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SPERM POLYMORPHISM IN AMPHIBIA- A FLUORESCENCE ACTIVATED CELL SORTING STUDY

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ABSTRACT

Spermatogonia and spermatozoan cells from three anuran and one urodele amphibian species were collected by differential centrifugation method and the cells were fixed in 80% ethanol, stained with ethidium bromide in sodium citrate buffer and scanned under FACS. The histograms obtained from *Bufo melanostictus, Bufo himalayanas, Bufo stomaticus*, and *Tylototriton verrucosus* exhibited normal haploid spermatozoa to hypohaploid and hyperhaploid spermatozoan cells. Similarly gonia cells exhibited normal diploid (2n) to hypodiploid (< 2n) and hyperdiploid (>2n) DNA values. The observations strongly support earlier observations on sperm polymorphism in amphibians made by karyological and ultrastructural technique/method.

Key words : Sperm, Polymorphism, FACS, Analysis, Amphibia.

INTRODUCTION

Existence of Sperm Polymorphism has been recorded in a number of invertebrates and vertebrates including Amphibia (Siebold, 1836; Ankel, 1924; Gupta, 1964; Ray *et al*, 1984; Roy and Ray, 1989) reported that in Amphibia there are more than one clone of stem cells and each such clone has different chromosomal set ranging from normal diploid number to hypo and hyper diploid sets . In the present investigation, an additional evidence is provided to substantiate the existence of sperm polymorphism in Amphibia using fluorescence activated cell sorting (FACS) technique.

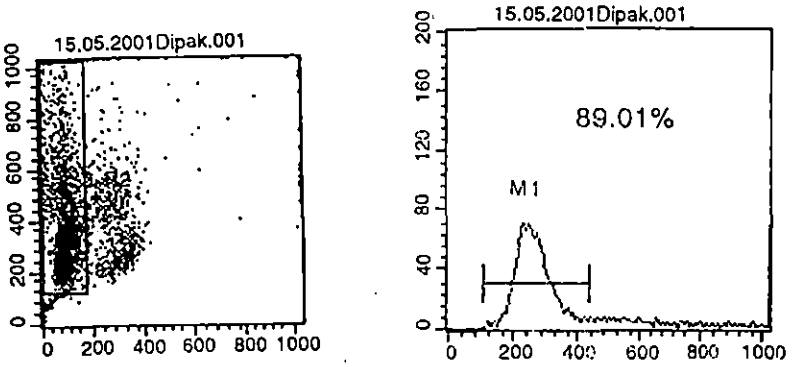
MATERIALS AND METHODS

Male adult toads of Bufo melanostictus, Bufo himalayanus, Bufo stomaticus and the urodele Tylototriton verrucosus were used as experimental animals. Spermatogonic cells were collected by using differential centrifugation technique (Ray *et al.*1984) and the fractions containing pure spermatogonia (2n) and sperm cells (n) were used for FACS study at IICB, Kolkata.

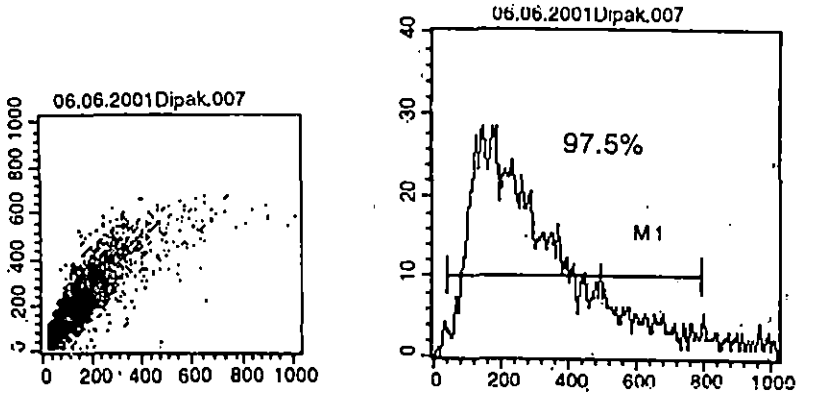
Spermatogonic cells were collected in phosphate buffer saline (PBS) and fixed in 80% ethanol overnight. Cells were then washed with citrate buffer(pH7) and mixed with 1% ethidium bromide in sodium citrate buffer prior to FACS study.

OBSERVATIONS

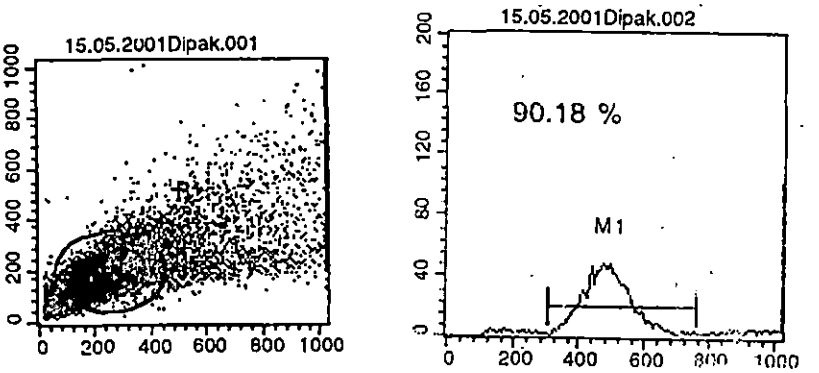
The histograms A, B, C, and D, suggest that in *B. melanostictus* 89.01% cells studied have normal haploid DNA value, while 10.9% cells have a range of below haploid DNA value to hyperhaploid DNA value. In *B. himalayanas* 97.15% cells have normal haploid value, while *B. stomaticus* 90.18% cells have normal haploid value. In *Tylototriton verrucosus*



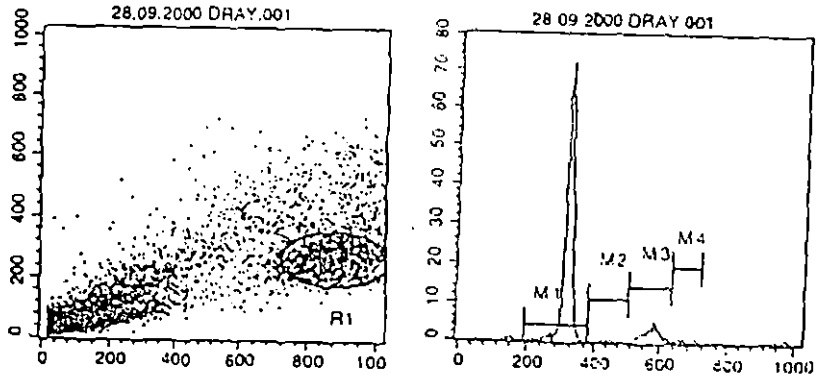
Histogram A - *Bufo melanostictus*



Histogram B - *Bufo himalayanus*



Histogram C - *Bufo stomaticus*



Histogram D - *Tylototriton verrucosus*

91.56% of cells have normal haploid value while 7.04% cells have normal diploid value. A small fraction of cells have hypo diploid (0.30%) and hyperhaploid (0.15%) DNA value. Similarly about 0.30% cells have hyper diploid DNA value.

DISCUSSION

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

Origin of sperm polymorphism has been described variously by different authors. Ray & Roy (1984), Roy (1989), Mukhopadhyay *et al*; (1999) reported that in amphibia gonia cells have different cytological and karyological features and gave the concept of 3 different clones of gonial cells; the normal diploid cell, hypo diploid cell and hyper diploid cells. The ultra structural features of such have been described in *B. himalayanus* by Mukhopadhyay (2002) and in *Tylototriton verrucosus* by Patra *et al* (2002).

In the present investigation, gonia cells (2n) were mixed with fractions of spermatozoa collected by differential centrifugation method theoretically to have haploid DNA content. However, the histograms exhibited an array of hypohaploid to hyperhaploid DNA values. Similarly hypodiploid and hyperdiploid DNA values were also recorded, unequivocally suggesting the existence of different gonia as well as spermatozoan cells in the four amphibian species investigated.

A further study is under process to establish the existence of different expression of sperm specific proteins (SP1, to SP6) in gonial as well as spermatozoan cells.

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SEXUAL DIMORPHISM IN ENZYME ACTIVITY PATTERN OF *Canis lupus chanco* (TIBETAN WOLF)

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A biochemical study was undertaken to reveal enzyme activity pattern of male and female captive wolves reared at Padmaja Naidu Himalayan Zoological Park, Darjeeling. The quantities of total protein, serum albumin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase were determined following standard biochemical techniques. The study indicated a strong sexual dimoephism in enzyme activity at normal physiological condition.

Introduction

Wolves are of generally two types – the Red wolves and the Grey wolves. The Tibetan wolf – *Canis lupus chanco* is one of the grey wolves. This Himalayan creature is found at an altitude between 7000 and 13000 feet in the Western Himalayas. These canines prey upon larger mammals, such as wild goats, sheeps and other high altitude herbivores to smaller ones like rodents besides birds (Annual Report PNHZP, 1995–1996).

Lots of information are available on the blood cells and various blood parameters of diverse vertebrates. Several workers have stressed upon the possibility of employing haematological indices as aids to the diagnosis and assessment of diseases. In the present work blood was collected from some of the captive wolves and was tried to estimate the total protein, serum albumin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase were determined following standard biochemical techniques. A biochemical study was undertaken to reveal the enzyme activity pattern in male and female wolves.

Materials and methods

Experimental animals

The specimens under experiment were from Padmaja Naidu Himalayan Zoological Park, Darjeeling, situated at an elevation of 2133.5 mts. Three Tibetan wolves were taken for the experiment.

Serum collection

10 ml of blood was drawn from individual wolf and centrifused (1 hr, 37°C) when the blood cells precipitated down. The supernatant was the only source for the serum which thereafter was centrifused (3000 RPM, 5 mins.).Discarding the unwanted materials the collected serum was immediately used for the assay.

Experimental procedure

The commercial kits were used for getting the results without delaying time as because the enzyme activity changes with the change of time. The experiments were done according to the protocols as provided by the kits (Qualigens diagnostics from Glaxo and Dr. Reddy's laboratory).

Results

After using the kits in required proportions and incubating in the prescribed order we recorded the results obtained from the spectrophotometric observations.

	Male	Female
Total Protein	8.29 gm%	8.79 gm%
Serum Albumin	4.13 gm%	3.82 gm%
Alkaline Phosphate	16.22 U/ml	14.24 U/ml
SGOT	16.25 U/ml	14.50 U/ml
SGPT	12.16 U/ml	10.92 U/ml

Discussion

From the observations recorded we noticed that all the blood parameters, except that of the total protein, show a greater value in male than that of the female wolf.

SGPT catalyses transfer of amino group from L–alanine to α–ketoglutarate with formation of pyruvate and glutamate. SGPT catalyses transfer of amino group from L–aspartate to α–ketoglutarate with formation of oxaloacetate and glutamate. Formed products are then used for citric acid cycle and protein biosynthesis (Das, 1995). The experimental result suggests that male wolves are metabolically more active than the females under captive condition.

SGOT, SGPT and alkaline phosphatase are enzymes which are globular in nature and supply a mass to the total protein.

A higher total potein level in female than the male may be due to the fact that the female bears the baby and so she feeds both for herself and the baby (Chatterjee, 1992). Again, a high total protein content in such carnivorous wild animals in respect to a normal human being (6.0–6.9 gm/100 ml, Chatterjee, 1992) suggests that it may help them during starvation period (there may be a chance of not getting adequate food everyday) as protein reservoir.

The serum albumin content in Tibetan wolves is in normal level (54–60% of the total plasma protein, Das, 1995) but the female shows a declined value than the male. We know that when the body fluid discharge or decreases the albumin level also decreases (McMurray, 1982). As the female in our experiment was pregnant, so the decreased albumin may be due to the placental supply to the baby.

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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., '0' limb stage, '2' limb stage, '4' limb stage and 'sub-adult' stage. The '0' 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 '0' membre, phase '2' membre, phase '4' membre et phase 'sous-adulte'. La gonade de la phase '0' 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 '0' 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 '0' 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.

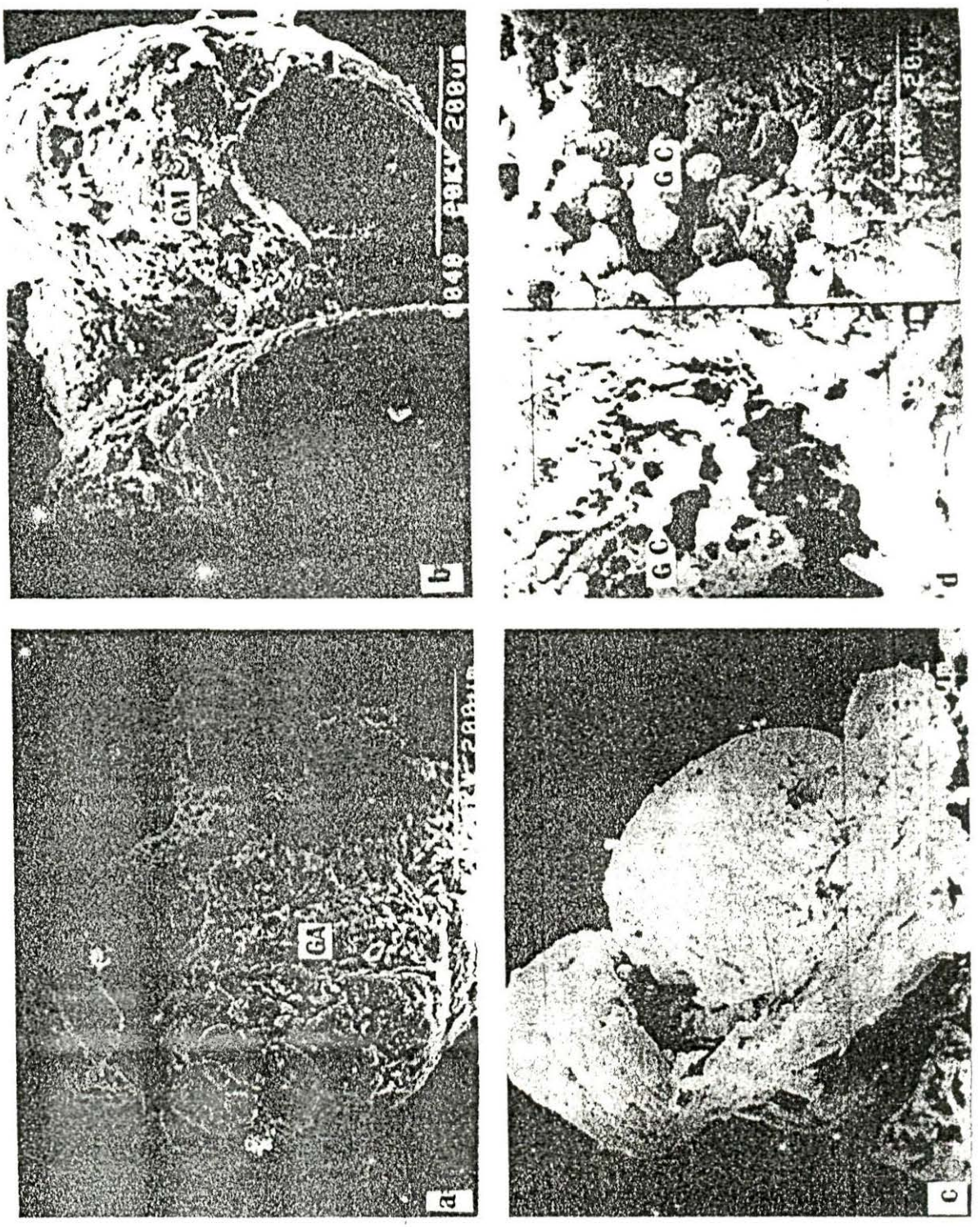
At '2' limb stage, the gonadal mass assumes a globular shape and is lodged at the anterior end of the kidney. The developing gonad is morphologically indistinguishable as Testis or Ovary. However, under SEM, the developing anlagen was exhibiting the appearance of the gonial cells as spherical irregular 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-c). 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).

Plate -1



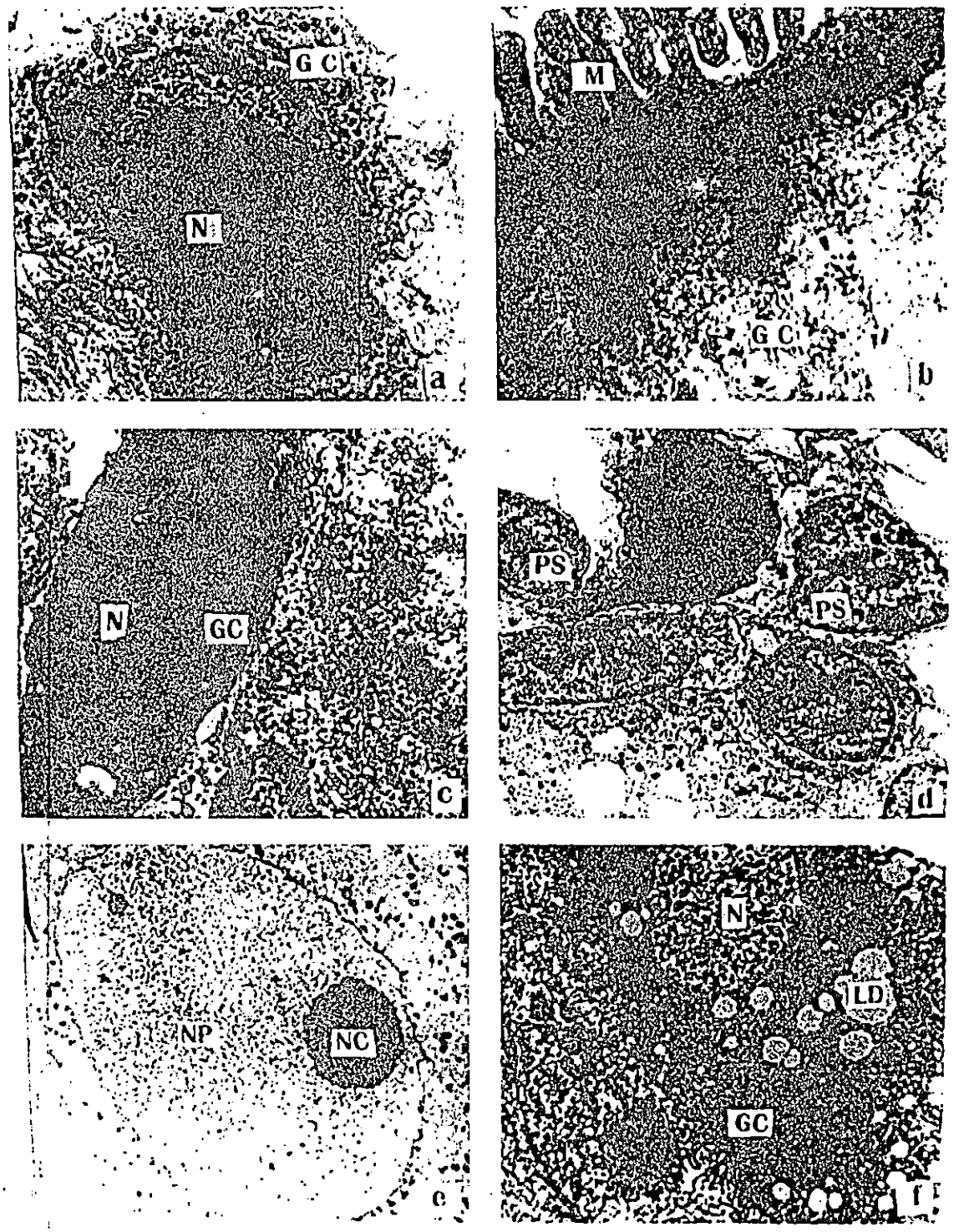
0 limb stage

2 limb stage

4 limb stage

4 limb stage (enlarged)

Plate -2



GC = Gonial cell PS = Primary spermatocyte N = Nucleus M = Micovilli
NC = Nucleolus NP = Nucleoplasm LD = Lipid droplet

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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