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RESEARCH ARTICLE

EFFECTS OF VITAMIN A AND THYROXINE ON THE METAMORPHOSIS OF INDIAN TREE FROG, *POLYPEDATES MACULATUS*

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ABSTRACT

The present study was carried out to investigate and examine the effects of vitamin-A and thyroxine on growth, development and regenerative processes of amputed tails in *Polypedates maculatus*. On exposure to different dosages of vitamin-A, prolonged metamorphosis, regeneration of the amputed tail with visible malformations like bending of tail, formation of bulbular mass and ectopic limb development due to homeotic transformations of tissues were observed. At the same time tadpoles reared in sub-lethal dosages of thyroxine showed enhanced metamorphic changes, disappearance of caudal fin, reduction in growth, abnormality in the development of limbs and loss of pigmentation. Simultaneous exposure of tadpoles to both vitamin-A and thyroxine induced abnormalities like delay in development of hind limbs, emergence of forelimbs, resorption of tail and reduced mortality in comparison to tadpoles of thyroxine group. This study reveals that vitamin-A and thyroxine are antagonistic in nature and on simultaneous exposure to both resulted in intermediate effects on life cycle of *Polypedates maculatus.*

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INTRODUCTION

The amazing discovery on regeneration in anurans under the influence of vitamin A made by Niazi and Saxena (1978) from University of Rajasthan and Mohanty-Hejmadi et al. (1992) from Utkal University open up a new dimension in the field of regeneration research. Since then several workers have started probing the effects of vitamin A on development and regeneration in amphibians. Niazi and Saxena (1978) showed that vitamin A has an inhibitory effect on the regeneration of tail and slowed down the process of metamorphosis in *Bufo andersonii.* Similar inhibitory effect of vitamin A on tail regeneration in *Xenopus laevis, Notophthalmus viridescens* and *Ambystoma mexicanum* was reported by Scadding (1987). Mohanty-Hejmadi et al. (1992) when amputed the tadpole tails of *Uperodon systoma* and exposed them to vitamin A, instead of tail regeneration, limbs appeared at the amputation site. This was for the first time homeotic transformation in anurans was demonstrated. They also reported that vitamin A also delayed the process of metamorphosis. The homeotic transformation was futher extended by Maden (1993) and Muller et al. (1994, 1996) in *Rana temporaria,* Mahapatra and Mohanty-Hejmadi (1994) in *Polypedates maculatus,* Muller et al. (1994) in *Rana ridibunda* and Das (1998) in *Bufo melanostictus.*

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Metamorphosis in amphibians, a complex developmental process is regulated by thyroid hormone (Brown and Cai, 2007). The period of metamorphosis can be differentiated into three stages: pre-metamorphosis, post-metamorphosis and climax. Thyroid gland develops and begins to release thyroid hormone during pre-metamorphosis stage. The level of thyroid hormone rise during post-metamorphosis and initiates morphological changes like development of hindlimbs. In climax stage, the level of thyroid hormone is at its peak and induces rapid metamorphic changes. Researchers like Furlow and Neff (2006) in *Xenopus laevis,* Badawy (2011) in *Ambystoma mexicanum* and Mahapatra et al. (2015) in *Duttaphrynus melanostictus* have shown that exogenous thyroid hormone treatment accelerates metamorphic changes. It is observed that vitamin A and thyroid hormone have opposite effects on metamorphosis in anurans. So far no study has been reported about the effect of both vitamin A and thyroid hormone when administered together on the life cycle of anurans. Keeping this in view, the present study was taken up to investigate the toxic and teratogenic effects of vitamin A and thyroxine administered separately and together on the metamorphosis of *Polypedates maculatus.*

MATERIALS AND METHODS

Polypedates maculatus, the tree frog is a seasonal breeder, breeding only during the monsoon. Depending on rainfall the breeding season extends from July to September. In July 2018, several foam nests of *Polypedates maculatus* were sighted in the science block tank of the Institute. The foam nests were found adhering to the wall of the tank slightly above the water surface. Some of them were brought to the laboratory and kept in plastic tubs with tank water for hatching. It took 2 to 3 days for hatching after the collection. The number of hatching per nest ranged between 100 and 150. They were fed with boiled spinach leaves as the sole food source. The tadpoles were reared to the hind limb bud stage in conditioned water i.e. de-chlorinated water. The temperature was maintained at 30 $\pm 2^0$C throughout the study period. Vitamin A and thyroxine was used for the study. For the thyroxine treatment, thyroxine was dissolved in the minimum amount of 1N NaOH solution and made to the concentration of 1 mg/ml solution with water. This thyroxine solution was added to water of pH 7.4 to give the desired final concentration of 1.5×10^{-8} mol/L and 3.0×10^{-8} mol/L. Three different concentrations i.e. 20 IU/ml, 30 IU/ml and 40 IU/ml of vitamin A (retinyl palmitate or vitamin A palmitate is the ester of retinol and palmitic acid) solution was used for the study. At the relevant hind limb bud stage, the tadpoles were anaesthetized by using MS222 and their tails were amputated about 4 to 5 mm from tip with a sterilized razor blade. The day of amputation of tail was considered as day 1. They were divided into ten groups, each group of 30 tadpoles. One group, the control group was maintained in conditioned water, while remaining eight groups were experimental groups exposed to different concentrations of vitamin A and thyroxine solutions. Three of these experimental groups were exposed to three different concentrations of vitamin A solutions i.e. 20 IU/mL, 30 IU/mL and 40 IU/mL, three groups exposed to both vitamin A and thyroxine i.e. 20 IU/mL vitamin A with 1.5×10^{-8} mol/L thyroxine, 30 IU/mL vitamin A with 1.5×10^{-8} mol/L thyroxine and 40 IU/ml vitamin A with 1.5×10^{-8} mol/L thyroxine and two groups were maintained in two different concentrations of thyroxine solutions i.e. 1.5×10^{-8} mol/L and 3.0×10^{-8} mol/L. In addition to these nine groups, a group of 30 non-amputed tail tadpoles were maintained in conditioned water for comparison. Every two days, water with relevant concentrations of vitamin A and thyroxine as per the study was changed and the tadpoles were grown until metamorphosis. Development of various stages (identified by following Gosner, 1960), changes in length (snout to tail tip), weight, mortality, limb development, tail regeneration and abnormalities were recorded of both control groups (non-amputated and amputated) and experimental groups.

RESULTS

Vitamin A and thyroxine had diverse effects on the metamorphosis of tadpoles of *Polypedates maculatus.* The following is a summation of results observed during the study.

Mortality: In general a positive relationship was found between mortality and the dose concentrations (Figure - 2). There was almost no casualty in different doses of vitamin A like both control groups (tail non-amputed and amputed). However, casualty was observed in experimental groups treated with thyroxine. Tadpole exposed to higher dose of thyroxine showed casualty on 4^{th} day of exposure and 90% died on 7^{th} day while there was delayed casualty in case of tadpoles exposed to lower dose. The experimental groups in which tadpoles were exposed to different concentrations of vitamin A with lower concentration of thyroxine showed reduced mortality. Tadpoles reared in water containing lower concentration of vitamin A (20 IU/mL) with lower concentration of thyroxine (1.5×10^{-8} mol/L) underwent 60% mortality on 10^{th} day while mortality was 100% in both 30 IU/mL and 40 IU/mL vitamin A with lower thyroxine concentration on the same day.

Figure 1. Blastema formation at the amputed site of the tail.

Weight: There was gradual increase in average body weight of tadpoles of control groups up to 9^{th} day and there after that gradual reduction was observed (Figure - 3). As thyroxine exposure enhanced metamorphosis, reduction in the average body weight of tadpoles was recorded from third and fourth day onwards in higher and lower doses respectively. In case of tadpoles reared in different concentrations of vitamin A with thyroxine showed no marked change in average body weight.

Length (Snout to tail tip length): The control group tadpoles (non-amputed) showed an increase in the average snout to tail tip length till emergence of forelimbs and was 39 mm while amputed control group was 35 mm (Figure - 4). There was decrease in average snout to tail tip length of tadpoles exposed thyroxine in comparison to the control group tadpoles. In case of tadpoles exposed to higher dose of thyroxine, the average length was 24 mm and lower dose of thyroxine was 26 mm on 8^{th} day. Tadpoles exposed to different concentrations of vitamin A showed increase in snout to tail tip length like that of control groups but showed an inverse relationship. In lower concentration of vitamin A (20 IU/mL), the average length was 36 mm which was more than the tadpoles exposed to higher concentration (40 IU/mL) of vitamin A i.e. 32 mm. Similar trend was observed in the average length of tadpoles reared in different concentrations of vitamin A with thyroxine (Figure 4).

Tail abnormalities: Normal regeneration of tail was observed in both control groups (non-amputed and amputed). Thyroxine induced reduction in tail and tail fins regeneration because of early metamorphosis. However, the tadpoles exposed to different concentrations of vitamin A showed abnormality in the tail. It includes suppression of regeneration of amputed tail, formation of bulbular mass, suppression of regeneration of dorsal, ventral or both tail fins and bending of tail. Tadpoles showed suppression of tail regeneration exposed to lower dose of vitamin A with thyroxine while in some tadpoles exposed to higher dose of vitamin A with thyroxine developed pouch like structures at the cut end of the tail.

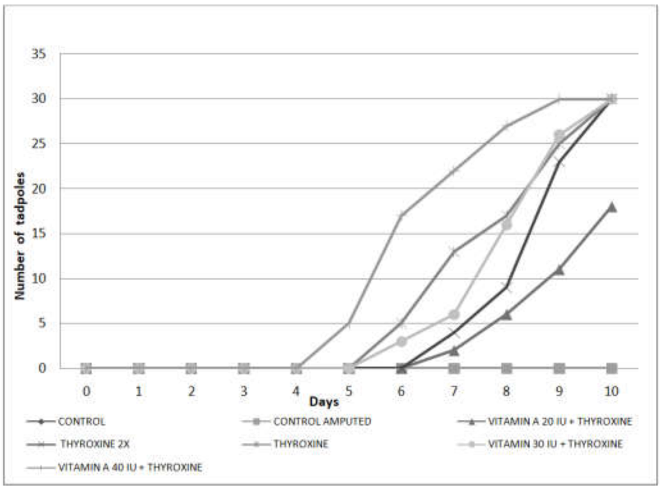

Figure. 2. Effect of different dosages of vitamin A and thyroxine on mortality.

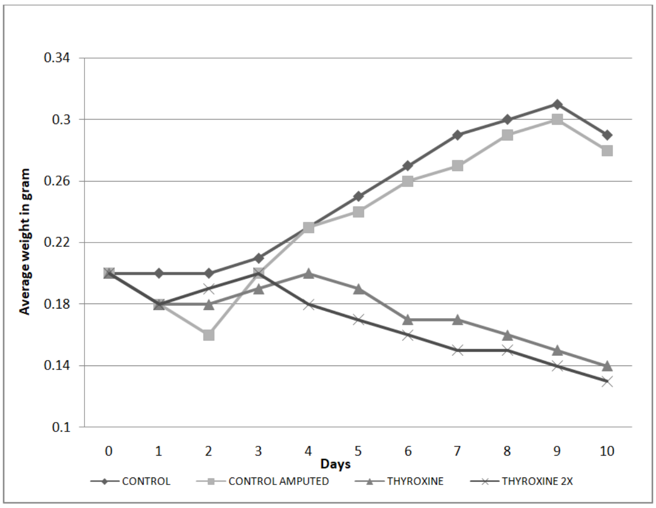

Figure 3. Effect of different dosages of thyroxine on average weight of tadpoles

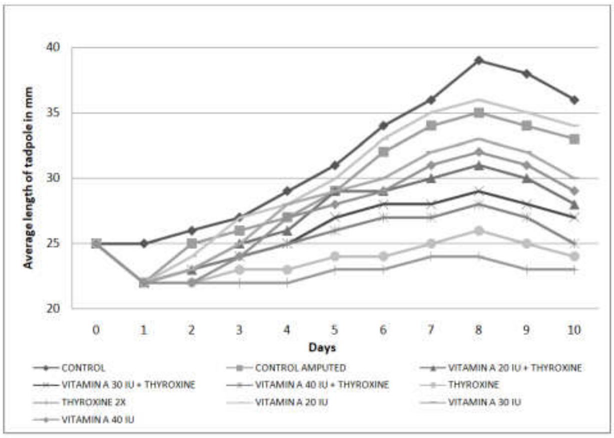

Figure 4. Effect of different dosages of vitamin A and thyroxine on length of tadpoles

Limb development: Tadpoles of both control groups showed normal hindlimb development on 13th day and fore limbs began to develop on 25th day. The most unexpected finding was suppression of hindlimb and forelimb development ranging from partial to complete suppression in tadpoles exposed to different concentrations of vitamin A (Figure - 7). The total suppression was observed in case of 30 IU/mL and 40 IU/mL but tadpoles exposed to 20 IU/mL showed partial development of hind limbs on 23rd day and few developed forelimb bud on 33rd day of the study (Figure – 9). Majority of tadpoles developed hindlimbs on 3rd day and forelimbs on 4th day exposed to thyroxine. However, inhibitory effect of vitamin A was counteracted by thyroxine in case of tadpoles exposed to both vitamin A and thyroxine. Majority of tadpoles developed both hindlimb and forelimb on 6th day of the study in all three concentrations of vitamin A with thyroxine (Figure – 7, 9).

Ectopic limb formation: Very few tadpoles (four) of the experimental group reared in 20 IU/mL vitamin A showed development of ectopic hindlimbs from the amputed tail stumps having all regular features i.e. thigh, shank, ankle and digits (Figure - 10). The sizes of ectopic limbs were comparatively smaller than the normal hindlimbs. This result clearly demonstrated the homeotic transformation of amputed tails into hindlimbs. No ectopic limb development was observed from the cut end of the tail in all other experimental groups.

DISCUSSION

Most anurans like frogs and toads undergo indirect development. Eggs hatch into free swimming larvae which metamorphose into juvenile frogs. Upon metamorphosis, virtually every organ system undergoes extensive morphological and functional changes with increase of thyroid hormone level. It has been known for long that excess of vitamin A delay the process of metamorphosis and causes a variety of malformations and defects in anuran tadpoles. Thus, vitamin A and thyroxine work opposite to each other. In the present study an attempt has been made to study the diverse effects of both vitamin A and thyroxine separately and together. Metamorphosis was normal in control group tadpoles and took 60 to 62 days. The tadpoles of experimental groups exposed to different concentrations of vitamin A showed delayed metamorphosis. A positive correlation was noticed between the doses and onset of metamorphosis. The increased from 20 IU/ml to 40 IU/ml. Prolongation of metamorphosis induced by vitamin A have also been reported by Niazi and Saxena (1972) in *Rana cyanophlyctis*, Mohanty-Hejmadi and Parida (1984) in *Bufo melanostictus*, Sharma and Niazi (1979) in *Rana breviceps,* Mohanty-Hejmadi et al. (1992) in *Uperodon systoma*, Mahapatra and Mohanty-Hejmadi (1994) in *Polypedates maculatus,* and Mahapatra et al. (2001) in *Microhyla ornata*. Niazi and Saxena (1972) stated that prolonged metamorphosis is due to inhibitory effect of vitamin A on the thyroid gland development and also reported that on exposure to higher concentration of vitamin A thyroid gland become progressively smaller leading to hypothyroidism. The slow metamorphosis of tadpoles in the present study may be due to inhibitory effect of vitamin A on the development of thyroid gland. On the other hand, enhanced metamorphosis was observed in case of tadpoles exposed to thyroxine which was completed in 12 to 15 days. Thyroxine promotes metamorphosis by inducing resorption of larval tissues, growth and differentiation of new adult tissues and remodeling of larval tissues to create new adult tissues and organs (Furlow and Neff, 2006). The results of the present study are in accordance with the findings of Keifer et al. (2012) in *Rana temporaria* and Mahapatra et al. (2015) in *Duttaphrynus melanostictus*.

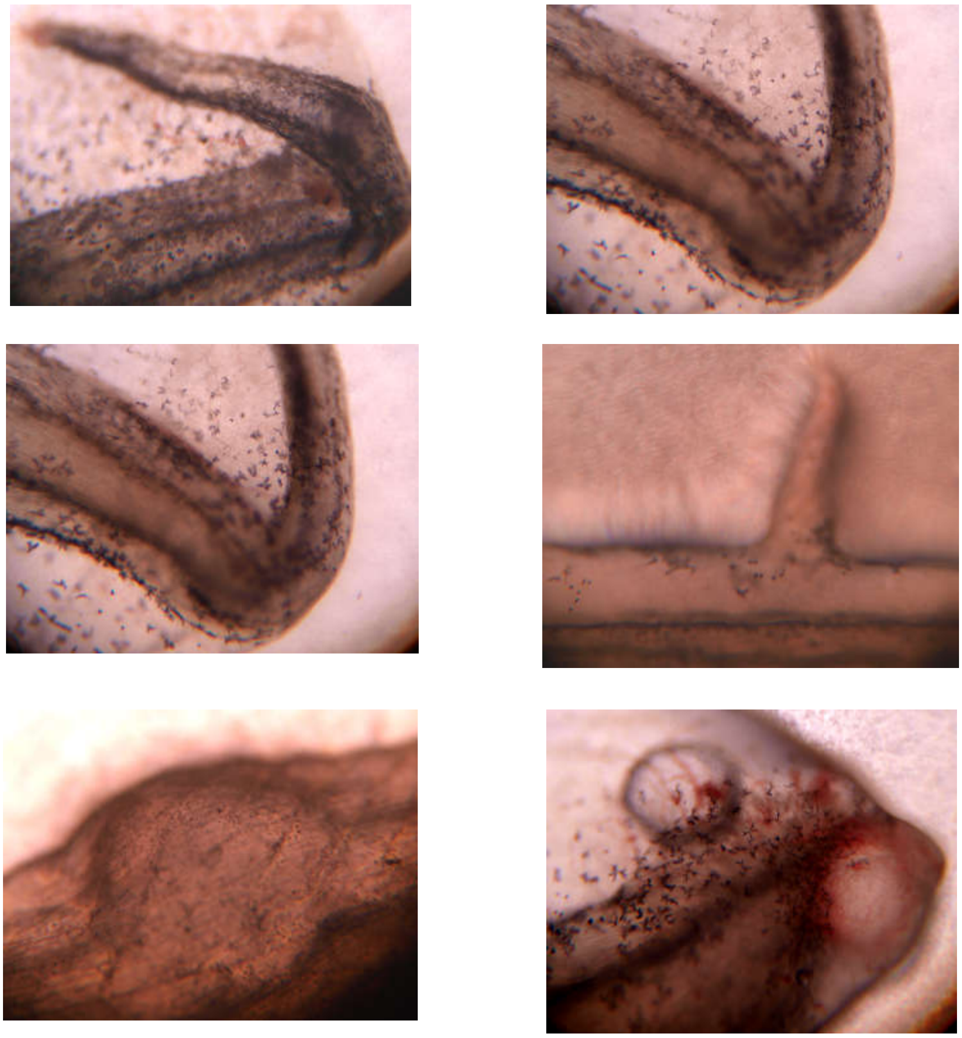

Figure 5. Tail abnormality showing bending, bulbular mass and outgrowths

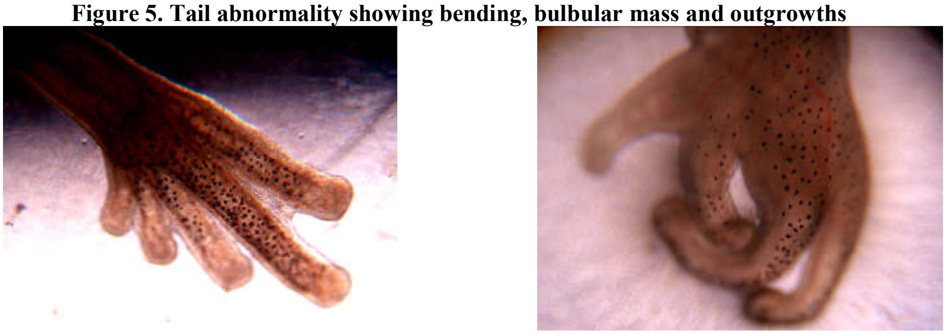

Figure 6. Normal digits in hindlimb of control group and abnormal digits in hindlimb of thyroxine exposed tadpoles

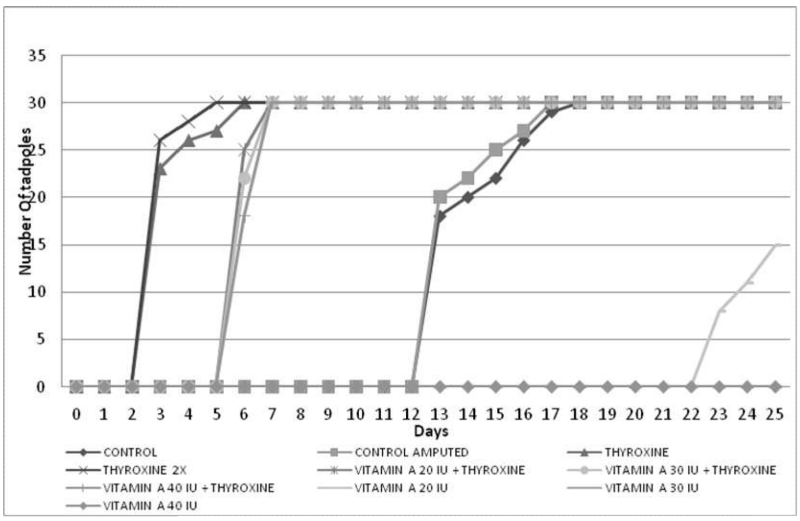

Figure 7. Effect of different dosages of vitamin A and thyroxine on hind limb development.

Figure 8. Abnormality in limb development exposed to thyroxine

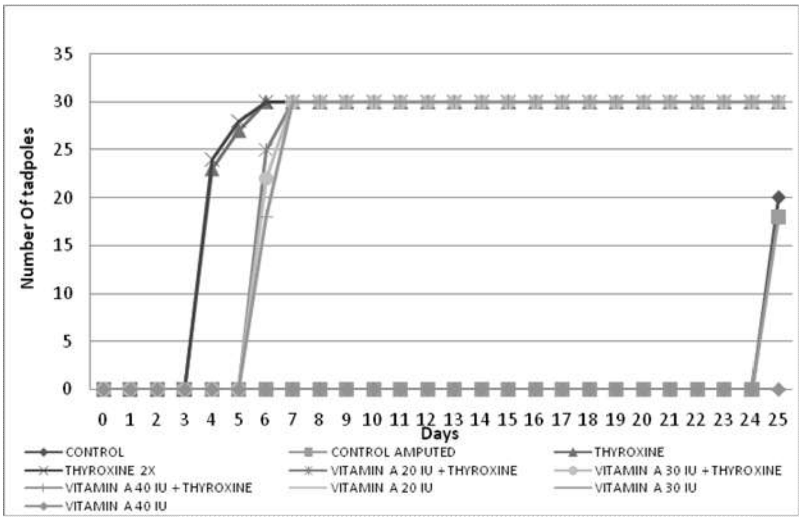

Fig 9. Effect of different dosages of vitamin A and thyroxine on fore limb development.

Figure 10. Ectopic hindlimb formation from the amputed tail stump

Interestingly, the tadpole exposed to both vitamin A and thyroxine showed enhanced metamorphosis in comparison to vitamin A exposed tadpole but slower than tadpoles exposed to thyroxine alone. This clearly demonstrates that thyroxine and vitamin A are counteracting each other and producing an intermediate effect. The results of the present study revealed that exogenous administration of thyroxine on one hand enhanced metamorphosis but on the other induced toxic effects. It resulted in high percentage of mortality (90 %) incase of tadpoles exposed to thyroxine alone and to both thyroxine and vitamin A (Figure – 2). A progressive increase in mortality occurred with increase of concentration of thyroxine. Our findings are consistent with earlier reports of Mukai et al. (1995), Beachy (2001) and Mahapatra et al. (2015). Almost no casualty was observed in tadpoles exposed to lower dose while 25 to 30 % casualty was recorded in tadpoles exposed to higher dose of vitamin A in the present study. Dose and duration of exposure related mortality has been reported in *Rana temporaria* (Maden, 1993 and Muller *et al.,* 1996), *Microhyla ornate* and *Hoplobatrachus tigerina* (Das and Dutta, 1996) and *Bufo melanostictus* (Mahapatra *et al.,* 2001a). Based on the morphological investigation of the study it was observed that there was gradual increase in snout to tail tip length and average body weight (mass) in the tadpoles of control group till emergence of forelimbs. But there was reduction in snout to tail tip length and average mass in the tadpoles exposed to thyroxine. Beachy (2001) reported similar result in *Bufo americanus* following thyroxine treatment and suggested that reduction in length may be due to dehydrating action of thyroxine. The results of the present study demonstrated that the reduction in snout to tail tip length and body mass is dose dependent i.e. higher the concentration more the reduction. The results are in line with the findings of Mahapatra *et al.* (2015). Mohanty-Hejmadi and Crawford

(2003) reported retarded growth of tadpoles upon exposure to vitamin A in four anuran species. The reduction in snout to tail tip length was dose and duration dependent. The investigators observed that the tadpoles reared in vitamin A and thyroxine together showed initial increase in length and body mass up to emergence of forelimbs and thereafter decreased but lesser than the control group. The tadpoles of the control group showed normal regeneration of tails from the amputated end. Within 3 days of amputation blastema developed at the site of amputation. Gradually the blastema disappeared and regeneration of tail was observed. However, the regeneration of tail was suppressed in tadpoles exposed to thyroxine and regression of tail was observed. This finding is in aggrement with the work of Dmytrenko and Kirby (1981) and Kiefer et al. (2012) in *Rana temporaria* and Mahapatra et al. (2015) in *Duttaphrynus melanostictus*. The resorption of tail is due to programme cell death. During metamorphosis, enzymes like cathepsin and acid phosphatases are actively involved in resorption of tail tissues in anurans (Mahapatra *et al.,* 2001; Das et al, 2006; Mahapatra and Mahapatra, 2011, 2012). The rapid regression of tail as observed in the present study may be due to exogenous thyroxine which further raises the level of lysosomal enzymes like acid phosphatases and cathepsin in the cells of tail tissues (Mukai et al, 1995; Berry *et al.,* 1998; Mahapatra et al, 2015). However, histological analysis of tail tissues by Nakai et al. (2017) suggested that regression of tail is due to autolysis rather than phagocytosis. Morphological observations of tadpoles exposed to different concentrations of vitamin A revealed suppression of tail regeneration and abnormalities like formation of bulbular masses and bending of tail. The degree of suppression was dose dependent. Only few tadpoles of the experimental group exposed to lower dose (20 IU/ml) of vitamin A developed blastema on 11^{th} day.

In higher doses, the wound healed up but there was no regeneration of tail. Niazi and saxena (1968) and Scadding (1987) are the pioneers in the study of tail regeneration. They have shown that vitamin A has an inhibitory influence on tail regeneration. Similar results have been reported by Mohanty-Hejmadi and Crawford (2003) in four anuran species. Several researchers have reported that vitamin A induces formation of bulbular mass in amputated tail (Das and Mohanty-Hemadi, 1998; Patnaik *et al.,* 2012; Mahapatra *et al.,* 2015). Similar formation of bulbular mas after exogenous vitamin A treatment was observed in the present study. This might be due to formation of extensive compact chorda cells resulting in the formation of bulbular mass (Maden, 1993; Das and Mohanty-Hejmadi, 1998). An interesting thing which was observed in the present study is that bulbular mass not only formed at the amputated tail end but also in other regions of the tail which needs further study. Bending of the tail and suppression of tail fins in tadpoles due to exogenous vitamin A in the present study confirms the findings of Mahapatra and Mohanty-Hejmadi (1994). The results of the present study revealed that vitamin A had an inhibitory effect on limb development. This unexpected result was ranging from partial to complete suppression in response to exogenous vitamin A treatment. Suppression of limb development following vitamin A treatment has also been reported in *Rana cyanophlyctis* (Niazi and Saxena, 1972), *Polypedates maculates, Microhyla ornate, Uperodon systoma* and *Bufo melanostictus* (Mahapatra and Mohanty-Hejmadi, 1994) and in *Rana breviceps* (Sharma and Niazi, 1983). Few tadpoles of the experimental group exposed to higher dose of vitamin A (30 IU/ml and 40 IU/ml) hindlimbs did not advance beyond the limb buds becoming somewhat elongated and conical. Exposure to lower dose of vitamin A (20 IU/ml), tadpoles developed defective hypomorphic hindlimbs. Suppression of limb development may be due to high level of vitamin A in the cells of tadpole because of both exogenous and endogenous vitamin A that reduced the expression of *Hox* genes responsible for limb differentiation. Tadpoles exposed to thyroxine and vitamin A with thyroxine developed both hindlimbs and forelimbs earlier to tadpoles of control group. However, in majority of tadpoles limbs were undifferentiated and in some defective being smaller in size with abnormal digits. Such teratogenic effects of thyroxine as observed in the present study are in accordance with the findings of Mahapatra et al. (2015) in *Duttaphrynus melanostictus.* Few tadpole of the experimental group exposed to 20 IU/ml of vitamin A developed several ectopic hindlimbs from the amputated tail stumps. This is regarded as an example of homeotic transformation. Maden (1993) has mentioned that *Hox* genes are responsible for homeotic transformation of the tail into limbs. This indicates that exogenous vitamin A treatment influences the expression of specific *Hox* gene. Ectopic hindlimbs did not develop in tadpoles exposed to higher concentrations of vitamin A. From this we concluded that higher concentrations of vitamin A completely suppressed the action of specific *Hox* gene and prevented ectopic hindlimb formation from the amputated end of tail. Our findings are consistent with earlier reports on homeotic transformation by Mahapatra and Mohanty-Hemadi, 1994; Muller *et al.,* 1994; Maden, 1996; Mahapatra *et al.,* 2001; Tazawa and Yaoita, 2017 and Morioka *et al.,* 2018.

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