CHAPTER 1

IN-OVO SCREENING OF TWO INSECTICIDE FORMULATIONS: STUDY OF GROSS MORPHOLOGICAL AND SKELETAL MALFORMATIONS

INTRODUCTION

The developmental toxicity takes into consideration all the abnormalities in the development of the organism i.e. malformation, functional deficits, developmental delays and death (**Wilson, 1973**) caused by any chemical or physical agent. In many organ systems the developmental abnormalities occur before birth, thus the teratogenic effect precedes, while in few others like the central nervous system (CNS) there are several sensitive processes such as cell migration, synaptogenesis, and apoptosis that occur even after birth and in the early years of life, and therefore the effects may be exhibited latently in the later part of the life (**Durisala, 1998**). The period of early embryonic development is highly vulnerable to the slightest of perturbations caused by certain extraneous agents, called as the teratogens. The developmental mechanisms are sensitive to a wide range of teratogens i.e. the pharmaceutical drugs, radiations, maternal infections and several environmental chemicals (**Brent and Beckman, 1990**). **Rodier (1976)** has shown that morphological defects in brain development of mice are induced by exposure at several specific times in late gestation to a chemotherapeutic agent, and that there are differences in the pattern of neurobehavioral effects associated with exposure at each time point. Therefore, teratogens can induce different types of effects based on the magnitude and time of dose. As major organ structure is completed, organization at the histological level as well as physiological and biochemical differentiation proceeds; in most mammals, these processes occur to a varying extent during pre- and postnatal development. Exposure to a number of agents during this period may result in alterations that are detected as histopathology, growth retardation, and/or functional changes.

One of the cardinal principles in determining whether or not an exposure to a particular agent is related to an effect is that there is a dose-response relationship between the two. **Wilson (1973)** originally described the overlapping dose-response relationship between

malformations, embryolethality, and maternal toxicity. In the case of an agent that causes malformations, as dose increases, the severity of malformations also tends to increase until death intervenes. If death of the conceptus occurs at higher doses as a result of malformations, a nonlinear dose-response relationship for malformations may result. Therefore toxicity testing and dose response relationship is a necessary part of understanding the cause and effect relationship between the chemical exposure and illness caused.

Of these numerous factors causing developmental toxicity, the pesticides have received considerable attention. Several studies have reported the embryotoxic and teratogenic effects of pesticides in fish (**Kang *et al.*, 2008**), amphibians (**Pawar and Katdare, 1984; Osano *et al.*, 2002**), aves (**Hoffman and Sileo, 1984; Anwar, 2003; Slotkin, *et al.*, 2008**), rhodents and other mammals (**Tocco *et al.*, 1987, Muto, *et al.*, 1992; Roy, *et al.*, 1998**). With regard to increasing number of pesticides being introduced into the market, and the considering their potency to meddle up with the developmental processes, it was felt crucial to conduct an evaluation and ascertain their toxicity.

Hence, in the current chapter, a preliminary range finding study was designed for the two different insecticides i.e. *Anaconda 505^{TM} and Tracer*, and an attempt was made to evaluate the developmental malformations induced by them. *Anaconda 505^{TM}* is a combination of two most commonly used insecticides, chlorpyrifos and cypermethrin, while *Tracer* is a naturalyte class of insecticide with Spinosad as the active ingredient. As described in the preceding chapter, the combination insecticide formulation was selected for the study owing to the following reasons:

1. Widespread usage (as individual ingredients) in agriculture and/or household.
2. Meagre details regarding the effects of combination insecticides.
3. Chances of an organism being exposed to more than one type of insecticide through the food chain accidentally or ignorantly.
4. The need for studying the commercial formulation rather than the active ingredients.

Tracer was included in the study because:

1. Scanty studies on embryotoxicity and teratogenicity, especially with an avian model.
2. Classified to a "reduced risk" category by the EPA, however the neurotoxic nature of this insecticide could not be overlooked for developmental toxicity studies.
3. Necessity to investigate the commercial formulations rather than only the active ingredient

4. To understand the differences in the toxicities induced by the chemical class (Combination insecticide) and naturalyte class (Spinosad) of insecticides.

The toxicity of a compound could be rated by identifying the amount of the dose which induces the lethality and the type of incongruity it induces. Therefore, as a preliminary step of toxicological investigation, a dose-ranging study was performed in order to understand the relation between the dose of the test chemical applied and the response shown by the test animal. For this, fertile RIR embryos were exposed to the concerned test insecticides prior to incubation and a stage-wise monitoring was done as follows, to evaluate the embryopathy:

1. To observe the dose response relationship and calculate the LD_{50}.
2. Visual (microscopically and /or by naked eye) examination of the embryos at various stages of development and, assess the crown rump length and embryonic wet weight.
3. Examine the morphological changes in the external and skeletal structures
4. Monitor the hatchling body weight and organ weight.

MATERIALS AND METHODS

Test chemicals

Two different commercial insecticide formulations, purchased from a local licensed pesticide vendor at Vadodara, were used for the present study. The first one was a combination insecticide (Ci) which constituted of chlorpyrifos (50%) and cypermethrin (5%); while the second one was a biological insecticide, Spinosad (Sp). A detailed description of these two pesticides is specified in earlier in this treatise under the section Materials and Methods.

Egg Procurement and Incubation:

Fertile RIR eggs were obtained from the Intensive Poultry Development Unit, Vadodara and refrigerated at 4^0C until use (stored not for more than four days). After an appropriate treatment (described below), the control and the insecticide treated eggs were set to incubation in the incubator (regulated to a temperature of 37.5 ± 0.5^0C and 75-80% relative humidity), for 21 days or as per the requirement of the experiment. The eggs were manually turned over an angle of 180° for seven times a day until 3 days prior to hatch.

In ovo Injections:

The eggs were injected through the air sac method as per **Blankenship *et al.* (2003)** on day '0' of incubation (detailed in the earlier section Materials and Methods).

Dose-ranging Study and LD_{50}

A preliminary range finding study was conducted with different doses of the insecticides as follows:

Combination insecticide (Ci): 0.001, 0.005, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5 and 0.8µg/egg diluted in the vehicle corn oil, dosed in volumes of 50µl/egg.

Vehicle control 1 (VC1): corn oil dosed in volumes of 50µl/egg.

Spinosad (Sp): 0.05, 0.1, 0.25, 0.5, 0.75, 1.5, 1.75, 2.0, 2.5 and 3.5 mg/egg diluted in 0.4% methylcellulose, in volumes of 50µl/egg.

Vehicle control 2 (VC2): 0.4% methylcellulose in volumes of 50µl/egg.

Sham: hole was drilled and an empty prick was given with syringe needle.

On 21st day the hatchability and mortality were observed and the LD_{50} was calculated by probit analysis using the software IBM SPSS statistics 19.0.0

Doses equivalent to $LD_{50}/10$, $LD_{50}/2$, and LD_{50} were chosen for further studies. Combination insecticide (Ci) was dosed in concentrations of 0.01, 0.05 and 0.1µg/egg; while *Tracer* (Spinosad) was dosed in concentrations of 0.15, 0.75 and 1.5mg/egg. Controls were dosed with respective vehicles alone. Sham injections were given to check if there was any stress on the embryo due to the egg injections.

Effect on Embryonic Growth and Development

The embryonic growth was monitored at various stages from 36hr after incubation till the 21st day. The embryos were assigned to the different stages as per the standard reference of **Hamburger and Hamilton (1951).** When control embryos were picked after a specific time point and assigned to a particular stage, the insecticide treated embryos were also picked up at the same time point of incubation. The following features were noted under each stage:

At stage-10, stage-13 and stage-20:

(i) Somite number
(ii) Abnormalities in the shape, size and position of the somite
(iii) Neural tube defects (NTD)

Owing the large number of somites, the somite counting was not considered for the stage-20 embryo.

At Stage- 26, 34, 38, 44 and 46:

(i) Viability at all these stages was monitored through candling the incubated eggs.

(ii) Morphological malformations were noted.

At Stage-34:

(i) Morphological malformations were noted.

(ii) Crown-rump length was measured

(iii) Embryonic wet weight was recorded.

At stage-46 (Hatchling on day 20-21):

(i) The percent hatch and mortalities in all the groups were assessed.

(ii) The hatchlings were weighed and their weight relative to the initial egg weight was calculated.

(iii) The weight of hatchlings, their liver and brain were also recorded and the relative weights were calculated.

(iv) The live hatchlings as well as dead embryos were examined and classified to the various forms of developmental abnormalities based on the severity and position the malformation, as follows:

a) Appendicular deformities: 1) Ap1: crooked legs, twisted phalanges and unsteady gait

2) Ap2: missing phalanges

b) Axial deformities: 1) Ax1: beak deformities or jaw prognathism

2) Ax2: defects in vertebral curvature, wry neck

3) Ax3: deformities in formation of skull, sternum and ribcage

c) Others: anophthalmia, umbilical hernia, edema

Bone and Cartilage Staining

For visualizing the skeletal development, the hatchlings as well as the unhatched or dead embryos of the various groups were collected on day 21 and stained for bone and cartilage as per **Lamb, (2003)**. Details of the method are described in elsewhere.

RESULTS

Dose Ranging Studies and LD_{50}

The range finding study was conducted by using the combination insecticide and the Spinosad in separate groups of fertilized RIR eggs by injecting different concentrations of the insecticides. The lethality and mortality of both the insecticides tested showed a dose-response relationship i.e. the lethality increased as the dose was increased (**Table 1.1a and 1.1b**). Probit analysis was done to find the median lethal dose, (LD_{50}) by injecting the fertilized RIR eggs prior to incubation and observing the hatchability on the 21st day. The LD_{50} of the combination insecticide was found to be 0.109µg/egg, while Spinosad was lethal to 50% of the dosed eggs at a concentration of 1.54mg/egg (**Table 1.2, Figure 1.1 and 1.2**). Once the LD_{50} was derived, three different doses of the insecticides i.e. $LD_{50}/10$, $LD_{50}/2$ and LD_{50} were chosen for further study, i.e. 0.01, 0.05 and 0.1µg/egg of combination insecticide and 0.15, 0.75 and 1.5mg/egg of Spinosad.

Effect on Embryonic Growth and Development

Early embryonic stages

Stage-10 chick embryos

As per **Hamburger and Hamilton (1951)** a stage-10 embryo shows first indication of cranial flexure, the three primary brain optic vesicles are clearly visible and the heart is bent slightly to right. The control embryos showed these described features. At stage-10 of incubation post dosing, the mean of somite number was found to be significantly less ($p \leq 0.01$) in the 0.1µg of Ci dosed embryos than in the controls, while 0.01 and 0.05µg of Ci did not induce comparable changes in the somite number. However, abnormal somite disposition and neural tube defects were sighted at both the 0.05 and 0.1µg of Ci (**Table 1.3**).

The abnormalities in somites were sighted in the form of distorted and scattered somites, and also a row of smaller sized somites on one side of the neural tube. Failure of neural tube closure and wavy neural tube observed in the present study were considered as neural tube defects.

The Spinosad treated embryos at a dosage of 0.15 and 0.75mg/egg did not show any considerable change in somite number though, a dose of 1.5mg/egg showed a lag in somite

development (**Table 1.3**). 10% of embryos both at 0.75 and 1.5mg of Spinosad showed abnormal somite disposition.

Stage-13 chick embryo

The standard features of stage -13 embryos matched to that of the control embryos here in the study i.e. the presence of twenty somites, broad curves in cranial and cervical flexures, primary optic vesicle and stalk are well established, head fold of amnion covers forebrain, midbrain, and anterior part of hindbrain.

The embryos treated with 0.1 and 0.05μg of Ci after incubation for 48hr (stage-13) showed a significantly lowered number of somites. The embryos treated with 0.01μg of Ci did not show any comparable changes. With 0.05 μg of Ci, 30% embryos showed abnormal somite disposition and 40% of them showed neural tube defects. The 1.5mg/egg Spinosad treated embryos showed a lag in somite development ($p \leq 0.05$) while no similar effect was observed in the 0.75 and 0.15mg Spinosad treated embryos (**Table 1.3**).

Three Siamese twin embryos were discovered among the combination insecticide treated group (one from the group of 0.05μg/egg of Ci and two other from the group of 0.1μg/egg of Ci). In all the three pairs of conjoined twins observed, one was always smaller in size compared to its counterpart (**Figure 1.3 and 1.4**). The split of the axis was limited to the anterior region. They were attached from posterior till the level of heart, had separate heads and attached heart primordia. The frequency of conjoined twins considering the entire number of embryos studied was 1.07%. If the combination insecticide treated group is considered exclusively, where the conjoined twining actually occurred, the frequency of occurrence was 2.7% (considering 280 embryos studied over a period of eight months, of which 30 embryos belonged to VC1, another 30 belonged to VC2, 110 embryos were Ci dosed and 110 were Sp dosed).

Stage-20 chick embryo

When the embryos were examined after 72hr of incubation, the controls showed normal development (**Figure 1.5**). No overt signs of abnormalities were observed in the group treated with 0.01μg per egg of combination insecticide except for a delayed development sighted in two of the ten embryos observed. The embryos treated with the combination insecticide at concentrations of 0.05 and 0.1μg per egg showed several developmental abnormalities like delayed growth relative to control, dispersed or distorted somites, smaller

somites on one side, incompletely developed heart primordium, wavy neural tube, delay or failure of neural tube closure and highly distorted cephalization, (**Figure 1.6, 1.7 and 1.8**). 20% of embryos dosed 0.05 μg showed abnormal somite disposition and/or neural tube defects. While the dose of 0.1μg of Ci induced abnormal somite disposition in 40% embryos and/or neural tube defects in 50% of the embryos.

Among the 0.15 and 0.75mg/egg of Spinosad treated embryos, no deformities were observed. However, 40% of embryo given 1.5mg/egg of Spinosad showed delayed development with relatively fewer numbers of somites or they resembled the embryos of a previous stage when observed in terms of the cervical flexion. A twin embryo was found in 0.75mg/egg Spinosad treated group (**Figure 2.9**).

Stage-34 (8 day) chick embryo

On day eight, the embryos treated with 0.01 and 0.05μg of Ci had no change in the crown-rump lengths and wet embryonic weights of the embryo. However the 0.1μg Ci dosed embryos showed a highly significant ($p \leq 0.05$) decrease in the crown-rump length and the embryonic wet weight (**Table 1.4 and Figure 1.10**). The morphological examination of the 0.1μg of Ci treated embryos on day eight also showed defects in the formation of beak and severe haemorrhages in the head and heart region (**Figure 1.11**).

The Spinosad dosed embryos at all the three tested doses of 0.15, 0.75 and 1.5mg/egg did not induce any changes in the crown-rump length and embryonic wet weight (**Table 1.4**). No morphological anomalies were observed on visual examination of these embryos, although the embryos treated with 1.5mg of Spinosad seemed to be oedematic.

Stage-46 (day old hatchlings)

The sham injected group did not show any considerable variations in hatchability compared to the vehicle control groups VC1 and VC2. When the viability and hatchability of the embryos were observed during the embryonic incubation period and post hatching; the combination insecticide dose of 0.01μg/egg caused a slow decline in viability from 80% on 5th day of incubation to 65% on 21st day. 0.05μg/egg of Ci showed 80% embryos to be viable on 5th day and later decreased to 60% post hatch i.e. on 21st day. The dose of 0.1μg/egg of Ci caused a highly declined viability of 50% on day 5 of incubation which further decreased to 35% post hatch (**Table 1.5, Figure 1.12a**).

On day 5 of incubation all the embryos dosed with 0.15 and 0.75mg/egg of Spinosad were found alive. However, on the 21st day, the hatchability was 75% among 0.15 mg/egg embryos while it was 55% among 0.75mg/egg treated embryos. The Spinosad dose of 1.5mg/egg showed a few early mortalities, 85% of them were alive on day 5 but the hatchability decreased to 60% (**Table 1.5, Figure 1.12b**).

There was a significant decrease ($p \leq 0.05$) in hatchling body weight relative to initial egg weight as well as in the relative weight of liver with the dose of 0.1µg. However, no significant changes occurred in hatchling body weight relative to initial egg weight, relative weights of liver or brain at 0.05 and 0.01µg when compared to the VC1 group. The hatchling body weight relative to initial egg weight or the relative weights of livers and brains among the VC2 and Spinosad treated groups showed no significant difference (**Table 1.6**).

Though there observed a significant difference in the egg weights and hatchling body weights between VC1 and VC2 groups, hatchling body weights relative to initial egg weights and also relative weights of liver and brain between the two control groups were found to be within the normal range.

Morphological and Skeletal Malformations

Significant embryonic malformations in axial and appendicular skeleton were observed in all the three different dose levels of the combination insecticide selected. At a dose of 0.01µg/egg, the effect was not very obvious morphologically, though an unsteady gait was observed occasionally. The abnormalities at 0.05µg/egg were higher by the exhibition of 10% of the treated group with crooked legs, twisted phalanges in one or both the hind limbs (**Figure 1.13 and 1.14**), beak deformities, microphthalmia and anophthalmia (**Figure 1.15**). At 0.1µg/egg the hatchlings showed overt signs of teratogenicity in more than 20% of the treated group that include wry neck (**Figure 1.16**), craniorachischisis, in which the brain and the spinal cord remained open (**Figure 1.17**), beak defects, bilateral anophthalmia, deformities in formation of sternum and ribcage, vertebral deformities (**Figure 1.18**), micromelia, missing phalanges and umbilical hernia. Thus the defects were more apparent as the dosage increased (**Table 1.7**).

Tracer, on the "other hand" dosed at much higher levels i.e. 0.15, 0.75 and 1.5mg/egg showed no visible skeletal deformities in the hatchlings, though the dead embryos seemed to be highly oedematic (**Figure 1.19**) and umbilical hernia was spotted at times (**Table 1.7**)

Neither of the vehicle control groups i.e. the VC1 or VC2 treated groups showed any sort of malformations or developmental anomalies.

DISCUSSION

Dose Ranging Studies and LD_{50}

From the observations made in the present study, very low doses of the combination insecticide were found to be highly teratogenic to the chick embryos. And the effects increased as the dose increased, showing a dose-response relationship. **Muscarelle *et al.* (1984)** reported a chlorpyrifos Ld_{50} of 1500µg per chick embryo (Cornell-K strain; observed after 17days of development). A study by **Anwar (2003)** reported an LD_{50} at 800ppm (40µg/egg) of cypermethrin in chick embryos. While in the present study, the combination insecticide showed a very low LD_{50} value of 0.109µg/egg, implying that the Ci is highly toxic to the developing embryos. Spinosad on the other hand was lethal to half of the dosed embryos at 1.54mg/egg, which is quite higher than that of the Ci dose. This gives a clear picture that Spinosad is relatively subtle on the chick embryos in terms of lethality.

Effect on Embryonic Growth and Development

A pre-hatch observation of the embryos at various stages of the development showed that the embryos treated with the combination insecticide exhibited abnormalities in somite disposition like fewer number of somites relative to control, dispersed or distorted somites and formation of diminutive somites on one side. Similar observation of somite abnormalities were reported by **Alhifi *et al.* (2004)**, where the organophosphate dimethoate induced abnormal developmental effects on the heart, brain, neural tube and somites. The classical studies on embryonic development show that the somites arise from the paraxial mesoderm adjacent to the neural tube and notochord in a cranio-caudal sequence. The budding off of the somites and their compartmentalization is mediated by local signals from adjacent structures through diffusible substances such as sonic hedgehog (Shh), Wnts and Bone morphogenetic protein (BMPs) or by cell–cell interactions via membrane-bound receptors and ligands such as *Delta* and *Notch* (**Christ *et al.*, 1998**). Compartmentalization of the somites and their derivatives is reflected by the differential expression of developmental regulatory genes such as *Pax-1, 3, 7* and *9, MyoD, paraxis, twist* and others. The combination insecticide might have interfered with one of these early signals and thereby lead to abnormalities in the somite formation.

Apart from the abnormalities in the somite disposition, wavy neural tube and neural tube closure defects were also sighted in embryos treated with 0.05 and 0.1 µg/egg of Ci treatment. And these outcomes of pesticide treatment could be matched with the investigations of **Rull *et al.* (2006)** which signify an elevated risk of neural tube defects in human population associated with exposures to agricultural pesticides.

Further, an interesting observation in the combination insecticide treated group is the Siamese twins. Identical or non-identical twins and conjoined/Siamese twins in various animals have been reported earlier either as natural occurrence or experimentally induced. Natural occurring twins in avian species were reported by many authors: **Levi (1941)** sighted twins in pigeons; **Griffith and Stewart (1998)** reported the occurrence of non-identical twins in house sparrow; twin embryos of hen were reported by **Bhatnagar and Bhatnagar (1998)** and identical emu twins were described by **Bassett *et al.* (1999)**. Conjoined twins or Siamese twins are a rare aspect of multiple birth. In an earlier report **Chai and Crary (1971)** described conjoined twins in rabbits. **Patwardan and Ghaskadbi (2000)** reported occurrence of conjoined twins and double embryos in chick. Experimentally induced twinning in chick embryos was depicted by **Eyal-Giladi (1970)**, **Peskova (1980)**, and **Naito *et al.* (1991)**. **Al-Jufaily *et al.* (2005)** reported wild siamese twins in black tip sea catfish and opined that pollutants such as DDT (organochlorine pesticide), heavy metals and hydrocrabons found in the waters of the gulf of Oman, might have induced this abnormal development. **Manna and Sadhukhan (1986)** reported siamese twins in Tilapia after injecting an organophosphate Roger 30E to the male parent.

The frequency of conjoined twin appearance in the present study was more than the spontaneous observations reported earlier by **Patwardan and Ghaskadbi (2000)**, which was 0.25% over a period of five years. Owing to the high frequency and considering the potential of organophosphate pesticide to induce siamese twin formation (**Manna and Sadhukhan, 1986**), it could be derived that the insecticide treatment might have induced the siamese twin formation in the present study. These malformations might be attributed to certain crisis developed during gastrulation due to insecticide treatment. Any complete separation of an embryo before gastrulation results in identical twins, however at a later stage incomplete separation or a complete separation and partial fusion would lead to siamese twins

(Sulzberger, 1994). Though the chick body axis specification occurs during early cleavage stage, the formation is accomplished during gastrulation **(Gilbert, 2006)**. Experiments on inductive capabilities of hypoblast reveal that, during gastrulation the mesoderm formation and axial patterning is controlled by the hypoblast **(Bertocchini and Stern, 2002; Bertocchini *et al.*, 2004; Idkowiak *et al.*, 2004)**. A report by **Bertocchini and Stern (2002)** describes that, if hypoblast is removed, it would lead to multiple axes formation and therefore hypoblast emits inhibitors of multiple axis formation. The molecular signalling expression *Nodal* is thought to initiate the formation of primitive streak and *Cereberus*, an antagonist of *Nodal*, secreted by the primary hypoblast prevents further primitive streak formation. As the primary hypoblast cells move away from the posterior marginal zone (PMZ), the absence of cereberus allows nodal to be expressed in the PMZ, stimulating the primitive streak formation. Once primitive streak forms, it secretes *Lefty* another *Nodal* antagonist, thereby preventing any future primitive streak **(Bertocchini and Stern, 2002)**.

Therefore, a possible conclusion to the observations followed by combination insecticide treatment might be that, the pesticide interferes with hypoblast signalling at some point of gastrulation. Thereby it produces anomalies in paraxial mesoderm and somite formation. Also, the insecticide might be interfering with the signalling of the hypoblast and the streak formation, and leading to multiple axes and conjoined twins.

Spinosad on the other hand did not reveal any abnormalities in the somite development except for a slow growth and relatively fewer numbers of somites, under the current tested levels of concentration. The normal twins which appeared in the Spinosad treated group could be only incidental and may not be attributed to insecticide treatment, since it occurred only once. The formation of double axes in this case might have happened in the early gastrulation stages before the egg was laid.

Morphological and Skeletal Malformations

Several types of axial and appendicular deformities with other types of malformations were encountered in the combination insecticide treated groups. **Moe and Co-workers (1978)** described the pathogenesis of vertebral development as defects of segmentation and defects of formation during embryonic development. The somites form a metameric pattern during embryonic development determining the appropriate arrangement of segmentally arranged structures like vertebral column, ribs, dorsal root ganglia, spinal nerves and blood vessels. The somites bud off from the paraxial mesoderm and under the influence of ventral signals

from the notochord and ventral neural tube, the ventro-medial somite differentiates into the sclerotome. The sclerotome gives rise to the vertebral column, ribs, connective tissue and meninges (**Yusuf and Brand-Saberi, 2006**). Therefore, the formation and patterning of the somitic cells play a crucial role in the formation of skeletal structures. During the early events of somite development, the vertebrate embryonic axial skeleton is most susceptible to the teratogenic effects of a variety of pharmaceutical and environmental agents (**Barnes *et al.*, 1996**). The early embryonic observations of the Ci treated embryos showed defects in the somite formation and patterning. Therefore this gives a direct clue to the formation of various skeletal deformities like crooked limbs, micromelia, missing phalanges, beak deformities, deformations in the vertebrae, sternum and ribcage and the craniorachischisis.

One of the malformations craniorachischisis, observed in this study is an indication of failure to initiate closure of neural tube at the start of neurulation. Wavy and open neural tubes were sighted in the present study even at an early stage of 36 and 48hr post incubation, in the 0.05 and 0.1μg of Ci treatment. Similar observations made by **Murdoch *et al.* (2001)** while explaining the neural defects in loop tail (Lp) mutant mouse give credence to the present notion. The process of neurulation in verterbrates involves a precisely orchestrated set of morphogenetic movements within the neural plate itself (intrinsic processes) and also within neighbouring tissues (extrinsic processes) (**Smith and Schoenwolf, 1997**). This process is complex and is regulated by many genetic and environmental factors. **Murdoch *et al.* (2001)** proposed that role of *Lpp1* (a novel gene) in neurulation may be to restrict the lateral extent of differentiation of the floor plate, thereby allowing precisely controlled midline bending of the neural tube closure. They also opined that *Shh* (sonic hedgehog) acts as a negative regulator of *Lpp1* expression. Therefore, it is logical to hypothesize that a flaw in the expression of either of these genes might have resulted in the neural tube defect like the currently observed craniorachischisis.

Morphological and skeletal malformations were reported by **Rao *et al.* (1992)** in hatchlings of white leghorns after dosing *in ovo* with RPR-V [E-2-butenoic acid 3-(diethoxyphosphinothionyl) ethylether], an organophosphate at 0.25, 0.5, and 1mg/egg. In another study, **Anwar (2003)** observed severe teratological abnormalities in chick embryos at 100, 200, and 400 ppm of cypermethrin treatment. Xenobiotic induced malformations were also reported by **Ahmad and Asmatullah (2007)**, in foetuses of pregnant mice treated with chlorpyrifos at 18, 36, and 72 mg/kg b.wt., which included head and skeletal abnormalities

such as, microcephaly, hydrocephaly, agnathia, anophthalmia, micromelia, hind limb twist, sacral hygroma, drooping twist, and kinky tail.

Organophosphates and pyrethroids are known to influence neurotransmission (**Rose *et al.*, 1999**). The vertebral defects are long been attributed to decrease in acetylcholinesterase and the associated disruption of cholinergic system (**Greenberg and La Ham, 1970; Walker, 1971; Landauer, 1975; Meiniel, 1978**). This inhibition during the phase of embryonic development becomes more lethal, because acetylcholine is one of the transmitters that provide neurotrophic input, regulating the proliferation, differentiation, and migration of its target cells (**Hohmann *et al.*, 1988, 1991; Bachman *et al.*, 1994; Hohmann, 2003**). Thus, at an early stage of cell development, a given neurotransmitter signal may activate the genes required for replication of the target cell, whereas, at a later stage, the same transmitter and receptor signal may initiate the transition from replication to differentiation (**Slotkin, 2005**). Hence, any hindrance to the functioning of AChE during early embryonic development would mean debilitation much severe than just neurotoxicity. The aforementioned developmental regulatory genes for the somite formation, compartmentalization and differentiation, and the encumbrance to the AChE synthesis and functioning could be a probable explanation to the presently observed malformations in the combination insecticide-treated embryos.

In this study, it is likely that the teratogenic propensity of the combination insecticide involves more than one kind of biochemical/molecular/cellular lesions, which may include an altered or interrupted cell signalling, inappropriate apoptosis, and/or defective closure of neural tube other than being just a cholinesterase inhibitor. And hence, a variety of anomalies were observed right from a decrease in hatchability and hampered growth to more serious conditions of skeletal malformations

Tracer, on the other hand, exhibited a toxicological profile relatively benign. Under the highest tested dose, i.e. 1.5mg/egg, only hydrocephaly and oedematic condition could be noticed. No axial or appendicular deformities were observed at the tested dose levels of Spinosad. No published data were obtained till date regarding avian *in ovo* studies dealing with Spinosad toxicity, although developmental toxicity studies were conducted on mammalian models. An 8-week study on male albino rats by **Mansour *et al.* (2008b)**, a commercial formulation of Spinosad dosed corresponding to Acceptable Daily Intake (0.30 and 0.02 mg a.i.kg^{-1}b.w.), No Observed Adverse Effect Level (29.0 and 9 mg a.i.kg^{-1}b.w.),

and 1/100 LD50 (13.75 and 37.38 mg a.i.kg^{-1}b.w.) lead to the inhibition of serum acetylcholinesterase. In this investigation, low levels of AChE inhibition or inhibition at a later stage might have occurred and possibly waived off the impediment to skeletal development. In another investigations by **Breslin and coworkers (2000)** on CD rats and New Zealand white rabbits with Spinosad, the maternal no-observed-effect levels (NOEL) were 50 and 10 mg/kg/day in rats and rabbits, respectively, and the embryonal/foetal NOELs were 200 mg/kg/day in rats and 50 mg/kg/day in rabbits. Moreover, they opined that the overall incidence of external, visceral, or skeletal malformations in rat foetuses was incidental and not treatment related.

SUMMARY

This study revealed that the combination insecticide induced a more pronounced teratological manifestations that include abnormal somite disposition, neural tube defects, morphological and skeletal malformations, decline in hatchability, hatchling body weight, and the liver weight, when compared with that of Tracer. In the light of the present investigation, it could be construed that the commercial combination formulation of chlorpyrifos and cypermethrin is a potential teratogenic and embryotoxic compound, whereas Tracer under the present experimental conditions seems to be relatively less toxic to the embryonic development. The concern regarding the array of teratogenic malformations induced by Ci gets compounded on observing the low doses (0.05 and 0.1μg/egg) which induced these effects. Further, it also puts forth the necessity to understand the mechanisms through which the Ci incites aberrances in the developmental mechanisms of the embryo. Therefore, an attempt was made to understand the mechanisms and further manifestations of these insecticides, in the succeeding chapters.

TABLE 1.1a Insecticide dose range studies and LD_{50} calculation

Treatment (50µl/egg)	Attribute		
	Dose	No. of dead embryos	Corrected % mortality
VC1	Corn oil	03	0[@]
Combination insecticide (µg/egg)	0.001	04	6
	0.005	05	12
	0.01	07	23
	0.05	08	29
	0.10	11	47
	0.15	13	59
	0.20	13	59
	0.25	14	65
	0.50	15	70
	0.80	16	76
	2.00	13	56
	2.50	14	62
	3.50	15	69

[@] Percentage mortality corrected to nearest whole number; n=20

TABLE 1.1b Insecticide dose range studies and LD_{50} calculation

Treatment (50µl/egg)	Attribute		
	Dose	No. of dead embryos	Corrected % mortality
VC2	0.4% methylcellulose	04	0[@]
Spinosad (mg/egg)	0.05	04	0
	0.10	05	6
	0.25	07	19
	0.50	09	31
	0.75	08	25
	1.50	12	50
	1.75	12	50
	2.00	13	56
	2.50	14	62
	3.50	15	69

[@] Percentage mortality corrected to nearest whole number; n=20

TABLE 1.2 Probit analysis and regression equation

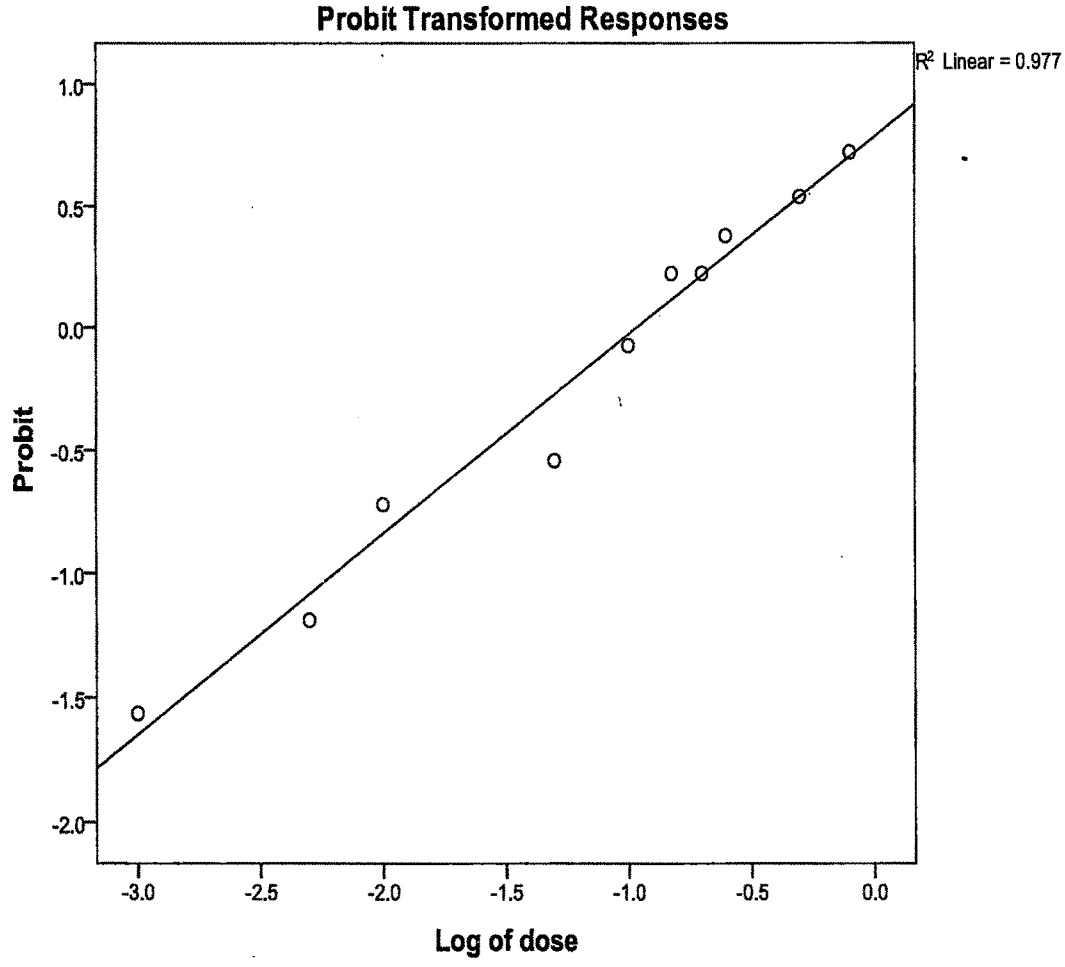
Treatment	Attribute			
	LD_{50}	Regression Equation	χ^2	p
Ci	0.109µg	Y = 0.76 + 0.26x	6.67	0.57[@]
Sp	1.54mg	Y = 0.43 + 0.37x	9.52	0.30[@]

[@] Since the significance level is greater than 0.150, no heterogeneity factor is used in calculation of the confidence limits.

Ci: Combination insecticide (µg/egg)

Sp: Spinosad (mg/egg)

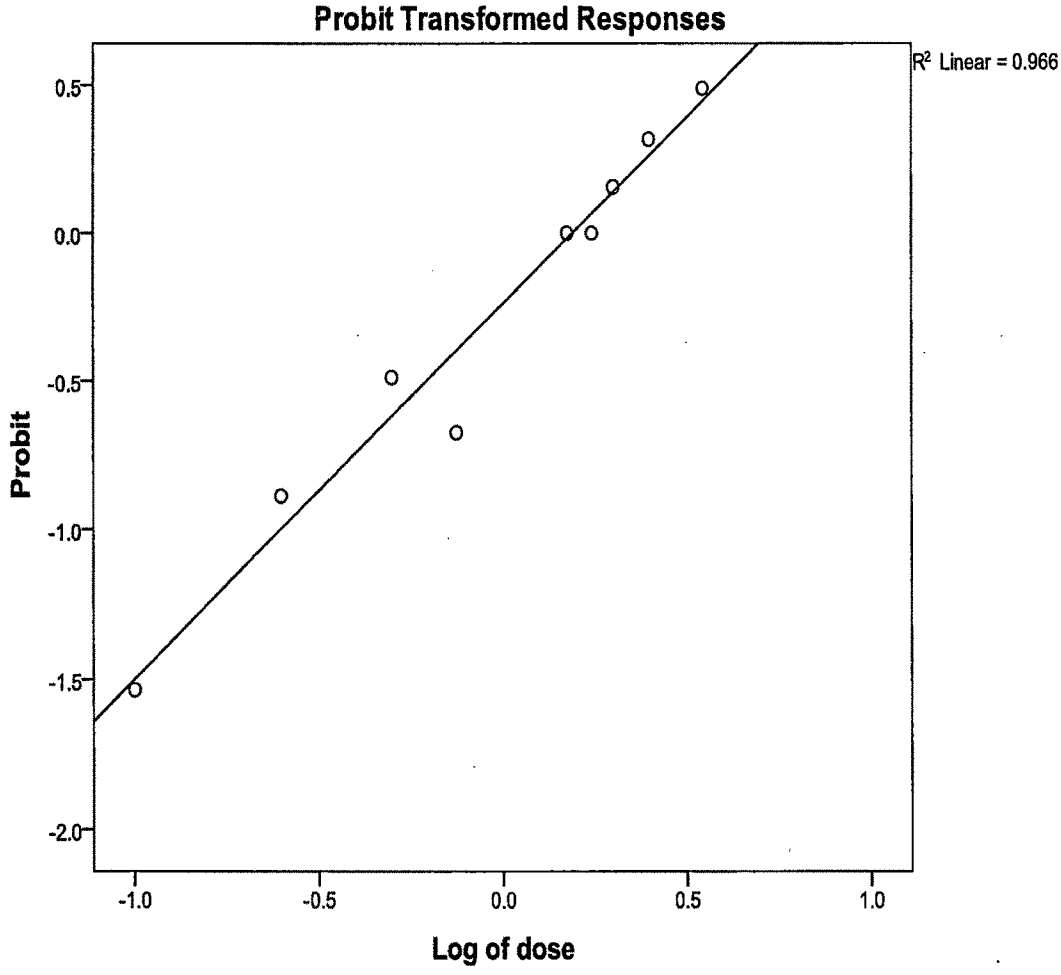
FIGURE 1.1 Probit transformed responses in chicks dosed with the combination insecticide



x- axis : Dose plotted as its log value

y-axis: Percent mortality converted into probits

FIGURE 1.2 Probit transformed responses in chicks dosed with the Spinosad.



x- axis : Dose plotted as its log value

y-axis: Percent mortality converted into probits

TABLE 1.3 Frequency of developmental anomalies during the early period of incubation

Treatment	Stage-10			Stage-13		
	SN	AS (%)	NTCD (%)	SN	AS (%)	NTCD(%)
VC1	@10.2±0.20	-	-	20.2±0.20	-	-
0.01Ci	9.8±0.20	-	-	19.5±0.22	-	-
0.05Ci	9.2±0.24	20	20	18.1±0.65↓*	30	40
0.10Ci	8.6±0.47↓**	40	50	17.5±0.70↓**	40	40
VC2	10.0±0.14	-	-	20.0±0.14	-	-
0.15Sp	9.9±0.17	-	--	20.0±0.14	-	-
0.75Sp	9.7±0.30	10	-	19.3±0.26	20	-
1.50Sp	9.0±0.33↓*	10	-	19.0±0.44↓*	10	-

@Values are expressed as mean ± SEM; n= 10; *p ≤ 0.05; ** p ≤ 0.01

↓ : Significant decrease

VC1: Corn oil; VC2: 0.4% Methyl cellulose; Ci: Combination insecticide; Sp: Spinosad

SN: Somite number; Number of embryos with abnormal somite disposition (AS) and/or with neural tube closure defects (NTCD)

TABLE 1.4 Crown rump lengths in 8 day embryo of control and insecticide treated groups

Treatment groups	Attribute			
	Crown-rump length (mm)	Crown length(mm)	Rump length(mm)	Embryonic wet weight
VC1	@7.66 ± 0.16	2.30 ± 0.09	5.36 ± 0.08	1.04 ± 0.04
0.01Ci	7.62 ± 0.09	2.38 ± 0.07	5.23 ± 0.06	1.10 ± 0.08
0.05Ci	7.33 ± 0.08	2.26 ± 0.04	5.06 ± 0.03	0.95 ± 0.06
0.1Ci	6.88 ± 0.84↓*	2.33 ± 0.12	4.55 ± 0.28↓**	0.75 ± 0.08↓*
VC2	7.56 ± 0.06	2.35 ± 0.07	5.22 ± 0.04	1.06 ± 0.03
0.15Sp	7.40 ± 0.08	2.36 ± 0.04	5.08 ± 0.09	0.99 ± 0.07
0.75Sp	7.33 ±0.09	2.16 ± 0.06	5.16 ± 0.04	1.09 ± 0.06
1.50Sp	7.42 ± 0.14	2.26 ± 0.08	5.15 ± 0.06	1.01 ± 0.09

@Values are expressed as mean ± SEM; n= 10; * p value ≤0.05; ** p value ≤0.01

↓: Significant decrease

VC1: Corn oil vehicle control; VC2: Methyl cellulose Ci: Combination insecticide (µg/egg)

Sp: Spinosad (mg/egg)

TABLE 1.5 Embryo viability and hatchability during the incubation period in control and insecticide treated groups

Treatment	Attribute				
	5th day (stage 26)	8th day (stage 34)	12th day (stage 38)	18th day (stage 44)	% hatchability on 21st day (stage 46)
Sham injected	100@	95	95	85	85
VC1	100	95	95	95	85
0.01Ci	80	75	75	65	65
0.05Ci	80	70	70	70	60
0.10Ci	50	50	45	45	35
VC2	100	95	80	80	80
0.15Sp	100	90	85	85	75
0.75Sp	100	85	85	85	55
1.50Sp	85	85	80	80	60

@ Values expressed as percentage of live embryos, n = 30

VC1: Corn oil ; VC2: Methyl cellulose

Ci: Combination insecticide (μg/egg)

Sp: Spinosad (mg/egg)

TABLE 1.6 Egg and hatchling weights and relative weights of liver and brain of control and insecticide treated groups of chick hatchlings (day old)

Treatment	Attribute				
	IE.Wt (gm)	H.Wt (gm)	H.Wt / IE.Wt (%)	R.L.Wt	R.B.Wt
VC1	@61.87 ± 1.23	42.63 ± 0.84	68.96 ± 0.94	2.38 ± 0.06	2.12 ± 0.08
0.01Ci	63.07 ± 1.44	43.67 ± 1.11	69.27 ± 0.97	2.19 ± 0.07	2.10 ± 0.06
0.05Ci	59.15 ± 1.27	40.37 ± 1.33	68.12 ± 1.11	2.31 ± 0.09	2.22 ± 0.05
0.1Ci	61.30 ± 0.89	39.17 ± 0.99	64.01 ± 1.80↓*	1.96± 0.19↓*	2.34 ± 0.07
VC2	55.48 ±2.00↓*	37.03 ±1.90↓*	66.99 ± 1.83	2.52 ± 0.09	2.40 ± 0.10
0.15Sp	61.21 ± 1.22	41.11 ± 1.06	66.11 ± 0.45	2.68 ± 0.18	2.54 ± 0.10
0.750Sp	57.90 ± 1.29	38.15 ± 0.75	65.22 ± 1.76	2.52 ± 0.05	2.37 ± 0.05
1.50Sp	60.80 ± 2.51	39.29 ± 1.21	67.80 ± 2.18	2.44 ± 0.14	2.26 ± 0.10

@ Values are expressed as mean ± SEM; n= 12; *p value ≤0.05

VC1: Corn oil vehicle control; VC2: Methyl cellulose vehicle control

Ci: Combination insecticide (µg/egg); Sp: Spinosad (mg/egg)

IE.Wt: Initial egg weight; H.Wt: Hatchling body weight; H.Wt/IE.Wt (%): hatchling body weight relative to initial egg weight; R.L.Wt: relative liver weight; R.B.Wt.: relative brain weight.

↓* marked on the VC2 row refers to the significant difference compared to VC1 after unpaired t test.

TABLE 1.7 Frequency of occurrence of abnormalities in different groups as observed on 21st day of incubation

Treatment	Appendicular deformities (Ap)		Axial Deformities(Ax)			Others
	Ap1	Ap2	Ax1	Ax2	Ax3	
VC1	-	-	-	-	-	-
0.01Ci	+	-	+	-	-	-
0.05Ci	+	-	++	+	-	+[b]
0.1Ci	+++	+	+++	+	+	+[a,b]
VC2	-	-	-	-	-	-
0.15Sp	-	-	-	-	-	-
0.75Sp	-	-	-	-	-	+[c]
1.50Sp	-	-	-	-	-	++[b,c]

n=30

VC1: Corn oil; Ci: Combination insecticide (μg/egg); VC2: methyl cellulose; Sp: Spinosad (mg/egg) Ap1: Crooked legs, twisted phalanges and unsteady gait

Ap2: Missing phalanges

Ax1: Beak deformities

Ax2: Defects in vertebral curvature, wry neck

Ax3: deformities in formation of skull, sternum and ribcage

Others: anophthalmia[a], umbilical hernea[b], oedem[a]

\+ : low incidence (≤5%); ++ : moderate incidence (≤10%); +++: high incidence (≥20%)

FIGURE 1.12a Percent hatchability in the vehicle control and combination insecticide treated group

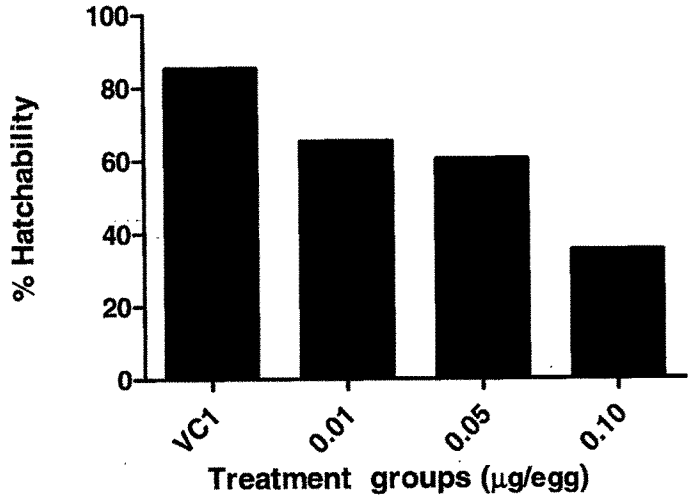
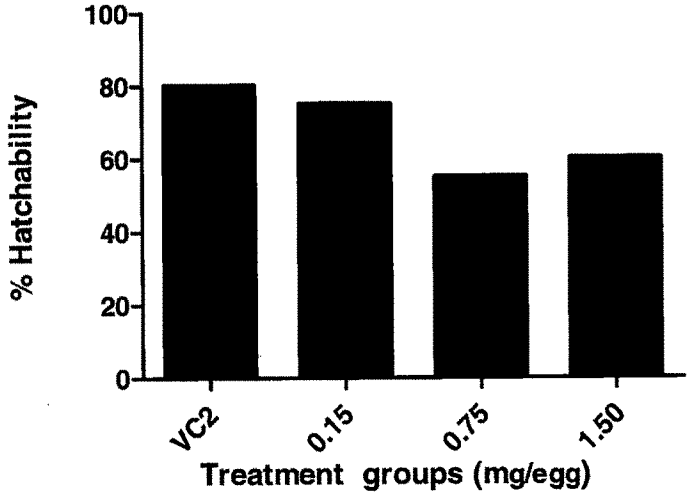


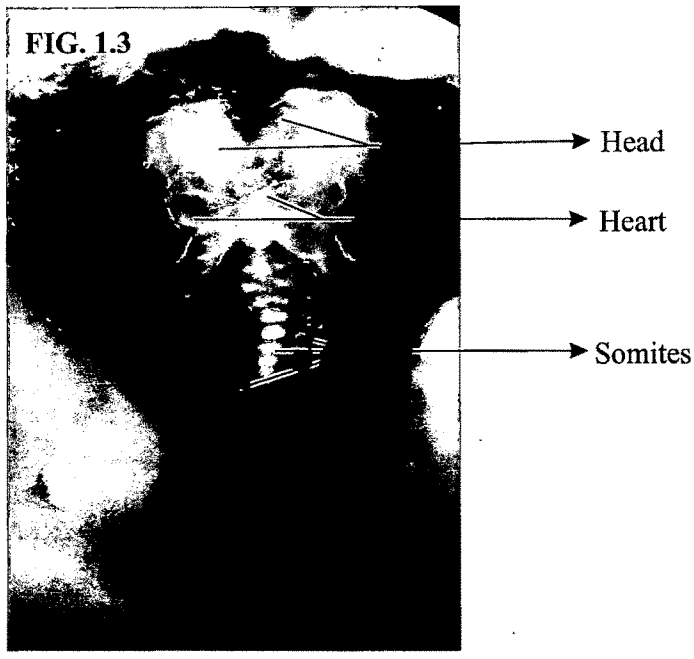
FIGURE 1.12b Percent hatchability in the vehicle control and Spinosad treated groups.



n= 20; Ci: combination insecticide; Sp: Spinosad

VC1: corn oil; VC2: methyl cellulose

Conjoined twin embryos in the Ci treatment group



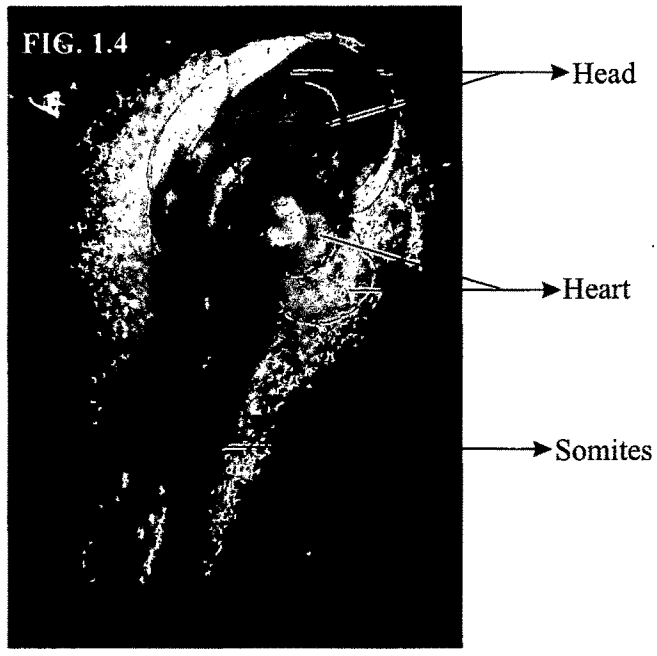


FIGURE 1.3 and 1.4 Conjoined twins from the Ci treated group of chick embryos. One of twin was always smaller in size compared to its counterpart. The split of the axis was more prominent in the anterior region. They were attached from posterior till the level of heart, had separate heads and attached heart primordia.

Control and insecticide treated chick embryos of stage-20

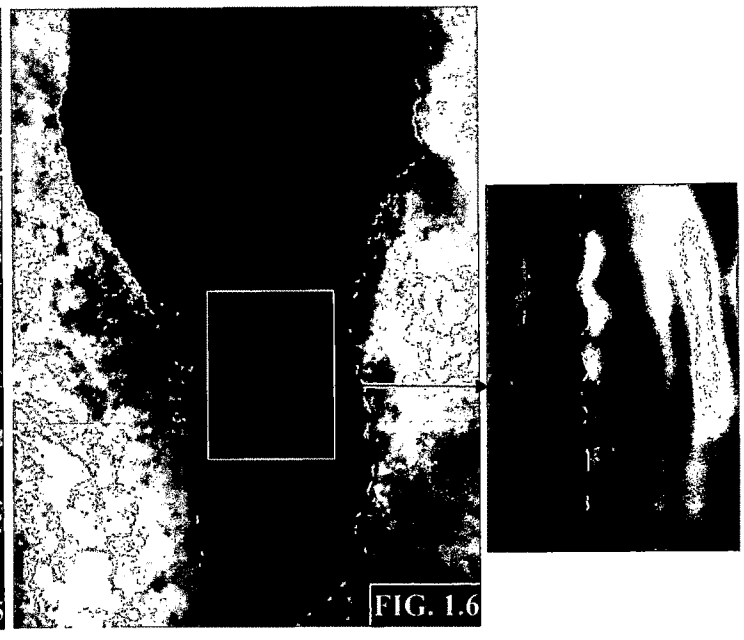


FIGURE 1.5 Control embryo (stage -20) showing normal development.

FIGURE 1.6 Ci treated (0.05µg/egg) chick embryo (stage -20) with inconspicuous row of somites on side of the neural tube.

Combination insecticide treated embryos with malformations

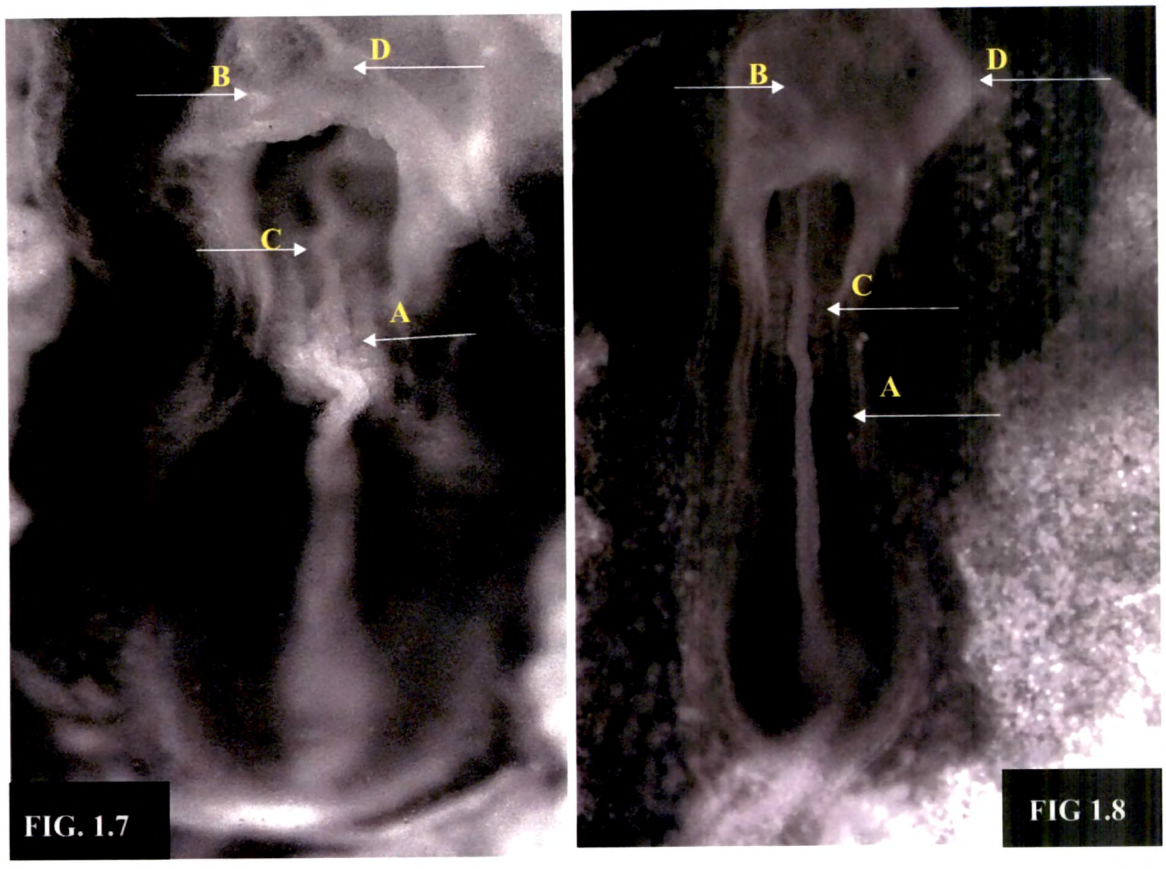


FIGURE 1.7 and 1.8 Ci treated embryos (0.1µg per egg) of stage-20 showing dispersed or distorted somites (A), anomalous location of the heart primordium (B), wavy neural tube, delay or failure of neural tube closure (C) and highly distorted cephalization (D).

Gross malformations in insecticide treated embryos

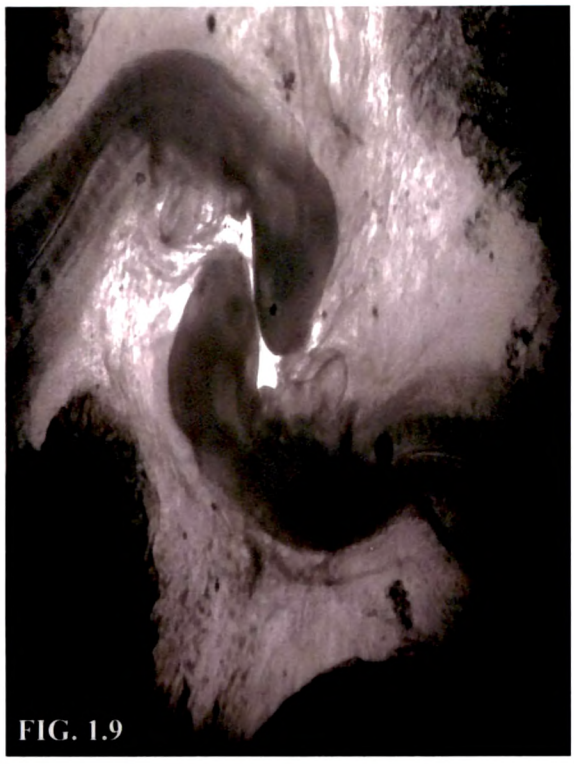


FIG. 1.9

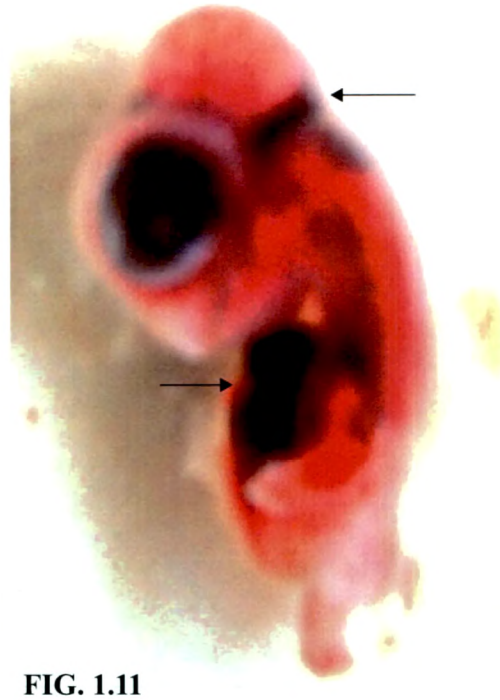


FIG. 1.11

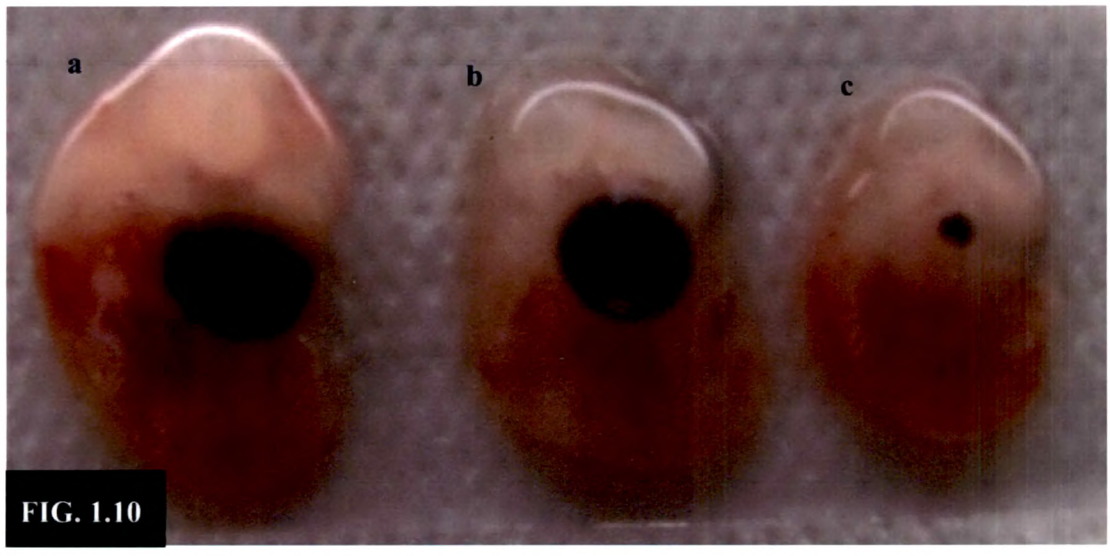


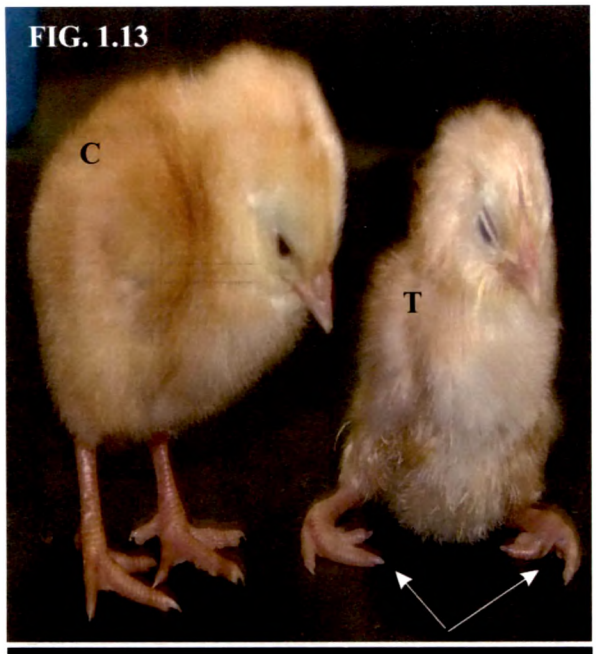
FIG. 1.10

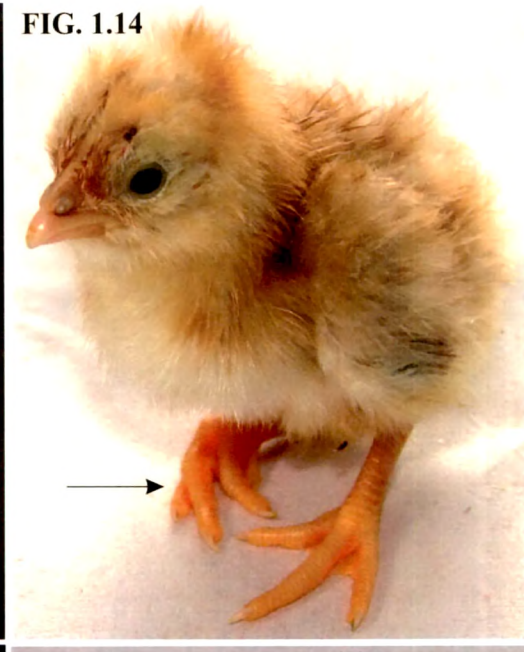
FIGURE 1.9 Twin embryos from the egg treated with 0.75mg of Spinosad

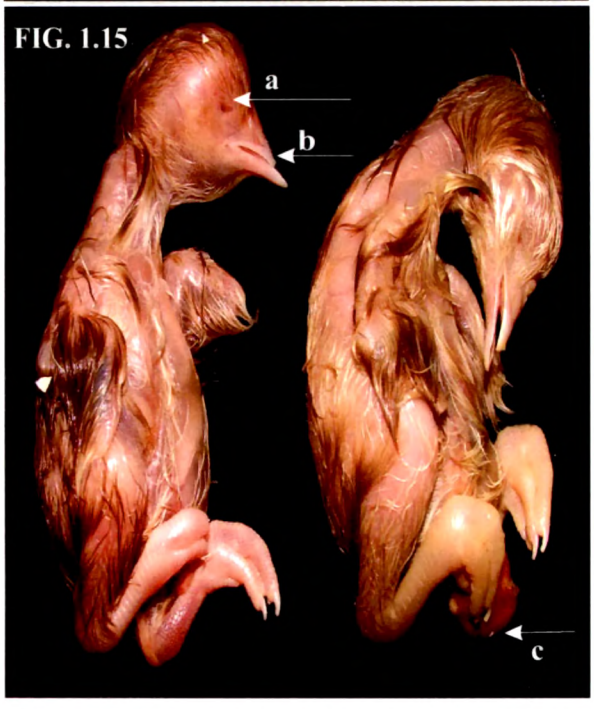
FIGURE 1.10 Day eight embryos control (a), 0.75mg Spinosad dosed (b), and 0.1µg Ci dosed (c) embryos examined for the crown rump length

FIGURE 1.11 Ci treated (0.1µg) embryo (day 8) with hemorrhages in the head and heart region

Morphological malformations in combination insecticide treated chick embryos







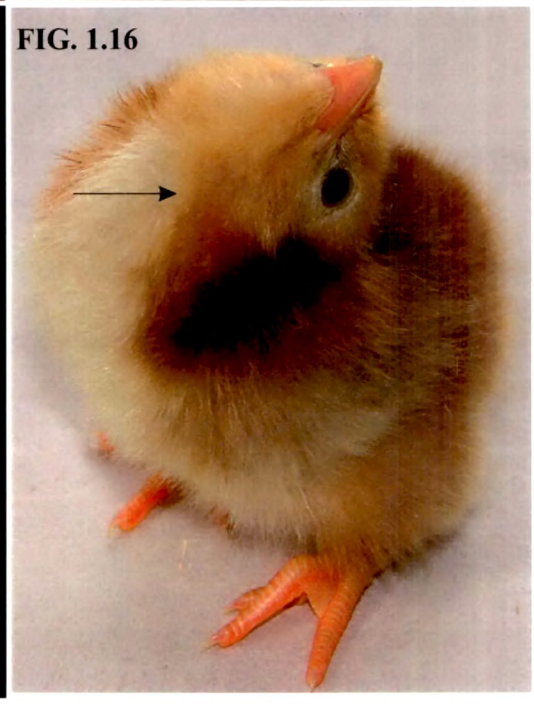


FIGURE 1.13 Control hatchling (C) and 0.05µg Ci treated chick (T) with crooked hind limbs and twisted phalanges

FIGURE 1.14 Twisted phalanges in a single hind limb of 0.05µg Ci treated chick

FIGURE 1.15 Anophthalmia (a), beak deformity (b) and umbilical hernea (c), in 0.1µg Ci treated embryos

FIGURE 1.16 wry neck in 0.1µg Ci treated embryo

Skeletal malformations in combination insecticide treated groups

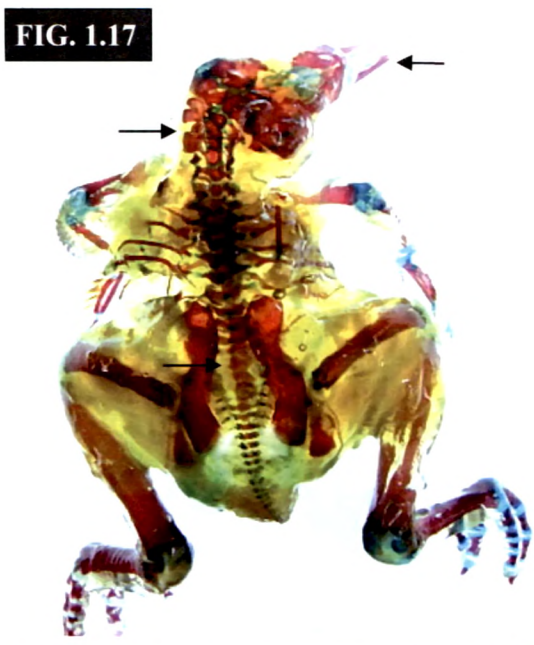


FIGURE 1.17 Craniorachischisis in embryo treated with 0.1µg/egg of Ci

FIGURE 1.18, 1.19 and 1.20 Malformations in sternum, ribcage and vertebral column in 0.1µg/egg of Ci treated chicks

Morphological anomaly in Spinosad treated embryo

FIGURE 1.21 Oedema in embryo treated with 1.5mg of Spinosad