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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

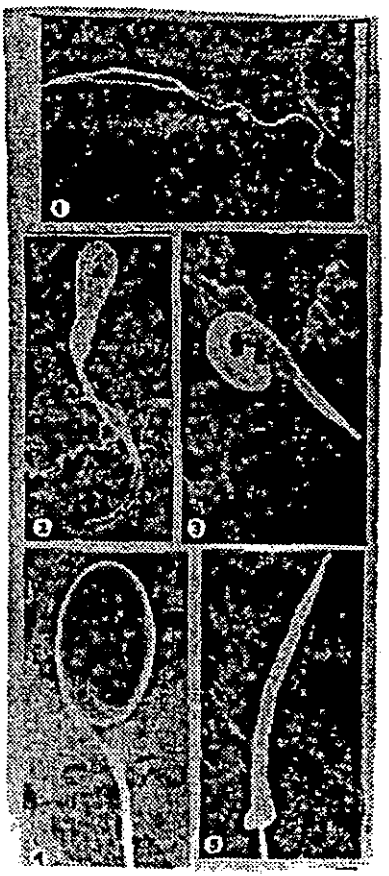

Fig. 1: Spermatozoon with elongated head, distinct middle piece and two axial tail filament.

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).

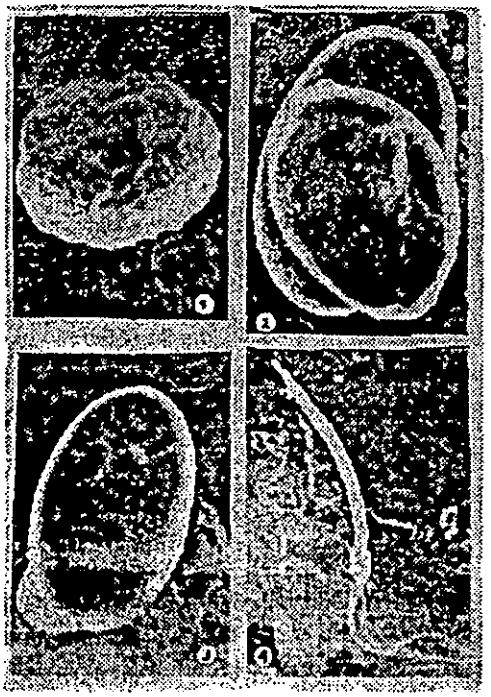

Plate 1. Photomicrographs of different stages of spermiogenesis of *Bufo himalayanus* under scanning electron microscope.

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)

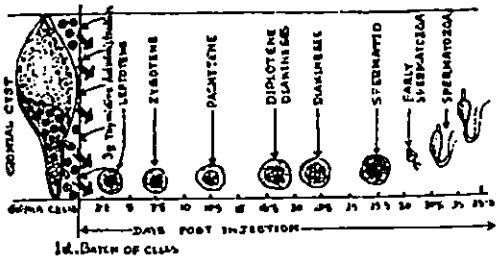

Fig. 1(a) Progression of ^3H - Thymidine labelled spermatogonia through various stages of meiosis and spermiogenesis in *Bufo himalayanas* (Breeding season, May–August)

(176)

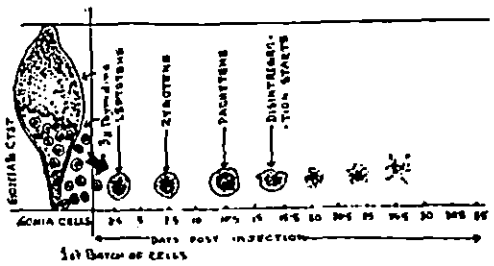

Fig. 1 (b) Progression of ^{3}H – Thymidine labelled spermatogonia during winter season through meiosis

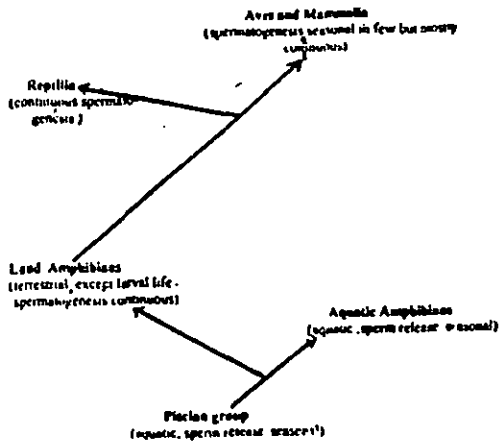

Fig. 2. Phylogenetic relationship between different vertebrates

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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