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RESEARCH ARTICLE

# Exposure to chlorantraniliprole leads to genotoxicity and behavioural changes in *Fejervarya limnocharis* tadpoles: an *in vivo* approach

**Sweety Nath Barbhuiya[1*], Dipsanu Paul[2], Neha Farzana[2], Abujam Romibala Devi[2], Utsab Singha[2], and Dharmeswar Barhoi[2]**

[1]Department of Zoology, Patharkandi College, Patharkandi, Sribhumi – 788724, Karimganj, Assam, India.

[2]Department of Zoology, The Assam Royal Global University, Betkuchi, Guwahati - 781035, Assam, India.

*Corresponding author email: sweetynath91@gmail.com
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## Abstract

The prevalent use of pesticides poses noteworthy threats to biodiversity, like in amphibians, which are critical indicators of ecological health. This study investigates the genotoxic and behavioural effects of chlorantraniliprole (CAP), an anthranilic diamide insecticide, on the tadpoles of *Fejervarya limnocharis*. *F. limnocharis* that were collected from natural territories were acclimatized under laboratory conditions and exposed to different concentrations of CAP based on 96 h $LC_{50}$ values. $LC_{50}$ exposes the dose-dependent toxicity in *F. limnocharis*. Behavioural changes were also monitored for 30 days, which highlights lethargy, reduced activity, and predator avoidance behaviours in treated groups compared to the control group. Potential genotoxic effects were also evaluated via micronucleus assays, which validated a substantial surge in erythrocyte abnormalities across different exposure periods. Similarly. statistical analyses showed a dose-dependent increase in genotoxic markers and behavioural alterations, suggesting hormonal imbalances or neurotoxic stress as potential mechanisms that further need to study. The findings underscore CAP's detrimental impact on tadpole survival, feeding, and physiological development, posing risks to amphibian populations in agrochemical-contaminated environments. Given the ecological significance of amphibians in pest control and food webs, the adverse effects of CAP could exacerbate population declines, affecting ecosystem stability. This study advocates for further research into the molecular pathways of CAP toxicity and calls for sustainable pest control alternatives to mitigate environmental and biodiversity impacts.

Keywords: Chlorantraniliprole; Coragen; Pesticides; *Fejervarya limnocharis*; Genotoxicity; Behavioural Study; Environmental Toxicity

## 1. Introduction

Asia uses more than half of the world's insecticides. After China and Turkey, India is the third in Asia and 12th globally in terms of pesticide consumption. The FAO reports that India has used about 58160 tons of pesticide in 2018 (Nayak and Solanki, 2021). There are currently 293 pesticides registered for usage in India as of March 1, 2021, and 61703 MT of technical-grade chemical pesticides were consumed annually in 2019–2020. Among the 293 pesticides, 130 are classified as extremely dangerous and are prohibited in nations other than India. Pesticides have long been used in the agriculture sector for managing crop pests (Stinson et al., 2022), but potential exposure to the ecotoxic impact of pesticides on the aquatic environment is increasing due to spray drift, surface runoff, or accidental leaching.

Diamides are a relatively recent class of chemical insecticides for managing myriad lepidopteron and are among the dozens of pesticides in use today (Yang et al., 2023). They are ryanodine receptor activators, inducing the uncontrolled release of calcium stores and the cessation of feeding, as well as the paralysis of muscle tissues, resulting in death (Cordova et al., 2006). An anthranilic diamide first developed by the DuPont Crop Protection company, chlorantraniliprole (CAP) is registered in many countries worldwide and represents 30% of global all pesticide sales (Song et al., 2019; Yin et al., 2023). Anthranilic diamides are among the most widely sold families of insecticides in many countries today (Hadiatullah et al., 2022; Zhang et al., 2022). CAP was the first anthranilic diamide to be registered in 2007 (Rezende-Teixeira et al., 2022; Fonseca and Brodeur, 2023) and is been in used regularly by the farmers for prevention of insect pests.

Amphibians are a paraphyletic group encompassing all tetrapods, they are ectothermic four-limbed vertebrate animals. The majority of amphibians have a biphasic life cycle, including both an aquatic and a terrestrial phase. Their life cycle typically starts as aquatic larvae with gills known as tadpoles. Over the past several decades, amphibian numbers have decreased globally. Among the primary possible causes of this reduction are pesticides and insecticides. The decline in amphibian populations in agricultural areas is likely caused directly by exposure to several chemicals and habitat loss (Collins and Storfer, 2003). Since then, amphibian diversity and redundancy in agricultural areas have declined relative to nearby non-agricultural zones (Davidson et al., 2001; Relyea and Jones, 2009).

According to Liu et al (2011), *Fejervarya limnocharis* is a widespread species that is distributed throughout Asia. Typically, it breeds in freshwater pools, such as rice pools and wayside pools. Even though they are mostly herbivores, most tadpole species also ingest animal tissue, and many of them display opportunistic cannibalism. Even though *F. limnocharis* can reproduce throughout the year. *Fejervarya sp.* is an early breeder, with mating occurring from March to August in response to the first monsoon rains (Lalfakawmi et al., 2019) because spawn must be deposited in standing water, the species breeds after rains. *Fejervarya limnocharis* breed successfully in the observed water temperature of 24 to 27°C, ambient temperature of 24-28°C, and pH (5.54 to 7.22). The reproductive phenology of numerous frog species is influenced by rainfall, especially in tropical woods where seasonal precipitation occurs abundantly. Rainfall and irrigation are connected with their breeding, which makes them easier to target for insecticides and herbicides used in irrigation techniques. The applications of pesticides typically coincide with the occurrence of tadpoles in shallow water in a rice field.

The amphibians living in agrochemical-polluted habitats exhibit high mortality, high abnormality, low hatching success, and small size at metamorphosis and this trait has led to physical issues in individual survival, such as weakened liver and kidney function

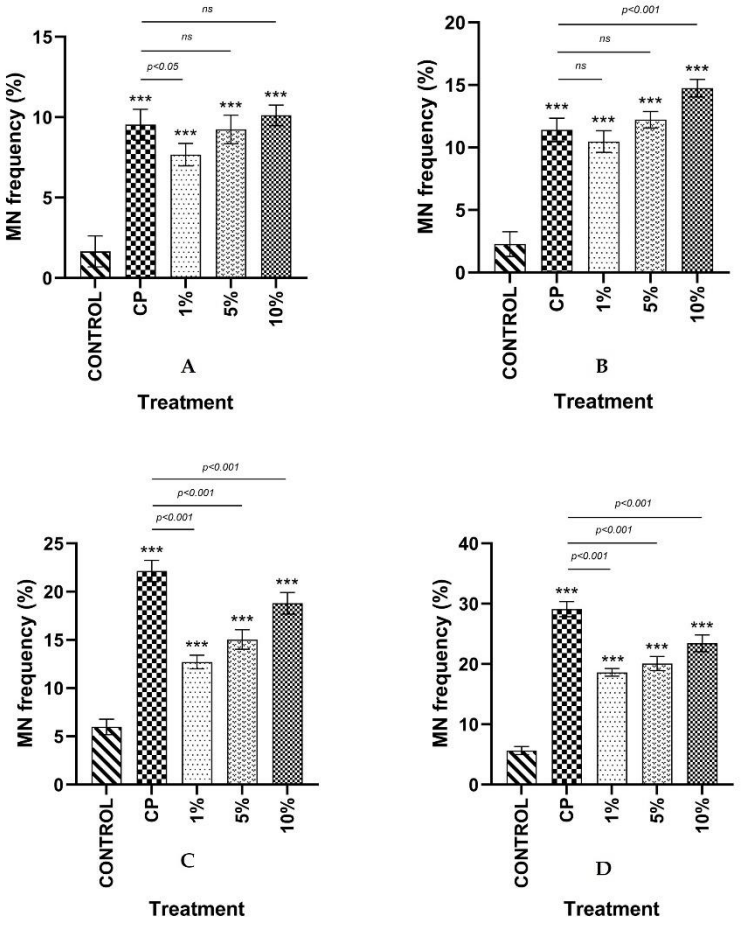


**Figure 1.** The histogram A, B, C, and D represent the prevalence of micronuclei (MN) frequency, at 24h, 48h, 72h, and 96h periods respectively, in the blood cells upon exposure to CP and different concentration of CAP. CP; *Cyclophosphamide (Positive control),* CAP; *Chlorantraniliprole*, CAP 1% (1% of 96 h $LC_{50}$; 0.02 mg/L); CAP 5% (5% of 96 h $LC_{50}$; 0.1 mg/L); CAP 10% (10% of 96 h $LC_{50}$; 0.2 mg/L). Each bar in the histograms represents the mean of ten (n=10) study animals and error bars reflect SD. Chlorantraniliprole (CAP) was used as a test chemical in the treatment group. Statistical analysis: One-way analysis of variance (ANOVA) was done by following Tukey's test for multiple comparisons. All the values are significantly different from the control group at p<0.001 (***) level.

and deformities and paralysis (Nielsen et al., 2003). Studies have suggested that tadpoles show a considerable number of behavioural changes and morphological alterations in the presence of risk or predatory cues (Barnett and Richardson, 2002). Amphibian larvae undergo massive morphological and ecological changes during their development hence, when exposed to toxicity and risky environment they show behavioural and morphological changes quickly (Simmons, 2019). The most frequently cited erythrocyte malformations are binucleated cells, nuclear buds, notched, lobed, reniform, nuclear bubbled, anucleated, picnotic, and apoptotic cells. Other erythrocyte malformations include nuclear bubbles and nuclear buds. The presence of chemical or physical agents, in addition to other disruptions of the ecosystem, can have a substantial impact on the life history of the species.

*F. limnocharis* is excluded from the endangered list of amphibians in the IUCN Red List category and considered "least concern" meaning that the *Fejervarya sp*. is widely distributed and stable at present (Thammachoti, 2012). Additionally, the species local abundance makes it a tadpole of the choice species when the toxicity of coragen is evaluated. Our study aimed to examine the behavioural impact of coragen on *F. limnocharis* in vitro, under laboratory conditions. In addition to this, the influences of this chemical on genetic material are also investigated using micronucleus assay in the blood cells of tadpoles. The primary method of determining genotoxicity is the evaluation of micronucleus frequency and its effects. It is also advised by numerous regulatory bodies that are found throughout the world and can be carried out as a part of the product safety assessment (Machin, 2001). The results will aid in comprehending the harmful effect of coragen (chlorantraniliprole 18.5% w/w) on tadpoles and the human population.

## 2. Materials and methods

### 2.1. Chemicals

Chlorantraniliprole (CAS No.- 500008-45-7) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd. Methanol (CAS No.- 67-56-1), Ethanol (CAS No.- 64-17-5), and Giemsa stains (CAS No.- 51811-82-6) were purchased from HiMedia Laboratories Pvt. Ltd. Chemicals used in this experiment are of analytical grade, and were prepared freshly.

### 2.2. Animals

Tadpoles of *Fejervarya limnocharis* were collected from the ponds near the Ahom Gaon, located in Guwahati, Assam. They were kept in tubs and acclimatized under laboratory conditions in a 12h light and 12 h dark cycle for 14 days. Tadpoles were screened to detect and segregate from Gosner stages 26 – 28 (Gosner, 1960). This time frame is equivalent to vigorous cell division and strong hematopoiesis in the blood which is appropriate for genotoxicity research (Singha et al., 2014).

### 2.3. Acute toxicity assessment

For evaluating the lethal concentration ($LC_{50}$), a total of 60 tadpoles (*F. limnocharis*) were divided into six experimental groups containing 10 tadpoles in each group (n=10). The experimental species were treated with an increasing concentration of Chlorantraniliprole (0.5mg/L, 1 mg/L, 2mg/L, 4mg/L, and 6mg/L) for a period of 96 h. The water was changed every 24 h intervals and fresh treatment of Chlorantraniliprole was applied to maintain the optimum level of Chlorantraniliprole. Species were monitored for every 24 h and the number of dead species was recorded if any death happened. The death and alive percentage for each group were calculated for 24 h, 48 h, 72 h, and 96 h exposure and lastly $LC_{50}$ value was calculated by using probit analysis.

### 2.4. Study groups

A total of 50 tadpoles (*F. limnocharis*) were divided into five study groups i.e. Group I (Control), Group II (Cyclophosphamide, CP, 2 mg/L), 1% of 96 h $LC_{50}$ (i.e. Group III, 0.02 mg/L), 5% of 96 h $LC_{50}$ (i.e. Group IV, 0.1 mg/L), 10% of 96 h $LC_{50}$ (i.e. Group V, 0.2 mg/L). Each experimental group consisted of 10 species (n=10). The doses of experimental groups were selected based on the 96 h $LC_{50}$ of Chlorantraniliprole in *F. limnocharis* as shown in Table 1.

### 2.5. Genotoxicity assessment

#### 2.5.1. Micronucleus assay

A micronucleus (MN) study was employed to assess the genotoxic potential of chlorantraniliprole using the guidelines of Singha et al (2014) with minor modifications. Ten tadpoles (n=10) from each group were anaesthetized with 30% ethyl alcohol, later the middle of the tail was amputated using a sterile needle for blood collection. Then blood smear was prepared in clean grease-free slides and were left to air-dry at room temperature. The slide was then dipped into an absolute methanol for 3 min. The very next day, the slides were stained for 20 min. with 10% Geimsa stain that was prepared freshly. 1000 erythrocytes per species were counted for the presence of micronucleus using a light microscope (Magnus MLXi-Plus LED, Magnus Opto Systems India Pvt. Ltd.). The MN frequency was calculated as:

$$MN\% = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells studied}} X\ 100$$

### 2.6. Behavioural study

For the assessment of the behavioural study, 40 tadpoles were randomly assigned into four different groups each containing 10 species, for 30 days to find valuable information regarding the prolonged effect of the pesticide on the tadpole species. The

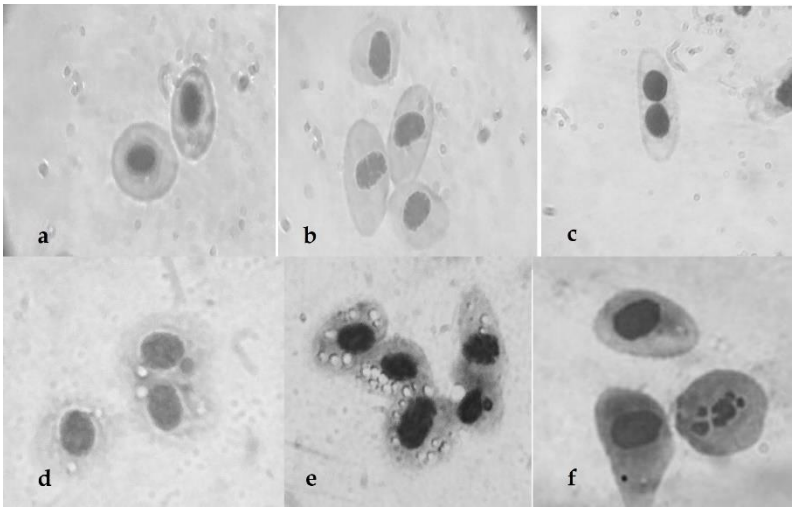


**Figure 2.** Photomicrographs demonstrate aberrant blood erythrocytes in *Fejervarya limnocharis* due to exposure to CAP; a & b represent normal blood erythrocytes; c represents bi-nucleated, d & e reflects stress vacuole with micronucleus, and f, showed multi-nucleated cells, respectively.

experimental group consists of the control group (without chemicals), and three concentrations of chlorantraniliprole i.e. 0.02mg/L, 0.1 mg/L, and 0.2 mg/L. Doses were based on previous 96 h $LC_{50}$ (1%, 5%, and 10%) data. Different patterns described by Brunelli et al (2009) were used to assess the altered behavioural patterns of tadpole species and noted down for further assessment.

*2.7. Statistical analysis*

Statistical analysis was done with the help of SPSS 21.0 (IBM Corp., Armonk, New York) and the experimental data were presented as the Mean±SD. Comparisons between the control and different treatment groups to test the significance were conducted using a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons, with a confidence level of 95%.

**Table 1.** Showing various lethal concentration ($LC_{50}$) of CAP to *Fejervarya limnocharis* at different exposure period. $LC_{50}$; Lethal concentration 50 describes the amount of chemical intake by test species that causes 50% death of that particular test species population throughout the experiment. CAP; Chlorantraniliprole.

| Exposure time | $LC_{50}$ (mg/L) |
|---|---|
| 24 h | 8.52±1.02 |
| 48 h | 4.42±0.20 |
| 72 h | 2.44±0.30 |
| 96 h | 1.77±0.03 |

# 3. Results

*3.1. Determination of lethal concentration of chlorantraniliprole in Fejervarya limnocharis*

To assess the $LC_{50}$ of chlorantraniliprole in *Fejervarya limnocharis*, the tadpole was exposed to increasing concentrations of chlorantraniliprole i. e. (0.5 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, and 6mg/L) for 24 h, 48 h, 72 h, and 96 h. The median lethal concentration ($LC_{50}$) was determined by using probit analysis, in MS Excel for Windows (MS Office 2021), calculated $LC_{50}$ values are shown in Table 1. The LC50 values of chlorantraniliprole at 24 h, 48 h, 72 h, and 96 h exposure period were observed as 8.52±1.02 mg/L, 4.42±0.20 mg/L, 2.44±0.30 mg/L, 1.77±0.03 mg/L. For further assessment of the toxicological potential of chlorantraniliprole, different concentrations of chlorantraniliprole were chosen based on the 96 h $LC_{50}$ of chlorantraniliprole in *F. limnocharis*. The selected test concentrations were 0.02 mg/L, 0.1 mg/L, and 0.2 mg/L.

*3.2. Exposure of chlorantraniliprole enhances the frequency of MN in Fejervarya limnocharis*

The genotoxic potential of chlorantraniliprole was assessed by using the micronucleus assay. Significant rises in the frequencies of MN in 24h chlorantraniliprole-treated species were observed as compared to the control group. The positive group, containing cyclophosphamide showed a rise in the MN frequency to 9.54±0.90% ($p< 0.001$) as compared to the control group (Figure 1A). Similarly, experimental species treated with 0.02 mg/L, 0.1 mg/L, and 0.2 mg/L concentrations of chlorantraniliprole showed a significant rise in MN (%) to 7.68±0.65% ($p<0.001$ vs control), 9.24±0.84% ($p<0.001$ vs control), and 10.12±0.60 ($p<0.001$ vs control), respectively (Table 2) (Figure 1A).

Similarly, the 48h treatment group also reflected a dose-dependent rise in the MN percentage. The MN% of the cyclophosphamide-treated group was found to be 11.40±0.88% ($p<0.001$) as compared to the control (2.28±0.93%) (Figure 1B). Experimental species treated with 0.02 mg/L, 0.1 mg/L, and 0.2 mg/L concentrations of chlorantraniliprole showed a significant rise in MN (%) to 10.48±0.81% ($p<0.001$), 12.22±0.62% ($p<0.001$), and 14.74±0.66 ($p<0.001$), as compared to the control (2.28±0.93%) (Table 2) (Figure 1B).

In the case of 72 h chlorantraniliprole-treated group also showed an overall rise in the MN frequency. Species exposed to cyclophosphamide reflected an increment in the MN% to 22.14±1.04%, which was significant ($p<0.001$) as compared to the control (5.98±0.76%) (Figure 1C). Treatment with 0.02 mg/L, 0.1 mg/L, and 0.2 mg/L concentrations of chlorantraniliprole showed a significant augmentation in MN (%) to 12.70±0.66% ($p<0.001$ vs control), 15.04±0.96% ($p<0.001$ vs control), and 18.80±1.05 ($p<0.001$ vs control), respectively (Table 2) (Figure 1C).

As expected, the 96h treatment group also showed a whole increase in the MN%. Species treated with cyclophosphamide showed an increase in MN frequency to 29.10±1.18% ($p<0.001$) as compared to the group (5.66±0.63%) (Figure 1D). Species exposed to 0.02 mg/L, 0.1 mg/L, and 0.2 mg/L concentrations of chlorantraniliprole showed a significant ($p<0.001$) increase in MN (%) to 18.62±0.61%, 20.08±1.10%, and 23.44±1.31, respectively as compared to the control group (5.66±0.63%) (Table 2) (Figure 1D). So, the insecticide chlorantraniliprole can cause genotoxicity in erythrocytes of *F. limnocharis* tadpoles (Figure 2). The control group showed normal erythrocytes (Figure 2a, b); in exposed groups not only micronucleus but several other erythrocyte abnormalities such as binucleate cells, trinucleate cells, and stress vacuoles are also seen. Stress vacuoles with MN are highest at 48 h of exposure (Figure 2d, e). Multinucleate cell was seen at 96h (Figure 2f). and binucleate cells at 72 h (Figure 2c). of exposure. MN along with these other abnormalities of erythrocyte cells can induce genetic, pathological and Para pathological damage which can cause mutation in these animals.

*3.3. Chlorantraniliprole alters the behavioural activity in Fejervarya limnocharis*

In this study, we found that after exposure of Chlorantraniliprole to the tadpoles at about the end of 14 days of treatment, tadpoles showed some abnormal behaviours. They primarily engaged in eating and predator avoidance behaviour after being exposed to coragen (chlorantraniliprole). Tadpoles in the control group were active and in their normal state whereas tadpoles in the treated groups were lethargic and movements were slowed down. In the dose 0.01 mg/L the tadpoles were slowly moving and were not active compared to the control group. But in doses 0.1 mg/L and 0.2 mg/L, the tadpoles were completely still and were lethargic. They lay still in the bottom of the tubs and showed very little movement sometimes. Amphibian larvae may show-evasive behaviours, shelter-seeking, and behavioural inhibition (such as freezing behaviour, and lethargy) to evade predators. Tadpoles probably assess the risk of predation using a mix of chemical and visual cues. It can be assumed from their changing behaviours that the coragen induces some kind of hormonal imbalance and

**Table 2.** Incidence of micro-nucleated erythrocytes in the blood of *F. limnocharis* tadpoles exposed to different concentrations of test agents. Values are expressed as Mean ± SD for 10 experimental animals per study group (n=10). CP; *Cyclophosphamide* (Positive control), CAP; *Chlorantraniliprole*, CAP 1% (1% of 96 h $LC_{50}$; 0.02 mg/L); CAP 5% (5% of 96 h $LC_{50}$; 0.1 mg/L); CAP 10% (10% of 96 h $LC_{50}$; 0.2 mg/L). Values are significantly different from control group at $p<0.001$ (***) level. Statistical analysis: One-way analysis of variance (ANOVA) was done by following Tukey's test for multiple comparisons.

| Groups | Exposure period | | | |
|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h |
| Control | 1.66 ± 0.91 | 2.28 ± 0.93 | 5.98 ± 0.76 | 5.66 ± 0.63 |
| CP 2 mg/L | 9.54 ± 0.90*** | 11.40 ± 0.88*** | 22.14 ± 1.04*** | 29.10 ± 1.18*** |
| CAP 1% | 7.68 ± 0.65*** | 10.48 ± 0.81*** | 12.70 ± 0.66*** | 18.62 ± 0.61*** |
| CAP 5% | 9.24 ± 0.84*** | 12.22 ± 0.62*** | 15.04 ± 0.96*** | 20.08 ± 1.10*** |
| CAP 10% | 10.12 ± 0.60*** | 14.74 ± 0.66*** | 18.80 ± 1.05*** | 23.44 ± 1.31*** |

neurotoxic stress in the tadpoles which contribute to their feeding avoidance and also significant changes to their activity.

# 4. Discussion

Studies with pesticides show that active chemicals and their formulations vary in terms of their mutagenicity (Grisolia, 2002). Though numerous researches have been done regarding the toxicity of chlorantraniliprole in vertebrates and some insects, not much adequate data is available in the case of tadpoles. Li et al (2014) found that chlorantraniliprole with some agro-chemicals increased mortality rates with the increase in the concentration of the chemicals in the Chinese tiger frog (*Hoplobatrachus chinesis*), they also studied the lethal concentration ($LC_{50}$) for chlorantraniliprole mixed with other chemicals, our finding of $LC_{50}$regarding chlorantraniliprole also supports their discoveries as the $LC_{50}$ reflected a sequential trend value. Wei et al (2021) also studied the survival and growth factors in *F. limnocharis* and *M. fissipes* exposed to chlorantraniliprole. He concluded that the mortality rate and toxicity of chlorantraniliprole to tadpoles in species specific and also facilitated by other factors. In our present study, it was observed that chlorantraniliprole is toxic and with the increase in concentration, it induces toxicity and affects their feeding behaviour which ultimately affects growth and survival rate.

Odetti et al (2020) did a study in hatchlings of *Caiman latirostris* exposed to chlorantraniliprole and imidacloprid formulations, they found out that both chemicals have the potential to induce oxidative stress and genotoxicity. Similarly, in our present study, we found out a dose-dependent trend in genotoxicity assessment, that is compared to the control that CAP 1%, CAP 5%, and CAP 10% showed an increase in the MN frequencies, Overall, it can be said that the insecticide chlorantraniliprole can cause DNA damage and alter their normal behavioural pattern. All of these aberrations can ultimately affect their health, which makes them physically unfit, and sometimes their death. Local diversity loss can also occur if a large amount of these chemicals is used for a chronic time.

For an animal to survive, it must be able to detect both food and the presence of predators and alter its foraging behaviour accordingly. The tadpoles gradually stopped consuming the items that were provided to them as the concentration and duration of the agrochemical increased. The tadpoles in the control group were consuming the feeds regularly. A feeding control centre in the hypothalamus/preoptic region of the brain is thought to exist in amphibians. Here, in the hindbrain's nucleus of the solitary tract, some neurons work together to form a neural circuit that regulates orexigenic (which increases feeding) and anorexigenic (which suppresses feeding) behavioural output (Serrano et al., 2011). A protein hormone called leptin that controls food intake is released by fat cells. Leptin may increase the activity of CRF neurons, which would result in CRF-mediated suppression of eating (Simkin, 2019). It can be concluded that the chemical coragen (chlorantraniliprole) has some type of hormonal or may induce neurotoxic stress that affects their feeding patterns. For proper growth and survival, food is a requirement and this inhibition of feeding of the tadpoles may affect their overall health and survival as a whole.

# 5. Conclusion

Laboratory-based toxicity test determines that chlorantraniliprole has the potential to damage the genetic material in *F. limnocharis*. As we know amphibians play an important role in the control of pests in both aquatic and terrestrial food webs, but due to the constant use of agricultural artificial chemicals and other toxicants, the amphibian population has declined in recent years. These chemicals have also the potential to alter the normal habits of amphibians. Eventually, they induce DNA damage, physical deformities, and other behavioural changes. Further research is needed to investigate via what potential pathways chlorantraniliprole alters normal cellular or genetic components. As a whole, it is imperative to find natural alternatives that can have the least effects as compared to the artificial ones, then only environmental stability can be maintained rationally.

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**Authors contribution**

Sweety Nath Barbhuiya- Resources, Writing - Original draft. Dipsanu Paul - Methodology, Formal analysis, Investigation, Visualisation. Neha Farzana and Abujam Romibala Devi - Conceptualisation, Validation, Data curation. Dharmeswar Barhoi and Utsab Singha - Writing – Review and Editing, Supervision, Administration.

**Declaration of conflict of interest**

Authors have no conflict of interest

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