Chapter-III

Developmental disruptions induced by insect growth regulator (Novaluron) in *Bufo melanostictus* tadpoles
Introduction

Many agrochemicals, which are commonly used as pesticides, weedicides, insecticides or fungicides found as contaminants in the water bodies are known to be potential endocrine disrupters for non-target aquatic organisms affecting their growth, reproduction and immune system (Pickford and Morris, 1999; Sparling et al., 2001; Hayes et al., 2002; Bevan et al., 2003). The exposure effects are found more severe on developing organisms than adults (Park and Kidd, 2005; Marquis et al., 2006; Sparling and Fellers, 2007; Allran and Karasov, 2000; Carr et al., 2003). Amphibians are considered to be indicator species for environmental in particular aquatic health as they typically reproduce and pass through all the developmental stages in the fresh water, and are highly vulnerable to chemical contaminants owing to their permeable skin; therefore, any change in the surrounding medium may significantly affect the individuals and populations of amphibians (Hayes et al., 2006; Sayim and Kaya, 2007). In recent years owing to the water pollution, there is an increase in the production of amphibian fauna with gross malformations affecting the survival ability leading to overall decline in their population (Wake, 1991; Hayes et al., 2003). These have prompted serious concern regarding the biological status of many anuran species (Green, 1999; Cohen, 2001; Daniels, 2003; Hayes et al., 2006).

During metamorphosis of amphibians, the tail of the tadpole degenerates completely only when the alternate means of locomotion (limbs) appear (Saxen et al., 1957; Kollros, 1961). Thyroid hormone is known to promote the amphibian metamorphosis in general (Hayes and Wu 1995; Opitz et al., 2006; Degitz et al., 2005; Oka et al., 2009) while, retinoids (vitamin A/retinoic acid/palmitic Acid) interfere with limb development and are known to induce homeotic transformation leading to the appearance of ectopic limbs at amputed end of the tail (Mahapatra and Hejmadi, 1994; Das and Hejmadi 1998, 1999, 2003; Hejmadi and Crawford, 2003). It has been reported that exposure of *Xenopus laevis* and *Bufo buto* larvae to methoprene (IGR) and its derivatives resulted in similar effects those reported for vitamin A, when the tail is amputated (Paulov, 1976; Degitz et al., 2003). Although a very few
CHEMICALS ARE TESTED, THERE MAY BE SEVERAL CHEMICALS/INSECTICIDES/PESTICIDES WHICH ARE ROUTINELY IN USE AND MAY HAVE SIMILAR i.e. DEVELOPMENTAL DISRUPTING/ENDOCRINE DISRUPTING ACTIONS EXERTING HARMFUL EFFECTS ON AMPHIBIAN POPULATIONS (Daniels, 2003).

Novaluron, an insecticide belongs to the class diflubenzuron used in many American, European, African and Asian countries against fruit-borers for crops like cotton, soybean, fruits and vegetables and domestic pests, belongs to a class of insect growth regulators (IGRs). It is known to inhibit chitin synthesis affecting moulting stages of insect development (Tawatsin et al., 2007).

In the present study, the effects of exposure to different concentrations of novaluron on the regeneration of amputated tail, limb development and completion of metamorphosis in tadpoles of common toad Bufo melanostictus were studied. Thyroxine and vitamin A were used for comparison, and tadpoles exposed to amphibian ringer alone served as controls.

![Chemical Structure of Novaluron](image)

Novaluron
Materials and Methods

**Procurement of eggs and rearing of tadpoles**

Egg clutches of *B. melanostictus* obtained from breeding grounds around Karnatak University campus, Dharwad in the early mornings of last week of August 2007, were transferred to glass aquaria and reared in conditioned water. The fertilized eggs underwent development and hatched into tadpoles within 3 or 4 days. The tadpoles were maintained on boiled spinach under natural temperature (23 ± 1º C) and photoperiod (11.5 – 12.5 hrs). The rearing medium (conditioned water) was changed every alternate day.

**Amputation of tail and Treatment**

In order to study the abnormality or the delay in tail regeneration, emergence of limbs and induction of polymelia if any, owing to the exposure of chemicals, tail amputation was done in all the tadpoles before starting the treatment. The tadpoles at hind-limb bud stage (Stage 26 of Gosner, Gosner 1960) were selected. The tail was amputated approximately at the middle of its length using a sterilized blade under ether anesthesia (Das and Mohanty-Hejmadi, 1999). Immediately after tail amputation, the tadpoles were transferred to trays (30 X 25 X 10 cms) containing treatment media (1 litre). Ten tadpoles were accommodated in each tray and three trays were used for each concentration of chemicals. All the doses of vitamin A and novaluron chosen were sub mortality concentrations as the objectives of the study were to observe the developmental disruptions if any, induced by the presence of lowest concentrations by the insecticide in comparison with vitamin A and thyroxine.

Following treatment groups were made.

- **Group 1**: Controls: tadpoles exposed to only amphibian ringer (AR)
- **Group 2**: Thyroxine (Sigma, USA) (tadpoles exposed to 1.0, 2.0, 3.5 and 5.0 µg thyroxine/litre of AR).
- **Group 3**: Vitamin A (Nicholas Piramal, India) (tadpoles exposed to 5, 20, 40 and 60 IU vitamin A/litre in AR).
Group 4: Novaluron (Rimon, Indofil Chemical Co. India) (tadpoles exposed to 0.5, 0.75, 1.0 and 1.5 µg novaluron/litre of AR).

**Statistics**

The data were analyzed using a non-parametric Friedman's Test. The results were judged significant at 5% level of significance (Steel and Torrie, 1980).
Results

Controls

In all the 10 tadpoles/tray exposed to ringer solution and subsequently reared in conditioned water the tail regenerated on the 5th day after amputation, hind-limbs appeared on 15th day followed by the development of fore-limbs on 24th day and the metamorphosis was completed by 30th day (Fig. 1). The tadpoles were active and exhibited normal movements and fed voraciously.

Thyroxine

In the tadpoles exposed to 1 and 2 µg thyroxine/L the development proceeded significantly (P < 0.05) faster compared to those of controls, i.e. the tail regeneration was observed on 4th day, hind limbs appeared on 11th / 12th day, fore limbs between 17-18 day and over all metamorphosis was complete between 23 and 24th day (Fig. 1). In higher dose (3.5 and 5 µg) thyroxine exposed tadpoles the development was slower and was comparable to controls (Fig. 1). All the tadpoles exposed to different concentration of thyroxine were healthy, fed on spinach voraciously and exhibited active swimming movement.

Vitamin A

In majority of the tadpoles exposed to different concentrations of vitamin A the tail regeneration was abnormal i.e., regenerated portion of the tails was twisted to lateral direction resulting in a drooped/crooked tail in 40 - 60% of tadpoles (Figs. 2 and 4). In such tadpoles hind limb developed normally on 15th day, while the appearance of fore limbs was hindered and the metamorphosis was delayed (Fig. 2). The movement and feeding rate of tadpoles decreased with the increasing concentration of vitamin A.

Novaluron

No mortality was observed for the exposed doses of pesticide. In lower dose (0.5µg/L) exposed tadpoles, the regeneration of tail, development of hind limbs, fore limbs
and completion of metamorphosis was comparable to those of control tadpoles (Fig. 3). But in tadpoles exposed to 0.75 - 1.0 µg/L of novaluron, a laterally drooped/crooked tail was regenerated (Fig. 4). In such tadpoles, hind limbs developed normally but their appearance was delayed compared to controls (Fig. 3). Fore limbs failed to develop and metamorphosis was hindered. Thus, with the increase in the concentration of novaluron the rate of metamorphosis was delayed. Feeding and movement were normal in all the novaluron exposed tadpoles.
Discussion

The widespread decline of amphibian populations in recent years is although attributed to habitat loss, UV radiation, global warming and diseases, the major cause appears to be chemical pollution owing to the increased contamination of natural water bodies by agrochemicals (Green, 1999; Cohen 2001; Daniels, 2003; Hayes et al., 2006; Wake, 2007). Field studies have indicated that there is a correlation between the use of chemical pesticides and emergence of the amphibian larvae with developmental deformities in Northern America (Glare and O’Callaghan, 1999). Experiments in the laboratory have confirmed that a mixture of pesticides has greater inhibitory effects than individual pesticide on growth and development of *X. laevis* and *Rana pipiens* larvae (Hayes et al., 2006). The developmental disruptions/anomalies induced by weedicide (atrazine), organophosphate insecticides, and environmental estrogens, in amphibians are well documented (Bevan et al., 2003; Hayes 2003; 2006; Ezemonye and Ilechie, 2007). However, the impact of insect growth regulators (which are also in use amply) on the amphibian development and metamorphosis are rarely reported except methoprene.

In the present study, the tadpoles reared in amphibian ringer alone, showed normal regeneration of tail and development of limbs and completion of metamorphosis. When they were exposed to various concentrations of thyroxine, lower (1-2 µg/L) doses caused tail regeneration, limb development and metamorphosis faster than higher (>3 µg/L) doses. Thyroxine (T4) is known to accelerate amphibian metamorphosis by regulating the gene expression and protein synthesis (Hayes and Wu 1995; Opitz et al., 2006; Degitz et al., 2005; Oka et al., 2009). The plastic monomer Bisphenol A and related chemicals are known to suppress metamorphosis in *Rana rugosa* by interfering the binding of hormone to its receptors (Goto et al., 2006). Similarly corticosteroids are reported to disrupt development in *Xenopus laevis* by modulating thyroid hormone effects (Lorenz et al., 2009). In the present study, tadpoles exposed to various concentrations of vitamin A exhibited developmental deformations such as delay in tail regeneration; tails regenerated were crooked/drooped.
laterally to either left or right direction, followed by the abnormal forelimbs and delay in metamorphosis. This is in agreement with the earlier studies that retinoids elicit frog malformations (Gardiner et al., 2003). Experimental studies on amphibian tadpoles have shown that treatment of amputed tails with retinoids leads to inhibition of regeneration in *Bufo andersoni*, (Niazi and Saxena, 1968), *Notophthalmus viridescens*, *Ambyostoma maxicanum* and *X. laevis* (Scadding, 1987). Further, in addition to inhibition of tail regeneration, ectopic supernumerary limbs generated at the amputed region was demonstrated in *Uperodon systoma* (Mohanty Hejmadi et al., 1992), *Polypedates maculatus*, *B. melanostictus*, *Microhyla ornata* and *Rana tigrina* (Mahapatra and Mohanty Hejmadi 1994; Das and Dutta 1996). The difference in results obtained in the present experiment compared to those of earlier workers may be attributed to difference in the vitamin A source, length of exposure and time of the exposure and the experimental design used (tadpoles were exposed to vitamin A only for first five days after amputation and then reared in conditioned water in the present study).

In India, novaluron is commonly used against fruit/ stem borers and houses hold pests. Studies involving measurement of environmental levels of this chemical reveal that the residues of novaluron are demonstrated in cesspits (1-15 m³), street drains (4-6 m³) and disused wells with effective duration of 11-13, 8-17 and 33-69 days respectively, when used at a dose of 1- 10 mg/m² against *Culex quinquefasciatus* for >80% inhibition of emergence; further, field studies in artificial and natural habitats have shown that novaluron (10% EC) was effective against populations of *Culex species* at application rates of 10-50 ppb (Jambulingam et al., 2009). In the present study, the exposure of tadpoles to novaluron (especially 0.75 µg and higher concentrations) elicited results comparable to the effects of vitamin A. However, polymelia was not observed in novaluron exposed tadpoles unlike reported by retinoids (Hejmadi and Crawford, 2003). The synthetic insect juvenile growth hormone methoprene (and its degradation products) used widely in west are known to mimic the actions of retinoids and induce similar developmental toxicity in *X. laevis* and *B. bufo* tadpoles by regulating the gene expression (Paulov, 1976; Degitz et al., 2003). It is well
known that pesticides, insecticides and endocrine disrupter chemicals are known to interfere with the reproduction, i.e. egg production and oocyte maturation of amphibians. (Pickford and Morris, 1999; Sower et al., 2000).

In conclusion, the study reveals for the first time that, novaluron an IGR, used widely in agriculture, disrupts the normal amphibian development when present in very minute concentrations in the surrounding aquatic medium where amphibians live and reproduce.
FIGURE LEGENDS

**Fig. 1.** Effects of different concentrations of thyroxine (1, 2, 3.5 and 5μg/L) on the regeneration of amputed tail, appearance of limbs and completion of metamorphosis in *B.melanostictus* tadpoles. Values Mean ± Standard Error.

*Significant at 5% level compared to corresponding controls.
Fig. 2. Effects of various concentrations (5, 20, 40 and 60 IU/L) of Vitamin A on the regeneration of amputed tail, appearance of limbs and completion of metamorphosis in *B. melanostictus* tadpoles. Values Mean ± Standard Error.

*Significant at 5% level compared to corresponding controls.
Fig. 3. Effects various concentrations (0.5, 0.75, 1.0 and 1.5 µg/L) of novaluron on the regeneration amputed tail, appearance of limbs and completion of metamorphosis in *B*. *melanostictus* tadpoles. Values Mean ± Standard Error.

* Significant at 5% level compared to corresponding controls.
**Fig. 4.** Shows regeneration of normal tail and completion of metamorphosis on day 30 in thyroxine treated tadpoles (A, D and G) and laterally drooped tails and delayed metamorphosis in Vitamin A (Retinol) treated (B, E, H) and Novaluron treated (C, F, I) *B. melanostictus* tadpoles (X 40).