Efficacy of Flubendiamide on the morphology and hematology of developing chick

INTRODUCTION

All abnormalities that have an effect on the growth of an organism are included in the category of developmental toxicity (Wilson, 1973). These abnormalities include malformations, functional deficits, developmental delays and mortality. A wide variety of chemical or physical elements may cause these problems. Prior to birth, these abnormalities manifest themselves in a variety of organ systems, which is indicative of early teratogenic consequences. The early stages of embryonic development are especially susceptible to disturbances brought on by elements from the outside world, which are referred to as teratogens. According to Brent and Beckman (1990), development is vulnerable to a wide variety of teratogens, including pharmaceutical medications, radiation, maternal illnesses and a variety of environmental pollutants. It was discovered by Rodier (1976) that brain development problems in mice may be generated by exposure to a chemotherapeutic agent at specific moments during late gestation. The neurobehavioral effects of the treatment varied depending on the timing of the exposure. This suggests that the impact of teratogens can differ depending on the dose and the timing of their administration.

Histological organization, physiological and biochemical differentiation and the completion of the formation of major organ structures are all ongoing processes that take place to variable degrees during prenatal and postnatal development in most animals. Histopathological abnormalities, growth retardation and functional alterations are all possible outcomes that might occur as a consequence of exposure to specific substances throughout this process.

It is important to consider the dose-response connection when attempting to ascertain whether exposure to a certain chemical is associated with a particular consequence or not. Malformations, embryo lethality and maternal toxicity were all parts of the dose-response relationship that Wilson (1973) defined as having an overlapping relationship. When it comes to drugs that cause malformations, administering increasing doses typically results in more severe abnormalities until death occurs. It is possible to observe a nonlinear dose-response

relationship when the conceptus dies while being exposed to greater dosages as a result of abnormalities. For this reason, toxicity testing and dose-response analysis are absolutely necessary in order to comprehend the causal connection that exists between chemical exposure and the subsequent health impacts.

Pesticides have garnered a large amount of attention as one of the many elements that contribute to developmental toxicity (Figure 3.1). Research has documented the embryotoxic and teratogenic effects of pesticides in various species, including fish (Kang et al., 2008), amphibians (Pawar and Katdare, 1984; Osano et al., 2002), birds (Anwar et al., 2004; Slotkin et al., 2008; Sharma et al., 2018), rodents and other mammals (Tocco et al., 1987; Muto et al., 1992; Roy et al., 1998). Because of the growing number of pesticides that are being introduced into the market and the fact that they have the ability to interfere with the processes of development, it is vital to examine and identify the toxicity of these pesticides.

Figure 3.1: Victim of endosulfan poisoning in Kerala
(https://organiser.org/wp-content/uploads/2022/08/endosulfan-affected-child.jpg)

The increased utilization of pesticides in agriculture has raised the likelihood of hazardous substances infiltrating the ecosystems of unintended species, thus impacting their overall health and welfare (Zaller & Zaller, 2020; Kalyabina et al., 2021). Diamides are a type of modern insecticide commonly utilized in present-day agriculture. Flubendiamide is a phthalic acid diamide that specifically affects lepidopterans by attaching to ryanodine receptors. This

interaction ultimately results in paralysis followed by the death of the affected organisms (Tohnishi et al., 2005; Aghris et al., 2022). Recent findings have raised concerns about the impact of flubendiamide on non-target species (Li et al., 2014; Sarkar et al., 2014, 2017, 2018). Consequently, there is a growing need to assess the safety of insecticides on non-target organisms due to reports of their potential harm.

Hematological parameters serve as sensitive indicators of systemic toxicity, organ dysfunction and immune system activation, providing valuable insights into the overall health of organisms (Witeska et al., 2023). Understanding the hematological profile is crucial for evaluating the health status and physiological responses of organisms exposed to environmental stressors, such as pesticides. By analyzing fluctuations in parameters such as red blood cell counts, hemoglobin levels and white blood cell counts, researchers can assess the influence of pesticides on critical physiological processes, including oxygen transport, immune function and tissue integrity (Witeska et al., 2023). Additionally, hematological analysis enables the early identification of adverse effects, facilitating timely interventions to mitigate potential health risks. Examining the hematological effects of pesticides on non-target organisms is essential for protecting ecosystem health and biodiversity. The research underscores the importance of hematological profiling in ecotoxicological studies, highlighting its role in evaluating the sublethal effects of environmental contaminants on wildlife populations (Pérez-Cadahía et al., 2020).

The chick embryo was chosen for this study due to its molecular composition, cellular structure and anatomical features, which are comparable to those of the human embryo. It is a valuable resource for the examination of developmental processes because of this similarity (Stern, 2018). The current study investigates the hematological consequences of in ovo flubendiamide administration to newly born chicks, with the objective of identifying potential systemic toxicity and organ-specific impairments. This research endeavors to identify the fundamental mechanisms of flubendiamide-induced hematological changes by evaluating parameters such as body and liver weights, albumin, globulin and total protein levels, as well as red and white blood cell counts. It is imperative to comprehend these effects to inform pesticide usage regulations, minimize environmental damage and ensure the health of the ecosystem and agricultural productivity.

MATERIALS AND METHODS

Fertilized Rhode Island Red (RIR) chicken eggs were sourced from the Intensive Poultry Development Unit in Vadodara, Gujarat, India. Before incubation, the eggs were disinfected with betadine and candled to verify the air sac. Following CCSEA guidelines, the IAEC (Approval No. MSU-Z/IAEC04/10-2020) approved the experimental protocols. The eggs were incubated in an automated incubator set at $37\pm0.5°C$ and 70-75% humidity (Scientific Equipments Works, New Delhi, India), positioned with broad ends up and rotated hourly. Nonviable eggs were removed every two days through candling.

Technical grade flubendiamide (CAS 272451-65-7) was obtained from Sigma-Aldrich. Based on a dose range study identifying a LOEC of 25μg/50μL (500 ppm), 30 eggs were divided into control and treatment groups, with three repetitions each. LC-MS/MS analysis confirmed the presence of flubendiamide in treated embryos. On the zeroth day of incubation, air sacs were located, eggshells were perforated, eggs were dosed using a sterile syringe and then sealed with paraffin wax. Treatment groups received 50 μl of 500 ppm flubendiamide in PBS, while control groups received only 50 μl PBS.

Following carbon dioxide euthanasia, chicks and livers were weighed and liver tissues were analyzed. Plasma glucose levels were measured using the Erba glucose kit (GOD-POD Method), serum albumin was calculated using the Erba albumin kit and serum globulin was calculated by subtracting albumin from total protein. Protein levels were determined using Bradford's assay. Blood from the chicks' brachial veins was collected with EDTA-rinsed syringes, refrigerated and processed within six hours. Hematological parameters were evaluated using a BC2300 analyzer. Hatchlings from both control and treatment groups were X-rayed for skeletal malformations (BPL Medical Technox-rayed Bangalore, India).

A digital balance (SF-400) was used to weigh 10-day-old embryos. A vernier caliper measured crown-rump length, head diameter (anterior-posterior), eye diameter, neck length and beak length. A scale and compass were used to measure the fore and hind limbs length of control and treated embryos. Morphological observations were recorded using Sony SLT-A58K cameras. Observed abnormalities included omphalocele, microphthalmia, short upper beak, edema, hematoma formation, anophthalmia and microcephaly (Table 3.6 describes the definitions of the morphological abnormalities). Data were presented as Mean ± SEM and analyzed using Student's t-test in GraphPad Prism v8.0, with significance defined as a 'p' value of less than or equal to 0.05.

RESULTS

Dose range study of flubendiamide

The death rate increased in a dose-dependent manner in the dose range study. The LD_{50} of flubendiamide was determined to be 1000 ppm using Probit and linear fit analysis (Figure 3.2). For further studies, the LOEC of flubendiamide was identified at 500 ppm, where 75% of the embryos survived (Table 3.1). Embryos exposed to this dose were isolated and targeted LC-MS/MS analysis confirmed the presence of flubendiamide in the tissues of treated embryos (Figure 3.3).
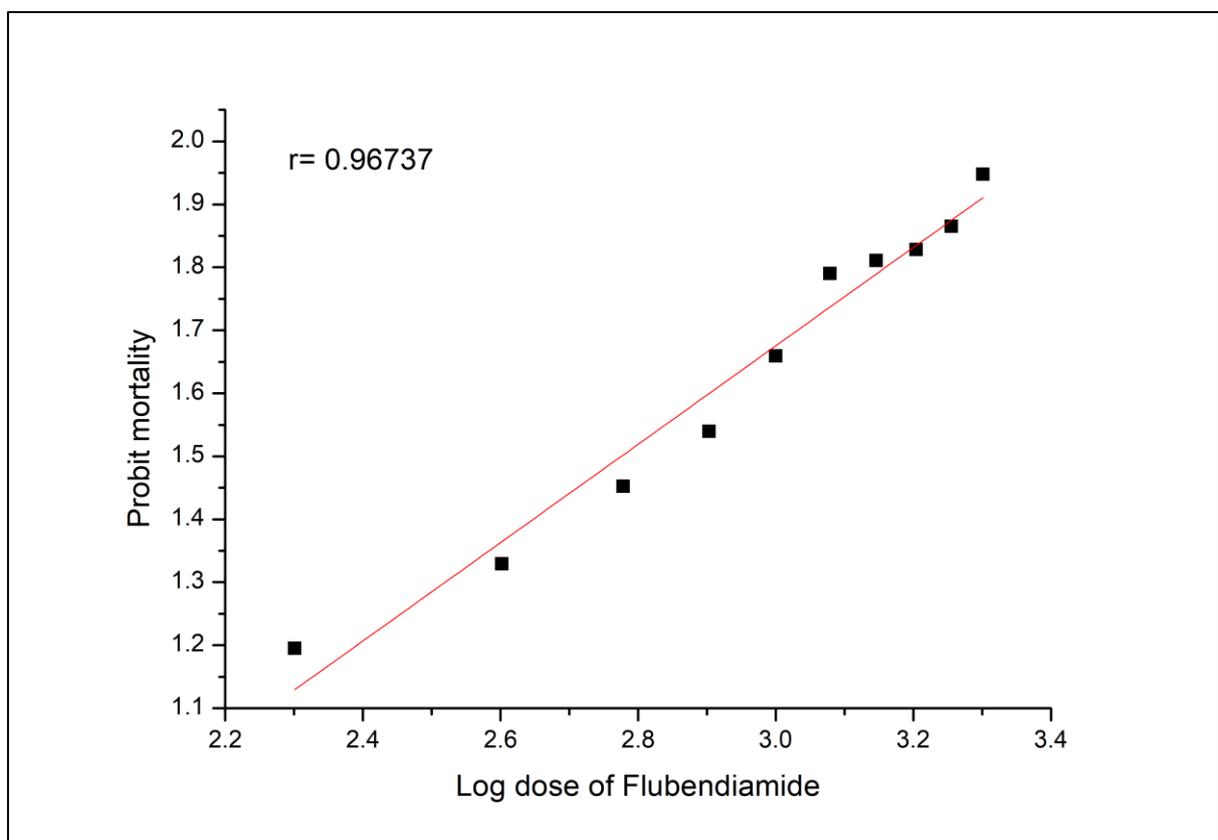

Figure 3.2: Median Effect Plot of Flubendiamide in chick embryos. Dose-range concentrations of 200 to 2000 ppm analysis of Flubendiamide in chick embryos. n=3 with 30 eggs per group per experiment.
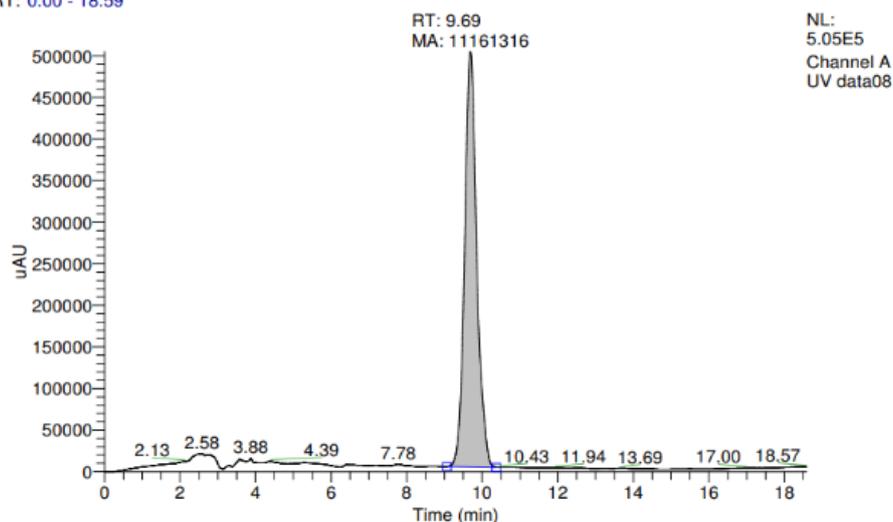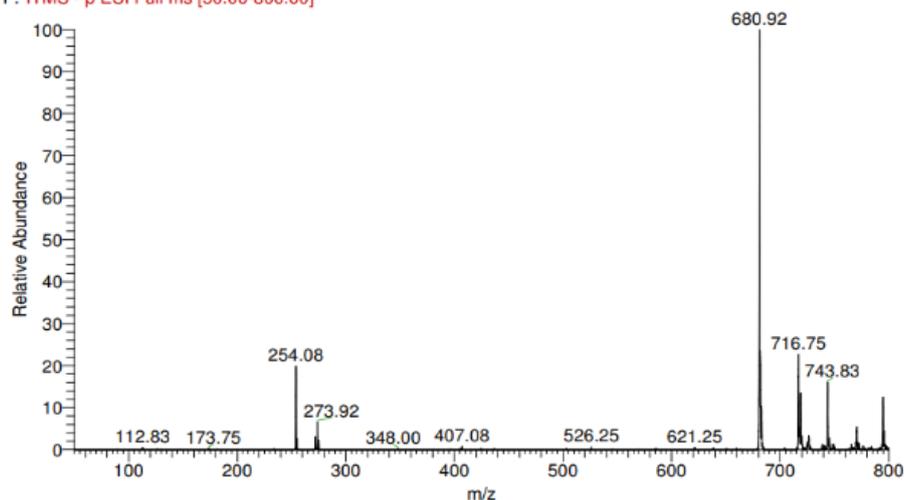
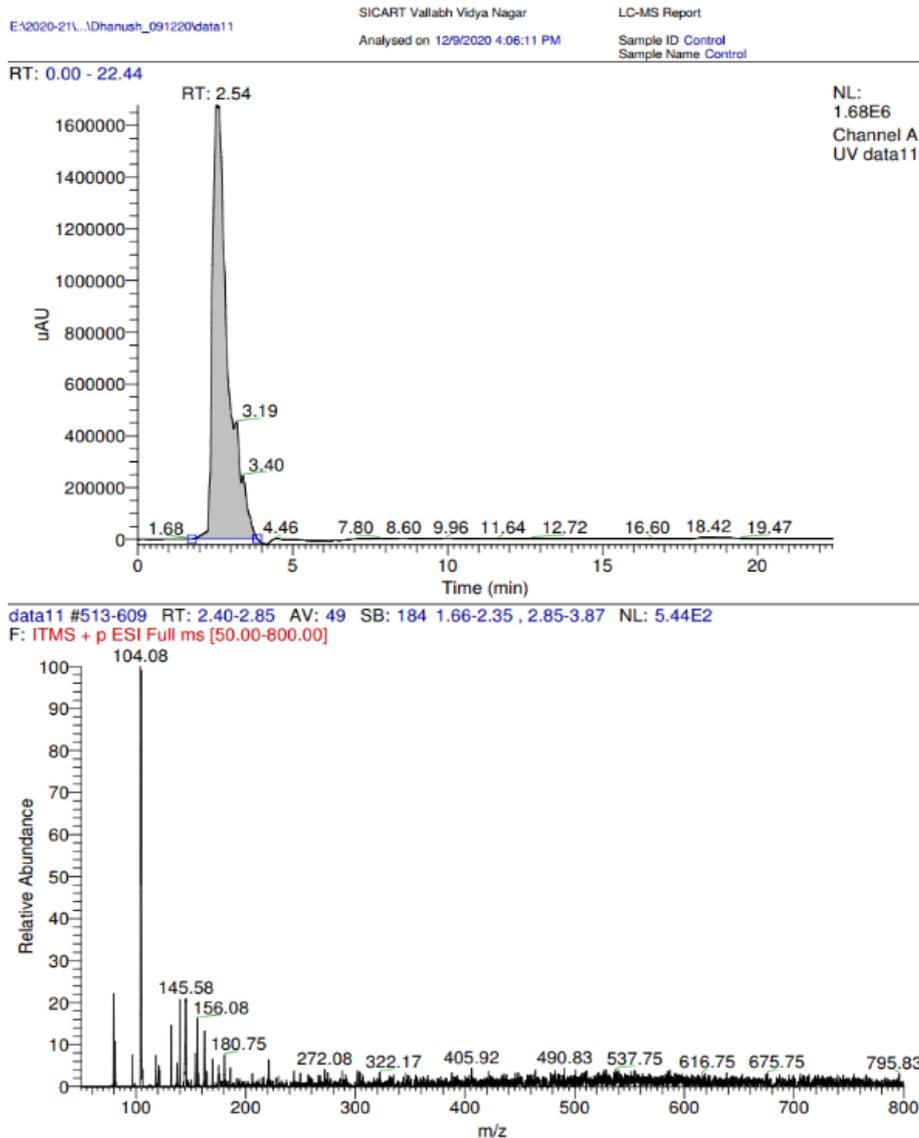**Figure 3.3A:** LC profile of a pure solution of Flubendiamide (standard group) along with the mass profiles of the compounds eluting at highlighted same time points in corresponding LC profile. Fig Top: Liquid chromatography profile of standard solution of Flubendiamide. The only peak present appeared at 9 minutes. The grey highlighted area was magnified to see the mass peak of chemicals eluting at that point in time. Unit of Y-axis: µAU: micro absorbance unit. Fig Bottom: Mass spectrum of compounds eluting within the highlighted area in figure top. A peak of 680.92 visible here is a mass peak appearing in positive mode for Flubendiamide, as its molecular weight is 682.39.
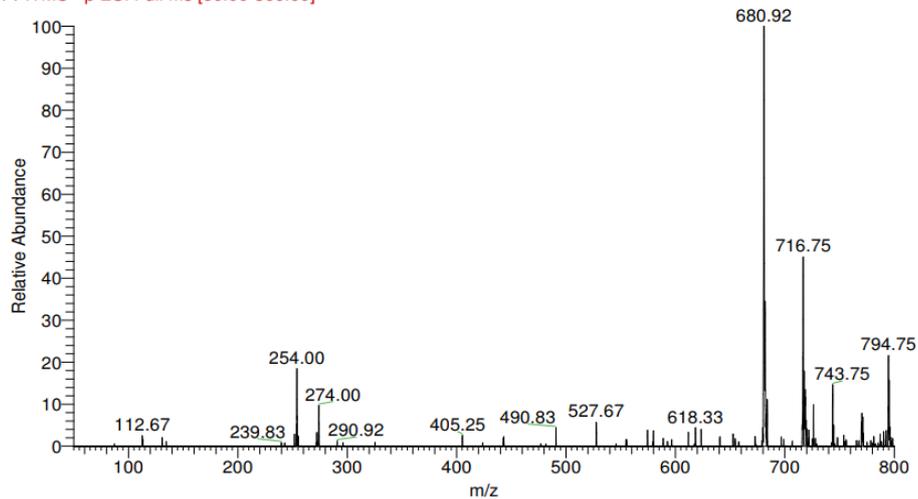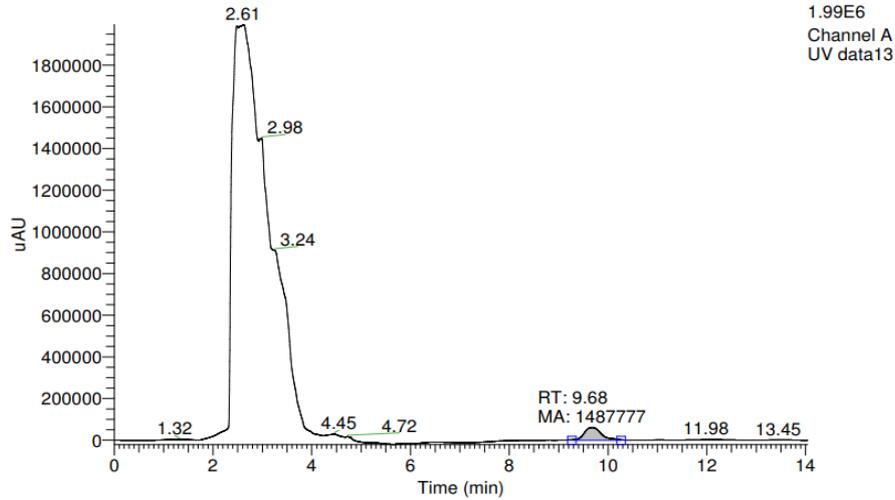
Figure 3.3B: LC profile of the treated group along with the mass profiles of the compounds eluting at highlighted same time points in corresponding LC profile. Fig Top: Liquid chromatography profile of the treated group of embryos. The day 2 embryonic sample showed the largest peak at 0.9 to 4 minutes. The flubendiamide peak appeared at 9 minutes. The grey highlighted area was magnified to see the mass peak of chemicals eluting at that point in time. Unit of Y-axis: µAU: micro absorbance unit. Fig Bottom: Mass spectrum of compounds eluting within the highlighted area in figure top. A peak of 680.92 visible here is a mass peak appearing in positive mode for Flubendiamide, as its molecular weight is 682.39.

Figure 3.3C: LC profile of the control group along with the mass profiles of the compounds eluting at highlighted same time points in corresponding LC profile. Fig Top: Liquid chromatography profile of Control group of embryos. The day 2 embryonic sample showed the largest peak at 0.9 to 4 minutes. The grey highlighted area was magnified to see the mass peak of chemicals eluting at that point in time. Unit of Y-axis: µAU: micro absorbance unit. Fig Bottom: Mass spectrum of compounds eluting within the highlighted area in figure top. A peak of 104.08 visible here is not a mass peak appearing in positive mode for flubendiamide in its standard solution. The relative abundance plotted on the Y axis showed that the peak present between 0.9 to 4 minutes had extremely low intensity compared to flubendiamide peak intensity in treated embryos. The peak did not correspond to flubendiamide conclusively. m / z on the X-axis stands for mass-to-charge ratio.

Estimations of body weight and liver weight

The embryos that were exposed to a concentration of 500 ppm of flubendiamide after hatching exhibited a lower body weight in comparison to the control group ($p \leq 0.05$). A statistically significant reduction in liver weight was also observed ($p \leq 0.05$). However, no perceivable difference was observed in the relative weight of the liver (Table 3.2).

## *Estimations of serum proteins*

The embryos treated with 500 ppm of flubendiamide after hatching showed significant variation compared to the control group after analyzing the results of serum albumin, globulin and total protein. There was a decrease in albumin ($p \leq 0.001$), globulin ($p \leq 0.01$) and total protein ($p \leq 0.001$) content (Figure 3.4; Table 3.3).
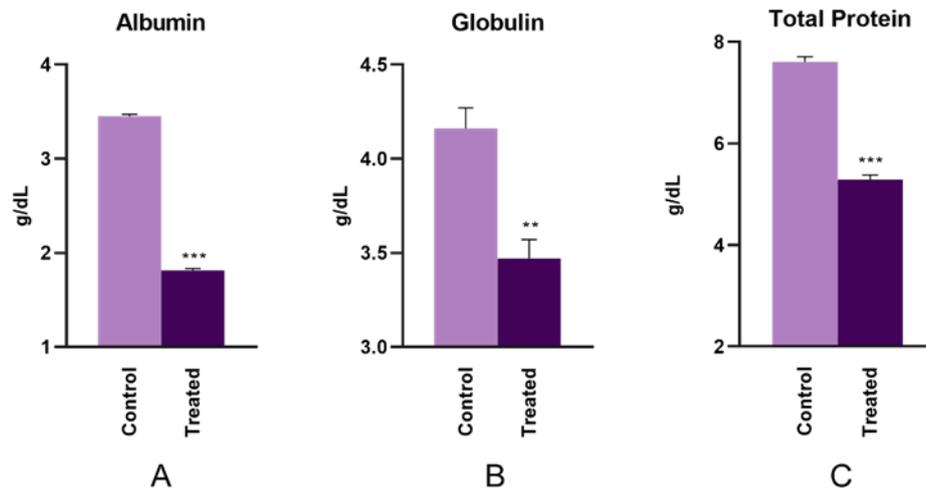
Figure 3.4: Blood serum protein estimation in the liver of newborn chicks treated with Flubendiamide. (A) Albumin (B) Globulin (C) Total Protein. All values are expressed as Mean ± SEM. n=3 with 30 eggs per group per day. **$p \leq 0.01$, ***$p \leq 0.001$.

Hematology profile of newborn chick

The blood analysis revealed a significant decrease in hemoglobin levels in the treated group compared to the control group ($p \leq 0.01$). Red and white blood cell counts decreased significantly ($p \leq 0.01$). In the differential leukocyte counts, there was an increase in eosinophils and polymorphs ($p \leq 0.05$), while basophils showed a non-significant increase. Conversely, lymphocyte and monocyte count decreased notably ($p \leq 0.05$). Furthermore, the platelet count exhibited a significant decrease ($p \leq 0.01$) (Figure 3.5; Table 3.4).
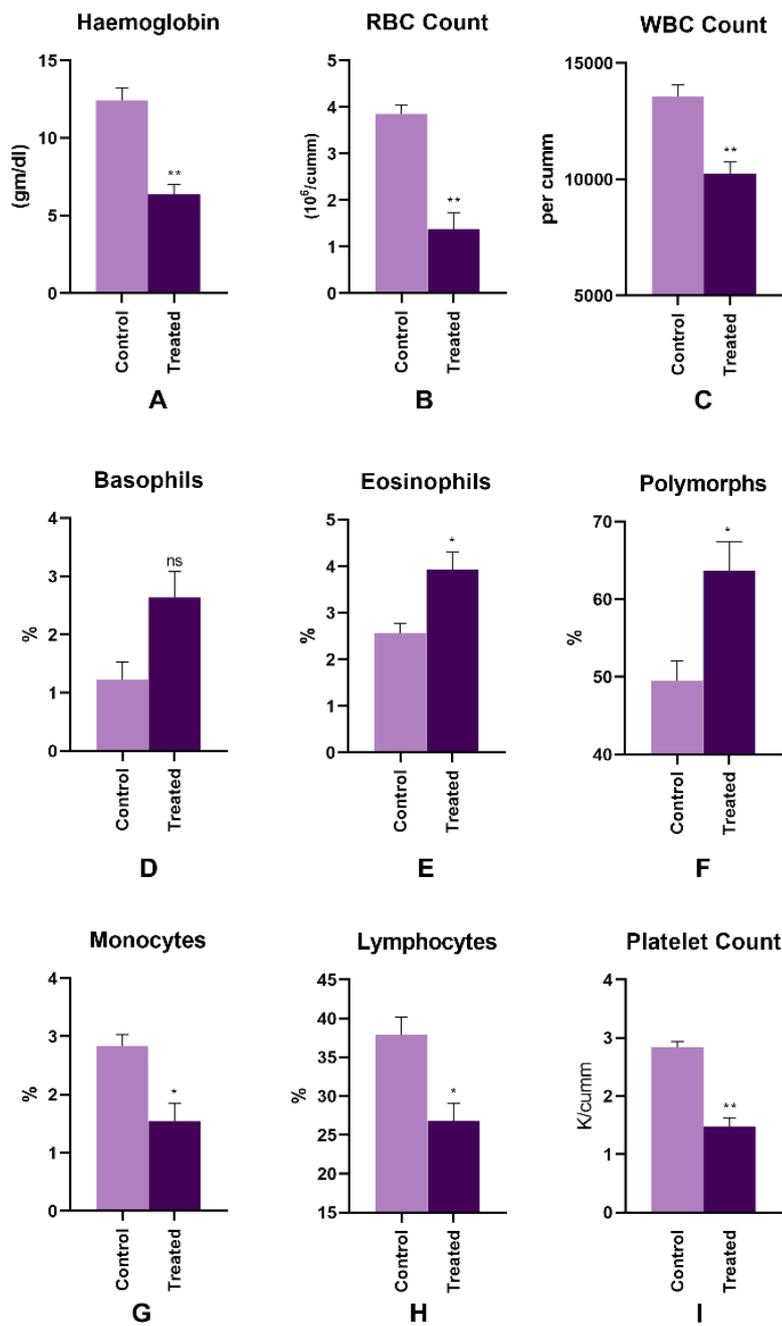
Figure 3.5: Blood cell counts of newborn chicks subjected to flubendiamide during embryonic development. The values are expressed as Mean ± SEM; n=3; ns = not significant; *p≤0.05; **p≤0.01.

X-Ray analysis

On day 21, the chicks were allowed to hatch and their morphology was observed. The treated chicks exhibited multiple defects, including an overall reduction in size, crippled limbs and an unsteady gait compared to the control group (Figure 3.6). The chicks were analyzed using an X-ray machine, revealing that the flubendiamide-treated chicks exhibited defective skeletal structures. Specific abnormalities included a kinked neck, crippled limb bones and edema in the abdominal region, indicating a ventral body wall defect (Figure 3.7).
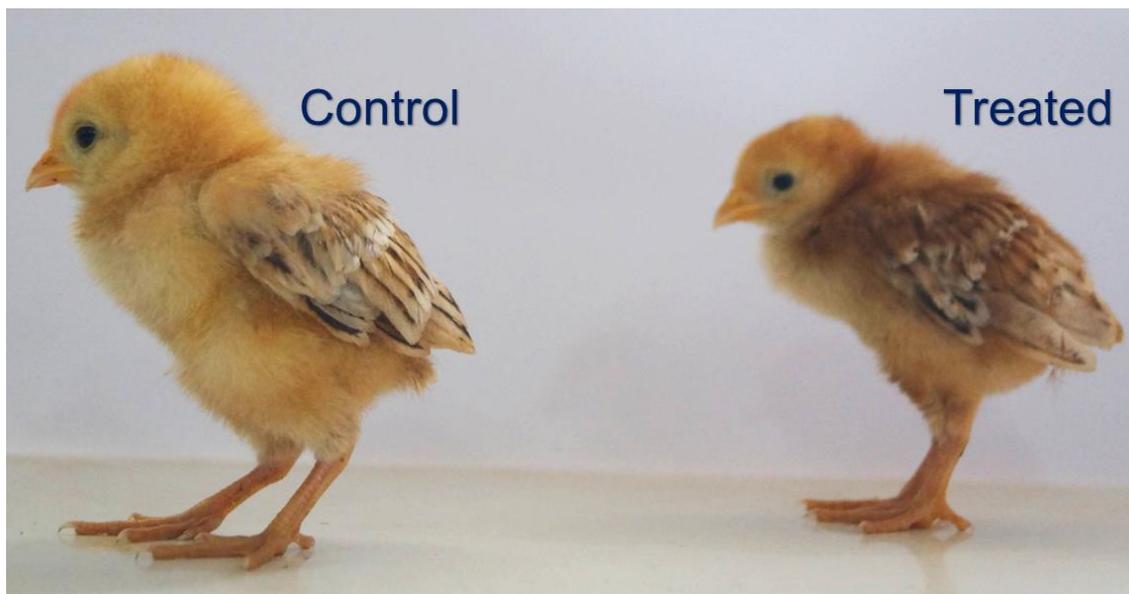
Figure 3.6: General morphology of 21-day hatched chicks in control and flubendiamide-treated groups. Compared to the control group, the treated chicks displayed multiple abnormalities, including a noticeable reduction in overall size and an unsteady gait.
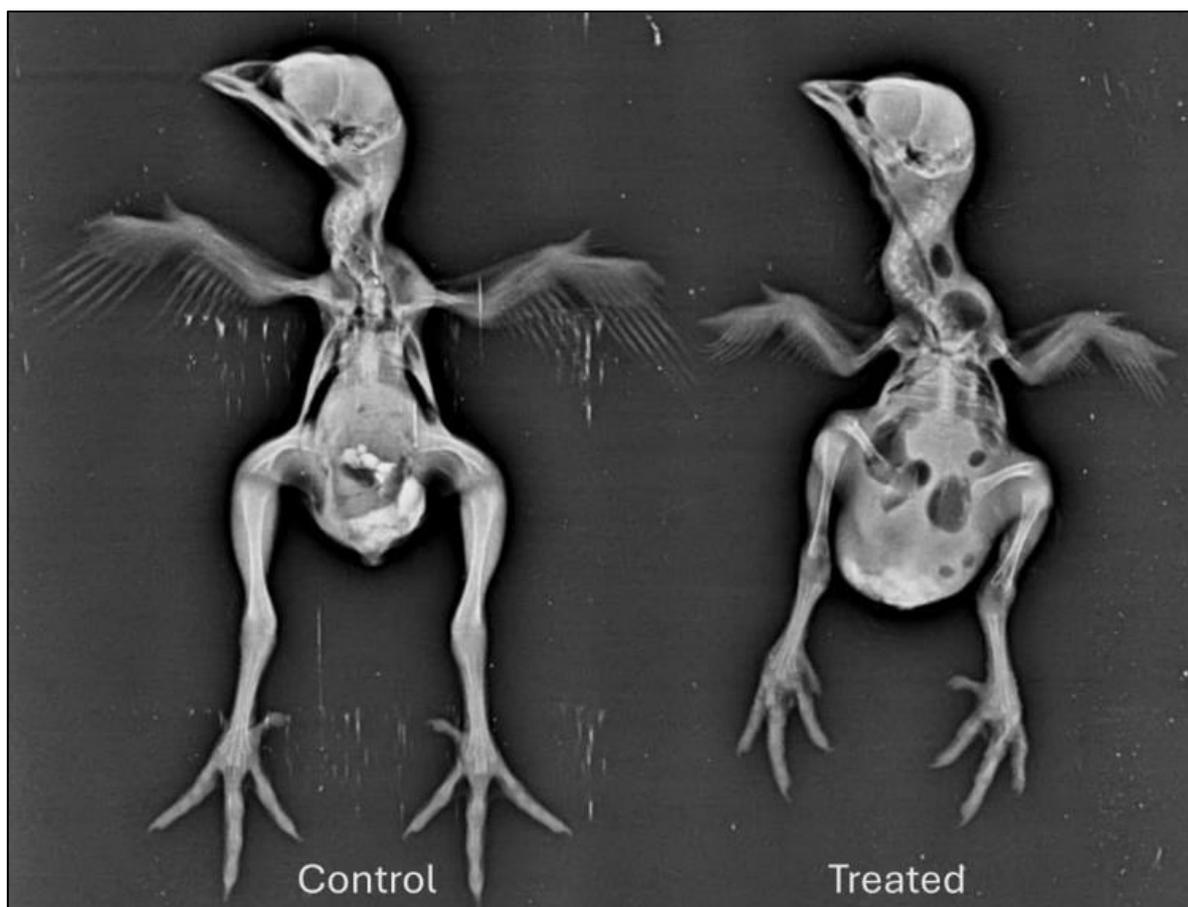
Figure 3.7: X-ray analysis of chicks in the control and flubendiamide-treated group. The flubendiamide-treated chicks exhibited defective skeletal structures, including a kinked neck, deformed limb bones and abdominal edema.

Morphometric analysis

A range of parameters, including wet body weight, head and eye diameter, neck length, humerus, radius and ulna, metacarpus, femur, fibula and metatarsus length, were measured for viable 10 days old chick embryos of control and treated groups. The measurements were recorded along with the mean and standard error of each parameter. The flubendiamide-treated chick embryos showed significant reductions in body weight, head diameter, crown-rump length, eyes diameter, neck length, beak length and the length of the forelimbs (humerus, radius and ulna, metacarpus) and rear limbs (femur, tibia and fibula, metatarsus) at all dose concentrations (Table 3.5).

Frequency of Malformation

By the tenth day of embryonic development, the treated groups showed certain abnormalities, while the control groups showed the regular form of normal chick embryos (Figure 3.8). There were no abnormalities in the embryos taken from the control group; they had all the normal features, such as the eyes, beak, legs and ears. In the treated group, certain very concerning abnormalities were discovered, such as hematoma formation, abnormal body pigmentation, microcephaly, microphthalmia, extra-mass growth behind neck, meromelia, micrognathia and meromelia (Table 3.7).
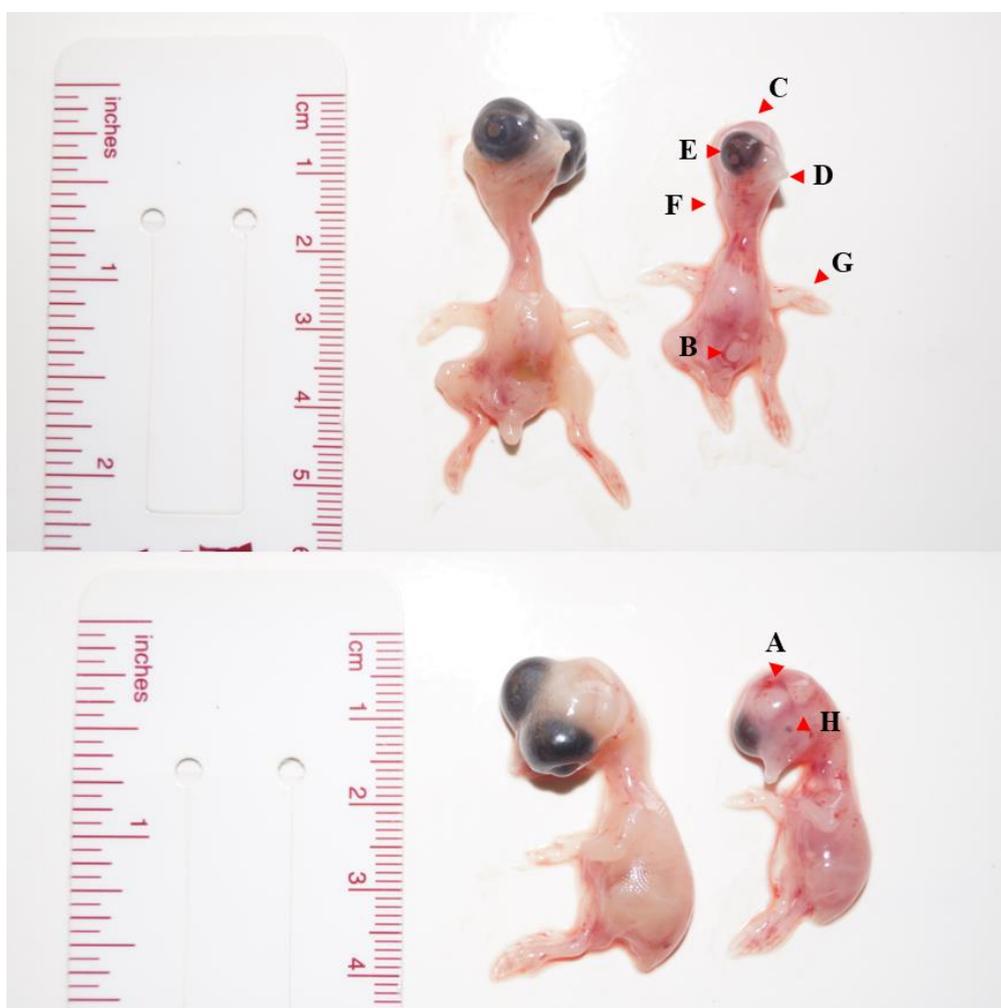
Figure 3.8: General morphology of embryos in control and flubendiamide-treated groups. The treated embryos exhibit several defects, including A) hematomas, B) omphalocele, C) microcephaly, D) micrognathia, E) microphthalmia, F) an extra mass growth behind the neck G) micromelia and H) anophthalmia. The regions of deformity are indicated with red arrows.

DISCUSSION

Pesticides unquestionably enhanced crop yield and diminished post-harvest losses. Nevertheless, the widespread use of pesticides significantly impacted the ecosystem and non-target organisms (Al-Saleh, 1994). One such pesticide, Flubendiamide is a novel pesticide that is recognized for its ability to target insects (Troczka et al., 2017). Recent findings have highlighted the interaction of flubendiamide with non-target organisms (Li et al., 2014; Sarkar et al., 2014, 2017, 2018). The current study examines the hematological effects of in ovo administration of flubendiamide on newly hatched chicks, disclosing several significant findings.

The dose range study revealed a dose-dependent increase in the mortality rate. Probit and linear fit analyses determined the median lethal dose of flubendiamide to be 1000 ppm. The lowest observed effect concentration was identified at 500 ppm, at which 75% of the embryos survived. Embryos exposed to this sublethal dose were subsequently isolated for further analysis. Targeted LC-MS/MS was employed to confirm the presence of flubendiamide in the tissues of the treated embryos, thereby verifying the internal exposure and potential bioaccumulation of the insecticide.

This investigation provides a comprehensive understanding of the hematological and morphological effects of in ovo flubendiamide injection on newly hatched chicks. The findings underscore significant concerns regarding the systemic toxicity and teratogenic potential of flubendiamide. Additionally, the data highlight the impact of flubendiamide on both physiological and developmental parameters in young birds.

Impact on the Hematological System

At a lower dosage (LOEC, 500 ppm), the treated embryos showed significant reductions in liver and body weights after hatching, which suggests that the substance in question is hazardous to the reproductive system. These findings are in line with those of earlier research that has demonstrated the detrimental effects of a variety of pesticides on liver function and the inhibition of overall growth (López et al., 2007).

In embryos that were treated with flubendiamide, the amounts of albumin, globulin and total protein were found to have significantly lower levels, as determined by hematological analysis. This points to the possibility of liver dysfunction, which could be caused by oxidative stress

and inflammation, either hindering the process of protein synthesis or increasing the rate at which proteins are degraded. The loss of albumin and globulins is indicative of reduced liver function and immunological competence, as indicated by other studies (López et al., 2007; Pathania, 2021). Albumin and globulins are essential for a variety of physiological processes, including the transport of substances and immune responses (Karami-Mohajeri & Abdollahi, 2011).

The observed anemia, which is characterized by lower hemoglobin levels and RBC count, is consistent with the available literature on insecticide-induced anemia (Barna-Lloyd et al., 1991; Goel et al., 2006). The evidence suggests that the hemolytic and myelosuppressive actions of flubendiamide are responsible for this anemia, which may result from decreased red blood cell production, death of red blood cells, or interference with the manufacture of hemoglobin. In addition, the findings of our study, which included leucopenia and decreased lymphocyte counts, are consistent with the findings of previous research on the immunotoxic effects of pesticides. These findings point out a decrease in immunocompetence and an increase in susceptibility to infections (Garg et al., 2004; Ojezele and Abatan, 2009; Gowri et al., 2010).

It has been demonstrated in the past (Araujo et al., 2008) that the oxidative stress brought about by flubendiamide is responsible for the drop in platelet counts found in our research process. On the other hand, an increase in basophils, eosinophils and polymorphonuclear leukocytes indicates an immunological response to flubendiamide exposure, which may indicate allergic reactions or inflammation. Gupta (2011) and Kim et al. (2017) are two examples of studies that have highlighted the immune activation and potential tissue harm that can occur as a result of pesticide exposure. These findings are in agreement with those studies.

Significant Effects on Morphology and Developmental Processes

In the X-ray examination of newly hatched chicks, a number of skeletal abnormalities were observed. These abnormalities included kinked necks, crippled limb bones and abdominal congestion. These structural anomalies suggest that exposure to flubendiamide causes disruptions in embryonic patterning and organogenesis, ultimately resulting in abnormalities of major developmental significance. The findings of this study are in line with those of past research on the teratogenic effects of malathion and cypermethrin, that observed similar defects (Asmatullah et al., 1993; Anwar et al., 2004).

A further revelation of the teratogenic effects of flubendiamide was provided by morphometric analysis, which showed significant reductions in body weight, head diameter, crown-rump length and limb lengths. These findings are consistent with those of other studies that were conducted on other pesticides, such as malathion and cypermethrin, which also exhibited similar developmental abnormalities (Pourmirza, 2000; Uggini et al., 2012). In addition, the qualitative abnormalities that were identified in embryos that were treated with flubendiamide, such as microcephaly, hydrocephaly and limb malformations, are consistent with the teratogenic effects that have been recorded for other pesticide chemicals (Pinakin et al., 2011).

The range of abnormalities identified in this study, from hematomas to microphthalmia, underscores the detrimental impact of flubendiamide on embryonic development. Previous research on the teratogenic effects of dithiocarbamates and diamides has highlighted the potential hazards of pesticide exposure during critical developmental periods (Van Steenis and Van Loghten, 1971; Kraggerud et al., 2010). Our findings align with these earlier studies, reinforcing the correlation between pesticide exposure and developmental risks.

The findings of this study indicate the considerable hematological and morphological impacts that flubendiamide exposure has on avian eggs. These findings underscore the necessity for rigorous controls and the administration of flubendiamide with caution. As a result of the hematotoxicity, immunotoxicity and developmental abnormalities that have been discovered, it appears that flubendiamide poses significant hazards to species that are not its intended targets, which could potentially result in long-term ecological and health effects. In addition, it is essential to conduct exhaustive risk assessments and implement regulatory measures in order to guarantee the sustainable utilization of pesticides, which will protect the health of both humans and wildlife.

TABLES

Table 3.1: Dose-range analysis of Flubendiamide in day 4 chick embryos.

Dose (µg/50µL)	ppm	% Survival (three replicates)			Average
Untreated	0	98	96	95	96.33
0 (vehicle control)	0	93	93	92	92.67
10	200	83	86	84	84.33
20	400	79	78	79	78.67
30	600	72	70	73	71.67
40	800	67	64	65	65.33
50	1000	54	55	54	54.33
60	1200	37	39	39	38.33
70	1400	35	36	35	35.33
80	1600	32	33	33	32.67
90	1800	27	28	25	26.67
100	2000	13	9	12	11.33

LD_{50} of the flubendiamide was found to be 50µg/50µL. A single dose of the insecticide which is less than the LD_{50} was chosen for further study i.e., $LD_{50}/2$ = 25µg/50µL (i.e., 500 ppm) of flubendiamide. n= 3 technical replicates of 30 biological samples each.

Table 3.2: Body weight and liver weight of the newborn chick.

Parameter	Control	Treated
Body weight (gm)	32.00 ± 0.90	$25.6 \pm 0.08^{*}$
Weight of liver (gm)	1.021 ± 0.08	$0.848 \pm 0.08^{*}$
Relative liver weight (%)	3.19 ± 0.24	3.31 ± 0.32^{ns}

Values are expressed as mean ± SEM of six experiments. ns - nonsignificant, * p≤0.05.

Table 3.3: Blood serum protein estimation on flubendiamide-treated newborn chick.

Attributes	Control	Treated
Albumin (g/dl)	3.45 ± 0.02	1.81 ± 0.01***
Globulin (g/dl)	4.16 ± 0.11	3.47 ± 0.10**
Total Protein (g/dl)	7.61 ± 0.10	5.29 ± 0.09***

The values are expressed as Mean ± SEM; n=3; **p≤0.01; ***p≤0.001

Table 3.4: Blood cells count of newborn chicks subjected to flubendiamide during their embryonic development.

Parameters	Control	Treated
Hemoglobin (gm/dl)	12.43 ± 0.81	6.38 ± 0.64**
RBC Count (10^6/cumm)	3.85 ± 0.24	1.38 ± 0.35**
WBC Count (/cumm)	13560 ± 2.27	10240 ± 0.18**
Basophils (%)	1.23 ± 0.31	2.64 ± 0.44^{ns}
Eosinophils (%)	2.57 ± 0.17	3.93 ± 0.38*
Polymorphs (%)	49.48 ± 0.62	63.74 ± 0.73*
Monocytes (%)	2.83 ± 0.19	1.54 ± 0.31*
Lymphocytes (%)	37.89 ± 0.12	26.83 ± 0.12*
Platelet Count (K/cumm)	2.84 ± 0.14	1.48 ± 0.14**

The values are expressed as Mean ± SEM; n=3; ns - nonsignificant, *p≤0.05; **p≤0.01.

Table 3.5: Morphometric parameters of control and Flubendiamide treated 10 days old chick embryos

Eggs Groups Parameters	Control	Flubendiamide-treated (μg/50μl)				
		5	10	15	20	25
Wet body weight (g)	6.67 ± 0.03	5.49 ± 0.04***	4.52 ± 0.05***	3.32 ± 0.05***	2.57 ± 0.03***	2.02 ± 0.06***
Crown RL (cm)	6.12 ± 0.03	5.05 ± 0.04***	4.18 ± 0.04***	2.93 ± 0.05***	2.25 ± 0.03***	1.78 ± 0.06***
Head Diameter (cm)	2.82 ± 0.06	2.49 ± 0.06*	2.27 ± 0.06**	2.29 ± 0.07**	2.07 ± 0.06***	2.04 ± 0.07**
Eye diameter (cm)	2.48 ± 0.05	1.96 ± 0.05**	1.72 ± 0.05***	1.76 ± 0.06***	1.52 ± 0.05***	1.47 ± 0.06***
Beak length (cm)	2.88 ± 0.07	2.31 ± 0.07**	1.93 ± 0.07***	1.83 ± 0.08***	1.61 ± 0.07***	1.56 ± 0.08***
Neck length (cm)	2.62 ± 0.07	2.23 ± 0.07*	2.11 ± 0.07**	2.05 ± 0.07**	1.76 ± 0.07***	1.66 ± 0.07***
Humerus length (cm)	2.39 ± 0.06	2.06 ± 0.06*	1.84 ± 0.06**	1.81 ± 0.06**	1.45 ± 0.05***	1.27 ± 0.07***
Radius and ulna length (cm)	2.37 ± 0.06	2.07 ± 0.06*	1.75 ± 0.06**	1.67 ± 0.07**	1.34 ± 0.05***	1.22 ± 0.07***
Metacarpus length (cm)	2.38 ± 0.06	2.05 ± 0.06*	1.71 ± 0.06**	1.57 ± 0.07***	1.33 ± 0.06***	1.29 ± 0.07***
Femur length (cm)	3.01 ± 0.07	2.55 ± 0.07*	2.33 ± 0.07**	2.29 ± 0.08**	1.88 ± 0.06***	1.66 ± 0.08***
Fibula length (cm)	2.49 ± 0.05	2.09 ± 0.05**	1.86 ± 0.05***	1.78 ± 0.06***	1.41 ± 0.04***	1.23 ± 0.06***
Metatarsus length (cm)	2.67 ± 0.07	2.32 ± 0.07*	2.14 ± 0.07**	1.96 ± 0.08**	1.62 ± 0.07***	1.48 ± 0.08***

Note: Values expressed as mean; n=3 with 30 eggs, *p≤0.05; **p≤0.01; ***p≤0.001

Table 3.6: Definition of the morphological defects.

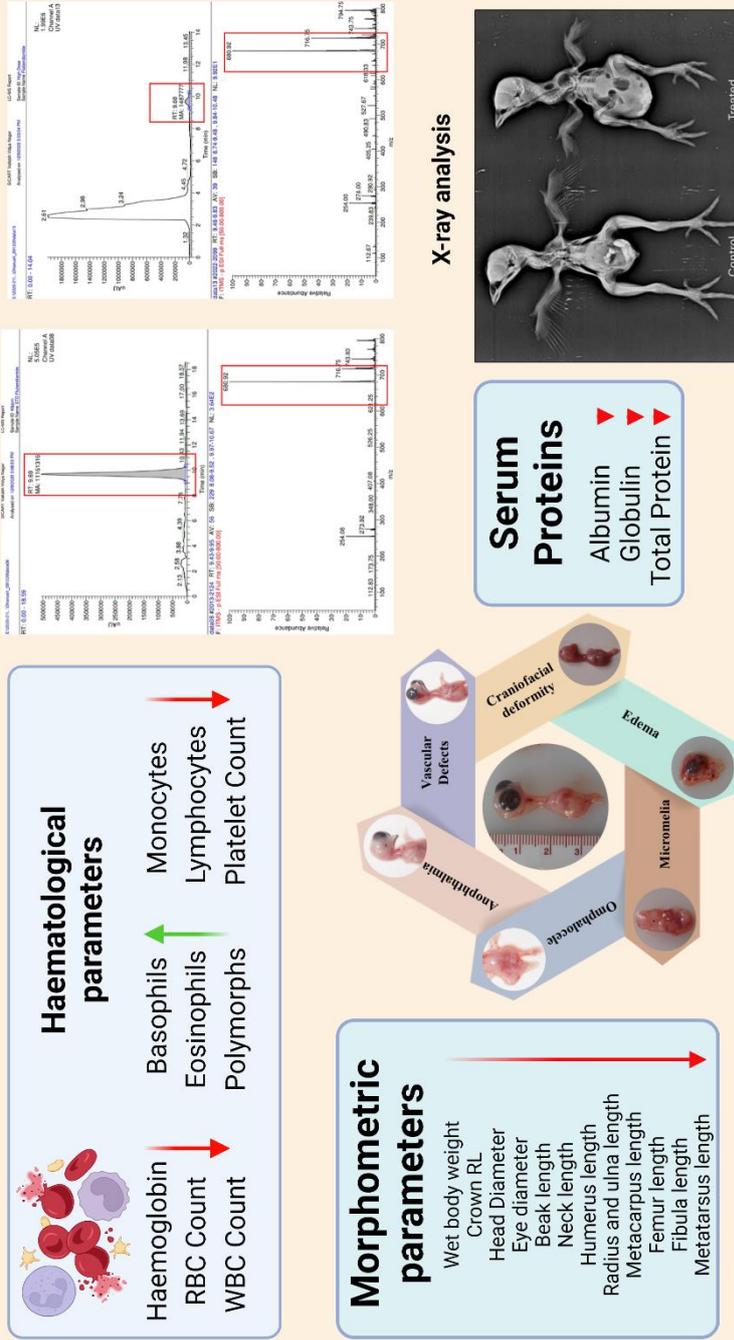| Disorder	Definition
Agnathia	A congenital condition where part or all of the lower jaw is absent.
Amelia	A congenital condition where one or more limbs are completely absent.
Anophthalmia	A condition characterized by lack of one or both of eyes.
Ectopia Cordis	A congenital condition where the heart is located partially or completely outside the chest cavity.
Edema	Swelling caused by excess fluid trapped in body tissues.
Hematoma	The localized collection of blood outside blood vessels, usually due to trauma or injury.
Hydrocephaly	A condition where there is an abnormal accumulation of cerebrospinal fluid in the brain's ventricles, leading to increased pressure and enlargement of the head.
Meromelia	A congenital condition where part of a limb is missing.
Micrognathia	A condition where the lower jaw is abnormally small.
Microcephaly	A condition where the head is smaller than normal, typically due to abnormal brain development in utero or during infancy.
Micromelia	A condition where one or more limbs are abnormally small.
Microphthalmia	A condition characterized by abnormally small eyeballs.
Omphalocele	A birth defect in which abdominal organs protrude into the base of the umbilical cord due to a defect in the abdominal wall.
Short Upper Beak	A congenital anomaly where the upper part of the beak or bill is shorter than normal, often seen in birds or animals.

Table 3.7: Frequency percentage of different anomalies observed in Flubendiamide treated 10-day-old chick embryos.

Anomalies	Treated Embryos (µg/50µl)				
	5	**10**	**15**	**20**	**25**
Abnormal body coloration	0	24.12	27	54.72	88.34
Agnathia	0	0	0	46.37	67.23
Amelia	0	0	0	66.67	86.35
Ectopia cardis	0	0	0	13	23
Edema	0	6.47	12.75	27.43	35.33
Hematoma	50	64.65	75	89.31	95
Hydrocephaly	0	10	28	34	42
Meromelia	0	18.32	45	70	82
Microphthalmia	43	76	82	90	92
Micrognathia	0	26.33	48	54	62
Microcephaly	0	33.33	25	33.33	45.33
Micromelia	0	10.74	30	53.47	65.12
Omphalocele	7	23	65	70	89
Short beak	0	47.83	65	78	82
Swelling around eyes	0	3.87	9.80	13.73	25.89

Note: All values are expressed in percentage (%).

Hematological, systemic and morphological toxicity of in ovo flubendiamide exposure in chick embryos (*Gallus domesticus*)

X-ray analysis

Haematological parameters

Haemoglobin
RBC Count
WBC Count

Basophils
Eosinophils
Polymorphs

Monocytes
Lymphocytes
Platelet Count

Morphometric parameters

Wet body weight
Crown RL
Head Diameter
Eye diameter
Beak length
Neck length
Humerus length
Radius and ulna length
Metacarpus length
Femur length
Fibula length
Metatarsus length

Craniofacial deformity
Edema
Vascular Defects
Anophthalmia
Omphalocele
Micromelia

Serum Proteins

Albumin
Globulin
Total Protein

Summary

The study highlights the teratogenic and systemic toxicity of flubendiamide in chick embryos, revealing significant dose-dependent morphological abnormalities, increased mortality rates, and hematological disruptions, underscoring the need for cautious pesticide use to protect non-target organisms from adverse health impacts.