MATERIAL AND METHODS

A. Material

The Himalayan newt, ***Tylototriton verrucosus*** Anderson (Urodela : Amphibia) was used as the experimental animal in this study.

a) Site of Collection

The male newts were collected from the mountain streams near the Darjeeling hill areas at an altitude of about 1660 meters (latitude 26. 95° East and longitude 88.27° North) in the Darjeeling district of West Bengal, India. Specimens were procured from their normal habitat round the year and maintained in the laboratory in glass aquarium provided with stones, water and plants. They were fed with normal fish meal, tadpole and earthworm.

b) Identification of Male Specimens

The male specimens may be clearly identified by their size , shape and body coloration. They are smaller in length (14 -16 cm) than females (18 -20 cm). The size, however, is a misleading criterion. Hence, other features were taken into consideration by the present worker. The males have slender body, a short blunt flat head and are dark brown in colour in contrast to the females which are characterized by broad body, narrow triangular head and bright brown body colour (Plate-1). Moreover, the males have a yellow rim at the ventral side along the length of tail.

PLATE - 1

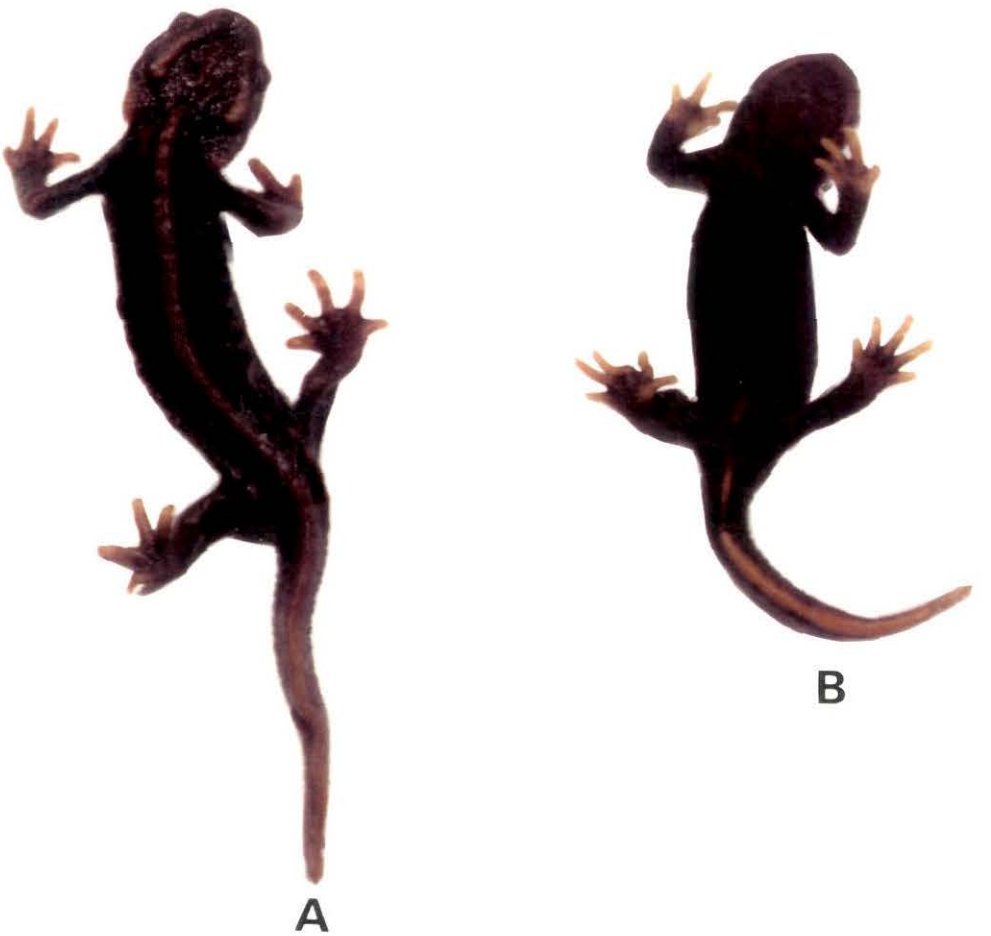

Figure A. Male *Tylototriton verrucosus* Anderson , dorsal view.
Figure B. Male *Tylototriton verrucosus* Anderson , ventral view.

B. Methods

a) Collection of testis samples

First year specimens were collected and five specimens of each season were sacrificed. Males are dissected ventrally to collect the testes. After collection blood, blood vessel and other tissue fragments were removed.

b) Histological Preparation

Immediately after the collection of testes the blood vessels were removed. Small pieces of gonadal tissues were fixed in Bouin's solution (aqueous) for 24 hours. Routine histological procedure (Baker, 1966) was followed. Tissues were embedded in paraffin. Sections were cut in 5μ thickness and stained with Periodic Acid-Schiff and haematoxylin (Ray, 1978) and Haematoxylin and Eosin.

c) Tissue preparation for scanning electron microscope study

Testes of pre breeding, breeding, post breeding and regression were surgically removed and each was medially cut into two halves. One half was fixed in Bouins solution (aqueous) for routine histological technique. The remaining half was fixed in 2.5 % glutaraldehyde and 1% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.1) for 4 hours at 4°C. After fixation, the materials were washed and transferred in 2% osmium tetroxide solution in the same buffer for two hours. The tissues were washed with cacodylate buffer and dehydrated through ascending grades ethyl alcohol. The tissues were treated with a mixture of absolute alcohol and amyl acetate (1:1) for 30 minutes and were kept in amyl acetate at 4°C (Tanaka et al., 1981). The materials were critical point dried (Tanaka et al., 1981) and coated with colloidal gold (Echlin et al., 1975).Surface morphology of the tissues were studied under Hitachi S530 Scanning Electron Microscope at Central Instrumentation Center of Burdwan University, Burdwan, West Bengal, India, where the subsequent

process was carried out.

d) Germ cells preparation for scanning electron microscope study

Testes of pre breeding , breeding , post breeding and regression were surgically removed and each was medially cut into two halves and the cut testes lobes were teased longitudinally into 0.1 M cacodylate buffer (pH 7.1).Then the resulting cloudy solution was centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was discarded, and the precipitate fixed in 2.5% gluteraldehyde with 0.1 M sodium cacodylate buffer (pH 7.1) for 4 hours at 4°C. After fixation, the material was transferred in 2% osmium tetroxide solution in the same buffer for two hours. The material was washed with cacodylate buffer and dehydrated through ascending grades of ethyl alcohol. Then the material was treated with a mixture of absolute alcohol and amyl acetate (1:1) for 30 minutes and was kept in amyl acetate at 4°C (Fujita et al., 1970). The materials were critical point dried (Tanaka et al., 1981) and coated with colloidal gold (Echlin et al., 1975).Then the germ cells were observed under a Hitachi S530 Scanning electron microscope at Central Instrumentation Center of Burdwan University, Burdwan, West Bengal, India.

e) Tissue preparation for transmission electron microscopy

The testes were surgically removed and dissected in amphibian saline (pH 7.1). They were reduced into fragments (1 - 3 mm.) and fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C (Karnovsky, 1965). After fixation, the materials were washed in cacodylate buffer (pH 7.1) and postfixed in 1% osmium tetroxide in the same buffer for 2 hours in the dark at 4°C , contrasted in block with aqueous solution of 5% uranylacetate for two hours, dehydrated in acetone and embedded in araldite. Thick sections (1μm) were cut, stained with aqueous toluidine blue and observed under a light microscope. Thin sections (60-70 nm) were cut in an

L.K.B ultramicrotome, mounted on copper grids and stained with a saturated solution of uranyl acetate in 50% alcohol and lead citrate (Reynolds, 1963). The grids were observed under a Philips CM 10 Transmission electron microscope at Regional Sophisticated Instrumentation Center, All India *Institute Of Medical Science (AIIMS), New Delhi, India.*

f) Preparation of spermatogenic cells for flow cytometric analysis

Testes were surgically removed and each was medially cut into two halves and the cut testes lobes were teased longitudinally in to 0.1 M phosphate buffer saline (pH 7.1). Spermatogonic cells were collected by using differential centrifugation technique (Ray et al., 1989) and the fraction containing pure spermatogonia (2n) and sperm cells (n). Spermatogenic cells were washed by PBS (pH 7.1) and centrifuged at 2000 r.p.m. for 5 minutes. The cells resuspended in 1 ml fresh PBS and fixed with 1 ml 80% ethanol at 4°C for overnight. The cells were spinned down and 250 μg of RNase in 500μl 38 m M sodium citrate buffer solution was added to it and kept at 37°C for 45 minutes. The cells were again spinned down and stained with 69μM ethidium bromide kept at room temperature for 15 minutes and then transferred at 4°C for 2 hours in a dark condition. The cells analyzed under a FACS CAN, Buton Dickensen machine at Indian Institute Of Chemical Biology, Kolkata, West Bengal, India.