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Blood Cell Profiles and Metamorphosis of Rice Field Frog (*Fejervarya cancrivora*) after Heavy Metal Copper (II) Sulfate Exposure

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Abstract

Tadpoles represent a critical stage in frog metamorphosis and are particularly susceptible to environmental stressors. Heavy metals, such as copper, are common industrial pollutants that adversely affect aquatic ecosystems. This study aimed to evaluate the effects of copper (II) sulfate on the blood cell profiles and metamorphic development of rice field frog (*Fejervarya cancrivora*) tadpoles. Experimental treatments included a positive control (mineral water), a negative control (Propylthiouracil / PTU 0.1 gL^{-1}), and exposure to heavy metal of copper (II) sulfate 0.01 mgL^{-1} (Cu (II)). Each treatment group comprised nine tadpoles, maintained for 14 days under controlled conditions. After 14 days, the survival rate of tadpoles exposed to Cu (II) was 66.67%. Analysis of variance (ANOVA) revealed that Cu (II) exposure significantly impacted blood cell profiles ($p < 0.05$). Specifically, Cu (II) exposure led to reduced nuclear size, increased immature erythrocytes, and a higher percentage of abnormal erythrocytes. Although metamorphic growth parameters did not differ significantly among treatments ($p > 0.05$), Cu (II) exposure accelerated skin pigmentation and induced tail abnormalities, including tumor-like formations. These findings demonstrate that Cu (II) is toxic to the blood cells and induces morphological abnormalities in *F. cancrivora* tadpoles, highlighting the potential ecological risks of copper contamination in aquatic habitats.

Keywords: copper (II) sulfate, erythrocytes, *Fejervarya cancrivora*, leukocytes, metamorphosis

Introduction

Global extinction rates are rising due to various environmental stressors, including chemical, physical, and biological contaminants. Environmental changes such as shifts in climate (temperature, precipitation, snow, and ice cover) and rare catastrophic events (e.g., meteorite impacts or large volcanic eruptions) significantly contribute to these extinction trends (Phillips et al., 2024). Furthermore, contamination of aquatic ecosystems by pollutants such as pesticides, herbicides, salts, fertilizers, heavy metals, and active pharmaceutical ingredients exacerbates these environmental challenges (Collins, 2010).

These stressors disrupt endocrine functions, leading to adverse effects on amphibians, whose metamorphosis is primarily regulated by thyroid hormones (THs). Thyroid hormones play a critical role in determining the rate of amphibian development. Disruption of endocrine regulation can inhibit the normal activity of THs, resulting in altered growth, development, and metabolism. Consequently, the metamorphic process may be delayed, and physiological abnormalities can emerge (Ruthsatz et al., 2020).

Tadpoles, the aquatic larval stage of frogs and toads, undergo dramatic developmental changes during metamorphosis, which involves the transformation of almost every organ. These changes include regressive processes, such as the loss of horn teeth and internal gills, and tail resorption, as well as progressive processes like limb development (Fauzi & Wibowo, 2021). However, these developmental stages are highly sensitive to environmental conditions, and stressors such as pollution can significantly alter the ontogenetic changes regulated by THs (Ojha et al., 2020). The permeable skin structure of tadpoles, coupled with their non-amniotic (shell-less) nature, makes them highly susceptible to pollutant exposure in their habitats.

Heavy metals, including copper, chromium, lead, and zinc, are prevalent in industrial processes such as metal finishing, mining, and chemical production. These metals are key indicators of water pollution and are highly toxic to aquatic life (Hartiningsih et al., 2024). Copper sulfate, widely used in industries like textiles, batteries, pesticides, and wood preservation, is a common pollutant in

aquatic environments (Briffa et al., 2020). While copper can also occur naturally due to environmental reactions (Shan et al., 2020), its elevated concentrations can have profound toxicological effects, impacting organisms at morphological, biochemical, and genetic levels.

Several studies have demonstrated the detrimental effects of copper exposure on amphibians. For example, exposure to copper has been shown to decrease hematological parameters in species such as *Channa punctatus*, with reductions in neutrophils, monocytes, and basophils (Singh, 2008). Higher concentrations of copper (>0.15 ppm) have been linked to physiological disorders, such as edema, resulting in high mortality rates (Dvorsky et al., 2023). Additionally, copper exposure has been reported to negatively impact frog metamorphosis, causing morphological abnormalities, reduced motility, and increased mortality (Ojha et al., 2021). Such toxic effects are not only detrimental to biodiversity but also impair biological control mechanisms in aquatic ecosystems (Thanigaivel et al., 2024).

Amphibians, particularly anurans, serve as important bioindicators due to the sensitivity of their blood parameters to environmental toxicants (Zhelev et al., 2017). Understanding the effects of copper contamination on blood cell profiles and metamorphosis is crucial for assessing its ecological impact. This study aims to evaluate the impact of copper (II) sulfate on the blood structure and metamorphic development of rice field frog (*Fejervarya cancrivora*) tadpoles. The findings are expected to contribute to the growing body of literature on amphibian ecotoxicology, highlighting the physiological abnormalities induced by copper exposure and the varying levels of tolerance influenced by species and habitat.

Materials and Methods

Materials

This study utilized 36 pro-metamorphic tadpoles with an average body length of 17.33 mm, collected from rice fields in Baleendah District, Bandung City, West Java. The tools employed included glass slides, coverslips, staining jars, blades, microscopes, millimeter block microscopes, and a camera for documentation. The materials used comprised ethanol (96%) for sample preservation and cleaning, ice cubes to maintain sample freshness during preparation, copper (II) sulfate (Cu (II), 0.1 gL^{-1}) as the experimental contaminant, and Giemsa stain for blood cell visualization under the microscope. Additionally, eosin (0.05%) was used as a counterstain, while propylthiouracil (PTU, 0.01 gL^{-1}, OGB Dexa) served as a control to inhibit thyroid hormone activity. These tools and materials were integral to conducting the experimental procedures effectively.

Methods

This study used an experimental method with Complete Randomized Design (CRD) with 3 levels of treatment, consisting of:

Control : Negative Control (mineral water)
PTU : Propylthiouracil 0.1 g L^{-1}
Cu (II) : Copper (II) sulfate 0.01 mgL^{-1}

Media preparation

Growth media for the study on the effects of toxicants on metamorphosis and blood profiles of tadpoles were PTU solution (0.1 gL^{-1}) and copper (II) sulfate solution (0.01 mgL^{-1}). To prepare the PTU solution, 100 mg PTU tablets were crushed into powder. A quantity of 0.1 g of the powder was weighed and dissolved in distilled water in 1.5 mL microtubes. For the Cu (II) sulfate solution, 0.01 mg of Cu (II) was weighed and dissolved in water in a 1.5 mL microtube.

Animals adaptation

The tadpoles underwent an adaptation period before treatment. During this stage, animal welfare, timing, and nutritional needs were carefully managed. The tadpoles were fed spinach that had been boiled and crushed. This research was conducted at the Biological Laboratory of Muhammadiyah University of Bandung. The experimental substrate was prepared using round basins with a 3 L capacity. Each basin was filled with nine tadpoles. Before starting the experiment, the tadpoles were acclimatized for seven days to ensure readiness for testing.

Research Treatment

A total of 27 tadpoles were divided into three groups: Group 1 (control) received water only, Group 2 received PTU treatment at a dose of 0.1 g L^{-1}, and Group 3 received Cu (II) sulfate solution at a dose of 0.01 mg L^{-1}. The treatment lasted for 14 days, with tadpoles fed boiled spinach and libitum. The substrate was replaced every three days, and treatment media were refreshed once per week. Observations on metamorphic growth were conducted on days 0, 7, and 14.

Growth measurement

Growth parameters measured included body length (BL), body width (BW), and tail length (TL). Tadpoles were placed on millimeter blocks for measurement, and the length was calculated by counting the number of squares on the block and converting the measurement by multiplying the units by 5 mm.

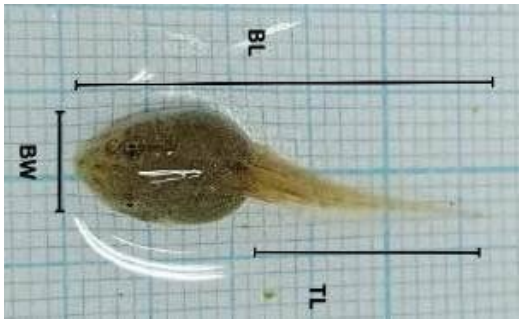

Figure 1. Growth measurement.

Morphology observation

Eye morphology was observed based on eye position and color. Tadpoles were placed on millimeter blocks, and their eyes were examined using a magnifying glass. The observations were recorded for further analysis. Pigmentation on the ventral side of the tadpoles was observed by placing them on millimeter blocks. Changes in pigmentation were noted and recorded during the study.

Anatomical observation

The anatomical observations were conducted on cytometric of blood cells, intestine length, and mebra structure. The tadpole dissection procedure followed the Institutional Animal Care and Use Committee guidelines at SUNY Fredonia. Tadpoles were immobilized in ice water for 15–20 minutes and then pierced with an injection needle (Medler, 2019). Blood was collected from the center of the tail on days 0, 7, and 14. Blood smears were prepared using the Edge Blood Smear (ADT) technique and stained with eosin for microscopic examination at 1000× magnification. Blood cells were identified in three fields of view, and 20 cells were counted in each field. Erythrocyte and leukocyte sizes, as well as nuclei, were measured using an ocular micrometer calibrated with an objective micrometer (Das & Mahapatra, 2012). The cytometric cell calculation followed this equation:

$$20\ OC = 5\ OB$$
$$1\ OC = \frac{5\ x\ 10}{20}$$
$$1\ OC = 2{,}5\ \mu m$$

Information:
OC: Ocular lenses
OB: Objective lens

After blood sampling, the tadpoles were dissected, and the spiral-shaped intestine was carefully removed. The intestine was extended without breaking, measured using millimeter blocks, and the length was recorded. The tail was observed under a microscope to evaluate the development of both the rear and front membranous structures. Observations included changes in tail structure and the formation of limbs.

Data analysis

Qualitative data collected in this study included observations of blood cells, skin pigmentation, and membranous structures. Quantitative data encompassed measurements of erythrocyte sizes, nuclei sizes, leukocytes per high-power field (HPF), the percentage of immature erythrocytes, the percentage of normal erythrocytes, body length, body width, and tail length.

The statistical analysis was performed using the SPSS software. The data were initially tested for normality using the Shapiro-Wilk test to ensure the appropriateness of parametric statistical methods. For normally distributed data, a one-way Analysis of Variance (ANOVA) was employed to identify statistically significant differences in blood profile parameters and morphometric traits between the treatment groups at a 95% confidence level. When the ANOVA results indicated significant differences, post-hoc analysis was conducted using Duncan's multiple range test to determine specific group differences.

Results and Discussion

Effect of copper (ii) sulfate exposure on the Blood Cell

The erythrocyte structure of pro-metamorphic *F. cancrivora* tadpoles exhibited variations in shape and nuclear position. Observed erythrocytes included round cells with centrally located nuclei

(Figure 2A), oval cells with centrally located nuclei (Figure 2B), and oval cells with eccentrically positioned nuclei (Figure 2C). Immature erythrocytes were characterized by larger nuclei that appeared less compact (Figure 2D), while abnormal erythrocytes displayed cytoplasmic droplets (Figure 2E) and swollen cells of increased size (Figure 2F). These findings align with previous research by Hota et al. (2013), who reported that 0.5–1% of tadpoles exhibit irregular (immature) erythrocyte shapes during stages 37 to 42, with erythrocytes varying in size compared to normal cells. Furthermore, erythrocyte morphology in anuran species is habitat-dependent, with aquatic species predominantly having rounded erythrocytes and terrestrial species exhibiting oval erythrocytes (Zhelev et al., 2017).

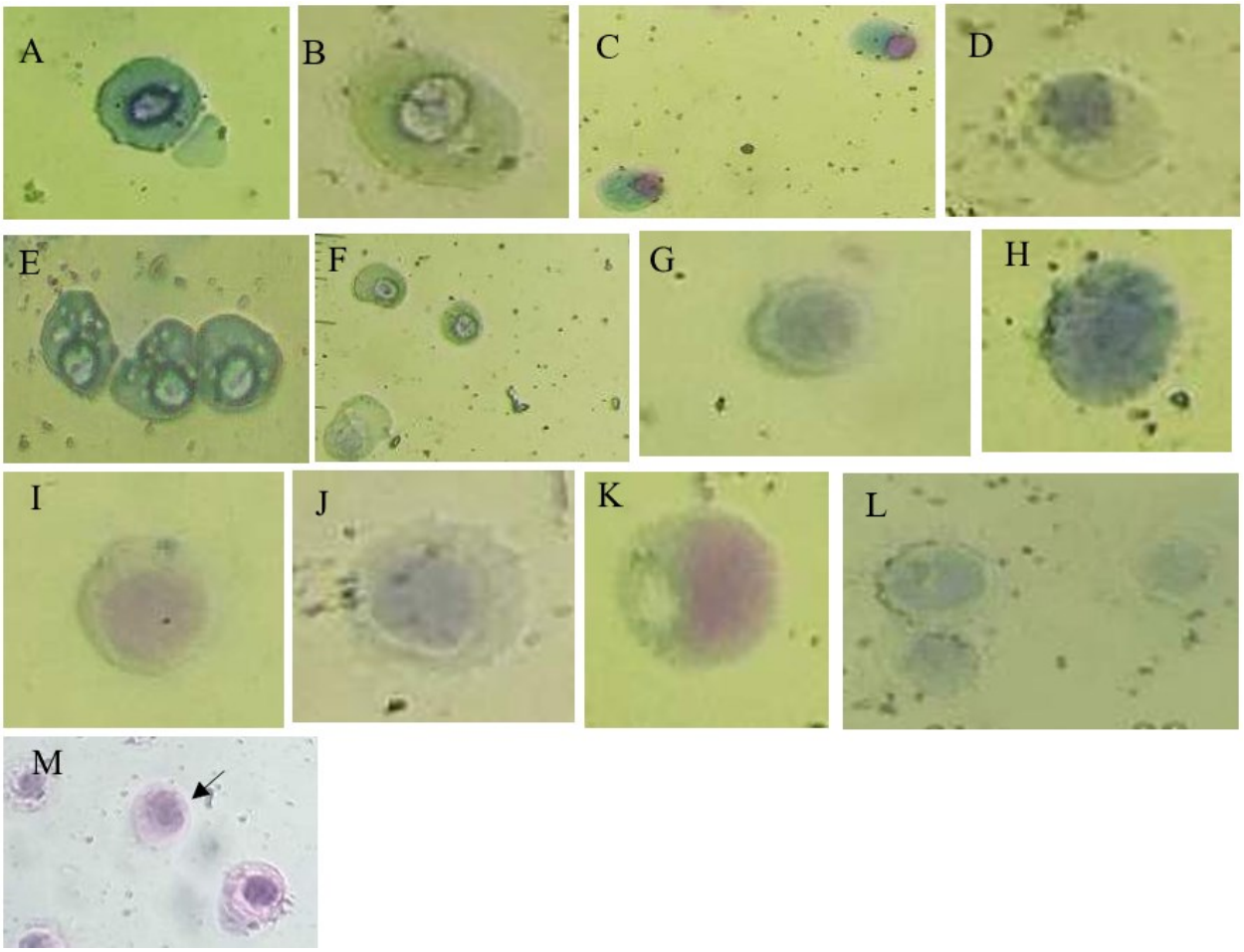

Figure 2. Photomicrographs of blood cells of *F. cancrivora* after copper (II) sulfate exposure. (A). round erythrocyte with nuclei in the middle, (B) oval erythrocyte with nuclei in the middle, (C) erythrocyte with an edged, (D) immature erythrocyte, (E) abnormal erythrocyte that has droplets, (F) swelling erythrocyte, (G) (H) lymphocytes, (J-I) monocytes, (K) basophils, (L) neutrophils, (M) eosinophils

Five types of leukocytes, or white blood cells, were identified in this study: lymphocytes, monocytes, basophils, eosinophils, and neutrophils. Lymphocytes were the most abundant, with spherical nuclei occupying approximately 95% of the cell (Figure 2A). Monocytes, which comprised around 80% of the cell structure, displayed round or horseshoe-shaped nuclei (Figures 3B and 3C). Basophils were spherical, with a large purple-stained nucleus and cytoplasmic granules (Figure 2D). Eosinophils had a U-shaped nucleus with granules (Figure 2E) or were spherical with the nucleus located at the cell's periphery (Figure 2F). These observations are consistent with Das and Mahapatra (2012), who described leukocytes in *Polypedates teraiensis* tadpoles. They reported spherical lymphocytes with varying nucleus sizes, monocytes with notched nuclei or pseudopod-like cytoplasmic extensions, eosinophils with lobed nuclei, and basophils with large, dark purple nuclei atop an irregular nuclear structure. Neutrophils exhibited bilobed, trilobed, or U-shaped (band) nuclei.

Compared to metamorphic stages, pro-metamorphic tadpoles exhibited less pronounced leukocyte differentiation, as also noted by Hota et al. (2013). The developmental stage influences the structure and functionality of leukocytes, which may reflect the tadpoles' adaptive responses to environmental stressors.

The results of ANOVA analysis showed that the erythrocyte sizes of *F. cancrivora* tadpoles did not differ significantly across treatments ($p > 0.05$), while the nuclei sizes were significantly different ($p < 0.05$). The average erythrocyte and nuclei sizes in the Cu (II) group were smaller compared to the control and PTU groups (Table 1). Specifically, in the Cu (II) group, the erythrocyte diameter decreased to 9.77 µm, compared to the normal size of 15.45 µm in pro-metamorphic tadpoles. Similarly, the erythrocyte nucleus size decreased to 4.19 µm from the normal size of 7.72 µm (Das & Mahapatra, 2012). This reduction in erythrocyte and nucleus size suggests cell deformation and damage, potentially leading to increased apoptosis and changes in blood cell structure. Exposure to heavy metals, such as Cu (II), has been shown to induce oxidative stress, shrink erythrocyte and nucleus size, and accelerate cell death (Zhelev et al., 2017).

Although copper concentrations below 0.1 mg/L are generally considered harmless in aquatic environments, concentrations exceeding 0.5 mg/L can cause oxidative stress in aquatic species, including tadpoles (Husain & Mahmood, 2019; Zheng et al., 2021). Heavy metal ions, including Cu (II), can speed hemolysis, alter cell size and shape, and disrupt ion channels in cell membranes. Environmental factors such as drought, pollution, and seasonal variations also influence erythrocyte size in Anura species (Zhelev, 2017). Other studies have reported that heavy metals like cadmium (Cd) and zinc sulfate ($ZnSO_4 \cdot 7H_2O$) directly decrease erythrocyte and nucleus size, leading to changes in cell proliferation and apoptosis (Stepanyan et al., 2011; Zhelev et al., 2017).

Table 1. Cytometric of erythrocyte and blood cell profiles of *F. cancrivora* after copper (II) sulfate exposure

Treatment	Erythrocyte Sizes (µm)	Nuclei Sizes (µm)	Percentage of Normal Erythrocyte	Percentage of Erythrocyte Immature	Leukocytes per high power field (HPF)
Control	11.26 ± 0.94^{a}	5.74 ± 1.35^{b}	91.00 ± 1.73^{c}	5.44 ± 2.70^{a}	1.59 ± 1.92^{ab}
Propylthiouracil	10.24 ± 1.65^{a}	5.06 ± 1.51^{ab}	70.00 ± 1.87^{b}	13.56 ± 3.81^{b}	2.77 ± 2.18^{b}
copper (ii) sulfate	9.77 ± 1.49^{a}	4.19 ± 0.66^{a}	17.83 ± 2.32^{a}	11.67 ± 1.63^{b}	0.71 ± 0.00^{a}

The same superscript letters are not significantly different by Duncan's test at a 95% confidence level

The percentage of immature erythrocytes in RFF tadpoles showed a significant difference across treatments ($p < 0.05$). The PTU group exhibited a higher percentage of immature erythrocytes compared to the control and Cu (II) groups (Table 1). PTU inhibits thyroid hormone synthesis, slowing erythrocyte maturation and reducing the production of adult erythroblasts, thereby delaying metamorphosis (Sachs & Buchholz, 2019). In the Cu (II) group, the release of immature erythrocytes into peripheral blood may represent a physiological response to toxic stress, increasing red blood cell counts to compensate for cellular damage (Zhelev et al., 2017).

Leukocyte counts per high-power field (HPF) in RFF tadpoles differed significantly among treatments ($p < 0.05$). The PTU group had the highest leukocyte count, followed by the control group, while the Cu (II) group had the lowest leukocyte count (Table 1). PTU exposure increased leukocyte counts, possibly as a response to delayed metamorphosis and immune system activation. Conversely, Cu (II) exposure suppressed leukocyte production, potentially due to toxic effects on immune system regulation. Leukocytes, including lymphocytes and neutrophils, play a primary role in immune defense during physiological stress. Monocytes, eosinophils, and basophils also contribute to nonspecific immune responses and early metamorphic development (Das & Mahapatra, 2012).

An increase in leukocyte counts during PTU treatment may reflect tissue remodeling and cellular debris clearance associated with metamorphic

changes. Leukocytes modulate tissue lysis, a critical process in structural reorganization during metamorphosis. The observed changes in blood cell profiles highlight the impact of Cu (II) toxicity and PTU treatment on the physiological and immunological status of RFF tadpoles.

Effect of copper (ii) sulfate exposure to the metamorphosis

The results of ANOVA analysis indicated that the rate of metamorphosis of rice field frog (*F. cancrivora*) tadpoles from day 0 to day 14 showed no statistically significant differences across treatments ($p > 0.05$) (Table 2). Tadpoles exposed to copper (II) sulfate (Cu (II)) showed slower growth in body length and width, likely due to the toxic effects of Cu (II), which inhibited metamorphic development. However, the dosage used in this study did not result in tadpole mortality. Similar findings were reported by Gurkan and Hayretdag (2012), where exposure to Cu (II) sulfate at 0.01 mgL^{-1} caused morphological abnormalities in Bufo viridis tadpoles but resulted in only 2 deaths out of 20 individuals, indicating that the dosage was relatively low.

Table 2. Effect of copper (ii) sulfate exposure to the metamorphosis of *F. cancrivora*

Treatment	Body Length	Body Width	Tail Length	Intestinal Length	Skin Pigmentation	Eye Morphology
Control	24.22 ± 1.75^a	5.67 ± 0.47^a	15.56 ± 1.42^a	90.33 ± 29.58^a	-	Lateral
Propylthiouracil	22.67 ± 2.58^a	5.67 ± 0.94^a	17.44 ± 2.99^a	79.00 ± 30.47^a	-	Lateral
Copper (ii) sulfate	22.00 ± 3.00^a	5.33 ± 0.47^a	14.33 ± 3.59^a	42.28 ± 14.75^a	+	Lateral

The same superscript letters are not significantly different by Duncan's test at a 95% confidence level

Propylthiouracil (PTU) treatment also delayed growth in body length and width due to the inhibition of thyroxine (T4) synthesis. PTU disrupts iodide absorption by follicular cells in the thyroid gland, which regulates morphological changes during early metamorphic stages, thus impairing tadpole development (Thambirajah et al., 2019; Miyata & Ose, 2012). Interestingly, while PTU typically reduces hind limb and tail growth, the current study observed faster tail growth after PTU exposure, likely due to the low dose used, which did not significantly inhibit tail development. Similarly, Cu (II) exposure did not significantly affect tail growth, consistent with findings by Stepanyan et al. (2011), where heavy metal ions such as Cr (VI) at 4.2 mgL^{-1} did not impair tail length due to its limited involvement in the tadpole's developmental process.

Observations of eye morphology (Table 2) indicated no abnormalities after 14 days of Cu (II) exposure at 0.01 mgL^{-1}. The tadpoles' eyes remained fully black and laterally positioned, maintaining a normal appearance. However, previous studies have shown that Cu exposure at higher concentrations can impair eye development and cause morphological abnormalities (Huang et al., 2020).

PTU treatment inhibited larval development and caused morphological disruptions in the thyroid gland, while Cu (II) exposure slowed overall growth and delayed metamorphosis into adult frogs (Miyata & Ose, 2012). No significant changes were observed in the development of the membranous structures during the tail-budding phase over the 14-day exposure period to Cu (II) and PTU. However, Cu (II) exposure caused notable morphological abnormalities, including pale, rough, damaged fins and inflammation on the tail skin, resembling tumor-like formations. These abnormalities are consistent with findings by Ojha et al. (2021), which linked heavy metal exposure to physiological disruptions, delayed metamorphosis, and increased mortality in amphibians.

Interestingly, Cu (II) accelerated the pigmentation process in tadpoles, an adaptive response to toxic environments for protection. Enhanced pigmentation is a well-known survival mechanism in tadpoles, enabling them to conform to environmental conditions and shield against UV radiation (Çömden et al., 2023; Belussi et al., 2016). However, Cu (II) exposure may also reduce melanin levels, altering pigmentation patterns more rapidly.

Regarding intestinal morphology, tadpoles in the control and PTU groups exhibited longer intestinal lengths compared to those exposed to Cu (II). Intestinal length was generally shorter in tadpoles exposed to Cu (II) over 14 days (Figure 3). In the early stages of metamorphosis, tadpoles typically have long, coiled intestines, which simplify as they

transition to adulthood and shift towards a more carnivorous diet.

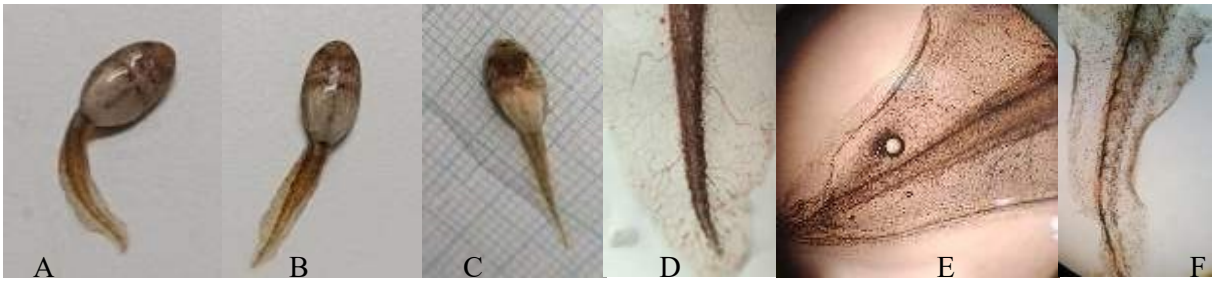

Figure 3. Morphology of *F. cancrivora* tadpoles: A - C: ventral structure, D - E: tail structure; (A, D) control treatment, (B, E) Propylthiouracil 0.1 g L^{-1}, (C, F) Copper (II) sulfate 0.01 mgL^{-1}

Cu (II) exposure acted as a stressor and endocrine disruptor, potentially damaging the hypothalamic-pituitary-thyroid (HPT) axis. Disruption of this axis during metamorphosis can impair thyroid hormone regulation, affecting physiological processes in later life stages. Altered thyroid hormone levels may influence metabolic functions, as previously reported by Ruthsatz et al. (2020). These effects are compounded by the high permeability and sensitivity of amphibian larval skin, making tadpoles highly vulnerable to environmental pollutants.

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