* CHAPTER - II *

* MATERIALS AND METHODS *

II. MATERIALS AND METHODS

A : RATE OF PASSAGE OF FOOD AND DIGESTIBILITY.

MATERIALS :

Live specimens of Rana tigrina were obtained from fresh water ponds near Barshi Town. They were brought to the laboratory of the Zoology Deptartment of Shri Shivaji Mahavidyalaya Barshi. The collected specimens were kept in large aquarium tanks (Measurement 60 x 30 x 30 cms.) containing fresh water. Approximate weights of these specimens were taken on a monopan balance.

Round glass containers of diameter 30 cms. and height of 25 cms. were used for experiments to determine rate of passage of food and digestibility. The experiments were conducted in rainy season as the frogs are active in this period and the range of variation of temperature is limited.

Live specimens of earth worms, cockroaches, beetles, and grasshoppers were collected from various places in or around Barshi and stored in small animal cages, for use in feeding experiments. Small live fishes and crabs were brought from the fishries centre at Chandani (Tal. Paranda) 18 kms. from Barshi. Raw meat and bread were obtained from the market.

METHODS :

Rana tigrina specimens were first kept in the aquarium tanks for two to three days to acclimatise them to laboratory conditions.

Before starting experiments on the rate of passage it was necessary to determine the time required for complete evacuation of the alimentary canal. To determine this time period some of the frogs from aquarium tanks were transferred to the glass containers containing filtered fresh water. These specimens were dissected at different intervals and it was noticed that after three days their alimentary canals never contain any food matter in any part of the alimentary canal. frogs used for the study of rate of food passage were starved for three days before starting the experiments.

METHOD OF FORCE FEEDING :

As frog is an active animal it was found that a feeding technique was necessary for force feeding. For this feeding experiment the frogs must be anasthetized suitably in such a way that the recovery will be fast. To determine adequate concentration of chlorofarm certain experiments were conducted using different concentrations of chlorofarm in a known quantity of water. Six liters of water were taken in the experimental tank because then the frogs remained sufficientely under water

surface. In the first experiment 20 Mls. of pure chlorofarm was added. The frogs which were active were anasthetized within one minute. To study its recovery it was transferred to a tank containing only fresh water, but no anasthetic. It was observed that the frog regained its normal activity within 15 minutes. In the next experiment 15 Mls. of chlorofarm were used. The frogs were anasthetized within one minute. In this experiment, frog regained its normal activity after 10 minutes. In the third experiment the concentration of chlorofarm used was 10 Mls. in 6 liters of water. The frogs were anasthetized within two minutes and the recovery was in five minutes. In the fourth experiment when concentration of chlorofarm used was 5 Mls. , then the frog was anasthetized within 5 minutes but as soon as it was removed from the tank it regained its normal activity. This suggests that with this concentration it was not properly anasthetized. On the basis of these observations the concentration of 10 Mls. of chlorofarm in six liters of water was selected as suitable concentration for anasthetizing frog for our feeding experiments, because this concentration will ensure that there will be no side effects of chlorofarm on frogs and their digestive process will not be affected, since the recovery was quick.

The weights of the frogs selected for experiments varied in the range of 80 gms. to 100 gms. The temperature of the tank water varied from only 27.5^{0}C to 29^{0}C through out the experiments.

The anasthetized frog was lifted with hand, then its mouth was opened widely, using fingers, and 5 gms. of food item was force-fed by using sterilized forceps. After this the frog was transferred to the experimental container with filtered fresh water to avoid any further entry of any other food particle in the medium. While recovering in fresh water the food item placed in the buccopharyngeal cavity was observed being swollowed and the frog then recovered its normal activity within 5 minutes. No vomiting of the food-item given was observed before or after its recovery. At a time four frogs were fed with the known quantity of the same food-item. Each of these specimens was kept in a seperate experimental tank.

The specimens were killed by pithing after the requisite time periods, and specimens were dissected after different time intervals and the food-item which was given, was located in the alimentary canal. The region where it was located, was recorded, the condition of the recovered food-item was observed and when the food item was recovered from rectum it was weighed for assessment of digestibility of that

food-item for the frog. As there is possibility that some of the faeces might have already been thrown into the surrounding water of the glass container containing filtered water, the water was refiltered & particles of faeces recovered, if any, were added to those recovered from rectum and then weight was taken to determine the amount of indigestible material.

B : STUDY OF CHITINOLYTIC ACTIVITY IN THE ALIMENTARY CANAL OF *Rana tigrina* :

A very simple method was devised to detect chitinolytic activity if any in the alimentary canal of *Rana tigrina*. The method is a slight modification of the method described by Jeniaux (1968).

PREPARATION OF ENZYME EXTRACTS :

The organs stomach, duodenum, ileum pancreas and liver were removed from the well fed frog with chitin containing food. (cockroach). The organs removed from the frog were kept in the ice cold distilled water. The gastric mucosa was removed and used for the preperation of extract. Similarly the mucosa of duodenum and ileum was used for preparing extracts. Liver and pancreas were used as a whole for preparing extracts. These parts were dried on the filter paper and ground with glass powder in a mortar with the help of a

pestle. The extracts were taken in the ice cold water in seperate test tubes and stoppered with cork. Then these tubes were kept at $4^{0}C$ for 24 hours. Then each extract was seperately centrifused for about 20 minutes. The supernatant was used to demonstrate chitinolytic activity.

The native chitin suspension was used as a substrate for determining chitinolytic activity. Native chitin was prepared by the method described by Jeuniaux, Ch. (1966) in ("Methods in enzymology" Vol. VIII by Neufeld and Ginsburg). The native chitin suspension was prepared from cuttle fish bones which were freshly collected from Ratnagiri. Dried bones were mechanically powdered and decalcified using .5N HCl it was then treated by .5 N NaOH at $100^{0}C$ and then washed. The resulting powder was used for preparing a suspension containing 5 mg/ml. (This powder is the native chitin).

To confirm whether the prepared product is chitin, it was tested by Van Wisselingh test which is based on its conversion into chitosan by saturated caustic potash at a high temperature and then identifying chitosan by its solubility in acids and by the rose violet colour it gives with iodine. Van Wisselingh Test: The material to be tested for chitin is treated with concentreated Potassium hydroxide, at 160°C for 20 minutes and then tested with 0.2% iodine in 1% Sulphuric acid, which gives a rose-violet colour if chitin is present (campbell,1929).

PREPARATION OF BUFFER SOLUTION :

It was prepared using citric acid .6 m and Na_2HPO_4 1.2m at p^H 5.1 .

PROCEDURE FOR DETECTION OF CHITINOLYTIC ACTIVITY :

The test tube containing 1ml. chitin suspension 1ml. buffer and 2ml. of enzyme solution (extract) was incubated at 37^0C for about 100 to 120 minutes. A control tube not containing enzyme extract was also incubated along with the other tubes. To determine whether there is any chitinolytic activity Van Wisselingh test for detection of chitin was then taken.

C : EFFECT OF DIFFERENT FOOD-ITEMS ON THE GROWTH RATE OF *Rana tigrina* UNDER LABORATORY CONDITIONS :

MATERIALS AND METHODS :

Live specimens of *Rana tigrina* were collected around Barshi Town and kept in aquarium tanks for few days to acclimatise them to laboratory conditions.

From this stock, specimens of approximately similar size and weight were transferred to experimental glass containers. Eight specimens were taken and only one specimen was kept in one container. The specimens were then starved for three days to ensure complete evacuation of alimentary canal. This period of three days was decided on the basis of our own previous experiments (Chapter II - A).

The experiments were conducted in rainy season as the frogs are active in this period and the range of variation of temperature is limited. Method for studing the comparative effects of different foods on the rate of growth was adopted from E.W. Shell (1966). For each food-item a duplicate experiment was also set up. The weight of each frog in each trough was recorded before the begining of the experiment. Eight troughs containing each a live specimen of Rana tigrina were fed on 2.5 gms. of each food-item twice a day i.e. 5 gms. in a day. Between two feedings of the day an interval of about 12 hours was kept. This ensures emptying of the stomach and this will ensure inclination of the frog to take food.

The food-items selected for study of effect on growth were cockroach, earth worm, meat and bread. For each food-item a duplicate experiment was set. Thus four food-items in all eight troughs were used.

The feeding of all specimens was continued for twenty days (from 1st August 1987 to 20th August 1987). Exactely similar experiments were repeated from 4th July 1988 to 23rd July 1988. After this time interval of 20 days each specimen was weighed again and the weights were recorded.

**