GONADAL DIFFERENTIATION AND SPERMATOGENETIC ACTIVITY IN HIMALAYAN TOAD, *Bufo himalayanas* (Gunther): ANURA : AMPHIBIA – AN ULTRASTRUCTURAL STUDY

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CERTIFICATE FROM RESEARCH GUIDE

This certifies that Mr. Shekhar Mukhopadhyay, M.Sc., carried out the investigations incorporated in the thesis entitled "GONADAL DIFFERENTIATION AND SPERMATOGENETIC ACTIVITY IN HIMALAYAN TOAD, *Bufo himalayanas* (Gunther) : ANURA: AMPHIBIA- AN ULTRASTRUCTURAL STUDY", under my supervision and guidance. This thesis which embodies the results of original investigations made by Mr. Mukhopadhyay, have been carried out during the period 1996 to 2001. He has fulfilled all the requirements and regulations relating the nature and prescribed period of research. This thesis is submitted in partial fulfillment of the degree of Ph.D. (Sc.) of the University of North Bengal. This thesis has not been submitted previously anywhere for any degree whatsoever either by him or anyone else.

It may please be noted that he shares equal authorship for all the published works appended to this thesis.

29/4/2002

Dated, Darjeeling
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DEDICATION

This thesis is dedicated to never fading memories of my beloved father late Sri Probodh Chandra Mukhopadhyay

and

My ever loving mother Smt. Lily Mukhopadhyay who created ever growing interest towards mystries of a living world in my mind.

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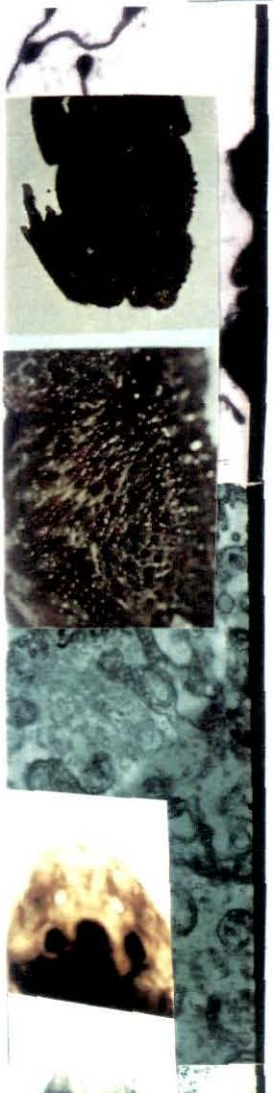
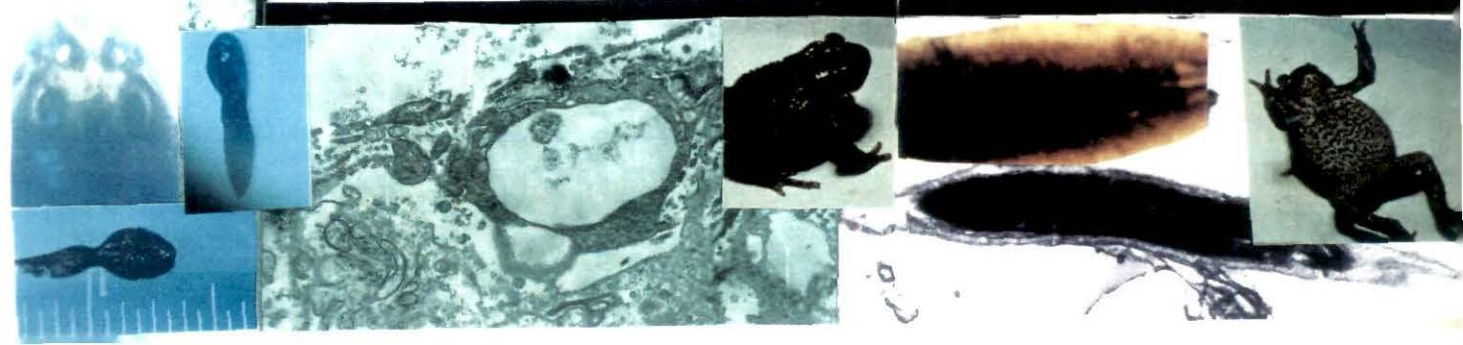




Preface

Bufo himalayanas is an endemic species of toad exists solely in the high altitude and specially at the different areas of greater Himalayan range. This himalayan toad is found in the North-Eastern range of himalayas. In my present study I have observed this species at different places ranging between 1500 meters and 2200 meters of altitude at Darjeeling town and its adjacent areas.This toad occurs in large number and breed naturally in stagnant pools of rain water. The natural developmental processes of this toad have also been monitored in laboratory. A normal table of development of this species was never done precisely before, has been worked out. From these observations it has been found that this species shows similarities with closely related species and other anurans. But temperature plays a very important role in their developmental process. Sometimes at the onset of winter even the larvae of this species undergo hibernation.

Their pattern of gonadal development and testicular development has also been observed. The observations reveal that this species has a similarity in developmental patterns of gonads as well as testes with that of teleosts and reptiles. Thus the observations provide proof of phylogenetic relationships with closely related classes of animals. The testicular development has also shown some relations with the environmental factors such as temperature, humidity, rainfall and others.

In the events of spermatogenesis some interesting observations were recorded. For example, this himalayan toad initiates their spermatogenesis at a very early stage. This was evident as early as 'O' limb stage suggesting a case of 'progenesis' in this species.The spermiogenesis in the adult takes mainly in the breeding season, however, in the non-breeding season spermatogenesis may also continue but ceases before the formation of spermatids.

The study of the kinetics of spermatogenesis reveals rapid transition and completion of spermatogenesis. It seems to be somehow related with the habit of seasonal breeding which has an extremely short duration of time as the areas of investigation are situated at high altitude.The kinetics of meiosis of this species is longer and shows similarities with *Xenopus* laevis and *Uperodon.* In contrast to meiosis, spermeiogenesis is remarkably short. The present investigation recorded the total duration of spermeiogenesis as about 9.5 days The total spermatogenesis process i.e. from the onset of meiosis to the completion of spermiogenesis is about 29 days.

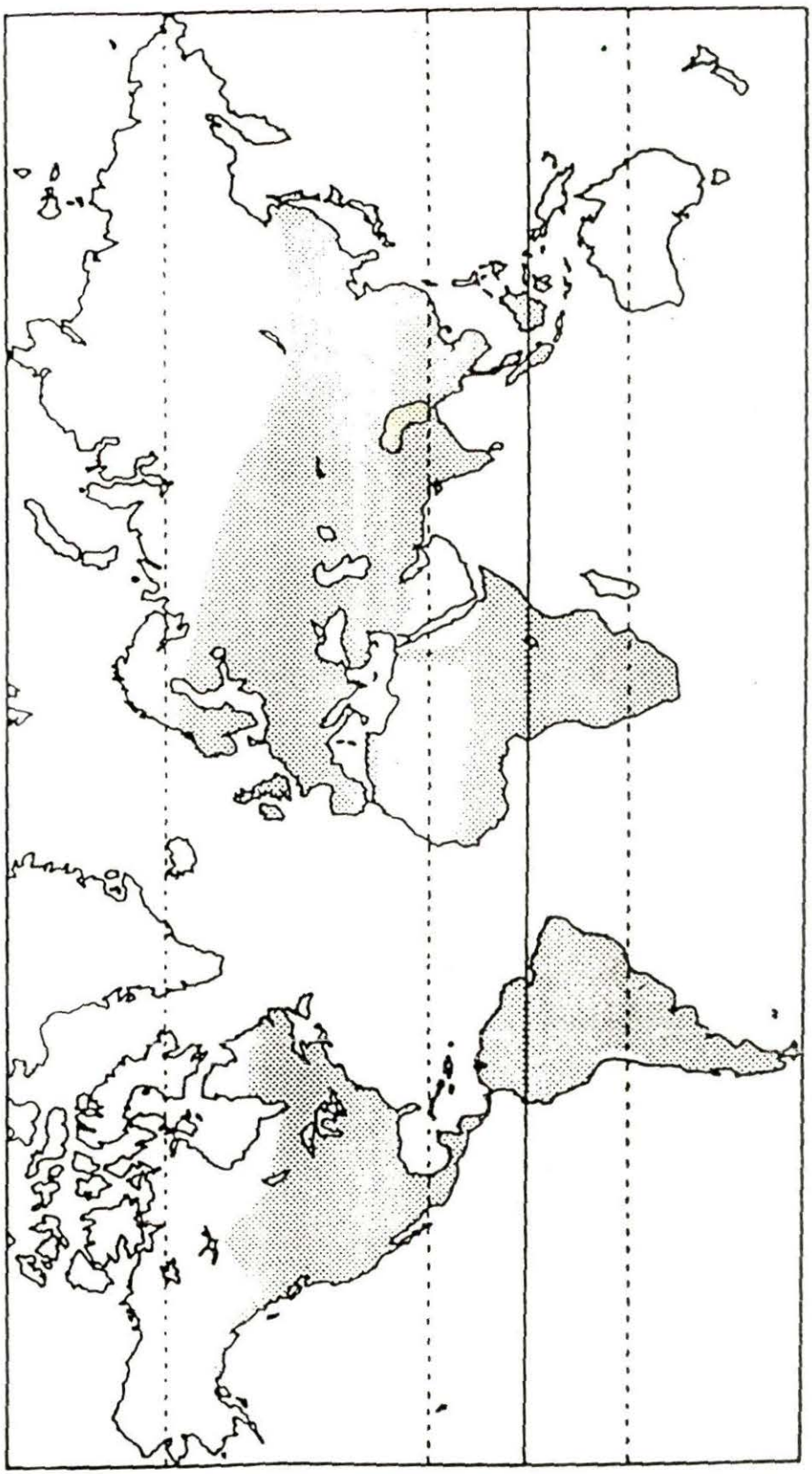
Therefore, the present material under investigation was proved to be an unique and special species which has to be investigated thoroughly in future, specially in their natural habitat to observe more interesting features regarding their breeding and spermatogenesis and other related characteristics . The present series of investigation also unveils some important aspects of their life cycle and spermatogenesis which was not known before. Electron microscopic studies also recorded their ultrastructural peculiarities related with the gonadal development and spermatogenesis.

The material for the present study

The material for the present study is an amphibian species belonging to the Order Anura, *Bufo himalayanas* (Gunther) and is commonly known as himalayan toad. This animal normally thrives in the himalayan hills at an altitude range of 1550 meters to 2200 meters. They are terrestrial in nature and love to inhabit moist and marshy areas of the hilly region. They have close similarities with their relatives of foothills and plains as *Bufo melanostictus*, though they are slightly larger in size and has some special characteristics. They are not only found in the hills of Darjeeling of West Bengal but also in other states such as Sikkim,Bhutan, Nepal and other North-Eastern Himalayan states of India which fall under similar range of altitude. Map-1 in the next page showing worldwide distribution of living species of Bufonidae family and distribution of *Bufo himalayanas.*

For the present study I have collected a good number of adult individuals as well as egg strips and also different stages of larvae from their natural habitat or breeding ground. Our collection spots were restricted to the Darjeeling hill in between the altitude of 1550 meters and 2200 meters.

MAP 1



worldwide distribution of living species of Bufonidae family

distribution of *Bufo himalayanas*.

(a) COLLECTION SPOTS.

My collection spots were mainly spread over an area of about 8 square kilometer per 'breeding zone'. These collection spots were selected for regular collection of adult specimens along with larvae and eggs (specially at breeding seasons). At the time of collection water was collected from their natural habitat for rearing of the larvae at the laboratory trays and aquarium. Temperature at the collection spots was recorded. (Table-1)

Water bodies were ditch like ground level water tanks like the tanks at Happy Valley Tea Estate (Plate 1,Figure C) or waste water reservoir surrounded by open drainage system at Sinchal Lake (Plate 1, Figure b) or a medium sized concrete water tank at Shrubbery Park etc.(Plate 1, Figure a).These water bodies are all situated in the Darjeeling hill. The water bodies had a number of aquatic weeds, algae and sometimes with floating vegetations. The strips of eggs and different stages of larvae along with adult male and female species were found to occur naturally in breeding seasons (May- August) in these collection spots.

(b) ADULT MALE AND FEMALE

Adult male and female have close similarities with common *Bufo melanostictus*. *Bufo himalayanas* or himalayan toad shows sexual dimorphism to some extent. These dimorphic characters became more prominent at the breeding season of this species.

PLATE- 1

FIGURE:a Collection spot at Shrubbery Park, Darjeeling

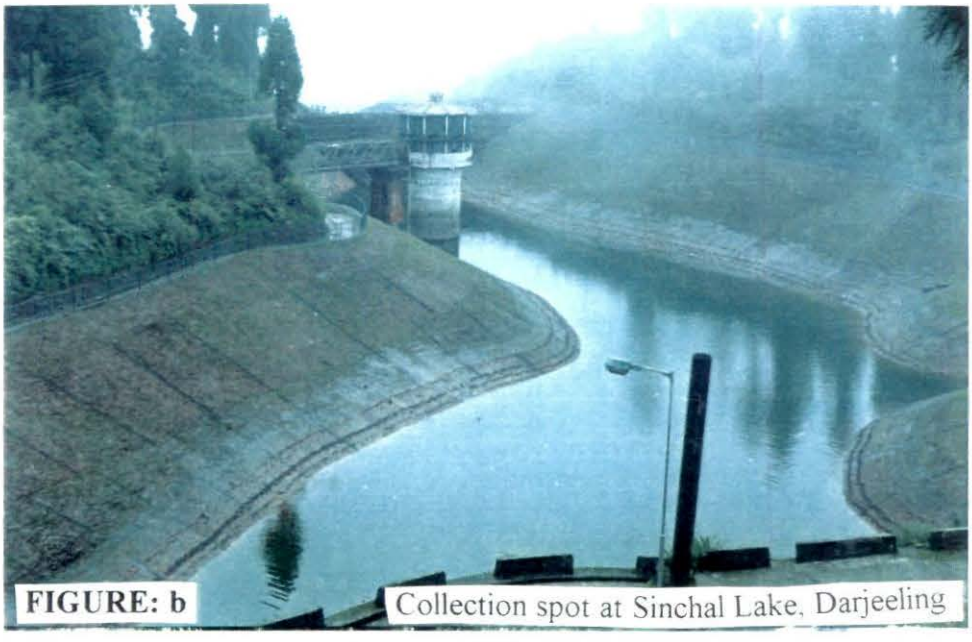


FIGURE: b Collection spot at Sinchal Lake, Darjeeling

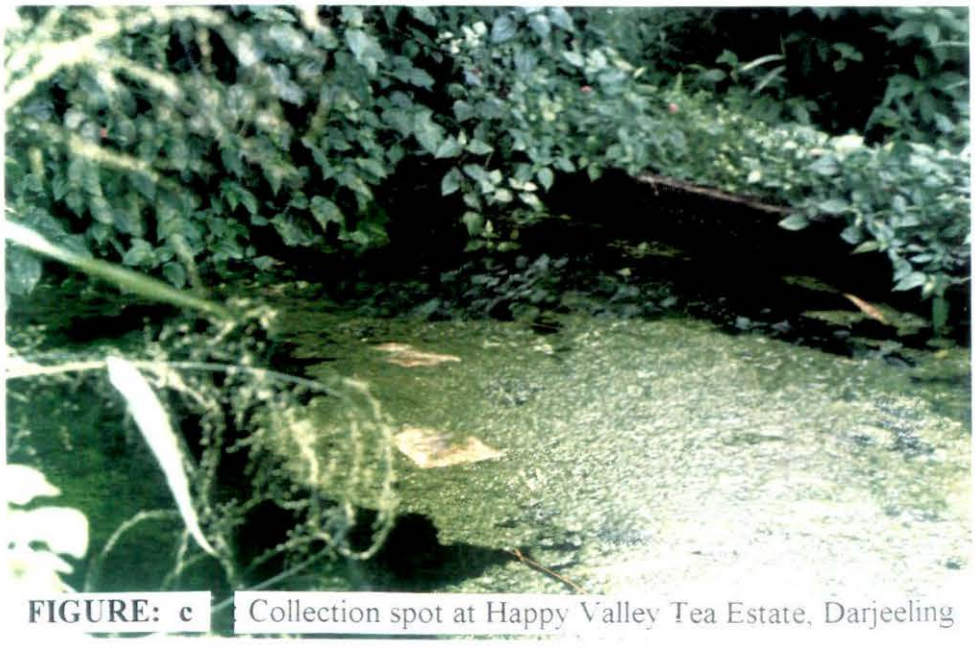


FIGURE: c Collection spot at Happy Valley Tea Estate, Darjeeling

The male toad is somewhat smaller in size and darker in appearance. They have small black and dark gray spots all over the body. Ventral side has lighter shade . Abdomen is slender in shape. Fore limb has a prominent thumb pad. A pair of bean shaped parotid gland is a characteristic of male (Plate 2, Figure a & b).

Table – 1

Recordings of temperature at the collection spots

Site of collection	Month of collection	Number collected	Temperature ° C	Remarks
Shrubbery Park	May	1st	16	Normal
		2nd	17.5	Normal
	June	1st	18	Normal
		2nd	18	Normal
	July	1st	18.5	Normal
		2nd	17	Normal
	August	1st	15.5	Normal
		2nd	16	Normal
Sinchal Lake	May	1st	17	Normal
		2nd	17.5	Normal
	June	1st	18	Normal
		2nd	19	Normal
	July	1st	16.5	Normal
		2nd	18	Normal
	August	1st	17	Normal
		2nd	16.5	Normal
Happy Valley Tea Estate	May	1st	21	Towards high
		2nd	22	Towards high
	June	1st	21	Towards high
		2nd	20	Normal
	July	1st	20	Normal
		2nd	19	Normal
	August	1st	18	Normal
		2nd	17	Normal

PLATE- 2

FIGURE:a

FIGURE: b

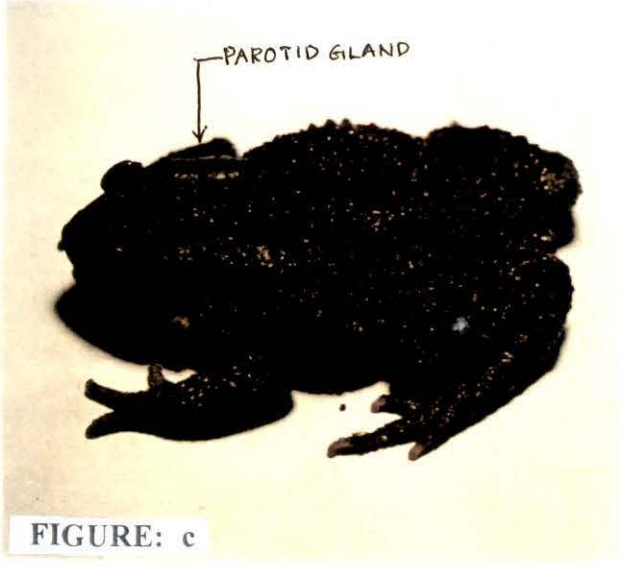


FIGURE: c

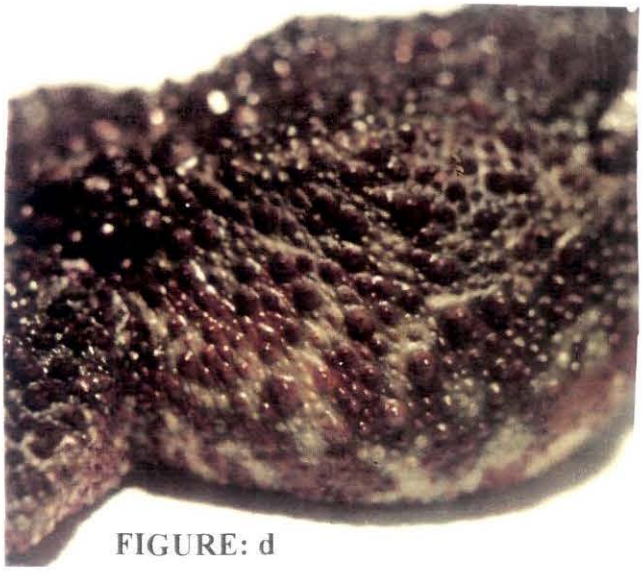


FIGURE: d

PLATE 2

Legends

Figure a: A mature male at breeding season

Figure b: Ventral side of the mature male at breeding season

Figure c: A mature female at breeding season

Figure d: Ventral side of the mature female showing pigmentation

The female toad is bigger in size in comparison to their males,and have bulging abdomen. Ventral side of the body develops characteristic reddish pigments in the skin and warts on the abdomen (Plate 2 & 3, Figure c and d). Thumb pads are absent and parotid glands are normal and slender in shape.

(c) BREEDING SEASON

Breeding season falls within May to August of the year. The most important controlling extrinsic factor of the breeding season is temperature. It has been observed that only favorable temperature can stimulate and regulate their breeding in normal habitat at high altitude. Thus sometimes the breeding season may start in April and it may be extended up to late September. They prefer the atmospheric temperature in between 15 ^{O}C and 22^{O}C.

At breeding season males and females of *Bufo himalayanas* were found in the natural water bodies which were usually full of aquatic weeds and semi-aquatic plants.

In the breeding season the male toad first select their suitable water pools and then started producing sex calls by inflating their vocal sac. Sex calls are species specific and this attracts a number of sexually mature female toad towards the males. The males copulate with the female by clinging at the dorsal side of the body of the female. Females stimulated by the act of copulation start liberating their eggs in a continuous strip or ribbon. Males at the same time discharge their sperm to fertilize the eggs externally as found in other species of toad.

CHAPTER - I

NORMAL TABLE OF DEVELOPMENT

CHAPTER- I

NORMAL TABLE OF DEVELOPMENT

CONTENTS

Introduction

Bufo himalayanus is an endemic species of toad exists solely in the high altitude of North-Eastern Himalaya. The animals are prevalent at the different areas of Greater Himalayan range. The specimen occurs in large number and breeds in stagnant pools of rain water under natural condition.

Under laboratory condition final duration of metamorphosis, on an average, is two and half a month with temperature preferably between 15^{O}-20^{O}C. While the metamorphosis time taken can be shortened under natural condition having relatively higher temperature between 22^{O}-24^{O} C.

The changing morphology of embryos, specially during organogenesis, necessites a method of quantifying the progress of development. Tables of normal stages of development have been worked out for a number of species of amphibians; normal developmental tables of *Icthyophis* (Sarasin,1980); number of species of salamanders and few species of anurans have been vividly described by various authors during 1887 to 1981. So nearly a century old tradition of investigating larval development reached its peak of accuracy at recent time.

What is normal table ?

Complete tables of develoment are necessary for accurate comparison of developmental stages of larvae in different organisms. Identification of each stage must be accompanied by a given temperature; the stages then can be identified mostly by external features.

During cleavage, the stages are identified from the number and size of the blastomeres. During gastrulation, the shape of the blastopores is used as an identifying character. After gastrulation the neural plate provides easily recognizable features.During the process of organogenesis, the progress in the formation of the tail, limbs, gills and mouth are convenient features.

Development is, of course, continuous, and the designated stages gradually grade into one another. The duration of time between two successive stages varies by different factors naturally. Gosner (1960) generalized and formatted the stages of larval development in anurans, which is still accepted as a standard Normal Table of development in any anuran species. This table is basically applicable to taxa that have aquatic eggs and larvae. Therefore my present study on the normal larval developmental stages of *Bufo himalayanas* is made according to the Standard Normal Table proposed by Gosner (1960).

Review of normal table studies

Various types of investigations have been made by different investigators regarding egg development , hatching and post – hatching developments of anuran larvae since a very long period of time. Physiology of anuran hatching mechanism was described by Miganti and Azzolina as long before as 1955. Presence of anti-proteolytic factors in embryo also reported in 1948 by Wu *et.al.,* in 1965 described different hatching enzymes at different stages of development in *Rana pipiens*. Anurans in which later development occurs in a capsule, hatch first from the vitelline membrane and much later from the capsule (Salthe,1963). Different types and patterns of hatching are also described by a number of investigators from as early as 1905 in *Xenopus laevis* by Bles; in *Bufo bufo* by Kobayashi (1954). In other anurans this has been investigated (Kobayashi,1954; Volpe *et. al.*, 1961; Kenny,1968; Petranka *et.al*,1982). A number of embryologists have extensively investigated the amphibian external eggs for both descriptive and experimental studies (Duellman and Trueb, 1986).

The morphological types of anuran larvae were described earlier y Orton (1953). Her classification of anurans was oversimplified and not related with correct phylogeny (Wassersug, 1984; Wassersug and Duellman,1984) it was used by many workers at that time . Mechanism of buccal pump in different species of anuran larvae was described by Kenny (1969) and Wassersug (1972,1980). Mouth disc along with labial papillae and denticles were throughly investigated and reported by Lutz and Orton (1946), Wager (1965) and others. Morphology of larval ventral sucker and its

adaptations were observed by Nobel (1929); Duellman and lynch (1969). Food taking behaviour of larval anurans and mouth adaptations were investigated by Altig and Brodie (1972); Gradwell (1973) and Wassersug (1980).

The most comprehensive treatment of amphibian development within a broad biological context is seen in the work of Salthe and Mecham (1974). It has been further appeared that a transition to a predominantly terrestrial life is accompanied by complete absorption of gills as observed by Harrison (1969) and Atkinson and Just, (1975) in *Rana.*

The transition from larva to adult is dramatic in anurans and definite changes take place during metamorphosis . The changes are most extensive in anurans in which the aquatic larvae undergo drastic transformation into a terrestrial adult or sub-adult.

From their larval forms of different stages it was evident that during development of larvae into a sub-adult a lot of morphological changes occur. The hind limb grows and matures. The fore limb develops within branchial chambers. The internal gills and associated blood vessels degenerate; lungs and pulmonary ventilation develop. The tail is resorbed. The skin thickens with glandular developments. Larval mouth parts degenerates and the adult mouth parts are formed. The eyes enlarge and eyelids develop. A comprehensive summary of the morphological changes during metamorphosis were described by Dodd and Dodd (1976) and Fox (1981,1984), Reilly et al, (1994) (Table-2).

TABLE – 2

Summary of some metamorphic changes in anurans

System	Larva	Adult
Locomotory	Aquatic, tail fin	Terrestrial, tail-less tetrapod
Respiratory	Gills, skin, lungs; larval hemoglobins	Skin, lungs; adult hemoglobins
Circulatory	Aortic arches, aorta , anterior,posterior and common jugular veins	Carotid arch, systemic arch, cardinal veins
Nutritional	Herbivorous: long spiral gut; intestinal symbionts, small mouth, horny jaws, labial teeth	Carnivorous: short gut, proteases, large mouth with long tongue
Nervous	Lack of nictitating membrane, porphyropsin, lateral line system, Mauthner's neurons	Development of ocular muscles, nictitating membrane, rhodopsin, loss of lateral line system, degeneration of Mauthner's neurons, tympanic membrane
Excretory	Largely ammonia, some urea (ammonotelic)	Largely urea, high activity of enzymes of ornithine-urea cycle (Ureotelic)
Integumental	Thin, bi-layered epidermis with thin dermis, no mucous glands or granular glands	Stratified squamous epidermis with adult keratins, well developed dermis contains mucous glands and granular glands secreting anti-microbial peptides

Data from: Turner and Bagnara , 1976 and Reilly et. al., 1994.

Aims and objectives of the present study

Although the occurrence of several amphibians as well as anuran species from the different altitude of Himalayas had been investigated and described earlier by a host of investigators (Anandale, 1909; Bhatt, 1969;Danial,1963; Limbaugh, and Volpe, 1957 and Smith,1924) but a comprehensive study on the life history of *Bufo himalayanas* , specially from north-Eastern Himalayas was yet to be done.

The present investigation thus endevours to draw a detailed account of larval development of *Bufo himalayanas* collected from Darjeeling , West Bengal from their natural habitat and breeding places, on the basis of the relationship between various body measurements to the total length according to procedure described by Martin and Little, (1966).

In the present thesis stages of developing larvae with special reference to their external feature changes during the course of metamorphosis had been described with various visual aids for easier comparative study of the normal table of this endemic, endangered, high altitude species. An attempt has been made to compare the life tables of various anuran species, particularly with that of related species of the same genus, *Bufo* (Khan, 1964)

Methodology

Different stages of developing larvae of *Bufo himalayanas* along with their eggs were collected from Sinchal Lake , Shrubbery Park, Happy Valley Tea Estate and from its adjacent areas of Darjeeling town. Specimens also collected from a few stagnant water logged places in and around the town . Sufficient amount of water was also collected from the natural habitat.

Larvae of *Bufo himalayanas* occur along with the other types of anurans ; for example, *Rachophorus* sp. and *Rana* sp. . Therefore the type under investigation were sorted out in the laboratory. The eggs of these species were laid on a gelatinous ribbon-like strips. These collected egg ribbons were kept in separate aquarium for studying the early stages of larval development. Further rearing at laboratory was made at room temperature on and around 18 degree centigrade. It was found that the larvae thrive well at a higher temperature but below $22^{O}C$. Rearing of the larvae were carried out in spacious aquariums partly filled with water collected from the collection spot.

Rearing of different stages of developing larvae were carried out in different enamel and plastic trays containing water collected from their natural habitat. Later stages of development were carried out within plastic trays to prevent extra chill of the metallic trays particularly at night. Furthermore during cold night warmth of hanging electric lamps were provided to the developing larvae.

Though the aquarium water of the larvae was provided with same vegetation and microorganisms present in their natural habitat, an artificial food for the larvae was also offered periodically. This artificial food was made with thoroughly mixed boiled lettuce, dry fish food and dry powdered fish together.

The larvae hatched out from the fertilized eggs were allowed to develop into early larval stages.They are now transferred to plastic trays to develop further. On every second / third day certain number of individuals were fixed in 4% formalin . The pigmentation of these larvae is high and thus dark black coloration obstruct perfect visualization and observation. Therefore instead of alcohol preservation , I have preferred formalin preservation to diminish the pigmentation to some extent.

Larvae collected from the natural habitat were also allowed to develop in separate trays to compare the specific identity of the different stages of the larvae. The adults were preserved in 70-80% alcohol.

Measurements of different larval stages were taken with the help of mm/cm scale and compass uniformly. Changes in the external features in accordance with the post-hatching larval stages were made following the method described by Gosner (1960) for subsequent observations.

Camera lucida drawings were made with simple and binocular microscope from stage-20 to stage 46. Photographs were also taken from binocular optical microscope with artificial light controls. Special emphasis was given to the development of limbs ,tail and mouth disc. Regression of tail was also recorded.

During this development different stages of larvae emerged and gradually developed into the next stages. These changes are descriptively summarized in a normal table of development.

OBSERVATION

[A] FIELD OBSERVATIONS:

(i) Behaviour

(a) Adult

The specimens were found in shallow water at about two feet depth. Some preferred to remain at the surface water. Very often they were found in small waterlogged pools with stagnant water provided with aquatic vegetations. Adult male and female were found in pairs. But in a small pool of water they were found in scanty numbers. Females are comparatively larger in size with reddish prominent pigmentation in the ventral surface of the body at breeding season. The breeding season starts from April and continues till August. During breeding season their courtship begins. Fertilization is external like other anurans.Therefore courtship only referred to the fertilization process. The males comes by the side of the water bodies and starts producing mating calls by sequential inflation of their vocal sacs. Many female toads come to the these sites being attracted by the male toad's mating calls. The male toad climb on the back of the female and clinged there by their limbs. The female starts producing strings of eggs linerely arranged on a gelatinous ribbon. Huge number of eggs were laid by a female in shallow water most of which were fertilized externally by the sperms discharged by the males over the eggs ribbons.

(b) Egg

Eggs are laid as a continuous gelatinous ribbon in linear fashion. Egg ribbons are found attached with aquatic vegetations. Most of the eggs are externally fertilized. Each egg has a central ovoid or spherical opaque body surrounded by semitransparent gelatinous mass (Plate 3, Figure a).

(c) Larva

The larvae are very sluggish in movement . They are poor swimmers due to the presence of heavier head and moderate tail. They are uniformly deep black in colour . As they are not good swimmers, they often need support of the aquatic plant or weed to remain near the surface of water. In natural as well as in laboratory condition, they are found near the surface of water at night , and often in cloudy weather probably by getting attracted by natural and artificial light. In natural condition larvae often found to stay in clutch near the surface water and never actively swim unless disturbed. The larvae are found in huge number soon after the onset of the breeding season upto the end of August which culminates the breeding season. However larvae in late October or early November are not uncommon in the field. These larvae may go for hibernation in the next stage before the onset of winter .

(ii) Feeding habit

[a] Adult and sub-adult:

In nature adult *Bufo himalayanas*(both male and female) are usually carnivorous. With their inverted and extended tip of the tongue, they procure small insects like lepidopteran moths and butterflies, orthopterans like small to medium sized grasshopper, dipteran flies ants and almost all types of terrestrial insects. They also feed on earthworms and other types of worms,snails and larvae of insects. When habituated with aquatic life in their breeding season, they also consume small fishes aquatic insect larvae and aquatic adult insects.

Post-metamorphic juveniles or sub-adults usually consume small bivalves, various forms of collembolans and lepidopteran larvae and some terrestrial forms of isopods.

Table-3

A comparative list of food items of larvae, sub-adult and adult of *Bufo himalayanas*

Stages	Food items
1. larvae (post-hatching)	Various bacteria,diatom,protozoa, phto- and zooplankton including cladocera, rotifera, nematocera, nematodes, chironomid larvae, tubifex, different types of algae and weeds' fragments
2.Sub-adult (post-metamorphic juvenile)	Small bivalves, various collembolans and lepidopteran larvae, small soil nematodes and some terrestrial isopods etc.
3. Adult	Small insects, viz. moths and butterflies, small to medium sized grasshoppers, dipteran flies and other small to medium sized insects, earthworms, nematodes, snails and insect larvae, aquatic insects and insect larvae, small fishes etc.

[b] Larvae

The larvae are presumably column or bottom feeder in habit. In natural habitat the larvae usually consume various bacteria, diatoms, protozoans, phyto- and zooplanktons including cladocera. rotifers, nematocera etc., which occur in the sediments of the bottom of water body.

Older larvae, however, consume several groups of soil nematodes, *Tubifex* and huge amount of chironimid larvae which are usually abundant in the natural habitat of this high altitude areas. A comparative list of food items naturally consumed by the larvae, sub-adult and adult are summarized in the Table- 3 .

[B] LABORATORY OBSERVATION

(i) Eggs

Eggs were arranged linearly and equidistantly within a common gelatinous ribbon which sometimes may be several feet long (but single if separated by mechanical manipulation shows an ovoid or spherical structure with a prominent opaque central developing embryo or ripe egg. Central egg is surrounded by semi-transparent gelatinous spherical mass (Plate 3, fig a).

PLATE 3

Legends

Figure a: An egg strip with gelatinous ribbon and a single separated egg enveloped by gelatinous mass

Figure b: Anterior part of a mature female enlarged to show the characteristic shape of its parotid gland

Figure c: Ventral side of the mature female showing bulging belly and pigmentation

PLATE-3

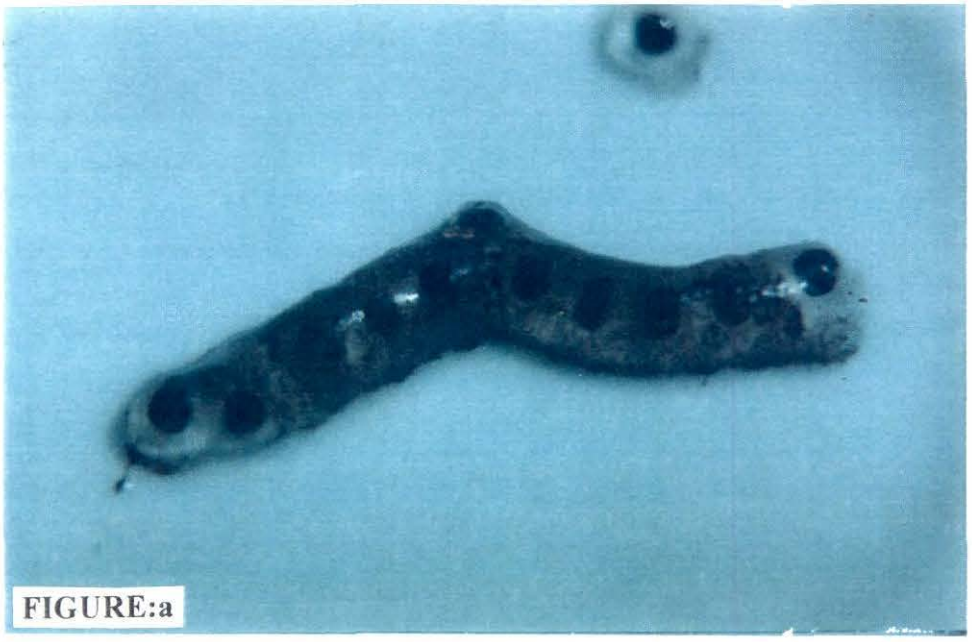


FIGURE:a

FIGURE: b

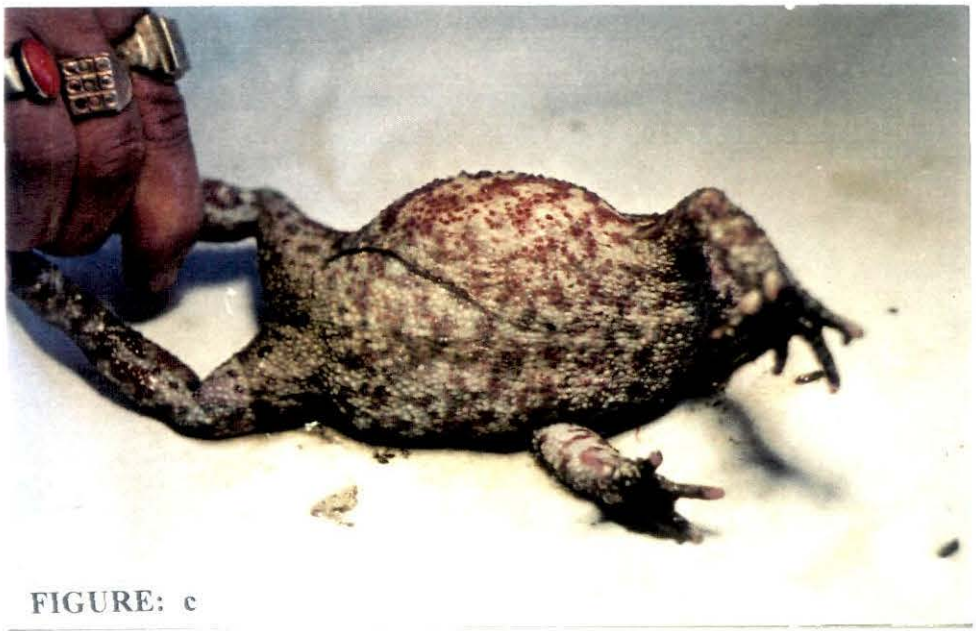


FIGURE: c

(ii) Early embryonic development

Detailed descriptions of these stages are not within our purview. However, these stages are included in the diagram 1.

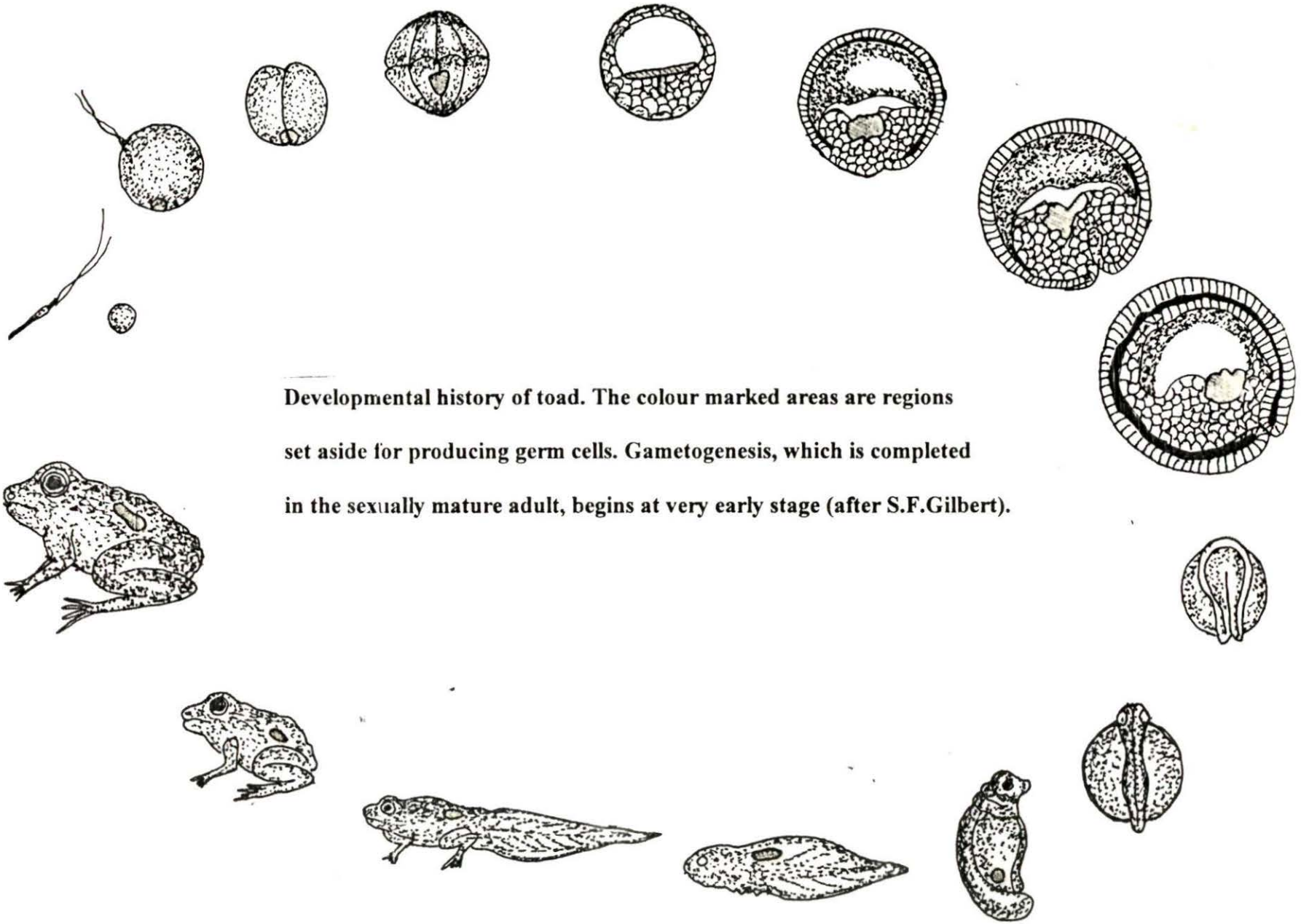
(iii) Pre-hatching embryo

The developing embryo at stage-18 are dark grey to balck bodies with a remnant of yolk sac attached to the body. A ventral notch is present in between the yolk sac and the small tail bud. Gill pouch , auditory vesicle and pronephros are also developed at this stage.
From stage –20 onwards the free living larval stages begin and the yolk sac is almost absorbed.

(iv) Post-hatching embryo

Hatching occurs at about stage-20. Due to intensive pigmentation , the larval colour is deep black. The tail fin is rather semitransparent with a round terminal end which later on became tappering and pointed at the end . Ventral sucker and three pairs of external gills are well marked from this stage.
A detailed morphological observations based on external features has beeen recorded in this current investigation which is followed by the measurement comparisons and ratio tables of the himalayan toad, *Bufo himalayanas.*

DIAGRAM : 1



Developmental history of toad. The colour marked areas are regions set aside for producing germ cells. Gametogenesis, which is completed in the sexually mature adult, begins at very early stage (after S.F.Gilbert).

(v) Post-hatching embryo's normal table (from stage-20)

In this current investigations description of normal table of *Bufo himalayanas* is based on the methods described and standardized by Gosner, K.L. (1960). For the present normal table here camera lucida drawings has been made of each stage of development up to post-metamorphic sub-adult stage-46. Special attention was given to the external morphology, limbs and tail development, changing pattern of mouth disc and tail regression process. Light microscopic and normal close-up photographs were also made for each individual case for a vivid and detailed study. Measurements were made in mm scale. Measurements were made on an average from at least five individual specimens of the same stage. Statistical analysis were made for each parameter, item and set as per conventional method suggested for biological specimens.

STAISTICAL INTERPRETATION

1. Body length (BL): *increases steadily upto stage* 29, decreases from stage 30 to 36 (not significant), remains almost stable till the end barring stage 44 when a sudden decrease occurs.

2. Tail length (TL): increases till stage 26 and then drops at stage 27, remains more or less stable till stage 32 and then decreases slowly. The decrease at stage 45 is remarkable.

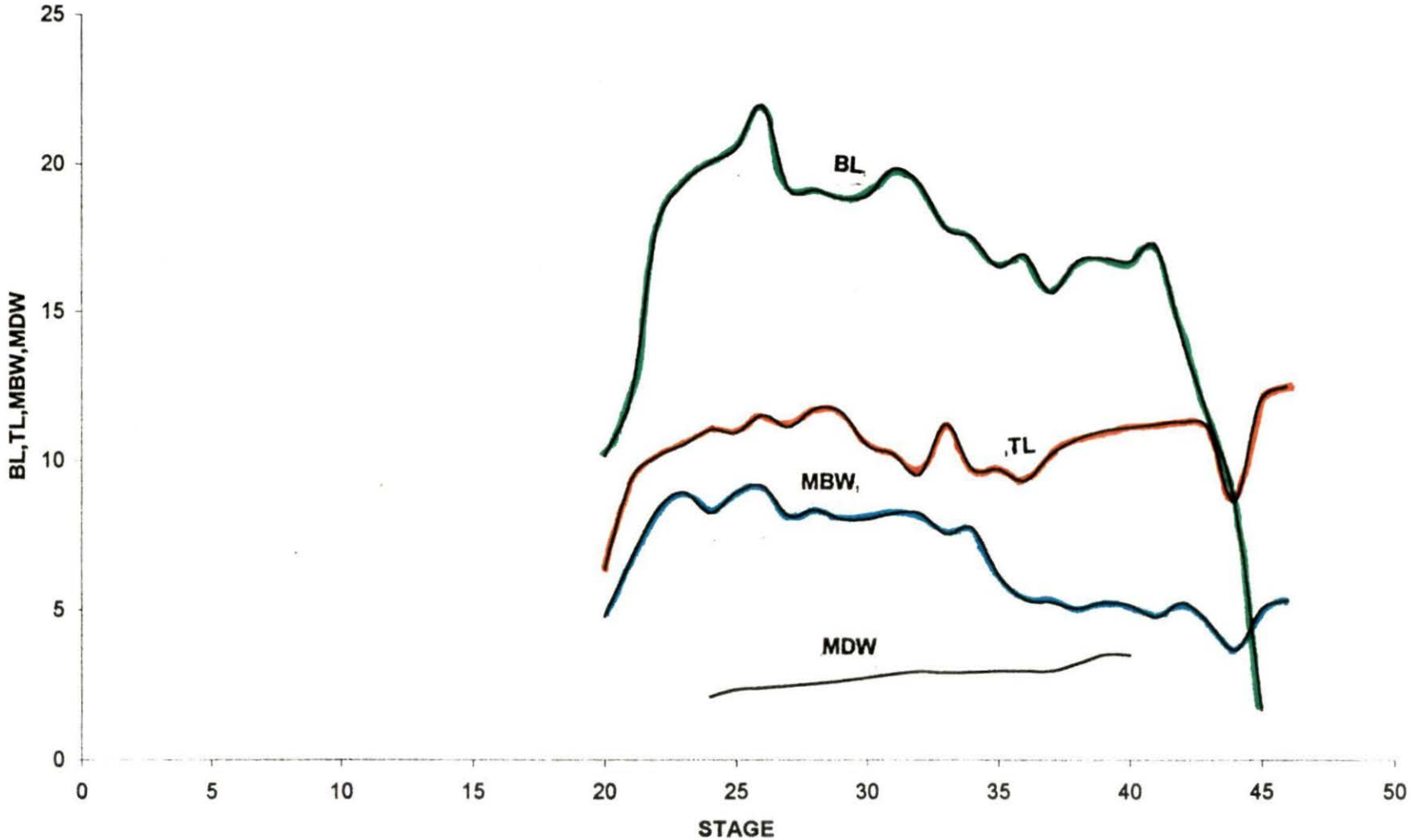
3. Maximum body width (MBW): increases till stage 26 and then decreases slowly in the subsequent stages. Significant decrease starts from 33 onwards (almost linear reduction).

4. Mouth disc width (MDW): Increase starts at stage 24, then increases linearly till stage 40.

From the above statistical interpretation, I may suggest that biologically this normal table has a significant gradual and linear decrease in the tail length of the developing tadpoles. In the maximum body width and mouth disc width there is a similar significant linear reduction and linear increment respectively. This pattern of linearity may be noticeable in other related groups of anuran species. Thus I may conclude that aquatic tadpoles of anurans species shows a significant correlation in the pattern of their developmental growth.

A comparative graph (Graph-1) showing the correlation and growth pattern in *Bufo himalayanas* is given in the next page.

GRAPH 1



GRAPH 1

Periodic life table of *Bufo himalayanas*

Stage 20: Measurements:

(i) Total length: An average of 16-17mm.

(ii) Body length: An average of 6.4mm.

(iii) Tail length: An average of 10.2mm.

(iv) Max. body width: An average of 4.8mm.

Special features: Ventral sucker and three pairs of external gills present. Operculum not distinguishible.Tail membrane (fin) broad and semi-transparent with rounded tip. Dark black in colour. (Plate 4, Figure 20/ABC, Diagram 2/20).

Stage :21 Measurements:

(i) Total length: An average of 21-22 mm.

(ii) Body length: An average of 9.4mm.

(iii) Tail length: An average of 12.2mm.

(iv) Max. body width: An average of 6.7mm.

Special features: Transparent eye, elongated body, tail membrane broad,dense pigmentation, body oval in shape (Plate 4, Figure 21/ABC, Diagram 2/21).

Stage :22 Measurements:

(i) Total length: An average of 28.2 mm.

(ii) Body length: An average of 10.2mm.

(iii) Tail length: An average of 18mm.

(iv) Max. body width: An average of 8.4mm.

Special features: Tail membrane more transparent and gradually tapering towards tip,opercular fold not distinguishable, body oval in shape (Plate 4, Figure 22/ABC, Diagram 2/22).

Stage :23 Measurements:

(i) Total length: An average of 30mm.

(ii) Body length: An average of 10.6mm.

(iii) Tail length: An average of 19.4mm.

(iv) Max. body width: An average of 9mm.

Special features: Mouth opening appears,opercular fold appear, three pairs of external gills seen,tail myotomes prominent,more elongated body (Plate 5, Figure 23/ABC, Diagram2/23).

Stage : 24 Measurements:

(i) Total length: An average of 31.2m.

(ii) Body length: An average of 11.1mm.

(iii) Tail length: An average of 20.1mm.

(iv) Max. body width: An average of 8.3mm.

(v) Max. mouth disc width : An average of 2.12mm.

Special features: Opercular folds tend to close; mouth disc disappear, mouth is with distinct lips and denticles; tail myotomes increase in number (Plate 5, Figure 24/ABC, Diagram 2/25).

Stage : 25 Measurements:

(i) Total length: An average of 31.6mm.

(ii) Body length: An average of 11mm.

(iii) Tail length: An average of 20.6mm.

(iv) Max. body width: An average of 9mm.

(v) Max. mouth disc width : An average of 2.36mm.

Special features: Spiracles appear,external gills disappear,mouth disc widens and get prominent jaws-like structure, eyes opaque and black (Plate 5, Figure 25/ABC, Diagram 2/26).

Stage : 26 Measurements:

(i) Total length: An average of 33.6mm.

(ii) Body length: An average of 11.6mm.

(iii) Tail length: An average of 22mm.

(iv) Max. body width: An average of 9.2mm.

(v) Max. mouth disc width : An average of 2.42mm.

Special features: Spiracles appear,external gills disappear,mouth disc widens and get prominent jaws-like structure, eyes opaque and black (Plate 5, Figure 26/ABC, Diagram 2/27).

Stage : 27 Measurements:

(i) Total length: An average of 30.4mm.

(ii) Body length: An average of 11.2mm.

(iii) Tail length: An average of 19.2mm.

(iv) Max. body width: An average of 8.2mm.

(v) Max. mouth disc width : An average of 2.5mm.

Special features: Hind limb bud increases in size and more or less than half half of its diameter, eyea prominent and black,body oval in shape (Plate 6, Figure 27/ABC, Diagram 2/27).

. **Stage : 28** Measurements:

(i) Total length: An average of 31mm.

(ii) Body length: An average of 11.8mm.

(iii) Tail length: An average of 19.2mm.

(iv) Max. body width: An average of 8.4mm.

(v) Max. mouth disc width : An average of 2.56mm.

Special features: Hind limb bud elongates,slightly greater than its diameter,mouth widens,jaws with denticles, body oval in shape (Plate 6, Figure 28/ABC, Diagram 2/28).

Stage :29 Measurements:

(i)Total length: An average of 30.6mm.

(ii)Body length: An average of 11.7mm.

(iii) Tail length: An average of 18.9mm.

(iv) Max. body width: An average of 8.1mm.

(v) Max. mouth disc width : An average of 2.66mm.

Special features: Hind limb bud elongates more and its length is greater than its diameter by 1.5 times; other features are as in stage-28. (Plate 6, Figure 29/ABC, Diagram 3/29).

Stage : 30 Measurements:

(i) Total length: An average of 29.6mm.

(ii) Body length: An average of 10.6mm.

(iii) Tail length: An average of 19mm.

(iv) Max. body width: An average of 8.1mm.

(v) Max. mouth disc width : An average of 2.76mm.

Special features: Hind limb bud elongates,slightly greater than its diameter,mouth widens,jaws with denticles, body oval in shape (Plate 6, Figure 30/ABC, Diagram 3/30).

Stage : 31 Measurements:

(i) Total length: An average of 30.2mm.

(ii) Body length: An average of 10.3mm.

(iii) Tail length: An average of 19.9mm.

(iv) Max. body width: An average of 8.3mm.

(v) Max. mouth disc width : An average of 2.88mm.

Special features: Hind limb tip flattened and conical, paddle shaped tip of hind limb without any digit, mouth with stout chitinious pair of jaws, tail with distinctly visible myotomes (Plate 7, Figure 31/ABC, Diagram 3/31).

Stage : 32 Measurements:

(i) Total length: An average of 29mm.

(ii) Body length: An average of 9.6mm.

(iii) Tail length: An average of 19.4mm.

(iv) Max. body width: An average of 8.3mm.

(v) Max. mouth disc width : An average of 2.98mm.

Special features: Hind limb tip or foot starts digitalization, 4^{th} and 5^{th} digit are visible now, tail is almost twice the body length, rigid chitinous pair of jaws guards the mouth aperture, operculam is now distinct (Plate 7, Figure 32/ABC, Diagram 3/32).

Stage : 33 Measurements:

(i) Total length: An average of 27.6mm.

(ii) Body length: An average of 11.3mm.

(iii) Tail length: An average of 17.9mm.

(iv) Max. body width: An average of 7.6mm.

(v) Max. mouth disc width : An average of 2.94mm.

Special features: More digits are distinguishable at the tip of the hind bud and this is more stout and well developed, operculam distinct, semitransparent and broad tail fin (Plate 7, Figure 33/ABC, Diagram 3/33).

Stage : 34 Measurements:

(i) Total length: An average of 27.4mm.

(ii) Body length: An average of 9.8mm.

(iii) Tail length: An average of 17.6mm.

(iv) Max. body width: An average of 7.8mm.

(v) Max. mouth disc width : An average of 2.96mm.

Special features: Digits or toes are more distinct, 1st and 2nd toes not separated yet, tail fin or membrane not so broad, hind limb is more developed with more joints (Plate 8, Figure 34/ABC, Diagram 3/34).

Stage : 35 Measurements:

(i) Total length: An average of 26.4mm.

(ii) Body length: An average of 9.8mm.

(iii) Tail length: An average of 16.6mm.

(iv) Max. body width: An average of 6.2mm.

(v) Max. mouth disc width : An average of 3mm.

Special features: Toes of the hind limb now distinguishable fully,all toes separated from each other,hind limb develops bends behind the foot, tail fin is thin,length of the tail is about twice the body length (Plate 8, Figure 35/ABC, Diagram 3/35).

Stage : 36 Measurements:

(i) Total length: An average of 27.2mm.

(ii) Body length: An average of 9.4mm.

(iii) Tail length: An average of 17mm.

(iv) Max. body width: An average of 5.4mm.

(v) Max. mouth disc width : An average of 3mm.

Special features: All toes are completely separated except 1st and 2nd which are slightly joined at their base, mouth is guarded by a pair of thick lips with cuticular thickening, body slightly elongated (Plate 8, Figure 36/ABC,Diagram 3/36).

Stage : 37 Measurements:

(i) Total length: An average of 26mm.

(ii) Body length: An average of 10.3mm.

(iii) Tail length: An average of 15.7mm.

(iv) Max. body width: An average of 5.3mm.

(v) Max. mouth disc width : An average of 3mm.

Special features: All 5 toes are free and completely separated, hind limb develops more joints and bends, mouth aperture broadens, body tail ratio is about 1:2 in length (Plate 9, Figure 37/ABC, Diagram 3/37).

Stage : 38 Measurements:

(i) Total length: An average of 27.6mm.

(ii) Body length: An average of 10.8mm.

(iii) Tail length: An average of 16.8mm.

(iv) Max. body width: An average of 5.1mm.

(v) Max. mouth disc width : An average of 3.24mm.

Special features: Hind limbs develop inner metatarsal tubercle, mouth widens more, body length is almost as in previous stage, tail membrane thin (Plate 9, Figure 38/ABC, Diagram 4/38).

Stage : 39 Measurements:

(i) Total length: An average of 28mm.

(ii) Body length: An average of 11.1mm.

(iii) Tail length: An average of 16.9mm.

(iv) Max. body width: An average of 5.3mm.

(v) Max. mouth disc width : An average of 3.54mm.

Special features: A whitish patch develop on ventral surface of toes and these pigment free patches will develop subarticular tubercles, body and tail length remains at 1:2 ratio (Plate 9, Figure 39/ABC, Diagram 4/39).

Stage : 40 Measurements:

(i) Total length: An average of 28mm.

(ii) Body length: An average of 11.2mm.

(iii) Tail length: An average of 16.8mm.

(iv) Max. body width: An average of 5.2mm.

(v) Max. mouth disc width : An average of 3.52mm.

Special features: Formation of hind limb almost completed, subarticular tibercles formed on toes, mouth disc now widens maximum, cloacal tail piece present,fore limbs develop within opercular folds (Plate 10, Figure 40/ABC, Diagram 4/40).

Stage : 41 Measurements:

(i) Total length: An average of 28.6mm.

(ii) Body length: An average of 11.3mm.

(iii) Tail length: An average of 17.3mm.

(iv) Max. body width: An average of 4.8mm.

(v) Max. mouth disc width : An average of 3.24mm.

Special features: Skin over fore limb thin and transparent, fore limb almost developed within the skin,larval mouth parts starts degeneration, tail starts regression (Plate 10, Figure 41/ABC, Diagram 4/41).

Stage : 42 Measurements:

(i) Total length: An average of 25.6mm.

(ii) Body length: An average of 11.4mm.

(iii) Tail length: An average of 14.2mm.

(iv) Max. body width: An average of 5.3mm.

Special features: Fore limb protrude out of the skin , fore limb almost developed fully, angle of mouth changed, mouth opening shifted upward anterior to nostril, horny beak like larval mouth disappear (Plate 10, Figure 42/ABC, Diagram 4/42).

Stage : 43 Measurements:

(i) Total length: An average of 22.8mm.

(ii) Body length: An average of 11.2mm.

(iii) Tail length: An average of 11.6mm.

(iv) Max. body width: An average of 4.6mm.

Special features: Mouth widens laterally, angle of mouth rests between nostril and midpoint of eye, bony jaws and muscular tongue formed, tail regression is more (Plate11, Figure 43/ABC, Diagram 4/43).

. **Stage : 44** Measurements:

(i) Total length: An average of 17.4mm.

(ii) Body length: An average of 8.7mm.

(iii) Tail length: An average of 8.7mm.

(iv) Max. body width: An average of 3.7mm.

Special features: Mouth widens further, angle of mouth rests at the end point of eye, body length and tail almost equal (1:1), tail further regressed to a small one, bulging eye developed (Plate 11, Figure 44/ABC,Diagram 4/44).

Stage : 45 Measurements:

(i) Total length: An average of 13.8mm.

(ii) Body length: An average of 12.1mm.

(iii) Tail length: An average of 1.7mm.

(iv) Max. body width: An average of 5.1mm.

Special features: Mouth widens to its maximum, angle of mouth crosses the eye, regression of tail is almost complete and reduced to a stub, fully developed fore and hind limbs with digits (Plate 11, Figure 45/ABC, Diagram 4/45).

Stage : 46 Measurements:

(i) Total length: An average of 12.4mm.

(ii) Body length: An average of 12.6mm.

(ii) Max. body width: An average of 5.4mm.

Special features: Tail completely resorbed, process of metamorphosis is completed, slightly elongated body with all internal and external organs, hind limbs more stout and more muscular than the fore limbs, grayish in color, a small toad which resembles the adult except the size, starts its terrestrial life (Plate 12, Figure A B C D, Diagram 4/46).

To summarize, in the himalayan toad (*Bufo himalayanas)* hatching starts at about stage-20, in laboratory condition. At this stage the larvae attains a length of 16-17 mm. Ventral sucker, olfactory pits and eyes are visible. Tail membrane is transparent. Three pairs of gills gradually get within the opercular folds. From stage 23 mouth disc starts developing and from this stage larvae begin its free swimming and feeding life. Development of mouth parts is completed by stage 25. Horney beak-like larval jaws are developed at and around stage 25 (Plate 5, Figure 25/ABC).

At the stage 26, the hind limb bud appears. The average measurements during these periods are described in the Table 3. Limb buds gets prominent shape at the stage 29. Up to stage 40 hind limb develops into its climax and size of the larvae gradually increases. Hind limb formation is completed at the end of stage 38. The larvae at stage 40 show deep black pigmentation both on dorsal and ventral surfaces. The myotomes of the tail with black pigmentation along the dorasl and ventral side is also observed (Plate 10, Figure 40/ABC,). Tail membrane becomes brown in color. Cornea tends to dialated and becomes larger than the eye ball itself. The mouth is sub-terminal with one upper and two lower rows of larval denticles. Body proportion of individual larva from stage 26-40 remain constant to some extent in *Bufo himalayanas* (Table 4 & 5: Plate 5-10). These findings are very similar as *Bufo valiceps* (Limbangh and Volpe, 1957).

Various body dimensions and body proportions of different stages of larvae are summarized in Table 4. Some unexpected results in the data may occur due to ill-fed or undernourished forms or due to specimens having stunted growth by some intrinsic and extrinsic factors in the laboratory conditions.

Total duration of the metamorphosis on an average is about two and a half months in the species described. However, this time of total metamorphosis can be shortened under higher temperature and natural conditions.

Fore limb protrusion out of the skin takes place in stage-42. Adult mouth complexity replaced the larval mouth parts at stage-44. Tail resorbed completely in stage-46. This stage is called sub-adult where metamorphosis is fully completed.

Drastic morphological changes occur mainly from stage 41 to stage 46. (Plate 10-12, Diagram 4).In other words, it can be said that metamorphosis is initiated at stage at stage 41 and completed by stage 46. In these four stages drastic changes took place and the animal dramatically changes its habitat, from aquatic to terrestrial life.

Periodic life table of Bufo himalayanas

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length	Body length	Tail length	Max. body width	Mouth disc width	Special features
	Units Av.	Units Av.	Units Av.	Units Av.	Units Av.	
20	16 17 16 (16.6) 18 16	6 7 6 (6.4) 7.5 5.5	10 10 10 (10.2) 10.5 10.5	5 4 5 (4.8) 5 5	.....	Ventral sucker and three pairs of external gills present. Operculum not distinguishable. Tail membrane(fin) broad and semitransparent with rounded lip (Plate 4, Figure 20/ABC, Diagram 2/20)
21	23 20 22 (21.8) 23 21	10 9 9.5 (9.4) 10 8.5	13 11 11.5 (12.5) 13 12.5	7 6 7 (6.7) 7 6.5	.....	Transparent eye, elongated body, tail membrane broad, dense pigmentation and body oval in shape (Plate 4, Figure 21/ABC, Diagram 2/21)
22	29 27 29 (28.2) 28 28	11 10 9 (10.2) 10 10	18 17 19 (18.0) 18 18	8.5 8 8 (8.4) 8.5 8.5	---------	Tail membrane more transparent and gradually tapering towards tip,opercular fold not distingushable,body oval in shape (Plate 4, Figure22/ABC, Diagram 2/22)

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length		Body length		Tail length		Max. body width		Mouth disc width		Special features
	Units	Av.	Units	Av.	Units	Av.	Units	Av.	Units	Av.	
23	30 30 31 30 29	(30.0)	11 11 10 10 10	(10.6)	19 19 20 20 19	(19.4)	9 9 9.5 9 8.5	(9.0)	-------		Mouth opening appears,opercular fold appear, three pairs of external gills seen, tail myotomes prominent, more elongated body (Plate 5, Figure 23/ABC, Diagram 2/23).
24	30 32 32 30 32	(31.2)	11 11.5 10 10.5 10.5	(11.1)	19 20.5 21 19.5 20.5	(20.1)	8 8 7 8.5 8	(8.3)	2 2 2.4 2.2 2	(2.12)	Opercular folds tend to close; mouth disc disappears, mouth with distinct lips and denticles; tail myotomes increase in number (Plate 5, Figure 24/ABC,Diagram 2/24).
25	31 32 32 32 31	(31.6)	10 11.5 11 11.5 11	(11.0)	21 20.5 22 20.5 20	(20.6)	9 9 32 9.5 8.5	(9.0)	2.5 2.3 2.4 2.3 2.3	(2.36)	Spiracles appear, external gills disappear, mouth disc widens and get prominent jaws-like structure, eyes opaque and black (Plate 5,Figure 25/ABC, DiaGRAM 2/25)..

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length		Body length		Tail length		Max. body width		Mouth disc width		Special features
	Units	Av.	Units	Av.	Units	Av.	Units	Av.	Units	Av.	
26	33		11		22		9.5		2.4		Emergence of hind limb buds, mouth widens with jaws, spiracles shifting to the ventral side of the body (Plate 5, Figure26/ABC, Diagram 2/26)..
	34		12		22		9		2.5		
	33	(33.6)	12	(11.6)	23	(22.0)	2.5	(9.2)	2.4	(2)	
	33		12		21		9		2.4		
	34		11		23		9		2.4		
27	31		11		20		8		2.5		Hind limb bud increases in size and more or less than half of its diameter, eyes prominent and black, body oval in shape (Plate 6, Figure 27/ABC, Diagram 2/27). .
	30		11		19		8.5		2.6		
	29	(30.4)	15	(11.2)	18.5	(19.2)	8.5	(8.2)	2.4	(2.5)	
	31		11.5		19.5		8		2.4		
	30		11		19		8		2.5		
28	31		12		19		8.5		2.5		Hind limb bud elongates, slightly greater than its diameter, mouth widens, jaws with denticles, body oval in shape (Plate 6, Figure 28/ABC, Diagram 2/28).
	30		11.5		18.5		8.5		2.6		
	30	(31.0)	11.8	(11.8)	18	(19.2)	8	(8.4)	2.5	(2.56)	
	31		11.5		19.5		8.5		2.6		
	31		12		19		8.5		2.5		

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length		Body length		Tail length		Max. body width		Mouth disc width		Special features
	Units	Av.	Units	Av.	Units	Av.	Units	Av.	Units	Av.	
29	31		11.5		19.5		8		2.6		Hind limb bud elongates more and its length is greater than its diameter by 1.5 times; other features are as in stage-28. (Plate 6, Figure 29/ABC, Diagram 3/29).
	30		11.5		18.5		8		2.7		
	30	(30.6)	10.5	(11.7)	18.5	(18.9)	8.5	(8.1)	2.6	(2.66)	
	31		12		19		8		2.6		
	31		12		19		8		2.7		
30	29		11		18		8		2.7		Hind limb bud elongates, slightly greater than its diameter, mouth widens,jaws with denticles, body oval in shape (Plate 6, Figure 30/ ABC, Diagram 3/30).
	30		10.5		19.5		8.5		2.8		
	30	(29.6)	11	(10.6)	19	(19.0)	8	(8.1)	2.7	(2.76)	
	29		10		19		8		2.7		
	30		105		19.5		8		2.8		
31	30		105		19.5		8.5		2.8		Hind limb tip flattened and conical, paddle shaped tip of hind limb without any digit, mouth with stout chitinious pair of jaws, tail with distinctly visible myotomes (Plate 7, Figure 31/ABC, Diagram 3/31).
	29		10		19		8		2.9		
	31	(30.2)	10.5	(10.3)	21.5	(19.9)	8.5	(8.3)	2.8	(2.88)	
	31		10.5		20.5		8.5		3		
	29		10		19		8		2.9		

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length		Body length		Tail length		Max. body width		Mouth disc width		Special features
	Units	Av.	Units	Av.	Units	Av.	Units	Av.	Units	Av.	
32	29 30 29 28 29	(29)	10 10 9.5 9 9.5	(9.6)	19 20 19.5 19 19.5	(19.4)	8 8 8.5 8.5 8.5	(8.3)	3 3 2.9 3 3	(2.98)	Hind limb tip or foot starts digitalization, 4th and 5th digit are visible now, tail is almost twice the body length, rigid chitinous pair of jaws guards the mouth aperture, operculum is now distinct (Plate 7, Figure 32/ABC, Diagram 3/32).
33	28 27 28 28 27	(27.6)	9.5 9.5 9 9.5 9	(11.3)	18.5 17.5 18 18.5 17	(17.9)	8 7.5 7.5 7.5 7.5	(7.6)	3 3 2.9 3 2.8	(2.94)	More digits are distinguishable at the tip of the hind bud and this is more stout and well developed, operculum distinct, semitransparent and broad tail fin (Plate 7, Figure 33/ABC, Diagram 3/33).
34	28 27 27 28 27	(27.4)	10 10 9.5 10 9.5	(9.8)	18 17 17.5 18 17.5	(17.6)	7.5 8 8 8 7.5	(7.8)	3 2.9 2.9 3 3	(2.96)	Digits or toes are more distinct, 1st and 2nd toes not separated yet, tail fin or membrane not so broad, hind limb is more developed with more joints (Plate 7, Figure 33/ABC, Diagram 3/34).

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length		Body length		Tail length		Max. body width		Mouth disc width		Special features
	Units	Av.	Units	Av.	Units	Av.	Units	Av.	Units	Av.	
35	27 26 27 26 26	(26.4)	10 9.5 9 9.5 10	(9.8)	17 16.5 17 16.5 16	(16.6)	6.5 6 6 6.5 6	(6.2)	3 3.2 3 3 2.9	(3.0)	Toes of the hind limb now distinguishable fully, all toes separated from each other, hind limb develops bends behind the foot, tail fin is thin, length of the tail is about twice the body length (Plate 8, Figure 35/ABC, Diagram 3/35).
36	27 28 26 28 29	(27.2)	10 9.5 9.6 9 9	(9.4)	17 18.5 17 16.5 16	(17.0)	5.5 6 4 5.5 5	(5.4)	3.0 2.9 3.2 2.9 3	(3.0)	All toes are completely separated except 1st and 2nd which are slightly joined at their base, mouth is guarded by a pair of thick lips with cuticular thickening, body slightly elongated (Plate 8, Figure 36/ABC, Diagram 3/36).
37	26 27 26 25 26	(26.0)	10.5 10 10.5 10 10.5	(10.3)	15.5 17 15.5 15 15.5	(15.7)	5.5 5.5 5 5 5.5	(5.3)	3.2 3 3.2 3 3	(3.0)	All 5 toes are free and completely separated, hind limb develops more joints and bends, mouth aperture broadens, body tail ratio is about 1:2 in length (Plate 9, Figure 37/ ABC, Diagram 3/37).

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length	Body length	Tail length	Max. body width	Mouth disc width	Special features
	Units Av.	Units Av.	Units Av.	Units Av.	Units Av.	
38	27 28 27 (27.6) 28 28	10.5 11 10 (10.8) 11.5 11	16.5 17 16 (16.8) 16.5 17	5 5 5.5 (5.1) 5 5	3 3.6 3.3 (3.24) 3 3.2	Hind limbs develop inner metatarsal tubercle, mouth widens more, body length is almost as in previous stage, tail membrane thin (Plate 9, Figure 38/ABC, Diagram 4/38).
39	27 27 28 (28.0) 28 28	11 11.5 11.5 (11.1) 11.5 10	16 17.5 16.5 (16.9) 16.5 18	5 5.5 4 (5.3) 5.5 5.5	3.9 3.6 3.4 (3.54) 3.6 3.2	A whitish patch develop on ventral surface of toes and these pigment free patches will develop sub-articular tubercles, body and tail length remains at 1:2 ratio (Plate 9, Figure 39/ABC, Diagram 4/39).
40	27 28 29 (28.0) 28 29	11 11.5 12 (11.2) 10.5 11	16 16.5 17 (16.8) 17.5 18	5 5 4 (5.2) 5.5 5.5	3.4 3.6 3.6 (3.52) 3.4 3.6	Formation of hind limb almost completed, sub-articular tubercles formed on toes, mouth disc now widens maximum, cloacal tail piece present, fore limbs develop within opercular folds (Plate 10, Figure 40/ABC, Diagram 4/40).

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length	Body length	Tail length	Max. body width	Mouth disc width	Special features
	Units Av.	Units Av.	Units Av.	Units Av.	Units Av.	
41	29 30 27 (28.6) 29 27	11 11.5 11 (11.3) 12 11	18 18.5 18 (17.3) 17 16	5 5 4.5 (4.8) 4.5 5	---------	Skin over fore limb thin and transparent, fore limb almost developed within the skin, larval mouth parts starts degenerating, tail starts regression (Plate 10, Figure 41/ABC, Diagram 4/41).
42	25 26 26 (25.6) 25 26	12 11 11.5 (11.4) 11 11.5	13 15 14.5 (14.2) 14 14.5	5.5 5 5.5 (5.3) 5 5.5	----------	Fore limb protrude out of the skin , fore limb almost developed fully, angle of mouth changed, mouth opening shifted upward anterior to nostril, horny beak like larval mouth disappear (Plate 10, Figure 42/ABC, Diagram 4/42).
43	23 24 22 (22.8) 23 22	11.5 12 12 (11.2) 11 10	11.5 12 11 (11.6) 12 11.5	4.5 5 4.5 (4.6) 4.5 4.5	---------	Mouth widens laterally, angle of mouth rests between nostril and midpoint of eye, bony jaws and muscular tongue formed, tail regression is more (Plate 11, Figure 43/ABC, Diagram 4/43).

contd.

Periodic life table of *Bufo himalayanas*

Table No.4

Stage	Total length	Body length	Tail length	Max. body width	Mouth disc width	Special features
	Units Av.	Units Av.	Units Av.	Units Av.	Units Av.	
44	18 17 17 (17.4) 18 17	9 9 8.5 (8.7) 9 8	9 8 8.5 (8.7) 9 9	4 3.5 3.5 (3.7) 4 3.5	--------	Mouth widens further, angle of mouth rests at the end point of eye, body length and tail almost equal (1:1),tail further regressed to a small one, bulging eye developed (Plate 11, Figure 44/ABC, Diagram 4/44).
45	15 14 12 (13.8) 14 13	13 12 11.5 (12.1) 12 12	2 2 1.5 (1.7) 2 1	5 5.5 4 (5.1) 5 5	-------	Mouth widens to its maximum, angle of mouth crosses the eye, regression of tail is almost complete and reduced to a stub, fully developed fore and hind limbs with digits (Plate 11, Figure 45/ABC, Diagram 4/45).
46	13 12 12 (12.4) 12 13	13 12 13 (12.6) 13 12	----	5.5 5.5 5 (5.4) 5.5 5.5	----	Tail completely resorbed, process of metamorphosis is completed, slightly elongated body with all internal and external organs, hind limbs more stout and more muscular than the fore limbs, grayish in color, a small toad which resembles the adult except the size, starts its terrestrial life (Plate 12, Figure A B C D , Diagram 4/46).

contd.

Table No.5

Periodic life table of *Bufo himalayanas*

Stage	Nos	Total length	Body length	Tail length	Body width	Mouth disc width	Body length/ Total length	Body width/ Body length	Mouth disc/ Body width
20	5	16.6 (16-18)	6.4 (5.5-7.5)	10.2 (10-10.5)	4.8 (4-5)	----	0.38	0.75	----
21	5	21.8	9.4 (20-23)	12.2 (8.5-10)	6.7 (11-13)	---- (6-7)	0.43	0.71	---
22	5	28.2 (27-29)	10.2 (10-11)	18.0 (17-19)	8.4 (8-8.5)	---	0.36	0.82	---
23	5	30.5 (29-31)	10.6 (10-11)	19.4 (19-20)	9.0 8.5-9.5)	---	0.35	0.84	----
24	5	31.2 (30-32)	11.1 (10.5-11.5)	20.1 (19-21)	8.3 (8-9)	2.12 (2-2.4)	0.35	0.74	0.25
25	5	31.6 (31-32)	11.0 (10-11.5)	20.6 (20-21)	9.0 (8.5-9.5)	2.36 (2.3-2.4)	0.34	0.81	0.26

Table No.5

Periodic life table of *Bufo himalayanas*

Stage	Nos	Total length	Body length	Tail length	Body width	Mouth disc width	Body length/ Total length	Body width/ Body length	Mouth disc/ Body width
26	5	33.6	11.6	22	9.2	2.42	34	0.79	0.26
		(33-34)	(11-12)	(21-23)	(9-9.5)	(2.4-2.5)			
27	5	30.4	11.2	19.2	8.2	2.5	0.36	0.70	0.30
		(30-31)	(11-11.5)	(18.5-20)	(8-8.5)	(2.4-2.6)			
28	5	31	11.8	19.2	8.4	2.56	0.38	0.71	0.30
		(26-32)	(11.5-12)	(18.5-20)	(8-8.4)	2.5-2.6)			
29	5	30.6	11.7	18.9	8.1	2.66	0.38	0.69	0.32
		(30-31)	(11.5-12)	(18.5-19.5)	(8-8.5)	(2.6-2.7)			
30	5	29.6	10.6	19	8	2.76	0.35	0.76	0.34
		(29-30)	(10-11)	(18-19.5)	(8-8.5)	(2.7-2.8)			

contd.

Table No.5

Periodic life table of *Bufo himalayanas*

Stage	Nos	Total length	Body length	Tail length	Body width	Mouth disc width	Body length/ Total length	Body width/ Body length	Mouth disc/ Body width
31	5	30.2 (29-31)	10.3 (10-10.5)	19.9 (18-19.5)	8.3 (8-8.5)	2.88 (2.8-3)	0.34	0.8	0.34
32	5	29 (28-29)	9.6 (9-10)	19.4 (19-20)	8.3 (8-8.5)	2.98 (2.9-3)	0.33	0.86	0.35
33	5	27.6 (27-28)	11.3 (9-10)	17.9 (17-18.5)	7.6 (7.5-8)	2.94 (2.8-3)	0.4	0.67	0.38
34	5	27.4 (27-28)	9.8 (7.5-10)	176 (17-18)	7.8 (7.5-8)	2.96 (2.9-3)	0.35	0.79	0.37

contd.

Table No.5

Periodic life table of *Bufo himalayanas*

Stage	Nos	Total length	Body length	Tail length	Body width	Mouth disc width	Body length/ Total length	Body width/ Body length	Mouth disc/ Body width
35	5	26.4 (26-27)	9.8 (9.5-10)	16.6 (16-17)	6.2 (6-6.5)	3 (2.9-3.2)	0.37	0.63	0.48
36	5	27.2 (26-28)	9.4 (9-10)	17 (16.5-18.5)	5.4 (5-6)	3 (2.9-3.2)	0.34	0.57	0.55
37	5	26 (25-26)	10.3 (10-10.5)	15.7 (15-17)	5.3 (5-5.5)	3 (3-3.2)	0.39	0.51	0.56
38	05	27.6 (27-28)	10.8 (10-11)	16.8 (16.5-17)	5.1 (5-5.5)	3.24 (3-3.6)	0.39	0.47	0.63

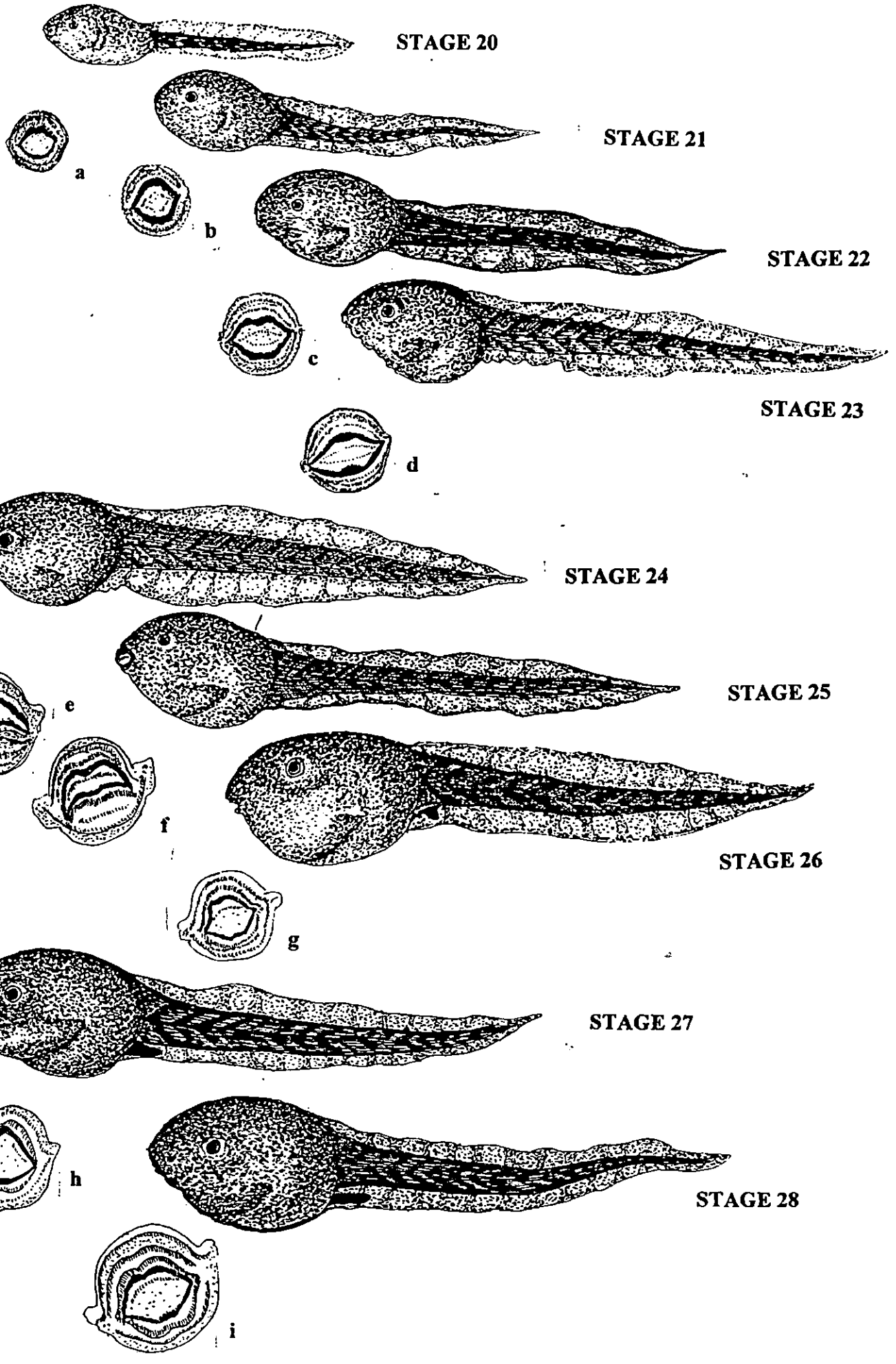
contd.

Table No.5

Periodic life table of *Bufo himalayanas*

Stage	Nos	Total length	Body length	Tail length	Body width	Mouth disc width	Body length/ Total length	Body width/ Body length	Mouth disc/ Body width
39	5	28	11.1	16.9	5.3	3.54	0.39	0.47	0.66
		(27-29)	(10-11.5)	(16-18)	(5-5.5)	(3.2-39)			
40	5	28	11.2	16.8	5.2	3.52	0.4	0.46	0.67
		(27-29)	(10.5-12)	(16-18)	(5.5-5)	(3.4-3.9)			
41	5	28.6	11.3	17.3	4.8	---	0.39	0.42	---
		(27-30)	(11-12)	(16-18.5)	(4.5-5)				
42	5	25.6	11.4	14.2	5.3	---	0.44	0.46	---
		(25-26)	11-12	(13-15)	(5-5.5)				
43	5	22.8	11.2	11.6	4.6	---	0.49	0.41	---
		(22-24)	(10.5-12)	(11-12)	(4.5-5)				
44	5	17.4	8.7	8.7	3.7	---	0.50	0.42	---
		(17-18)	(8.5-9)	(8.5-9)	(3.5-4)				
45	5	13.8	12.1	1.7	5.1	---	0.87	0.42	---
		(13-15)	(11.5-13)	(1-2)	(5-5.5)				
46	5	12.4	12.6	---	5.4	---	1.01	0.42	---
		(12-13)	(12-13)	---	(5-5.5)				

DIAGRAM : 2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a
b
STAGE 22
c
STAGE 23
d
STAGE 24
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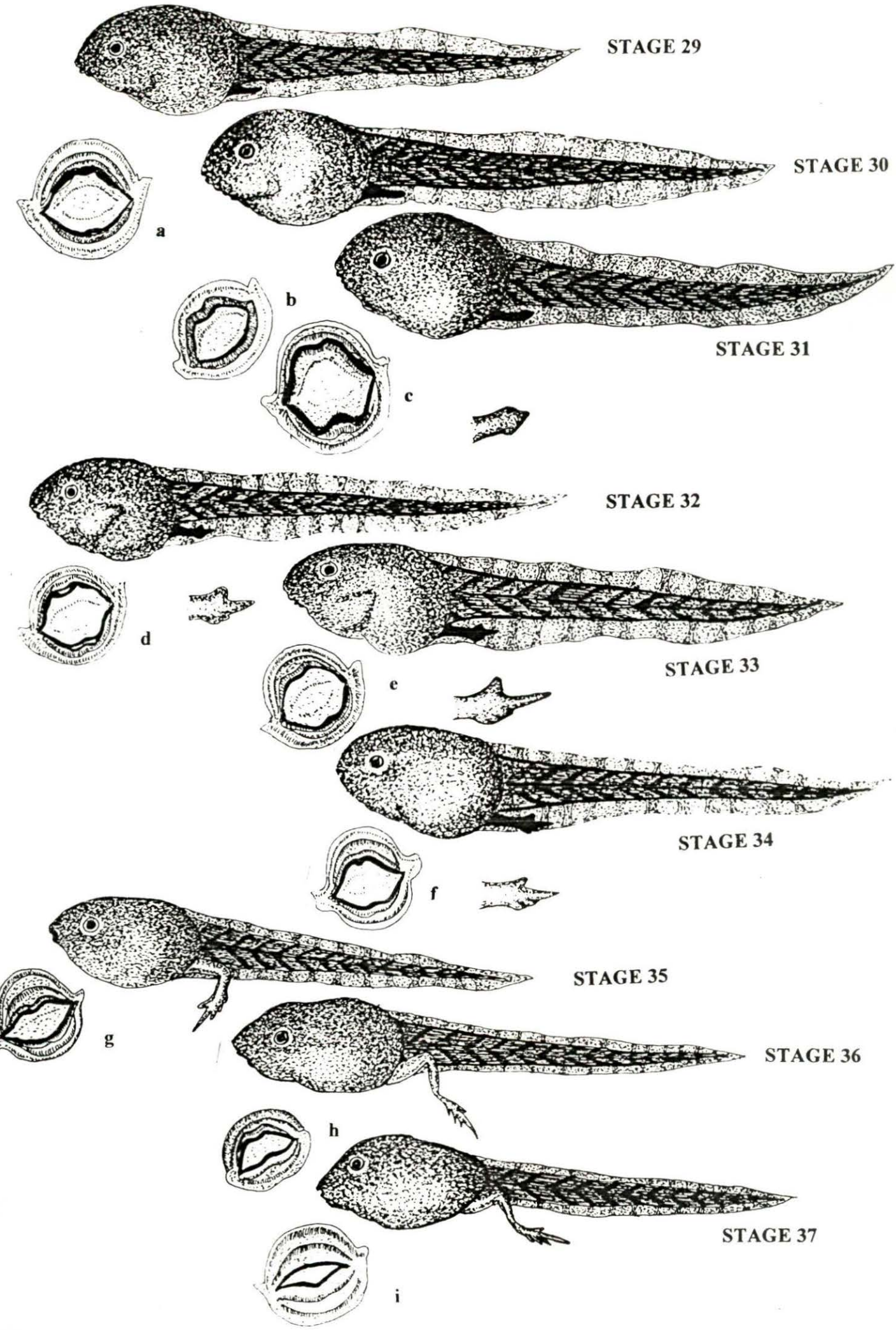
e
f
STAGE 26
g
STAGE 27
h
STAGE 28
i

DIAGRAM : 3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STAGE 30
a
b
STAGE 31
c
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d
STAGE 33
e
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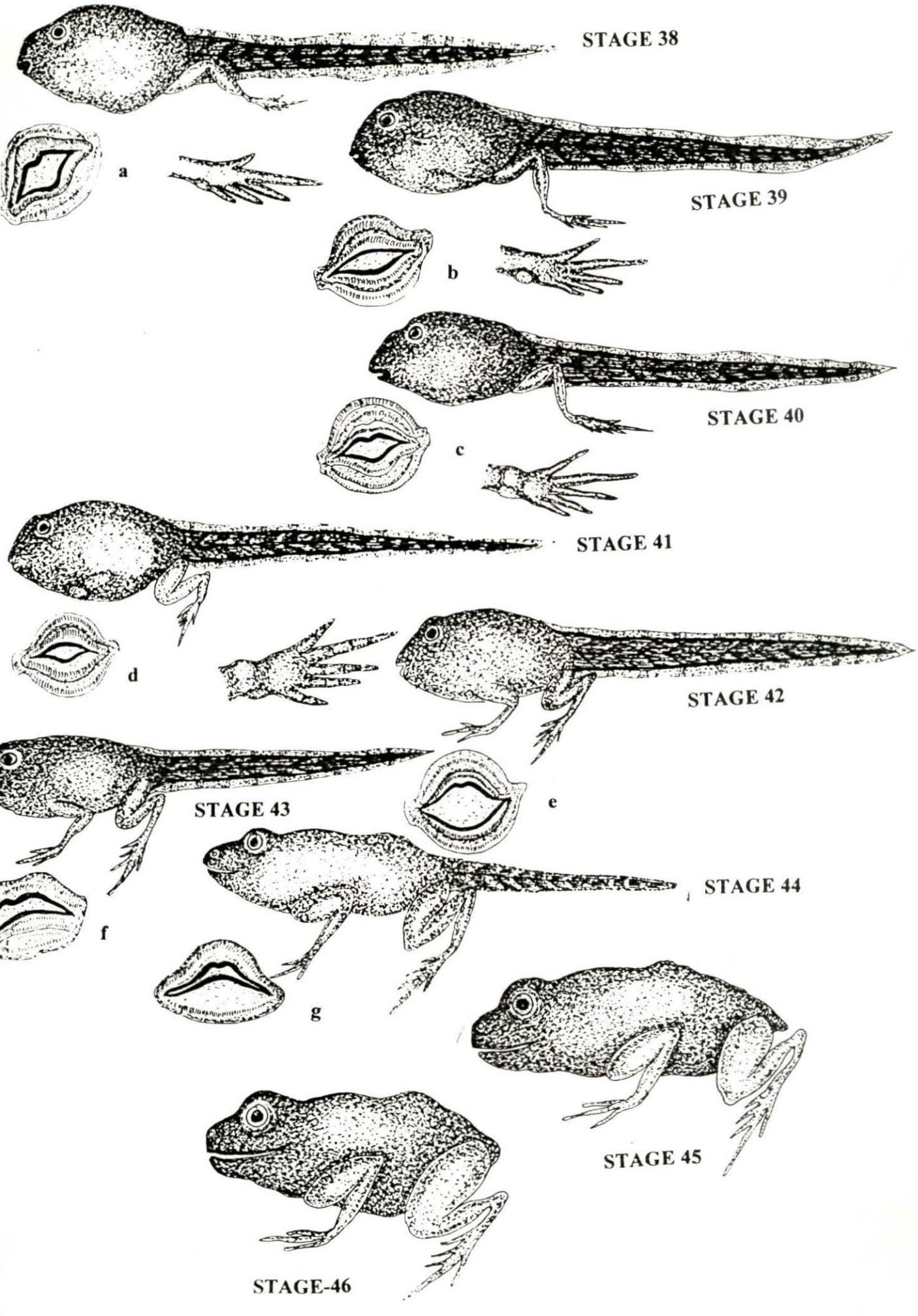
f
STAGE 35
g
STAGE 36
h
STAGE 37
i

DIAGRAM : 4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a
STAGE 39
b
STAGE 40
c
STAGE 41
d
STAGE 42
STAGE 43
e
STAGE 44
f
g
STAGE 45
STAGE-46

PLATE 4

Legends

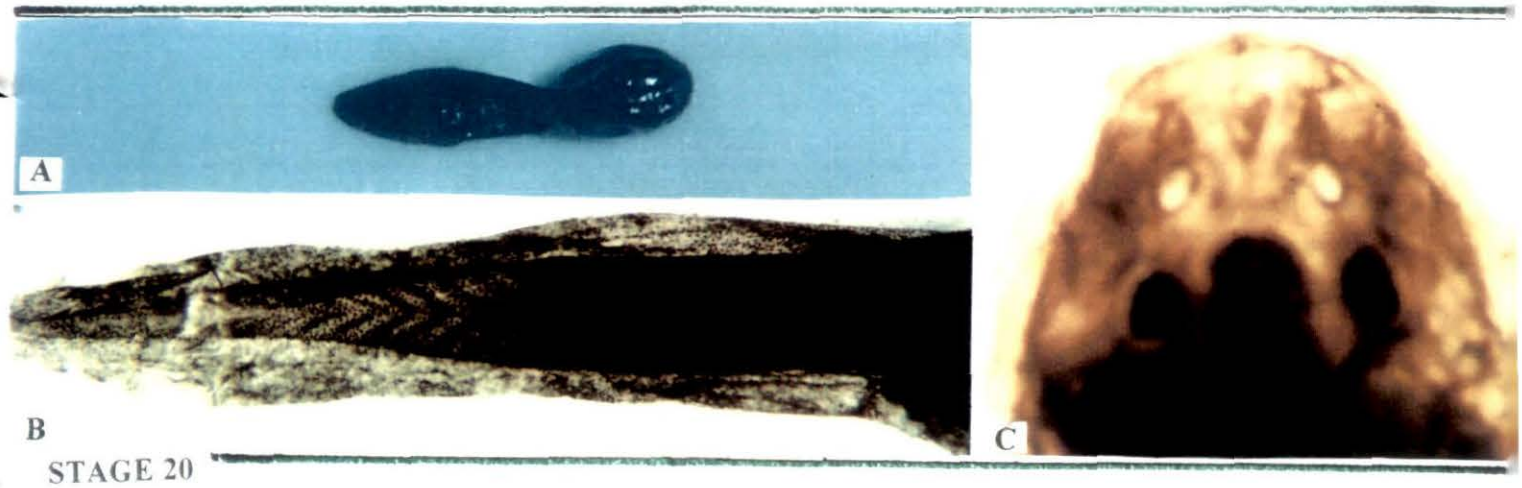
Normal life table of *Bufo himalayanas*

STAGE 20: A. Entire larva B. Tail portion of the larva enlarged
C. Dorsal view of the head enlarged

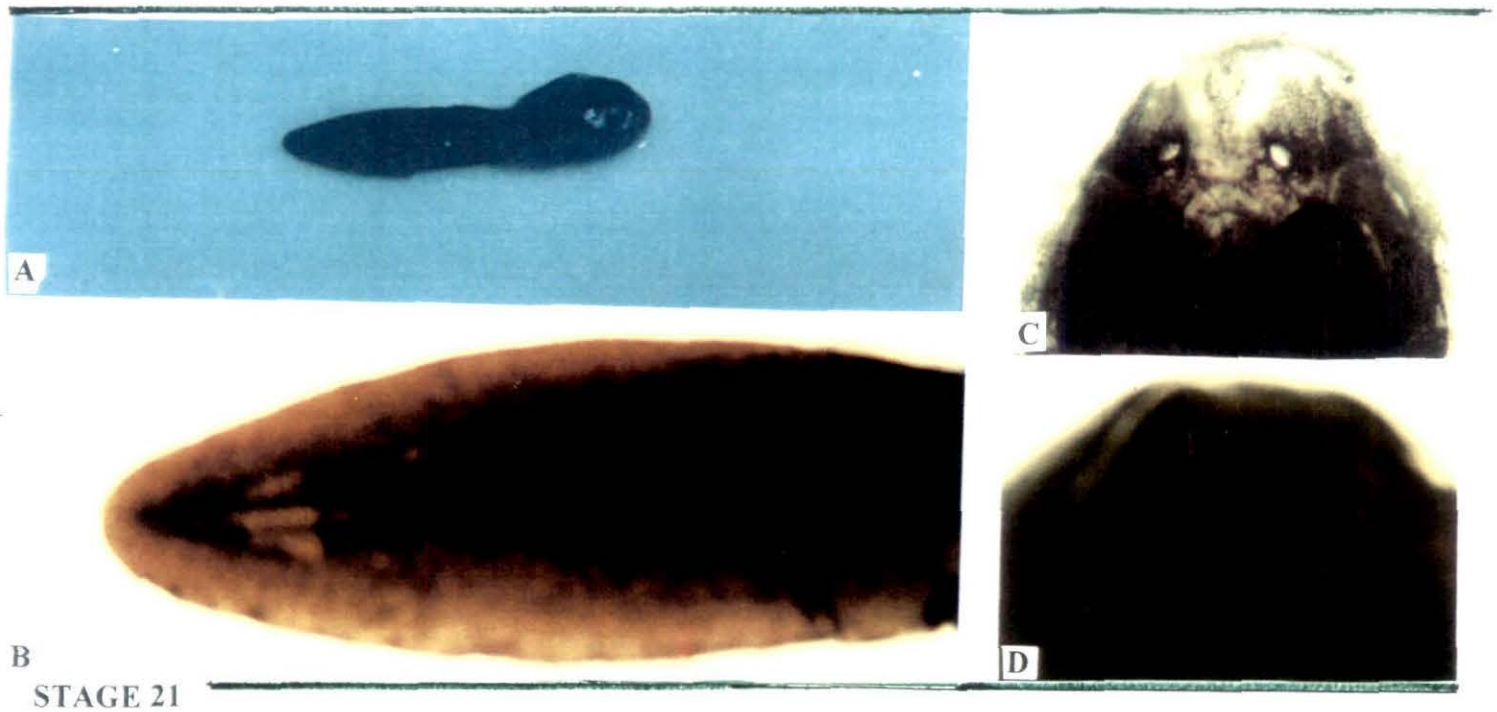
STAGE 21: A. Entire larva B. Tail portion of the larva enlarged
C. Dorsal view of the head D. Mouth disc of the larva

STAGE 22: A. Entire larva B. Tail portion of the larva enlarged
C. Dorsal view of the head

PLATE-4



STAGE 20



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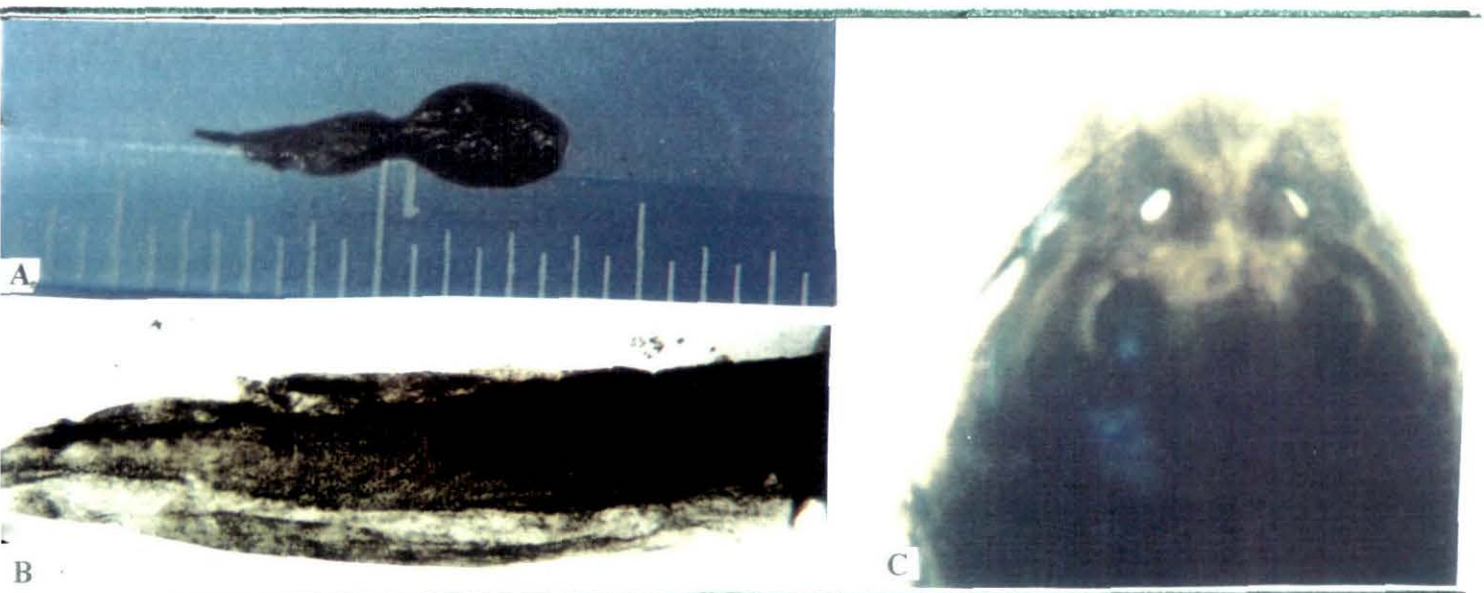


STAGE 22

PLATE 5

Legends

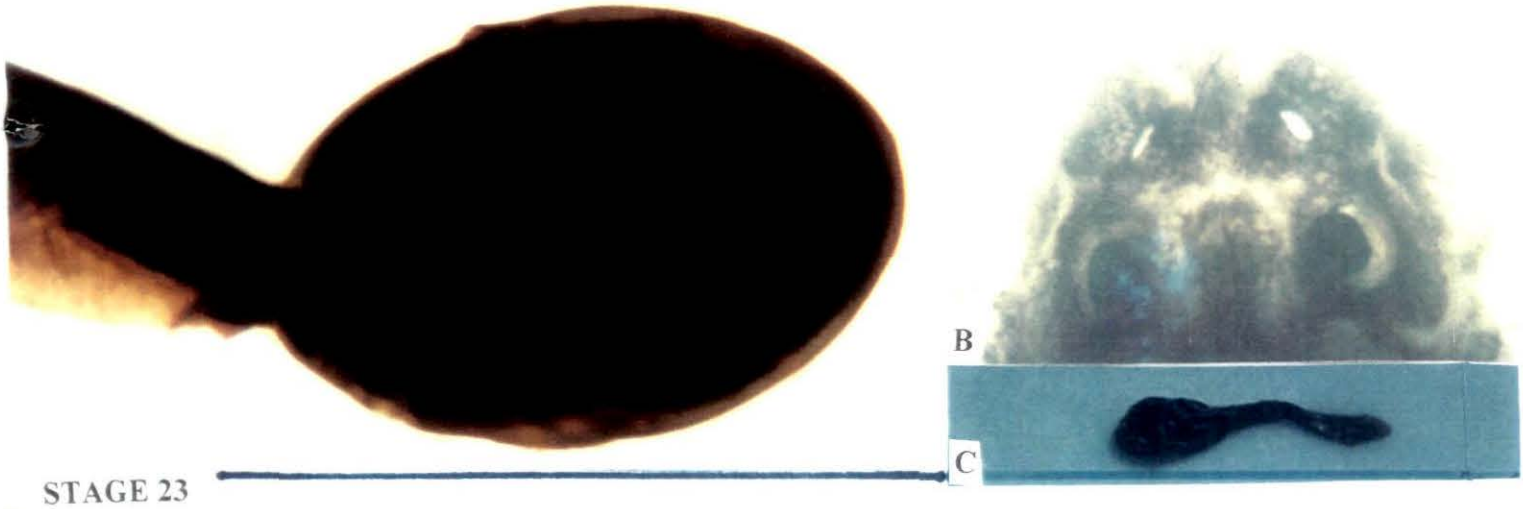
Normal life table of *Bufo himalayanas*

STAGE 23: A. Head and portion of head enlarged
B. Dorsal view of the head C. Entire larva

STAGE 24: A. Tail portion of the larva enlarged
B. Dorsal view of the head C. Entire larva

STAGE 25: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva

STAGE 26: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva



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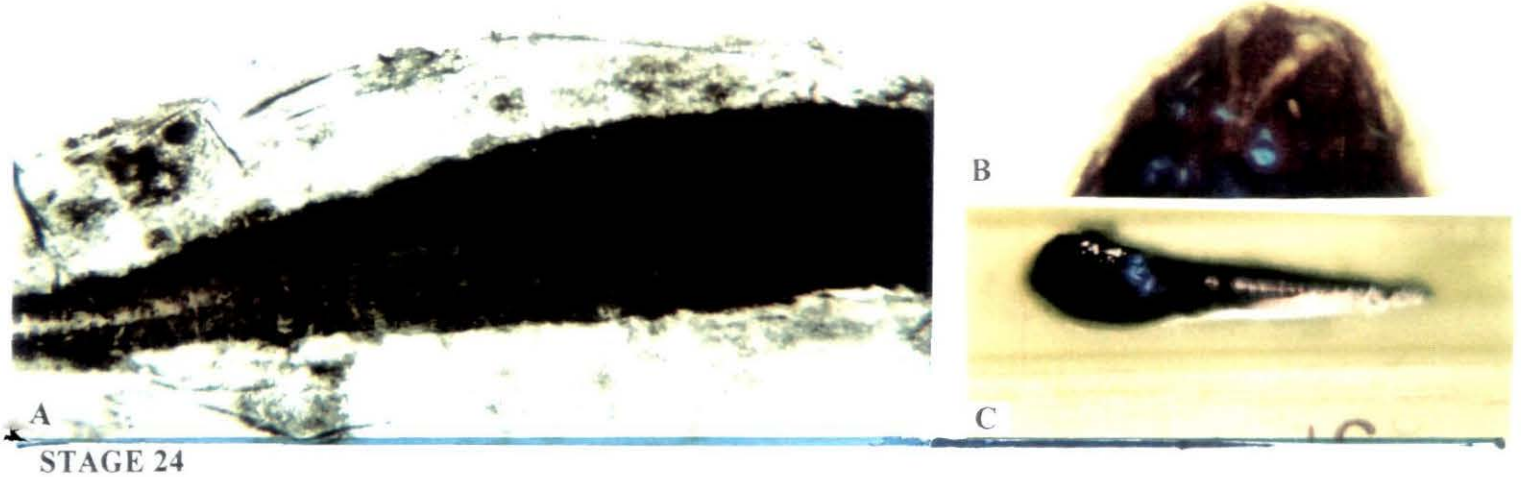

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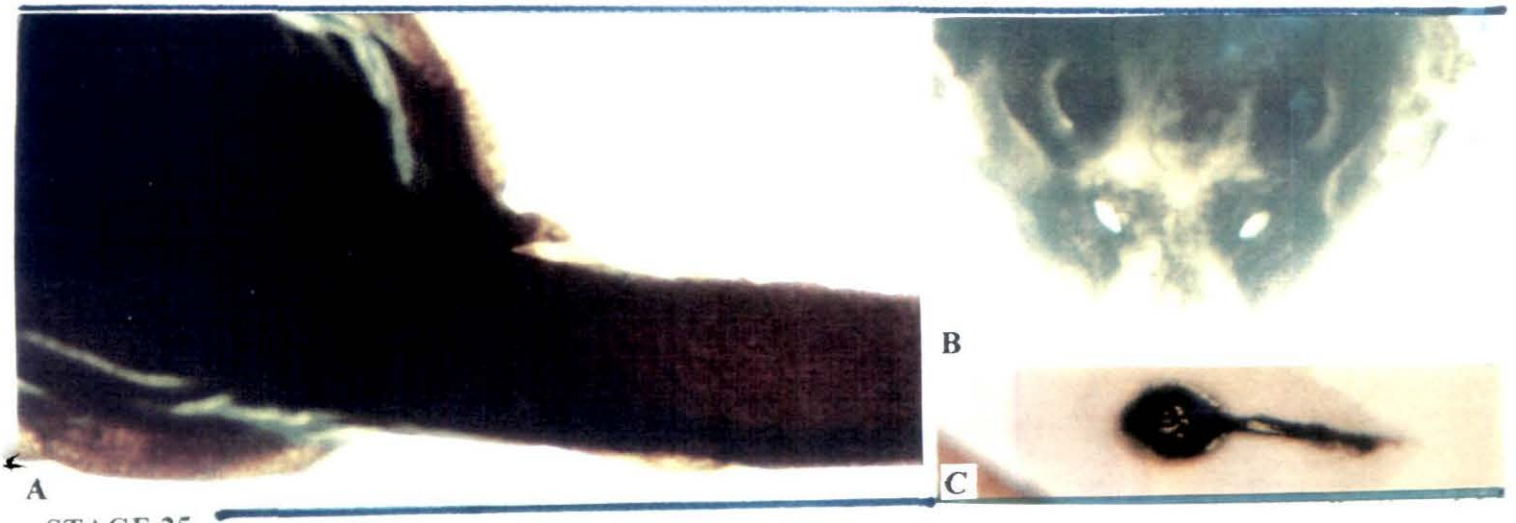
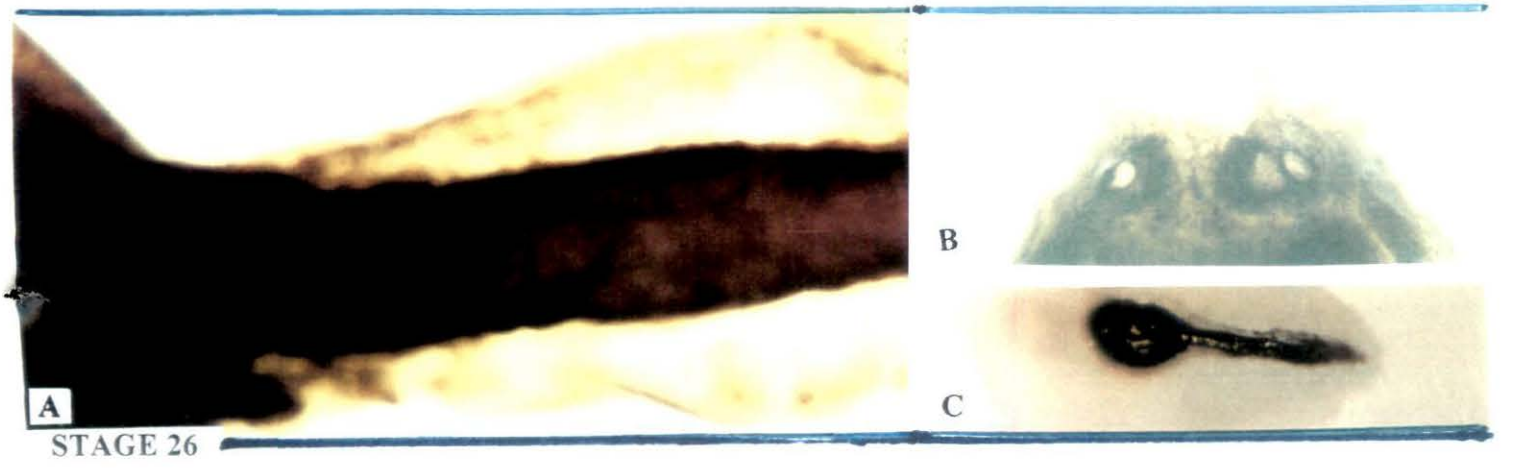


STAGE 25



STAGE 26

PLATE 6

Legends

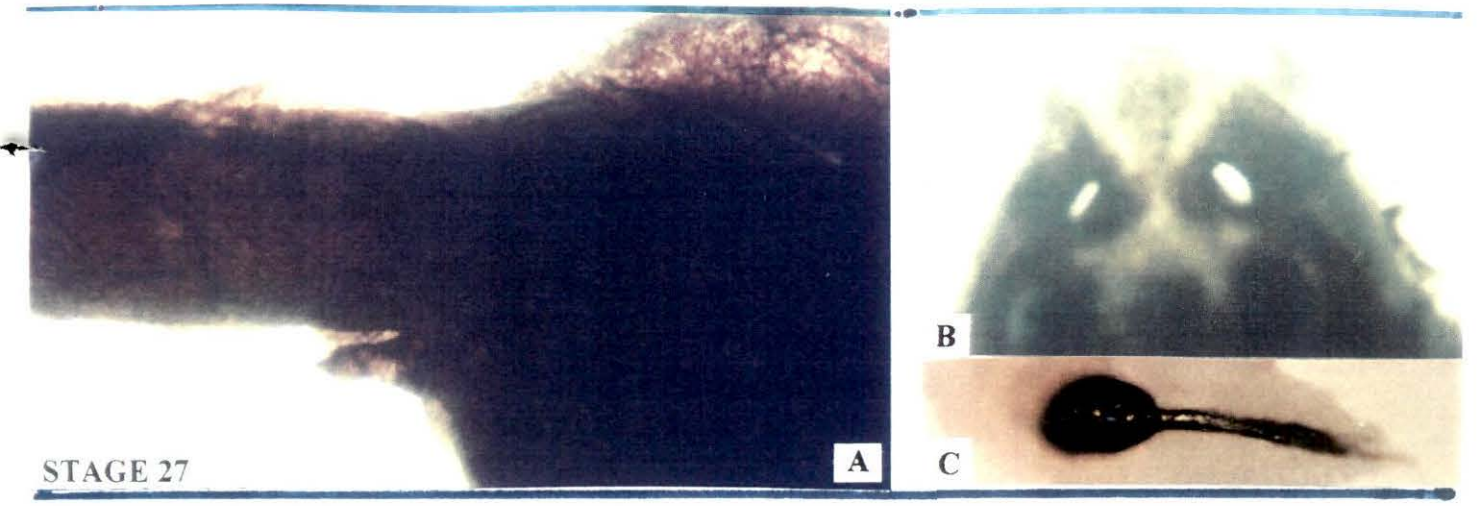
Normal life table of *Bufo himalayanas*

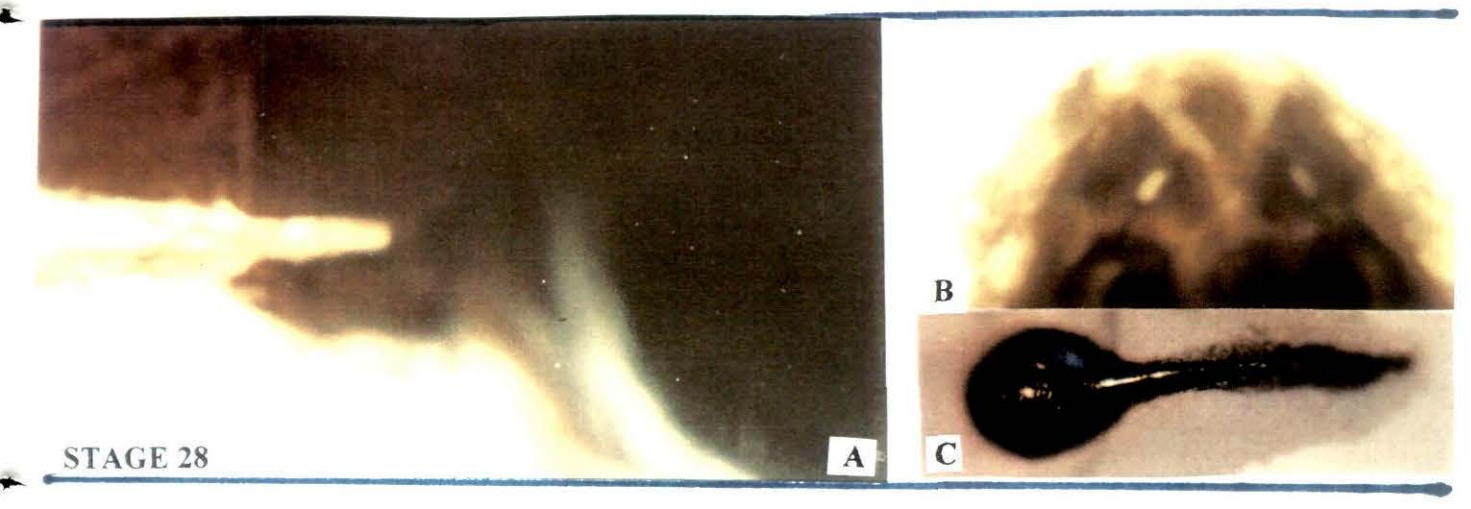
STAGE 27: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva

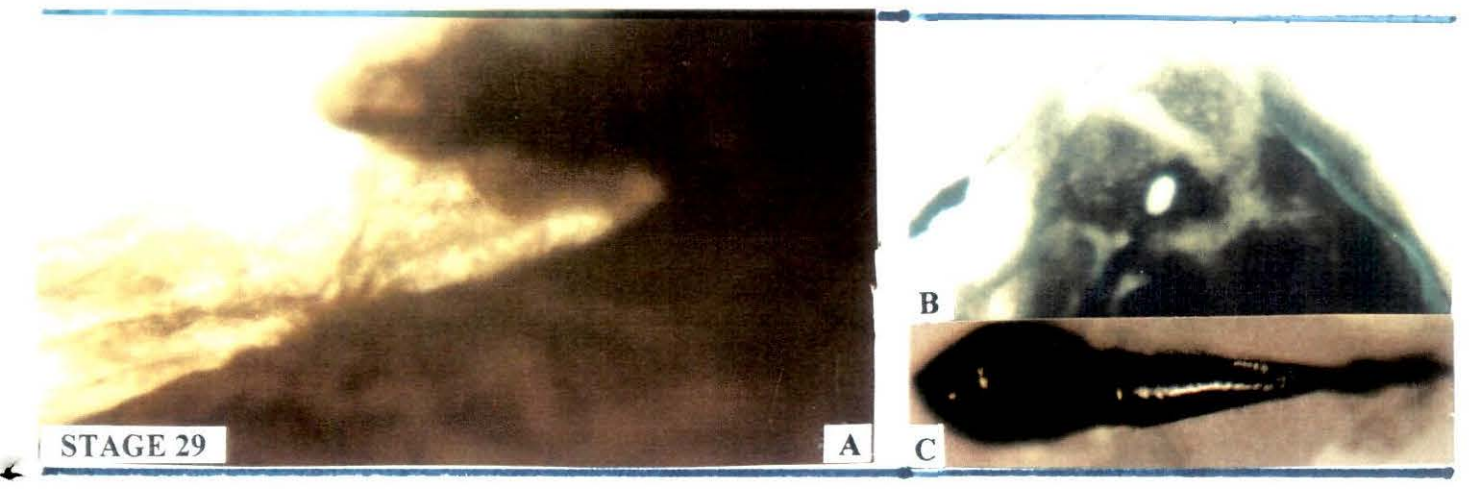
STAGE 28: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva

STAGE 29: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva

STAGE 30: A. Junction of head and tail enlarged to show hind limb bud B. Dorsal view of the head C. Entire larva


STAGE 27
A
B
C


STAGE 28
A
B
C


STAGE 29
A
B
C

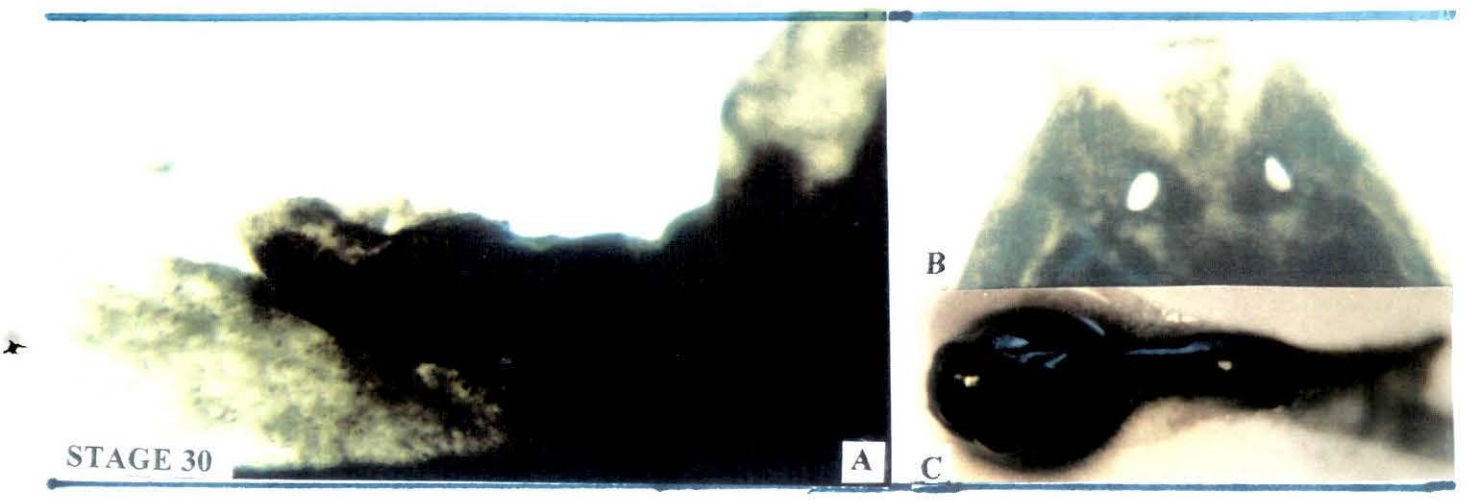

STAGE 30
A
B
C

PLATE 7

Legends

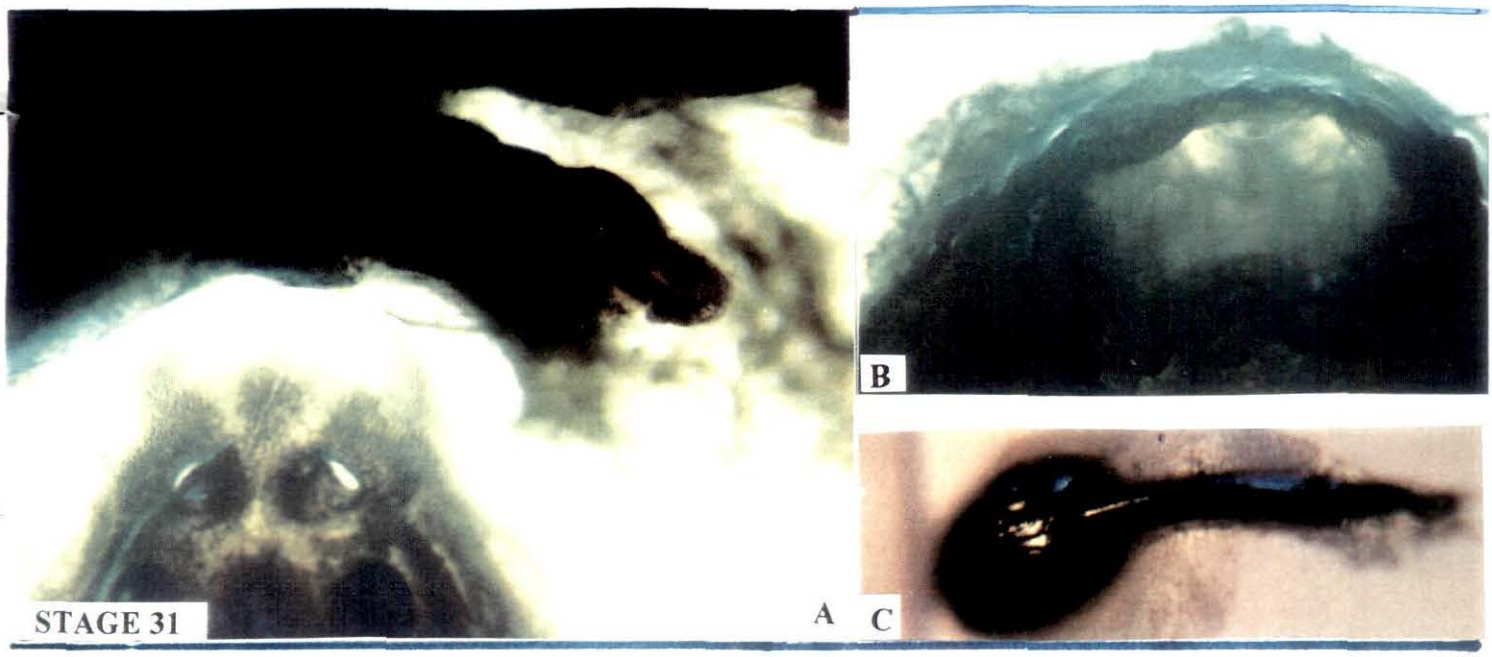
Normal life table of *Bufo himalayanas*

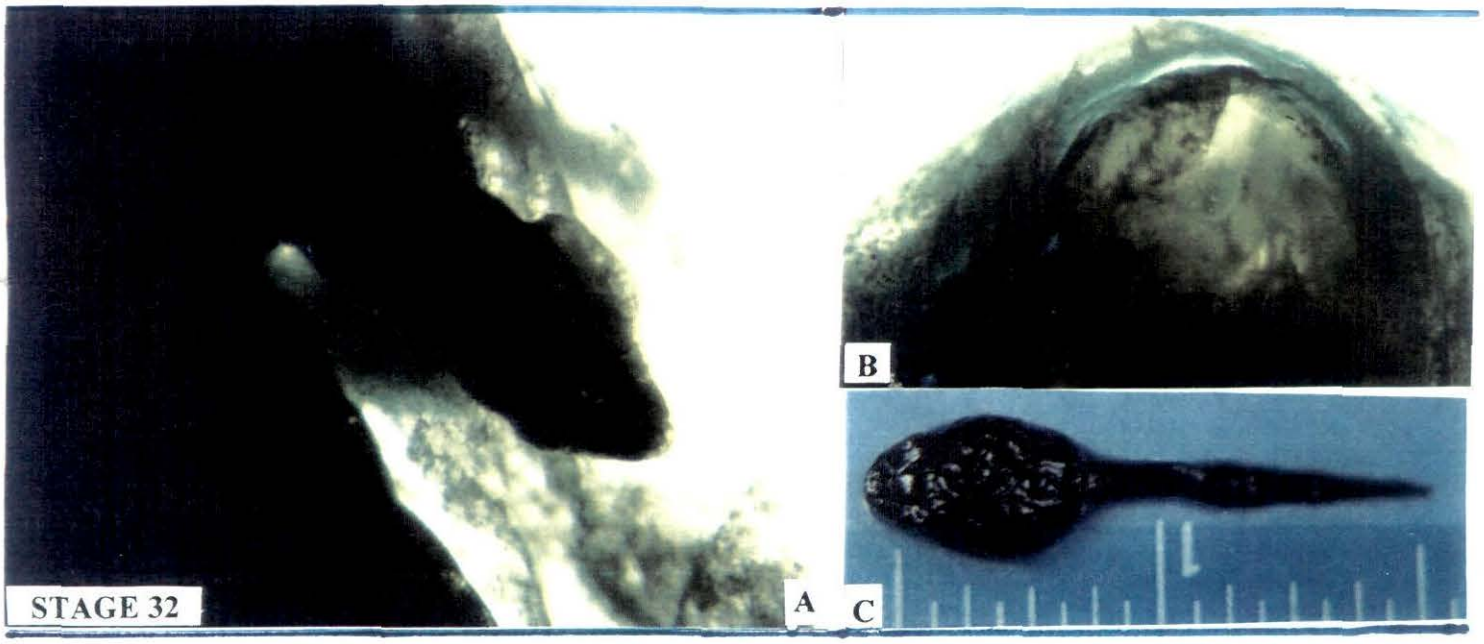
STAGE 31: A. Developing hind limb highly enlarged; dorsal view of the head (inset) B. Mouth disc enlarged C. Entire larva

STAGE 32: A. Developing hind limb highly enlarged
B. Mouth disc enlarged C. Entire larva

STAGE 33: A. Developing hind limb highly enlarged
B. Mouth disc enlarged C. Entire larva

PLATE-7





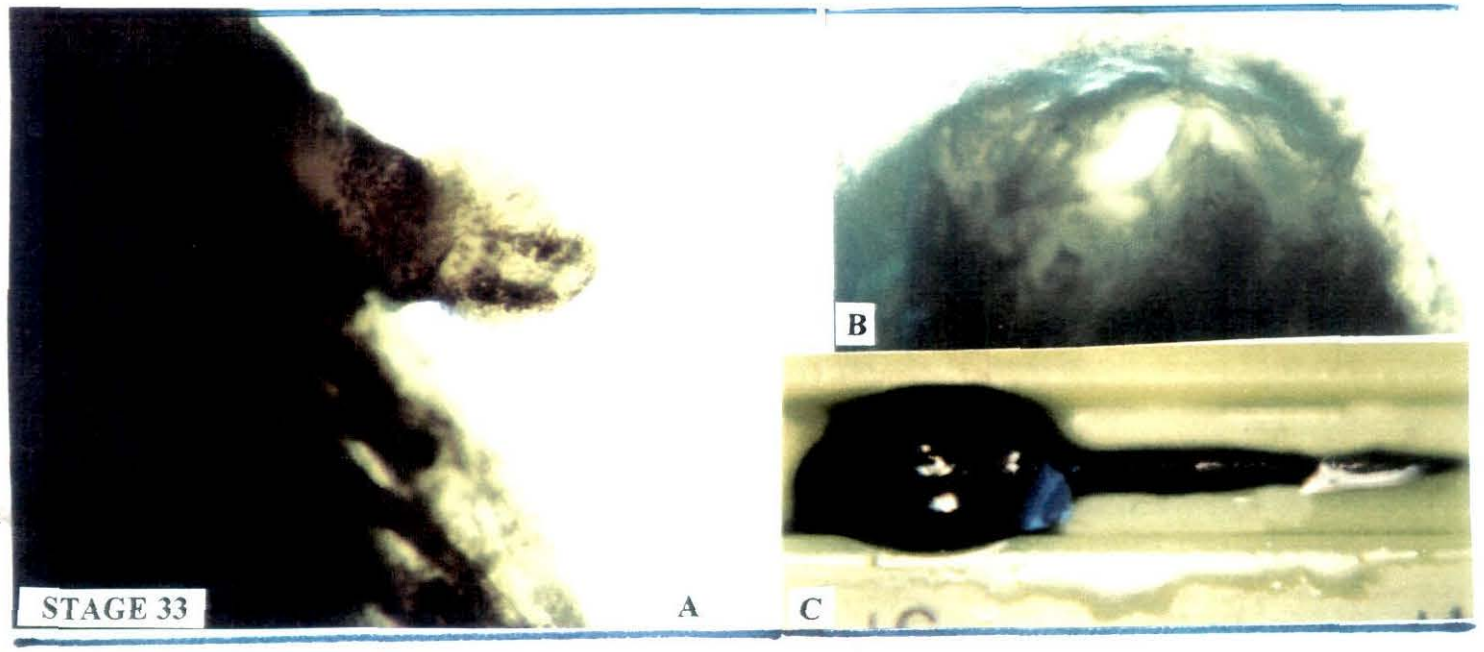


PLATE 8

Legends

Normal life table of *Bufo himalayanas*

STAGE 34: A. Developing hind limb highly enlarged

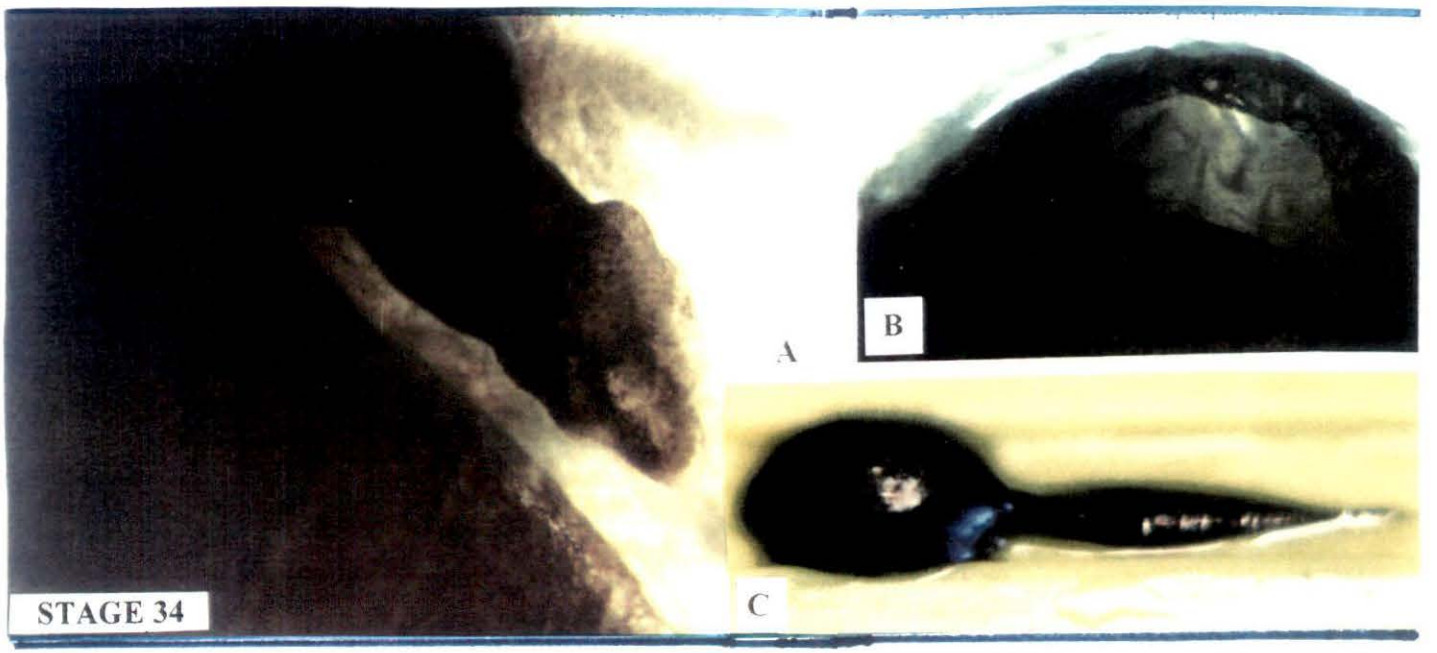
B. Mouth disc enlarged C. Entire larva

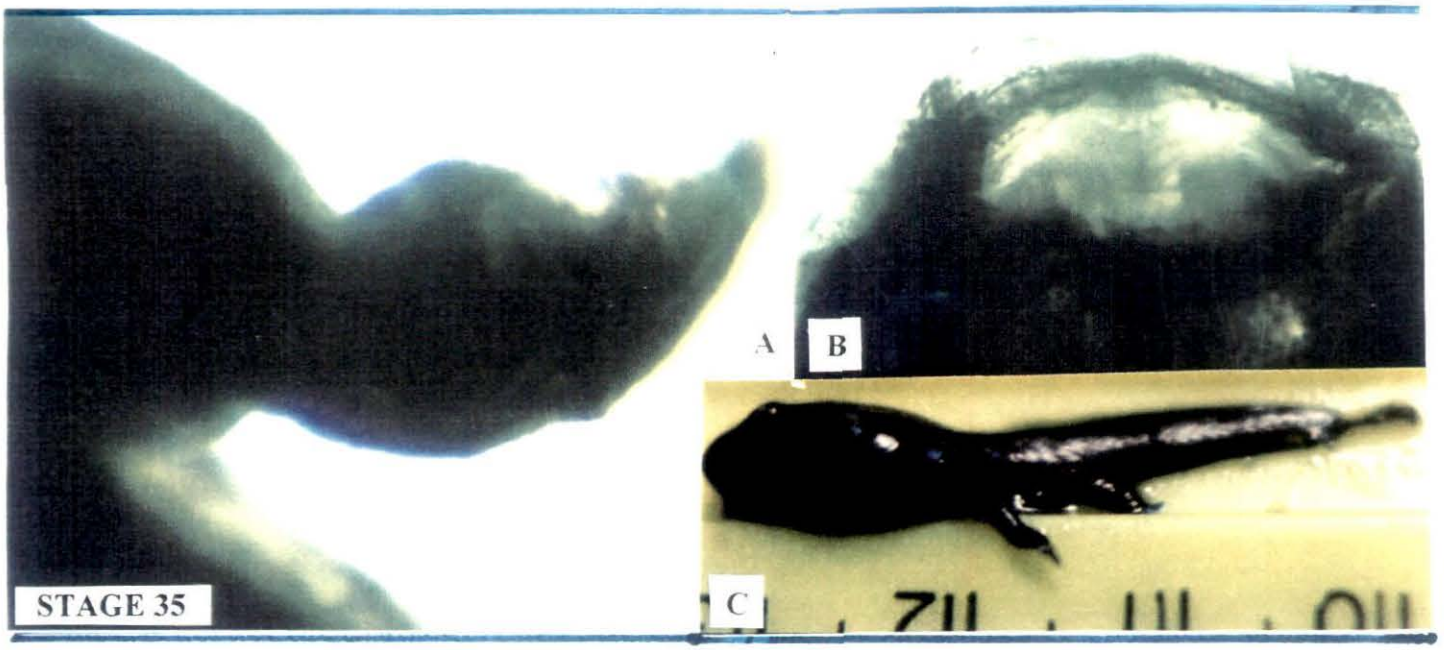
STAGE 35: A. Developing hind limb highly enlarged

B. Mouth disc enlarged C. Entire larva

STAGE 36: A. Developing hind limb highly enlarged

B. Mouth disc enlarged C. Entire larva


A
B
C
STAGE 34


A
B
C
STAGE 35

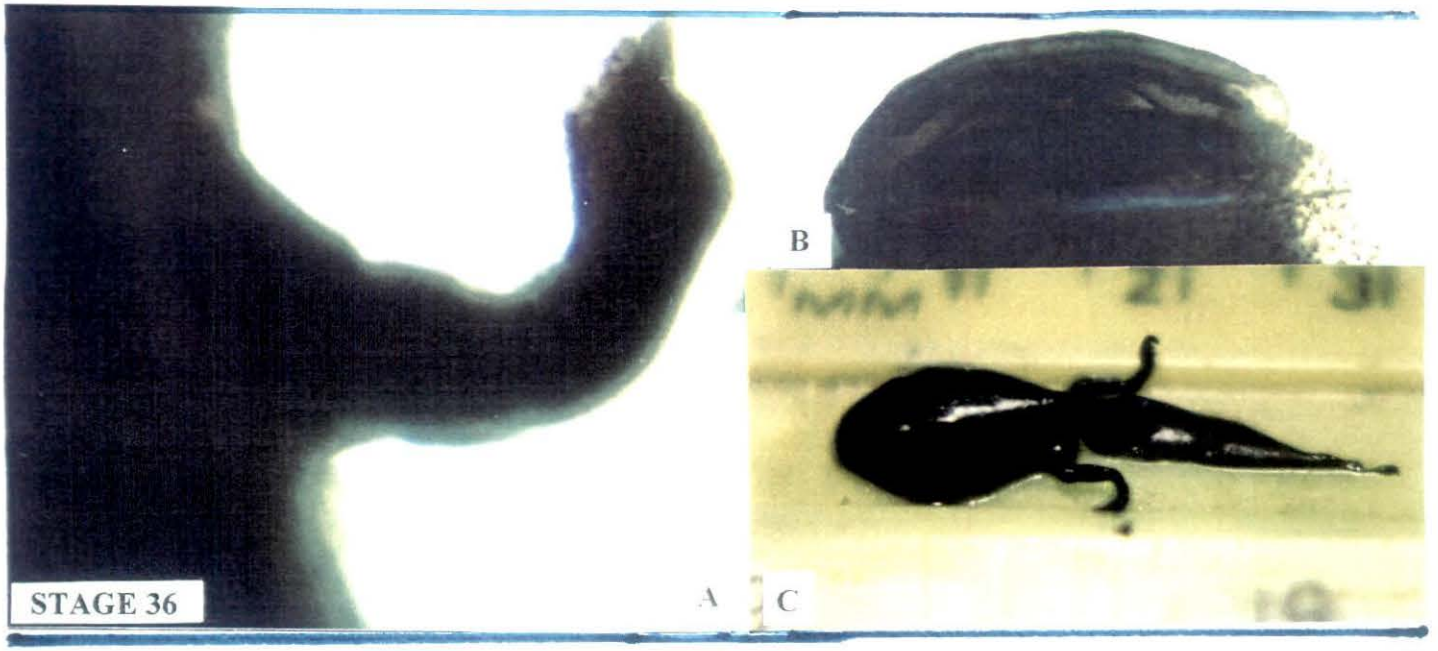

B
A
C
STAGE 36

PLATE 9

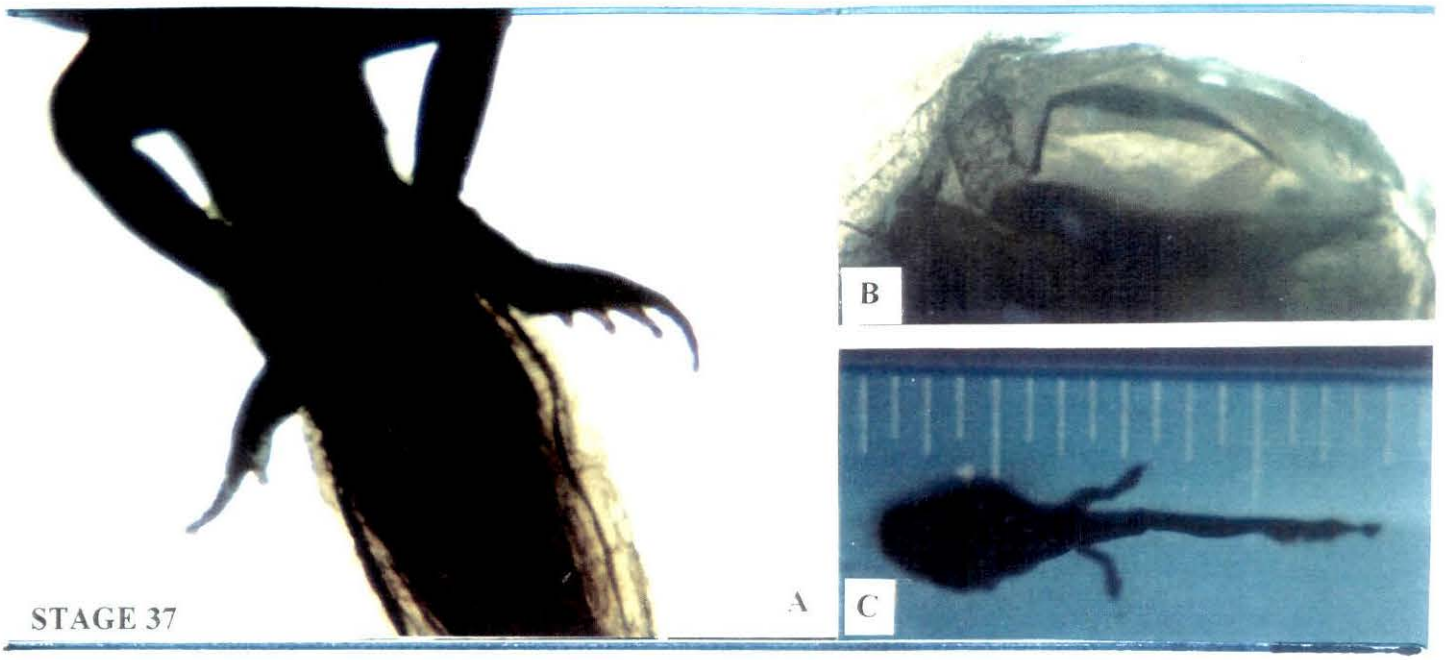
Legends

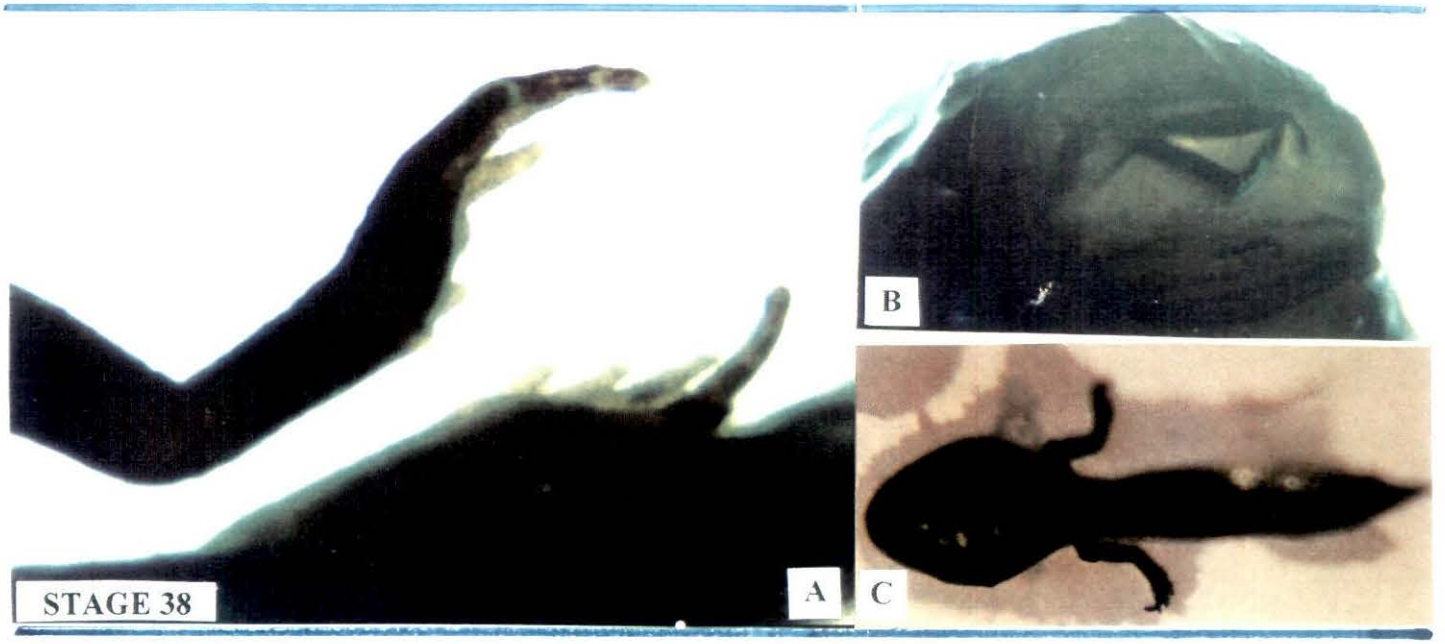
Normal life table of *Bufo himalayanas*

STAGE 37: A. Developing hind limb enlarged to show digits
B. Mouth disc enlarged C. Entire larva

STAGE 38: A. Developing hind limb enlarged to show digits
B. Mouth disc enlarged C. Entire larva

STAGE 39: A. Toe of the hind limb highly enlarged to show complete digitalization B. Mouth disc enlarged C. Entire larva


STAGE 37
A
B
C


STAGE 38
A
B
C

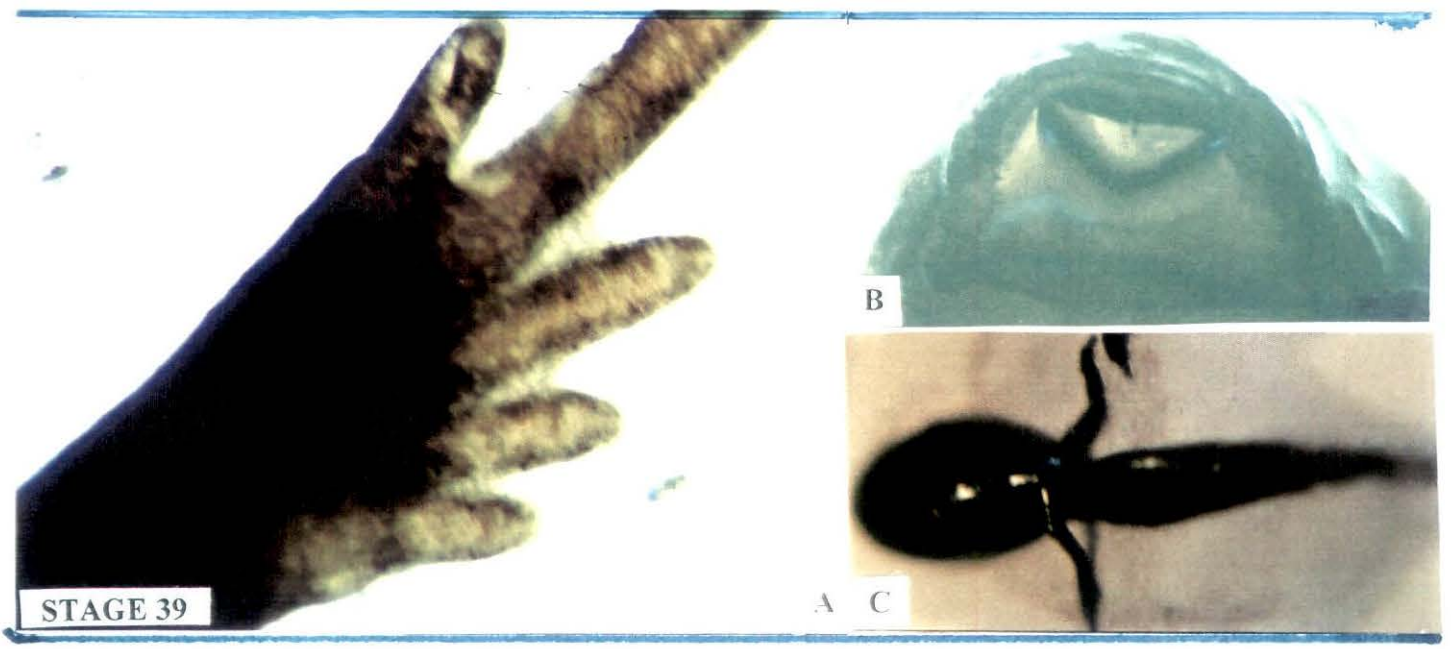

STAGE 39
A
B
C

PLATE10

Legends

Normal life table of *Bufo himalayanas*

STAGE 40: A. Both hind limbs along with tail enlarged

B. Mouth aperture enlarged C. Entire larva

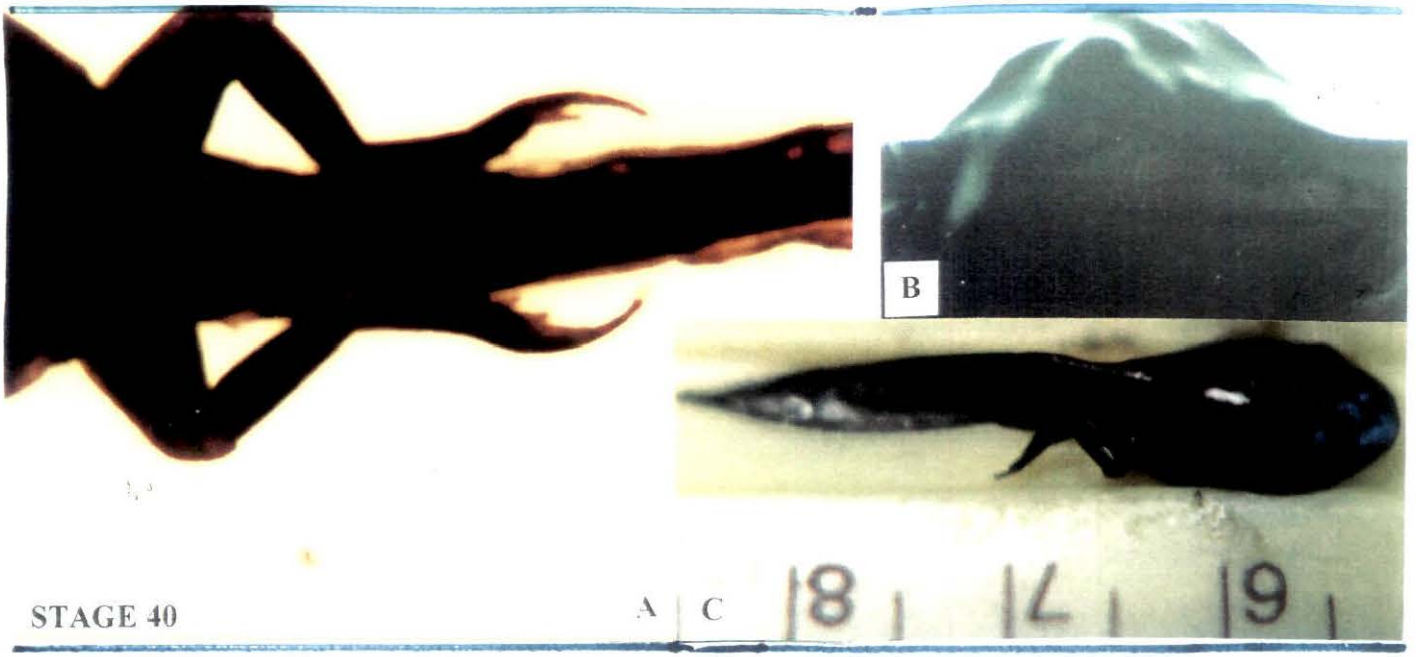
STAGE 41: A. head and body highly enlarged to show the forelimb protrusion on the side of the head

B. Mouth aperture enlarged C. Entire larva

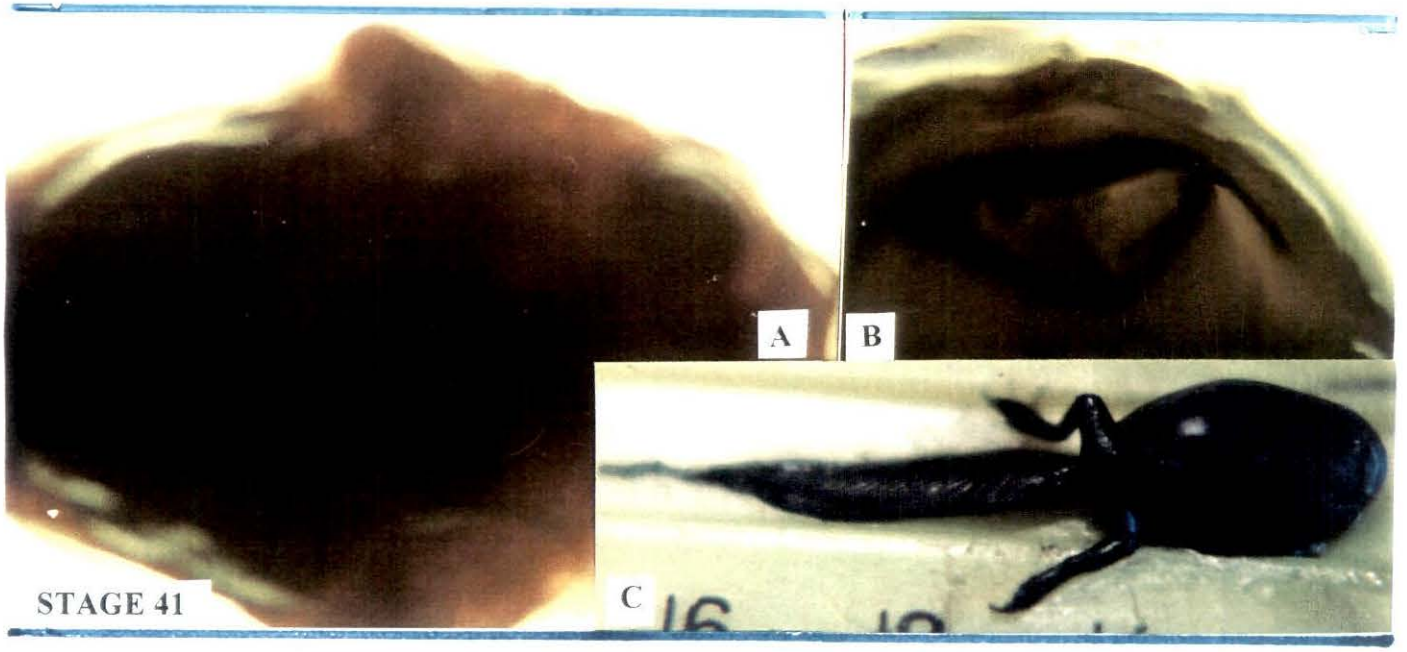
STAGE 42: A. Forelimb protruded out with complete digitalization

B. Mouth aperture enlarged C. Entire larva

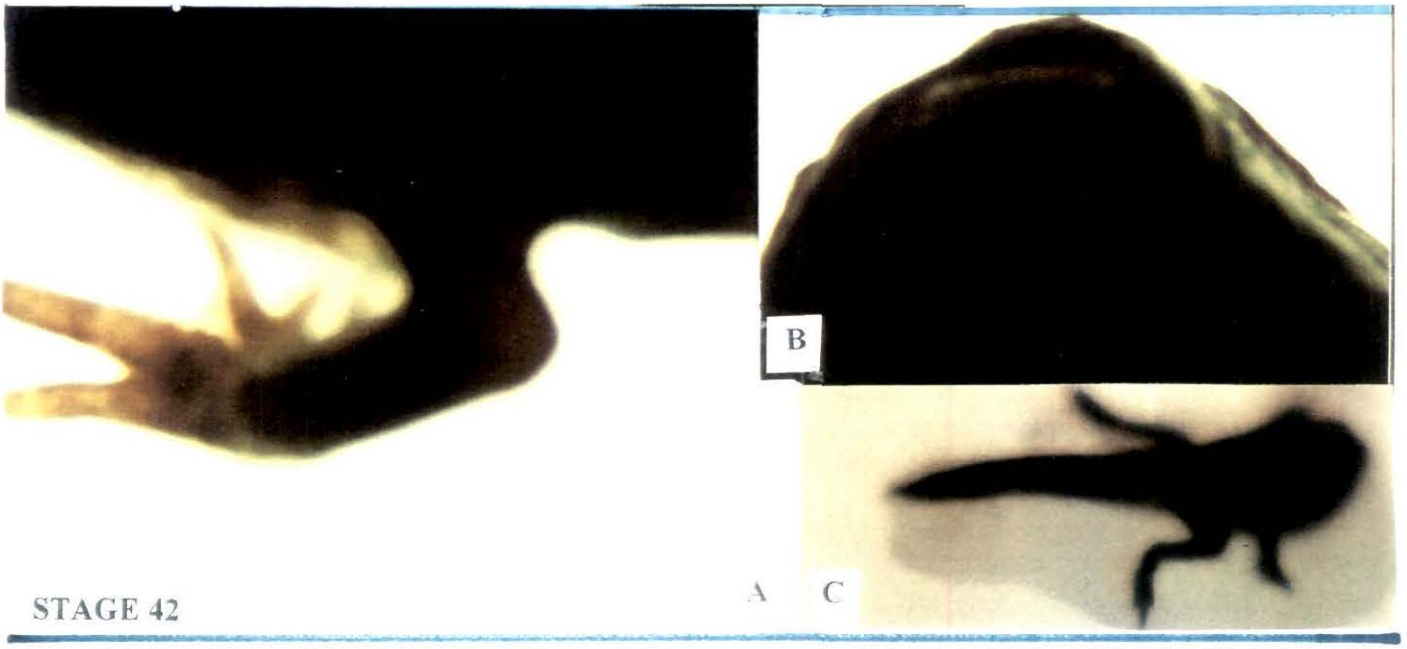
PLATE-10



STAGE 40



STAGE 41



STAGE 42

PLATE11

Legends

Normal life table of *Bufo himalayanas*

STAGE 43: A. Dorsal view with limbs and regressing tail

B. Mouth aperture enlarged C. Entire larva

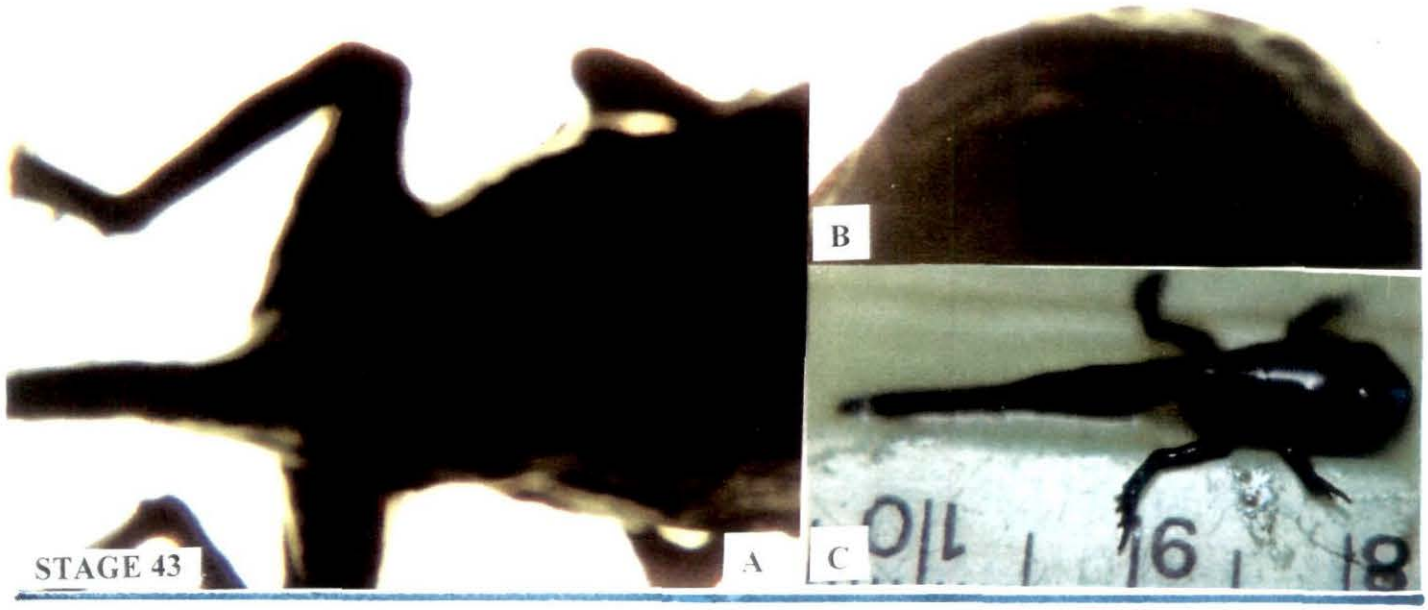
STAGE 44: A. Anterior part, dorsal view, head and body

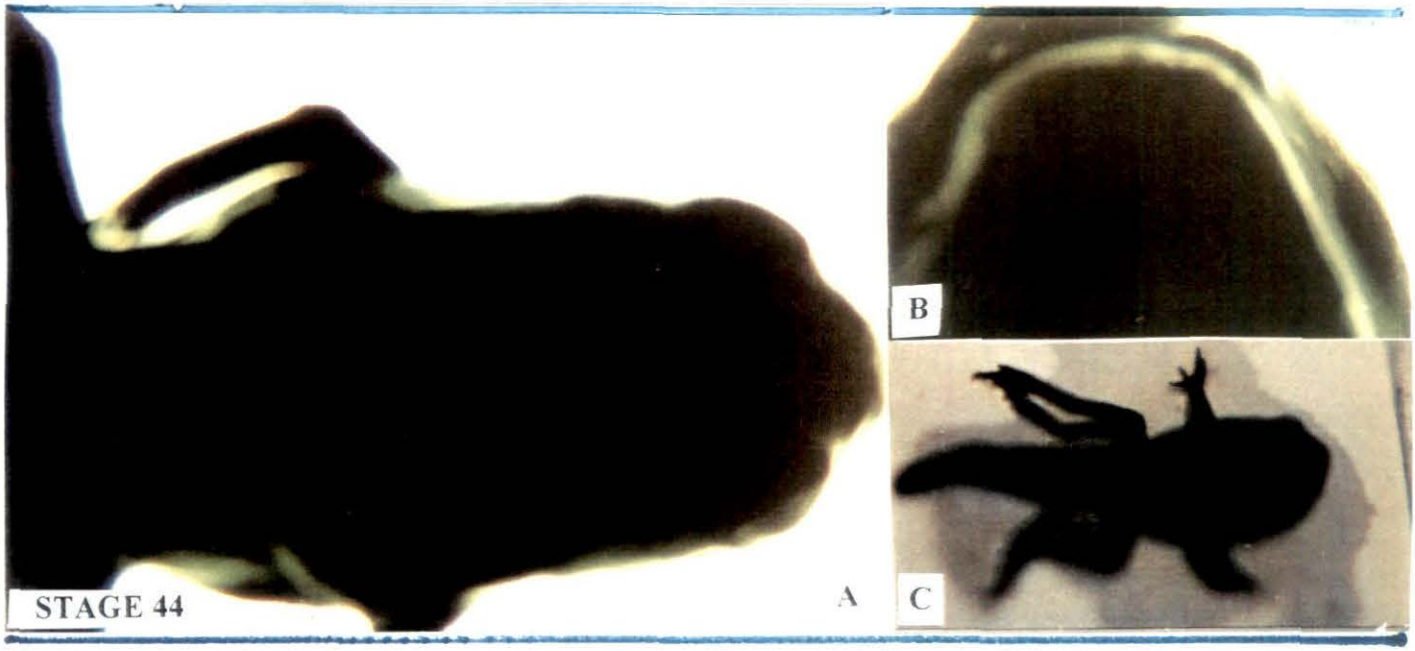
B. Mouth aperture, ventral view C. Entire larva

STAGE 45: A. Hind portion of the body showing tail knob

B. Head enlarged showing mouth and eyes.

C. Entire larva, tail almost totally regressed


STAGE 43
A
B
C


STAGE 44
A
B
C

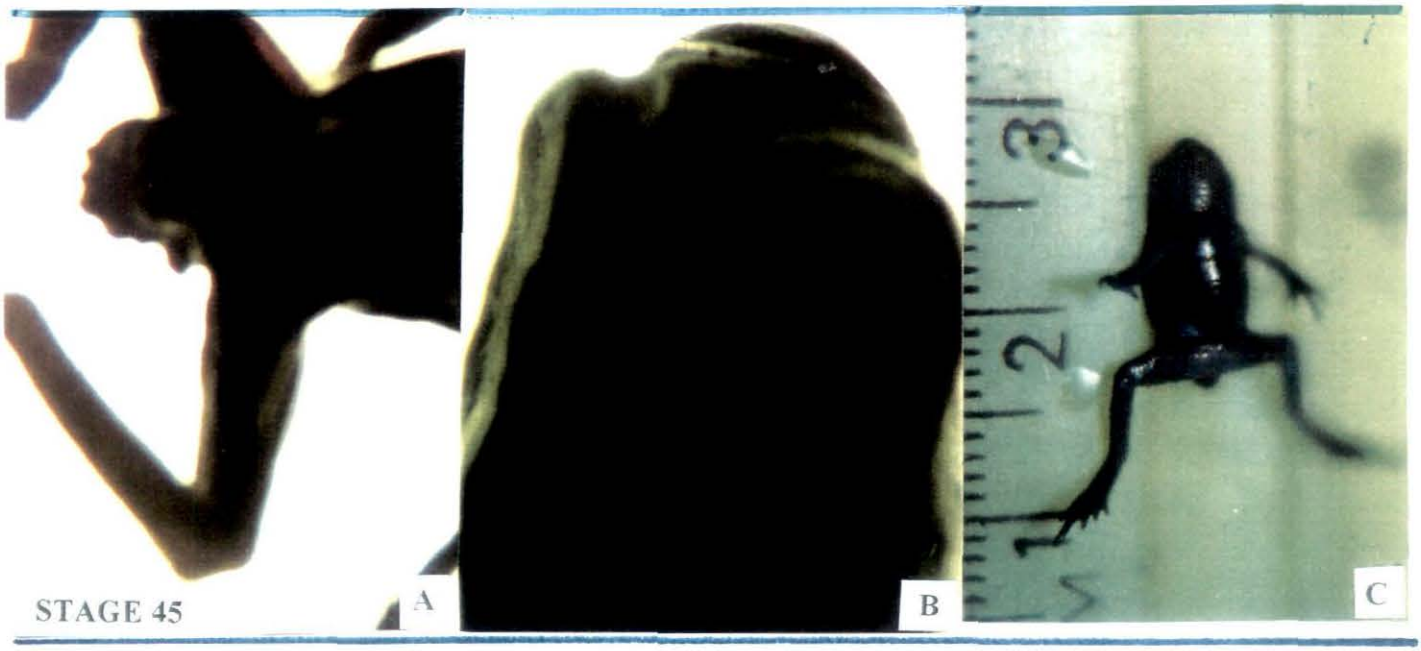
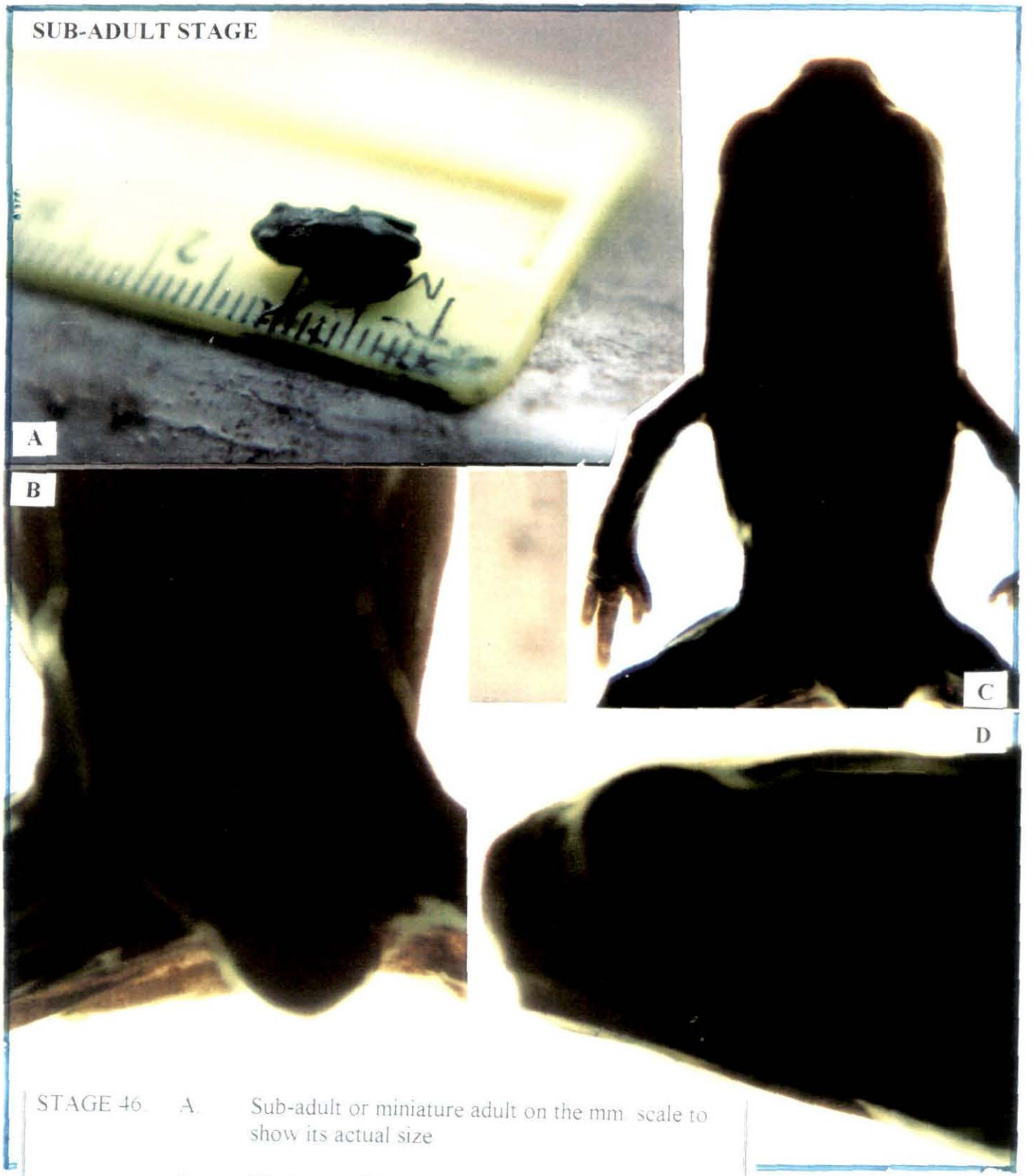

STAGE 45
A
B
C

PLATE-12



STAGE 46

A. Sub-adult or miniature adult on the mm. scale to show its actual size

B. Hind part of the body enlarged to show the fully regressed tail

C. Body with head and portion of the limbs

DISCUSSION

Metamorphosis in anurans involves the most comprehensive and most dramatic transformations of all major living chordate groups. This post-embryonic process systematically transforms most, if not all, organs of a tadpole to their adult forms.It also brings about the development of organs that only functions in the adult stage. The changes that are found during metamorphosis can be resolved into three main categories:

(a) Resorption of tadpole specific organs:
The tadpole specific structures, such as tail, gillS etc. are removed during metamorphosis. The two major tadpole organs,i.e., tail and gill, degenerate completely during this transition. Of the two, tail resorption has been extensively studied; but the gill degeneration has received little attention (Dodd and Dodd,1976; Atkinson,1981; Yoshizato,1989).

The tail represents a simple but complex organ of larval population makeup of a diverse type of tissue,viz., epidermis, connective tissue, blood vessel, notochord and muscle. Despite such diversity in tissue types, all tail tissues are resorbed by the end of metamorphosis.

Two different processes appear to contribute to tail resorption These are condensation and histolysis (Yoshizato,1989). Condensation is an important factor contributing to the length reduction and is believed to be caused by water loss resulting in the compaction of cells and the extracellular matrix (Frieden,1961; Lapiere and Gross, 1963; Yoshizato, 1989,1996).

Histolysis in the tail has been studied in the skin and fin (Kerr et. al.,1974;Kinoshita et. al.,1985; Yoshizato,1989). It has been found that hitolysis starts at the outermost layers and propagates towards the inner layer.

In recent years the resorption of tail has been equated with the programmed cell death or apoptosis. Kerr et. al., (1972,1974) first demonstrated that in the tail resorption apototic like changes like nuclear and cellular fragmentation, formation of membrane bound vessicles (apototic bodies) containing cellular and cellular fragments, including condensed chromatin fragments etc. occur. These observations have later been supported by other workers, viz., Willy et.al., (1980); Kinoshita et. al.,(1985); Nishikawa and Hayashi (1995); Nishikawa et.al., (1998) and others.

In anurans the time and stage of tail resorption varies among species within a narrow range. In *Xenopus laevis*, the most noticeable early change in the tail degeneration occurs around stage 60 (Nieuwkopp and Faber,1956), around stage 60-61 in *Rana catesbiana* (Atkinson,1981).

Similarly the gill degeneration follows a similar fashion in anurans. Gill resorption begins around 61-62 stage in *Rana catesbiana* and by stage 64-66, complete resorption of gill takes place (Atkinson and Just,1975; Atkinson,1981). The total resorption of the gills occour relatively late, around the same stage or slightly earlier than tail resorption (Atkinson,1981) (Table-6).

(b) De novo development of organs

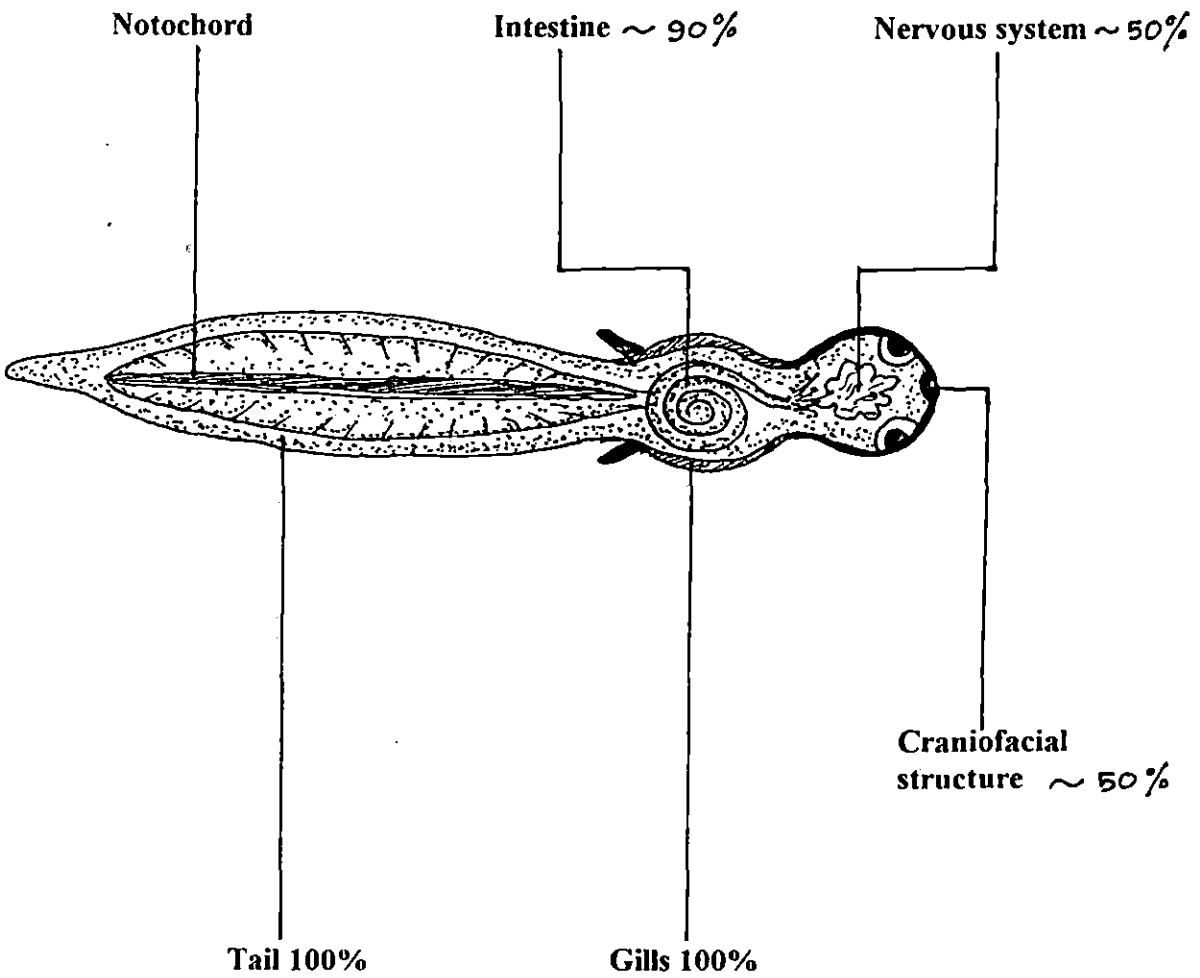
The development of limbs in an adult specific structure that develop during metamorphosis. Even though the limbs are made of similar tissue/ cell types as the tail, including skin muscle, connective tissue and cartilage, they develop progressively whereas the tail resorbs. Shi (2000) has stated that limb development is a thyroid hormone (TH)-dependent process while the tail resorption takes place when TH is present.

The requirement for TH in hind limb morphogenesis by experiments by manupulating the levels of TH in premetamorphic tadpole resulting faster morphogenesis of hind limb (Kattenbach,1953; Dodd and Dodd,1976) or inhibiting the systhesis of endogenous TH (Nieuwkoop and Faber, 1956).

(c)Remodelling of existing organ for adult use:

The majority of the organs are present in both tadpole and adult toad or frog. During metamorphosis, they undergo partial but profound transformations (Dodd and Dodd,1976; Gilbert and Frieden,1981; Fox,1983; Balls and Bownes,1985).
Several excellent reviews have described these processes representing different types of remodelling. For example, in case of liver remodeling the biochemical changes associated with the transition from ammonotelism to ureotelism thought to be due to reprogramming of the gene expression profiles of the existing hepatocytes into an adult like profile (Atkinson et. al., 1994,1996; Chen et.al.,1994,1998). Similarly remodeling in the nervous system (Dodd and Dodd,1976; Fox,1983; Gona et.al.,1988;Tata,1993) and that of the intestine(Glass,1968;

DIAGRAM : 5



Schematic representation of the major tissues of an anuran tadpole undergoing degeneration during metamorphosis. The numbers indicate the extent of programmed cell death expressed as percent of larval cells lost upon completion of metamorphosis

Segal and Petras,1992; Shi and Ishizuya-Oka,1996) and other organs have been encountered (Diagram 5).

Resorption of tadpole specific structures and remodeling of the existing organs for adult life form in recent years have been reviewed under a very specialised genetically controlled process called 'Programmed cell death'(PCD) or 'Apoptosis'.

Kerr et.al.,(1972) first through a series of microscopical observations found that cell death under physiological and pathological conditions occurs with distinct morphological changes, including membrane blebbing, chromatin condensation and cytoplasmic and nuclear fragmentation to form membrane enclosed vesicles containing chromatin fragments called 'apoptotic' bodies. Programmed cell death or apoptosis has now been recognized and unrevealed in tail resorption (Kerr et.al.,1974; Wyllie et. al.,1980; Nishikawa and Hayashi,1995; Nishikawa,1998), intestinal remodeling (Smith-Gill and Carver,1981; Dauca and Hourdry,1985; Yashizato,1989; Shi nad Ishizyya-Oka,1996; Shi,1998, Shi,2000).

In this current investigation on larval development of *Bufo himalayanas* (Gunther), a himalayan high altitude toad, reveals that the developmental stages are similar with other anurans as well as urodel species which are found in high altitude as observed by Harrison (1969); Dasgupta (1988) and others.

From the normal table of development of this species it has been found that breeding season starts before the onset of winter within April to August. Developmental changes in larval morphology and their measurements of size tabulated in the format described by Gosner (1966). From this normal table it is

observed that after hatching the Stage-20 emerge and upto Stage –25 only the length is increased, no special morphological changes observed except the formation and development of larval mouth.

At stage 26, hind limb bud appears. Hind limb shows prominent appearance and develops faster at stage 29. It is important to note that from stage 29 to stage 40 no morphological change happens except the limb development or digitalisation. This observation has similarities with other species of *Bufo* (Limbaughand Volpe,1957). The unique phenomenon of metamorphosis or drastic morphological changes occurred during developmental stage 41 to stage 46. This characteristic is also common with other anuran species. A comparison of larval anuran stages (Just et al.,1981) has been presented here in Table –6. Here the developmental sequences have a keen relationship with environmental temperature. This high altitude species thus shows its normal course of development at laboratory conditions where temperature is always kept below 20 °C to 22 °C. Studies in their habitat will establish the normal duration of the larval phase of the species especially, which will ensure non-interference of laboratory factors that may influence the result to some extent.

TABLE- 6

COMPARISON OF LARVAL ANURAN STAGES (Just et.al., 1981)

	STAGES			
SPECIES	LIMB BUD GROWTH	TOE DIFFERENTIATION	RAPID HIND LIMB GROWTH	TAIL RESORPTION
Bufo bufo (Rossi,1959)	23 24 25 I II III IV	V VI VII VIII VIII VIII IX	X X X XI	XI XII XIII XIV XIV XIV XV
Rana pipiens XXIV XXV (Taylor and Kollros,1946)	I II III IV V VI VII	VII IX X XI XII XIII XIV	XV XVI XVII XVIII	XIX XX XXI XXII XXIII
Xenopus laevis (Nieuwkoop and Faber,1956)	46 47/48 49/50 51 52 53	53 54 55 55 56 57 57	57 58 59 60	60 61 62 63 64 65 66
	PREMETAMORPHOSIS	PROMETAMORPHOSIS		CLIMAX

Metamorphic Subdivisions (Dodd and Dodd, 1976)

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CHAPTER- II

GONADAL DIFFERENTIATION PATTERN AND TESTICULAR DEVELOPMENT

CHAPTER- II

GONADAL DIFFERENTIATION PATTERN AND TESTICULAR DEVELOPMENT

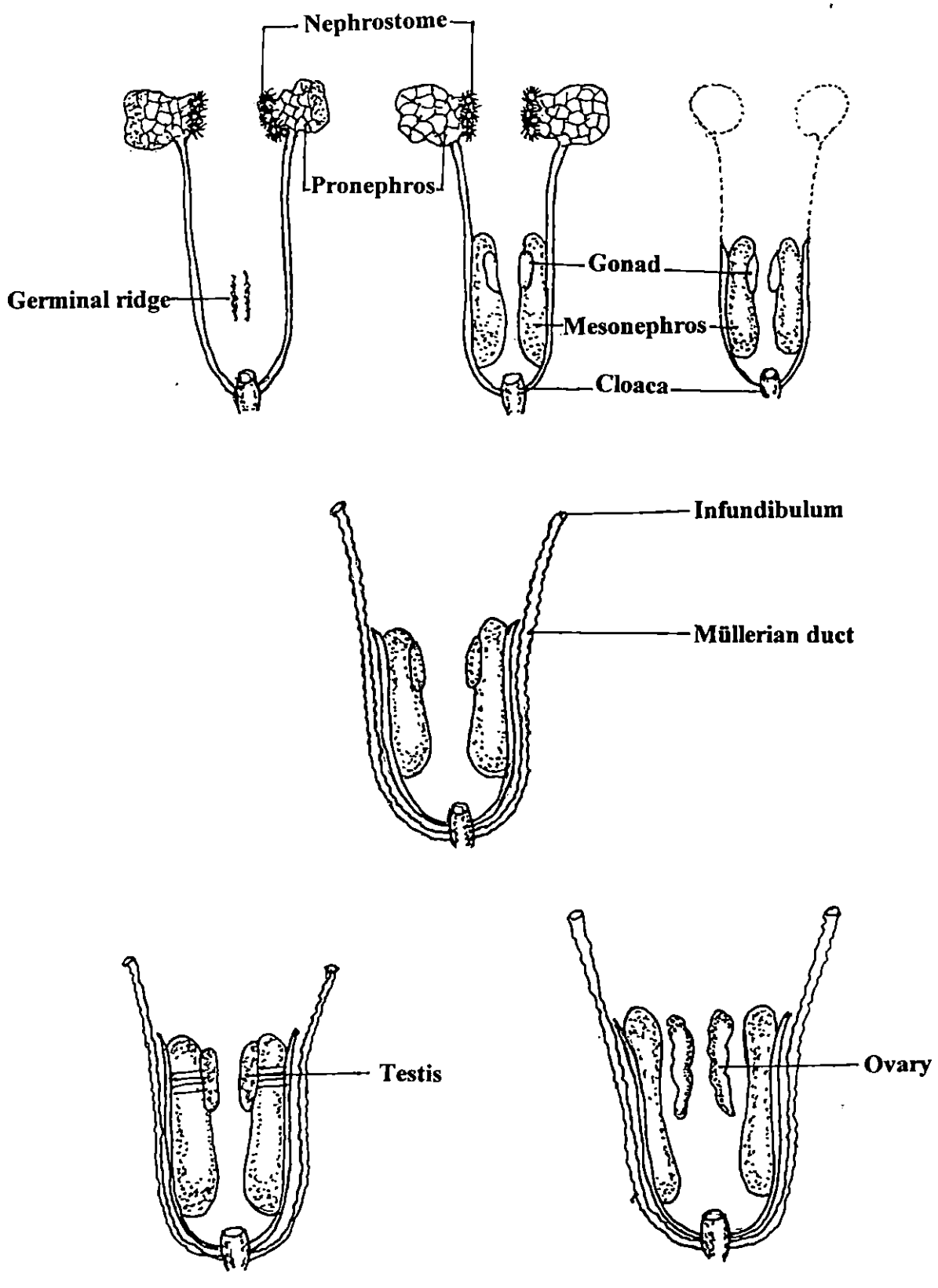
CONTENTS

INTRODUCTION

Amphibians have paired gonads. Male and female gonads develop from sexually undifferentiated 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 ventro-lateral aspect of mesonephric tissue. Finally genital ridges are formed as bulge from the dorsal wall of the coelomic cavity (Diagram 6). The caudal part of the genital ridge eventually gives rise to the gonads and cephalic area from the fat bodies (Franchi et. al., 1962) (Diagram 6).

In amphibia genital ridge develops in a caudal direction in both sexes. Two distinct areas become clearly distinguishable as peripheral cortex and inner medullary zones. At first, the cortical and medullary zones are similar in appearance in both sexes. The latter differentiation follows different patterns. The proliferation of the cortex with a concomitant regression of the medulla marks the transition into a presumptive ovary in a female embryo, while the reverse occurs in the male. This is the time when the genital ridges are developing.

DIAGRAM : 6



Development of urinogenital system in amphibia

Based on Gallien (1958)

primordial germ cells, which originate from extra-gonadal source, migrate into the ridges by passive movements induced by differential growth of the embryonic tissues. In anurans the germ cells are derived from presumptive endoderm (Franchi et.al.,1962) (Diagram-1).

In anurans the testes may differentiate and mature well before metamorphic climax as in *Pleurodema cinerea* , shortly before metamorphic climax as in *Ceratophrys ornata* and *Rana catesbeiana* or long after metamorphosis as in *Bufo arenarum* (Lofts,1974).

In the present investigation an attempt has been made to visualize the structural changes vis-à-vis ultra-structural changes that occur during different stages of metamorphosis.

MECHANISM OF GONADAL DIFFERENTIATION

[1] Early differentiation

Larval amphibians have a relatively primitive type of kidney (pronephros) that functions primarily in the excretion of water and ammonia , whereas in terrestrial adults the kidney functions to conserve water and excrete urea. Concomitant with the morphological metamorphosis of the kidney is the development and differentiation of the gonads (diagram 6).

The larval kidney or the pronephric kidney regresses and disappear by the completion of metamorphosis. Genital ridges are formed by sexually undifferentiated primordia in the peritoneal cavity in close association with the with the nephrostomes. Parts of these ridges give rise to the gonads, while the anterior parts form the fat bodies (Frachi *et al.*,1962). The genital ridges develop in a posterior direction, and two distinct zones appear - a peripheral cortex derived from peritoneal epithelium and an inner medulla.

Concomitant with the development of the genital ridges, primordial germ cells, which originate from extra-gonadal sources, migrate into the ridges by passive movements induced by differential growth of the embryonic tissues. In the anurans the germ cells are derived from presumptive endoderm (Franchi *et al.*,1962)

Differentiation of the gonads follows different patterns in males and females. Proliferation of the cortical cells and at the same time with the regression of the medulla forms hollow ovaries by folding of the germinal ridge ;the internal lining of the ovaries is thus medullary in origin.

In males, the medulla develops into testes, while the cortex regresses. In the undifferentiated gonads, mesenchymal cells separate the medullary tissues from the cortex; these cells eventually give rise to the outer sheath or tunic of the testes. In Bufonidae family, cortical rudiments of the germinal ridge form Bidder's organ in male.

During development, two pairs of ducts are derived from primitive kidney and develop in both sexes to become the presumptive adult genital ducts. These are Müllerian ducts and Wolffian ducts. Each pair of these ducts extend from the primordial gonads to the cloaca. In larvae, both sets are present and during metamorphosis the Müllerian ducts tend to degenerate in males whereas, in female they become the functional oviducts. Usually the Müllerian ducts tend to regress to small rudiments in male anurans (Diagram 6).

[2] Bi-potential nature of the differentiating gonads

Several studies have suggested that some amphibian species display environmental sex determination. A lot of review works has been done in this aspect (Dodd,1960; Gallien,1974; Dournon and Houillon,1984 Dournon *et al.*,1990).

In frogs, *Rana* (Witschi,1929; Piquet,1930; Yoskikura,1959; Hsu *et al.*,1971) and one species of *Bufo* (Piquet,1930) studies showed that the temperature of the rearing water can alter the sex ratio of larvae; But when these species reared at temperature experienced naturally by the species a 50:50 sex ratio was obtained (Witschi,1929; Wright and Wright,1949; Herreid and kinney,1966; Scale,1982;Waldman,1982).

Therefore, at early larval stage the larvae always have a bi-potential nature of the developing gonadal tissue which may develop either into testes or ovary. The gonads originates from an out-pocket of cells on the ventral surface of kidney. There are initially no histologically observable differences between males and females. The undifferentiated gonad is a solid structure with an intact cortex and medulla. In females, later on, the cortex develops and grows into ovarian tissue, whereas the medulla regresses leaving a hole which later develops into ovarian vesicle and is observable upon histological analysis (Plate 13). The cells in the cortex become large follicles and oocytes can be observed during fairly early stage of differentiation.

PLATE13

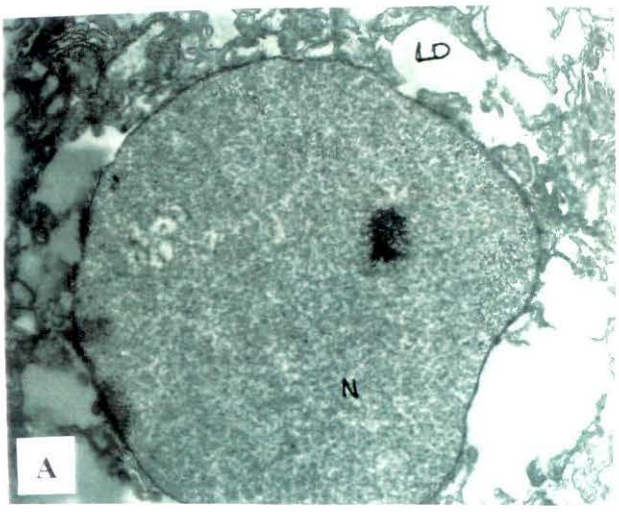
Legends

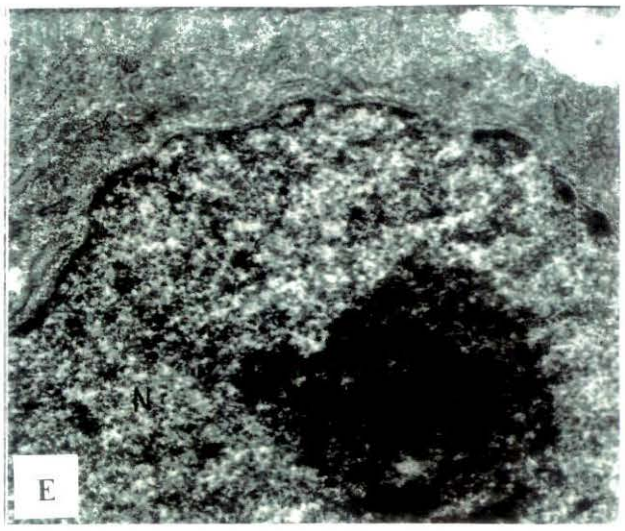
Electron micrograph of 'O' limb stage gonial anlagen

A,B,C and D: Peripheral or cortical cells showing gonial cells along with conspicuous nucleus made of compact electron dense materials. Cytoplasm shows prominent golgi apparatus, lipid droplets and mitochondria which all clearly indicates the nature of the oocytes.

E,F,G and H: Medullary cells and cell organelles. Secondary gonial cells with prominent nucleus and nucleolus. Smooth walled endoplasmic reticulum showing golgi vescicles and lysosomal vescicles. Highly enlarged E.R. shows granules alongside the reticulate vesicles.

N- Nucleus NU- Nucleolus ER- Endoplasmic reticulum
M- Mitochondria LD- Lipid droplets.


LD
N
A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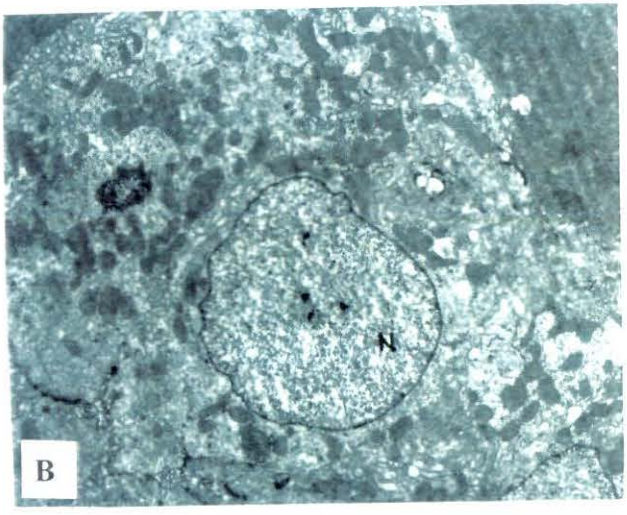
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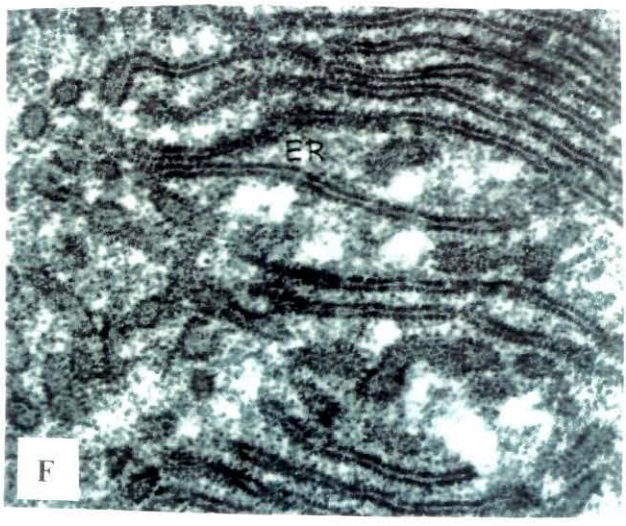
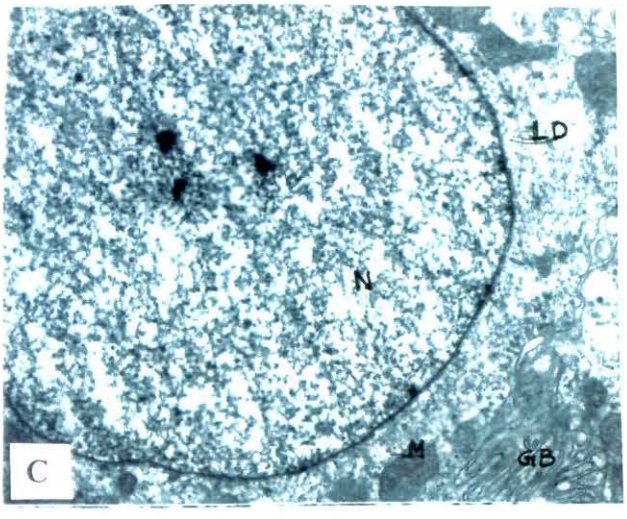

ER
F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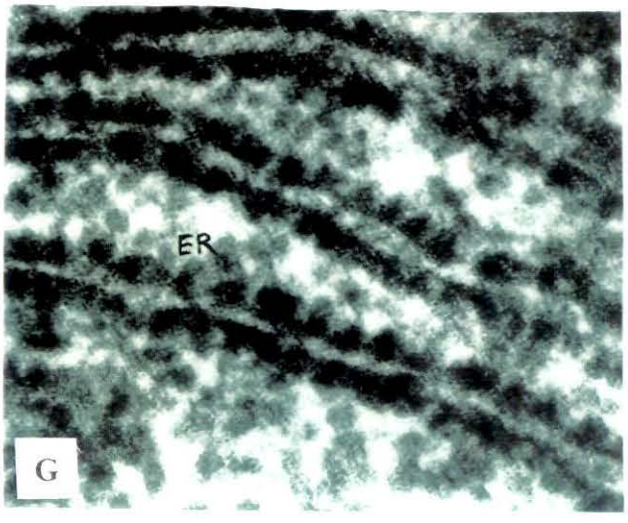
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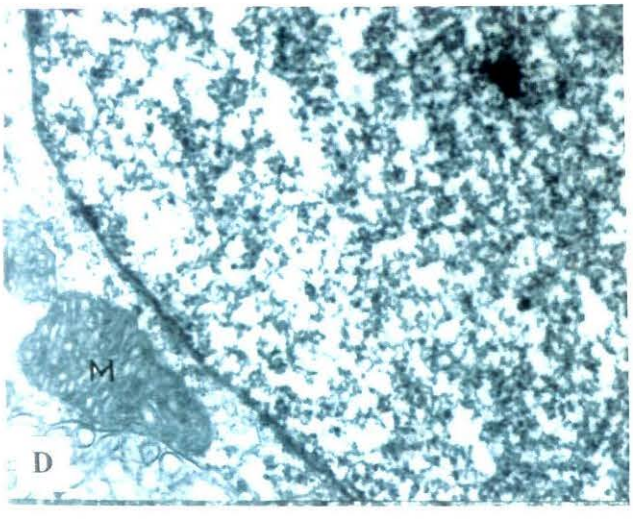
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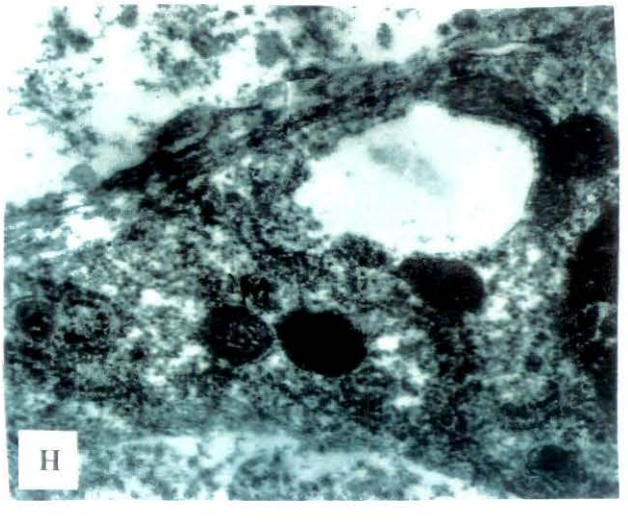
D


M
H

[3] Origin of the somatic gonadal tissues:

There are differences in opinions regarding the origin of the cells that form the gonadal ridge, which later on develops into an undifferentiated gonadal mass in amphibia.

In anurans, the development of the gonadal ridges and the tissue of the cortex and medulla has been observed by different investigators. In *Rana sylvatica , Rana arora and Hyla regilla* (Witschi,1927,1931) it was observed and reported that the cortex, which is the primordium of the ovary is derived from coelomic epithelium and that the medulla , which is the primordium of the testes, originated from the mesonephric blastema, without the contribution from the coelomic epithelium.

Several later studies and reviews have similar finding (Witschi,1967; Mittwoch,1973;Deuchar,1975).But according to Vannini (1941,1942), the medulla was derived from the interrenal blastema and not from the mesonephric blastema in *Rana agilis.*

Studies in Bufo bufo and *Bufo viridis* (Vannini and Busetto,1945) in *Bombina pachypus* ((Vannini, 1947) produced similar findings as in *Rana agilis.* Later studies in *Rana esculenta* (Sabbadin,1951; Vannini and Sabbadin,1954) and *Rana dalmatina* (Sabbadin,1951) suggested that the internal blastema contributed cells to the cortex, which is derived primarily from the cells of the coelomic epitheilium and to the medulla of the developing gonads. In this

early stages of the gonadal development, the cortex and the medulla are reportedly not distinct (bi-potential state) and migration from interrenal blastema to the cortex do not cease after the separation of the two layers (Vannini and Sabbadin,1954).

Recent investigations on the origin of the gonadal ridge of *Xenopus laevis* and *Rana pipiens* suggested that the mesonephric blastema may not contribute to the gonadal medulla instead they originate from the coelomic epithelium of the gonadal medulla , giving the medulla and cortex a common origin (Merchant-Larios and Villalpando,1978;1981). Studies on *Bombina orientalis* suggested that there was no medulla initially , and the germ cell-filled cortex was invaded by the medullary tissues which again originated from cells of the mesonephric blastema (Lopez,1989).

In *Rachophorus arboreas* investigation suggested that the entire gonad was formed by the cells segregating within the primordial gonad (Tanimura and Iwasawa,1987,1989). Further studies also suggested that cells migrate from the cortex into the medulla during testicular development (Vannini And Sabbadin,1954; Amanuma,1963).

[4] Role of hormones in gonadal differentiation:

Obviously there were some hormones which play a vital role in the process of gonadal differentiation in all vertebrates as well as invertebrates.

In amphibians gonadal or specially anuran 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 development of 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 the metamorphic changes.

It has been observed that the follicle stimulating hormone (FSH) is mainly responsible for the differentiation of the indeterminate (bi-potential) gonad into a testes, 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 produce complete spermatogenesis (Pisano and Burgos,1962). *Bufo arenarum* is a

species where the gonads differentiate long after the completion of metamorphosis and 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).

External factors such as temperature and internal influences such as endogenously produced androgenic and estrogenic steroids, can influence the genetically induced direction of development in the primordial gonads (Gallien, 1955). Estrogen induces a sex-reversal phenomenon in genetic males in a number of species, where either an ovotestis 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.

[5] Annual testicular cycle :

Testes exhibit distinct seasonal variations in amphibia. The common frog provides an example of a species which displays annual testicular cycle. In this frog enhancement of spermatogenic activity takes place after the spring breeding periods, so that by summer months the seminiferous tubules are packed with a large number of germinal cysts in different stages of development. By the autumn, the propagation of new cysts decline while the existing cysts produce bundles of spermatozoa. During winter individual testis becomes endowed with spermatozoa destined to be extruded during the forthcoming breeding season or spring (Van Oordt , 1956). Similar annual testicular cycle has also been recorded in other species of frog, for example *Rana arvalis* and *Rana dalmatina* (Cei, 1944). Annual testicular cycle of some Indian anuran species are shown in Table – 7.

Interistitial cells of the testis also exhibits morphological and cytochemical changes along with annual testicular cycle of amphibians. In *Rana temporaria* seasonal variation in the nuclear size of the interstitial leydig cells is distinctly visible and exhibits a sharp drop in number after spermiation in March-April period indicating a sudden ceasation of androgen secretion during that particular period of the cycle.

Table 7

Annual testicular cycles of some Indian anurans

Species	Spermatogenetic activity	GSI ratio Max. Min	Locality of study/ state	Reference
R.cyanophlyctis	Continuous	1.7	Dharad,Kanataka	Saidapur & Nadkarni,1975a
	Potentially continuous	3.0	Jaipur,Rajasthan	Bohra & Niazi.1984
R.hexadactyla	Continuous	2.3	Pondicherry	Basu,1962
R. tigerina	Potentially continuous	----	Calcutta,W.Bengal	Basu & Mondal,1961
	-do-	9.0	Dharwad,Karnataka	Saidapur & Nadkarni,1975
	-do-	10.5	Karwar, Karnataka	Kurian & Saidapur,1983
B. melanostictus	Continuous	----	Calcutta,W.Bengal	Bandopadhyay,1991
	-do-	1.5	Mysore,Karnataka	
	-do-	1.8	Dharwad	
U.globulosum	Discontinuous	----	Calcutta,W.Bengal	Ray et.al.,1981
Philatus anandali	Discontinuous	---	Darjeeling,W.Bengal	Ray & Sarkar,2001
B.assamensis	Continuous	----	Guwahati;Dhubri,Assam	Roy & Ray,2001
B.marinus	Potentially continuous	----	Ludhiana,Punjab	Saxena & Lal,1981
Rachophorus maculatus	Discontinuous	----	Calcutta,Darjeeling	Midya et.al.,1984

Interstitial cell cycle also coincides with the thumb pad development of this species (Lofts et. al., 1972). Testicular cycle is also correlated with seasonal variation of cholesterol content levels. It possibly may happened that seasonally altering pattern of intratubular lipid and cholesterol accumulation and depletion are indicative of some endocrinological function (Lofts et.al.,1972).

In *Rana* during the winter months preceeding the spawning or resting period sperm bundles in supporting cells line the open cysts which at this stage constitute the wall of the seminiferous tubules. At the periphery, primary spermatogonia surrounded by cyst cells are occasionally seen in preparation for the next reproductive period which begin in coming summer (Witschi,1924). Ultrastructural studies with electron microscope revealed that towards the end of the resting period the supporting cells are connected by multiple desmosomes. The nuclei are large , lobed and contain a distinct nucleolus as well as glycogen accumulation is also evident. The cytoplasm is somewhat vacuolated and shows large masses of glycogen granules. The mitochondrial cristae are usually tubular (Brokelmann,1964).

In the spawning period the cells appear even more rich in inclusions. Vacuoles accumulate mainly around the head of the spermatids and often show tubular evaginations. After spermiation the supporting cells contain many phagosomes filled with membranes and vacuoles. Glycogen disappears from the cells in the spermiation state. It is released with the spermatozoa (Van Dongen, Ballieux and Geursen,1960).

[6] Factors affecting testicular cycle:

The seasonal development and activity of the male gonads are under the direct control of adenohypophysis and accordingly regulated by the central nervous system via hypothalamic neurosecretions transported to the pituitary gland in the portal vessels. The extensive works on experimental endocrinology and amphibian testicular cycle have been reviewed by several authors including Van Oordt (1960) and Lofts (1974).

There is a correlation between the secretion of the pituitary gonadotropin and seasonal changes in the germinal epithelium and in the secondary annual characteristics in the male anurans and male urodels. Ablation of the entire pituitary or only the pars distalis results in the atrophy of the reproductive organs of these species. Androgenic hormone production rate by the interstitial tissue cells in the testes declines during the reproductive season. Experimental data have suggested a gonadal feed-back regulation of gonadotropin production by the pars distalis (Van Oordt,1961; Rastogi and Chieffi,1970; Vijaykumar *et al.*,1971).

[5] Adult amphibian testicular structure :

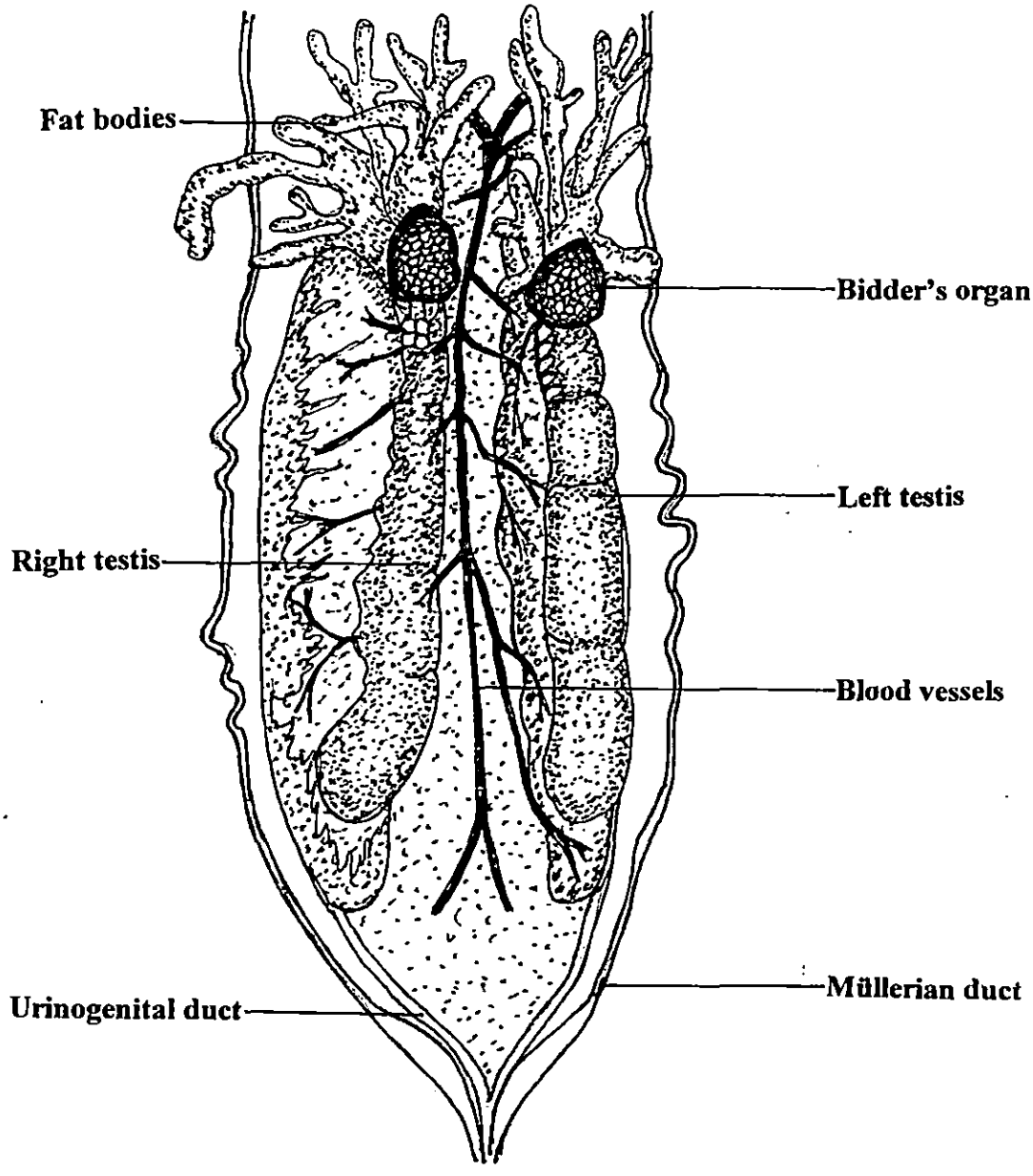
The primary sex organs in male amphibians are the paired testes. These structures are located permanently in the body cavity. The anuran testes are represented by two ovoid bodies surrounded by an elastic fibrous coat. They are attached to the dorsal body wall by a short membrane, the mesorchium through which vasa efferentia run posteriorly to reach Wolffian ducts and cloaca.

The testes increase in size during the breeding season, and in some anuran species they become pigmented. In anurans the testes usually are spheroid or ovoid or elongated structures that are ventral to the anterior half of the kidneys. But in some species, the testes are much larger and longer and thus extend to about posterior end of the kidneys (Diagram -7).

In larval Bufonids, the anterior end of each developing gonads has a growth of ovarian tissue. This is retained in male adult Bufonids as the Bidder's organ, which usually represented by a peripheral or lateral band or an anterior cap on the testes.

Fat bodies associated with the gonads are characteristic of all amphibians. In anurans the fat bodies are in the form of many finger like projections aggregated at the anterior end of the gonads. These bodies are usually larger and more pointed in males than in females. Fat bodies are a source of nutrients of the gonads (Noble,1931) (Diagram –7).

DIAGRAM : 7



THE URINOGENETAL SYSTEM OF MALE *Bufo himalayanas*

Histologically anuran testes display a specialized cystic arrangement. The primary spermatogonia, largest of the various germ cell generations, lie adjacent to the basement membrane together with their companion follicular cells and are usually eosinophilic in nature. The secondary spermatogonia are smaller than the stem cells and with spherical nuclei. Spermatocytes and spermatids are found towards the lumen and are characterized by vesicular and compact nuclei respectively (Plate 15, Figure E, F).

AIMS AND OBJECTIVES

Gonadal differentiation here concerned with testicular differentiation and the text has discussed about testicular development. In the present study attempts have been made to understand the gonadal differentiation in the early stages (i.e. larval stages) of *Bufo himalayanas* and also the testicular differentiation in male toad (both in larval as well as in adult stages).

In anurans the testes may differentiate and become mature well before the completion of metamorphosis in some species (Lofts,1974). Therefore in my present study verification of the 'Progenesis' event or phenomenon (juvenile spermatogenesis) has been studied.

Testicular morphology of adult himalayan toad and their larval stages have been equated with the process of metamorphosis (different stages of larval forms) as well as changes from sub-adult to sexually mature adult have been investigated under electron microscope to work out the pattern of gonadal differentiation in this species.

In this investigation I also will try to observe the developmental changes in developing testes of *Bufo himalayanas* both by external and internal morphology specially through scanning and transmission electron microscopy.

The present study mainly aimed to focuss on the gonadal histological and structural peculiarities and pattern of differentiation at the ultra-structural level to compare the findings with the existing other anurans as well as other ultrastructural observations made by various authors in the subject of amphibian gonadal differentiations (Swingle,1921; Rastogi *et al.*,1983; Jorgenson,1986 Hayes,1998). The observations that would be made will be a commentary on the 'bi-potential nature' of gonadal anlagen as follow up cascade mechanism(s) of testis or ovarian development

MATERIAL AND METHODOLOGY

For our purpose to observe different stages of larvae of *Bufo himalayanas*, I classified the larvae according to their morphological characteristics which change from time to time during the developmental period. Here I marked four distinct larval stages which can easily be identified by external morphology as follows:-

(i) 'O' Limb stage; where the larvae are without any distinct limbs, only hind limb bud persists.
(ii) '2' limb stage: where the larvae are with only hind limbs.
(iii) '4'limb stage: where the larvae posses both pairs of limbs (hind and fore).
(iv) Sub-adult stage: Stages of larvae just after the culmination of metamorphosis.
(Plate 14, Figure a,b,c,d)

(1) HISTOLOGICAL PREPARATIONS

Immediately after the collection of testes the blood vessels are removed from the sample. Small pieces of gonadal or testicular tissue were the fixed in Bouin's solution (aqueous) for 24 hours. Routine histological procedures described by Baker (1966) were followed. Then tissues were finally embedded in paraffin. Sections were cut by microtome at 5 micron and stained with Periodic acid-Schiff and hematoxylin (Ray,1978) and hematoxylin-eosin.

PLATE14

Legends

(a) 'O' Limb stage larva showing no limb

(b) '2' Limb stage larva showing only hind limb

(c) '4' Limb stage larva showing both hind and fore limb

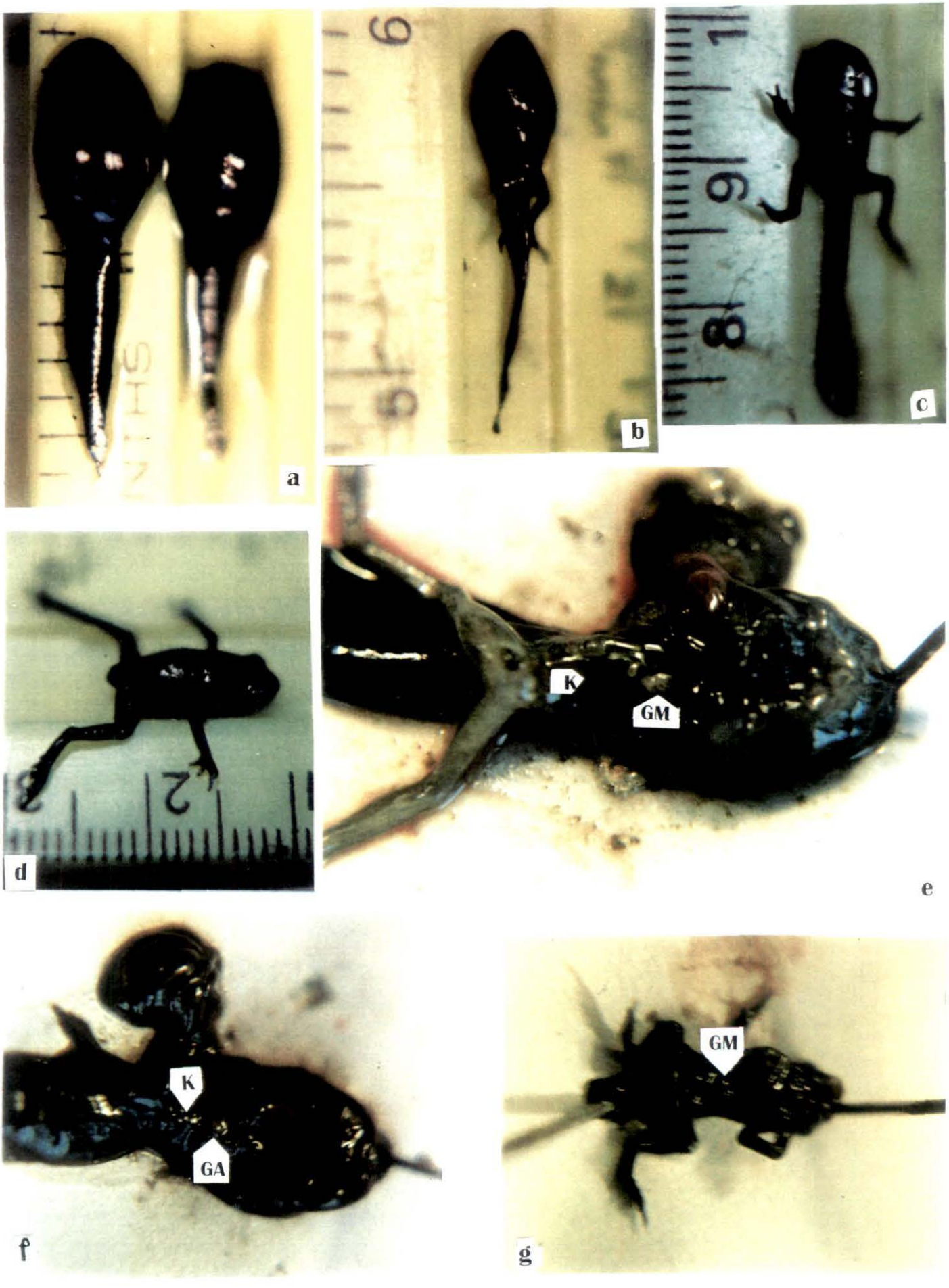
(d) Sub-adult stage showing miniature adult form with fully regressed tail

(e) Dissected body of a '2'limb stage larva showing gonial mass and kidney

(f) 'O' limb stage larva showing position of kidney and gonial anlagen

(g) Sub-adult stage of a larva dissected out to show slightly elongated gonial mass

PLATE-14



(2) LIGHT MICROSCOPIC STUDY

This study was made from histological section which were cut by microtome with acid-Schiff and hematoxylin (Ray,1978) and widely used hematoxylin-eosin technique. Then the sections are observed under light microscope (Leitz) at low and high power and photographed with colour film to identify different histological structures.

Another method of light microscopy was used from semi-thin stained histological sections obtained during the preparation of ultra-thin sections for transmission electron micrography. These semi-thin sections were stained with Toluidin Blue and were observed under light microscope and photographed .

STUDY ON TESTICULAR HISTOLOGY OF LARVAL STAGES AND ADULT SPECIMENS BY ELECTRON MICROSCOPY

a) Scanning electron microscopic study:

After dissection the testis lobes were collected from different larval stages as well as adults. Lobes were collected in normal saline (0.67% Sodium chloride w/v) solution and teased longitudinally. These were then stirred in saline solution. The resulting translucent solution was then centrifused , supernant discarded and the thick precipitate fixed in 2.5% glutaraldehyde with 0.1 M Sodium cacodylate buffer (pH-7.4) for 4 hours. After fixation the materials were transferred to 2% Osmium tetraoxide (OsO_4) solution in same buffer for 90 minutes. The material thereafter was dehydrated through graded alcohol, treated with a mixture of absolute alcohol and amylacetate (1:1) for 20 minutes, and then kept in absolute amylacetate overnight. The preparation was placed on metallic slab and coated with gold and observed through scanning electron microscope (Hitachi S 530) at the Burdwan University Instrumentation Center.

b) Transmission electron microscopic study

The gonadal mass dissected in amphibian saline (pH 7.2) and washed in 0.1 M cacodylic buffer (pH 7.2) (Plate-14, Figure e, f & g). Then they were reduced into fragments (1-3 mm in size) and fixed overnight in 4% paraformaldehyde and 2.5% gluteraldehyde solution solution mixture in 0.1M cacodylate buffer at 4^0 C (Karnovsky,1965). After that, the materials were post-fixed in 1% osmium tetraoxide in the same buffer for 2 hours in dark Then contrusted in block using 5% uranyl in aqueous solution for 1 hour , dehydrated in acetone and embedded in araldite. Sections are post-contransted in saturated solution in uranyl acetate in 50% alcohol and lead acetate(Reynolds,1963). These prepared materials then examined under Philips M10, transmission electron microscope at Regional Sophsticated Instrument Center, AIIMS, New Delhi.

OBSERVATIONS

LIGHT MICROSCOPIC OBSERVATIONS

In 'O' limb stage

The gonadal anlagen exhibit two types of cells:

(a) Eosinophilic cells with oval and conspicuous nucleus along with distinct hyperpycnotic chromatin dot(s) at the center, and

(b) Pale, irregular cells with conspicuous nuclei, which are less basophilic in nature. The cytoplasm exhibits vacuolation and granules.

The eosinophilic cells are found towards the periphery of the developing gonad while the pale type of cells are found in the medullary region (Plate 15, Figure A, B).

In '2' limb stage

The developing gonad exhibits the similar characteristics except the preponderence of a specific type of cells i.e. the gonads which have instructed to be the future testes exhibits higher frequency of medullary cells but a very few cortical cells. The reverse is true in case of anlagen destined to be an ovary (Plate 15, Figure C & D).

PLATE15

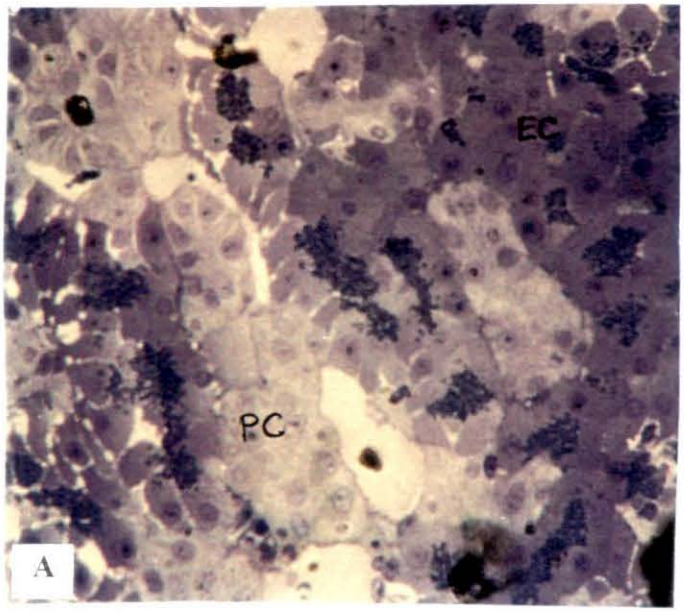
Legends

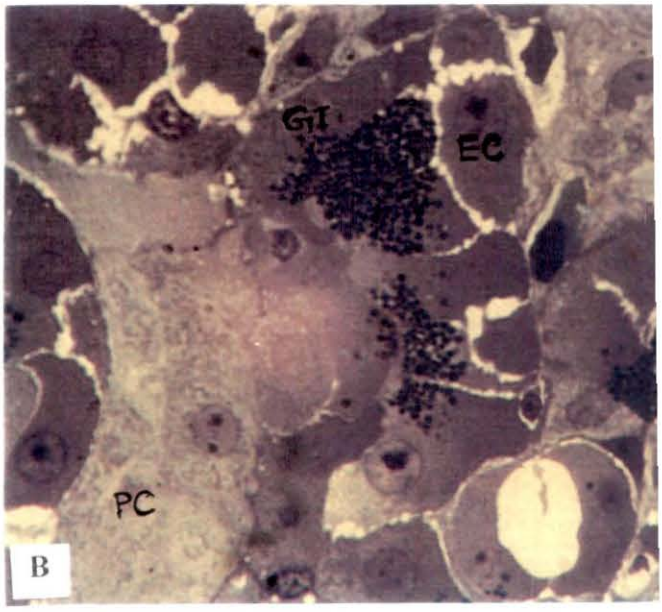
Light micrographs of the sections through the gonial tissue of different developmental stages of larva

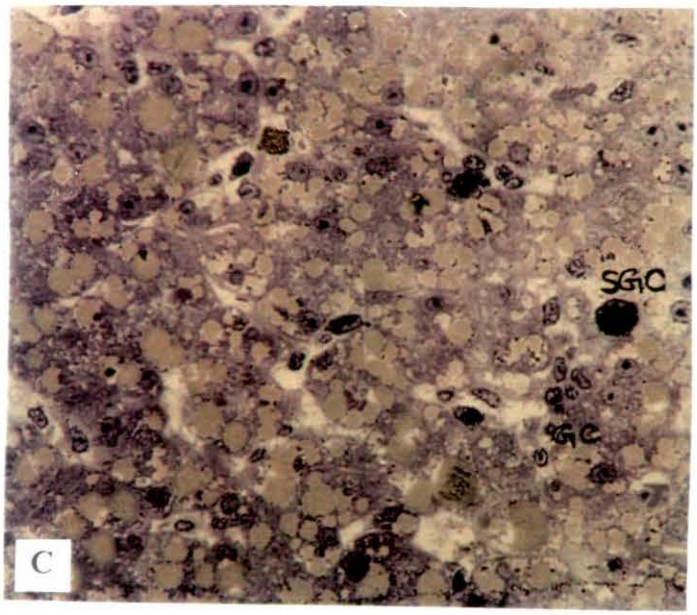
A. Eosinophilic cortical cells with dense granular inclusions and the pale medullary cells with conspicuous nucleus of 'O' limb stage larva
B. Enlarged view of the above field showing the same type of cells of 'O' limb stage larva
C. Lipid droplets, primary and secondary gonial cells in the '2' limb stage larva
D. Lipid droplets with secondary oogonial cells in the future ovary of a '2'limb stage larva
E. Spermatogonial cysts in seminiferous tubule showing different stages of spermatogenesis in a '4' limb stage larva
F. A single tubule enlarged showing cyst wall, spermatogonial cells and spermatids and hollow cyst lumen in a '4' limb stage larva

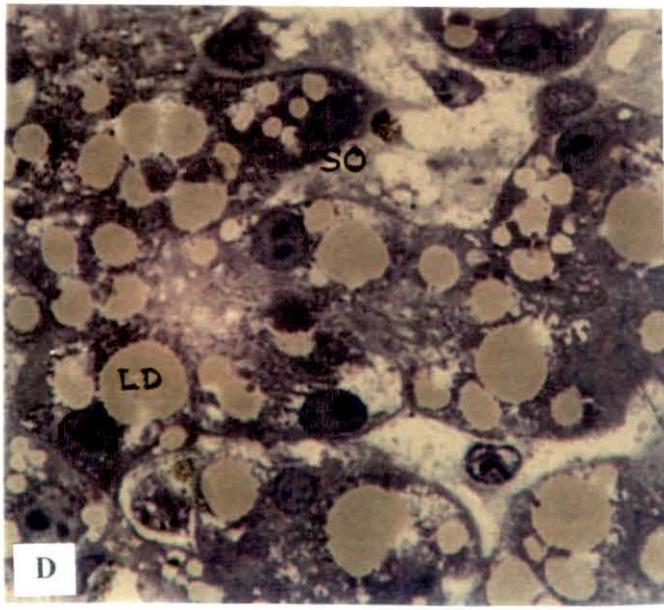
PC- Pale cells EC- Eosiniphilic cells . GC- Granular inclusions SGC- Seconadary gonial cells GC- Gonial cells LD- Lipid droplets SO- Secondary oogonia ST- Spermatids TC- Testicular cysts SG- Secondary spermatogonia CW- Cyst wall CL- Cyst lumen

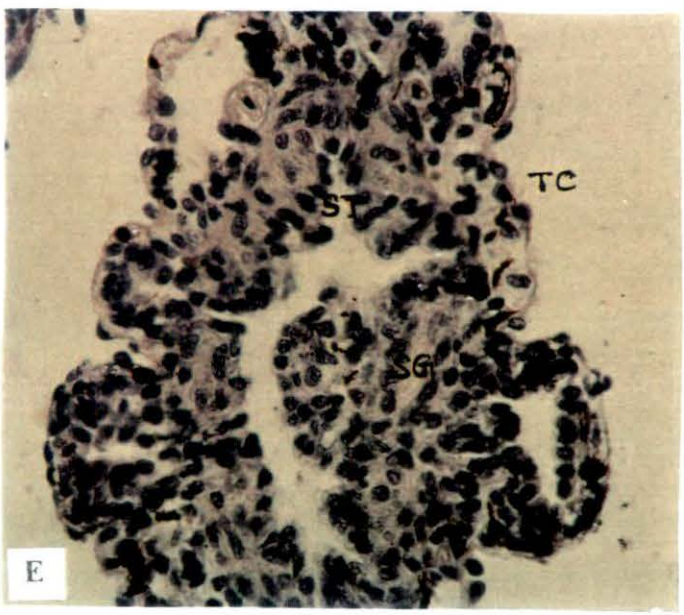
PLATE-15

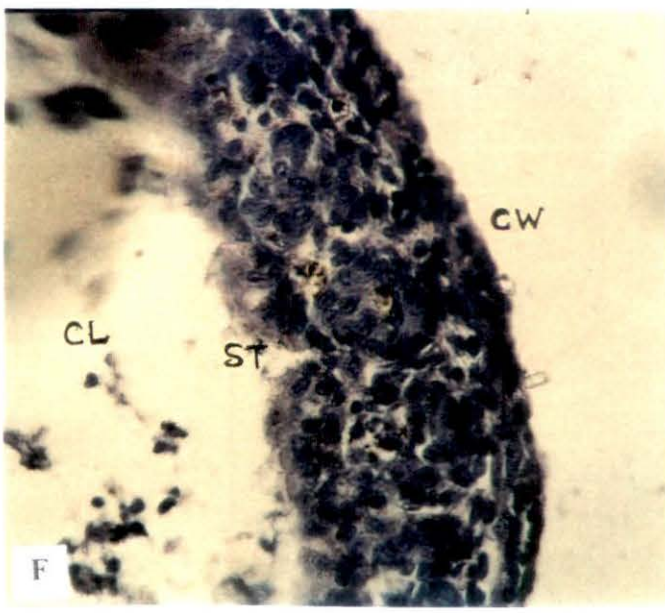












In '4' limb stage

In this stage the regression of cortical or medullary cells continue as per their directed fate. In case of a developing testis the medullary cells become conspicuous, more basophilic and the nuclei of the cells become compact and densely basophilic while in case of a developing ovary the cortical cells proliferate and vaccuolations become distinct in each cell (Plate 15, Figure E & F).

In Sub-Adult stage

The developing testis exhibits the initiation of spermatogenesis showing distinct characterization of gonia cells which are found near the basement membrane of the seminiferous tubule. However no cystic arrangement could be recognized. (SEM and TEM observations of this stages have been described separately). (Plate 16, Figure A & B).

In adult testis

The sections of the entire testis exhibits cystic arrangement, i.e. the cells present in a cyst are in the same stage of development and accordingly various cysts are recognizable (Plate 16, Figure C & D). The primary spermatogonia are the largest germ cells located adjacent to the basement membrane with oval to irregular nuclei and eosinophilic cytoplasm. No morphological variations could be recognized (Plate 16, Figure E & F).

PLATE16

Legends

Light micrographs of the sections through the gonial mass of sub-adult and testis of mature adult *Bufo himalayanas*

A. A testicular cyst (part) showing different stages of spermatogenesis with a wide lumen at its center

B. Enlarged view of a testicular cyst which clearly shows primary and secondary spermatogonia with binucleated dividing stages and primary-secondary spermatocytes

C. Testcular cyst of a mature male showing different stages of spermatogenesis including spermatids and spermatozoa

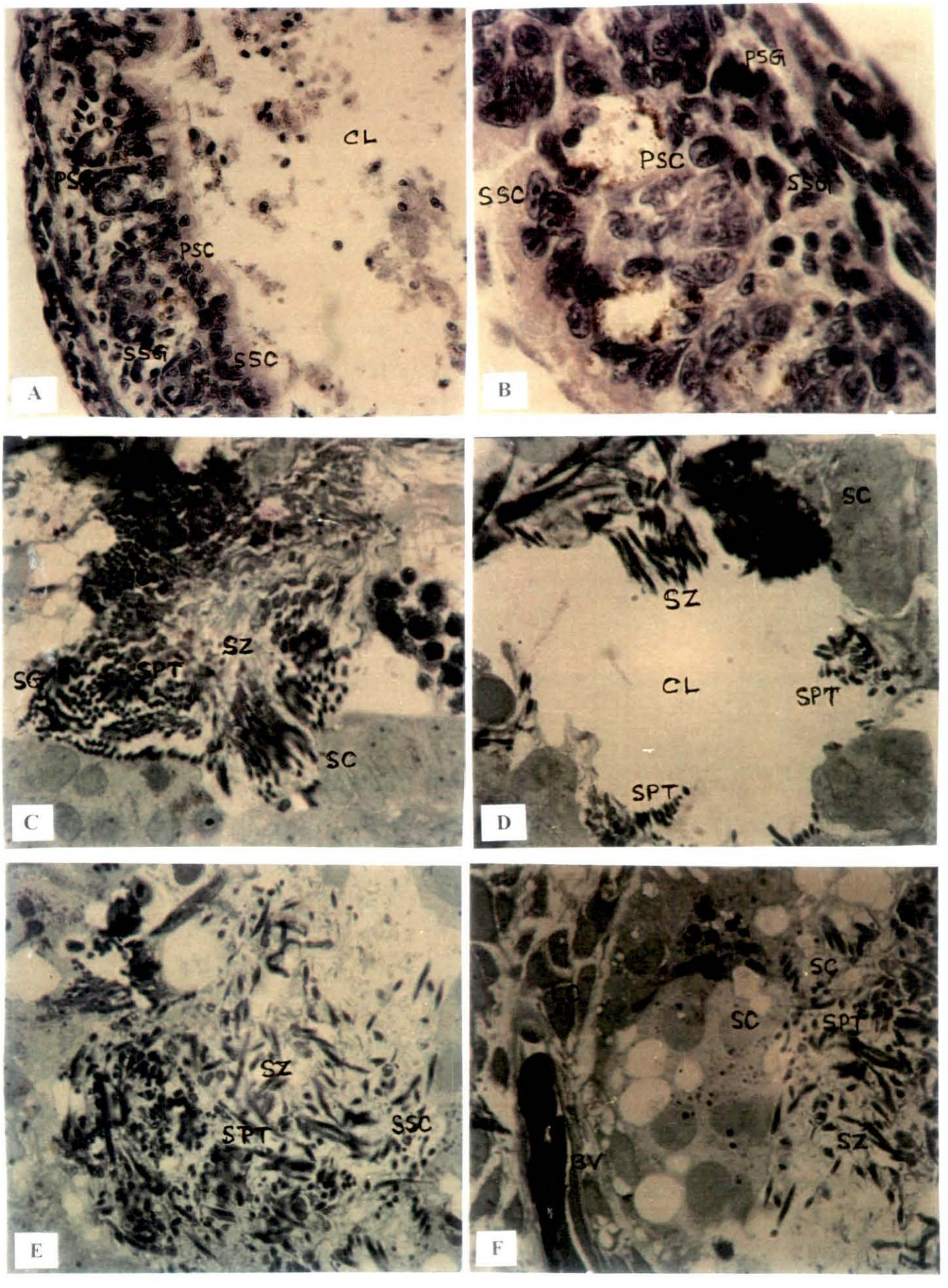
D. A cyst with wide lumen. At periphery there are bundles of spermatozoa attached to the Sertoli cells and free in the lumen

E. Compact cyst of different spermatogenic stages in the periphery and amture spermatozoa at the center

F. Spermatozoa and other stages with Leidig cells and Sertoli cells along with blood vessels

PSG- Primary spermatogonia SSG- Secondary spermatogonia PSC- Primary spermatocytes SSC- Secondary spermatocytes SG- Spermatogonia SPT- Spermatids SZ- Spermatozoa SC- Sertoli cells CL- Cyst lumen LC- Leidig cells BV- Blood vessel

PLATE-16


CL
PSC
PSC
SSG
SSC
A
PSG
PSC
SSC
SSG
B
SG
SPT
SZ
SC
C
SC
SZ
CL
SPT
SPT
D
SZ
SPT
SSC
E
SC
SC
SPT
SZ
BV
F

The secondary spermatogonia are smaller than primary spermatogonia and both cytoplasm and nuclei are basophilic. Primary spermatocytes are found irregularly disposed and with various features of meiotic activities. The secondary spermatocytes are smaller than primary spermatocytes with nucleus showing condensed chromaatin material and are strongly basophilic (Plate 16, Figure C).

The developing spermatids are of various shapes and sizes and have been recognised as oval, spherical, elliptical or elongated depending on the various stages of development (Plate 16, Figure E).

The maturing sperm cells are found as bundles and the heads of the maturing sperm cells are found embedded in Sertoli cells. Such nest cells are found attached to to wall of the seminiferous tubules (Plate 16, Figure C & D).

The various cell nests are usual characteristics of adult testis obtained from the specimens of the breeding seasons. However, at non-breeding seasons, sperm nests are found more frequently than other nest forms suggesting a ceasation of spermatogeneic activity.

The various cell nests present in the testis round the year have been summarized in the Table-8.

TABLE-8

Examples of animals in which degeneration is found regularly during metamorphosis

Phylum & Class	Stages of degeneration	Reference
Platyhelminthes		
Cestoda	Cytes I	Child (1907)
Turbellaria	Tids	Czernosvitov (1931)
Turbellaria	Meiosis	Lentali (1970)
Turbellaria	Gonia (& cytes?)	Schleip (1907)
Trematoda	Gonia	John (1953)
Trematoda	Gonia	Guilfard (1955)
Aschelminthes		
Nematoda	Meiosis	Schleip (1912)
Nematoda	Meiosis	Tretajakoff (1905)
Nematoda	Gonial mitosis	Fauré-Fremiet (1913)
Protozoa	Tids	Grellet (1957)
Mollusca		
Prosobranchia	Tids	Tuzet (1930)
Prosobranchia	Tids/other stages	Ankel (1924)
Prosobranchia	Tids	Aubry (1954)
Prosobranchia	Tids	Qua ttrini (1958)
Prosobranchia	Meiosis	Bulnheim (1962)
Bivalvia	Many stages	Strogonova (1963)
Cephalopoda	Gonia,cytes,tids	Thesing (1904)
Annelida		
Oligochaeta	All stages	Voigt (1885)
Oligochaeta	Tids	Chatton & Tuzet (1941)
Oligochaeta	All stages	Tuzet (1945)
Arthropoda		
Chilopoda	Gonia	Tönniges (1902)

TABLE – 8 (contd.)

Examples of animals in which degeneration is found regularly during metamorphosis

Phylum & Class	Stages of degeneration	Reference
Insecta		
Coleoptera	Early cytes I	Henderson (1907)
Coleoptera	Cytes	Demandt (1912)
Coleoptera	Gonia	Bonhag & Wick (1953)
Lepidoptera	All stages	Ammann (1954)
Diptera	Early cytes, tids	Boinen (1914)
Diptera	Tids	Bairati (1967)
Vertebrata		
Fishes	Gonia	Billard (1969)
Amphibia	Cytes	Flemming (1887)
Amphibia	Many stages	Champy (1913)
Reptilia	Gonial mitosis, meiosis	Dalcq (1921)
Mammalia	Gonia, cytes,tids, Meiosis	Roosen-Rung (1955)
Mammalia	Gonia, cytes	Ortavant (1958)
Mammalia	Gonia	Clermont (1962)
Mammalia	Meiosis	Swierstra & Foote (1963)
Mammalia	Gonia, other stages	Hochereau-de Reviers (1970)
Mammalia	Meiosis	Skakkebach et. al.(1973)
Mammalia	Tids	Holstein (1975)

ELECTRON MICROSCOPIC OBSERVATIONS

A. Scanning electron microscopic observations:

1. On larval gonads:

The larvae of the male *Bufo himayanas* from hatching to emergence of sub-dults can be categorized into 39 stages (Gosner,1960). However, the gonadal mass can only be recognised visually on and from satge 21 as 'O' limb stage.

At this 'O' limb stage, the gonadal undifferentiated mass of tissue or anlagen is visualized as an oblong mass at the antero-median part of the developing kidneys. Under electron microscope the anlagen mass exhibits primordial germ cells of spherical shape. The gonads originates from an outpocket of cells of the ventral surface of the kidney. There are no observable differences between males and the females at this stage and no sexual dimorphism has been observed (Vannini,1941,1942,1947; Deuchar,1975). The undifferentiated anlagen mass is kept within solid oblong gonads with clear cortex and medulla portion (Plate 17, Figure a).

At '2'-limb stage, the gonadal mass assumes a globular shape and is lodged at the anterior end of the kidney. The developing gonad is now also morphologically indistinguishable as testis or ovary.

PLATE17

Legends

Scanning electron micrographs of gonadal mass and gonial anlagen of different stages of development and stages of spermatogenesis

Fig. a. Gonial anlagen of 'O' limb larval stage

Fig. b. Gonadal mass of '2' limb larval stage

Fig. c. Gonadal cell inside the gonadal mass of '2' limb stage

Fig.d. A gonadal mass of '4' limb stage of larva

Fig. e. Enlarged view inside the gonadal mass of '4' limb stage larva showing round gonial cells

Fig. f. Developing testicular mass in a sub-adult

GA- Gonial anlagen GM- Gonadal mass GC- Gonial cell

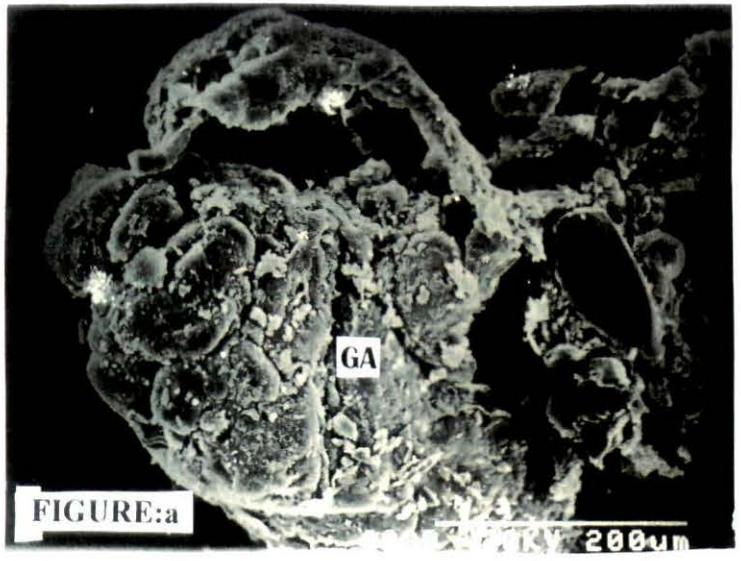


FIGURE:a

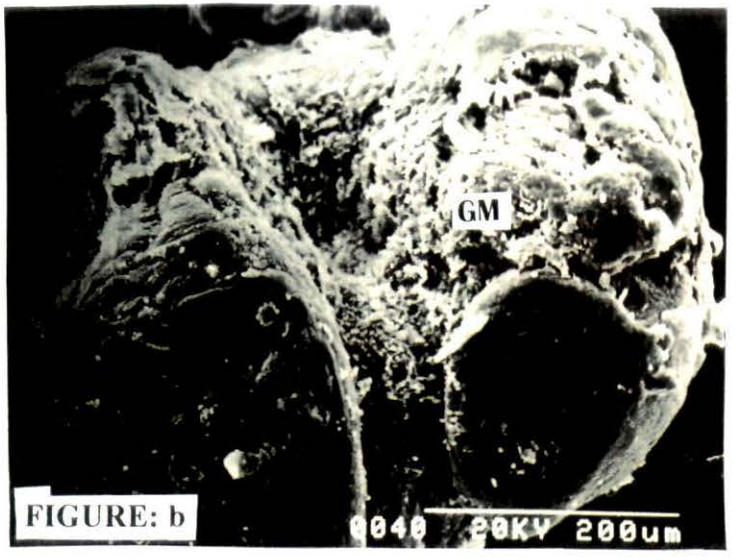


FIGURE: b

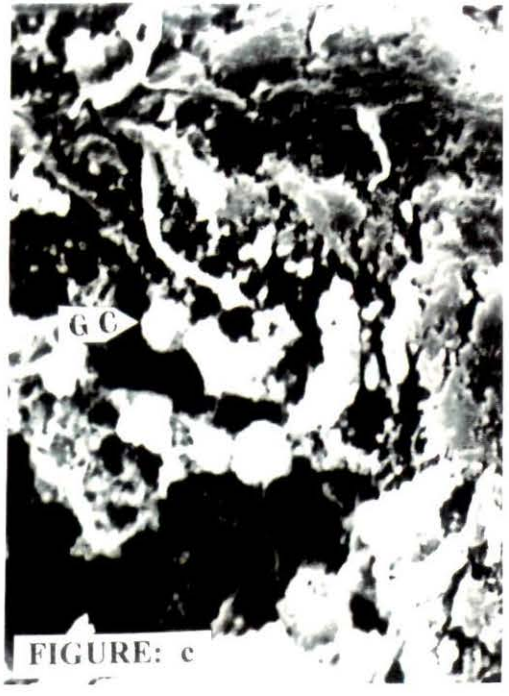


FIGURE: c

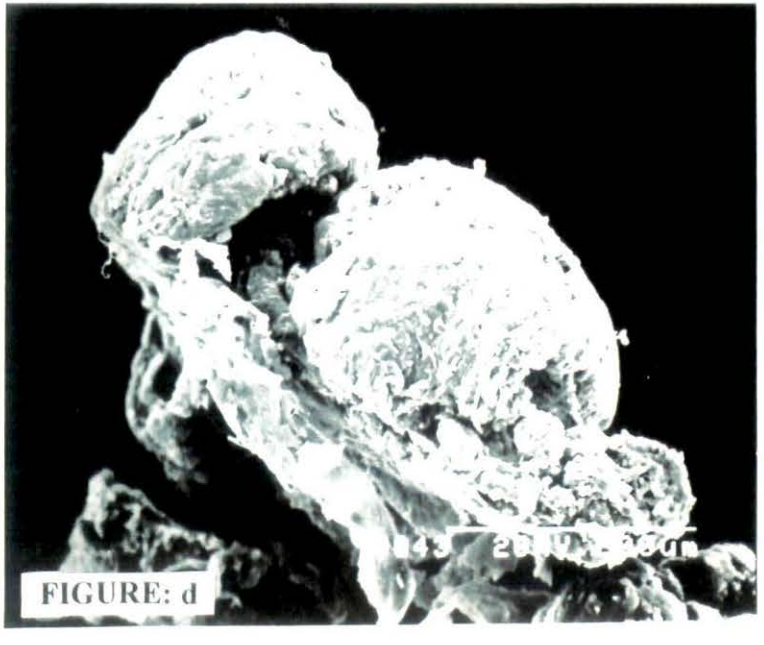


FIGURE: d

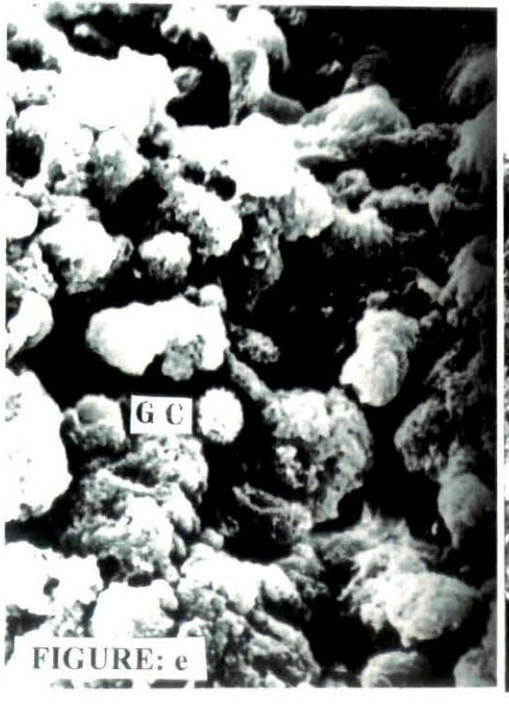


FIGURE: e

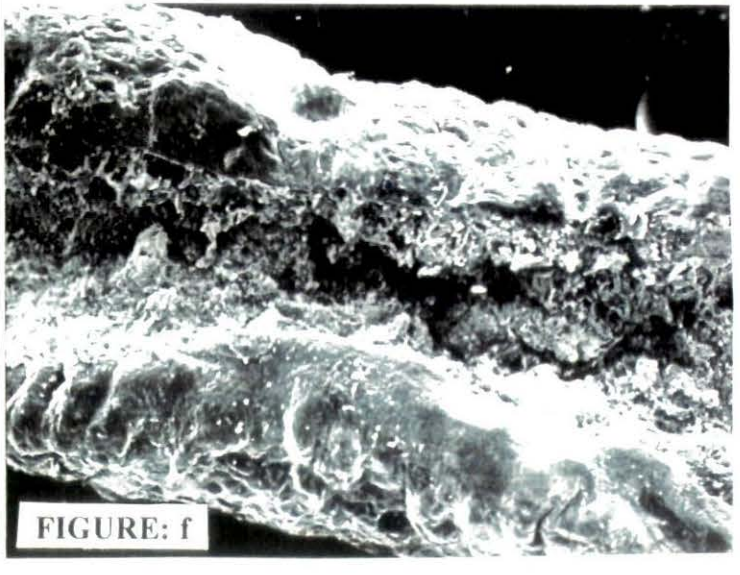


FIGURE: f

However, under scanning electron microscope, the developing undifferentiated mass or anlagen was exhibiting the appearance of the gonial cells as spherical irregular cells of about 7-10 millimiron in diameter. The surface morphology of gonial cells is characterized by rough and irregular surface texture with numerous depressions and ridges at regular intervals (Plate 17, Figure b & c).

At '4'-limb stage, gonial cells exhibit some characteristics as found in '2'- limb stage. Under scanning electron microscope, gonial cells show irregular surface morphology and oval and spherical shape of about 5-8 millimicron in diameter. Here at this stage, gonial cells along with primary and secondary spermatocytes are seen. And from this stage differentiation of sexes are clearly distinguishable. It may occurred just after the '2'- limb stage develops further (Plate 17, Figure d & e).

At sub-adult stage, metamorphosis has been culminated altogether and here more advancement of spermatocytes occurred. Here at this stage, scanning electron microscope shows surface morphology of the testis, which is similar to that of the adult. Gonial cells show association of primary and secondary spermatocytes along with spermatids. But no further advancement of the spermatogenic cycle is observed; instead this juvenile spermatogenesis usually ends with degeneration of spermatogenic nests as observed by Iwasawa and Kobayashi (1984) (Plate 17, Figure f).

2. On adult testes

Under scanning eletron microscope the surface morphology of the entire testis appears honeycomb in structure. Each unit of the honeycomb is oval or irregular in shape. The anteriormost part is somewhat narrow and shows scar suggesting the site of Bidder's organ attachment, which has been removed prior to SEM preparation (Plate 18).

The middle part exhibits distinct honeycomb units of similar shapes and sizes. The posteriormost part is oval, broad and made up of honeycomb units of various shapes and sizes.

Each honeycomb unit may be comparable with the cyst harbouring different cellular elements as observed under light microscope (Plate 16, Figure C).

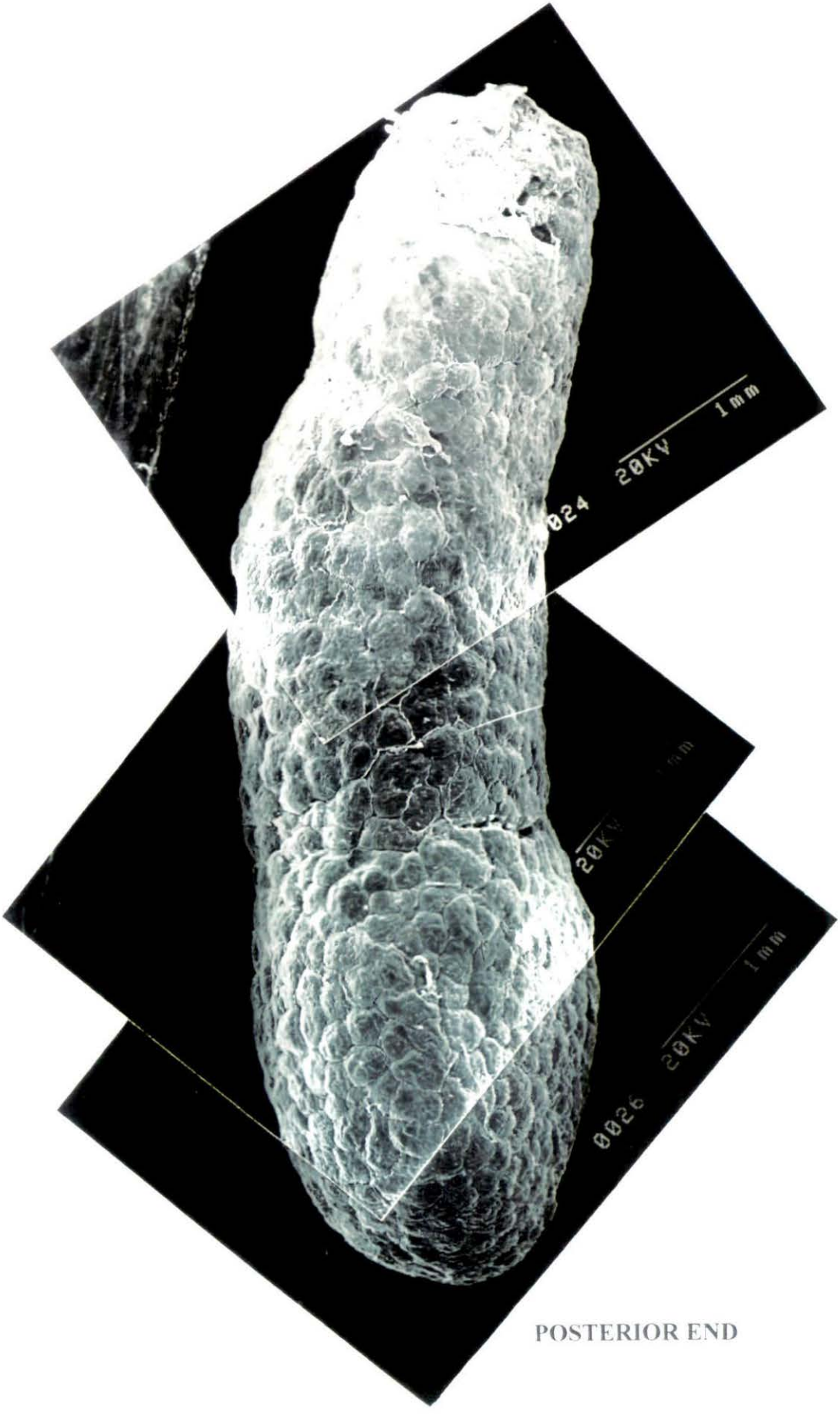
The inner morphology of the whole testis has been revealed from whole testis preparation opened medially by incision. The cystic arrangements visualized from light microscopy have also been revealed under SEM. The anterior part exhibits various forms of germ cells association ranging from spermatogonia to spermatozoa (Plate 19, Figure A B C D).

In the middle part more advanced stages *viz.* Spermatocytes, spermatids and spermatozoa could be revealed. The posterior part exhibits sperm nests with distinct association of Sertoli cells (Plate 19, Figure a & b).

However the non-breeding testes do not show any such cystic arrangement and empty vacuoles are more frequent throughout the entire testis suggesting the completion of spermiation prior to this phase (Plate 20, Figure a, b, c, d).

PLATE-18

SCANNING ELECTRON MICROGRAPH OF AN ADULT TESTIS



POSTERIOR END

PLATE19

Legends

Scanning electron micrographs of adult testicular tissues of *Bufo himalayanas*

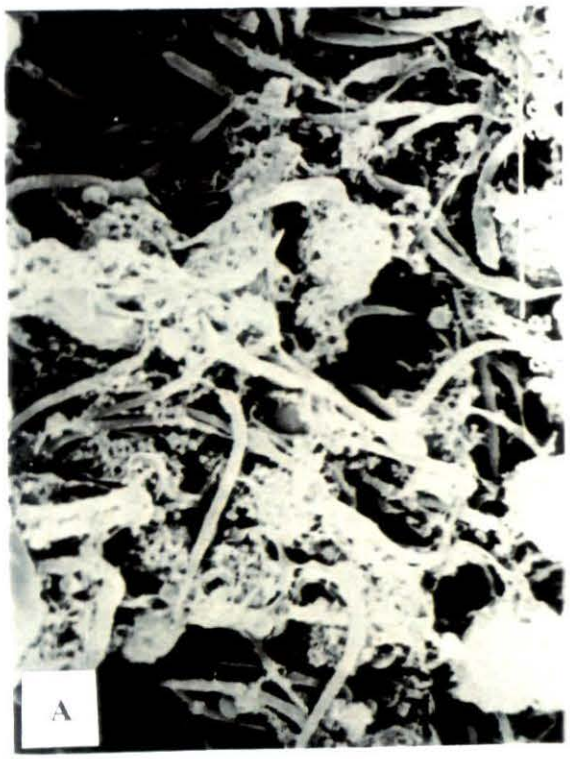
A. Spermatocyte with mature spermatozoa (elongated)

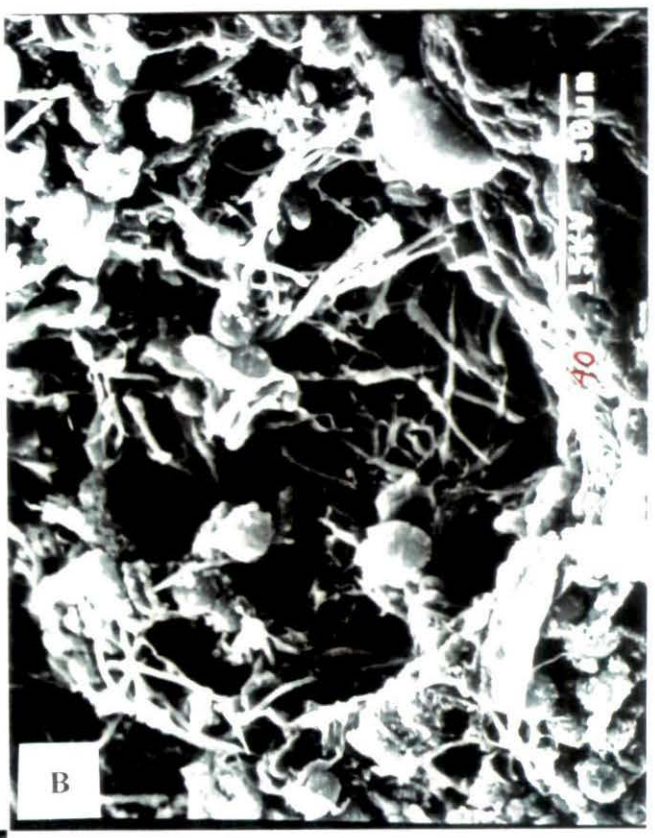
B. Gonial cells and spermatocytes (primary and secondary)

C. Spermatids and Sertoli cells

D. Primary and secondary spermatogonial cells

PLATE-19





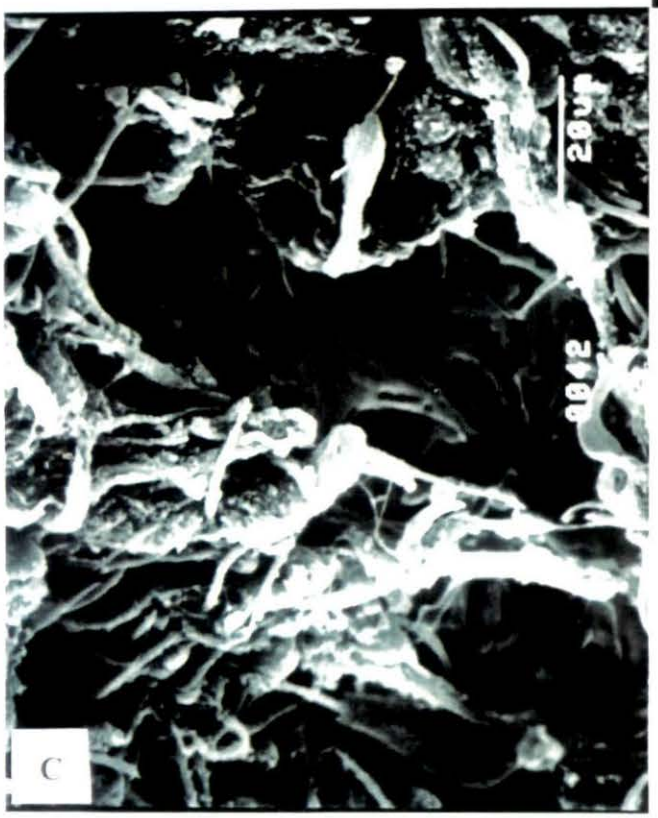


PLATE 20

Legends

Scanning electron micrographs of adult testicular tissues of *Bufo himalayanas* during non-breeding season (winter)

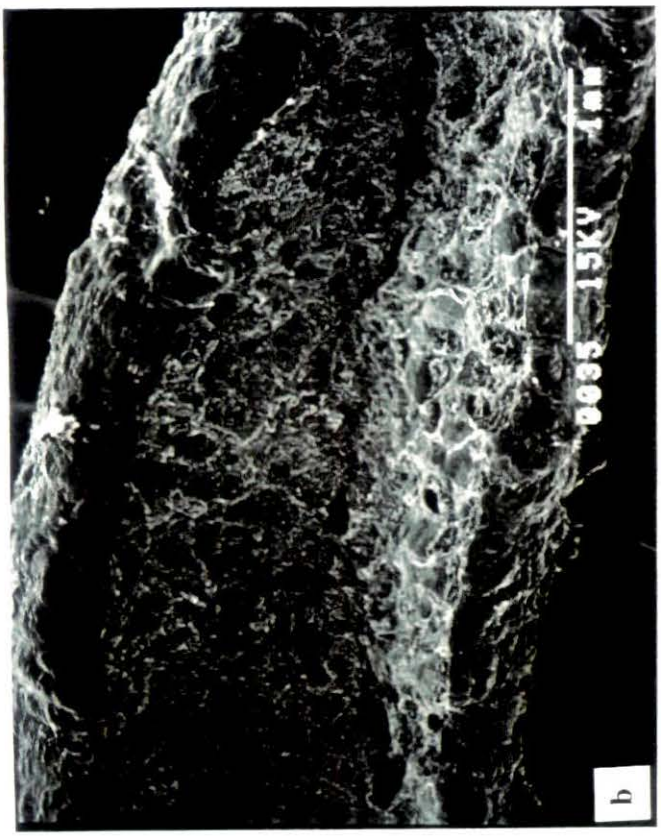
A. Testes cut open to show cyst formation inside

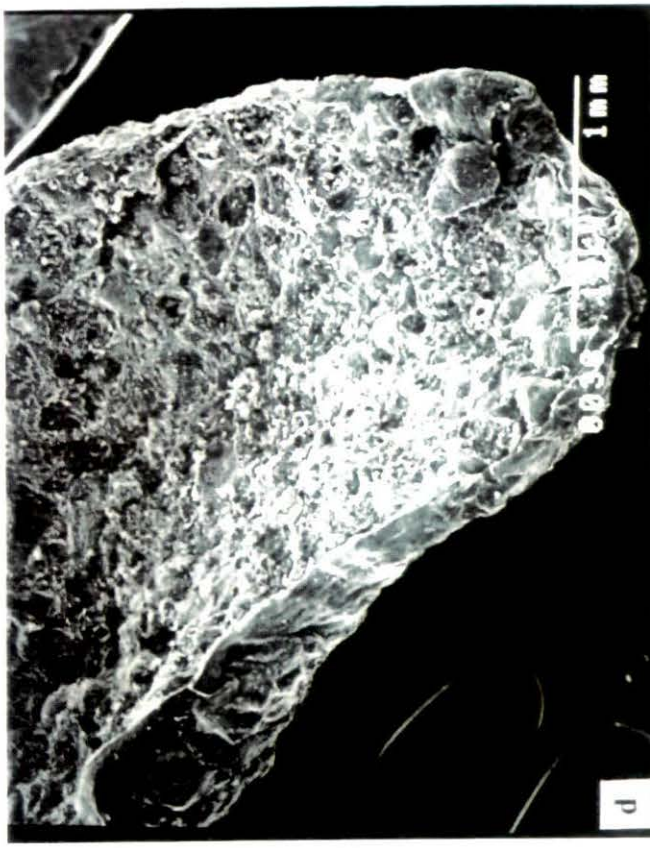
B. Testes cut open to show cysts without any spermatogenic activity

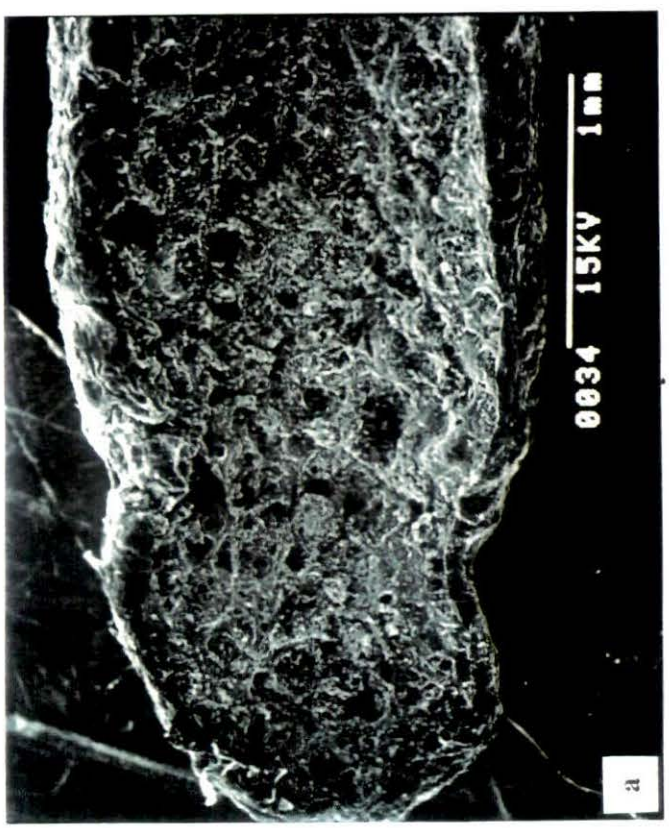
C. Testes cut open to show primary and secondary gonial cells

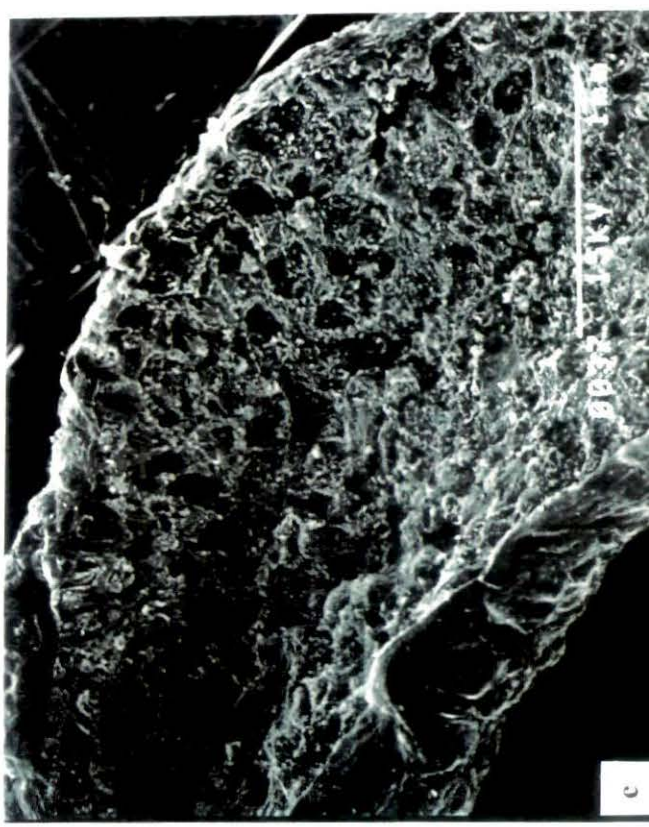
D. Testes cut open to show spermatogonial cells and spermatocytes only within the cysts

PLATE-20









B. Transmission electron microscopic observations:

(1) On larval gonads

At 'O' limb stage

Under transmission electron microscope the peripheral cells exhibited conspicuous nucleus with distinct nuclear boundary, homogenic nucleoplasm with small and large vaccuolations, distinct nucleolus with heterochromatic mass. The cytoplasm exhibited vaccuoles of various shapes and sizes, club shaped mitochondria, annulate bodies, lysosomes and vesicular structures (Plate 13, Figure A B C D).

The medullary cells has conspicuous nucleus with discontinuous nuclear membrane, granular nucleoplasm with distinct chromatic dots and a very conspicuous nucleolus with central condensed chromatin materials. Cytoplasm exhibited stacks of granular endoplasmic reticulum, lysosomes with coated materials, mitochondria of oval to elliptical shapes, collagen fibers and some conspicuous annulated bodies. The vacuoles are distinct and surrounded by a rim of mitochondria. Cell granules are distinct and filled with either lipid or glycogen. The existence of some macrophage cells with characteristic elongated or irregular nucleus and irregular cell boundary are found in between the peripheral and medullary cells (Plate 13, Figure E, F, G, H and Figure-21, a-f).

At '2' limb stage

Under TEM the peripheral cells exhibited elongated nucleus with patches of chromatin material, distinct nuclear lamella. Cytoplasm exhibited granular appearance with distinct vacuoles, mitochondria and very few lysosomes. Boundary of the peripheral cells exhibits projections or microvilli interdigited with adjacent cell.

The medullary cells are characterized by single or bi-nucleate cells, cytoplasm studded with mitochondria of oval to elliptical shape and granules. This morphology is comparable with gonia cells found in sub-adult and adult stage (Plate 23, Figure A, B, C, D).

At '4' limb stage

Medullary cells further exhibited proliferation. Nucleus of each cell is distinct with conspicuous chromatic masses and nuclear bodies. Cytoplasm exhibited elaboration of microtubules, mitochondria, lysosomes and granules of various forms(Plate 24, Figure A, B, C, D).

The peripheral cells exhibit further elaboration of nuclear material, nucleolus and elaboration of lipid filled granules in the cytoplasm (Plate 25, Figure A, B, C).

At sub-adult stage

The gonadal tissue of sub-adult received signal for ovarian development exhibits characteristic features of ovarian follicles viz. Vesicular nucleus, cytoplasm enriched with mitochondria, lipid droplets and glycogen granules (Plate 26, Figure A, B, C).

Whereas the gonadal mass which received signal for testicular development exhibits existence of flagellar bodies, centriole, spermatids of various shapes and granular cytoplasm (Plate 27, Figure A, B, C, D).

(2) On adult testis

The adult testis exhibits two compartments.

1] The seminiferous compartment- containing cysts filled with germ cells, spermatogonia, spermatocytes, spermatids, spermatozoa and Sertoli cells.

2] The interistitial compartment- outside the tubule containing elements from the connective tissue viz. Fibrocytes, collagen fibers and myoid bodies .

The seminiferous compartment

Germ cells: The germ cells are found in the testis of *Bufo himalayanas* in four stages of development, such as:

Stage 1: Spermatogonia- These are the largest cells of the testis with the spherical nucleus, granular chromatin and electron dense spherical prominent nucleolus. The cytoplasm contains spherical mitochondria, free ribosomes, agranular and granular endoplasmic reticulum and well developed system of annulate lamellae (Plate 28, Figure a).

Stage 2 : Primary and secondary spermatocytes- Primary spermatocytes show spheric center nucleus, indistinct nucleolus and chromatic masses. The secondary spermatocytes have a comparatively large nucleus with irregularly distributed chromatin masses and cytoplasm enriched with mitochondria, ribosomes, agranular and granular endoplasmic reticulum. The synaptonemeal complex, characteristics of spermatocytes could not be visualized (Plate 28, Figure b).

Stage 3: Spermatid- The spermatids are seen in different stages of spermiogenesis as indicated by the degree of chromatin condensation. Initially , they contain chromatin consisting of evenly distributed coarse granules. With the progress of spermatogenesis , the chromatin condenses into dense clots and the cell becomes smaller and more electron dense (Plate 28, Figure c & d.).

Stage 4 : Spermatozoa: Consists of head,neck and tail (the further details of description given in Chapter III, Plate –29, Figure d).

Sertoli cells: The Sertoli cells are distributed in the periphery of the cyst or around the spermatogonia. They show an irregular shape nucleus with condensed chromatin and prominent nucleolus; cytoplasm with desmosomes, mitochondria and irregular vesicles (Plate 29, Figure a, b).

The interstitial compartment

Leydig cells: These cells are interistial in location and are small, compact and ovoid in shape, distributed between the semeniferous tubules. The size and shape of the cells show seasonal changes in some anuran species including *Bufo himalayanas.*

At breeding season, Leydig cells are spherical in shape with large nucleus, conspicuous nucleoli, coarse chromatin granules and are considered to secret androgen actively. The Leydig cells observed here at non-breeding season are flat, sunken with reduced nuclear size and compact chromatin as reported by Saidapur, 1989 (Plate 29, Figure c).

PLATE 21

Legends

Electron micrographs of medullary cells of 'O' limb stage

a. Cytoplasmic inclusions of a medullary cell showing lysosomes, rough walled endoplasmic reticulum, mitochondria, vacuoles etc.

b. Macrophage cells in between peripheral and medullary cells showing lysosomal activity

c. Medullary cell cytoplasm with numbers of lipid droplets and glycogen reserves

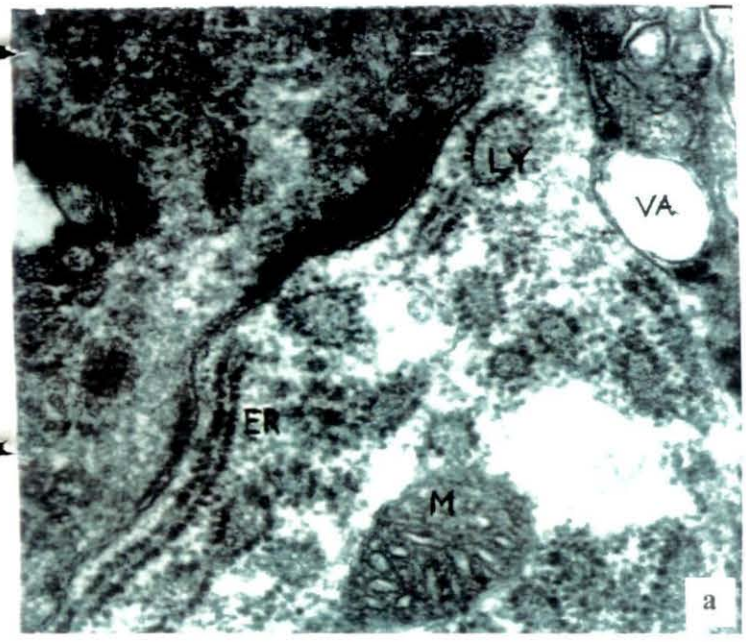
d. A round nucleus of a medullary cell with conspicuous nucleolus, granular nucleoplasm and irregular nuclear boundary

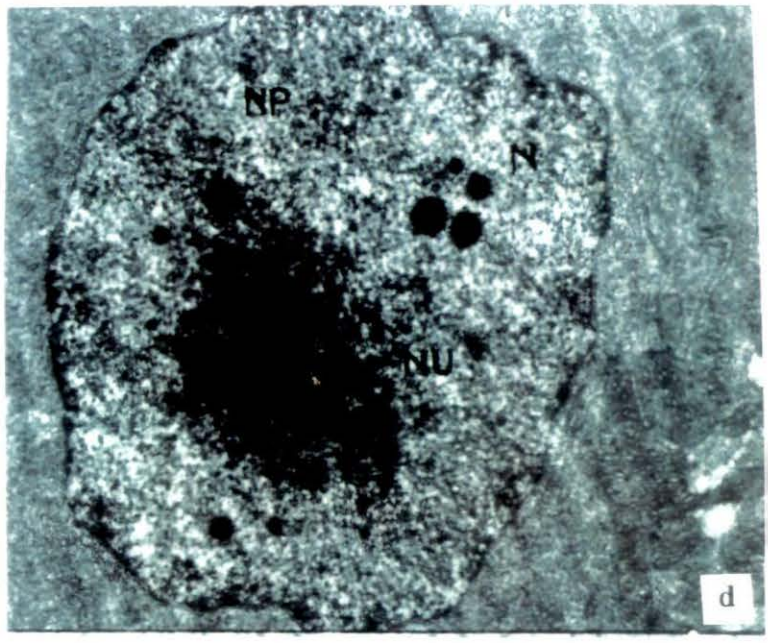
e. An elongated nucleus of a macrophage cell with numerous lysosomal vesicles

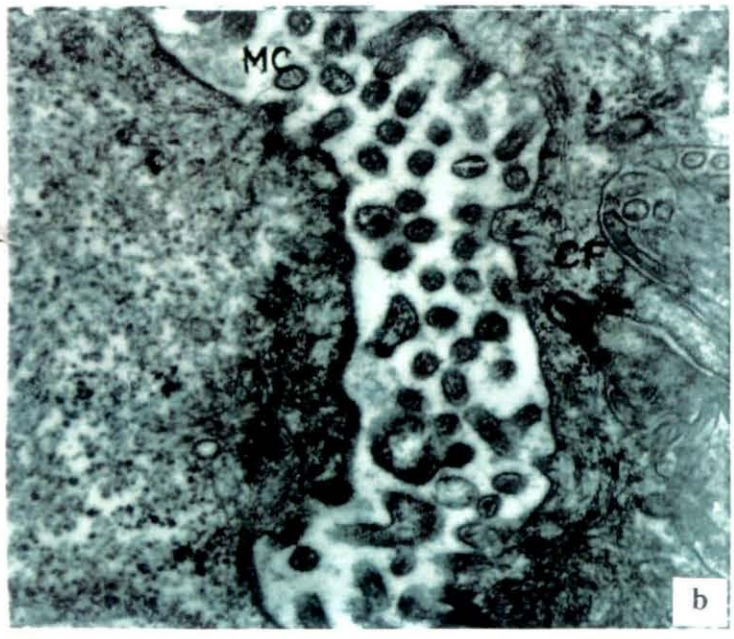
f. Cytoplasm of a medullary cell with lipid droplets, lysosomes and conspicuous mitochondria

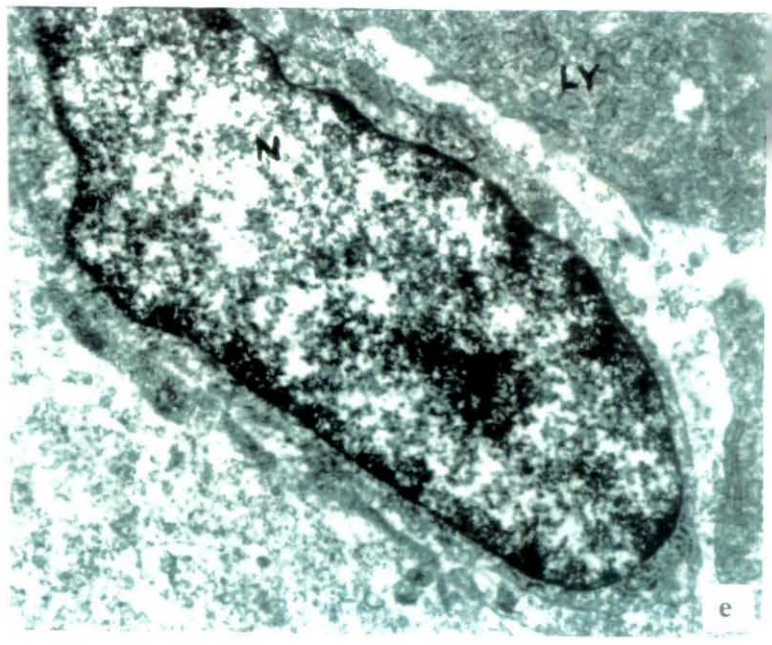
LY- Lysosomes ER-Endoplasmic reticulum VA- Vacuole
M-Mitochondria N-Nucleus NU- Nucleolus NP- Nucleoplasm
CF-Collagen fiber LI-Lipid droplets

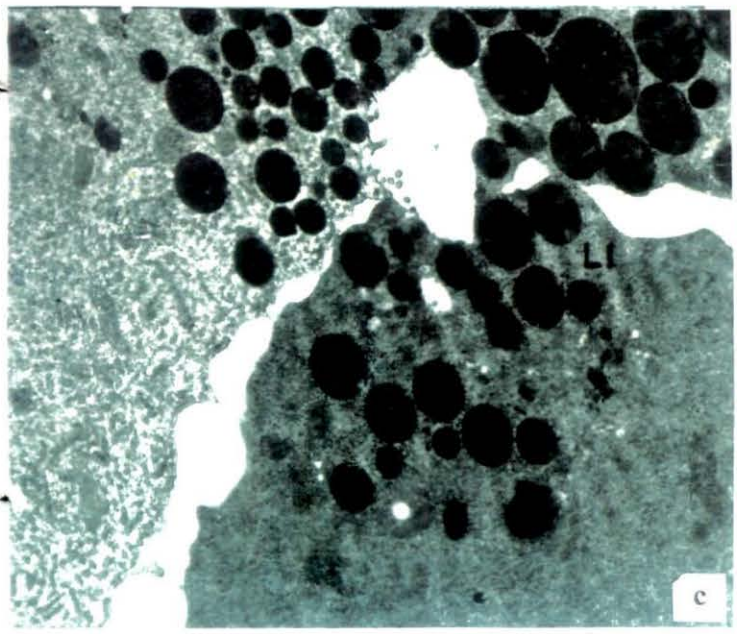
PLATE-21











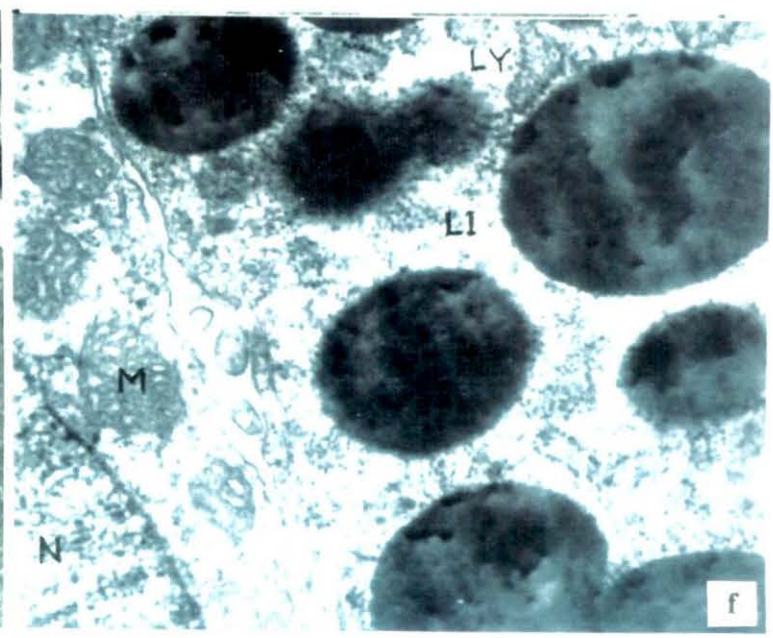


PLATE 22

Legends

Electron micrographs of cortical cells of '2' limb stage

a. Cortical cell's cytoplasm with cytoplasmic granules and vacuoles

b. Cortical cell showing distinct elongated nucleus with patches of chromatin materials, granular cytoplasm with distinct vacuoles

c. Boundary of a cortical cell with projection of microvilli interdigited with adjacent cell. Cytoplasm with round and elongated mitochondria

d. Cytoplasm showing distinct granular inclusions scattered within and a mitochondria

VA- Vacuole M-Mitochondria N-Nucleus CNL- Cytoplasmic granules
CM- Chromatin materials MV- Microvilli

PLATE-22

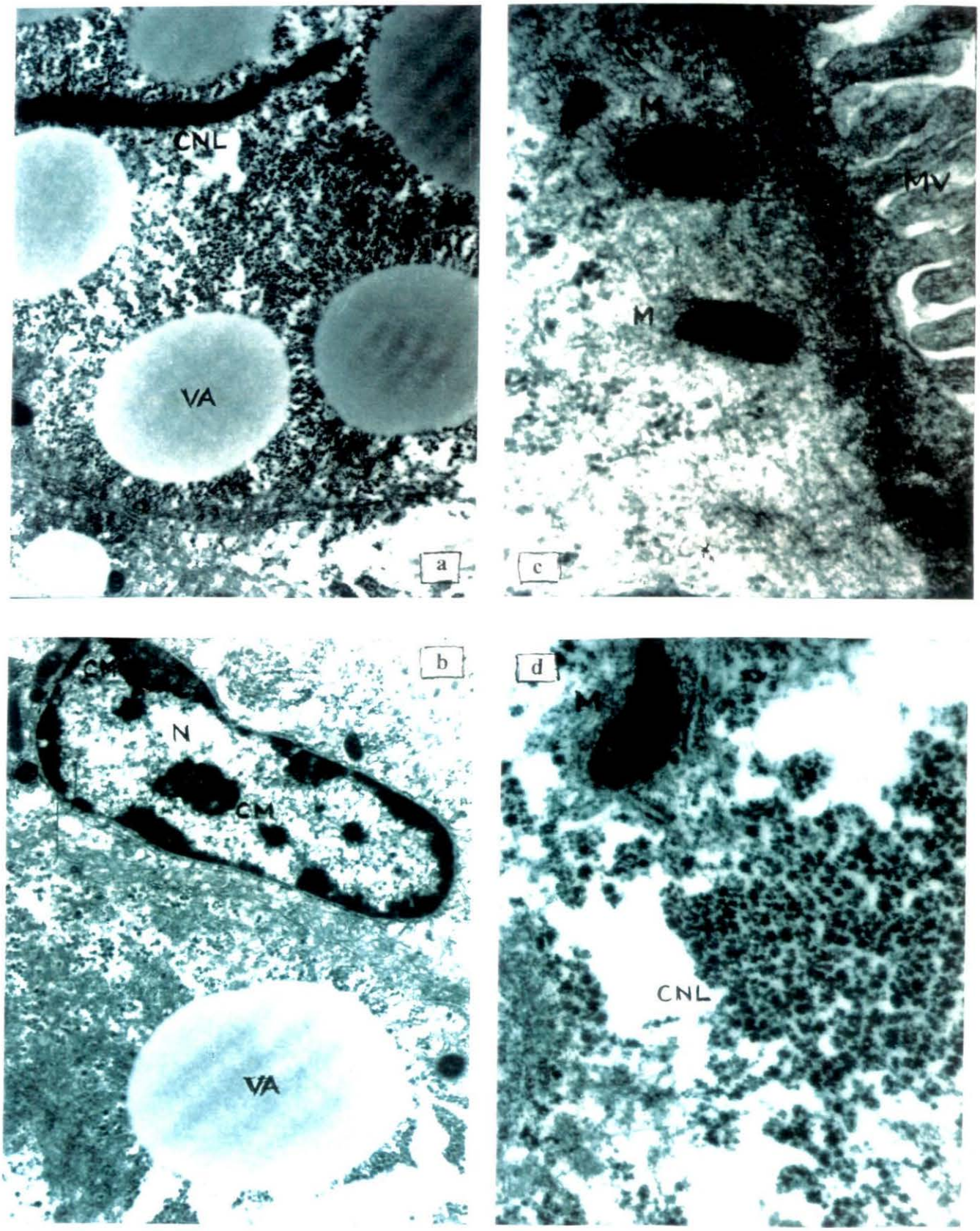


PLATE 23

Legends

Electron micrographs of medullary cells of '2' limb larval stage

A. Cytoplasm with round shaped mitochondria

B. A single cell with conspicuous nucleus and scattered chromatin materials within. Cytoplasm with round and elongated mitochondria and collagen fibrils

C. A bi-nucleate cell. Nuclei with peripheral chromatin materials

D. Cytoplasm with cytoplasmic granules and round shaped mitochondria

M- Mitochondria CM- Chromatin material N- Nucleus
CF- Collagen fibrils BNC- Bi-nucleate cell CYG- Cytoplasmic granules

PLATE-23

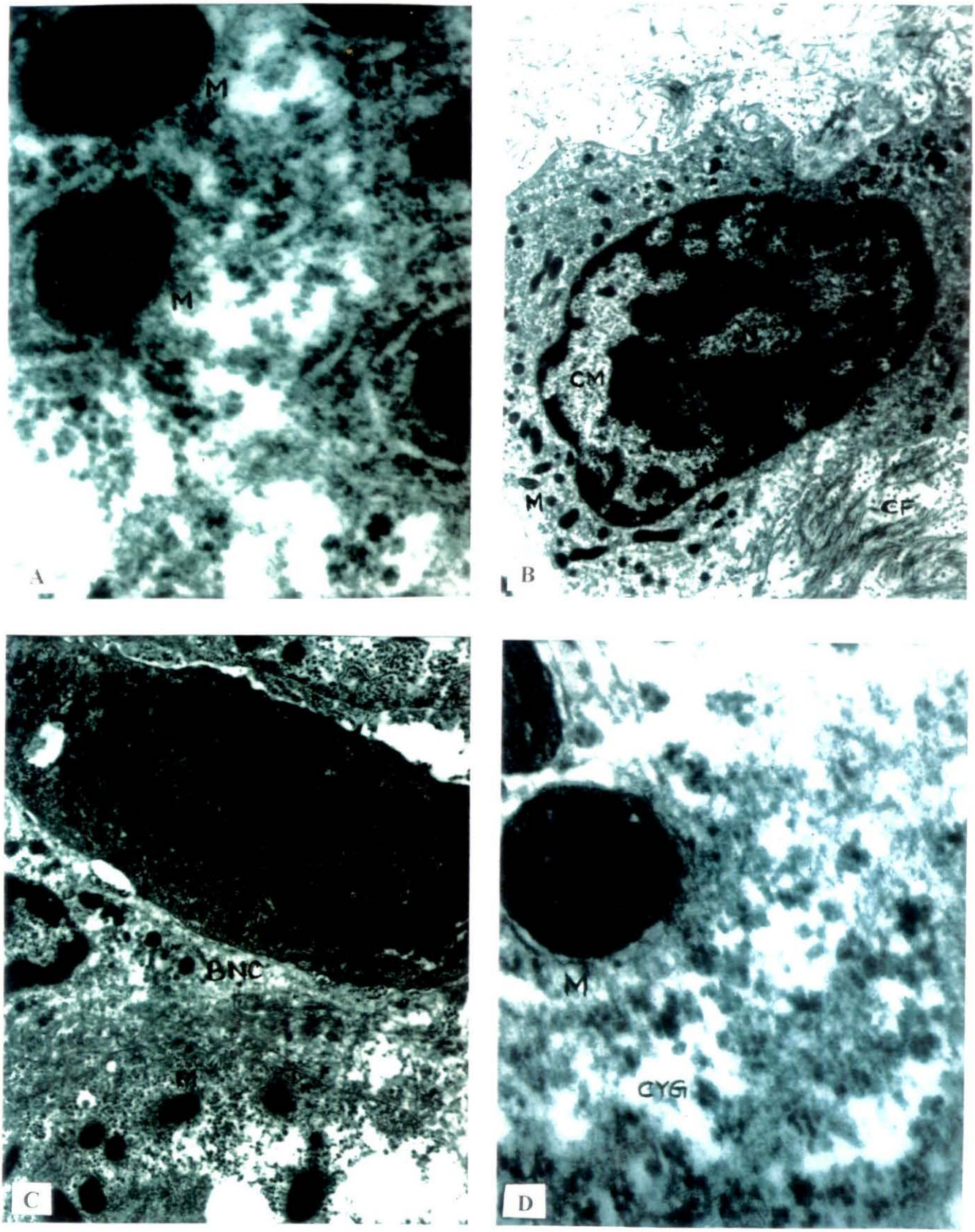


PLATE 24

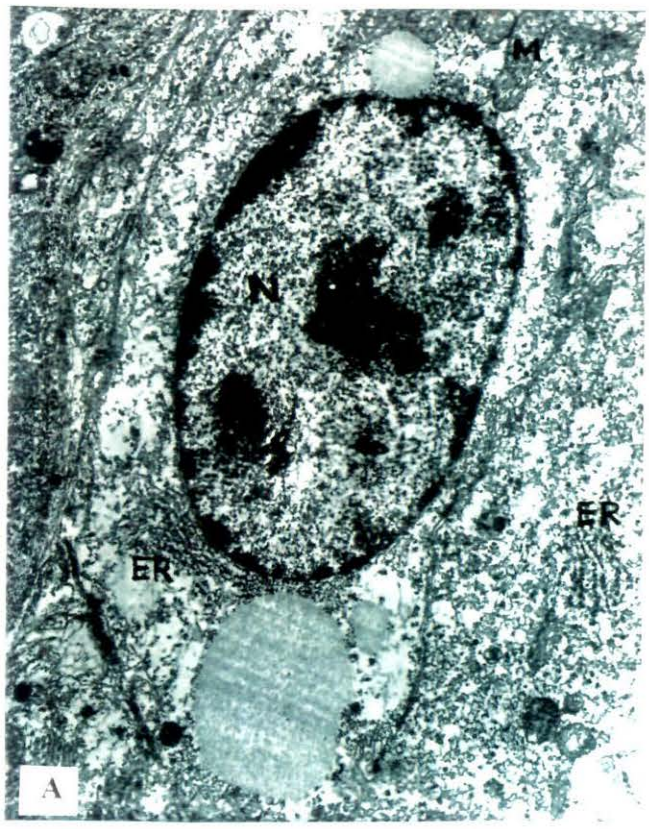
Legends

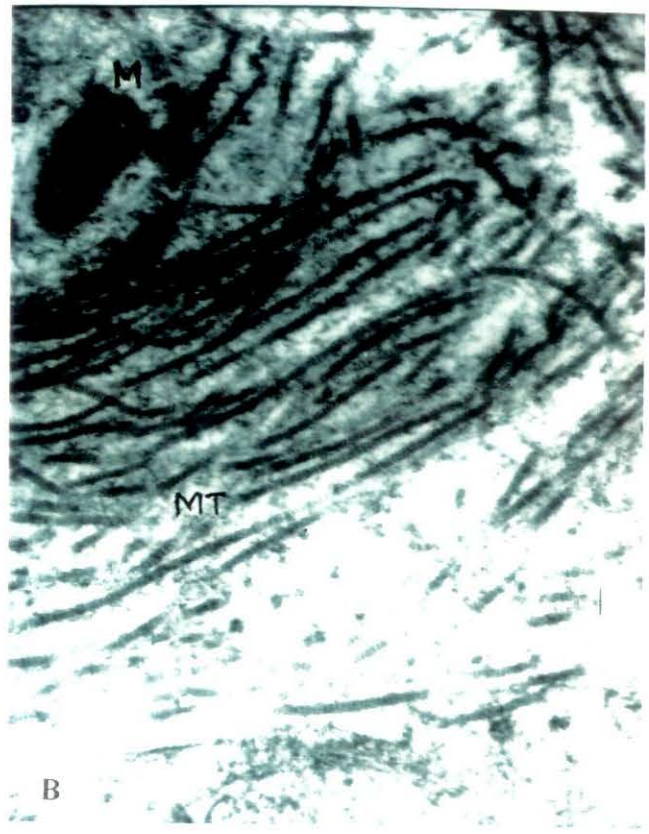
Electron micrographs of medullary cells of '4' limb larval stage

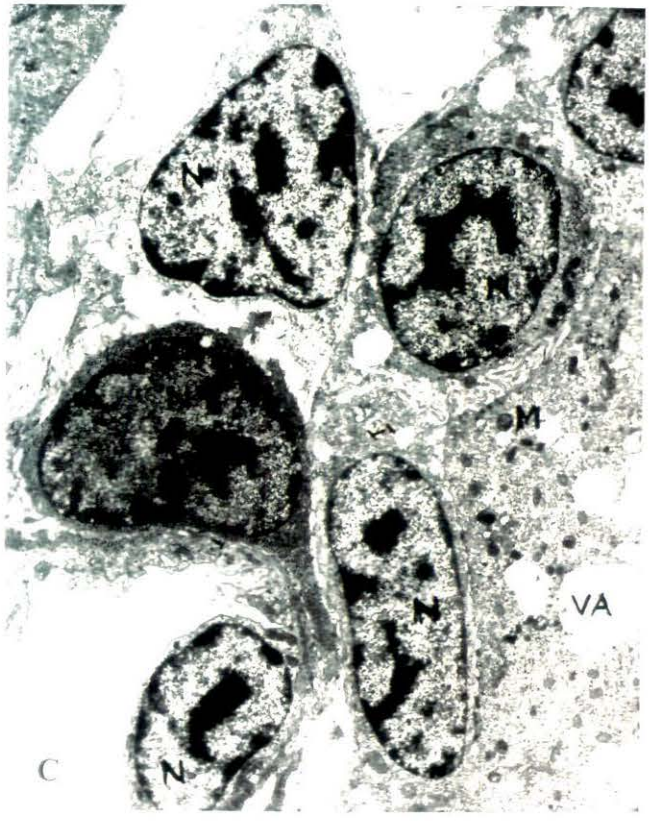
A. A cell with distinct oval nucleus with scattered chromatin materials, endoplasmic reticulum, mitochondria etc.
B. Cytoplasm of the medullary cells showing characteristic microtubules
C. Number of medullary cells with distinct nucleus with scattered chromatin materials. Cytoplasm with vacuoles and mitochondria
D. Cytoplasm shows different stages of lysososmal activity. Cytoplasm with cytoplasmic granules

M- Mitochondria N- Nucleus ER- Endoplasmic reticulum
CYG- Cytoplasmic granules MT- Microtubules VA- Vacuoles
LY- Lysosomes

PLATE-24







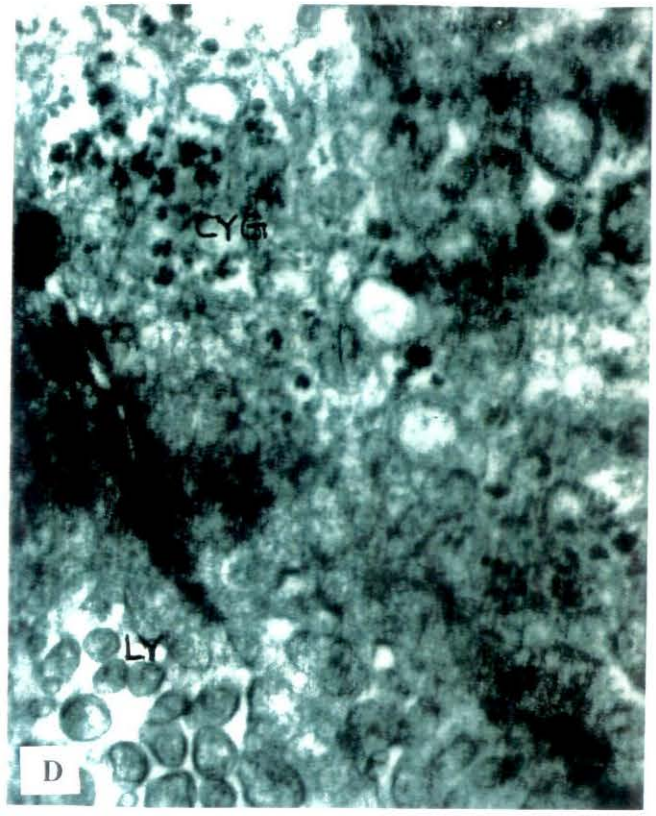


PLATE 25

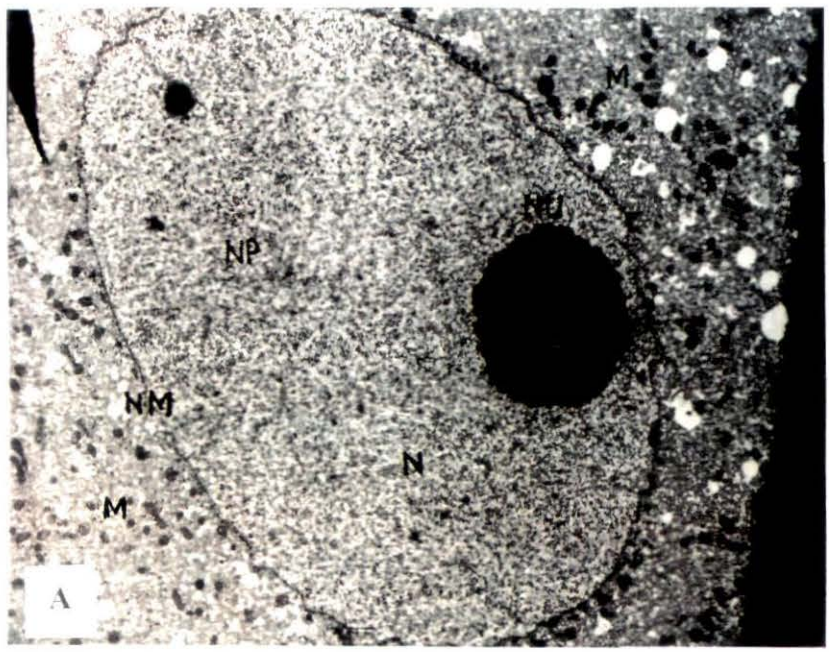
Legends

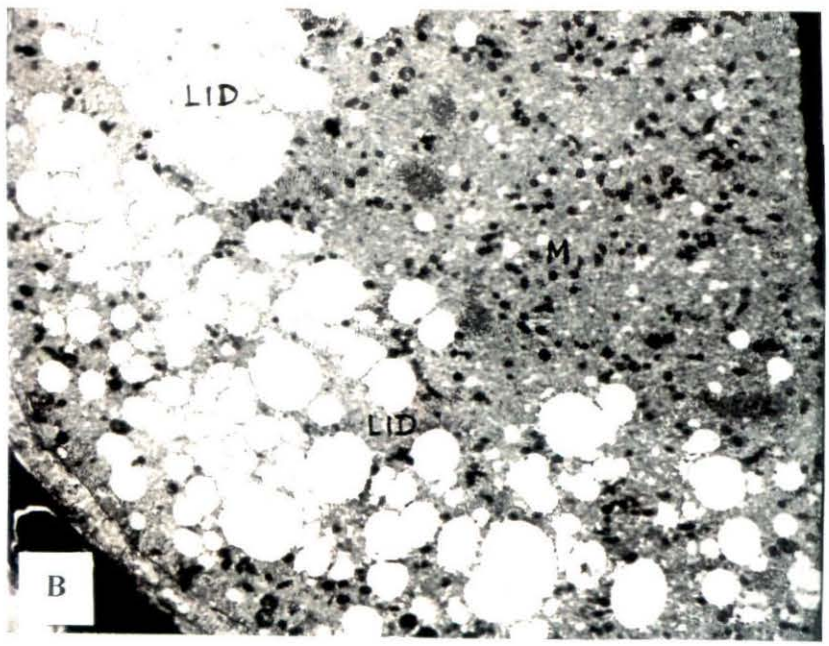
Electron micrographs of peripheral cells of '4' limb stage)

A. A peripheral cell showing large and distinct nucleus with conspicuous nucleolus, granular nucleoplasm, nuclear membrane surrounded by a number of cytoplasmic mitochondria and lipid droplets
B. Cytoplasm filled with numerous lipid droplets at the periphery and numerous mitochondria towards center
C. Enlarged lipid droplets and mitochondria at the periphery of a peripheral cell

N- Nucleus M-Mitochondria NU-Nucleolus LID-Lipid droplets NM-Nuclear membrane

PLATE-25





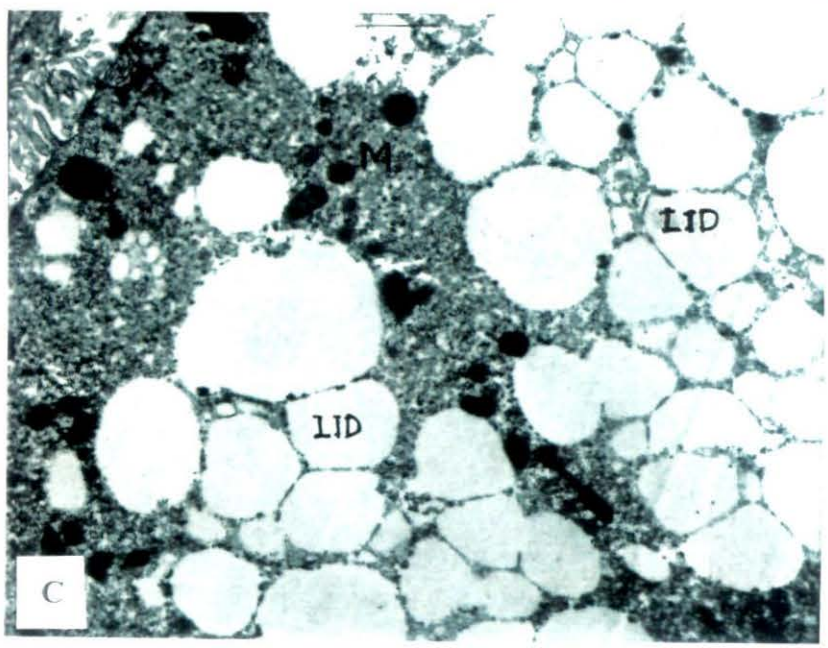


PLATE 26

Legends

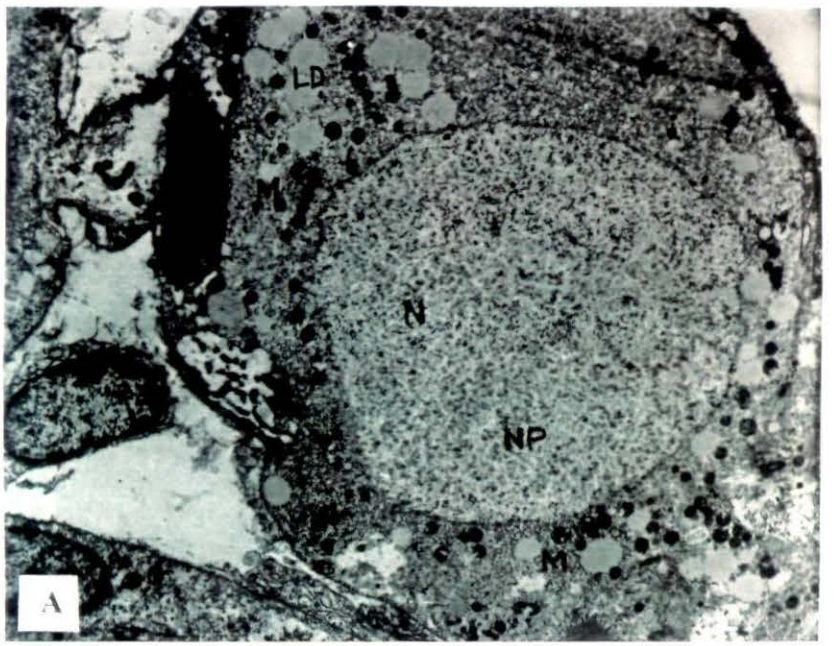
Electron micrographs of gonadal cells of sub-adult stage showing ovarian follicular features

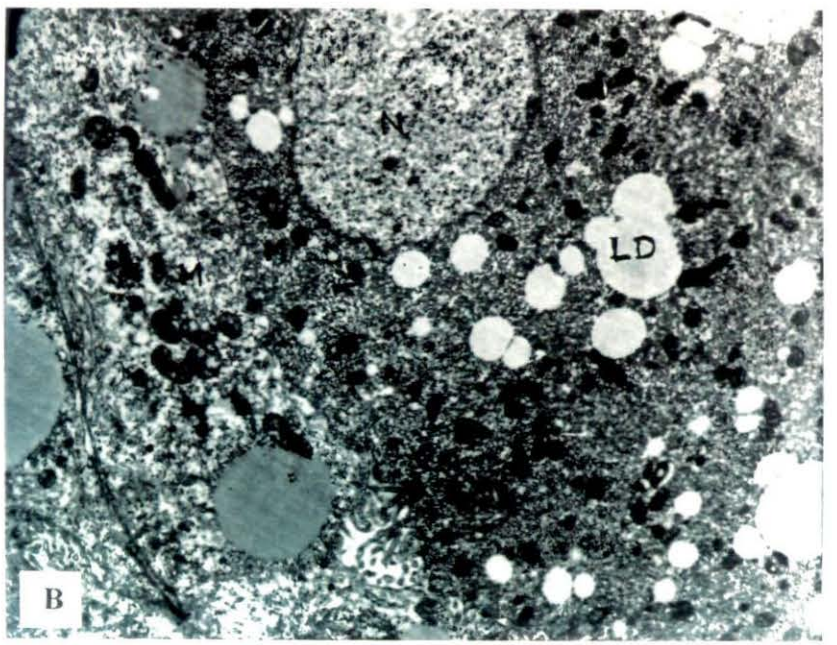
A. A cell with a vesicular nucleus without nucleolus. Nucleoplasm with diffused chromatin materials . Numerous mitochondria and lipid granules

B. Enlarged view of the nucleus along with lipid and glycogen granules. Mitochondria scattered all over the cytoplasm
C. Highly enlarged glycogen granules accumulated together

M- Mitochondria N- Nucleus NP- Nucleoplasm LD- Lipid droplets GG- Glycogen granules

PLATE-26





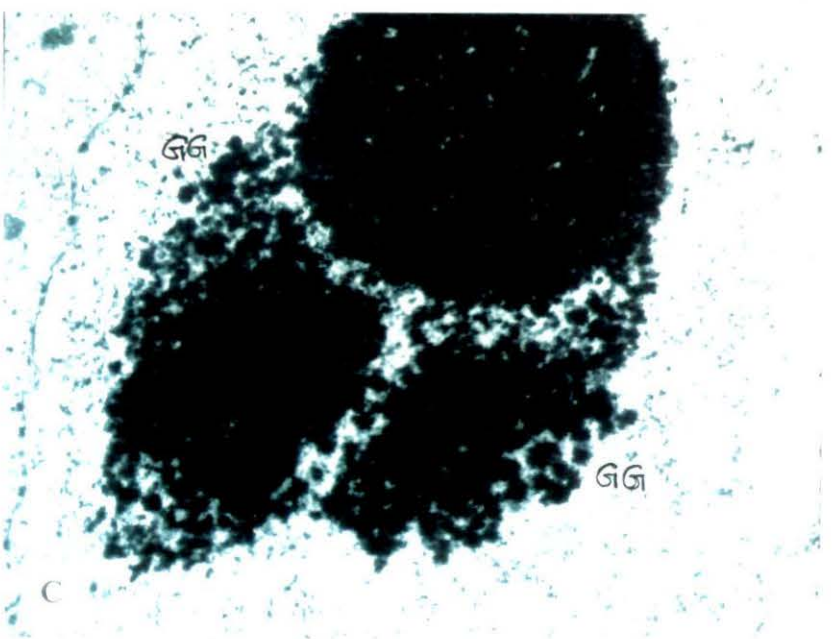


PLATE 27

Legends

Electron micrographs of gonadal cells of sub-adult stage showing testicular features

A. A mature sperm head with elongated nucleus and middle piece
B. Flagellum with typical arrangement of microtubules
C. In cross section different parts of developing spermatids
D. Microtubular arrangement in flagella of spermatids

N- Nucleus MP- Middle piece MT- Microtubules FL- Flagella ST- Spermatids

PLATE-27

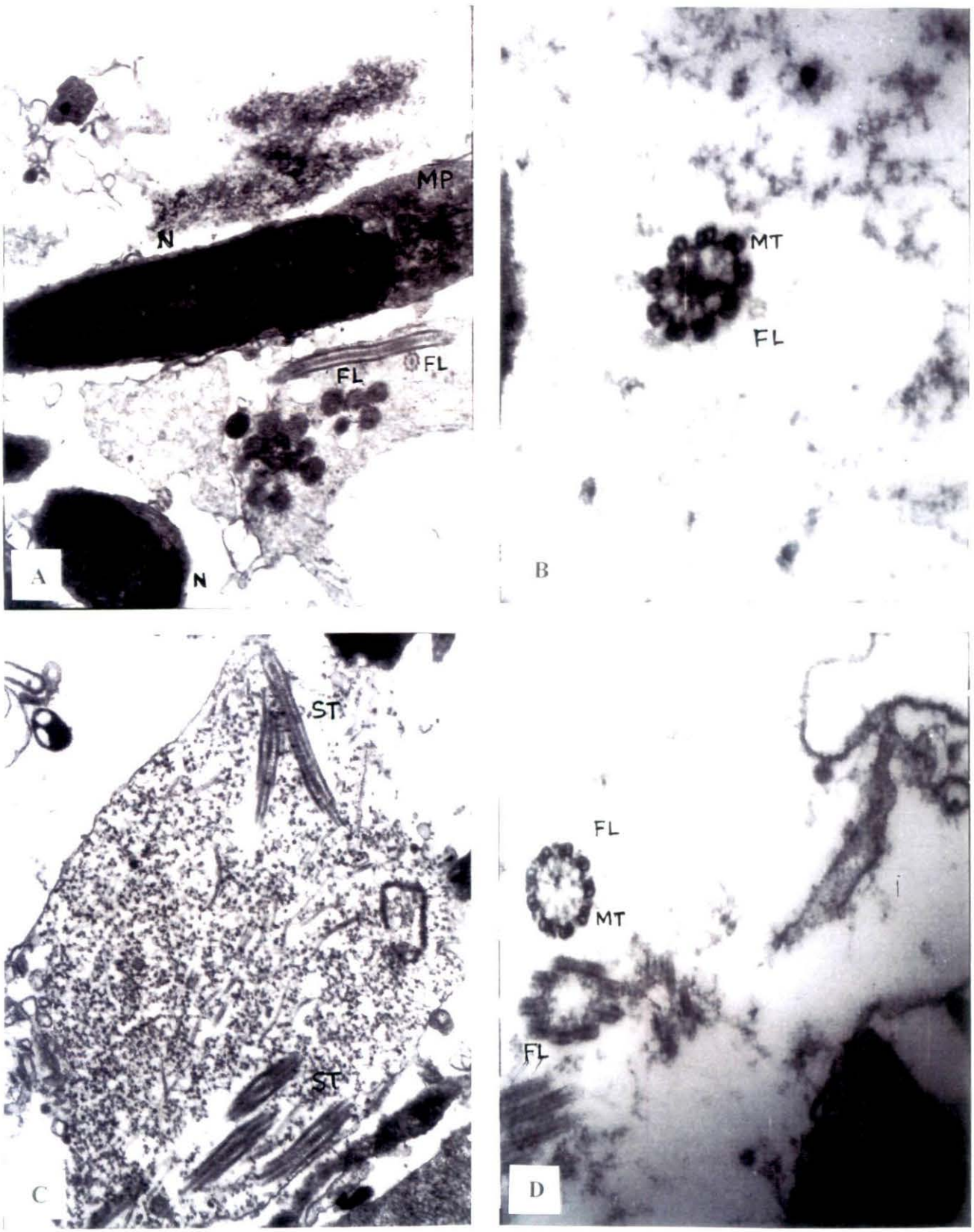


PLATE 28

Legends

Electron micrographs of adult testicular cells showing different stages of spermatogenesis

Fig. a. Spermatogonial cells with large spherical nucleus and dense chromatin material filled nucleoplasm

Fig. b. Primary spermatocytes and secondary spermatocytes with large spherical and elongated nucleus having irregularly distributed chromatin bodies

Fig. c. Spermatids in different stages of spermeiogenesis with oval and elongated chromatin dense nucleus

Fig. d. Late spermatid with the compact and highly electron dense chromatin bodies within the nucleusl

N- Nucleus MP- Middle piece SG- Spermatogonia M-Mitochondria
SPT- Spermatids ER- Endoplasmic reticulum
PRS- Primary spermatocytes SDS- Secondary spermatocytes

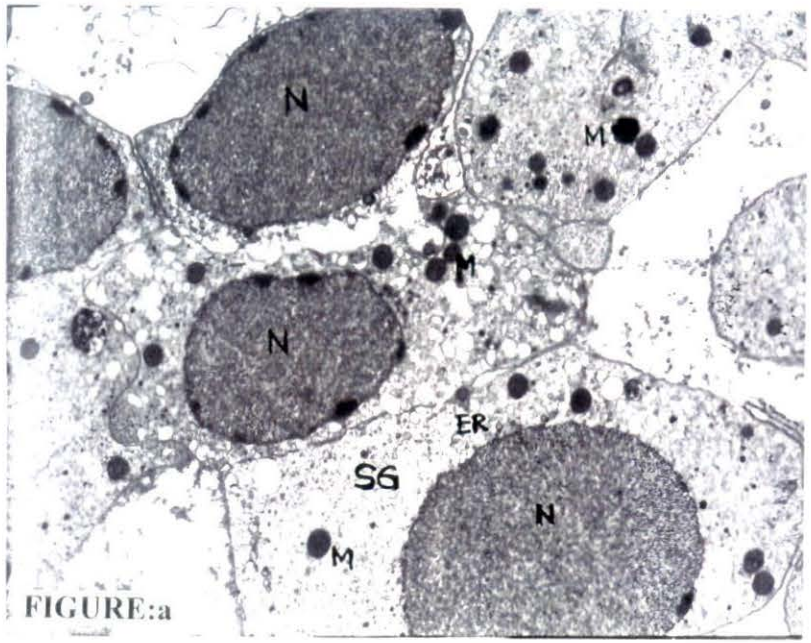


FIGURE:a

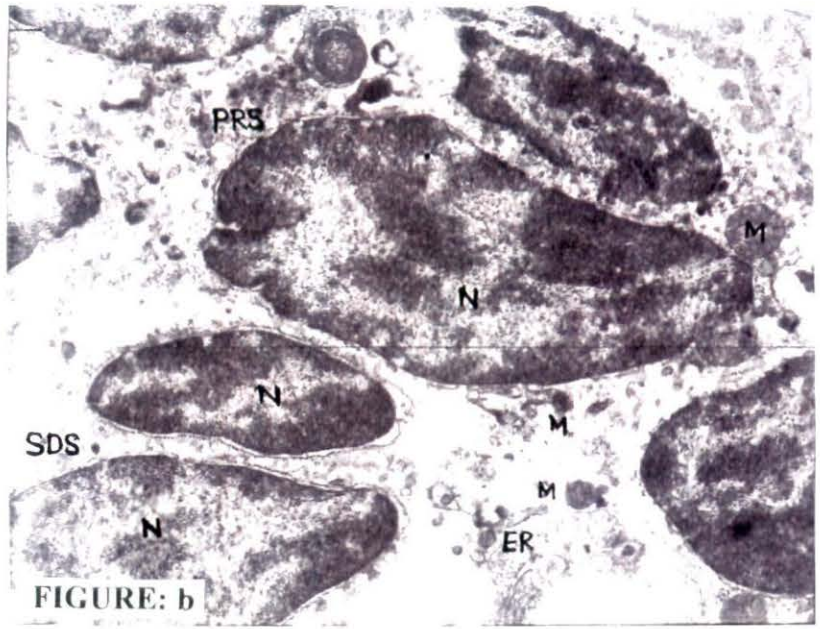


FIGURE: b

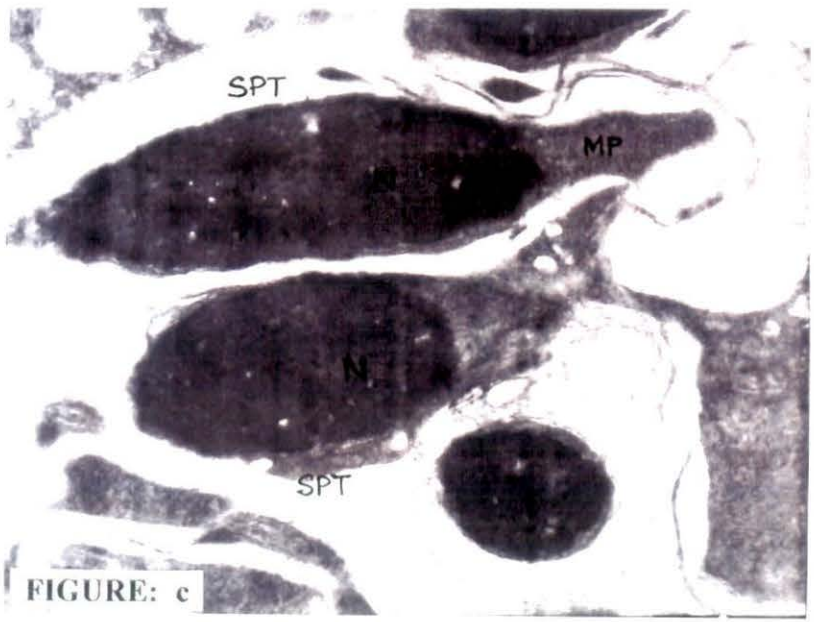


FIGURE: c

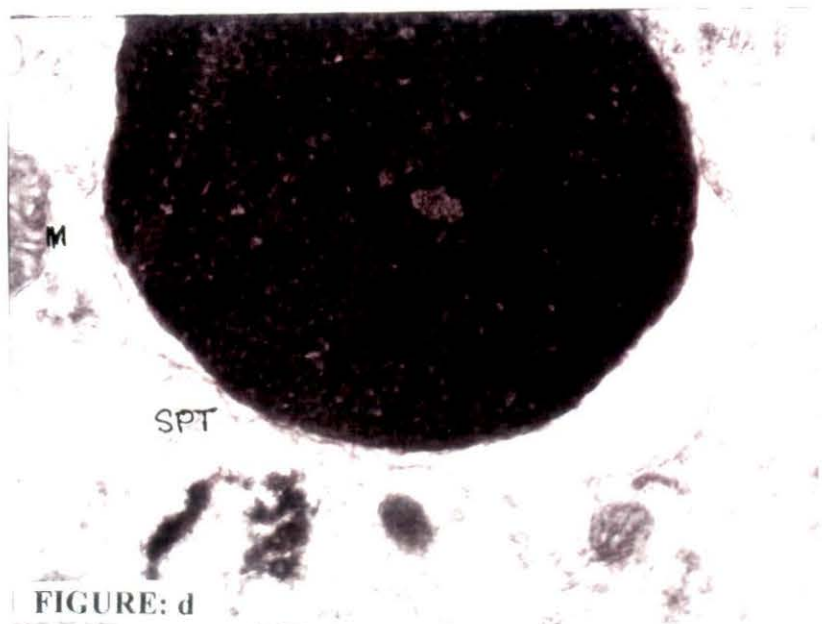


FIGURE: d

PLATE 29

Legends

Electron micrographs of adult testicular cells showing mature spermatozoa and associated Leydig cells

Fig. a. A Leydig cell with irregular vesicles, mitochondria and desmosomes

Fig. b. A Leydig cell with desmosomal complex and other vesicular Structures

Fig. c. A Leydig cell in non-breeding season

Fig. d. A mature sperm head showing acrosome, perforatorium and elongated nucleus

N- Nucleus M-Mitochondria DS- Desmosomes VS- Vesicles
LC- Leydig cell AC- Acrosomes P- Perforatorium

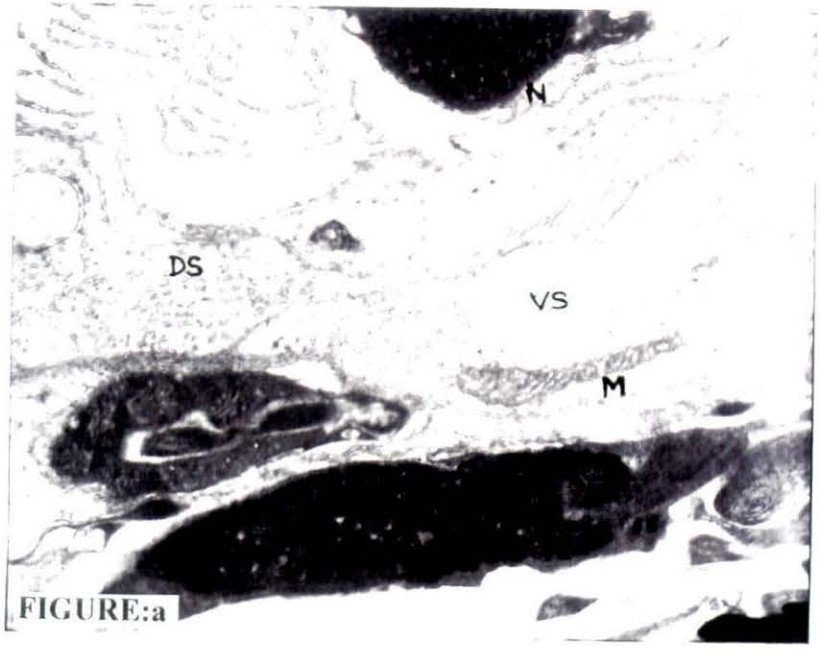


FIGURE:a

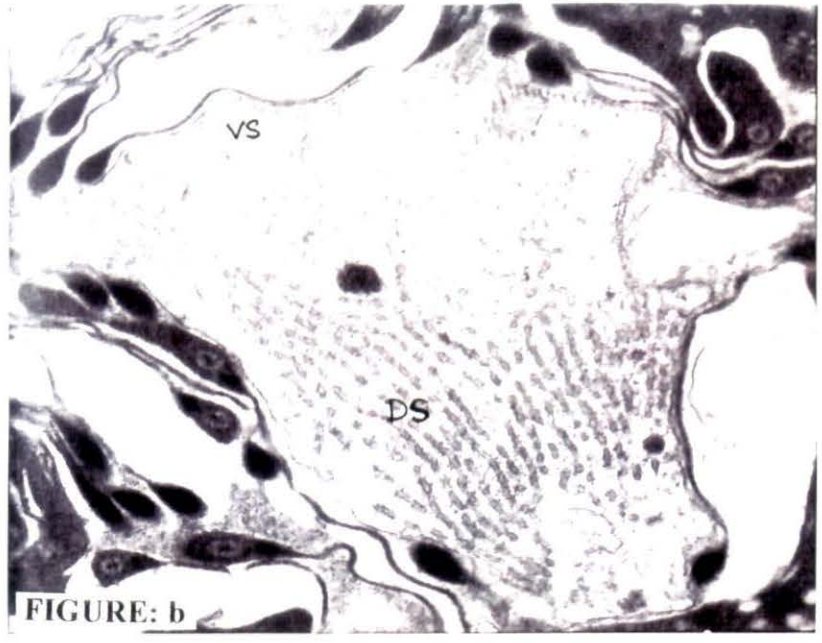


FIGURE: b

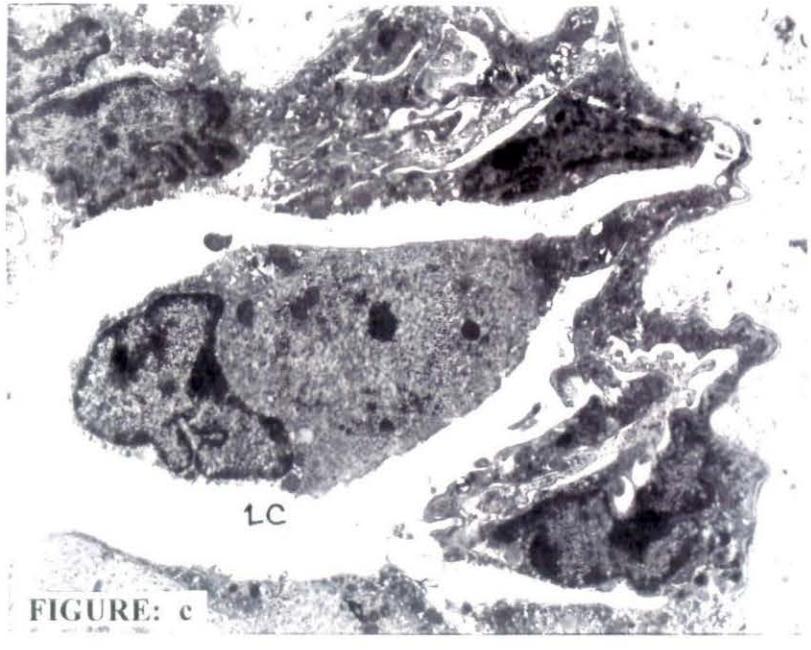


FIGURE: c

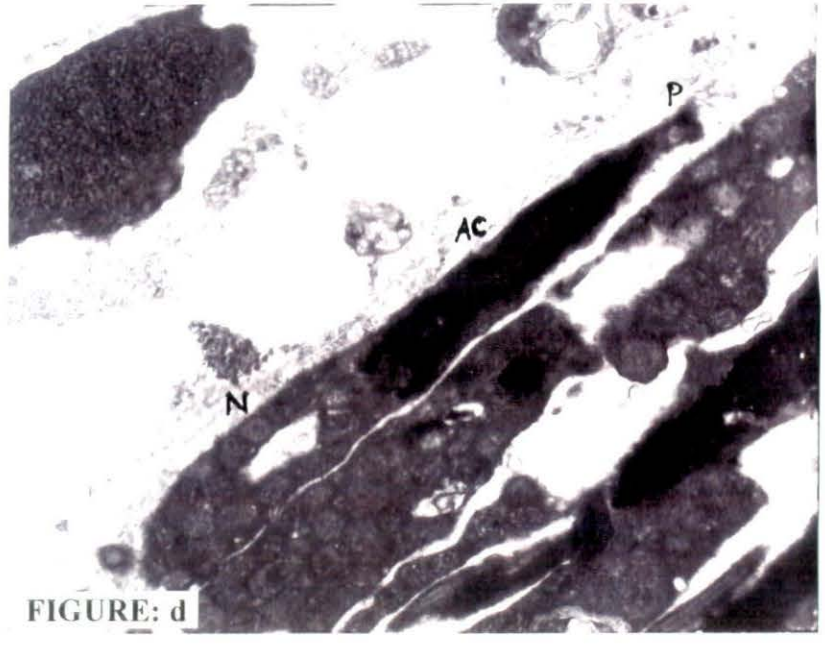


FIGURE: d

CHAPTER- III

EVENTS OF SPERMATOGENESIS

CHAPTER- III

EVENTS OF SPERMATOGENESIS

CONTENTS

Introduction

For their easy maintenance in the laboratory and relatively large size of their germ cells have made amphibians very resourceful objects from the very beginning of cytological and genetical investigations and then they have proved to be excellent experimental animal for research in endocrinology, reproductive and developmental biology. A number of important contribution to basic biology have been achieved in the course of investigation of spermatogenesis in amphibians. Long ago in 1876 George described various stages of spermatogenesis in five species of amphibians from observations on testicular tissues, fresh or fixed in osmium tetraoxide and laid the ground work for modern terminology in this field, coining the terms 'spermatocyst', 'spermatogonium' and 'spermatocyte'. Fleming (1887) used the spermatocytes of *Salamandra* in his meticulous study of the meiotic divisions and Witschi (1914) first recognized in *Rana temporaria* that the male sex in frogs is digametic. Yet the literatures lack a single article which might serve as a classic comprehensive description of amphibian testes and spermatogenesis.

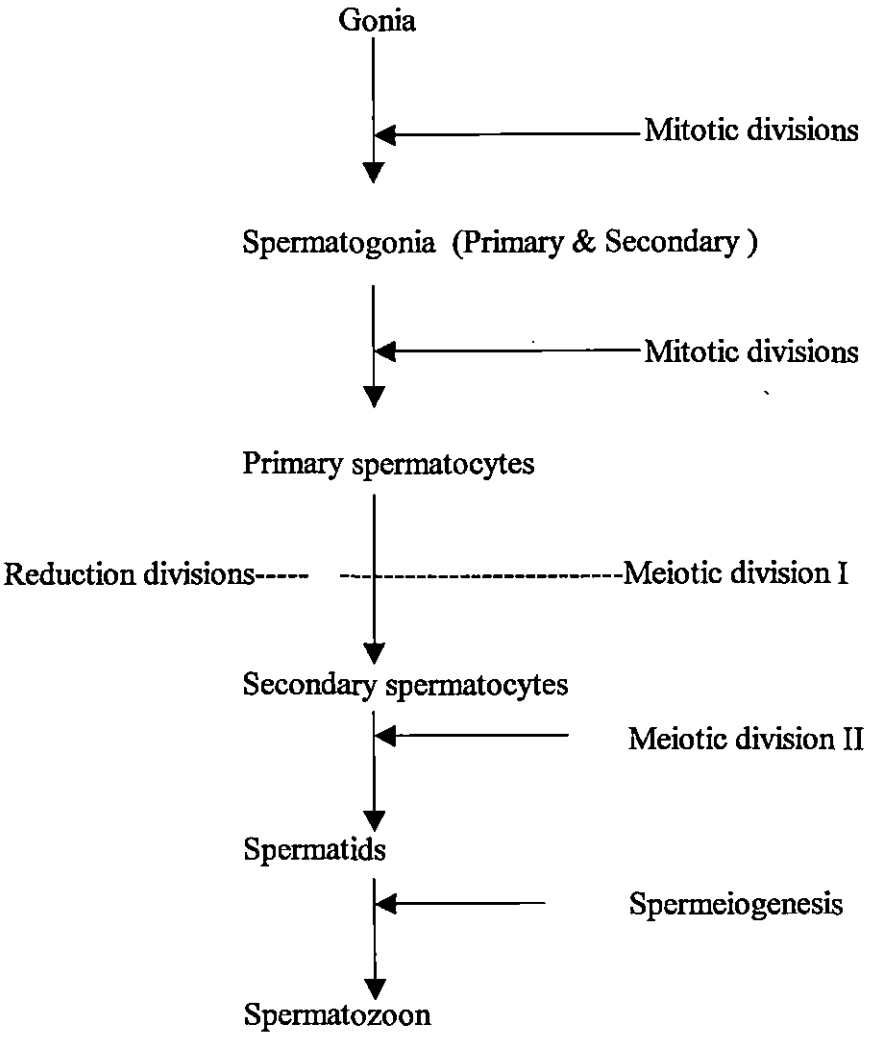
The patterns of cyclical activity and their variations and distribution have been thoroughly reviewed by Van Oordt (1960).

Spermatogenesis is a dynamic process of cellular differentiation (Clermont and Leblond,1953) and is divided into three stages : (i) spermatocytogenesis –which includes development and differentiation of germ cells; (ii) spermatocytic stage- which includes multiplication of primordial germ cells and reduction in chromosome number , and (iii) spermeiogenesis- which includes transformation of spermatids into spermatozoa (Roosen-Runge,1977). The events of spermatogenesis in general found in amphibian species are shown in Table-9.

There are considerable number of notes on investigations on the above parameters in mammals, birds, reptiles and salientian amphibians.. Nuclear polymorphism of anuran species has been observed by (Poska-Teiss,1933). A detailed description of Spermatogenesis in *Rana temporaria* was given by Witschi (1924). Electron Microscopic studies were also made by Brokelmann (1964) in frog. Biochemical studies were made by Van Dongen, Balliex and Geursen (1960).Olivieri (1965); Gould-Somero and Holland (1974); Erickson(1980). Effect of hormones on spermatogenesis and specially on the morphology of the spermiation has been investigated thoroughly by Burgos and Vitale-Calpe (1967); Pisano and Burgos (1971). However, study on sequential events of morphological changes through scanning and transmission electron microscope is meager and obscure (Picheral, 1979). The detailed ultra-structural studies of spermatogenesis in *Bufo himalayanas* were not reported earlier.

Table-9

Events of spermatogenesis



Aims and objectives of the present study

Various authors investigated amphibians and anuran spermatogenesis thoroughly. However, this particular high altitude species, *Bufo himalayanas* lacks proper attention before. So the present study has definite objectives or aims to describe the events of spermatogenesis in this himalayan toad. Specially the ultrastuctural studies of morphology and histology of this particular species may reveal some interesting findings.

The present study has a specific objective to show ultrastructural characteristics and peculiarities, if any, of spermatogenesis which include meiotic stages and the process of spermiogenesis.
The current investigations concentrates on the seasonal cycles of the events of spermatogenesis which is usually evident in the high altitude toad or in the anurans of temperate region (Van Oordt,1960).

In my present study, I want to reveal the basic morphology of the mature spermatozoa as the morphology of the spermatozoa is highly variable in amphibians. Ray et. al.,(1986) and others have reported an unique phenomenon of hypo- and hyperdiploid gonial population in amphibians apart from diploid gonial cells, which may be the reason for the variations in spermatozoan morphology. Thus in the present study an attempt has been made to record variations in sperm morphology as revealed from surface topographies of mature spermatozoa through electron microscopy.

Materials and methodology

Materials

In the present study the adult and larval stages of himalayan toad, *Bufo himalayanas* have been used as material. In case of adults only males are used.

Methodology

1. Study of the spermatogametic cycle:

Animals, both adults and larval stages were sacrificed and their testes or gonadal masses were collected from the dissected specimens (Plate-14, Figure e, f, g). Gonadal tissues and testicular tissues then preserved in normal saline solution or Bouin's solution(aquous) after removing blood vessels and other undesirable tissue parts. The tissues were prepared for cytological, histological as well as for scanning electron microscopy and transmission electron microscopy as per conventional techniques described in earlier chapter.

After the preparations of suitable sections for both types of microscopies, stained sections were examined and photographed for further examinations and cellular identifications.

2. Cytological preparation of testicular cells:

Permanent squash preparation:

The convenient squash technique for studying meiotic chromosomes (Book and Kjessler, 1964; Ohno,1965; Eicher,1966) was followed in the present study.

The testes were dissected out from the specimens and the blood vessels as well as testicular covering were removed from the adult testes. The gonads thereafter were quickly collected in the saline solution (0.67% Sodium Chloride W/V). Then the tissue was cut into small pieces and treated with 0.5% hypotonic solution for 30 minutes. The tissues were then fixed in freshly prepared aceto-alcohol (Glaciel Acetic acid:Absolute Alcohol, 1:3) for 2-3 hours. Then the fixed tissues were transferred to 45% Glaciel Acetic Acid and placed on a clean grease free albuminised slide, coverslipped and squashed by applying gentle pressure of thumb (Smith's technique, Sharma and Sharma,1975).

3. Identification of cell types:

Spermatogonial cells were divided into two categories for the identification of gonial cells.

(a) *Primary spermatogonia*: Smaller in size and contain chromatin in condensed and dusty appearance.

(b) Secondary spermatogonia: Relatively larger in size and contain chromatin in dispersed condition.

Spermatocytes were classified into leptotene,zygotene,pachytene and metaphase stages according to their chromatin condensation. Diplotene and diakinesis, not being clearly distinguishable in histological sections, were recorded in single category (Dip-Met) along with metaphase-I stage.

On the basis of their gross similarity to the mammalian spermatids, the spermatids were classified into:

a) early or Golgi phase- where PAS positive granules are present in the idiosomes.

b) Mid or cap phase- where PAS positive granules were fused and spread like a cap covering the nucleus and,

c) late spermatids- where definite acrosome formed over the nucleus.

4.Study of spermatogenesis by light, scanning and transmission electron microscopy.

a) **Light microscopic study**: Conventional squashed (Sharma and Sharma, 1975) technique was followed with 2% Lacto-aceto-orcein staining.

b) **Scanning electron microscopic study**: After dissection,the testes lobes were collected in normal saline(0.67% Sodium Chloride W/V) solution and teased longitudinally.These were then stirred in saline. The resulting cloudy solution was centrifused, supernatant discarded and the precipitate was fixed in 2.5% gluteraldehyde with 0.1M Sodium cacodylate buffer (pH 7.4) for four hours. After fixation, the materials were transferred to 2% osmium tetraoxide (OsO4) solution in the same buffer for 90 minutes. Then the material was dehydrated through graded alcohol, treated with a mixture of absolute alcohol and amylacetate (1:1) for 20 minutes and then kept in absolute amylacetate overnight. The preparation was placed on metallic stub and coated with gold and was observed under scanning electron microscope (Hitachi S 530). The selected frames were photographed for further investigations.

c) Transmission electron microscopic study: Methodology of this process was discussed in details in the earlier chapter (Chapter- II.).

Observations

Studying mammalian spermiogenesis by the conventional method on the basis of distribution and concentration of acid mucopolysaccharide in spermatids as detected by PAS reaction (Leblond and Clermont,1952) is not convenient for the study of spermiogenesis in *Bufo himalayanas*. The different stages of spermiogenesis have been studied from smear (air dried) and electron microscopical preparations. Ultra-thin sections prepared for transmission electron microscopy were also studied for the same purpose.

[a] Primary and secondary spermatogonia:

The existence of gonial cells in the developing testes has been observed from transmission electron microscope. The gonial cells are similar to the other anurans and exhibit the characteristic features of gonial cells. According to the size and the nuclear condensation, gonial cells may be categorized into primary and secondary spermatogonia. These gonial cells exhibit irregular surface morphology with myriad of convolutions (Plate 30, Figure a). Transmission electron micrograph shows oval or elliptical shape with oval nucleus and uniformly distributed chromatin granules. The nucleus is prominent, spherical; and electron dense in nature. Cytoplasm is homogeneous and contains oval mitochondria, free ribosomes, granular and agranular endoplasmic reticulum (Plate 28, Figure a).

[b] Spermatocytes:

Light microscopy reveals primary and secondary spermatocytes exist together.Primary spermatocytes, under scanning electron microscope,exhibit spherical and centrally placed nucleus without nucleolus. They have irregular surface morphology. Transmission electron microscopy shows electron dense heterochromatin masses of nucleoplasm that are irregularly distributed and condensed. (Plate 28, Figure a, b). Secondary spermatocytes are more condensed and smooth in appearance (Plate 28, Figure b).

[c] Spermatids

For the sake of easy identification from morphological structures, spermatids undergoing spermiogenesis are categorized into :

(a) early or round shaped spermatids
(b) Mid or oval shaped spermatids
(c) Late or elongated spermatids (elliptical or rod shaped)
(d) Mature spermatozoon.

From the light and electron (scanning and transmission) microscopic observations, the following features of the spermatids undergoing spermiogenesis have been recorded

(i) Each of **round spermatids** has a compact round nucleus and a thin rim of cytoplasm around its nucleus. Under scanning electron microscope these round and early spermatids show very irregular surface texture. Invaginations and buldges are the most prominent features (Plate 30, Figure B). Through TEM, these early spermatids appear as round shaped bodies with prominent nucleus (10μm) with nucleolus having electron dense granules.

(ii) A **mid spermatid** has an ovoid or elongated body with oblong nucleus. Its cytoplasm is condensed in nature. Under scanning electron microscope, apart from its general features, it shows smooth appearance. No invagination or protrusions are found (Plate 30, Figure C, D).Through transmission electron microscope, it has been revealed that acrosome cap spread over the primary surface of the spermatids. So a smooth and continuous texture is superficially observed (Plate 28, Figure c, d).

(iii) At the **late stage**, neck part originates from the more broad posterior end as irregular bands. Under scanning electron microscope, this irregular bands appear distinctly and under higher resolution neck part is found to be featured by transversely arranged tubules in a zig-zag fashion (Plate 30, Figure E, F, G). This is probably due to spirally arranged multiple cords at the neck region. Further condensation of the apical region along with the elaboration of the neck region is found under the transmission electron microscope. Cytoplasmic organelles were also elaborated at this stage (Plate 31, Figure c, D, E).

[d] Spermatozoon

The **mature spermatozoa** are found at the end of spermiogenesis . The spermatozoa show appearance of two axial filaments coiled upon one another. The head part becomes more elongated to assume a rod like shape, tapering at the apex area (Plate 30A, Figure A, B). Under scanning electron microscope a barb like perforatorium appears from the pointed anterior end (Plate 32, Figure b). The head measures about 18 micromillimeter in length, a short neck and a long filamentous and twin tail of about 25 micromillimeter in length. Under transmission electron microscope, these features revealed some more structural details and also about cytoplasmic inclusions which were seen in the ultra-thin section (Plate 33, Figure A, B, C, D).

The mature spermatozoa shows polymorphism in sperm morphology as revealed from surface topographies specially from scanning electron microscopy. Under scanning electron microscope, it was evident that sperms have different shapes and morphologies. Apart from normal spermatozoon, I have recorded at least four different types of spermatozoon as followings:

a) Spermatozoon with ovoid head, without perforatorium and ill-recognizable neck region (Plate 30A, Figure C).

b) Spermatozoon with sickle shaped head, without neck and tail (Plate 30A, Figure E).

c) Spermatozoon with round and highly conspicuous head and a long tail (Plate 34, Figure A).

d) Spermatozoon with a tail consisting of a single axial filament (Plate 30A, Figure D).

PLATE 30

Legends

Scanning electron micrographs of different developmental stages of spermatogenesis

A. Primary spermatogonia with irregular surface

B. Round spermatids with myriad convolutions

C. Mid-spermatids with smooth surface

D. A mid spermatid (ovoid) with smooth surface

E. Spermatid with a bulging protrusion

F. Late spermatid with growing neck region

G. Later spermatid with developing neck and tail region

PLATE-30

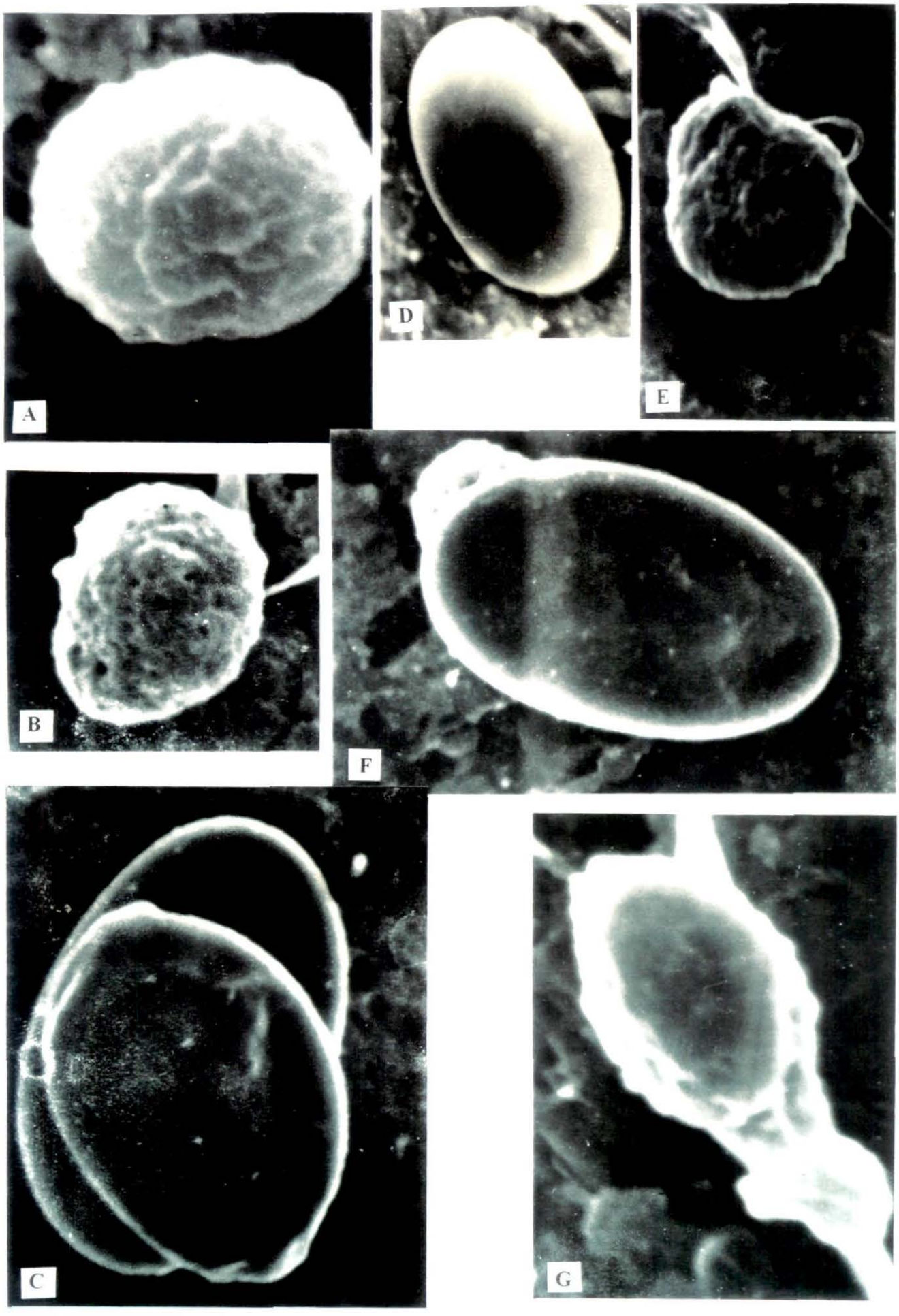


PLATE 30A

Legends

Scanning electron micrographs of mature spermatozoa and different atypical spermatozoa

A. A normal mature spermatozoon showing head, neck and typical bi-flagellate tail
B. Enlarged view of a spermatozoon's head, mid-piece and tail
C. An atypical spermatozoon with ovoid head, ill-developed neck and without perforatorium
D. An atypical spermatozoon's head, ill-developed neck and mono-flagellate tail
E. An atypical spermatozoon with sickle shaped head, without neck and tail
F. A normal spermatozoon with a spermatid

PLATE-30 A

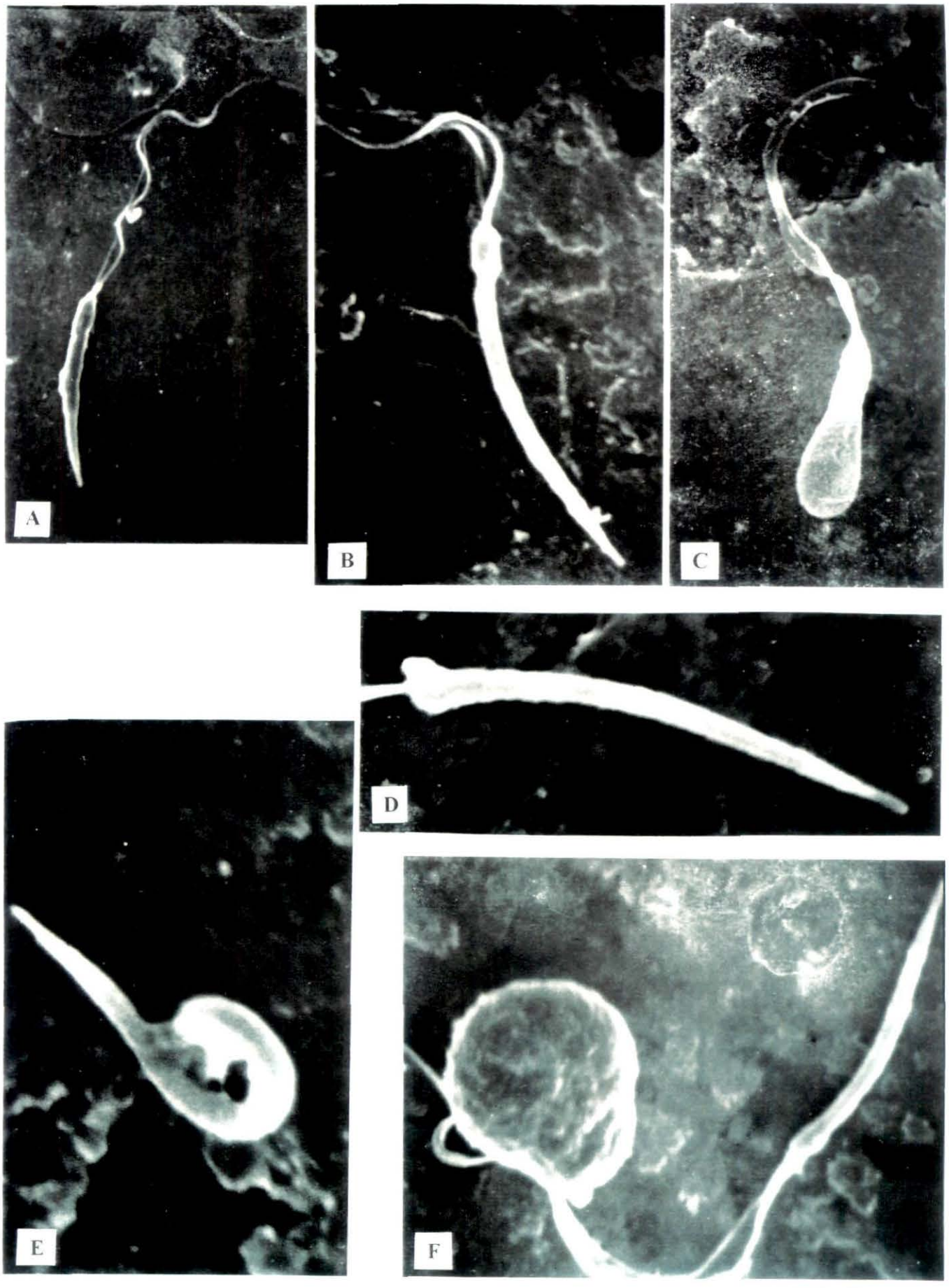


PLATE 31

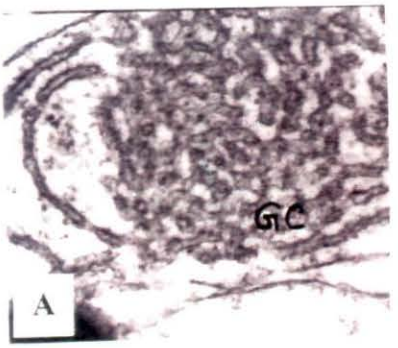
Legends

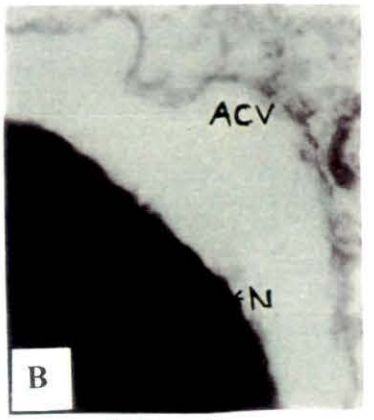
Electron micrographs of different parts of maturing spermatids and mature spermatozoa

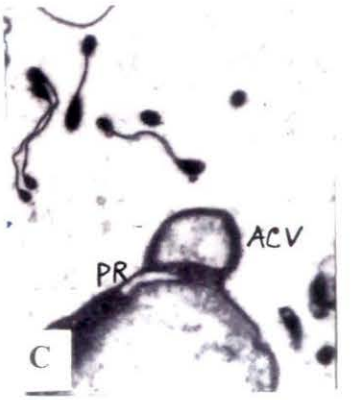
A. Golgi complex of an early spermatid
B. Acrosomal complex developing in late spermatid
C. Origin of acrosomal cap and perforatorium of a spermatozoon
D. Elongated and late spermatid with developing neck region
E. Late spermatids
F. Pre-mature spermatozoon
G. Maturing spermatozoon (posterior part)
H. Maturing spermatozoon (anterior part)

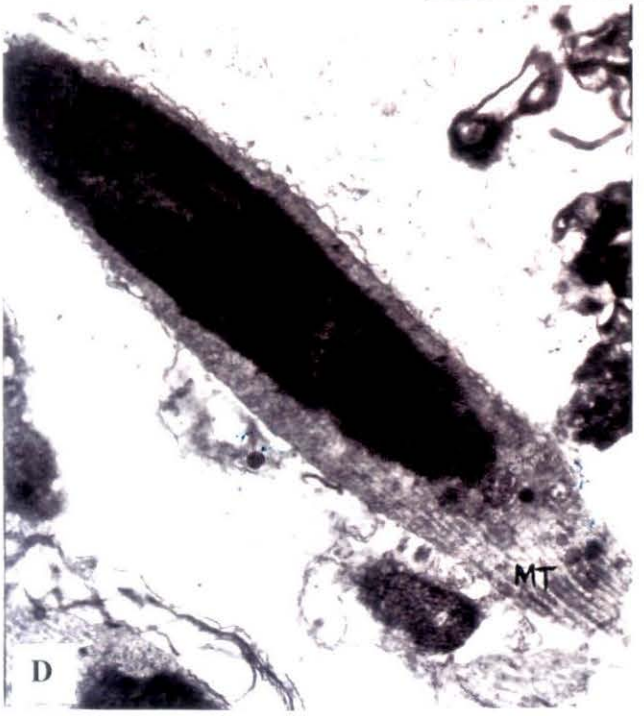
N- Nucleus GC- Golgi complex ACV- Acrosomal vesicle
PR- Perforatorium MT- Microtubules TL- Tail AC- Acrosome
M- Mitochondria DN- Developing neck

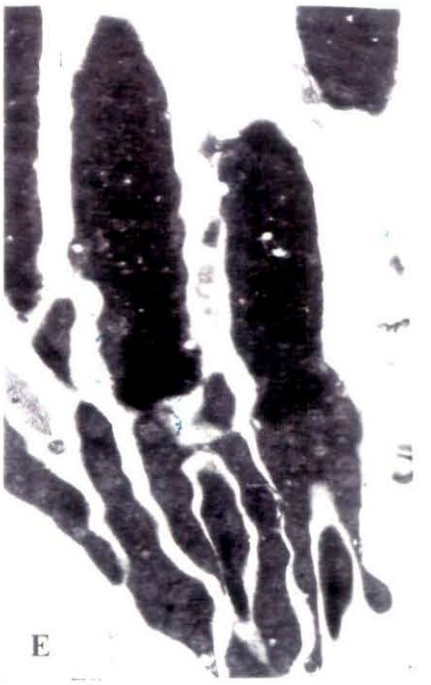
PLATE-31

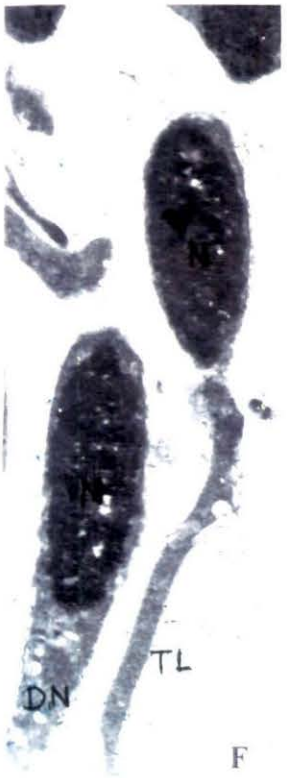












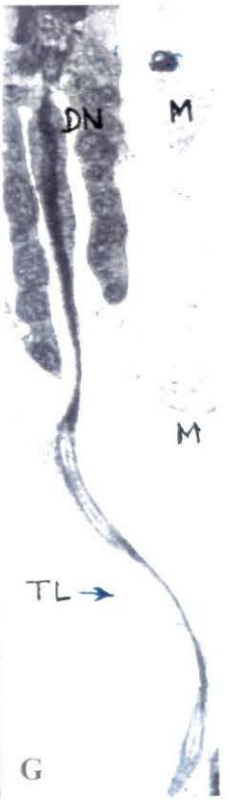


PLATE 32

Legends

Light and scanning electron micrographs showing different parts of mature and normal spermatozoa

Fig.a. Light micrograph of a normal, mature spermatozoon showing typical features of this species

Fig.b. Enlarged view of the anteriormost part of the spermatozoon showing a barb-like perforatorium

Fig.c. Scanning electron micrograph of a typical spermatozoon

Fig.d. Part of the tail highly enlarged to show bi-flagellate tail connected by a membrane medially

Fig.e. End portion of the tail highly enlarged to show the discontinuation of the membrane towards the end

PLATE-32

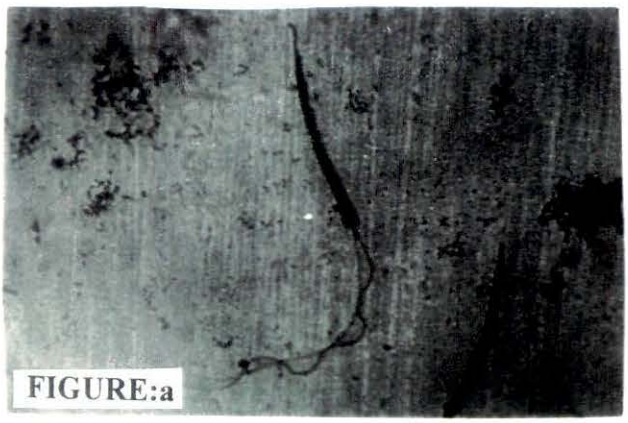


FIGURE:a

FIGURE: b

FIGURE: c

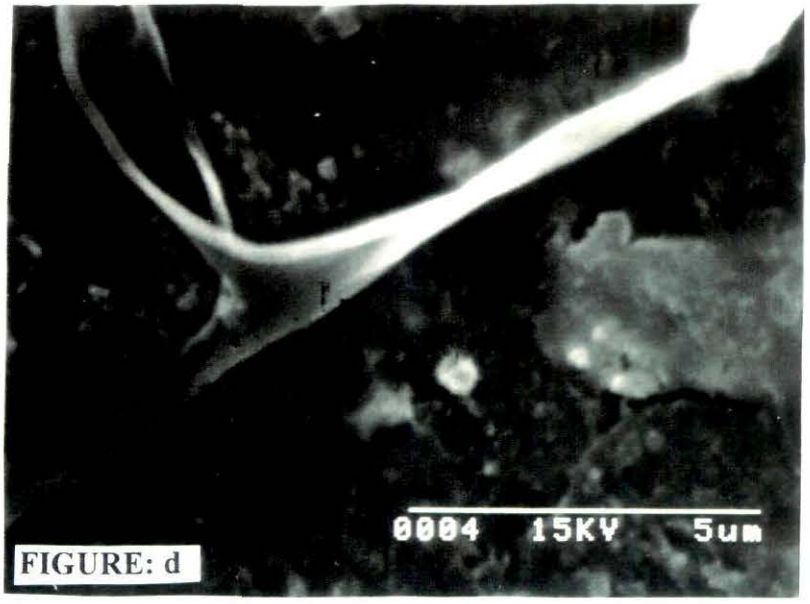


FIGURE: d

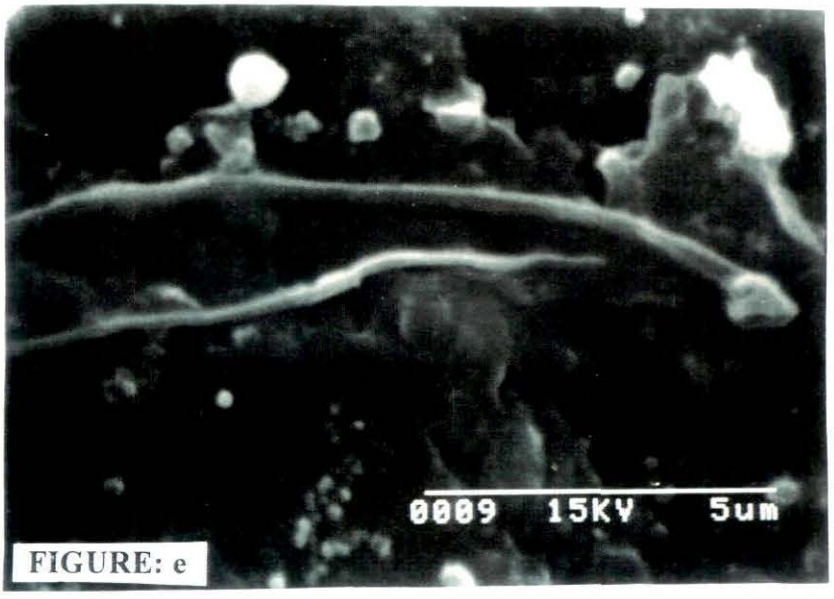


FIGURE: e

PLATE 33

Legends

Electron micrographs showing different parts of mature spermatozoa

A. Enlarged head of a spermatozoon showing perforatorium, acrosome and elongated nucleus
B. Numerous sperm heads in sperm bundle in cross section
C. Section through middle pieces of spermatozoa
D. Section through tail portion showing microtubules in cross section

N-Nucleus PR- Perforatorium AR- Acrosome SPH- Sperm heads MT- Microtubules

PLATE-33

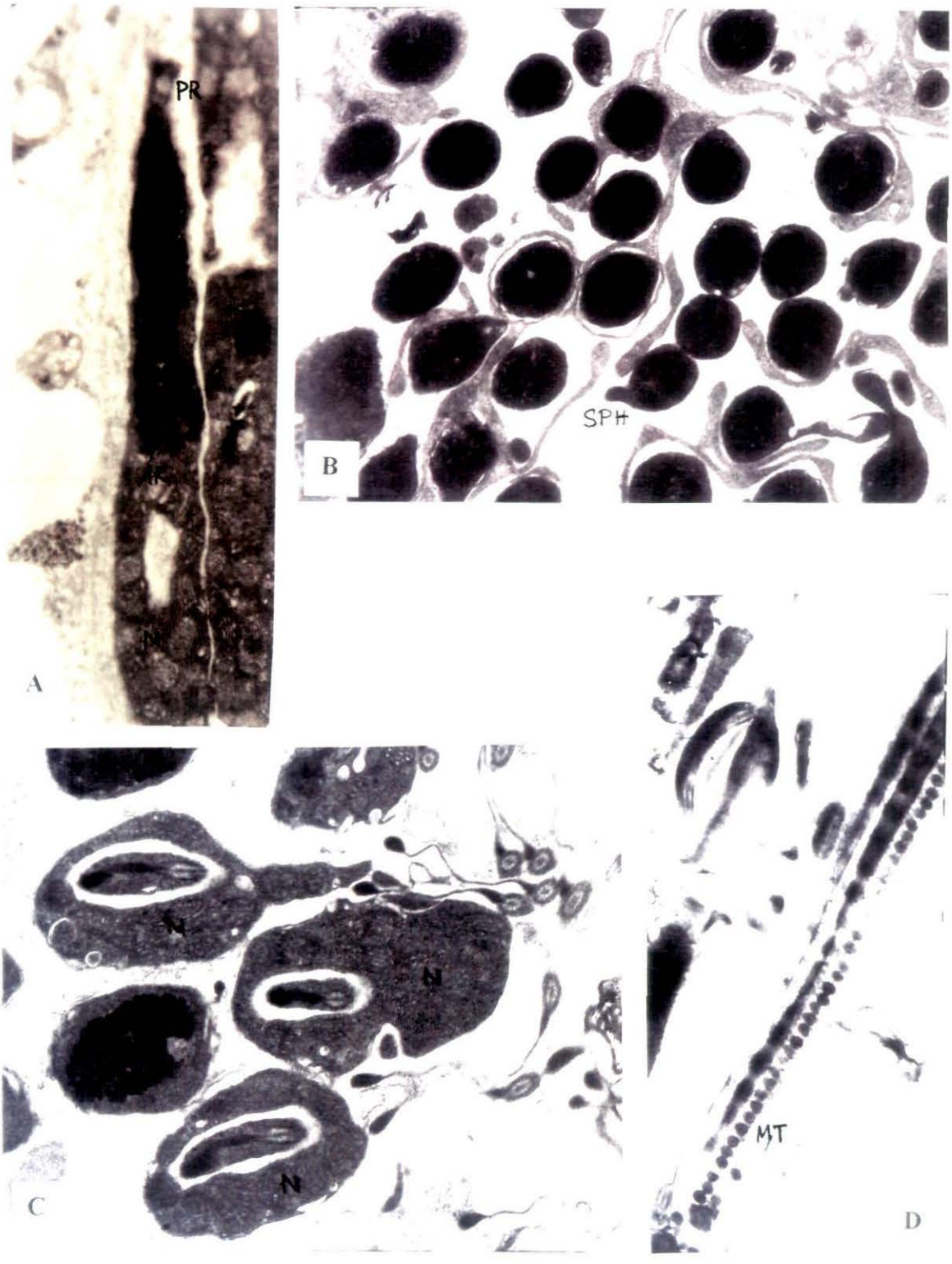


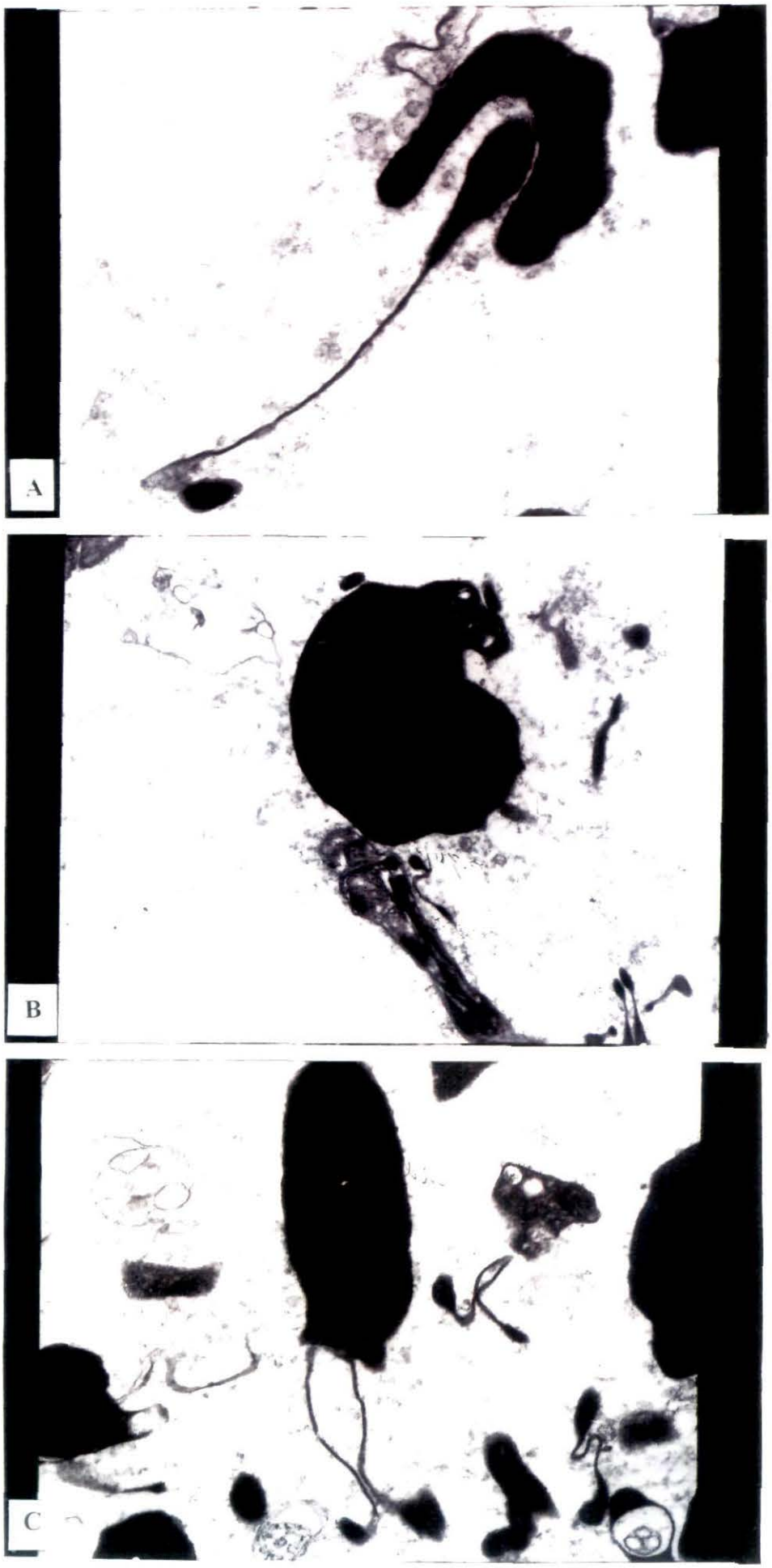
PLATE 34

Legends

Electron micrographs of some atypical spermatozoa

A. Megacephalic sperm with large and conspicuous head
B. Microcephalic sperm with highly condensed head region
C. Globular sperm with large oval head and ill- developed neck and tail region

PLATE-34



Discussion

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.

The identification of various stages of spermatogenesis in amphibians, particularly anurans, is done following the description of van Oordt (1956). However, in recent years, a modified method of qualitative assessment of spermatogenetic activity is adopted which is as follows:

Stage O: Primary spermatogonia
Stage I: Secondary spermatogonia
Stage II: Primary spermatocytes
Stage III: Secondary spermatocytes
Stage IV: Spermatids
Stage V: Sperm bundles attached to the Sertoli cells.

The primary spermatogonia are the largest germ cells located adjacent to the basement membrane of the seminiferous tubule. Such cells are with voluminous eosiniphilic cytoplasm and with an irregular nucleus. Rastogi et.al. (1985), based on the nuclear characters, recorded two types of primary spermatogonia in the adult testis of *Rana esculenta* and designated them as pale and dark types of primary spermatogonia comparable with A0 to A4 types of primary spermatogonia of mammals (Ray et.al.,1975; Ray et.al., 1978, 1979 and 1980).

The primary spermatogonia divide mitotically to form secondary spermatogonia. **The secondary spermatogonia** are smaller than primary spermatogonia and both cytoplasm and nuclei are basophilic in nature.

The secondary spermatogonia again undergo mitosis to form **primary spermatocytes**. The primary spermatocytes are larger than secondary spermatocytes with eosiniphilic cytoplasm and basophilic nuclei. Meiotic features are noted in the primary spermatocytes. Primary spermatocytes are found in the central part of the cross section of the testis or found attached to the wall of the tubule. The primary spermatocytes usually form a nest and small intracellular vacuoles may be detected in the cell nest (Saidapur,1989).

After the first meiotic division, primary spermatocytes give rise to **secondary spermatocytes**. They are smaller than primary spermatocytes with eosinophilic cytoplasm, basophilic nuclei and condensed chromatin. The intracellular vacuoles increase in size and become more evenly distributed. The cell nests are usually situated in the central part of the cross section of the testis (Saidapur,1989).

After the second meiotic division, the spermatids are formed. The **spermatids** are small globular cells, distinctly smaller than secondary spermatocytes, with eosinophilic cytoplasm and a spherical basophilic nucleus (Saidapur,1989).

With the initiation of spermiogenesis, the spermatids change morphology from globular to oval to elliptical and ultimately more and more elongated. The intracellular vacuoles first fuse into one big central vacuole and the cells are situated against the wall of the cysts. During spermiogenesis, the heads of the **maturing sperm** cells are found embedded in the Sertoli cells. Such cell nests are often found attached to the wall of the seminiferous tubule (Saidapur,1989).

The various cell nests may or may not be present in the testis round the year depending on the species or pattern of the spermatogenetic activity. In specieses like *Rana cyanophlyctis, Rana hexadactyla, Bufo melanostictus* which exhibit continuous type of spermatogenesis, all the stages are present throughout the year. Whereas *Rana tigerina, Bufo marinus* exhibit potentially continuous spermatogenic activity or discontinuous type as in *Rana temporaria* , the various stages of spermatogenesis undergo distinct changes.

The event of spermiogenesis has been studied in a number of amphibian species following conventional technique (Sharma and Sekhri,1955; Hirschler, 1928; Mc Gregor, 1899; Terni, 1914; Sharma and Dhindsa,1956; Baker,1963; Bandopadhyay,1977; Roy,1978; Midya et.al,,1981; Mallick,1987; Roy,1989).

Sharma and Dhindsa (1955) have provided an excellent account of spermiogenesis in Indian toad, *Bufo stomaticus*. At early spermatid stage deeply stained Golgi granules uniformly spread out throughout the cytoplasm. During the maturation of the spermatid the Golgi granules begin to fuse to form bigger granules which in turn form still bigger granules by fusion. One of these bigger granules now come in close contact with the end of the nucleus which is opposite the axial filament and form proacrosome. To begin with the proacrosoome satins deeply and uniformly. But as it grows it becomes differentiated in an outer chromophilic cortex and inner chromophobic core. As the maturation of the sermatid continues, its nucleus begins to elongate with the proacrosome at its anterior end and the cluster of small mitochondria at the posterior end . The proacrosome form the acrosome; it first becomes triangular and then pointed and needle like. It may be noted that no acrosomal granule is formed in the toad (Diagram 11). The ripe spermatozoon possess a deeply stained head with cylindrical nucleus and pointed acrosomal tip, small middle piece and two axial filaments joined together by a rudimentary undulating membrane.

Burgos and Fawcett (1956) have given a remarkable account of the electron micrographs of the maturing spermatids of *Bufo arenarum* including formation of the acrosome changes in the nucleus, formation of perforatorium, centriole and tail and undulation membrane. Their account confirms the main observations of Sharma and Dhindsa (1955) with light microscopy.

Burgos and Fawcett (1956) described that at the very onset of spermiogenesis, the spermatids lack a conspicuous Golgi complex such as occurs in the mammalian spermatids; instead it possesses one or many very small aggregation of spherical vesicles of varying sizes. According to the authors,no acrosomal granule is differentiated in the acrosomal vesicles, contrary to the mammalian spermiogenesis where one or more acrosomal granules are found. The acrosomal granules gradually increase in area of contact with the nuclear membrane, extending further down over the elongated nucleus at its anterior pole.

Burgos and Fawcett (1956) have described progressive condensation of the nucleus during spermiogenesis. At the early stages, the nucleus is spherical and in the course of spermiogenesis it becomes ovoid, then elongated and finally rod like in the spermatozoon. Burgos and Fawcett (1956) have described some striking changes in the fine structure of the nuclear content. At the early spermatid stage the nuclear contents are " homogeneous" and consists of very fine granules of about 100 to 150 Å in diameter. Later these very fine granules become randomly distributed and form coarse granules. However, the authors have not provided any explanation of such changes and their fate.

Burgos and Fawcett (1956) described for the first time a "perforatorium" in the maturing spermatid of the toad. They described that in the later stages of spermiogenesis, when the nucleus is progressively becoming elongated, a narrow, conical cleft appears between nuclei and head cap as assign of perforatorium appearance. It consists of a number of coarse dense strands which arise around the tapering end of the nucleus to converge in front of it to form a dense pointed structure.

Burgos and Fawcett (1956) described two centrioles of cylindrical shape in the maturing spermatids of toad. In cross section, each centriole appears "as thick walled moderately dense ring with a scalloped outline". About nine scallops are distinguished at the periphery and within each of these one or more minute mass of low density has been described. Centrioles have typical structure as found in others.

Sharma and Dhindsa (1955) first described in *Bufo* the existence of two centrioles in the middle piece which are located side by side. Each gives rise to individual axial filament.

Burgos and Fawcett (1956) described in the axial filament in case of toad of a bundle of 20 submicroscopic longitudinal fibrils. Of these, there is a centrally located fibril and nine pairs of evenly spaced circumferential fibrils. All the 20 fibrils appear tubular in cross section since their core seems to be of low density.

The undulating membrane is made up of a thin ribbon like band of the dense fibrous substance (Burgos and Fawcett,1956)

In the present investigation, all the changes that occur in a normal spermatogenic event have a conformity with the earlier observations made from light and electron microscopy. However, the following changes may be described as 'unique' for the species:

(i) Presence of primary and secondary spermatogonia with rough and irregular surface morphology. Cytoplasm homogeneous with oval mitochondria, free ribosomes and other organelles.

(ii) Spermatogonial cells exhibit hypo- and hyperdiploid gonial cells along with normal diploid gonial cells. These polyploidy may be an important factor for sperm polymorphism in this species.

(iii) Spermatids also exhibit different types of stages according to the degree of development. Anuploidy may also contribute a factor for the variations in the spermatids.

(iv) Mature sperms show polymorphism clearly in their morphology. Few types of morphologically different spermatozoa is an unique characteristic of this species.

(v) A mature normal spermatozoon exhibits an unique biflagellate tail with undulating membrane present in between except the posterior-most end. The flagellum is provided with inner core of axial filament which has submicroscopic microtubules.

(vi) Neck region or middle piece part of a mature spermatozoon is provided with a pair of centrioles. These centrioles are the site of origin of the two axial filaments or flagella.

CHAPTER IV

SPERM POLYMORPHISM

CHAPTER IV

SPERM POLYMORPHISM

CONTENTS

INTRODUCTION

The polymorphism of sperm i.e. existence of more than one type of morphological varieties of sperms in a species is a subject of long discussion and thought. Siebold (1836) discovered two types of spermatozoa in pond snail *Pabedina* sp.. Since then many cases of conspicuous polymorphism have been found in invertebrates and in some vertebrates. However systematic studies on this phenomenon have not been made until very recently.

In an acoelan flatworm, Hendellberg (1969) found two kinds of spermatozoa , a very peculiar "typical" one with two flagella and a smaller atypical one which during spermiogenesis cast off its nucleus with residual cytoplasm and remains in the semen as short cytoplasmic rod. Earlier Meves (1903) recorded diminution or loss of chromatin as a frequent characteristic of atypical sperm.He coined the term 'eupyrene' for cells with normal chromatin content; 'oligopyrene' for those with subnormal amounts and ' apyrene' for cell without chromatin . Chromatin may be absent or occur in abnormal amount in flatworms, mollusks, annelids, insects and vertebrates (Roosen – Runge,1977).

Sperm polymorphism has also been found in a number of amphibian forms (Ray 1978; Ray et.al.,1989; Roy and Ray 1994; Mukherjee et.al.,1999; Patra et.al., 2001).

AIMS AND OBJECTIVES

In the present study an attempt has been made :

1. To record various forms of sperms and their characteristics at light microscopic level.
2. To find out surface topography of each such form.
3. To account for various ultra-structural features, and
4. To workout the possible explanation of origin of various forms, behavior and functional aspect during the reproduction process, i.e. to accomplish normal fertilization.

MATERIALS AND METHODOLOGY

MATERIALS

Adult male Himalayan toad ,*Bufo himalayanas* males were collected during two distinct phases of reproduction, i.e. during breeding season (May to August) when spermatogenic activity is high and during non-breeding season (October to February) when spermatogenic activity ceases totally in high altitude forms and takes place in a low wave in the specimens inhabiting in low altitude (1500-2000 mt.). This is done in order to study the seasonal effect, if any, on sperm morphology.

METHODOLOGY

(1) Collection of spermatogenic cells:

Spermatogenic cells were collected following the differential centrifugation technique proposed by Roy *et.al.*,(1989). The testes were dissected medially and tissue materials were obtained, minced by a pair sharp edged scissors into small fragments in amphibian saline (pH 6.9). The tissue materials were suspended for a brief period to obtain a milky white suspension and were centrifuged at 100rpm to remove the debris. The supernatant was immediately transferred to gluteraldehyde-paraformaldehyde mixture for preservation.

A portion of the supernatant was centrifuged at 1000rpm, 2000 rpm, 3000 rpm and 4000 rpm to collect various stages of spermatogenic cells depending on their sizes. The residual supernatant centrifuged with different grades of solution ranging from 2.5% - 5.0 % to obtain pure sperms and were examined under light microscope to evaluate the degree of contamination. The best result obtained at around 3.0% - 3.5% gluteraldehyde solution.

(2) Fixation of cells for SEM and TEM study:

The details of the process of fixation was described in the chapter II of this thesis.

(3) Method of studying:

Sperm morphology

(A) Light microscopic study:

A drop of milky white supernatant suspension was spread carefully over a clean grease-free slide and after air drying the preparation was fixed with methanol for one minute. After drying Leishmann stain (E.Marck) was added and carefully spreaded with the help of a clean glass rod to cover the film entirely. This was kept for 10 minutes and then double distilled water was added in equal volume of the stain for maturation. After 15 minutes the preparation was washed thoroughly in running water for a considerable period of time.

Then the preparation was made dry and covered with DPX (E.Marck) mounting medium for permanent mounting with a glass coverslip. Observations were made under a light microscope (Leitz).

(B) Scanning electron microscopic study:

The detailed description has been given in chapter III of his thesis.

(C) Transmission electron microscopic study:

In this case the initial process of tissue preparation is somewhat dissimilar with the normal gonadal tissue preparations. As the spermatozoa were present within the fluid suspension a different procedure for the tissue block preparation was made. The maximum pure suspension with a good number of expected spermatozoa was fixed within a capsule made from araldrite mixture for block making. Then the block was cut as per procedure described in details in the chapter II of this thesis.

OBSERVATIONS

A. Light microscopic

A mature spermatozoon of *Bufo himalayanas* shows typical characteristics of an anuran sperm. It has a slender, anteriorly tappering heterochromatic head with a long slender deeply stained heteropycnotic nucleus . The nucleus gradually flattens from the tip to the broad base or posterior end. The tip of the nucleus covered with the acrosomal material, somewhat conical in shape and laterally overflows on the nuclear tip. Acrosome is achromatic in nature and lightly stained. Acrosome is also provided with a typical hook like curved structure or acrosomal barb at its anteriormost end (Plate 32, Figure a & b). Size of the head nucleus is 29X2 μm in diameter.

The middle piece is short and stout and is provided with centrosomal bodies within. From this part a flagellar tail emerge. The tail is typically biflagellate, which is also an important characteristic of this species. The length of the two long filamentous flagella is unequal (Plate 32, Figure a). In this work it has been observed that different types of morphologically non-identical mature sperms are present. Thus polymorphic nature of the spermatozoa is evident in this species.

B. Scanning electron microscopic

Under SEM all the morphological details revealed under light microscope could be visualized. The head, neck, middle piece and tail part are well documented and recognizable. **Head** is elongated with compact, elongated electron dense nuclear part of uniform diameter. Head surface is smooth

i.e. without any protrusions or accessory structures as found in related species. The anterior part of the head elongates gradually to form a slender structure called acrosome. The acrosomal barb is hook like with a cup like depression at its anterior-most end (Plate 32, Figure b).

The middle piece is situated in the somewhat flattened posterior portion and is laterally bulged out suggesting the accumulation of mitochondria and centrioles within (Plate 32, Figure c).

The **tail piece** is biflagellated and is connected by a membrane medially which is discontinuous at the posterior end. The end portion of the flagellum bears a knob like structure (Plate 32, Figure d & e).

Like light microscopy, several morphological varieties in terms of acrosomal vesicles, head structure and tail are also documentable (Plate 30A, Figure A,B, F).

C. ***Transmission electron microscopic***

Under transmission electron microscope similarly well differentiated head, neck and tail parts are visible.

Head is conical in shape, tapering towards the anterior end and with compact chromatin material. However in transverse sections, electron dense granules are found throughout the length of the nucleus and has different degree of compactness at various level (Plate 35, Figure A, B).

The anterior part of the head terminates in a electronlucent acrosomal cap. The cup shaped acrosomal barb is also visible and exhibits wooly appearance and with several discontinuous filaments in it (Plate 35, Figure D).

The middle part is elongated with median electron lucent vacuole surrounded by heavily packed multilayer of distinctly visible mitochondria that are almost circular in cross section. The two centrioles respectively called proximal and distal centrioles are also visible (Plate35,Figure C, E, F).

The proximal centriole is lodged in the nuclear pocket situated at **posteriormost part** of the head nucleus. The distal centriole is situated at the base of the middle piece and is not perpendicular to the proximal centriole. In tranverse section both the proximal and distal centrioles exhibits typical 9+2 arrangement of microtubules (Plate 35, Figure F, G).

The tail is biflagellated and each flagellum consists of an axial filament made up to 20 (18+2) sub-microscopic longitudinal microfibrils. The presence of undulating membrane has been documented by the presence of a wire like structure with electron dense materials at both ends (Plate 35, Figure G, H).

Apart from typical spermatozoon structure, megacephalic sperm with condensed head is evident. Similarly globular as well as microcephalic sperm head were also observed under transmission electron microscope (Plate 34, Figure A, B, C).

PLATE 35

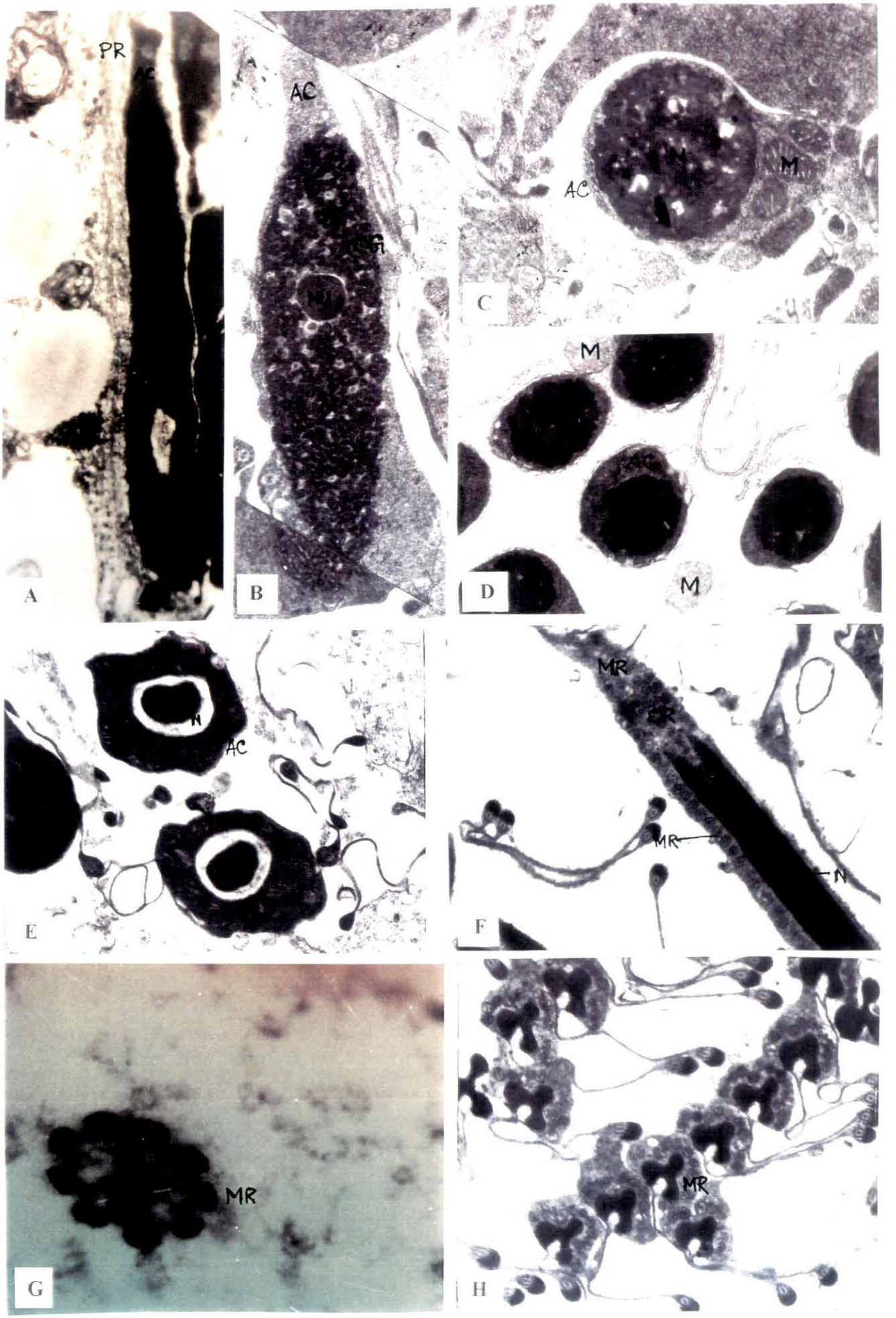
Legends

Electron micrographs of different parts of mature spermatozoa

A. A mature sperm with head and middle piece
B. Head shows compactation of nucleus and acrosomal materials
C. Head in cross section showing acrosome and middle part with a number of mitochondria
D. Heads in cross section in a sperm bundle showing nucleus and acrosomal envelop
E. Middle piece of a mature sperm in cross section showing centrosomes and microtubules
F. Tail showing filaments with typical (9+2) arrangement through its cross section
G. Tail pieces of mature sperms in a sperm bundle through cross section

N- Nucleus AC- Acrosome PR- Perforatorium NU- Nucleolus
CG- Chromatin granules M- Mitochondria MR- Microtubules

PLATE-35



DISCUSSION

Sharma and Dhinsda (1955) first provided structural details of ripe spermatozoa of toad, *Bufo stomaticus* and described that such spermatozoon consists of deeply stained head with a cylindrical nucleus and a pointed needle like acrosome situated at its anterior tip . Immediately behind the head and with its intimate contact a small deeply staining middle piece with clumped mitochondria and two centrioles lie side by side, were also described. Two axial filaments joined together by a rudimentary undulating membrane and each such filament arises from its own centriole.

Burgos and Fawcett (1956) has given a remarkable account of electron micrographs of the maturing spermatids of *Bufo arenarum* including the formation of acrosome, changes in nucleus, centriole, tail and undulating membrane. They first described the existence of a perforatorium, hitherto not described by any author in genus *Bufo* . These observations confirmed the earlier findings of Sharma and Dhindsa (1955) with light microscopy (Diagram-11).

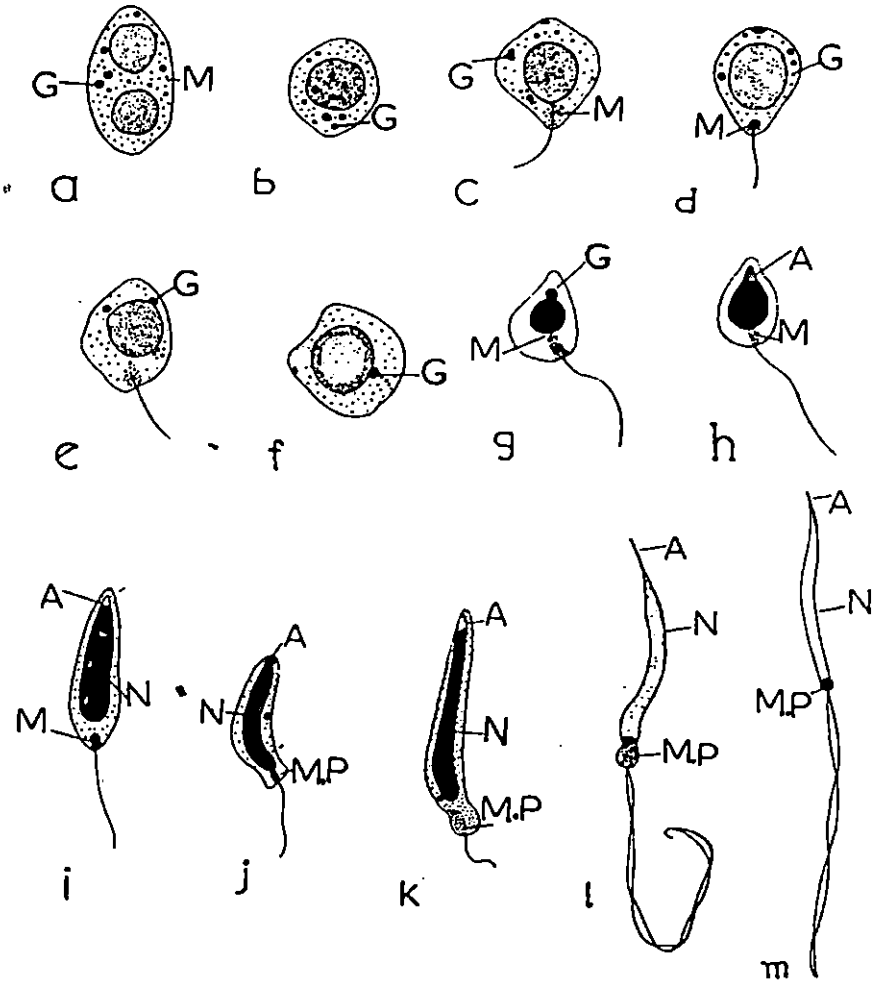
The present observations on *Bufo himalayanas* similarly observe confirmatory of the earlier descriptions of the mature spermatozoa of various species of genus *Bufo.*

In many cases of polymorphism, the aberrant types are morphologically very characteristics and sometimes show a behavior which suggest that they have become functional components of the reproductive process, although they have never been seen to accomplish normal fertilization. In the rotifer *Asplanchna* a typical spermatid produces immotile 'rod spermatozoa' which are not cells but cellular products of spermatids (Kochler and Birky,1966), the function of these rods is unknown. It may be related to the peculiar intradermal fertilization of these animals. Gupta (1964) reported the alternating development of two kinds of spermatids in *Diatomus*, one with and one without a chromatoid body. The first evelops into the typical fertilizing sperm and the second into the 'swelling spermatozoa'.

Various authors have reviewed the origin of atypical spermatozoa. Ankel (1924,1930,1933 and 1958) was able to show that in many species of viviparous *Chenopus vermetus* and some others the atypical series is recognizable already in the spermatogonial stage. In *Bythinia* and closely related species a atypical spermatozoa is morphologically very similar to typical ones, but they are oligo- or hyperpyrene and all of them arise in meiosis. Roy and Ray (1984) however recognized that in vertebrates there is a possible existence of different clones of spermatogonia mother cells and a concept of hypo-hyper diploidy was suggested. In *Bufo himalayanas* three such clones of spermatogonial cells were recorded having normal diploid chromosome sets (2n=24), hyper-diploid chromosomal set (2n=26/28) and hypo-diploid chromosomal set (2n=20-22). They recorded that there was a preponderance of particular chromosome 'duplication' or 'loss'. Roy and Ray (1989) have also established similar such clones of spermatogonial cells in the Himalayan newt, *Trilototriton verrucossus.*

The total cell loss during spermatogenesis in every case is the result of a series of specific degenerative events which occur at several identifiable stages. There are certain critical phases at which a proportion of gamets may begin to detiorate. Meiosis, specially in leptotene, zygotene and diakinesis is most frequently identified as a critical event. Spermatogonial mitosis, perhaps only certain one in the sequence, is a close second.

DIAGRAM : 11



SPERMATIDS AND SPERM OF TOAD
(after Sharma and Dhindsa, 1955)

G- Golgi bodies M- Mitochondria

N- Nucleus M.P.- Middle piece

A- Acrosome

Dalcq (1921) concluded that the most probable cause in an imbalance in the mechanism of division and the distribution of chromosomes. The failures in gene distribution do not always lead to immediate cell death. Often their consequences lead to the phenomenon of polymorphism. The various stages of spermatogenesis at which cell death occurs are summarized in Table-8.

In recent years, germ cells' degeneration has been equated with apoptosis. Miething (1992), Sinhahikim (1997) and others opined that germ cell death during spermatogenesis is a classical example of apoptosis as found in other developmental events like organogenesis of liver, kidney, lymphocytes, nerves cells and others.

The sperm morphology has been evaluated in the context of serial and coordinated action of sperm specific genes and a number of mutations, artificial and 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 these proteins by the spermatogonia. When BMP8B reaches a critical concentration, the spermatogonia differentiate into spermatids (Zhoe et.al.,1996). Mice lacking BMP8B do not initiate spermatogenesis. Similarly in man DA2 gene locatd on the long arm of Y-chromosome when deleted causes infertility. The DA2 gene is exclusively expressed in the gene cell line, specially in the spermatogonin and it appears to encode an RNA binding protein (Reijo et.al.,1995; Menke et.al.,1997). In *Drsophila,* similarly,two genes Rb97D and boule are essential for spermatogonial kinetics and

when mutated causes degeneration spermatogonia and the cells do not enter meiosis (Karsh-Mizrachi and Haynes,1993; Eberhart et.al.,1996). Some genes are also specific for male meiosis (Hoyle and Raff,1990; Nishioka et.al.,1990 etc..) In addition to gene transcription in diploid cells during meiotic prophase, certain genes are transcribed in the spermatids (Palmites et. al.,1984; Peschon et.al.,1997; Nantel et. al.,1996).

In the present investigation the frequency of atypical sperm is biologically significant. The typical sperms in these species are produced in large quantities in a comparable manner to fertilize huge number of eggs produced during the breeding seasons. But till date none has attempted to visualize the process along with the normal tadpoles vis-à-vis atypical tadpoles that are also produced and survive in the nature. The origin of such atypical forms of the same species may suggest a continual case of natural selection in action. The atypical toads may not survive in the existing situations but may be fit for a new condition in future. Rossen-Runge (1977) thus suggested "like the polymorphism of sperm structure by all appearances the effect is large and highly significant and deserves much more attention than it has received hitherto".

CHAPTER - V

THE KINETICS OF SPERMATOGENESIS

CHAPTER - V

THE KINETICS OF SPERMATOGENESIS

CONTENTS

INTRODUCTION

The investigation on the duration of the meiotic events and spermatogenesis has revealed a number of interesting findings. The cycle of seminiferous epithelium in mammal is a phenomenon of fixed duration (Clermont and Trot, 1969). In rat testis, hypophysial hormones altogether (Clermont and Harvey,1965) and increase in temperature (Chu et. al. 1974;Waites and Ortavant,1976) do not influence the rate of development of the germ cells and the duration of the cycle.

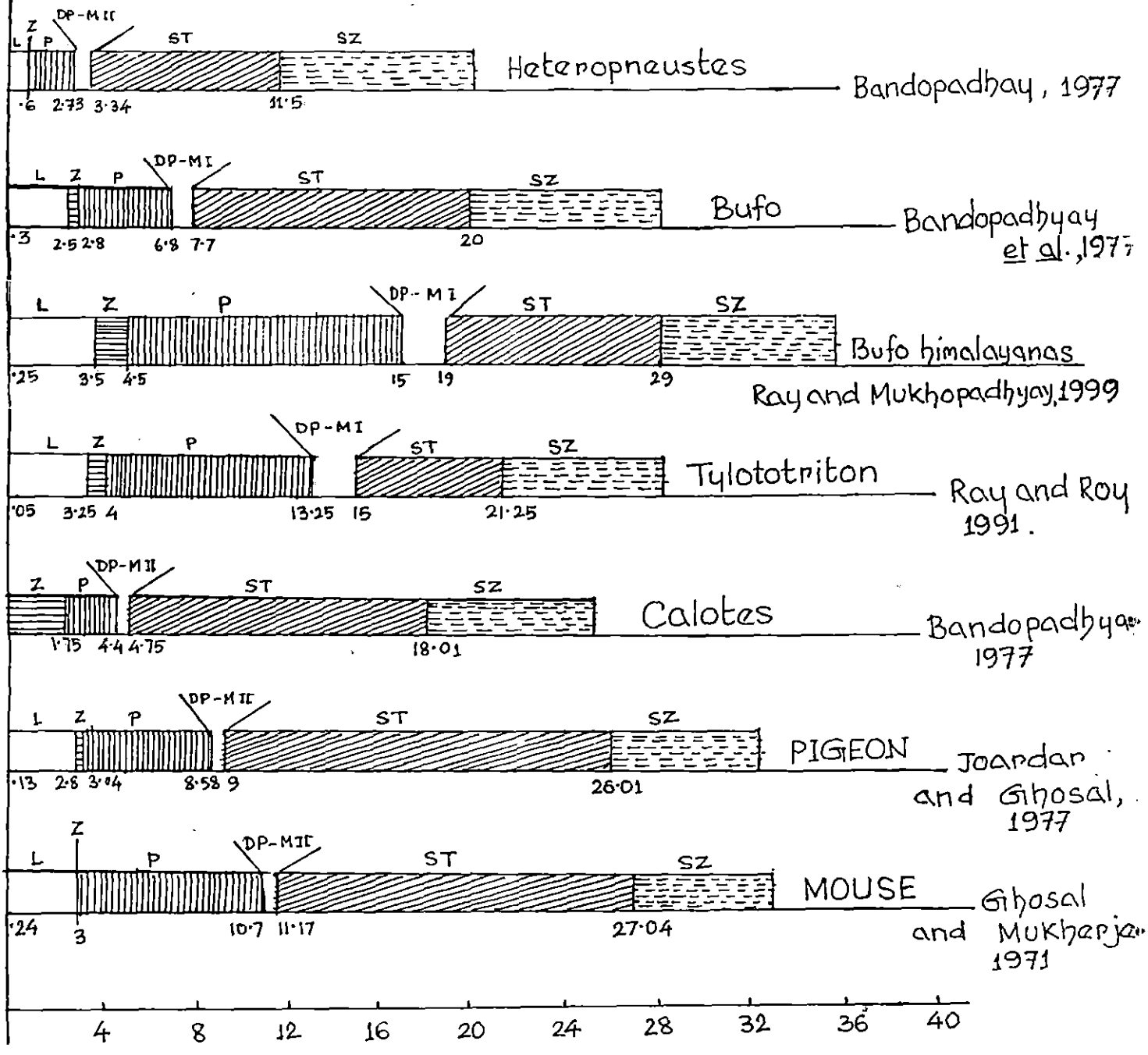
The time cycle of spermatogenesis has been estimated by several investigators using various techniques, such as the colchinine injection (Roosen-Runge,1951) , X-ray exposers (Oakberg,1955), heat-shock (Mcleod and Hotchkiss,1941; Young, 1927) and the injection of toxic substances (Drobeck and Coulston,1962; Muzzanti *et al.* ,1964) to the gonadal tissue. Probably the best of these methods is the one employing a lebelled precursor of DNA. After incorporation of the labels into the DNA of the germ cells, their kinetics till the spermatozoa formation could be traced autoradiographically.

Radioactive tracers such as ^{14}C –adenine, ^{3}H –thymidine and ^{32}P-phosphate has been used widely for determination of the duration of the meiotic stages and spermiogenesis in the mouse(Pele 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 different investigators are shown in Diagram-10.

However 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), and Mallick and Ghosal (1988) have recorded the duration or time cycle of spermatogenesis in some salientians and urodel species.

DIAGRAM : 10

A COMPARATIVE DIAGRAM OF THE KINETICS OF MEIOSIS AND SPERMIOGENESIS IN DIFFERENT VERTEBRATES



ABBREVIATIONS

L = Leptotene Z = Zygotene P = Pachytene
DP-MI = Diplotene to Metaphase-I
DP-MII = Diplotene to Metaphase-II
ST = Spermatid SZ = Spermatozoa

AIMS AND OBJECTIVES OF THE PRESENT STUDY

The present study involves the estimation of the total duration of the meiosis and spermiogenesis in Himalayan toad, *Bufo himalayanus* (Gunther) [Anura: Amphibia].

The present study has been engineered to see whether the spermataogenetic cycle of this particular species is continuous or discontinuous in nature and how the duration or time cycle of meiosis differs with other amphibian species.

The present study is also aimed to establish relation ship of the kinetics of meiosis and spermiogenesis with the seasonal breeding habit of this high altitude species.

The present study also will focus on some idea whether extrinsic factors (viz. temperature) has any effect on the kinetics of spermatogenesis. The present study also has an intention to show the phylogenetic significance of the meiotic kinetics and also the phylogenetic relationships between the closely associated classes of animals on the basis of available records.

MATERIALS AND METHODS

Material

Mature and developing testes of the himalayan toad *Bufo himalayanas* were collected from the dissected animals for various methods of preparations for further study and observations.

Methodology

For the study of the kinetics of meiosis and spermiogenesis radio-autographic method suits best. It has also been prooved by various investigators working on this type of experiments.

Radio-autographic method

a) **Selection of tracer :** Different types of labelled precursors like ^{32}P-phosphates (Ortavant,1958) , ^{14}C-adenine (Benette *et al.* 1960), ^{3}H-thymidine (Pollister,1969 ; Wright *et al.*,1970), etc. are used for lebelling DNA when it is synthesized during the premitotic as well as premeiotic S period. The use of ^{32}P- phosphate is not very suitable for our present study as it is incorporated into a variety of compounds besides DNA , while ^{14}C- adenine labels both DNA and RNA. ^{3}H- thymidine is , however, very suitable for its specificity for labelling DNA(Reichard and Estbor, 1951; Mukhopadhyay *et al.* ,1986). The ^{3}H-thymidine has been used in the present investigation, as tritium has additional advantages. It produces very low energy beta rays (a maximum output of energy of 0.018 MeV; Perey, 1964) that are recorded on the overlying nuclear track emulsion or striping film very close to their sites of emission (Messier and Leblond, 1960) resulting in a fine autoradiographic resolution (Taylor *et al.* ,1957). After the injection the tracer diffuses very rapidly throughout the gonad (Utakoji and Hsu, 1965).

b) Administration of tracer : Conventional dosages of 20 μci of tritiated thymidine (Bhabha Atomic Research Center, Trombay) was injected intraperitoneally (sp.act. 10,000 μci/mM) to the animal for investigation at normal breeding season and at the onset of winter.

c) Methods for obtaining the testicular samples: The testis from these specimens were collected after 0-25 days post-injection period. After collection squash preparation was made .

d) Methods for filming with AR-10 stripping film. Slides enshrining squashed testicular material were coated with Kodak-AR –10 autoradiographic stripping film in the dark room conditions (Stevens, 1966) and stored in light proof box containing silica gel (Pfizer) inside the refrigerator at 4 degree centigrade. A At every 5 days interval, used silica gel was replaced by freshly prepared gel. After 4 weeks of exposure, slides were developed for two minutes duration in Kodak D –19 or D- 19b developer (prepared in laboratory) , rinsed in distilled water, fixed in Kodak acid fixer also for two minutes duration, rinsed once again in distilled water and then washed thoroughly for half an hour in running tap water. Throughout the experiment the temperature was maintained at 19 degree Centigrade (Marchant *et al* . , 1965). Then the labelled stages were examined under microscope and selected frames were photographed. In a few cases where identification of the cell or the ellucidation of the archetechture of the chromosome was not possible due to accumulation of grain, the grains were removed and the particular cell then re-examined to identify the stages of the meiosis and spermiogenesis (Plate-36, Figure A-K).

e) Removal of silver grains: There were some case where huge amount of autoradiographic silver grains were accumulated in the slide. These grains were removed by the following procedure (Blanchi *et al.*,1964).

i) Slides were immersed in a slution of 7.5% potassium ferricyanide $[K_3Fe(CN)_6]$ for 15minutes.

ii)Then slides were placed in a 24% solution of sodium thiosulphate for 5 minutes, and

iii) washed in running tap water for 5 minutes and subsequently restained with Azur B Bromide (Gurr) or haematoxyline.

f) Specimen used: Animals were sacrificed at different time intervals (Table-TRK-1). Stages of spermatogenesis were collected by the usual standard technique and radioactivity was recorded by tracing the progression of each stage at various intervals.

OBSERVATIONS

The radioactivity of the gonial cells recorded soon after radioisotope application (i.e. 0-25 days post-injection). The labelled meiotic stages as leptotene, zygotene, pachytene, diplotene and diakinesis were recorded first on 2^{nd}, 4^{th}, 5.5^{th}, 18^{th} and 19^{th} day post- injection respectively.

The spermatids labelled only on 22^{nd} day post-injection and continued to be labelled till 30^{th} day post-injection at when the spermatozoa found labelled for the first time. Duration of leptotene, zygotene , pachytene, diplotene, diakinesis calculated on the basis of the method as stated earlier and the durations are shown in the table (Table-10). The total spermatogenesis process i.e., from onset of meiosis to completion of spermatogenesis is about 29 days (Plate-36, Figure A-K).

In amphibians spermatogenesis exhibits cyclic changes and different cycles were described by different investigators (Bohra and Niazi ,1984; Ghosal *et al.* 1981; Rastogi *et al.* 1985 Ray, *et al.*,1998). According to them the three different cycles were as follows:

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

(b) *Discontinuous type*: In *Rana temporaria* and *Rana pipens* this process is strictly seasonal and never found through the year.

(c) *Continuo - discontinuous type* : In *Rana esculenta, Rana trigrina,* etc., spermatogenesis depends invariably on environmental factors. In winter season spermatogenesis progresses only upto spermatid stage in these species.

Spermatogenetic activity in some anurans with their geographical distribution has been shown in Table-12.

Bufo himalayanas in present study exhibit a discontinuous cycle. Here *Bufo himalayanas* elaborates the production of spermatozoa only in its breeding season which is May- August of the year. This phenomenon also has been reviewed (Laskar,B.1997) (Diagram 8 & 9).

PLATE 36

Legends

Autoradigraphs of developing gonial cell and other stages of meiosis during spermatogenesis through ^{3}H- thymidine treatment

A. Leptotene phase B. Zygotene phase C. Early pachytene phase

D. Late pachytene E. Diplotene F. Diakinesis-Metaphase I

G. Early spermatids H. Early spermatids I. Oval spermatid

J. Late spermatid K. Mature spermatozoa

PLATE 36

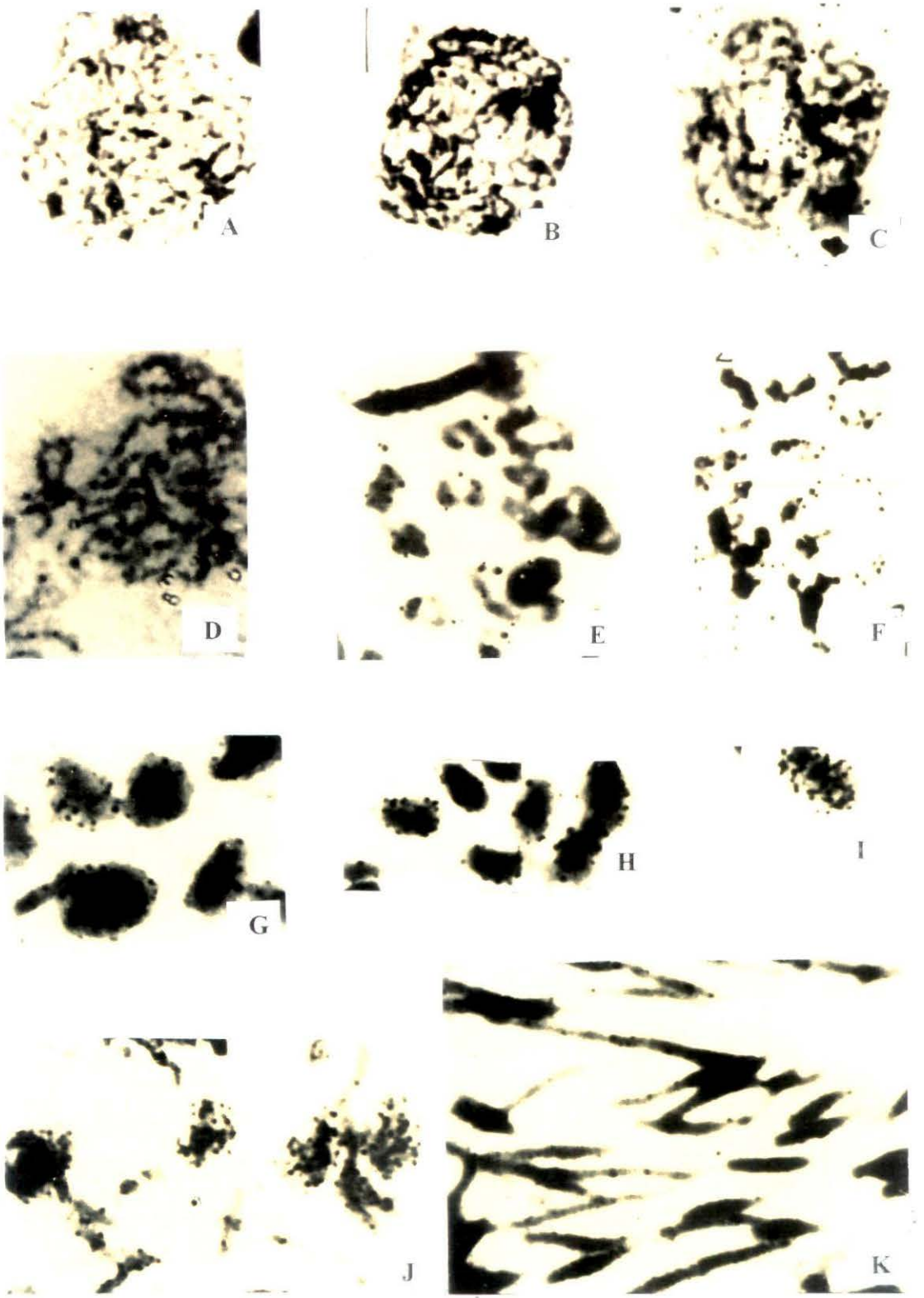


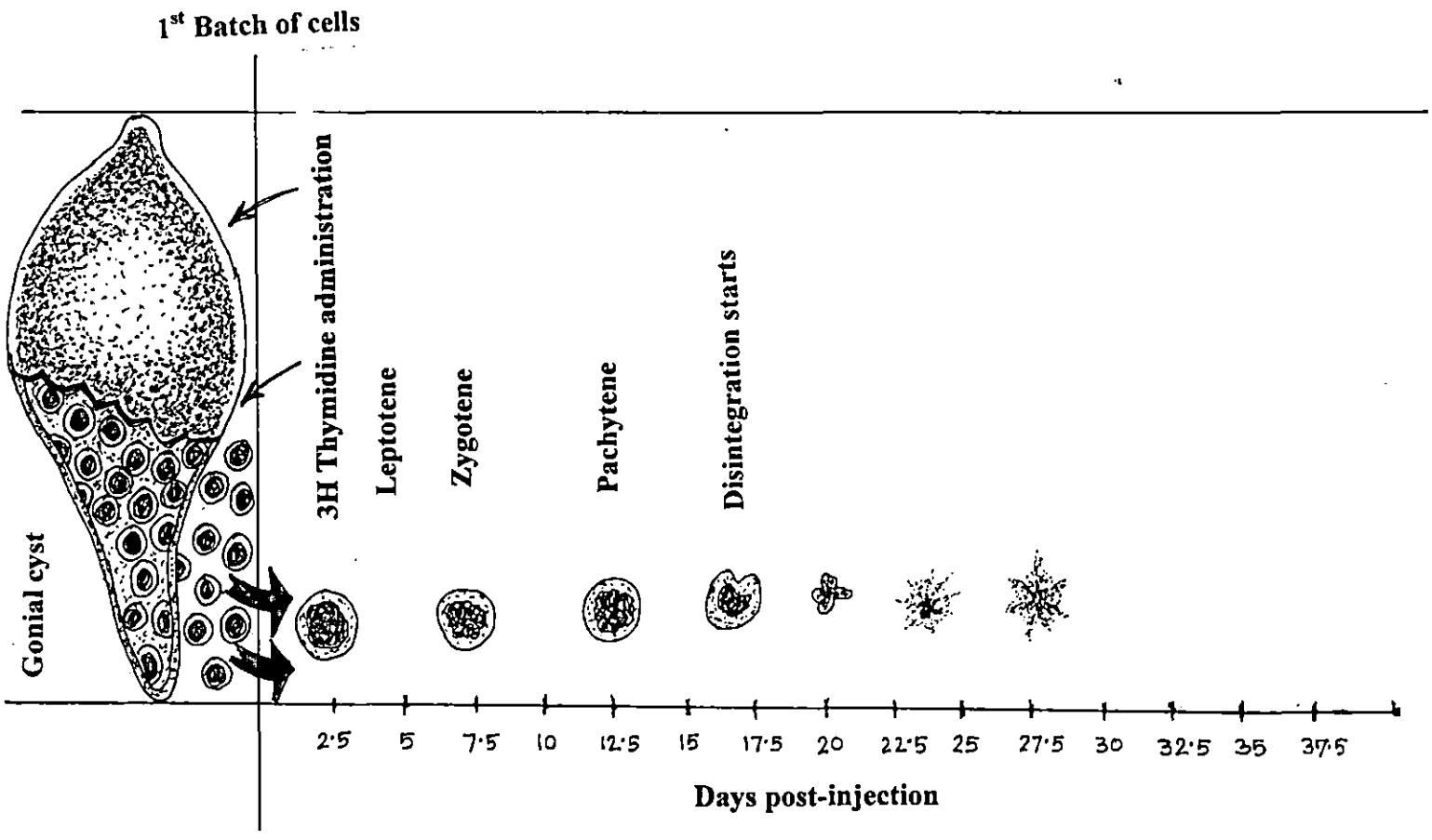
TABLE-12

Spermatogenetic activity in some anurans with their geographical distribution

Species	**Habitat**
Continuous type	
Bufo arenarum	South America
Bufo parachemis	South America
Bufo melanostictus	India, Java
Rana cancrivora *	Java
Rana hexadactyla	India
Pseudis mandidactyla	India
Discontinuous type	
Rana temporaria	Europe
Rana pipens	U.S.A.
Hyla crucifera	U.S.A.
Pleurodema bufonia	South America
Continuo-discontinuous type	
*Rana esculenta**	Europe
Rana gracea	South America
Rana tigrina	India
Rana nigromaculata	Japan

*In this species spermatogenesis goes only upto spermatid formation in winter season.

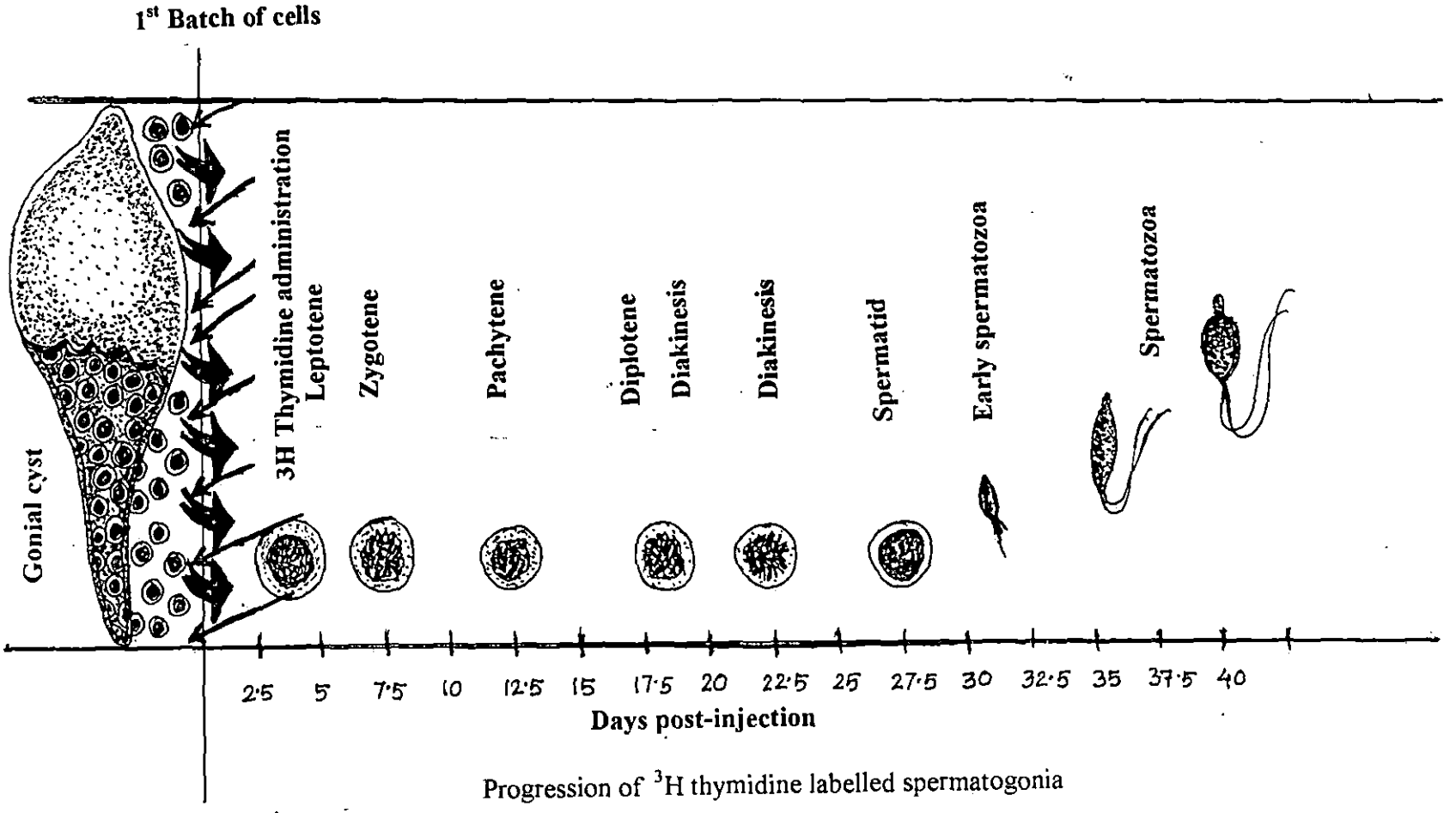
DIAGRAM : 8



Progression of 3H thymidine labelled

spermatogonia in *Bufo himalayanas* during winter season through meiosis

DIAGRAM : 9



Progression of ^{3}H thymidine labelled spermatogonia through various stages of meiosis and spermeiogenesis in *Bufo himalayanas* (breeding season, May-August)

Therefore, kinetics of the spermatoagenesis has been studied only during the breeding season. However the specimens collected from lower altitudes (i.e., below 4000ft. or below) exhibit continuation of spermatogenic activity till September and spermatocytes exhibit anarrest of meiotic transition beyond pachytene stage (Table 11).

The kinetics of meiosis of this species is somewhat is different from other anuran species found in tropical and subtropical regions. The longer duration of each letotene, zygotene, pchytene, diplotene and diakinesis is somewhat similar to *Xenopus laevis* (Kalt, M.R., 1976). In contrast to meiosis , spermatogenesis is remarkably short and is only 9.5 days in duration (Diagram 9). In some species the pachytene last for few months until they degenerate at the onset of winter (Ray and Mukhopdhyay, 1999), (Diagram 8).

Seasonal variation of kinetics of spermatogenesis

In order to study the seasonal variation of the meiosis and spermeiogenesis duration, testicular samples were collected from the specimens which were injected with ^{3}H thymidine both during the normal breeding season when they are actively busy with meiosis and spermeiogenesis (May to August) as well as durig the onset of the winter season when activity of the spermatogenesis seems to be ceased due to temperature drop (September-December).

Animals were sacrificed every month at different time intervals as shown in the Tables 10 and 11. It was found that labelled leptotene stages were

appeared on 2.0 day post-injection in May, June, July and August specimens. Whereas on the 3rd day post-injection, it appeared in September, November and December specimens.

There were some significant changes in duration of other meiotic stages also evident between May-August and September- December specimens. Appearance of first lebelled Zygotene, pachytene, diplotene and diakinesis and spermatids were found respectively on 4.0, 5.5,18.0, 19.0 and 22.0 day post-injection in May –August specimens. But in September-December specimens first labelled stages of pachytene appeared on te 5th day of post-injection. No zygotene phase was recognisable in these winter specimens. It is very significant to note that meiotic process exhibits an arrest of normal meiotic transition beyond pachytene stage (Table 11) in these September-December specimens probably due to fall of environmental temperature at the onset of winter season Ray and Mukhopadhyay, 1999). However the specimens collected from lower altitude (below 5000 ft.), the kinetic pattern remain more or less similar to that of breeding season.

TABLE 10

The progression of lebelled ^{3}H thymidine gonia cells through different stages of meiosis and spermeiogenesis in *Bufo himalayanas* (Gunther). (May-August season)

Days post-injection	Most advanced lebelled stage	Other labelled stage(s)
0.25	Gonia	-----
0.5	Gonia	-------
0.75	Gonia	-------
1	Gonia	------
1.5	Gonia	-----
2	Leptotene	------
2.5	Leptotene	Gonia
3.5	Leptotene	Gonia
4	Zygotne	Gonia,Leptotene
4.5	Zygotene	Gonia,Leptotene
5	Zygotene	Gonia,Leptotene
5.5	Pachytene	Gonia,Leptotene,Zygotene
7.5	Pachytene	Gonia,Leptotene,Zygotene
8.5	Pachytene	Gonia,Leptotene,Zygotene
9.5	Pachytene	Gonia,Leptotene,Zygotene
10.5	Pachytene	Gonia,Leptotene,Zygotene
11.5	Pachytene	Gonia,Leptotene,Zygotene
13.5	Pachytene	Gonia,Leptotene,Zygotene

TABLE 10 (contd.)

The progression of lebelled ^{3}H thymidine gonia cells through different stages of meiosis and spermeiogenesis in *Bufo himalayanas* (Gunther) . (May-August season)

Days post-injection	Most advanced lebelled stage	Other labelled stage(s)
15	Pachytene	Gonia,Leptotene,Zygotene
18	Diplotene	Gonia, Leptotene,Pachytene
19	Diakinesis	Gonia, Leptotene,Pachytene
22	Spermatid	Gonia,Leptotene,Pachytene, Diplotene
23	Spermatid	Gonia, Leptotene,Pachytene, Diplotene
25	Spermatid	Gonia, Leptotene,Pachytene,Diplotene
27	Spermatid	Gonia, Leptotene,Pachytene,Diplotene
29	Spermatid	Gonia, Leptotene,Pachytene,Diplotene
30	Spermatozoa	Gonia, Leptotene,Pachytene,Spermatid
32	Spermatozoa	Gonia, Leptotene,Pachytene,Spermatid
35	Spermatozoa	Gonia, Leptotene,Pachytene,Spermatid

Dose: 20 micro Ci/animal : Sp. Activity-1000Mci (BARC,Trombay)

TABLE-11

The progression of ^{3}H thymidine labelled gonia cells through meiosis in *Bufo himalayanas* (Gunther). September- December season)

Days post-injection	Most advanced labelled stage	Other labelled stage(s)
0.25	Gonia	-----
3.5	Gonia	------
0.75	Gonia	------
1	Gonia	-----
3	Leptotene	Gonia
5	Pachytene	Gonia
7	Pachytene	Gonia
10	Pachytene	Gonia
15	Pachytene	Gonia
20	Pachytene	Gonia
25	Pachytene	Gonia
30	Pachytene	Gonia
35	Pachytene	Gonia

Dose: 20 micro Ci/animal Sp.activity-1000 Mci (BARC,Trombay)

DISCUSSION

Chronology of meiosis and spermeiogenesis

The estimation of the duration of premeiotic 'S' period and the chronology of meiosis in both mammalian and sub-mammalian species have been worked out by various workers by using the technique which involves :

(i) Lebelling of the pre-leptotene spermatocyte's DNA during the premeiotic 'S' period and

(ii) Following the course of migration of these labelled spermatocytes through different stages of meiosis and spermeiogenesis (Crone *et al.* ,1965; Clermont, 1972; Foote *et al.* ,1972; Ghosal *et al.*, 1975; Kalt,1976; Joardar nad Ghosal, 1977; Sinha *et al.*, 1979,1982,1983; Ghosal *et al.*, 1981; Ray *et al.*, 1984; Bandopadhyay and Ghosal ,1985; Ray and Mukhopadhyay,1999).

In mammalian species leptotene duration varies from 3-5 days in different species. For example, leptotene stage lasts for 3 days in rat (Clermont, *et al.*,1959), mouse and golden hamster (Ghosal and Mukherjee 1971) while a little above 5 days in man (Heller and Clermont,1963). In contrast to mammalian species this duration is 2.71 ± 0.09 days in *Calotes* (Bandopadhyay,1977), 2.5 days in *Bufo melanostictus* (Bandooadhyay, 1977), 4 days in *Xenopus laevis* (Kalt,1976), 2.75 ± 0.25 days in Uperodon (Ray *et al.*, 1984), 0.4 day in *Colisa* (Sinha *et al.*, 1979), 0.5 day in *Heteropneustes fossilis* (Bandopadhyay and Ghosal, 1985), 3 days in *Tylototriton verrucossus*

(Ray and Roy , 1989) . In the present investigation we found the leptotene duration is 2.5 days. It first appeared on the second day and lasts upto 3.5 days during breeding season (May-August).

Though the zygotene duration is short as observed in various vertebrates with the exception of *Xenopus* where this stage lasts for as long as 6 days . In amphibian species the duration of zygotene varies from 0.79 to 0.92 day (Mallick,1987). In other sub-mammalian species shorter duration of zygotene has also been recorded (Kalt, 1976; Bandopadhyay *et al.*,1977; Sinha *et al.*, 1979; Ray *et al.* ,1984; Bandopadhyay and Ghosal,1985; Mallick, 1987).

In the present study, we observed longer duration of meiosis in *Bufo himalayanas*. Thus this is somehow similar to *Xenopus laevis* (Kalt, M.R.,1976) . Here zygotene first observed at the 4^{th} day of post-injection and lasts till the 5^{th} day. Thus the total duration of this stage is about two days in the normal breeding season (May- August).

The pachytene stage has got characteristically longer duration. Pachytene lasts for 16.65 ±0.3 days in dog (Ghosal *et al.*,1983), at least 7 days in golden hamster (Ghosal and Mukherjee,1971) and mouse (Ghosal and Mukherjee,1971), 14 days or more in chinese hamster and 10.5 day in bull (Bandopadhyay,1977). Among amphibian species the duration is somewhat variable . It lasts for 12 days in *Xenopus laevis* (kalt.1976), 4 days in *Bufo* (Bandopadhyay et.al.,1977), 5.63 ± 0.38 days in Uperodon (Ray et. al., 1984), 2.92 day in *Rana limnocaris* (Mallick,1987), 6.17 days in *Rana verrucosa* (Mallick,1987).It may be briefly mentioned that among the fishes pachytene duration is very similar with that of amphibian pattern

During the present investigation the pachytene duration appears to be about 10.5 days in *Bufo himalayanas* during its normal breeding season.

The kinetics of transition from diplotene to diakinesis and metaphase- II is extremely rapid in nature and individual stages like diplotene, diakinesis, metaphase –I and metaphase-II lasts for few hours to a day or two in all mammalian and sub-mammalian species studied so far (Bandopadhyay,1977; Bandopadhyay et.al.,1977; Joardar and Ghosal, 1977; Ghosal and Mukherjee,1971).

The present investigation observed longer duration similar with that of *Xenopus laevis* (Kalt,1976). It has been found that the total duration of diplotene to metaphase –I transition is about 2 days in *Bufo himalayanas* in its normal breeding season.

Duration of spermeiogenesis in vertebrates

The total duration of spermeiogenesis is considerably long in mammalian species. It lasts for more than 15 days in golden hamster and mouse (Ghosal and Mukherjee,1971), 21 days in bull (Bandopadhyay,1977), 20 days in dog (Ghosal *et al.*,1983) and about 20 days in lamb (De, 1978). Submamalian species too have longer spermeiogenesis duration , as for example 15 days in quail (Follett,1977),13 days in cock (Joardar,1977), 17.6 days in pigeon (Joardar and Ghosal,1977), 13.26 days in *Calotes* (Bandopadhyay,1977), 8 days in *Heteropneustes* (Bandopadhyay and Ghosal, 1985) and *Colisa* (Sinha *et al.*, 1979).

Amongst amphibia total duration of spermeiogenesis is 12 days in *Xenopus laevis* (Kalt,1976) , 13 days in *Bufo* (Bandyopadhyay et.al.,1977), 9 days in *Uperodon* (Ray *et al.*, 1984), 4.8 days in *Rana*

limnocaris (Mallick,1987), 5.95 days in *Rana verrucosa* (Mallick,1987), 6 days in *Rana cyanophlyctis* (Mallick,1987).

The present investigation recorded the total duration of spermeiogenesis is about 9.5 days *Bufo himalayanas* (Gunther) which shows similarities with *Xenopus* and *Uperodon.*

Kinetics of total spermatogenesis

The total duration of spermatogenesis (meiosis and spermeiogenesis) are characteristics in mammalian species. In man (Helter and Clermont, 1963) the total process is completed in 48 days. The total duration of spermeiogenesis in chinese hamster is 35 days (Utokoji,1966), in mouse it is 25.01 days and in golden hamster it is 27.04 days (Ghosal and Mukherjee,1971). In bull the total spermatogenic duration is about 37 days (Bandopadhyay,1977) and in dog it is 42 days (Ghosal et.al., 1983).

In the present observation the total duration of spermatogenesis is about 9.5 days. The kinetics of the total process of spermatogenesis i.e., from the onset of meiosis to completion of spermatogenesis is about 29 days where kinetics of meiosis only lasts for about 19.50 days which has a close similarity with that of *Xenopus laevis* as described by Kalt (1976). A comparative diagram of the meiosis and spermatogenesis in different vertebrates including *Bufo himalayanas* has been shown in Diagram-10. The total time required for spermatozoa production from any given point i.e., from any specific form of spermatogonia or from pre-leptotene spermatocytes, is of fixed duration in a particular mammalian species (Clermont,1972). Elevation of temperature or hypophysectomy or hypophysectomy-gonadotropin treatment (Clermont and Morgentaler,1955) or hormonal treatment alone (Ray *et al.*, 1981,Ray and Roy,1988) does not alter this total duration. Similarly gonadotrpin

antagonist have no effect on individual duration of meiosis and their transition (Ray and Roy,1988) in the first wave of spermatogenesis following treatment. However prolonged treatment prevents transition of pachytene spermatocytes to diplotene stages (Ray and Roy,1988).

FACTORS CONTROLLING SPERMATOGENETIC ACTIVITY

The seasonal development and activity of the male gonads of amphibians are under the direct control of various endogenous and exogenous factors . The extirpation of the pars-distalis of the hypophysis of the adult male of *Rana nigromaculata* exhibits inhibition of spermatogenesis (Iwasawa and Tojo,1976) at high ambient temperature.However it has been prooved by many investigators that the progress of spermatogenesis and activity of spermatozoa are not influenced by hypophysectomy. Effect of testosterone and gonadotropin on amphibian species has been reviewed by many authors (Basu and Nandi,1965; Iwasawa and Michibata,1972 ; Lofts,1974). Temperature is the major factor controlling spermatogenesis in amphibians. Effect of high temperature on testis and spermatogenetic activity has been investigated by various authors (Kort and Oordt,1965; Oordt and Lofts,1963; Lofts,1974). Rainfall also has similar influence on spermatogenic activty of amphibian species.

The present investigation also suggest that temperature plays an important role in meiotic transition and spermiogenesis. The degeneration of pachytene spermatocytes or arrest of further development during winter season (October-December and onwards) suggest that at low temperature (below10^0C) retards meiotic transition and as a result spermatids and spermatozoa are not formed. Whereas the spermatogenic activity continues at low level in the specimens obtained from lower

altitude where ambient temperature prevails at and around 20^0 C during day time.

In the present study, kinetics of spermatogenesis shows rapid transition and completion of spermatogenesis. It seems to be somehow related with the habit of the seasonal breeding which has an extremely short duration of normal breeding time at this areas of high altitude (4000-8000 ft.). Therefore the Himalayan toad, *Bufo himalayanas* normally has to complete its spermatogenesis within the normal breeding season of this species which ranges between May to August. Seasonal variation as observed is due to low temperature.

Phylogenetically amphibians are related to piscian group on one hand and with reptiles on the other. Their phylogenetic relationship has been established by various methods and techniques. So the kinetics of spermatogenesis can be used as a method to establish such relationship (Laskar,B.,1997;Saidapur,1983; Whittigton and Dixon,1975).

Meiotic duration in piscian group is the shortest among the vertebrates and similar short duration of meiotic stages are found in aquatic amphibian forms such as *Rana, Bufo, Tylototriton* etc. (Oordt and Van,1956; Roy,1990; Saidapur,1983). On the other hand spermatogenesis of terrestrial amphibians is much longer in duration as found in reptiles. Therefore the findings of this current investigation may also have significance in the establishing phylogenetic relationship between such closely related groups of animals.

SUMMARY

GONADAL DIFFERENTIATION AND SPERMATOGENETIC ACTIVITY IN HIMALAYAN TOAD *Bufo himalayanas* (Gunther) : ANURA:AMPHIBIA – AN ULTRASTRUCTURAL STUDY

Bufo himalayanas , the material for this study is an endemic and endangered species of toad that exists in the high altitude areas of the greater Himalayan range. In my present study observations have been made on the adult and larval forms of this species, all of which were collected from different places of their normal habitat ranging between 1500 meters and 2200 meters altitude at Darjeeling town and its adjoining areas. The natural developmental processes of this toad have been monitored in laboratory. Atmospheric temperature was recorded at the time of collection, which showed a clear range between 16^0-22^0 C in different seasons of the year.

A normal table of development of this species, which was not done precisely before has been worked out and tabulated in a table to observe the pattern of development and the climax of metamorphosis. Statistical analysis were also done to reveal significances of their developmental pattern. It has been observed that the normal table of development has a close similarity with other closely related anuran species. Observations further confirmed that the effect of environmental temperature plays a very significant role in their developmental process. Preferably the temperature for their normal development under laboratory condition was recorded between 16^0-22^0 centigrade. The duration of total larval development up to the final meramorphosis is nearly two and half month. At slightly higher temperature range between 22^0-24^0 centigrade , this duration of metamorphosis can be

shortened. In laboratory, their pattern of growth, feeding habit, morphological features and sizes also recorded. Detailed study was made specially of the post-hatching larvae of *Bufo himalayanas*.

In the field, observations were made on the behavior and courtship pattern of the adult male and female of this species. Their eggs and larval developmental stages and characteristic features of the mature male and female were also recorded.

The study was planned and framed accordingly to draw a detailed account of larval development of this species to compare it with other related species and other closely associated classes of animal to show any significant relations with them from the evolutionary or phylogenetic point of view. For this purpose a number of related references consulted. All these were incorporated in the first chapter, named as 'NORMAL TABLE OF DEVELOPMENT'. The normal table was made according to the standard proposed by Gosner (1960).

In the second chapter 'GONADAL DIFFERENTIATION PATTERN AND TESTICULAR DEVELOPMENT' has been observed through light, scanning and transmission electron microscopy to reveal their structural and ultra-structural details. These observations indicated that there was a similarity in developmental pattern of gonads as well as testes with that of teleosts and reptiles which has a clear indication on their phylogenetic relationships between closely related groups of animals.

When observing the mechanism of gonadal differentiation, the evidence of bi-potential nature of differentiating gonads were also recorded. Furthermore it has been observed that developing gonads of this species has two distinct areas as peripheral cortex and inner medullary zones. The proliferation of

the cortical cells with a medullary regression marks the transition into a presumptive ovary in a female embryo, while the reverse occurs in the male.

Like many other anurans, the testes of this species differentiate and mature well before metamorphic climax (as early as four limb stage of developing larva). Thus the study further suggested, through observations, that there is a clear case of progenesis in this species.

Third chapter is concerned with the 'EVENTS OF SPERMATOGENESIS' with the aid of structural and ultra-structural observations made through light, scanning and transmission electron microscopy. A lot of unique and characteristic structural observations have been made. Here in this species, spermatogonial cells with hypo- and hyperdiploid configurations have been observed. Further observations revealed polyploidy along with polymorphic sperms in this species.

In the developing spermatogonia it has been observed that the spermatogenesis is of cystic type like other amphibians. Different stages of spermatogenesis were also recorded. Here in this species stages like primary and secondary spermatogonia, primary and secondary spermatocytes, spermatids and mature spermatozoa have been found. Detailed light and electron microscopical observations revealed structural and ultra-structural characteristics of the cells. Here in this species a perforatorium, which consists of a number of coarse dense strands and located at the end of the nucleus of the mature spermatozoa has been recorded. Observation also confirms the presence of a bi-flagellate tail with an undulating membrane in between, except the posterior end.

The fourth chapter 'SPERM POLYMORPHISM' deals with this unique feature of polymorphism of spermatozoa in this species. The structural and ultra-structural peculiarities have been observed in the mature spermatozoa

of this species. Further study confirms that the mature sperm of this species has a characteristic biflagellate appearance. It has along slender head followed by a short mid-piece.

Transmission electron microscopic observations reveal that the electron dense materials are spread in the entire nucleus and have a different degree of compactness at various levels. Nucleus has, at its anterior end an acrosomal cap of barb like appearance with several discontinuous filaments in it. The elongated middle part has a electron lucent vacuole surrounded by heavily packed multiplayer of conspicuous and circular mitochondrions. The middle part also has two distinct centrioles.

The tail is uniquely bi-flagellate and each flagellum consists of an axial filament consisting of (18+2) sub-microscopic longitudinal microfibrils. Both types of electron microscopies show different forms of atypical spermatozoa like megacephalic, globular, sickle shaped and microcephalic sperm head.

The fifth and final chapter named as " THE KINETICS OF SPERMATOGENESIS" observed the results of autoradiographic study to know the kinetics of spermatogenesis and spermiogenesis in particular. It shows that the total duration of spermiogenesis in this species is about 9.5 days and the kinetics of the total process of spermatogenesis is about 29 days which has a close similarity with related species as well as other amphibian, teleost and reptilian species which may has a strong phylogenetic significance.

The present investigation also suggests that temperature plays an important role in meiotic transition and spermatogenesis. The degeneration of pachytene spermatocytes indicate the arrest of further development during winter season of low temperature suggest that below 10^0 C meiotic transition

retards and so spermatids and spermatozoa are not formed in this high altitude dwelling species.

Kinetics of spermatogenesis shows rapid transition and completion of spermatogenesis within the short normal breeding season (May- August). Seasonal variation observed in this species due to temperature difference of the various seasons which also has a similarity with other high altitude species of anurans.

Therefore the entire present study revealed a lot of characteristics of the normal table of development, gonadal differentitation pattern as well as testicular development. Various events of spermatogenesis, sperm polymorphism and the kinetics of the spermatogenesis of this high altitude dwelling endemic species of toad found in the Himalayan range only are also observed. Observation shows similarities with the other closely related species as well as classes of animal which suggest a definite phylogenetic significance of the present study. Recorded observations indicate the development as well as structural characteristics of this unique species of toad, which has not been done before and importance of further study specially in the field conditions.

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POLYMORPHISM IN SPERM MORPHOLOGY - A SCANNING ELECTRON MICROSCOPIC STUDY ON *Bufo himalayanas* (ANURA : AMPHIBIA)

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Sperm morphology of *Bufo himalayanas* (Anura : Amphibia) has been studied from surface morphology revealed from Scanning Electron Microscopic observation. Sperm were isolated by gradual centrifugation technique, fixed in 2.5% Glutaraldehyde with 0.1 M sodium cacodylate buffer. Post fixation was done with 2% osmium tetraoxide with same buffer. Typiocal sperm have a pointed head, a very short perforatorium, coiled tubules around neck and tail consisting of two axial filaments. Apart from pointed head, sperm with round, oval or elliptical head, absence of perforatorium and neck, tail with single axial filament etc. are some polymorphic features. A probable origin of such sperm have been suggested from hypo and hyperdiploid gonial cells. Genetic control on sperm morphology has been suggested.

Introduction

The basic morphology of an amphibian spermatozoon involves following structures in a linear anteroposterior sequence - acrosome, head, middle piece and tail. The morphology of spermatozoa is highly variable in amphibians. Ray et. al. (1986) and others have reported an unique phenomenon of hypo and hyper diploid gonial population in amphibians apart from diploid gonial cells. The fate(s) of these gonial population in germ line propagation has a special significance in conservation of diploid chromosomal status of the species.
In the present study an attempt has been made to record variations in sperm morphology as revealed from surface topographies.

Materials and Methods

Male toads, *Bufo himalayanas* (Anura : Amphibia) were collected from different location of Darjeeling district. Sperms were collected by gradual centrifugation after preservation in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer for 4 hours. Post fixation was done in 2% osmium tetraoxide with same buffer. Surface morphology was studied under scanning electron microscope (Hitachi S530).

Observation

The study shows a unique array of sperm morphology. Under phase contrast microscope, a normal spermatozoon has deeply staining head consisting of cylindrical nucleus and pointed needle like acrosome situated at its anterior tip. A short neck or middle piece is followed by head, where mitochondria are clumped together. Tail consisting of two axial filaments joined together as if an undulating membrane. The surface morphology as revealed from electron microscope unequivocally supports this observation (Plate 1; Fig. 1). The head region shows irregular elevations and avery rudimentary perforatorium like structure. The neck shows spiral arrangement of tubules.
Polymorphioc nature of the sperm morphology can be boiled down as -

a) Spermatozoon with ovoidal head, without perforatorium and ill recognizable neck (Plate 1; Fig. 2).
b) Spermatozoon with sickle shaped head, without neck and tail (Plate 1; Fig. 3).
c) Spermatozoon with round highly conspicuous head and long tail (Plate 1; Fig. 4).
d) Spermatozoon with tail consisting single axial filament (Plate 1; Fig. 5).

Discussion

The biological significance of differences in size and structure of spermatozoa in amphibians is unknown. Species specific differences in spermatozoa may be correlated with differences in the structure of egg membrane (Kawanura, 1953). There is also a positive correlation between spermatozoan head length and

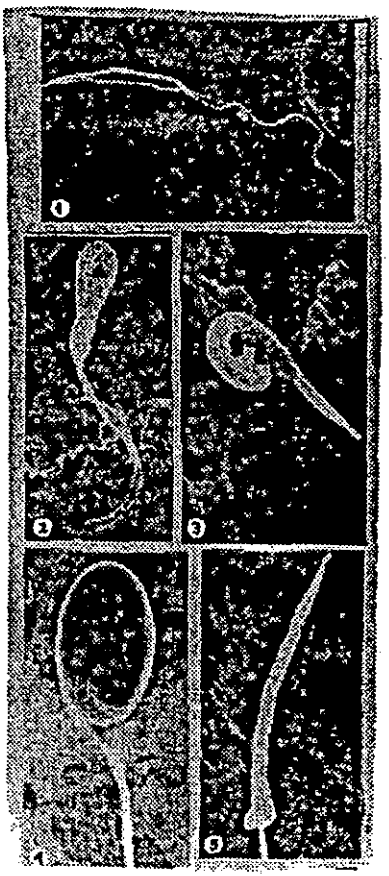

Fig. 1: Spermatozoon with elongated head, distinct middle piece and two axial tail filament.

Fig. 2 : Spermatozoon with ovoidal head, without perforatorium and ill recognizable neck.

Fig. 3 : spermatozoon woth sickle shaped head, without neck and tail.

Fig. 4 : Spermatozoon with round highly cnspicuous head and long tail.

Fig. 5 : Spermatozoon with tail consisting single axial filament.

amount of nuclear material (Fawcett, 1970). Wortham et. al. (1977, 82) suggested that the long head is correlated with more nuclear material as well as evolutionary plasticity of the species or group.
However, the polymorphism in sperm structure within a species be looked at from a different angle, i.e. from their ontogenic source. Roy and Ray (1989) from karyomorphological studies have shown a possible existence of polymorphic cell population in amphibians. Such hypo and hyperdiploid gonial cells apart from normal diploid cell continue normal gametogenic progression will lead to a polymorphic structures in terms of sperm morphology. In no sense it be assumed that morphology of sperm is a mere developmental pattern without genetic control.

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SCANNING ELECTRON MICROSCOPIC STUDY OF SPERMIOGENESIS OF HIMALAYAN TOAD, *Bufo himalayanus* (ANURA : AMPHIBIA)

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Stages of spermiogenesis of Himalayan toad, *Bufo himalayanus* have been studied from scanning electron microscopic observation. The early spermatids have round irregular surface structure as depression and evaginations. Mid spermatids show smooth appearance of the surface. Late spermatids have 'short neck'. Tail appears as axial filaments. Mature spermatozoon consists of needle like head with barb like perforatorium, a very short neck with tubules and a tail with two axial filaments without an undulating membrane. Polymorphic nature of head, neck and tail structure are also recorded.

Introduction

Spermatogenesis, a process of cellular differentiation (Clermont and Leblond, 1953) is divided into three stages : i) Spermatocytogenesis, ii)Spermatocytic stage and iii)Spermiogenesis (Rossen - Runge, 1977). The events of spermiogenesis includes the differentiation with extensive morphological changes that convert the haploid spermatid into a mature spermatozoa. There are extensive literature on nuclear condensation or changes (Dixon, G.H. 1972), elaboration of cytoplasmic organelles (Bloom and Fawcett, 1975) and biochemical events during spermiogenesis (Olivieri, 1965; Gould-Somero and Holland, 1974; Erickson, 1980). However, study on sequential events of morphological changes through scanning electron microscope is mere and obscure (Picheral, 1979).

Material and Methods

Male specimens of June, July and August were sacrificed and sperms were collected by mild centrifugation in amphibian saline. Then fixation was done in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer. Post fixation was done 2% osmium tetraoxide with same buffer and preserved in amylacetate. Surface morphology of different stages of spermiogenesis was studied under scanning electron microscope (Hitachi S530).

Observation

Cytologically spermiogenesis of an amphibia is not so convincing as in mammals. Therefore, depending on morphological shapes, it is divided into early, mid, late and mature stages having respectively round, oval, elliptical and rod like appearances.

Under scanning electron microscope our observations are as follows :

i) The early so called round spermatids have as usual round but surface shows very irregular appearance. Invaginations and buldges are the most prominent features.(Plate 1; Fig. 1).

ii) At mid stage, the spermatids become somewhat elongated and become smooth in appearance. No invaginations or protrusions are found. This may be due to spreading of acrosomal cap over the primary surface (Plate 1; Fig. 2).

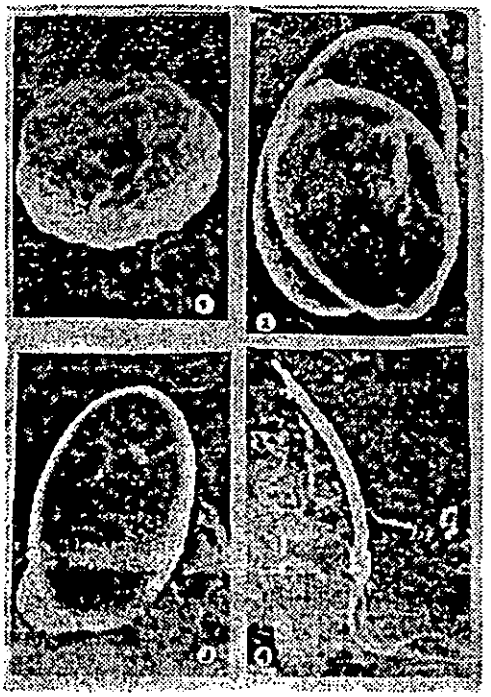

Plate 1. Photomicrographs of different stages of spermiogenesis of *Bufo himalayanus* under scanning electron microscope.

Fig. 1 : Early (round) spermatid showing characteristic invaginations on the surface.

Fig. 2 : Mid (ovoid) spermatid showing smooth suface.

Fig. 3 : Late (elongated) spermatid showing transveres tubules in the neck region.

Fig. 4 : A mature spermatozoon showing two axial filaments and rod like head with perforatorium at its tip.

iii) At late stage, neck part originates from the more broad posterior end as irregular band. Under higher resolution neck part is found to be featured by transversely arranged tubules in a zig-zag fashion (Plate 1; Fig. 3).

iv) The mature stage which marks the end of spermiogenesis shows appearance of two axial filaments coiled upon one another. The head part become more elongated to assume a rod like appearance. A barb like perforatorium appears from the pointed anterior end (Plate 1; Fig. 4).

Sperms have different shapes and morphologies — oval megacephalic, tail with single axial filament etc. are most common apart from the rod like sperm with double axial filaments.

Discussion

Sharma and Dhindas (1955) recorded that in *Rana* the events of spermiogenesis started with condensation of the nucleus together with elaboration of cytoplasmic organelles, particularly the appearance of PAS positive acrosomal granule as acrosomal cap. The tail part originates from the posterior or distal centriole. The neck part marks the accumulation of mitochondria etc.

Surface structures as revealed from scanning electron microscopic in present case unequivocally suggest that during spermiogenesis a progressive condensation of nuclear head takes place causing thining of the nucleus and sperm head assumes a rod like onfiguration. The presence of two axial filaments may have lead the postulation of presence of an undulating membrane in anuran sperm by earlier workers (Sharma and Sekhri, 1955).

Morphological variations of sperm structure may be correlated with chromosomal variation found in spermatogonial cell population (Ray et al., 1986) or with morphogenetic factors (Fawcett et. al., 1971; Risley, 1981) influencing spermiogenesis (McIntosh and Porter, 1967).

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Kinetics of meiosis and spermiogenesis in Himalayan Toad *Bufo himalayanas* (Gunther)

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ABSTRACT

The kinetics of spermatogenesis in Himalayan toad, ***Bufo himalayanas*** has been studied by monitoring the progression of labelled gonial cells through different stages of meiosis and spermiogenesis. The total duration of meiosis and spermiogenesis respectively, 19.50 and 9.5 days. In winter species the meiotic transition found arrested at and around pachytene stage. The phylogenic significance of kinetics of spermatogenesis in vertebrates has been discussed.

The kinetics of spermatogenesis have been studied in a number of amphibian species by using various techniques and methods[1,3,5,10,11]. However, such account is meager in anuran species found at high altitudes. The present communication narrates the total duration of meiosis and spermiogenesis of Himalayan Toad, *Bufo himalayanas* (Gunther) [Anura: Amphibia] *Bufo himalayanas* is found in the hilly regions of Darjeeling district and adjoining region of Sikkim at an altitude of 4000–8000 ft. Male toads were collected during breeding season (May – August) administered with ^{3}H- Thymidine at a dose of 20 μCi (Sp. act. 10,000 m Ci / m M , BARC , Trombay) / animal and were sacrificed at different time intervals (Table-1). Stages of spermatogenesis were collected by the usual standard technique and radio activity was recorded by tracing the progression of each stage at various intervals.

The radioactivity of gonial cells recorded soon after radioisotope application (0–25 d p.i.). The labelled leptotene, Zygotene, pachytene, diplotene and diakinesis were recorded first on 2,4,5.5,18,19 day post injection respectively. The spermatids labelled only on 22 days post-injection and continued to be labelled till 30 days post injection at when the spermatozoa found labelled for the first time. Duration of leptotene, zygotene, pachytene, diplotene, diakinesis calculated on the basis of the method as stated earlier and the durations are shown in the Table-1. The total duration of spermiogenesis is about 9.5 days. The kinetics of total spermatogenesis process *i.e.*, from on set of meiosis to completion of spermiogenesis is about 29 days.

In amphibians spermatogenesis exhibit Cyclic changes and three different cycles are known : continuous, discontinuous and contineo-discontinuous[2,4,9,10,11].

(175)

Table-1. The Progression of labelled ^3H - thymidine gonia cells through different stages of meiosis and spermiogenesis in *Bufo himalayanas* (Gunther). (May–August season)

Days post injection	Most advanced labelled stage	Other labelled stage (s)
0.25	Gonia	–
0.5	Gonia	–
0.75	Gonia	–
1	Gonia	–
1.5	Gonia	–
2	Leptotene	–
2.5	Leptotene	Gonia
3.5	Leptotene	Gonia
4	Zygotene	Gonia, Leptotene
4.5	Zygotene	-do-
5	Zygotene	-do-
5.5	Pachytene	Gonia, Leptotene, zygotene
7.5	Pachytene	-do-
8.5	Pachytene	-do-
9.5	Pachytene	-do-
10.5	Pachytene	-do-
11.5	Pachytene	-do-
13.5	Pachytene	-do-
15	Pachytene	-do-
18	Diplotene	Gonia, Leptotene, Pachytene
19	Diakinesis	-do-
22	Spermatid	Gonia, Leptotene, Pachytene & Diplotene
23	Spermatid	-do-
25	Spermatid	-do-
27	Spermatid	-do-
29	Spermatid	-do-
30	Spermatozoa	Gonia, Leptotene, Pachytene & spermatid
32	Spermatozoa	-do-
35	Spermatozoa	-do-

Dose : 20 μCi / animal
Sp. activity - 1000 Mci (BARC, Trombay)

Table-2. The progression of ^3H- thymidine labelled gonia cells through meiosis in *Bufo himalayanas* (Gunther) (Sept. - December)

Days post injection	Most advanced labelled stage.	Other labelled stage(s)
0.25	Gonia	-
3.5	Gonia	-
0.75	Gonia	-
1	Gonia	-
3	Leptotene	Gonia
5	Pachytene	Gonia
7	Pachytene	Gonia
10	Pachytene	Gonia
15	Pachytene	Gonia
20	Pachytene	Gonia
25	Pachytene	Gonia
30	Pachytene	Gonia
35	Pachytene	Gonia

Dose : 20 μCi / animal
Sp. activity - 1000 Mci (BARC, Trombay)

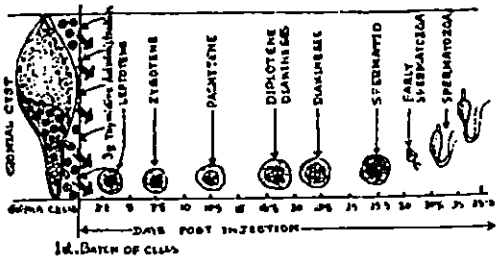

Fig. 1(a) Progression of ^3H - Thymidine labelled spermatogonia through various stages of meiosis and spermiogenesis in *Bufo himalayanas* (Breeding season, May–August)

(176)

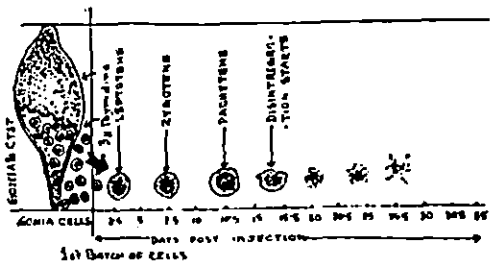

Fig. 1 (b) Progression of ^{3}H – Thymidine labelled spermatogonia during winter season through meiosis

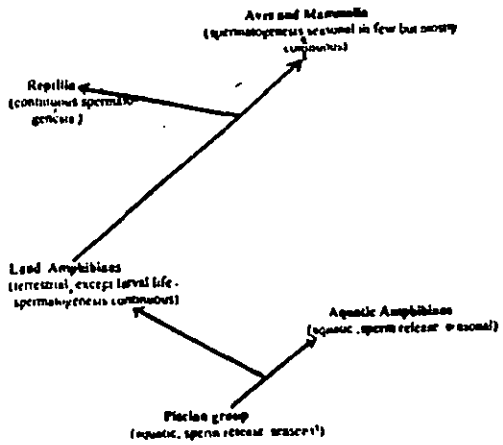

Fig. 2. Phylogenetic relationship between different vertebrates

Bufo himalayanas exhibits a discontinuous cycle, *i.e.*, in such species production of spermatozoa is confined to limited period of the year (breeding season, May-August)[6]. Therefore, kinetics of the spermatogenesis has been studied only during the breeding season. However, the species collected from lower altitudes (4000 ft. or low) exhibit continuation of spermatogenic activity till September and spermatocytes exhibit an arrest of meiotic transition beyond pachytene (Table-2).

The kinetics of meiosis of this species is somewhat different from other anuran species found in tropical and subtropical regions. The longer duration of each leptotene, zygotene, pachytene, diplotene and diakinsis is somewhat similar to *Xenopus laevis*[5]. In contrast to meiosis, spermiogenesis is remarkably short and is only 9.5 days in duration (Diagram-1a). In some species the pachytene may last for few months until they degenerate at the onset of winter (Diagram-1b).

The rapid transition and completion of spermatogenesis is somehow related with the habit of seasonal breeding which has an extremly short duration at high altitude.

Phylogenetically amphibians are related to piscian group on one hand & with reptiles on the other. Their phylogenic relationship has been established by various methods and techniques. The kinetics of the spermatogenesis can be used as a method to exhibit such relationship[6,13,14].

Meiotic duration in piscian group is the shortest among the vertebrates and similar such duration of meiotic stages is found in aquatic amphibian forms viz., *Rana. Bufo, Tylototrition* etc.[8,12,13]. On the other hand spermiogenesis of terrestrial amphibians is much longer as in reptiles (Diagram-2).

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Gonadal Differentiation Pattern of Himalayan Toad, *Bufo himalayanas* (Gunther) : ANURA, AMPHIBIA-An Ultrastructural Study

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Abstract

Gonadal differentiation pattern during larval development period has been studied from SEM and TEM observations. The larval period has been categorized into four stages, viz., 'O' limb stage, '2' limb stage, '4' limb stage and 'sub-adult' stage. The 'O' limb stage gonad exhibits no sign of gonadal differentiation and cells exhibit uniformity at ultra-structural levels. Gonadal differentiation becomes evident around '2' limb stage and '4' limb stage. Gonial cells also appear at late '4' limb stage. 'Sub-adult' stage exhibits appearance of primary and secondary spermatids. Appearance of scanty sperms is also evident at this stage which suggest a probable case of progenesis in this species.

Abstrait

Le motif de différentiation gonadale pendant la période du développement larvaire a été étudié des observations SEM et TEM. La période larvaire a été catégorisée en quatre phases, c'est-à-dire, phase 'O' membre, phase '2' membre, phase '4' membre et phase 'sous-adulte'. La gonade de la phase 'O' membre ne nontre aucun signe de différentiation gonadale et les cellules montrent l'uniformité aux niveaux ultra-structuraux. La différentiation gonadale est évidente vers les phases '2'-membres et '4'-membres. Descellules gonadiques apparaissent aussi vers la fin de la phase '4'-membres, La phase 'sous-adulte' expose l'apparence des spermatides primaives et secondaires. L'apparence des spermes insuffisants est aussi évidente à cette phase, ce qui suggère un cas probable de progénésis dans cette espèce.

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Spermatogenesis in amphibians is usually identified with sexual maturity. However, in several anuran species, spermatogenic waves have been reported well before the sexual characters developed and even in the larval stage. This phenomenon has been named as 'pre-spermatogenesis' or 'precocious spermatogenesis' or 'juvenile spermatogenesis' (5, 6).

In the present text, a phemonon of juvenile spermatogenesis has been described in an anuran species, *Bufo himalayanas*, found at high altitudes of Darjeeling hills. The study has been made under Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

Materials and Methods

Tadpoles of *Bufo himalayanas* were collected from different places of Darjeeling hills at an altitude of 1800-2300 mts. Tadpoles were reared in the laboratory conditions (temperature 20°C). Following the metamorphic events, the tadpoles were classified as 'O' limb stage, '2' limb stage, '4' limb stage and 'sub-adult' stage (Plate 1, Fig. a-d). Gonadal masses from each stage were collected by surgical operations for routine histological and ultra-microscopic studies.

A part of gonadal mass were fixed in 2.5% Glutaraldehyde in Cacodylate buffer (pH 7.1) for SEM studies, while the rest mass was fixed in Glutaraldehyde-Paraformaldehyde mixture for TEM observations. In both the cases, the post-fixation was made with 0.01 M Osmium Tetr oxide in cacodylate buffer (pH 7.1). Before critical point drying, the gonadal masses were superficially teased to expose the internal mass for SEM observations. SEM and TEM observations were made under Hitachi S-530 and Philips CM-10 Microscopes, respectively.

Observations and Discussions

The larvae of the *Bufo himalayanas* from hatching to emergence of sub-adults can be categorized into 39 stages. However, the gonadal mass can only be recognized visually on and from stage 21, called '0' limb stage, as a median oblong mass around the developing kidneys. For the convenience of the study, the larval developmental events has been summarized as '0' limb stage (i.e., with limb bud), '2' limb stage (i.e., with hind limbs only), '4' limb stage (i.e., with both hind and fore limbs) and 'sub-adult' stage (i.e., just after culmination of metamorphosis).

At 'O' limb stage, the gonadal anlagen is visualized as an oblong mass at the antero-median part of the developing kidneys. Under the SEM, the anlagen mass exhibits primordial germ cells of spherical shape. The gonads originate from an outpocket of cells on the ventral surface of the kidney. There is no observable differences between males and females. The undifferentiated anlagen mass is kept within solid oblong gonads with clear cortex and medulla portion.

At '2' limb stage, the gonadal mass assumes a globular shape and is lodged at the anterior end of the kidney. The developing gonad is morphologically indistinguishable as Testis or Ovary. However, under SEM, the developing anlagen was exhibiting the appearance of the gonial cells as spherical irregular cells of about 7-10 mμ in diameter. The surface morphology of the gonial cells is characterised by rough and irregular surface with numerous depressions and ridges at regular intervals (Plate 1, Figs. a-d).

The existence of gonial cells in the developing testis has been sustained from TEM observations. The gonial cells exhibit the characteristic features of gonial cells as in other anurans (Plate 2, Figs. a-e). These gonial cells exhibit irregular surface morphology with myriad convolutions. TEM ultra-structure shows oval or elliptical shape with oval nucleus and uniformly distributed chromatin granules. The nucleolus is electron dense and spherical. Cytoplasm is homogeneous and contains oval mitochondria, free ribosomes, granular and agranular endoplasmic reticulum (Plate 2, Figs a, b & c).

At '4' limb stage, gonial cells show some characteristics as in '2' limb stage. Under SEM observations, gonials cells show irregular surface morphology and oval and spherical shape of about 5-8 mμ in diameter. Here, at this stage, gonials cells along with primary and secondary spermatocytes are seen. TEM observations show gonial cells of similar nature as found in '2' limb stage. Primary and secondary spermatocytes show spherical central nucleus without nucleous. The electron dense heterochromatin masses of nucleoplasm irregularly distributed and condensed at the periphery of the nucleus. The cytoplasm shows characteristic electron dense droplets and vacuoles (Plate 2, Figs. d, e, & f).

Spermatogenesis is usually identified with sexual maturity. However, in several anuran species, spermatogenic activity has been recorded during early development before secondary sexual characters developed. Spermatogenesis has been recorded even in the larval conditions (1, 2, 4).

Iwasawa and Kobayashi (2) have described that the juvenile spermatogenesis usually ends with degeneration of spermatogenic nests before completion of spermatogenic cycle. However, others (3, 4, 5, 6, 7) have recorded that in some anuran species, the juvenile spermatogenic cycle may proceed to the formation of spermatozoon with no clear difference from the adult spermatogenic cycle.

The present study indicates that in Himalayan toad (*Bufo himalayanas*), the spermatogenic activity starts in the larval stage, when the differentiation of the gonadal anlagen of '0' limb stage takes place into a distinct gonadal fate (i.e., either testis or ovary) around '2' limb stage, which is supported by the experiments of Hayes (8). Therefore, critical point of gonadal differentiation in this species is likely to take place at around '2' limb stage.

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