Materials and methods

*Bufo melanostictus* (Schn.) the common Indian toad, is a large-sized toad measuring for about six inches in its snout to vent length. It is common throughout Indian subcontinent. It is a commensal of humans, and information on its distribution, morphological features, identification, breeding behaviour etc. is easily found in literature (Ref.: Boulenger, 1890; Inger and Dutta, 1987; Kanamadi and Hiremath, 1989).

The fertilized eggs needed for the present research work, were obtained by collecting the amplexed pairs of toads from the field, in the rainy season (April to July) and allowing them to spawn in the laboratory aquarium. Amplexed pairs were collected from the temporary ponds, in the surrounding area of the laboratory, at Dharwad (15° 17'N, 75°3'E). If the amplexed pair was collected at about 7 a.m. in the morning and brought to the laboratory, the spawning would take place by 11 a.m. in the laboratory. Live embryos and larvae of the toad, in the laboratory were cared as per the guidelines given by Rugh (1962).

The amplexed pair in the laboratory was kept in aged tap water, in a glass aquarium of size 15 X 12 X 9 inches. The height of the water level in aquarium was maintained, so that the amplexed pair remained half submerged, its body touching the bottom of the aquarium. *B. melanostictus* layed a large number of eggs at a single spawning. According to Kanamadi and Yamakanamaradi (1970), it lays about 9000 to 51000 eggs. The eggs were laid in the form of two separate strings of jelly, both strings simultaneously coming outside the animals body. Each string consists of a single row of eggs. Each string remains as a single, continuous thread of jelly, throughout the spawning process. Whole spawn of
the toad, thus consists of a large mass of eggs, in the form of two separate strings of jelly. As the sufficient amount of spawn became collected in the aquarium, still spawning amplexed pair was removed from the aquarium. Spawn remaining in the aquarium was left there for some time. During this, the spawn was gently agitated in the aquarium water, to ensure fertilization process.

After some time, the long strings of egg mass were cut into small pieces and pieces were transferred to petri-dishes of diameter of 6 inches, containing aged tap water. In each dish only few pieces were put, so that pieces were sparsely distributed throughout the dish, for facilitating the respiration of developing embryos.

The eggs were left in the petri dishes, for about 5 days after fertilization process. During this period, the water was changed daily. At about 5th day, larvae appeared to have started feeding, as indicated by appearance of the faecal matter in the dishes. (Faecal matter appeared in the dish, although no food was added to the dishes during the period of five days. This seemed that the larvae, at 5th day, which already had developed mouth parts, might have used as food, the jelly coverings which remained after hatching, and to which the hatched larvae remain attached for long time, after hatching). Also, at 5th day, the larvae started to move more actively across the tray. Thus, appearance of their faecal matter and their active movement indicated the necessity of feeding the larvae, after 5th day.

Some anuran larvae release a substance that hinders the growth of other larvae in the same container (Dent, 1968). This was also observed in the larvae of the B. melanostictus, in my earlier attempts to rear a group of larvae, in a single tray. Larvae
from same batch of eggs, appeared to be of more dissimilar sizes, from each other, and to 
be more dissimilar in their stages of development. This was observed, irrespective of 
factors like tray size, frequency of change of water in the tray, etc. Thus, to provide all the 
larvae the same environmental conditions, the larvae of the toad were reared individually, 
after 5th day, till completion of their metamorphosis. When cultured individually, all larvae 
developed almost with the same speed, all larvae of a stage, attaining similar sizes, at 
particular stages of their development.

From start of spawning to completion of metamorphosis, all larvae were reared 
under the same laboratory conditions. Photoperiod of about 12hrs. light and 12hrs. dark, 
and temperature of about 24.5°C was maintained in the rearing room. Tap water was used 
for rearing, and it was made dechlorinated by allowing it to stand for two to three days in 
an open cement tank, before use. All larvae were reared individually, on fifth day onwards, 
and also were fed from the same day onwards. The small trays used for individual rearing 
were of plastic white colored trays of size 3.5 X 3.5 X 1.5 inches. Each was filled with 
water, almost fully. Larvae were fed on varieties of food, like green colored debris 
collected from the bottom of the pond, the algae like spirogyra, white and yellow portions 
of boiled egg, boiled vegetable leaves like, spinach, cabbage, etc. and some times frog 
thigh muscles also. Faecal matter along with half water was removed from the tray by 
sucking tube, and half fresh water and food was added to the tray, at the same time, usually 
once in a day.

Totally, egg layings of three different pairs of amplexed toads were reared in the 
laboratory for collecting the different stages of development for histological work and to 
observe the duration of different stages of development. For convenience three rearings
were started at different times, with a gap of six to seven days in between. But, all larvae from eggs of a pair, were always fed at the same time, with same quantity and same kind of food. The developing embryos and larvae were frequently observed under stereoscopic binocular dissection microscope, for identification of different developmental stages. Cold light was used during observation, esp. on earlier stages of development. If more than seven embryos / larvae of ten randomly selected ones were in required stage of development, a particular stage was assigned to those ten embryos / larvae. Noting the duration of stages, those ten embryos / larvae of each stage were fixed and preserved. One which was of medium size, of all ten, was measured for its size. Sizes of the embryos of earlier stages were measured by micrometer fitted to the dissection microscope. As the embryo / larval size was increased, in later stages of development, they were measured by putting them on the graph paper. Selecting one specimen from each stage of development its lateral, ventral and dorsal views were drawn with the help of camera lucida.

The values of durations of different stages of development and metamorphosis, and values of normal sizes of the embryos / larvae of each stage, were obtained from the egg layings of first pair of amplexed toads. Those are the values mentioned in the text. However, those values were confirmed by the egg layings of second and third pairs of toads, while collecting the embryos for histological work, and also by observations done on their larvae, but observations were done occasionally. Eggs of all three pairs of toads developed almost with the same speed, and larvae metamorphosed at about 48th day of egg laying. All embryos and larvae of three pairs of toads at particular stages of development appeared to be approximately of similar sizes, to each other, during development. About 500 larvae, from first pair of amplexed toads were cultured individually. Of them, about
350 larvae were used for collecting different stages of larvae, till completion of metamorphosis. Majority (about 85%) of the remaining larvae were metamorphosed at 48th day of their development, after egg laying. Likewise, about 200 larvae from each of second and third pair of amplexed toads were cultured individually. Majority of them also metamorphosed at about 48th day of their development.

For histological work, about 30 embryos, ten from each pair of amplexed toads were collected for each stage of development. Embryos and larvae, from two-cell stage to early hind limb bud stage, were fixed in smith’s fluid. The jelly covering around the embryos till hatching stage were removed before fixation. The embryos, with the help of a brush were rolled over the wet, blotting-paper, to remove the jelly coverings. After fixation in smith’s fluid, the embryos were thoroughly washed in running tap water and preserved in 4 % formalin. The larvae from hind limb bud-stage onwards, were fixed in Bouin’s fluid and washed and preserved in 70 % alcohol.

External features of the embryo, are described as they were visible, mainly at 10 X 2 magnification of stereoscopic binocular dissection microscope. Internal features were studied in embryos from two-cell stage to the advanced neural tube stage. For this, totally nine embryos of a stage were sectioned into serial sections. Of nine, three were sectioned into cross sections, three into lateral, and another three into horizontal sections. Before selecting the embryos for sectioning, each of ten embryos of a particular stage, collected from three pairs of toads were mixed randomly. Of these total 30 similar embryos, ten embryos that appeared to be furthermore similar in their external features and in their sizes were selected for sectioning. As mentioned above, these embryos were fixed in smith’s fluid and were preserved in 4 % formalin. In further histological process, they were washed
in distilled water, dehydrated in alcohol grades, cleared in benzene and embedded in paraffin wax. Serial sections were cut at 5 – 10 μ thickness. But, always, one specimen was cut only at one thickness. Sections were stained with eosin and hematoxylin. Observations were done with the monocular compound microscope, mainly at 5 X 10 and 10 X 10 magnifications.