasm nor the primordial germ cells are associated with in these species. However, if the lateral mesoderm is removed, in almost all of the larvae the gonads lack the germ cells (Gilbert, 1997).

## B. MIGRATION OF GERM CELLS IN TO GONADAL ANLAGEN

In almost all vertebrates germ cells of gonads are of extragonadal in origin but in later part of development they are found in the developing gonad called gonadal anlagen. Therefore, it is conventionally clear that a developing gonad has two components - the gonadal own part which arises from endodermic cells and germ cell part which arises extragonadally from the lateral mesoderm and later migrates into the gonadal anlagen.

Gonads are paired structures and develop from sexually undifferentiated embryonic primordia located in the peritoneal cavity in close association with the presumptive kidneys. As the gametic event is on progress, the germ cells migrate along the developing ridges and harbor into the forming gonad. How the germ cells migrate is not very clear. The available evidences clearly advocate that the primordial germ cells move 'inmassae' by active amoeboid movement, under the influence of some guiding chemicals strictly express on the gonadal anlagen cells (Gilbert, 1997).

## C. DIFFERENTIATION OF THE GONADAL ANLAGEN INTO TESTICULAR STRUCTURE

Male and female gonads are paired structures and develop from sexually undiffertiated embryonic primordia located in the peritoneal cavity in close association with the presumptive kidneys. The presumptive gonads appear as bilateral thickenings of the coelomic epithelium at the ventrolateral aspect of mesonephric tissue. Finally genital ridges are formed as bulging from the dorsal wall of the coelomic cavity . The caudal part of the genital ridge eventually gives rise to gonads and cephalic area from the fat bodies (Franchie et al ., 1962).

In amphibia, genital ridge develops in a caudal direction in both sexes. Two distinct areas become already distinguishable as peripheral cortex and inner medullar zones. At first, the cortical and medullar zones are similar in appearance in both sexes, but soon follows different patterns in later differentiation process. The proliferation of the cortex with a concomitant regression of the medulla marks the transition of undifferentiated gonad into a presumptive ovary in a female tadpole, while the proliferation of the medulla to gather with the regression of the cortex cause the differentiation of a gonadal anlagen into the testis in the male tadpole (Witschi,1967, Mittwoch,1973, Deuchar,1975). Hayes, 1998, has clearly established that lateral differentiation of gonad is under entire control of genetic regulation, sex chromosome as well as autosomes.

## D. HORMONAL AND ENVIRONMENTAL ROLE IN TESTICULAR DIFFERENTIATION

### 1. Hormonal role

In the process of gonadal differentiation in all vertebrates as well as invertebrates there are some hormones which play a vital role. In amphibians, gonadal testicular differentiation is also influenced by the hormones.

The pituitary appears to be less involved in the very early stages of sexual differentiation and the mammalian gonadotropin fails to influence the larval gonads. The effect of exogenously administered hormones are only well observed if they are given after metamorphosis, but before the completion of the differentiation process, just to avoid the unwanted interaction between the injected hormones with the naturally secreted thyroid hormone which are responsible for metamorphic changes.

It has been observed that the follicle stimulating hormone (FSH) is responsible for the differentiation of the intermediate (bi- potential )gonad into a testis, but different effects are produced in different species depending on the time of treatment in relation to the time of gonadal development. In male *Ceratophrys ornata* gonads differentiates shortly before metamorphosis., the treatment with FSH immediately after metamorphosis hardly stimulates spermatogenic activity (Pisano and Burgos,1971). But in *Pleurodema cinerea* differentiation of gonads occur long before metamorphosis. Here FSH treatment also produces complete spermatogenesis (Pisano and Burgos,1962). In *Bufo arenarium*, in which the gonads differentiated long after completion of metamorphosis as in *Ceratophrys*, FSH administration immediately after metamorphosis provokes no spermatogenic stimulation (Pizarro and Burgos,1963). In both species, FSH produces an increase in the testicular size, but when the apparently stimulated gonad is examined histologically it displays a sponge-like network of fluid-filled ampullae and the spermatogonia appear unchanged (Pisano and Burgos,1971).

### 2. Environmental role

External factors such as temperature and internal influence such as endogenously produced androgenic and estrogenic steroids, can influence the genetically induced direction of development in the primordial gonads (Gallien, 1955).

Estrogen induces sex-reversal phenomenon in genetic male in a number of species, where ovo-testis or a complete and often permanent femaleness occurs. Androgen-induced sex reversal in females is, however, less frequent, with the exception of ranids who are also susceptible to progesterone.

## E. TESTICULAR CYCLE AND SPERMATOGENIC PATTERNS IN AMPHIBIA

Amphibians are poikilothermic (ectothermic) and as well as majority of them depend upon water for breeding. Their reproductive strategy is greatly affected by the changing climatic factors such as the temperature, rainfall, day light length and relative humidity. By and large, amphibians are therefore, seasonal breeders.

Depending on environmental cues the testicular morphology vis- a- vis the spermatogenic activities greatly show seasonal variations and are conventionally studied as testicular cycles.

The testicular cycles encompass the kinetics of gonial mitosis, meiotic drive, transformation of spermatids into spermatozoa, release of spermatozoa together with changes of Sertoli cells and endocrine elements , the interstitial cells.

The "innate" gametogenetic cycle, operating despite the constraints of the local environment, characterizes the annual patterns of reproductive cycle. Among the amphibians several distinct spermatogenetic patterns are

recognized.

In Caecilians active spermatogenesis takes place throughout the year (Wake, 1980).

Basu ( 1969) recorded four distinct spermatogenetic activity in Anurans in reference to their geographical distribution :

**a) Continuous type :** In Bufo arenerum, *Bufo melanostictus, Rana cancrivora and Rana hexadactyla* the process of spermatogenesis continues throughout the year.

**b) Discontinuous Type :** In *Rana temporaria* and *Rana pipens* this process is strictly seasonal.

**c) Continuo-discontinuous type:** In *Rana esculenta* and *Rana tigrina* spermatogenesis depends invariably on environmental factors. Particularly in *Rana esculenta,* spermatogenesis progresses only up to the spermatid stage in winter.

**d) Variable type:** In *Rana esculenta ridibunda* and *Discoglossus pictus* spermatogenesis is of discontinuous type in Europe while, strikingly enough in the Mediterranean region spermatogenesis in the same species is of continuous type. The patterns of spermatogenetic activity in different anuran species collected from different geographical areas are summarized in Table 1.

## Table - 1

Spermatogenetic Activity in some anurans with References to Their Geographical Distribution

| 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 |
| *Hyla crucifer* | USA |
| *Pleurodema bufonia* | S. America |
| **Continuo – discontinuous type** | |
| *Rana esculenta*[1] | Europe |
| *Rana gracea* | S. America |
| *Rana tigrina* | India |
| *Rana nigromaculata* | Japan |
| **Variable Type** | |
| *Rana esculenta ridibunda*[2] | Europe<br>Mediterranean region |
| *Discoglossus pictus*[3] | Europe<br>Mediterranean region |

[1] In this species spermatogenesis goes only up to spermatid formation in winter.

[2] and [3] In Europe, spermatogenesis is of discontinuous type while in Mediterranean region, it is of continuous

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 ***Tylototriton verrucosus***, the onset of spermatogenetic activity is palpable in the April testes. In apical lobe of the April specimens, the emergence of spermatocytes is restricted to some particular lobules. Meiosis begins in the May testes where spermatogonial cells are present only in the peripheral lobules while primary spermatocytes are restricted to the inner lobules of the testes. The caudal lobe also exhibits spermatogonial differentiation into spermatocytes. Meiotic upsurge is observed in the apical and caudal lobes of June and July specimens where about 80% of the lobules are studded with spermatocytes at different divisional stages. Spermiogenesis commences in the June specimens and continues during the months from July to December. Spermiogenesis however, is not a synchronous event in all lobules 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 continues up to December. However, in some species spermatozoa remain within the lobules up to February. The seasonal variation in testicular weight and composition of testicular lobe (s) are summarized in the figure -1. It seems that the spermatogenesis in ***Tylototriton verrucosus*** follows an annual pattern.

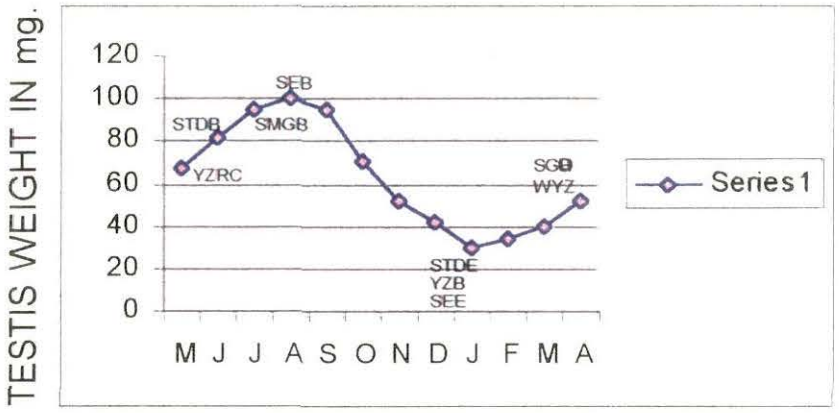
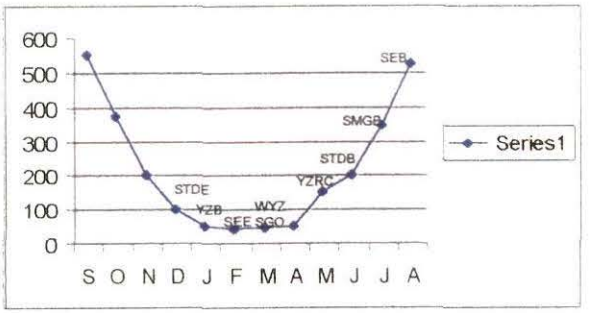



FIGURE - A *Tylototriton verrucosus*


FIGURE - B *Tylototriton verrucosus*

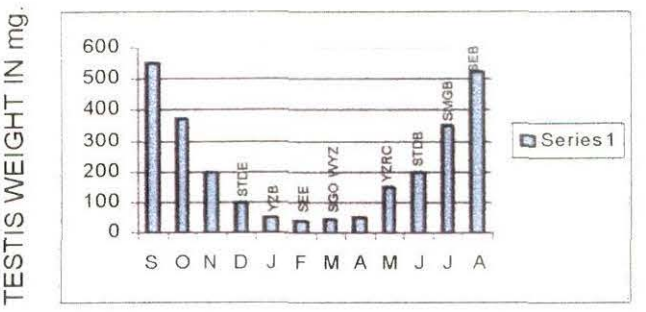
**FIGURE - 1**

SEB : sperm evacuation begins, SEE - Sperm evacuation ends,
SGO - sperm ontogenic onset, SMGB - Spermiogenesis begins,
STDE - spertid formation ends, STDB - spermatid formation begins,
WYZ - well developed yellow zone, YZB Yellow Zone formation begins,
YZRC - Yellow Zone resorption complete.



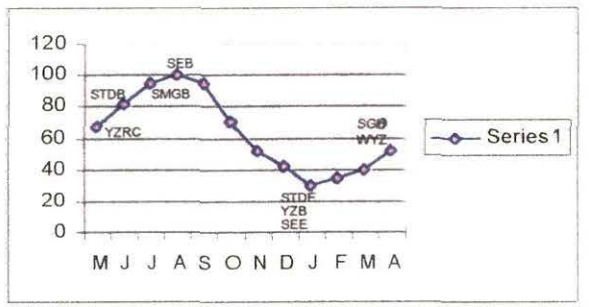

MONTHS

FIGURE - A *Trituroides hongkongensis*



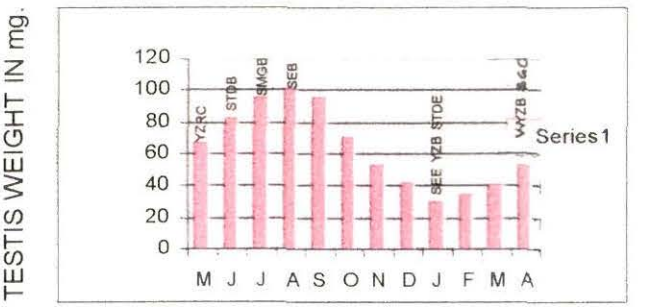

MONTHS

FIGURE - A *Trituroides hongkongensis*



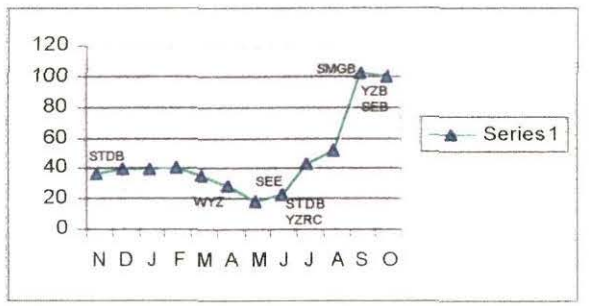

MONTHS

FIGURE - B *Tylototriton verrucosus*



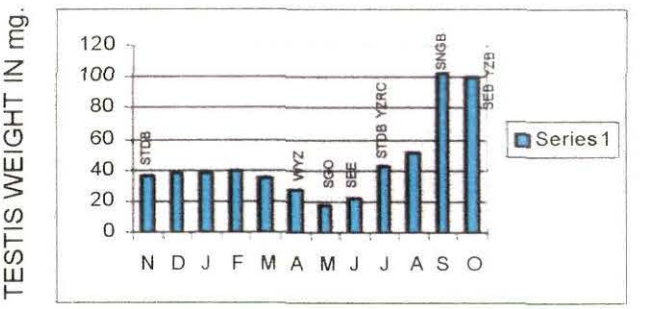

MONTHS

FIGURE - B *Tylototriton verrucosus*




MONTHS

FIGURE - C *Cynop pyrrohgaster pyrrohgaster*




MONTHS

FIGURE - C *Cynop pyrrohgaster pyrrohgaster*

## FIGURE - 2

SEB : sperm evacuation begins, SEE - Sperm evacuation ends, SGO - sperm ontogenic onset, SMGB - Spermiogenesis begins, STDE - spertid formation ends, STDB - spermatid formation begins, WYZ - well developed yellow zone, YZB Yellow Zone formation begins, YZRC - Yellow Zone resorption complete.

The annual testicular pattern in *Trituroides hongkongensis* (Tso and Lofts, 1977) and *Cynop pyrrhogaster pyrrhogaster* (Tanaka and Iwasawa, 1979) closely resembles the same in *Tylototriton verrucosus.* However, testicular composition and events of spermatogenesis differ among these three species, whose testicular composition has been summarized in the figure - 2.

The "Yellow Zone" composed of residual Sertoli cells (Vellano,1969) is well developed in the March and April testes. The resorption of the Yellow zone takes place during May and spermatogenetic activity begins. In *Trituroides hongkongensis,* the "yellow zone" resorbs during May. This results in the swelling of the testicular lobe with lobules filled with spermatogonial and spermatocytic germinal cysts ( Tso and Lofts, 1977). The "yellow zone" might be associated with the secretion of testicular hormones and be involved in the final development of secondary sexual characters (Aron, 1924). Steroigenic activity of the "yellow zone" has been evidenced from a 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, 1977) *Trituroides vulgaris* (Verrell et al., 1986).

## F. PATTERNS OF SPERMATOGENESIS IN AMPHIBIA

Reproductive cycles in amphibians are subject to hormonal controls, which within genetic limitations respond to environmental variables and produce certain patterns.

Among the Caecilians, spermatogenesis is a seasonal process as found in *Ichthyophis glutinosus* (Breckenridge and Jayasingha, 1979) and *Dermophis mexicanus* (Wake, 1980)

Amongst the anurans two distinct cycles are noted. In most temperate zone species the production of spermatozoa by the somniferous elements is

confined to a limited period of the year. Hence, the cycle is discontinuous one (Cei, 1944; van Oordg, 1956; Klug, 1981). In the species inhabiting equatable tropical and subtropical areas where environmental temperatures are not subject to great seasonal fluctuations, spermatogenesis continues throughout the year (Hing, 1959; Church, 1960, Inger and Greenberg, 1963, Berry, 1964, Inger 1967, Crump, 1974; Duellman, 1978). However, in upper Amazon Basin four patterns of spermatogenesis are evident among the anuran species :

1. **Continuous :** The spermatogenesis is continuous with the exception of clear, dry nights with intense moonlight.

2. **Opportunistic :** The spermatogenesis takes place regularly after heavy rains throughout the year.

3. **Sporadic wet :** The spermatogenesis takes place sporadically after heavy rains.

4. **Sporadic dry :** The spermatogenesis takes places sporadically during infrequent dry periods (Duellman and Trueb, 1985)

Two major reproductive patterns are exhibited by salamanders. The classical annual pattern is found in aquatic breeders viz *Ambystoma* (Bishop, 1941, Baldauf, 1952; Hasinger et al. 1970), *Pleurodeles* (Thorn, 1968; Steward, 1970), *Amphiuma* ( Cagle, 1948), *Siren* (Hanlin and Mount, 1978) , *Andrias* (Chang, 1936) etc. The second major pattern is biannual and is characteristic of the terrestrial plethodontid salamanders.

## G. FACTORS AFFECTING TESTICULAR CYCLE

The hormonal and environmental factors controlling or affecting the annual changes in the testis have been well established in several temperate zone amphibians (Jørgensen et at., 1979; Lofts, 1984). Due to the inadequate thermoregulatory mechanisms amphibians inhabiting temperate region are

greatly affected by the seasonal changes in the ambient temperature. Consequently, spermatogenesis is interrupted during cold winter months. This gametogenetically inactive period is known as the resting period. The spermatogenetically active period is restricted to spring-summer months. Further, spermatogenesis may be prenuptial or postnuptial type depending upon the species (Lofts, 1984). In general, spermatogenetic activity in the temperate zone anurans is high in summer while in winter the rate of spermatogonial division is extremely low (*R. esculenta*) or absent (*R. temporaria*). Consequently, they show either potentially continuous or discontinuous type of spermatogenetic cycles (Van Oordt, 1960; Lofts, 1984). The testicular cycles of temperate species have recently been reviewed (Jørgensen et al., 1979 ; Rastogi and Iela, 1980; Lofts, 1984).

The testicular cycle in amphibians is controlled by both intrinsic and extrinsic factors as in other vertebrates. However, there are few studies dealing with the control of testicular cycles in the Indian representatives. The available literature is summarized below.

## 1. Endocrine Control

### a) Role of Hypophysis

It is well established that hypophysial gonadotrophins play an important role in the regulation of spermatogenesis in all vertebrate species studied so far. The Individual role of gonadotrophins and testicular steroids in the control of spermatogenesis are better understood in mammals. In the non-mammalian vertebrates it is not clear whether the hypophysial control of spermatogenesis is direct or mediated through the testicular steroids (Rastogi and Iela, 1980; Callrad et al., 1978; Saidapur, 1983). Also until recently it was not known whether amphibian pituitary elaborates one or two gonadotrophins.

The fractionation studies of pituitary hormones from anuran and urodelan

species have now revealed two chemically distinct gonadotrophins (Licht, 1979). Purified amphibian FSH and LH are not commercially available. Hence, mammalian gonadotrophins (LH, FSH, HCG and PMSG) have been generally used in the study of spermatogenesis of Amphibia.

The limited number of studies made to elucidate the hormonal control of spermatogenesis in amphibians have yielded confusing results (Basu, 1969; Lofts, 1974; Callard et al., 1978; Rastogi and Iela , 1980; Saidapur, 1983). It is generally believed that in adult amphibians the early phases of spermatogenetic cycle, the mitotic proliferation of primary and secondary spermatogonia are gonadotrophin-dependent (Lofts. 1974). In *R. esculenta* hypophysectomy results in the loss of mitotic activity in primary spermatogonia (Sluiter et al., 1950). However, according to Rastogi et al., (1976 ), in the same species the primary spermatocytes are the most sensitive elements and hence degenerate soon after hypophysectomy. The spermatids, if present at the time of operation, are not affected. The primary spermatogonia increase in number but lose their capacity to form secondary spermatogonia which decrease but do not undergo massive degeneration. In the toad , Bufo bufo, 4 weeks of hypophysectomy had no measurable effect on spermatogenesis from the stage of primary spermatocytes and onward, whereas production and survival of secondary spermatogonial cysts were strongly impaired (Guha and Jørgensen, 1979). Gonadotrophin independence during the process of spermatogenesis in *Bufo bufo bufo* is acquired late in the secondary spermatogonial stage (Guha and Jørgensen, 1978). Interestingly, in juvenile *Alytes obstericans* (Guha and Jorgensen, 1978) and *Rana nigromaculata* (Iwasawa and Kobayashi, 1976) spermatogenesis occurs even in the absence of gonadotrophins. It appears, therefore, that hormone dependent stages of spermatogenesis may be different in different species.

It was formerly believed that in Amphibia ICSH acts on the Leydig cells to stimulate androgen synthesis and on Sertoli cells to induce spermination while FSH stimulates only spermatogenesis (Lofts, 1974) . Recently, it has been shown that HCG promotes complete spermatogenesis in the hypophysectomized *R. hexadactyla* (Kasinathan and Basu, 1973), and *Bufo* (Guha and Jørgensen, 1978) . In the Indian bull frog *R. tigerina* during the post-breeding regression phase both HCG and PMSG induced spermatogenetic activity but the effect of PMSG was less than that produced by the same dose of HCG (Kurian and Saidapur, 1982). In the only study of homologous hormones in the American bull frog, *Rana catesbeiana* no difference was evident between FSH and LH effects on spermatogenesis, both caused an increase in the number of germ cell cysts in the adult hypophysectomized individuals. Thus, the findings on *R. hexadactyla*, *R. catesbeiana* and *R. tigerina*, indicate that both HCG and PMSG are capable of stimulating spermatogenesis in anurans.

In mammals, it is known that ICSH stimulates androgen synthesis by the Leydig cells which in turn regulates meiotic divisions. The FSH is needed for spermiogenesis (Steinberger and Steinberger, 1974). Thus in mammals, ICSH and FSH have sequential rather than synergistic effects on spermatogenesis. Since HCG induces complete spermatogenesis as well as the Leydig cell activity in anurans, it is necessary to determine whether its effects are direct on spermatogenetic cells or mediated through androgen production by the Leydig cells. Even though earlier workers attributed more importance to FSH and ICSH in spermatogenesis the future years may prove contrary to this belief. The FSH may have a supporting role.

The annual changes in the testis may result due to the changes in the pituitary gonadotrophic activity or response of the germinal epithelium to gonadotrophins as shown in some temperate anurans (Van Oordt. 1960; Lofts, 1974). In the Indian green frog, the sensitivity of the germinal epithelium and

also the gonadotrophic content of the pituitary gland were found to be greater in summer months. (Kasinathan and Basu, 1972). In *R. tigerina* hypophysial gonadotrophs exhibit very distinct seasonal changes in their secretary activity (Kurian and Saidapur, 1984) which accounts for the annual testicular cycle. However, the frog testes maintain their sensitivity to gonadotrophins even during the postbreeding regressive phase (Kurian and Saidapur, 1982). A similar situation may be encountered in other species showing potentially continuous type of spermatogenetic activity. In view of the continuous spermatogenetic activity in *B. melanostictus* and *R cyanophlyctis* the sensitivity of the germinal epithelium to gonadotrophins, and gonadotrophin content of the pituitary, may not drastically very seasonally. In these species, pituitary seems to secrete tonic levels of gonadotrophins throughout the year.

The Leydig cell activity in anura is also regulated by the hypophysial gonadotrophins. Recent studies on *R. tigerina* show that annual changes in Leydig cell activity is correlated with the changes in the hypophysial gonadotrophs (Kurian and Saidapur, 1984). These findings support the above view. Therefore, hypophysectomy causes regression of these cells which can be prevented by pituitary homogenates, LH or HCG (Kasinathan and Basu, 1973). Similarly, treatment with anti-gonadotrophic substances such as methallibure (Saidapur et al., 1975; Kanamadi and Saidapur, 1981) and steroids (estrogen and androgen) which block pituitary gonadotrophin secretion also cause their regression (Saidapur and Nadkarni, 1975; Kanamadi and Saidapur, 1982).

**b) Role of Androgen**

In amphibians as in other vertebrates the Leydig cells are the main source of androgen in the testis. In anurans, androgen is known to perform diverse roles such as stimulating specific stages of spermatogenesis, development and maintenance of the secondary sexual structures such as the thumb pads and

vocal sacs and feed back regulation of hypophysial gonadotrophin secretion.

The development and regression of thumb pads indicate fluctuation in the androgen secretion by the Leydig cells of the testis in anurans. The seasonal changes in the blood and tissue (testis) levels of androgens are not known for the Indian amphibians. Nevertheless, seasonal study of Leydig cell morphology and 3ß-HSDH activity and associated changes in the androgen dependent thumb pads provide clues regarding the pattern of changes in the androgen levels in frogs and toads. Accordingly, absence of seasonal changes in the Leydig cells and thumb pads in *R. cyanophlyctis* suggests that basal level of androgen is secreted year round. Whereas, in R. tigerina there is a characteristic post-breeding regression of both Leydig cells and thumb pads, The regression of thumb pads is, therefore, attributed to an interruption of androgen synthesis. In the common toad, Leydig cells and thumb pads undergo correlative seasonal changes but they never regress completely, suggesting that low amounts of androgens may be produced during the non-breeding months also (Kanamadi et al., 1983). Thus the three sympatric species living in the same areas environment show diverse patterns in their Leydig cell secretary activity.

It is interesting to note that in a number of temperate zone anurans, the annual cycle of Leydig cell activity (androgen secretion follows a different pattern from that of the spermatogenetic cycle). For instance, in the European species *R. temporaria* and *R. esculenta* thumb pad development and Leydig cell activity are high when spermatogenetic activity is nil, but during the period of spermatogenetic recovery the thumb pads atrophy and the Leydig cells become inactive (Lofts, 1974). In *Icthyophis glutinosus*, an Indian apodan species studied in detail, the spermatogenetic and Leydig cell cycles are likewise dissociated (Seshachar, 1941). On the contrary, in the Indian anurans studied so far spermatogenetic and Leydig cell activities and thumb pad development run parallel (Saidapur, 1983). These observations suggest that

in these anurans androgen may be needed for spermatogenesis.

The effect of exogenous androgen on the spermatogenesis in anurans have been reviewed (Callard et al., 1978; Rastogi and Iela, 1980; Saidapur, 1983). Basu and co-workers have reported that implantation of 15 mg testosterone pellet in the dorsal lymph sac in *R. tigrina*, *B. melanostictus* and *R. hexadactyla* inhibits spermatogenesis (Basu, 1962 a, b, 1968, 1969; Kasinathan and Basu, 1971, 1973, 1975, 1977). Apparently they used a massive pharmacological dose of testosterone in view of the fact that circulating levels of the hormone may never exceed 20-30 ng/ml blood plasma as shown in *R. esculenta*. Basu and his colleagues opine that androgen inhibits spermatogenesis, by directly acting on the germ cells regardless of the presence or absence of the gonadotrophins. Interestingly Basu ( 1968) also states that testosterone has no inhibitory effect on spermatogenesis in vitro as revealed in the organ culture studies. Recent studies also on the toad show that testosterone in low doses does not inhibit spermatogenesis ( Kanamadi and Saidapur, 1982). Further the inhibitory effect of estradiol – 17ß on spermatogenesis in the toad were not only overcome by pituitary homogenate but also by testosterone ( Kanamadi and Saidapur, 1982) Earlier, Thyagaraja and Sarkar ( 1972) studying the effects of steroids on the compensatory hypertrophy of the remaining testis following hemi castration in the same species (*B. melanostictus*) found that testosterone propionate – treatment markedly increased the mitotic activity of the cell nests (as revealed by the total increase in the number of cell nests of all stages). However, administration of cyproterone acetate (CPA) or cyproterone (CP) along with HCG blocked cell nests of stages III-V which suggests that meiotic division and spermiogenesis in the frog require androgen (Kurian and Saidapur, 1985). Further, in the same study, it was shown that administration of HCG + testosterone significantly increased the primary spermatogonial number without affecting the subsequent stages thereby suggesting that androgen may have a synergistic effect with gonadotrophin in affecting the mitotic proliferation

of spermatogonia as in *R. esculenta* (Rastogi, et. al ; 1976).

**c) Role of Fat Body and Other Factors:**

The possible involvement of fat bodies in the regulation of gonadal functions in amphibians was suggested several decades ago. According to Chieffi et al. (1980), there exists a portal of two tissues. These authors also suggest that the pituitary secretes a factor which mobilizes the fat body contents. In *R. esculenta* and *R. hexadactyla* the fat body ablation selectively impaired spermatogenesis in the homolateral testis (Chieffi et al., 1975; Kasinathan et al., 1978). The atrophic changes in the testis of fat body ablated frogs was prevented by extracts of fat body (Kasinathan et al., 1978; Chieffi et al., 1980), prostaglandin and cAMP (Kasinathan et al., 1979). In *R. hexadactyla* fat bodies are therefore supposed to secrete cAMP and prostaglandin which are transported to the homolateral testis (through portal system). Further prostaglandin are considered to have a role in spermatogenesis while cAMP has to in testicular steroidogenesis ( Kasinathan et al., 1979) On the other hand, in a study on *R. cyanophlyctis*, fat body ablation of 45 or 92 days in different seasons did not affect spermatogenesis or Leydig cell activity (Kanamadi and Saidapur 1988) Therefore, additional studies on different species are needed to determine more convincingly the role of fat bodies in spermatogenesis and also species variation, if any.

That the ascorbic acid and / or its derivative, dehydroascorbic acid may have a role in the testicular physiology of anurans has been recently suggested. In the common toad , *B. melanostictus* 10 days after hypophysectomy there was marked decrease in the testicular ascorbic acid, dehydroascorbic acid and in the Leydig cell 3ß-HSDH ( Biswas, 1969, 1970, 1971; Biswas and Deb 1970). Administration of 01 mg LH to hypophysectomized toads increased both ascorbic acid and 3ß HSDH of the

testis (Biswas and Deb, 1970). Interestingly FSH given along with dehydroascorbic acid stimulated Leydig cell 3ß HSDH (Biswas, 1970). That the toad testis is capable of catalyzing the conversion of ascorbic acid into dehydroascorbic acid has also been reported (Biswas, 1971). However, these studies do not clarify the role of ascorbic acid and its derivative dehydroascorbic acid in spermatogenesis.

## 2. Environmental Control

The relationship between environmental factors such as temperature, light, rainfall and the testicular cycles of Indian anurans has not been experimentally studied. Therefore, only tentative conclusions are possible regarding the role of these extrinsic factors in controlling the testicular cycles of Indian anurans, based on correlative observations.

In temperate zones the ambient temperature may fluctuate between 5 to 25°C depending upon the season. In anurans living there, high temperatures (20 – 25°C) stimulate spermatogenesis but suppress the Leydig cell activity . In winter or at low temperature changes range from 20 – 30°C, and therefore favorable temperature becomes available for most part of the day. Consequently, the anurans living in these regions have evolved either continuous or potentially continuous type of spermatogenesis depending upon whether they undergo hibernation / aestivation or not. In temperate zone environmental temperature is also known to regulate breeding of amphibians. However, in the tropics rainfall seems to be very important factor controlling the breeding activity since all the anurans studied so far in southern India are known to breed mainly during the rainy months of monsoon. In species with continuous gametogenetic activity at the individual or at population level may exhibit scattered breeding activity during other months as well.

The importance of day length, if any, in the control of hypophysial gonadal axis, is not known. In the South Indian regions from where the above studies

were made sufficient day length is available throughout the year with only minor fluctuations. Therefore, what effects temperature and light may have on pituitary gonadotrophic activity in the Indian amphibians needs to be studied.

## H. Gene Function in Spermatogenesis

As a differentiation process, Spermatogenesis is regulated by the genomes and is dependent upon coordinated expression of selected portion of the genome (Ghosal, 1971, Ives et al., 1971; Ghosal and Mukherjee , 1977). Spermatogenesis in unique in that, the chromatin condensation takes place prior to sperm formation. Consequently, transcription that is necessary for sperm differentiation must occur before the major differentiation events. The loss of transcriptional capacity during late spermiogenesis has been documented in a number of species (Geremia et al., 1977).

Although RNA synthesis stops, completion of sperm formation depends on the continued synthesis of protein. In absence of concurrent synthesis of RNA, protein synthesis is supported by stable RNA ( Post-meiotic RNA) Produced during early stages of spermatogenesis and stored for translation during spermiogenesis (Monesi, 1965; Brink, 1968; O'Brien and Bellve, 1980).

In trout, Iatrou and Dixon (1978) and Iatrou et al., (1978) have recorded the replacement of histones by protamines in spermatids.

The premeiotic synthesis of DNA occurs only at pre-leptotene spermatocytes (Lima–de-Faria and Borum, 1962). Nearly all the nuclear DNA is replicated during the premeiotic interphase (Taylor and McMaster, 1954; Monesi, 1962; Pearson, 1973) although a very small amount of DNA synthesis is expected to occur during zygotene and pachytene stages (Whitehouse, 1963; Meselson, 1964). An extraordinary low level of DNA synthesis has been reported during meiotic prophase of the male mouse (Mukherjee et al. 1966,

67, 68; Mukherjee and Cohen, 1968; Meistrich et al., 1975) and rat (Soderstrom and Parvinen, 1976).

In recent years, 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 stage specific. The initiation of spermatogenesis during puberty in mammals is probably regulated by a gene called BMP8B which enhance the synthesis of their protein by the spermatogonia. When the product of BMP8B reaches a critical concentration the spermatogonia differentiate into spermatid (Zhao et al., 1996). Mice lacking BMP8B gene do not initiate spermatogenesis. Similarly in man DAZ gene located on the long arm of Y chromosome when deleted causes infertility. DAZ gene is exclusively expressed in the 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.( Palmiter et al., 1984, Ray et al., 1999, Peschon et al., 1997).

## I. Kinetics of Spermatogenesis

The investigation on the duration of meiosis and spermiogenesis has presented several interesting findings. The cycle of the seminiferous epithelium in mammals is a phenomenon of fixed duration (Clermont and Trott, 1969). In rat testes, hypophyseal hormones themselves (Clermont and Harvey, 1965) and elevation of temperature (Chu et al., 1974;) do not influence the rate

of development of the germ cells and the cycle duration.

The duration of spermatogenesis has been estimated by several investigators using various techniques, such as the colchicine injection (Roosen – Runge, 1951). X-irradiation (Oakberg, 1955) , heat – Shock (Mcleod and Hotchkiss, 1941; Young, 1972) and the injection of toxic substances (Drobeck and Coulston, 1962; Muzzanti et al., 1964) to the gonadal tissue. Probably the best of these methods is the one employing a labelled precursor of DNA. After the label is incorporated into the DNA of the germ cells their kinetics till the spermatozoa formation could be followed autoradiographically.

Radioactive tracer such as $^{14}$ C- adenine, $^{3}$H-thymidine and $^{32}$P-phosphate have been widely used for determining the duration of the meiotic stages and spermiogenesis in the mouse (Pelc and Howard, 1956; Sirlin, 1958), rat (Clermont et al., 1959), rabbit ( Swierstra and Foote, 1963, 1965), the chinese hamster (Utakoji, 1966). Golden hamster (Ghosal and Mukherjee, 1971), man (Heller and Clermont, 1963) stallion (Swierstra et al. 1974) and *Mus booduga* (Basu Roy et al., 1987). The details of the duration of individual stages of meiosis and spermiogenesis in various vertebrates as estimated by various investigators are shown in Table 2.

## Table 2

Duration (in days ) of Individual Stages of Meiosis and Spermiogenesis in Different Vertebrates.

| Spices | | | | | | | AUTHORS |
|---|---|---|---|---|---|---|---|
| | L | Z | P | DP-MI | DP-MII | S | |
| *Heteropneustes fossilis* | 0.5 | 0.1 | 2.1 | | 0.61 | 8 | Bandopadhyay, 1977 |
| *Bufo melanostictus* | 2.5 | 0.3 | 4 | 0.9 | | 13 | Bandopadhay et al., 1977 |
| *Rana limnoceris* | 2.29 | 0.5 | 4.7 | 1 | | 6 | Mallick ,1987 |
| *Uperodon globulosum* | 2.7 | 0.75 | 5.5 | | 0.5 | 8.3 | Ray et al., 1984 |
| *Rhacophorus maculatus* | 1.88 | 0.84 | 4.78 | 0.5 | | 9.75 | Ghosal et al., 1981 |
| *Tylototriton verrucosus* | 0.05 | 0.75 | 9.25 | 1.75 | | 6.25 | Roy, 1989 |
| Mouse | 3 | 0.05 | 7 | | 0.47 | 15 | Ghosal and Mukherjee, |

Abbreviations: L, Leptotene; Z, Zygotene; P, Pachytene; DP-MI, diplotene to metaphase – I; DP-MII, diplotene to metaphase – II; S, Spermiogenesis.

Information on the details of the amphibian spermatogenic chronology is somewhat sporadic. Gurwitsch (1911), Stieve (1920), Kalt (1976), Bandopadhyay et al., (1977), Ray (1978), Ghosal et al., (1981), Ray et al., (1984), Mallick (1987), Ghosal and Mallick (1988), Mallick and Ghosal (1988) and Roy, (1989) have recorded duration of spermatogenesis in some salientian and urodele species.

## J. SPERM POLYMORPHISM

Polymorphism was first recorded in the pond snail *Paludina* by Siebold (1836) and he described two types of spermatozoa in that species. However, systematic studies on this issue have been made only in recent years. Hendelberg (1969) found two kind of spermatozoa in an acoelan flatworm one with two flagella called 'typical' and a smaller 'atypical' without nucleus. In many other cases of polymorphism the aberrant types are morphologically very characteristic and sometimes show a behavior which suggested that they have become functional components of the reproductive process although they have never been successful to accomplish normal fertilization ( Roosen - Runge, 1977 ).

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 hyperpyne 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 originate 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 evacuated 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 is recognizable early. In contrast to typical spermatogonia which is 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. he suggested that such atypical spermatozoa develop from the same primordial cell along with typical ones but a set of complex enzyme system operate 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, Roy et al., 1989; Mukherjee et al., 1999, Patra et al., 2002; Chatterjee et al., 2002).

## K. SCOPE OF THE STUDY OF THE PRESENT INVESTIGATION

Review dealing with the reproductive cycles of amphibia have published periodically since 1950. (Marshall, 1956; Gallien, 1959; Oordt, 1960; Wake, 1968,1980; Wortham et al.,1977,1982; Lofts, 1968,1974,1984; Duellman et al., 1986; Saidapur and Nadkarni, 1975; Saxena and Lal, 1981; Kurian and Saidapur, 1983; Kanamadi et al., 1983; Bohra and Niazi, 1984; Jamieson et al., 1993; Kwon et al., 1995; Selmi et al., 1997; Lee et al., 1992, 1993; Sheltinga et al., 2001; Garda et al., 2002).

It is evident from these studies that much of the work on amphibian reproduction related to temperate species. Tropical and subtropical amphibian fauna gained attention through Japanese and Brazilian scientists ( Hanlin,

1978; Ifft, 1942; Iwasawa et al., 1972; Kort et al., 1965 ; Wake, 1980; Tanaka, 1979; Lee et al., 1992, 1993; Garda et al., 2002). In comparative recent years several papers dealing with gonads of some South Indian amphibians that enjoyed the tropical climate are published (Saidapur and Nadkarni, 1975; Kanamadi et al., 1983; Kurian and Saidapur,1983). Of the three orders, of present day amphibians studies on the gonad of Indian urodela are scanty or almost lacking. Therefore, the present investigation has a potential scope for study on following aspects of urodelan reproductive biology.

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

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

3. 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).

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

The knowledge that would be obtained from the observations on the above parameters will bridge the gap in our understanding of the various aspects related to the reproductive biology of urodela, particularly that of our sole urodelan representative.