DISCUSSION

Metamorphosis exemplifies the most profound change in the form found during the postembryonic development of all animals. Perhaps the oldest and most widely used model system to study metamorphosis has been the amphibians. The most commonly known form of amphibian metamorphosis is the anuran metamorphosis. Essentially every organ/tissue of the tadpole undergoes a transformation and consequently an aquatic herbivorous tadpole gets converted into a terrestrial carnivorous frog.

Since amphibian metamorphosis can be used as a model system to study postembryonic development in vertebrate animals, it has always attracted the interest of biologists. A wide spectrum of life scientists, from developmental biologists to biochemists to cell biologists to more recently molecular biologists have focussed their attention to this complex but systematic process.

In its simplest form, metamorphosis can be perceived as a postembryonic period of profound morphological changes by which the animal alters its mode of living. Metamorphosis is known to occur in all major living chordate groups except amniotes (Just et. al. 1981). However, the most comprehensive and most dramatic transformations occur in anurans. This postembryonic process systematically transforms most, if not all, organs of a tadpole to their adult forms. Essentially, three primary changes in tadpoles take place during metamorphosis. The first is the complete resorption of tadpole-specific organs such as the tail. On the other extreme, frog-specific organs like the hind limb develop de novo from undifferentiated cells in a process that involves first the proliferation of cells and subsequent cell differentiation and
tissue morphogenesis. The last major type of transformation is the partial but profound remodeling of the existing organs like the liver and intestine into their adult forms.

Likewise, the other organ systems also undergo transformations which can be either degenerative, modification or proliferative. Thus, the gills degenerate completely, while the nervous system is restructured, the exocrine pancreas change in shape, larval hemoglobins and red blood cells convert to adult types.

Not only do different organs undergo different changes, but they also occur at distinct developmental stages to coordinate the effective transition of a tadpole to a frog. Furthermore, even within a single organ, different tissues undergo specific transformations.

Amphibian metamorphosis provides an excellent model system to understand the basis of thyroid hormone action in vertebrates, particularly during development. The process of metamorphosis is broadly separated into three specific periods: premetamorphosis, prometamorphosis and metamorphic climax. Premetamorphosis refers to a period when embryogenesis and early tadpole growth and development take place in the absence of thyroid hormone. However, some morphological changes such as limited development of the hind limbs do occur. During prometamorphosis, hind limbs undergo morphogenesis as exemplified by the differentiation of the toes and rapid and extensive growth of the hindlimbs. The period is characterized by rising concentrations of endogenous thyroid hormones. Metamorphic climax is the period when endogenous TH is at highest levels and when rapid morphological changes, such as tail resorption take place.
Thyroid hormone has long been known to be important for vertebrate development and adult organ function. However, the role of the thyroid in metamorphosis was first demonstrated by Gudernatsch (1912), who discovered that tadpoles metamorphosed prematurely when fed with sheep thyroid gland. Shortly thereafter, it was shown that the active ingredient in the thyroid gland is thyroid hormone. Subsequent studies led to the isolation and structural characterization of two natural thyroid hormones and demonstration that TH is the causative agent of amphibian metamorphosis. It is now well known that the rising level of TH from the developing tadpole's thyroid gland induces a timed series of functional and morphological changes leading to the formation of a frog.

Both the naturally occurring thyroid hormones 3,3',5,5'-tetraiodothyronin (T4), commonly known as thyroxine, and 3,3',5'-triiodothyronin (T3) are synthesized in the thyroid gland. The T3 and T4 secreted from the thyroid gland are subsequently carried by the plasma to different organs/tissues where they exert their biological effects. Elevations in the circulating plasma concentrations of thyroid hormone T3 and T4 correlate with metamorphosis. Studies have established the fact that TH is a causal agent for metamorphosis. Another major characteristic of metamorphosis is that very different types of developmental changes are initiated in different tissues by the same hormonal signal. Thus thyroid hormones control such diverse processes as induction of morphogenesis in limb buds, cell death underlying the regression of tail and gills and excessive morphological and biochemical remodeling of the liver, intestine, pancreas, skin and central nervous system.
The TH-induced morphogenetic changes are the result of regulated gene expression. Further the biological effects of TH are mediated by TH receptors (TRs). TRs belong to the superfamily of nuclear receptors and two TR isoforms have been identified, TR $\alpha$ and TR $\beta$. Metamorphosis is probably initiated by the binding of TH to TR $\alpha$ which appears during embryogenesis and is present throughout the tadpole life. TR $\beta$ is presumed to play a role later in development and follows the endogenous TH concentration.

**TAIL**

During anuran metamorphosis, almost every organ undergoes modification, such as limb development, intestinal remodeling and the loss of the tail. The completion of tail resorption marks the end of metamorphosis in anurans. Tail resorption is by far the most spectacular yet the simplest of metamorphic programs because there is no concomitant growth and development.

The degenerative changes in the tail take place in conjugation with other changes in the tadpole body, like the eruption of the forelimbs and changes in shape of the head.

Even though the tail structurally consists of a variety of tissue and cell types, they all have a single uniform fate, namely death and resorption. Thus by the end of metamorphic climax, both the tail fins, epidermis, connective tissue, blood vessels, notochord and muscles are all resorbed. This degeneration process in tail proceeds proximally from the tip of the tail.
As in spontaneous degeneration, the first grossly observable sign of TH induced changes is the darkening and retraction of the tail fin, followed shortly thereafter by a progressive decrease in its length. Likewise T3 induced tail muscle degeneration indicates that it exhibits an ultrastructural pattern of degeneration similar to that detected in spontaneously regressing tail muscle.

Electron microscopic studies on regressing tail demonstrated that both TH induced and spontaneously degenerating tail exhibit the same sequence of ultrastructural transitions. Further, in both spontaneous and TH induced tail muscle degeneration, the loss of myofibrillar I-band integrity appears to be an initial and conspicuous sign of myofibrillar degeneration. The specific changes in the fine structure of the tadpole tail muscle cells are the early signs of tissue regression at metamorphosis. The more advanced stage being marked by atrophy of the tissue in tail at metamorphic climax.

At fine structural level, the tail cells undergo programmed cell death or Apoptosis with distinct sequential morphological changes. The degenerative changes are comparable in both spontaneous as well as TH induced metamorphosis.

It is known that tail resorption is under the direct control of TH. It is now generally believed that the biological effects of TH are mainly mediated through gene regulation by nuclear thyroid hormone receptors (TRs). The TRs in turn modulate gene expression by binding to specific DNA sequences in target genes.

The observations of the present work are consistent with previous studies on Xenopus laevis and on Rana catesbeiana. (Atkinson, 1981). The resorption of tadpole tail is autonomous and is highly specific being
unaffected by other neighbouring tissues. The tadpole tail being genetically predetermined toward destruction awaits only for sufficiently high levels of TH to trigger the process. This being further substantiated by induction of tadpole resorption by exogenously added TH. However, the coordinated elimination of the different tissues of the tail occurs by different processes. Some tissues are directly removed by autonomous programs (the suicide model involving apoptosis, Rowe et al., 2002), others being killed by surrounding proteases of the extracellular matrix (the murder model, Berry et al., 1998). In tail muscle it has been suggested that apoptosis induced by TH acts around the onset of metamorphic climax and thereafter by both apoptosis and phagocytosis.

Studies in the recent past, on amphibian metamorphosis are directed towards addressing the molecular and developmental roles of TR in post-embryonic development. TR is generally believed to be the main mediator of T3 action through transcriptional regulation. This is supported by the fact that TR has been shown to be both necessary and sufficient for the metamorphic effects of TH (Shi, 2000).

Recently co-cultural system using myogenic cells and non-myogenic cells have shown that notochord cells suppress adult myogenesis during *Xenopus laevis* metamorphosis (Nishikawa, 2012).
INTESTINE

There are a majority of organs present in both tadpoles and frogs which undergo partial but profound transformations during metamorphosis. One such organ with drastic morphological changes during metamorphosis is the intestine. During metamorphosis there is a general shortening of intestine as well as cell degeneration and regeneration.

The structural differences between larval and adult intestine presumably reflect changes in the physiological functions between herbivorous tadpoles and carnivorous frogs. The longer but simpler tadpole intestine consists of a single layer of columnar epithelium surrounded by thin layers of muscle with little intervening connective tissue. During premetamorphosis and prometamorphosis, the intestinal connective tissue is immature and mostly localized in a single fold. However, at the beginning of metamorphic climax, the connective tissue cells actively proliferate and differentiate into several types of cells. By the end of metamorphic climax, the adult intestine has elaborate connective tissue and muscles, at the same time the epithelium also forms multiple circular folds. The result of the present study are consistent with other studies on tadpole intestine pertaining to transition from primary to secondary epithelium.

Electron microscopic studies show that the larval epithelium degeneration accompanies decrease in number and height of microvilli composing the brush border. It was observed that the larval epithelial cells undergo apoptosis and the apoptotic bodies are removed by phagocytosis by macrophages. There is also an increase in the number
of lysosomes as well as apoptotic bodies which is again consistent with degeneration.

The changes observed in the TH induced intestine were similar to that observed during spontaneous metamorphosis. There was a gradual replacement of the larval/primary epithelium by adult/secondary epithelium.

During the recent past there have been numerous studies on the cellular mechanism for intestinal remodelling. It has been observed that at the cellular level, the larval to adult epithelial transformation during the intestinal remodeling involves two main processes (1) Apoptosis of the primary larval epithelium (Ishizuya-Oka et.al., 1992) and (2) Development of the secondary adult epithelium by active proliferation and subsequent differentiation. (Ishizuya-Oka et.al., 1994). Several researchers have described nests of adult cells in the tadpole epithelium that proliferate at climax to replace the degenerating larval epithelium (Ishizuya-Oka et.al., 1997). However a few studies considered that at least some adult cells are derived from larval epithelial cells (Amano et.al. 1998). The debate continues as studies provide direct evidence that stem cells that generate the adult intestinal epithelium originate from the larval epithelium, through thyroid hormone induced dedifferentiation (Ishizuya-Oka et.al. 2009). Other studies have shown that the adult intestinal stem cells develop de novo and are distinct from the larval cells (Shi et.al, 2011).

Like other organs, the intestine can also be induced to undergo precocious remodeling by treating premetamorphic tadpoles with TH. The experiments with precocious metamorphosis yield similar results as spontaneous metamorphosis. It has been shown that the larval-to-
adult epithelial transformation can be organ-autonomously induced by TH in vitro just like in vivo (Ishizuya-Oka et al., 1991). More recent studies on terminal differentiation pathway in the small intestine of Xenopus involve examination of apoptosis and cell proliferation by using in situ nick end-labeling of genomic DNA (TUNEL) and bromodeoxyuridine (Brd U) immunohistochemistry (Ishizuya-Oka et al., 1996).

The molecular mechanisms of intestinal remodeling had been sought to be clarified (Ishizuya-Oka et al., 2005) by assessing functions of TH genes that are endogenously expressed in the X.laevis intestine. Heimeir, et al. (2010) have observed that during intestine development 3,132 genes were up regulated while 1,624 genes are down regulated with the most number of up- and down-regulations occurring at the climax stage.

**LIVER**

The developing amphibian tadpole has lent itself admirably to the study of a possible evolutionary course of nitrogen excretion mechanism. When water dwelling R.tigerina tadpoles metamorphose to land dwelling frogs, there is a shift from ammonotelism to ureotelism. While ammonia can be readily excreted in the aquatic habitat, it cannot be done in the terrestrial and so the need to excrete urea.

During the metamorphosis of R.tigerina there are cytological alterations in the liver cells. These changes provide cytological criteria for differentiation in cells during metamorphosis. The nuclei of the hepatic cells are euchromatic in early metamorphic stages and become heterochromatic about the time of limb development and thereafter
remain so. They become more irregular in shape, and the number of nucleoli increases.

While no unique changes are apparent in stained sections of liver during metamorphosis as seen under light microscope, striking cytological changes are observed with electron microscope. During the early stages of metamorphosis, direct association between endoplasmic reticulum and mitochondria occurs frequently. The mitochondria and endoplasmic reticulum profiles of adult frog liver appear to have a more random arrangement. Moreover there is an apparent proliferation of endoplasmic reticulum and rather marked morphological alteration of both mitochondria and endoplasmic reticulum.

A comparison of the fine structural changes in liver cells during natural metamorphosis with those which occur during TH induced metamorphosis reveals that the alterations are similar in the two types of metamorphosis. Although these changes are subtle, they provide cytological criteria for the differentiation of liver cells during metamorphosis.

Earlier studies have shown that during metamorphosis there are drastic increases in the biosynthesis of nucleic acids and proteins in the liver, which account for, among other things, the dramatic increase in albumin levels in the blood as well as a number of liver enzymes. (Smith-Gill and Carver, 1981). The most extensively studied are the urea cycle enzymes, which are responsible for the synthesis of urea for excretion of nitrogenous waste in frogs. The five urea cycle enzymes are carbamyl phosphate synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase and arginase. The role
of TH in the regulation of the rate-limiting enzyme hepatic carbamyl phosphate synthetase I activity in *R. catesbeiana* has been studied (Galton V.A. et.al.,1991). During natural and TH induced metamorphosis, the genes encoding all the 5 urea cycle enzymes are up-regulated in the liver which in turn leads to higher levels of translation to produce corresponding enzymes.

Histochemical alterations in the lipid content in the liver of growing and metamorphic tadpole of *R.tigerina* have been carried out previously in our laboratory. These studies revealed that lipids tend to accumulate during late prometamorphosis. The lipid content was further found to be increased during the late metamorphic climax, after a lowering at the early metamorphic climax. The enhancement in the lipid levels of liver during prometamorphosis and late metamorphic climax may be due to the import of end products of lipolysis in extrahepatic tissues (Sawant and Varute, 1973).

**Limb**

During metamorphosis the organism is faced with behavioral consequence of alteration in habitat. Critically the organism is also required to change its locomoter strategy from axial-based body undulations to a limb based mode.

Hind limb development is one of the earliest changes during frog metamorphosis. In *R.tigerina*, the hind limb buds are first visible, about 2 weeks after the embryogenesis, during premetamorphosis. During the early development, the limb bud consists of undifferentiated loose mesenchyme covered by epithelial tissue. As the tadpole grows, the limb bud increases in size. Little morphological change takes place in
the hind limb buds before prometamorphosis. Since the thyroid glands do not become fully functional before prometamorphosis, it can be concluded that the hind limb bud formation and growth does not require TH.

During prometamorphosis the hindlimb buds begin to differentiate and undergo morphogenesis. The tadpole epidermis which is a few cell rows in thickness differentiates into a multilayered epidermis. The thickening proceeds through the later stage of metamorphic climax. The mesenchymal cells begin to acquire specific differentiated character during prometamorphosis. This stage is marked by chondrogenesis, myogenesis and ossification. The limb bones ossify in proximo-distal sequence. After the onset of metamorphic climax the hind limbs increase in size but do not change much morphologically.

While most frog organs that are formed from pre-existing tadpole organs serve a similar function, the developing limb has no function in a tadpole. In that respect, the limb's developmental profile resembles that of an insect imaginal disk that resides in the larva in an undifferentiated yet fully determined state until the metamorphosing hormone stimulates its development to an adult structure (Brown et.al., 2005). Studies have also shown a correlation between structural changes and functionality of limbs. Abdala et.al. (2012) have shown that reduced mobility delayed the onset of skeletogenesis and produced shorter long bones.

It has further been demonstrated that in the absence of thyroid hormone, limb bud does not develop further and has no terminally differentiated muscle or cartilage (Brown et.al., 2005). Further Marsh-Armstrong et.al., (2004) have shown that both, the muscles formed
during limb growth as well as the spinal cord cells that innervate the limbs are a direct target of TH control. Another peculiar feature of TH-controlled limb development is the continuous requirement of TH. Interruption of TH synthesis by the goiterogen methimazole during metamorphosis of *Xenopus laevis* at anytime from NF 52 to 57 arrests limb development at that stage (Elinson et al., 1999).

**LUNGS**

Successful adaptation from aquatic to terrestrial life in *R. tigerina* is manifested in the respiratory system. While skin and gills are the main respiratory structures of the water-breathing tadpole stage, cutaneous and pulmonary respiration characterizes the air-breathing frogs. During the course of metamorphosis, gills which are the main respiratory organs prior to the development of frog lungs, undergo degeneration. The function of respiration is primarily taken over by the lungs during metamorphosis.

Lung morphology indicates changes in its composition during metamorphosis. The premetamorphic tadpoles have lungs that are simple thin-walled sacs with fewer septa. The tadpole lung is made up of an epithelial lining which is supported by smooth muscle and blood vessel. The cells forming this epithelium are sometimes referred to as pneumocytes as they appear to be homologues of the alveolar cells in mammalian lung. As the metamorphosis progresses, the lung histology shows the formation and extension of numerous septa which increase the surface area. These septa are more or less club shaped and divide the air space into alveoli. These septa were seen to be provided with branches from pulmonary blood vessels.
The developmental transition from water to air breathing is accompanied by modifications in the ventilatory mechanisms and respiratory surfaces. Most of the studies in the past are pertaining to the gill and/or lung ventilation. In larval amphibians, gas exchange is accomplished primarily by rhythmic ventilation of gills, but as development progresses, lung ventilation assumes a greater fraction of overall gas exchange. (Burggren and West, 1982). Upon metamorphic climax, gill ventilation is replaced by rhythmic buccal movements characteristic of adults, with lung ventilation occurring episodically (Broch et al., 2002).

Further as the transition from aquatic to terrestrial mode of respiration involves a drastic change in the surrounding environmental conditions. Studies have been carried out on the defects in amphibian respiratory system due to altered environmental factors and it has been observed that dry conditions had adverse effect on amphibian respiration (Oztay, 2002).

Of the pulmonary cells, pulmonary neuroendocrine cells (PNEC) have drawn some the attention. PNEC in tadpole and adult Rana ridibunda have been studied. These cells produce and release regulatory factors in the respiratory tract of larval lungs and have been shown to play an active role during the embryonic development of lung (Oztay, 2008).

**BLOOD**

There are striking changes in the proteins in the blood during metamorphosis. Quantification of total serum proteins reveal that serum proteins concentration doubles during metamorphosis and that
this change also occurs during TH induced metamorphosis. The increase in the serum proteins is believed to play an adaptive role as the tadpole is transformed into the frog. One of the changes that occur in the blood is the switch in the type of hemoglobin (Hb) in the circulating RBCs. The metamorphic Hb transition is accomplished by a switch from larval type to adult type Hb. The transition of Hbs during metamorphosis in Xenopus has been reported to involve replacement of the larval RBCs by adult RBCs (Weber et al. 1989).

Morphological differences between larval and adult RBCs were observed. During Metamorphosis, it was observed that the larval RBCs were larger. These larger larval RBCs were subsequently replaced by a population of smaller adult RBCs.

Studies on erythrocyte differentiation during metamorphic Hb switch of R. catesbeiana have shown that this accomplished through (1) the differentiation of new erythrocyte population that synthesizes only adult Hb and (2) the selective removal of tadpole Hb-containing erythrocytes from the circulation (Dorn et al., 1982). The role of apoptosis in the removal of mature larval-type RBC from circulation during and after metamorphosis has been proposed (Tamori et al., 2000). There is also sufficient evidence that developmental effects of TH in frogs are modified by other hormones for example prolactin retards and cortisteroids accelerate some components of TH induced metamorphosis. However, glucocorticoid is found to have an inhibitory effect on cultured RBCs (Schneider et al., 1995)
EYE

The development and differentiation of the eye of *R. tigerina* is of interest as it involves the transformation from primary optic vesicle to a fully functional eye.

By premetamorphosis, the characteristic histological structure of the eye is already attained. As metamorphosis progresses the eye itself increases in size but the overall morphological organization of lens and retina does not change. During the course of metamorphosis the diameter of the lens increases. The lens fibers develop until they nearly fill the lumen and eventually form at the equator of the lens. The retina is divided into a central differentiated and peripheral undifferentiated area. As the metamorphosis progresses the retinal layers increase in size and attain proper organization.

Early during metamorphosis, the lens is well formed and acquires the adult crystalline structure. The optic fibers reach the brain, forming a fibrous layer above the tactual cells. The steady increase in the number of retinal cells and optic nerve fibres during larval life is accompanied by a marked thickening of the retina. Morphological changes in the developing retina results in accompanying modifications of the receptive field properties.

In tadpoles, the two eyes are placed laterally with no binocular overlap. As the skull changes shape during metamorphic climax, laterally positioned eyes migrate dorsofrontally. Concomitant with the metamorphic change in eye position, a new pattern of retinal projections develop, connecting the retina to the ipsilateral thalamus. These uncrossed retinal projections, together with the change in eye
position, subserve the new acquisition of binocular vision, appropriate to the predatory lifestyle of the adult frogs. (Grant and Keating, 1986).

Retinal growth results from the proliferation of cells located at the periphery of the retina, in the ciliary marginal zone (CMZ). A shift to an asymmetrical growth pattern is observed as more cells are added to the ventrotemporal portion of the retina than to dorsonasal portion. The CMZ is spatially ordered with respect to cellular development and differentiation, with the youngest and least determined stem cells closest to the periphery, the proliferative retinoblasts in the middle and postmitotic cells at the central edge (Wetts et.al. 1989).

The pattern of retinal vasculature in the frog Litoria moorei has been studied and it reflects the topography of cells in the ganglion cell layer throughout development. It was reported that during the early tadpole stages the retinal vasculature increased only to be remodelled by the loss of the capillaries by early post-metamorphic life (Dunlop et.al 1997).

Earlier studies have reported that light and its spectra influence reproduction and metamorphosis. Exposure to red light has been reported to result in precocious metamorphosis of tadpoles of Rana cyanophlyctis (Joshi and Mohinuddin, 2003).

The role of thyroid hormone and its receptors (TR) in frog metamorphosis is well established. It has been shown that TR is essential for Xenopus laevis embryogenesis, involving retina and neural tube, although its physiological role remains to be defined. (Havis et.al. 2006).
BRAIN

The tadpole brain undergoes extensive reorganization during metamorphosis. The gross changes in the central nervous system, which is well developed by prometamorphosis, include reduction in size but thickening of the walls of the ventricles of the cerebellum and medulla oblongata. There is a widening and shortening of the diencephalon and narrowing and shortening of the fossa rhomboidalis of the medulla. In tadpoles the cerebellum remains in a relatively early developmental stage during premetamorphosis and has a more rapid development during metamorphic climax.

Many studies on the metamorphic transitions of the nervous system have mainly focussed on individual neuron types. Two classes of neurons that disappear or regress markedly during metamorphosis are the Mauthner and Rohon-Beard neurons. Mauthner neurons of the hind brain are drastically reduced in size during metamorphosis, whereas the numerous Rohon-Beard neurons in the spinal cord disappear completely. Some studies have established that brain mast cell population is not static in the sense that it is influenced by the developmental, physiological and behavioral status of the animal. The population of mast cell and their distribution has been studied and it has been observed that they are more numerous in the adult brain than in premetamorphic and post-metamorphic tadpoles (Monteforte, 2010). Study of mast cells have gained attention because the sensory products of mast cells released in the central nervous system can alter the function of both neural and vascular elements (Khalil et.al., 2007).

Amphibians have large quantities of neuromelanin distributed throughout their CNS. The localization of neuromelanin has been
studied in the CNS of *Rana esculata* and its distribution is studied in tadpoles at various stages of development (Kemali and Gioffre', 1985). Since mammals also have a large quantity of neuromelanin in a few areas of the CNS, such studies give a phylogenetic and ontogenetic meaning to their distribution.

Previous studies have shown that the developing and metamorphosing brain of tadpoles show characteristic alterations in the lipids. The alterations in the total lipids and the individual phospholipid and neutral lipid components seem to be related to the growth of brain and myelination of neurons as well as the changes due to induced metabolic stress during nonfeeding period of metamorphosis (Sawant and Varute, 1973).

Like other processes during metamorphosis, the transformation of the brain also depends upon TH. Administration of T3 to tadpoles results in precocious maturation of the nervous system (Kollros, 1981). The TH induced changes involve extensive remodeling of regions of the CNS that are necessary for the shift from one stage of life history to another. Denver et.al. (1997) have predicted the gene regulatory networks underlying TH action on brain and understanding the basic molecular mechanism underlying neural cell differentiation.

Advances in imaging technology and the accessibility of internet have led to the introduction of digital brain atlases which allow better temporal and spatial resolution. Horowitz and Summons (2007) have used still images from cresyl violet stained material to highlight key periods in tadpole brain development. They have further emphasised the utility of morph videos in examining dynamic aspects of brain of developing bull frog.