INTRODUCTION

Life is limited to a functional component of the planet earth known as the biosphere. It is the home of many living organisms like plants, animals and also that of humans. These living organisms in the biosphere are surrounded by natural environment where they interact with the environment and also with each other (Johnson *et al.*, 1997). The interactions of the organism with its environment are forming a necessary base to the survival of that organism and the functioning of the ecosystem as a whole (Elton, 1968).

Apart from the interactions, development of an organism is significant and for development a living organism has to undergo a series of biological changes associated with information transfer, growth and differentiation during its life cycle to sustain this world. The development of a new life is a spectacular process. This process of development is well distinguished in all the species including human beings.

Human beings are the most important part of the biosphere. They can alter the environment according to their needs and when it comes to their development it should be happening in favorable conditions. Until and unless conditions are playing vital role in normal development, an organism including man attains natural shape of body performing normal functioning but when any kind of teratogen interferes with the development so many unacceptable circumstances arise which result in death, malformation, growth retardation and functional deficits (Wilson, 1973).

By far there can be several teratogens to cause malformations and it is known that 65% of malformations are caused by unknown agents, 20-25% are due to genetic reason which comprise the largest group of congenital malformations with known etiology, 10% or lesser are due to environmental teratogens and less than 1% malformations are related to drug exposure, radiations and chemicals. But studies of environmentally induced malformations are of significance to analyze teratogenicity and to prevent malformations in humans (Brent and Beckman, 1990).

As per Brent and Beckman (1990) there are two etiological categories of teratogenic agents namely environmental and genetic each have specific pathologic mechanisms that result in deviant development. As far as congenital malformations due to genetic etiology is concerned these result from gene deficiency, gene abnormality, chromosome rearrangement, chromosome deletion, or chromosome excess, which results in abnormal development. Congenital malformation due to environmental etiology is when environmental agents or factors such as drugs, chemicals, radiation, hyperthermia, infections, abnormal maternal metabolic states, or mechanical factors interact with an embryo during its developmental period and lead to any kind of abnormality.

It has been manifested that human beings and other living organisms in the environment are commonly exposed to mixtures of different environmental teratogens or pollutants either simultaneously, sequentially, or both (Lokke *et al.,* 2013). Indeed, pesticides are regarded as the most ubiquitous environmental teratogens due to their extensive uses in all aspects of human endeavor (Ambali *et al.,* 2011). Pesticide is a chemical or biological agent (such as a virus, bacterium, antimicrobial or disinfectant) that deters, debilitates, kills or otherwise discourages pests. Although pesticides have benefits, some also have drawbacks such as potential toxicity to humans and other desired species (Tebourbi *et al*., 2011). According to the Stockholm Convention on Persistent Organic Pollutants, 9 out of the 12 most dangerous and persistent organic chemicals are pesticides (Gilden *et al.,* 2010).

The most popular classes of pesticides are organophosphates (OP) and synthetic pyrethroids (PYR),which are marketed in many formulations. In the current study, focus was made on two insecticides representing these groups, utilized as single compounds or as a mixture of both – cypermethrin and chlorpyrifos. Nurelle D 505 EC (Chlorpyrifos 50% EC + Cypermethrin 5% EC) was selected in the study.

Chlorpyrifos– Introduction

Chlorpyrifos (CPF) is a broad spectrum organophosphate pesticide used extensively for domestic as well as industrial applications (Mansour and Mossa, 2010; Lee *et al.,* 2012). The routes of exposure to CPF are inhalation, ingestion of contaminated food and by dermal contact (Yan *et al.,* 2012). The IUPAC name of Chlorpyrifos is O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate.Chlorpyrifos is produced by reacting 3,5,6-trichloro-2-pyridinol with diethylthiophosphoryl chloride.

Chlorpyrifos – Physical and Chemical Properties

Chlorpyrifos is a colorless to white crystalline solid (USEPA, 2006; Tomlin, 2006).It was introduced in 1965 by Dow Chemical Company. It has a mild mercaptan odor, which resembles the smell of sulfur compounds found in rotten eggs, onions, garlic and skunks (Tomlin, 2006), with a molecular weight of 350.6 (Mackay *et al.*, 1999) and melting point of 42°C (Lide, 1998). Chlorpyrifos is formulated as an emulsifiable concentrate, wettable powder, granule, and microencapsulated formulations. It is moderately soluble in water, with a solubility of 2 mg/L at 25ºC (Kidd and James 1991; Mackay *et al.*, 1999) and is relatively stable in acidic medium but the stability of chlorpyrifos decreases as the pH increases (Hui *et al*., 2010).

The empirical formula of chlorpyrifos is $C_9H_{11}C_{l3}NO_3PS$ and the chemical structure is O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothioate (Kidd and James, 1991) (Figure 1).

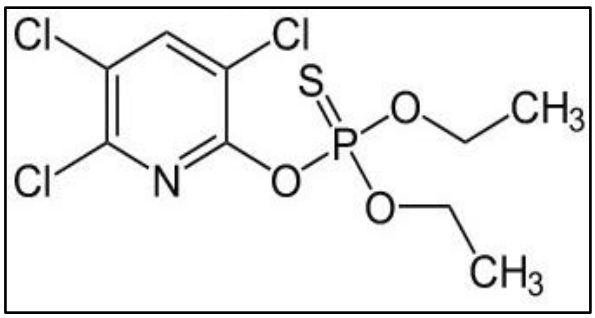

Figure1. Structural formula of chlorpyrifos

Chlorpyrifos - Applications

According to the United States Environmental Protection Agency (USEPA, 1996), CPF is registered only for agricultural use, where it is "one of the most widely used organophosphate insecticides". Chlorpyrifos is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea, beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton field, fruit, nut and vegetable crops as well as on lawns and ornamental plants (NASS, 2003). An example of consumption patterns of chlorpyrifos is given in figure 2.

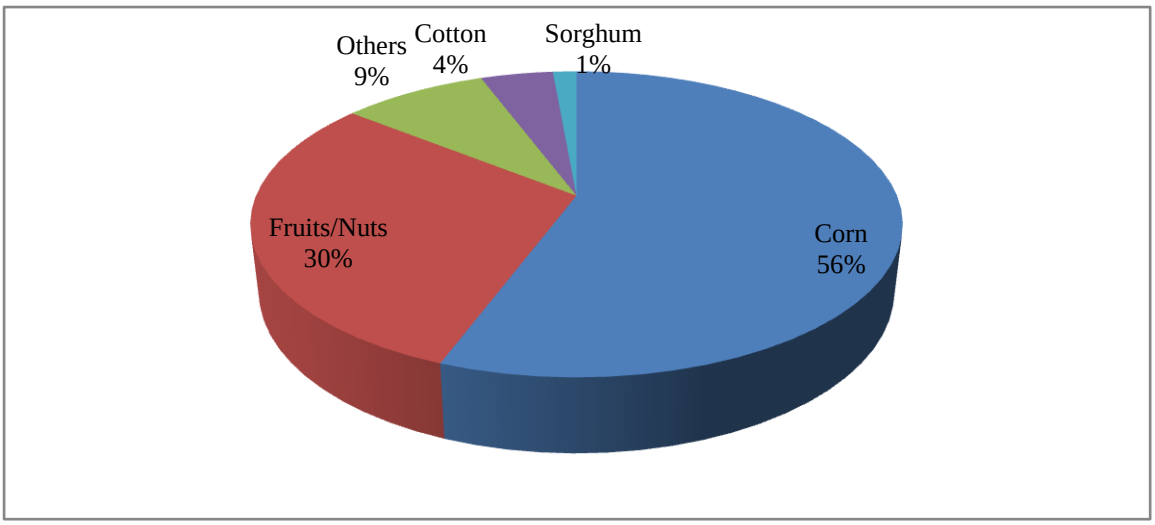

Figure2. Consumption pattern of chlorpyrifos in India (Venugopal *et al*., 2012)

Chlorpyrifos - Mode of Action

The primary mechanism of toxicity for chlorpyrifos is cholinesterase (ChE) inhibition. Chlorpyrifos affects the nervous system by inhibiting the breakdown of a neurotransmitter called acetylcholine (ACh) (Smegal, 2000). When insects are exposed to chlorpyrifos, it binds to the active site of the cholinesterase (ChE) enzyme, which prevents breakdown of ACh in the synaptic cleft (Karanth, 2000). The resulting accumulation of ACh in the synaptic cleft causes overstimulation of the neuronal cells, which leads to neurotoxicity and eventually death (Karanth, 2000).

Chlorpyrifos - Environmental Fate

Chlorpyrifos is moderately persistent in soils. The half-life of chlorpyrifos in soil is usually between 60 and 120 days, but depending upon the type of the soil half-life can range from 2 weeks to over 1 year (Howard, 1991; Wauchope *et al.*, 1992). In the water column chlorpyrifos is non-persistent due to its volatility in water, low water solubility, and strong affinity for sediments and suspended solids. Sediment-water half-lives for chlorpyrifos range from 1.2 to 34 days (Schimmel *et al.*, 1983).

Chlorpyrifos may be toxic to some plants, such as lettuce (McEwen and Stephenson, 1979). Residues of Chlorpyrifos remain on plant surfaces for approximately 10 to 14 days. Data indicate that this insecticide and its soil metabolites can accumulate in certain crops.

Chlorpyrifos- Effects and Toxicity

Chlorpyrifos is one of the most widely used organophosphorous insecticide and belongs to class II of World Health Organization's classification of pesticides (Cox, 1997). Chlorpyrifos is moderately toxic to humans; moderately to very highly toxic to birds and very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms. Acute and chronic exposure to CPF can elicit several adverse effects like neurological effects, developmental disorders, autoimmune disorders and oxidative stress. Chlorpyrifos is reported to induce oxidative damage in the hepatic and renal tissue of Kunming mice (Ma *et al.,* 2013) and hepatic and immunological modulation, genotoxicity, embryotoxicity in other test systems (Yin *et al.,* 2009). Further, Chlorpyrifos inhibits acetylcholine decomposition and therefore it increases the acetylcholine level in the synaptic cleft and over stimulates cholinergic receptors (Jeyaratnam and Maroni, 1994; Nostrandt*et al*., 1997). Prolonged exposure to Chlorpyrifos has been reported to cause anemia although the mechanism has not been elucidated (Ambali *et al*., 2010a).

Cypermethrin – Introduction

Cypermethrin (CYP) is a composite pyrethroid insecticide and a fast-acting neurotoxin with good contact and stomach action (Jin and Webster, 1998) and has become one of the most important insecticides in wide-scale use (Leahey, 1985). Cypermethrin was synthesized in 1974 (WHO, 1989) and its structure is based on pyrethrum, a natural insecticide which is obtained from chrysanthemum flowers, but it has a higher biological activity and is more stable than its natural model.

Cypermethrin - Physical and Chemical Properties

Cypermethrin is an odourless, yellowish brown and viscous semisolid at ambient temperature with molecular weight of 416.3. Its melting point is 60º- 68°C and it decomposes at 220°C. Photodecomposition of cypermethrin has been observed in field tests with no reduction in biological performance. The optimum stability occurs at pH 4 while decomposition occurs under alkaline conditions. Cypermethrin may be formulated as an emulsifiable concentrate, ULV, and wettable powder formulations.

Technical cypermethrin is a mixture of eight different isomers; each of them may have its own chemical and biological properties. Molecular formula of cypermethrin is $C_{22}H_{19}Cl_2NO_3$ and the chemical structure of cypermethrin is [(±)-α-Cyano-(3-phenoxyphenyl) methyl (±)-

cis/trans-3-(2, 2-dichlorovinyl)-2,2dimethylcyclopropanecarboxylate] and is represented in the figure 3.

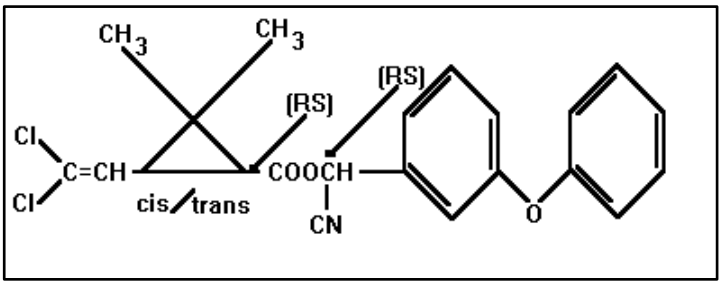

Figure3: Structural formula of cypermethrin

Cypermethrin - Applications

Over 90% of the manufactured Cypermethrin is applied to kill cotton pest (WHO, 1989). Cypermethrin is also used to control many pests of fruit and vegetable crops. In addition to its agricultural use in major crops like cotton, fruit and vegetables, cypermethrin is also used in public health programmes and for the control of flies, cockroaches, etc. An example of consumption patterns of cypermethrin is given in figure 4.

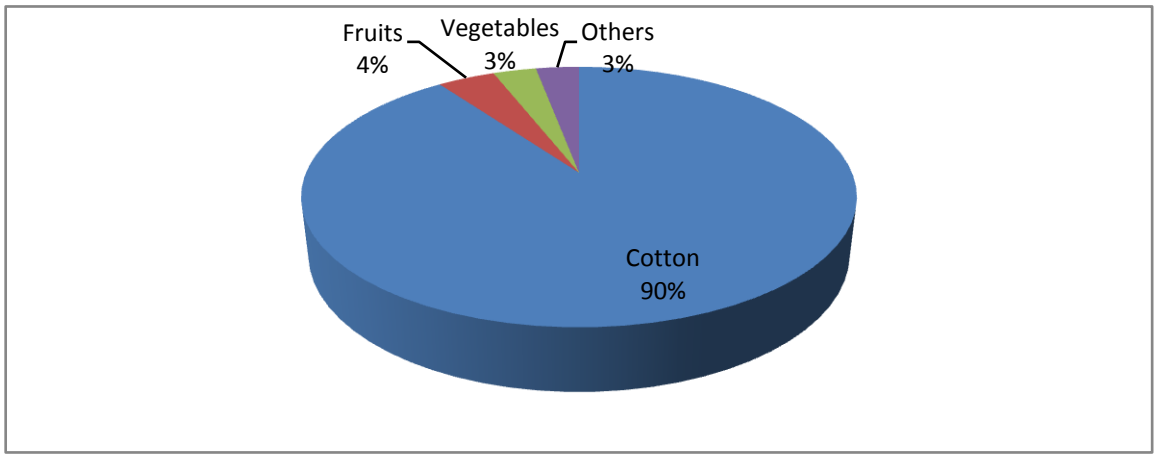

Figure: 4 Consumption pattern of cypermethrin (NASS, 2003)

Cypermethrin - Mode of Action

Cypermethrin is classified by the World Health Organization (WHO) as 'moderately hazardous' (Class II). Cypermethrin, like all synthetic pyrethroids, kills insects by disrupting normal functioning of the nervous system. In humans, handling or working with cypermethrin sometimes developed tingling, burning, dizziness and itching (WHO, 1989). Also, impairment in thedevelopment, maturation, and functioning of the blood-brain barrier (BBB) (Gupta *et*

al., 2004)and the decrease in the glial fibrillary acidic protein (GFAP) in the rat brain affectingastrocytes (Malkiewicz *et al*., 2006) been reported to have caused by cypermethrin.

Moreover, cypermethrin has been reported to induce a dose-dependent apoptotic cell death in the central nervous system (CNS) of the tadpoles of the toad *Bufoarenarum* (Casco *et al*., 2006) and also in mice (Singh *et al*., 2011). However, very little is known about the mechanism of cypermethrin-induced apoptosis in mammalian CNS in general and astrocyte in particular.

Synthetic pyrethroids are known to stimulate nerves by causing pronounced repetitive activity (Narahashi, 1996; Narahashi, 2000). Cypermethrin is a cyanopyrethroid which induces long-lasting trains of repetitive nerve impulses. The major effect of cypermethrin is to delay sodium channel closure so that a prolonged sodium tail current persists, after the membrane repolarization (Vijverberg and Van den Bercken, 1982; Narahashi 1996;Vijverberg and Van den Bercken, 1990) and the mechanism may cause paraesthesia. At relatively high concentrations, pyrethroids can also act on GABA-gated chloride channels, which may be responsible for the seizures seen with severe type II poisoning (Bradberry, *et al*., 1989).

Cypermethrin - Environmental Fate

Cypermethrin has a very low vapor pressure and is not readily volatilized into the atmosphere. Ithas a moderate persistence in soil due to its strong affinity to bind to the organic matter. pH and microbes affect the degradation of cypermethrin in soils. Higher pH and clayey soil increase adsorption of cypermethrin and hence it degrades more slowly. Isomers of cypermethrin occur as a mixture of both the cis and trans forms. In technical grade cypermethrin the cis/trans ratio is 1:1 (Kidd and James, 1991). The cis isomers are more active than the trans by a factor of two. No significant difference was observed between the photodegradation rates of the two isomers in soil, although the trans-isomer was hydrolyzed 1.2-1.7 times faster (Takahashi *et al.,* 1985). Hydrolysis and photolysis play vital roles in the degradation of cypermethrin in soil to its byproducts with half-lives of 8-16days (Walker and Keith, 1992). The water solubility of cypermethrin is very low, 4 ppb at 20^{o}C (Kollman and Segawa, 1995). Since cypermethrin is not soluble in water it quickly moves from an aqueous solution to suspended particulates (Fitzpatrick, 1982). Thus, a significant amount of cypermethrin from the aqueous phase can be removed by relatively small amounts of suspended matter in natural bodies of water. Soils and sediment are the main environmental

reservoirs for cypermethrin (Bacci *et al.*,1987). It is therefore, unlikely to cause groundwater contamination (Kidd and James, 1991).

Cypermethrin - Effects and Toxicity

Cypermethrin is reported to be highly toxic to fish and aquatic invertebrates (Bradbury and Coats 1989), and it is also highly toxic to bees (Waller, 1988). It is carcinogenic and long term repeated dose study showed that cypermethrin induces benign lung tumors in female mice at the highest dose tested (229 mg/kg/day) (USEPA, 1989).

Effectsof Mixture of Cypermethrin and Chlorpyrifos:

As mentioned previously, the most popular classes of insecticides are organophosphates and synthetic pyrethroids, which are offered in many formulations; and their main mechanisms of action are well known on individual basis especially in the field of Neurotoxic long-term effects and developmental toxicity. It is also reported that when they applied together, the organophosphates enhance pyrethroids toxicity (Ray and Forshaw, 2000; Tiwari *et al*., 2008). Pyrethroids like cypermethrin are broken down by esterases enzymes and the same enzymes are phosphorylated and therefore made dysfunctional by organophosphate. Hence, the conclusion is that two kinds of insecticides are in synergism and the toxicity of cypermethrin in combination with an organophosphate insecticide is greater than the toxicity of either insecticide alone (Gaughan *et al.,* 1980).

It is observed that mixed pesticides have high price for their high efficiency, convenience and rapid actions, and are becoming increasingly popular in agricultural use. Mixed pesticides, compared with single pesticides, could generally cause significant synergistic effects of toxicity on target species, while they are also effective to beneficial species in most cases. Zhou *et al*. (2011) reported combined toxic effects of cypermethrin and chlorpyrifos on earthworm. Karabay and Oguz (2005) reported that imidacloprid in combination with methamidophoscould cause cytogenetic and genotoxic effects in Wistar albino rats. Hussain (1987) indicated that the combined anticholinesterase effect of insecticidal formulation of acephate and methamidophos on enzymes in insects and mammals. Siroki *et al*. (2001) reported mutagenic and clastogenic activities of propoxur and pirimicarb insecticide mixture in rats. Anderson and Lydy (2002) reported that atrazine in combination with three organophosphate insecticides (chlorpyrifos, methyl parathion, and diazinon) could cause a significant increase in toxicity to *H. azteca* compared with individual pesticides. Clark *et al*.

(2002) indicated that toxicity of chlorpyrifos was enhanced by atrazine and cyanazine to the fourth-instar larvae of the aquatic midge, *Chironomustentans*. Neurotoxic effect of Cypermethrin and Profenofos combination in Wistar rat was reported by Rajesh and Pilo (2009). Thus the study of pesticide mixtures is even more important in evaluating ecological risk of pesticides on ecosystems.

It has been reported that the Plasma ChE activity is not affected alone by cypermethrin but in combination with chlorpyrifos plasma ChE gets inhibited. Researchers have observed a significant decrease in plasma and brain ChE activity by the toxicity of cypermethrinand chlorpyrifos combination in Wister rats (Latuszynska *et al*., 2001). Therefore, cypermethrinand chlorpyrifos combination was introduced in the agricultural world to assure the synergistic effect on the pests against their resistance to either of the pesticides in isolation (Tiwari *et al*., 2008; Rajesh and Pilo 2009).However, recent studies conducted in our lab showed that treatment of chorpyrifos and cypermethrin in combination induces widespread developmental anomalies in the F1 generation of chicks (Uggini *et al.,* 2012; Uggini and Suresh, 2013). The treatment of the combination insecticide (chorpyrifos and cypermethrin) induced structural malformations like craniorachischisis, crook leg and wryneck. The researchers also reported compromised immunity and subdued redox physiology in chorpyrifos and cypermethrin treated chicks (Gowri *et al.,* 2010; Uggini *et al.,* 2012).

The developmental anomalies, induced by combination insecticide treatment, reported so far is limited only to the malformation and/or its mechanism of onset observed in the first generation. Examples of such studies are cited earlier. However, it will be of great significance to know the adverse effect, if any, of the pesticide treatment in the second generation of organism which may receive a compromised signal from the intoxicated parents (largely maternal) resulting in deviant development.

Therefore the present study was envisaged wherein three sub-acute doses of a commercial formulation of chlorpyrifos (50%) and cypermethrin (5%) (Nurelle D 505; manufactured by Dow AgroSciences India Pvt. Ltd; Mumbai) was administered to the air cell of fertilized RIR eggs and the developmental toxicity of the compound was studied in the first and second generations of chicks.

Rationale behind the selection of egg as a model for study

If we look at the research models used throughout the history of biology, the domestic fowl and their eggs have been extensively used by the researchers. The embryo of the chick holds the record as the animal with the longest continuous history as an experimental model for studies in developmental biology (Needham, 1959).Moreover, for the past few decades, researchers are preferring chick embryos as a model for assessing toxicant induced teratogenic studies (McLaughlin *et al*. 1963; Walker 1971; Greenberg and LaHam 1969).

Furthermore, for various reasons like the availability of fertilized eggs, the rapid growth of the embryo, the ease in manipulation and cell lineage analysis for assessing morphological, biochemical and functional studies on growth, differentiation and organogenesis have made the chick embryo considerably less expensive model system than the live animals. During the period of development, when the egg progresses from a single cell to a hatched one, all the complexities of development and differentiation can be observed and analyzed which are similar to mammals (Karnofsky, 1965).Above all the selected model (fertilized egg) will eliminate the wasteful killing of a mother. Therefore, in the current study, the effect of pesticide toxicity and its possible transformation from one generation to next generation was evaluated in freshly collected fertilized eggs of RIR fowl.

Objective of the Study:

Recent advancement in the science and technology has enabled human beings to develop new technologies to exploit the natural resources to fulfill their ever increasing needs. To an extent the use of natural resources is necessary and their exploitation beyond limits becomes quite dangerous and if not curtailed might disturb the delicate balance across the ecological unit. As far as the technological advancement is concerned, people in the past centuries have observed the creation of three ages which are the Nuclear Age, the Electronic Age and the Chemical Age and each with its profound social implications. The chemical age is the oldest one and chemical tools like detergents, adhesives, lubricants, solvents, pesticides, pharmaceuticals, cosmeceuticals, etc. have deeply permeated in our day to day lives.

Among these chemical tools if the credits of pesticides include boosted economic potential in terms of increased production of food and fibre, and amelioration of vector-borne diseases, then their debits have resulted in serious health implications to man and his environment. The health hazard due to exposure to the toxicity of pesticides has to be understood well and minimized as far as possible. Hence, here in this study combination of chlorpyrifos and

cypermethrin has been used to explore its hazard threshold and transformation of pesticide induced toxicity from one generation to the next. The flow of the experimental procedure is outlined below for easy comprehension.

Steps oftheexperiment

STEP 1: Freshly Fertilized RIR Eggs
STEP 2: Grouped in 3 experimental + 1 Control
STEP 3: Injected with appropriate dose
STEP 4: Incubated and Hatched (F1)
STEP 5: Vaccinated and wing banded F1 generation
STEP 6: Rearing of chick up to adult stage (25 weeks)
STEP 7: Collection of Fertilized eggs
STEP 8: Incubated and Hatched (F2)
STEP 9: Vaccine and wing band F2 generation
STEP 10: Rearing of chick up to week 4 of age

After step four and step eight the hatchlings of F1 and F2 generations respectively were visibly observed for Ci induced gross structural anomalies and the results are discussed in chapter 1. Subsequently, six representatives from each group (3 experimental and 1 control) of F1 generation chicks of twenty five week old and F2 generation chicks of four week old were sampled for the analysis of haemogram (chapter 2) and histophysiology (chapter 3) to comprehend the extend two generation developmental toxicity of Ci in RIR chicks.