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DIFFERENTIAL ALTERATIONS IN PHYSIOLOGICAL AND BIOCHEMICAL TRAITS OF ROHU (*Labeo rohita*) EXPOSED TO EXPERIMENTAL DOSES OF CARBOFURAN PESTICIDE

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Abstract

Carbofuran is a common and widely used pesticide for agriculture (growing different types of crops) across Bangladesh. The ultimate destination of pesticides is aquatic environments through run-off, having a long-term adverse effect ecosystem and aquatic biodiversity. The present study was conducted to determine the effects of different doses of carbofuran on a major carp fish, Rohu (*Labeo rohita*). Four different doses of carbofuran were maintained including: 0 mg/L (control), 0.5 mg/L (T1), 1 mg/L (T2) and 2 mg/L (T3) to investigate the biological changes in Rohu for 60 days. Carbofuran treatments resulted in 20 – 30% lower growth, 20 – 48% lower survival rate and 10 – 20% lower blood cell counts compared to the control. In contrast, 30 – 60% higher oxygen consumption rate, 15 – 50% higher blood cortisol and 20 – 80% higher blood glucose levels were obtained for the three carbofuran treatments. Significantly higher ($P < 0.05$) growth, survival and blood cell counts in contrast to lower levels of O_2 consumption, blood glucose and cortisol levels indicate no imposed stress on Rohu at the control condition. Large scale changes in growth, survival, O_2 consumption, blood cell counts, blood cortisol and glucose levels are indicative of the intensity of stress imposed on the experimental fishes. Types and number of deformed blood cells were also found to vary significantly ($P < 0.05$) among the treatments. Gill ultra-structural view also exhibited considerable damage in gill lamellae and filaments with increasing pesticide concentrations. Overall, findings of this study imply that lower carbofuran dose (T1 = 0.5 mg/L) initiate the slight damage in different biological traits of Rohu while higher doses (T1 = 1 mg/L and T2 =

2 mg/L) cause severe damage. Thus, pesticide pollution is a severe threat to aquatic biodiversity and appropriate measures must be taken to control the excessive use of this chemical to protect the entire aquatic ecosystem as well as biodiversity.

Key words: Major carp, pesticide pollution, aquatic toxicology, anthropogenic stressor

Introduction

Rohu (*Labeo rohita*) is the most popular freshwater carp species in Bangladesh due to its good aquaculture attributes including faster growth (compared to other species), delicious taste, higher market price, availability of hatchery produced seed and wild brood stocks (Ali et al., 2008; Afroz et al., 2021; Mridul et al., 2024). Therefore, *L. rohita* is widely farmed in different water bodies of Bangladesh (Shah et al., 2011; Sabbir et al., 2017). In addition, this is a major capture fishery from different wild habitats (Rahi & Shah, 2012; Islam et al., 2015). Unfortunately, the wild abundance of this fish has been declining due to various anthropogenic activities (Ali et al., 2015; Siddika et al., 2025). Aquatic pollution is a major threat to the decline of the wild stocks of this species; in particular, run-off from agriculture (pesticide pollution) is the major contributor to the pollution of freshwater habitats and subsequent mortality of different species (Ullah & Zorriehzahra, 2014; Kumar et al., 2021). Pesticide pollution is extremely harmful for the early developmental stage of fish and also known to reduce reproductive performance (Kamble & Shinde, 2012). Recently, the widespread uses of pesticides have been increased tremendously in order to produce more crops (Svobodová et al., 1993; Kalyabina et al., 2021; Rani et al., 2021). Most of the pesticides are non-biodegradable, persist in the environment for a long time and cause permanent damage to aquatic ecosystem (Clasen et al., 2018; Ray & Shaju, 2023). Moreover, aquatic environments continuously receive pesticides and different types of pollutants, causing gradual increase/accumulation in the aquatic habitats that is extremely harmful to the aquatic biodiversity (due to bioaccumulation) and entire ecosystem (Sabbir et al., 2010; Rahi et al., 2013; Ballesteros et al., 2017). Therefore, use of pesticides is major concern around the world due to their bio-accumulation into different fish tissues and subsequent spread in the food chain (through the process of bio-magnification) which will ultimately result in serious health concern for the consumers (Hampel et al., 2015; Hader et al., 2020; Rahi et al., 2020). To reduce the harmful effects of pesticides, some biodegradable pesticides have been developed but still remain harmful because these pesticides produce different metabolites and heavy metals following degradation; impose another threat (Muhammad et al., 2023; Lema et al., 2024). Carbofuran is a cypermethrin group pesticide, widely used in Bangladesh for agriculture which is biodegradable and is known to be less harmful compared to the non-biodegradable groups (Poorbagher et al., 2018; Ahmed et al., 2020; Hasan et al., 2023). Some earlier studies reported that cypermethrin (carbofuran) type pesticide also induce relatively higher levels of toxicity in fish including adverse effects on different cells and tissues like gonadotrophic cells, gonad, gill, eye, kidney and also blood cells (Nath et al., 2008; Singh & Singh, 2008; Magar et al., 2012; Rahi et al., 2021a). The physiological damage includes growth retardation, reduced fecundity and offspring survival (Moshtaghi et al., 2017; Tang et al., 2018). Moreover, pesticide exposure causes DNA damage and large scale alterations in different blood parameters as well as behavioral changes (David et al., 2002; Zeehad et al., 2024). It was found that lower concentrations of pesticides initiate aquatic pollution that organisms can tolerate at the expense of some compensatory mechanisms while increasing concentrations results in toxicity (massive mortality that puts different species to extinction) (Sauco et al., 2010; Proterro et al., 2011; Rohani, 2023).

Laboratory investigations on various species have already showed that this group of pesticide is also hazardous to aquatic animals (Santhakumar, et al, 2000; Farag et al., 2021). Similar to the other pesticides, carbofuran is found to adversely affect the growth, different tissues, blood parameters and reproduction (fecundity) of different fish species (Kim et al., 2017; Martyniuk et al., 2020; Rahi et al., 2022). Although widely used in Bangladesh, no studies have been performed to test its adverse effect on any freshwater species for a longer experimental exposure. A few studies have been conducted to test the effects for a short time for determining the lethal and sub-lethal doses (Carvalho, 2017; Cowie et al., 2017; Kumar & Mukherji, 2018; Maurya et al., 2019). A long-term exposure to carbofuran experiment will be helpful in understanding the mechanism of damage occur in fish and broadly other aquatic species (intensity of adversity on overall biodiversity). Therefore, the current study was conducted to investigate the cellular (gill ultra-structure), physiological (growth, survival performances and O_2 consumption rates) and biochemical (blood parameters, glucose and cortisol hormone levels) changes/damages in Rohu (*Labeo rohita*) due to different doses of carbofuran pesticide exposure for 60 days.

Materials and Methods

Sample Collection and Maintenance

In total, 600 Rohu fry (≈5 g) were collected from a commercial hatchery for this study. Fishes were maintained under four pesticide doses (Carbofuran), including a control (no carbofuran). Different experimental conditions were maintained as 0 mg/L (as control), 0.5 mg/L (T1), 1 mg/L (T2), and 2 mg/L (T3). In total, 12 experimental glass tanks (50 L) were maintained for this experiment, including three replicated tanks for each experimental condition. In each experimental tank, 50 fish individuals were maintained with continuous aeration (150 fish per treatment). At first, a stock solution was prepared by mixing Carbofuran with water. An appropriate amount of the solution was added in each tank to achieve the target doses of carbofuran. Fishes were maintained under the pesticide doses for 60 days.

Growth, Survival, and O_2 Consumption

Mean body weight of experimental Rohu was measured at every 15 days interval. For growth evaluation, 30 individuals were randomly sampled from each treatment (10 from each replicate tank). Survival rates were estimated by deducting the number of individuals from the beginning to the end of this experiment. Further growth-related parameters were measured according to the following equations (Zeynali et al., 2020; Rahman et al., 2022):

$$DWG\ (\%) = \{(BWf - BWi) / (BWi \times t)\} \times 100$$

$$SGR\ (\%) = \{(\ln BWf - \ln BWi) / t\} \times 100$$

$$FCR = \text{feed intake (g)} / \text{weight gain (g)}$$

$$FI\ (\text{g fish}^{-1}\text{day}^{-1}) = (\text{dry diet given - dry remaining diet recovered}) / \text{number of fish}$$

Here, DWG: daily weight gain, BWf: final body weight, BWi: initial body weight, t: total experimental time, SGR: specific growth rate, FCR: feed conversion ratio, FI: Feed intake.

Experimental Rohu was sampled at different time intervals to measure the carbofuran dose-specific changes in O_2 consumption rates. The sampling times for measuring O_2 consumption rates were on Day 1 (immediately after achieving the target carbofuran doses), Day 2, Day 3,

Day 4, Day 5, Day 10, Day 20, Day 30, and Day 60. Rates of O_2 consumption were estimated according to the methods outlined in Rosas et al. (2001).

Blood Cell Counts

Total number of blood cells was counted according to the methods of Witeska et al. (2022) and Akram et al. (2023). In brief, three replicate fish were collected from each experimental condition (one from each of the replicated tank). From each fish, 50 µL of blood was collected using heparinized micro-injection and immediately transferred in Eppendorf tubes containing an equal volume (50 µL) of 20 mM EDTA. Following this step, 100 µL of 10% neutral buffered formalin was added to each blood sample and maintained at ambient temperature for 30 minutes to fix the samples. Samples were then be serially diluted 2, 4, 8, 16, and 32 times using ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.2). Finally, the total number of normal blood cells were counted using a hemocytometer (Boeco, Hamburg, Germany) and checked under a microscope (SOLARIS-TLED, Rome, Italy) at 100x magnification.

Determining Blood Glucose and Cortisol Levels

A total of 200 µL blood was collected from each fish (three replicate fish from each experimental condition) for estimating blood glucose and stress hormone (cortisol) levels. Collected blood samples were immediately transferred in 1.5 ml tubes containing 200 µL of heparin (Zentiva, Czech Republic) to avoid blood clotting (Pravda & Svobodová, 2003). 100 µL of the anti-coagulated blood was centrifuged at 800g (4°C) for 10 minutes to isolate blood plasma for determining glucose levels. Glucose assays were performed using a commercial kit (Glu L 1000, PLIVA-Lachema, Czech Republic) as outlined in Rahi et al. (2021b). The remaining 300 µL blood samples were used for obtaining adequate quantities of plasma for the assay of cortisol levels. Each of the blood sample was centrifuged at 16000 g (at 4 °C) for 2 minutes to obtain plasma. Finally, 80 µL plasma samples was used for determining cortisol levels according to Fuchs et al. (2015), Bögner et al. (2018), and Islam et al. (2020) by using a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) kit (Enzo et al., USA).

Tissue Ultra-structure through Scanning Electron Microscopy (SEM)

Gill tissues were dissected out from fish of each treatment. Dissected tissues were preserved in 2.5% glutaraldehyde solution (diluted in PBS) and maintained in the refrigerator (4 ºC) for subsequent analysis. Preserved tissues were dehydrated gradually through a series of ethanol (different concentrations of ethanol: 30 – 100%) washing steps. Samples were then bathed serially in hexamethyldisilisane (HDMS) several times at various concentrations (25 – 100%) according to Akram et al. (2023) and Zeehad et al. (2024). Different tissue samples were air-dried at room temperature overnight. Following which samples were gold coated for 180 S (~40 mA) by using the Edwards Sputter Coater for examining the samples with a FEI Quanta 200 ESEM using the conventional mode (high vacuum) and the Thornley–Everhart secondary electron detector.

Statistical Analysis

Different types of data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively, using the software package (SPSS (Version 23). Results were also evaluated for one- and two-way analysis of variance (ANOVA) using a 5% level of significance ($P < 0.05$). Pesticide treatments and sampling times were considered independent variables for two-way ANOVA, while comparisons were made only between treatments for one-way ANOVA. The dependent variables were different physiological (growth and O_2 consumption) and biochemical (number of blood cells, glucose, and cortisol levels) parameters. Comparisons between the means of different parameters were

evaluated using the Tukey HSD test. Results presented in the tables and graphs as mean ± standard error (SE). R package (version 3.5.1) was used for correlation plotting among different parameters: i) growth and O_2 consumption, ii) growth, glucose, and cortisol levels, iii) blood cell counts and cortisol levels.

Results

The present study compared the physiological and biochemical alterations in Rohu (*Labeo rohita*) due to carbofuran pesticide treatments (T1 = 0.5 mg/L, T2 = 1 mg/L, and T3 = 2 mg/ L) compared to the control (no pesticide) for a period of 60 days.

Carbofuran-induced changes in growth and survival Performance

Crabofuran treatments significantly ($F(5, 120) = 97.6$, $P < 0.05$) affected the growth and survival performance of experimental Rohu (*Labeo rohita*) individuals (Table 1 and Figure 1). No significant differences were observed among treatment groups (T1, T2 and T3) from 1st day up to 15th day (Figure 1). Significant differences ($P < 0.05$) were observed among the three treatments from 30th day to the end. Different doses of carbofuran also found to significantly ($P < 0.05$) affect various growth-related parameters (DWG, SGR, Feed Intake and FCR) and survival rate of the experimental fishes (Table 1).

The highest level of growth was obtained at the control condition while the lowest growth was observed at T3 = 2 mg/L. At the start of this experiment, all of the experimental fish had a similar body weight, no significant differences between control and treatment groups. Carbofuran challenge test significantly ($P < 0.05$) reduced the growth performance of Rohu with the progress of time. Levels of growth among the experimental groups were ranked as T3 < T2 < T1 < control. Survival rate was found to vary significantly ($P < 0.05$) among all four experimental groups (Table 1).

Table 1: Effects of carbofuran treatments on different growth-related parameters of Rohu (*Labeo rohita*). Different superscripts indicate significant differences at 5% level of significance.

Growth Parameters	**Control**	**T1 (0.5 mg/L)**	**T2 (1 mg/L)**	**T3 (2 mg/L)**
Initial Weight (BW_i) (g)	1.4^a	1.41^a	1.41^a	1.42^a
Final Weight (BW_f) (g)	5.34^a	4.92^b	4.16^c	3.81^c
Daily Weight Gain (DWG) (%)	4.96^a	3.83^b	3.71^b	3.29^c
Specific Growth Rate (SGR) (%)	2.30^a	2.05^b	1.91^c	1.82^d
Feed Intake (g g^{-1} day^{-1})	0.152^a	0.157^b	0.159^b	0.161^b
Feed Conversion Ratio (FCR)	3.06^a	4.10^b	4.28^b	4.89^c
Survival Rate (%)	96^a	77^b	64^c	51^d

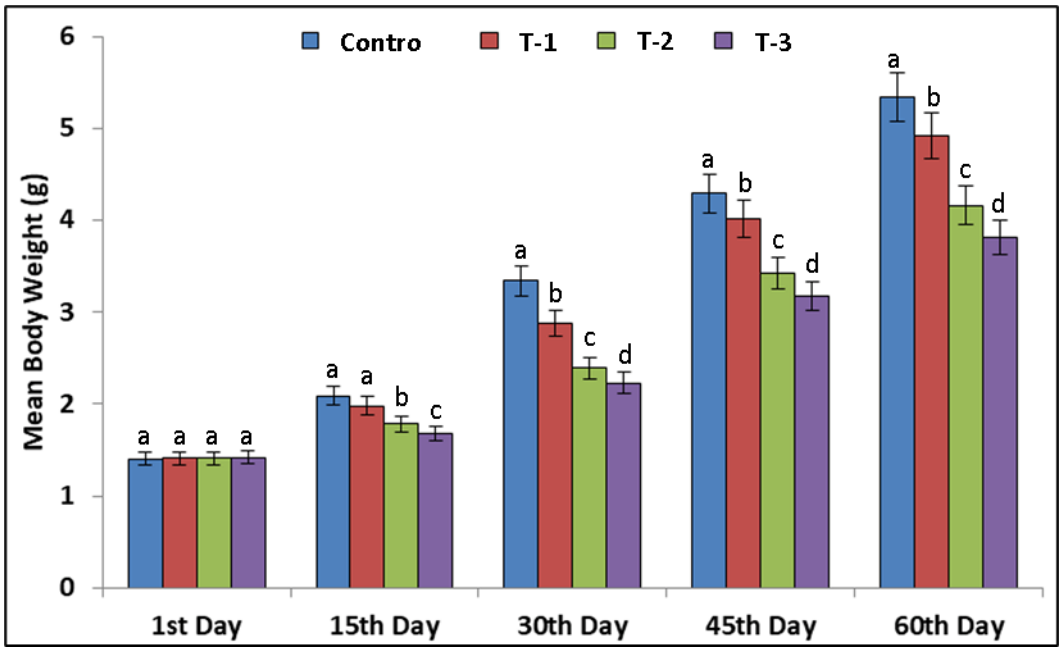

Figure 1: Mean body weight (±S.E.) of experimental Rohu at 15 days interval (N = 30 fish samples per sampling time). Different letters above the bars indicate significant difference at 5% level of significance. T1 = 0.5 mg/L Carbofuran, T2 = 1 mg/L Carbofuran and T3 = 2 mg/L Carbofuran

Carbofuran induced change in oxygen consumption rate

Carbofuran treatment significantly increased ($F(9, 108) = 89.4$, $P < 0.05$) the O_2 consumption rates of the experimental Rohu *(Labeo rohita*). Initially (Day 1), no significant differences were observed among the control and treatments (Figure 2). No significant differences were observed for O_2 consumption rates among the three carbofuran treatments, following which, significant differences were observed among the three treatments from 3rd day to the end. Figure 2 clearly implicate that the highest level of O_2 consumption was at T3 (2 mg/L) and the lowest level at the control.

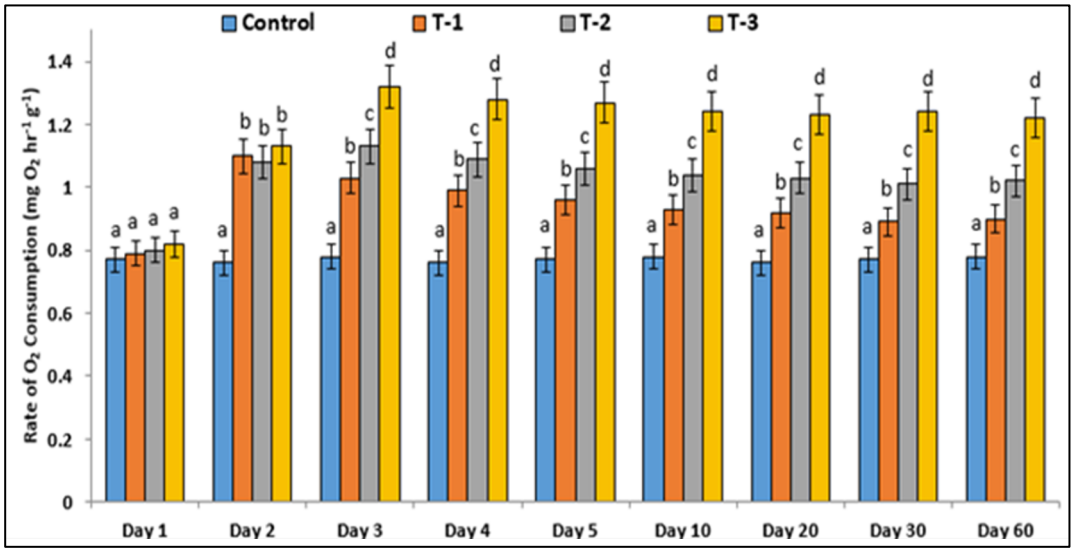

Figure 2: Carbofuran induced changes (Mean ±S.E.) in the rate of O_2 consumption in experimental Rohu (*Labeo rohita*) individuals across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

Carbofuran induced changes in total blood cell counts

Carbofuran treatments significantly altered ($F(9, 108) = 102.3$, $P < 0.05$) the blood cell counts of the experimental Rohu (*Labeo rohita*) (Figure 3). Significantly higher ($P < 0.05$) levels of total blood cell counts were obtained for the control group from 2nd day to the end of this study. No significant differences were observed between the three carbofuran treatments from beginning to the end of this experiment (only exception was for T3, showed significantly lower blood cell counts on 3rd day compared to T1 and T2).

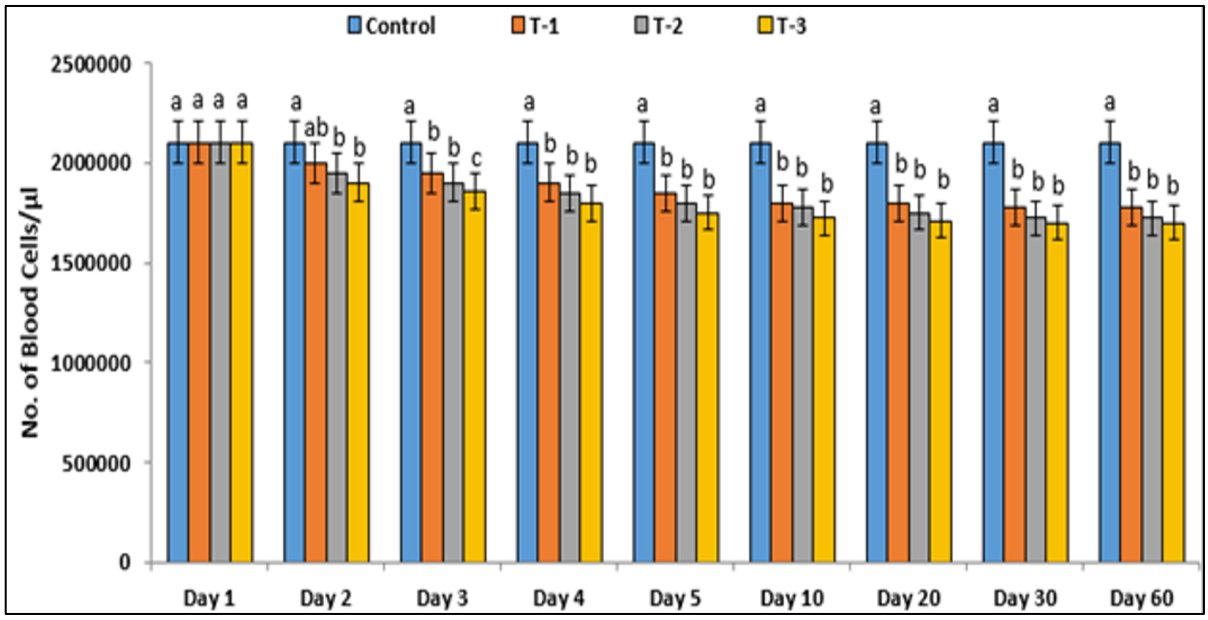

Figure 3: Changes in the number of total blood cell counts for experimental Rohu (*Labeo rohita*) across the sampling times.

Carbofuran treatments significantly ($P < 0.05$) altered the types and number of deformed blood cells (Table 2 and Figure 4). No deformity type or deformed blood cell was detected for the control. Each of the treatment group had differential types and numbers of deformed blood cells; higher number of deformed blood cells and deformity types were observed with increasing carbofuran dose/concentration. The lowest number of deformed blood cells and deformity types were detected at T1 while the highest numbers were at T3. Types of blood cell deformity and number of deformed cells were found to be significantly different among the three carbofuran treatments.

Table 2: Types of deformity and number of deformed blood cells in Rohu (*Labeo rohita*) exposed to different experimental carbofuran doses.

	Control	**T1 (0.5 mg/L)**	**T2 (1 mg/L)**	**T3 (2 mg/L)**
Types of deformity	0	4^{a}	5^{ab}	6^{b}
Number of deformed cells	0	5^{a}	8^{b}	10^{c}

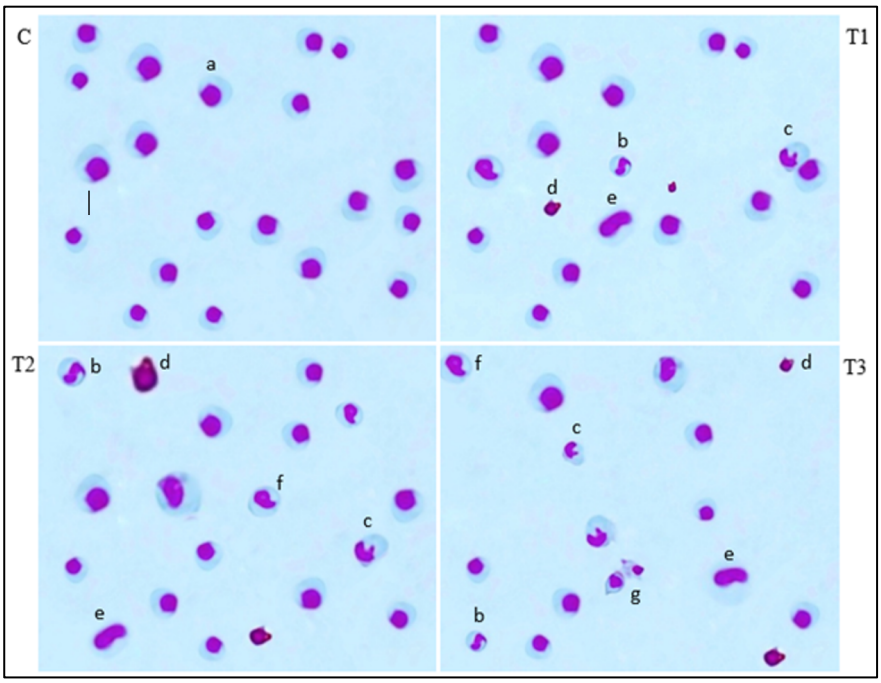

Figure 4: Types of blood cell deformities of experimental Rohu (*Labeo rohita*) at different doses of Carbofuran (imaging at 100x magnification on 5µm area). Here, a) normal blood cells of control group (no deformity of blood cells), b) change in shape/size of nucleus, c) breakdown of nucleus, d) complete lysis/breakdown of cell wall, e) kidney shaped nucleus, f) micro nuclei formation, g) double nucleus formation.

Changes in blood cortisol and glucose levels

In contrast to the other parameters, significantly higher ($P < 0.05$) blood cortisol (stress hormone) and glucose levels were observed for the three carbofuran treatments over the control throughout the experiment (Figures 5 and 6). Both the cortisol and glucose levels were found to increase gradually up to the 5th day, followed by a gradual decrease from 5th to 10th day, and finally stable levels for the remaining time frame. Blood glucose levels were found to vary significantly among the three treatments groups from 4th day to the end of this study while blood cortisol levels showed very inconsistent pattern between the three treatments in terms of significance (significant differences were found between the treatments only on 4th and 5th day).

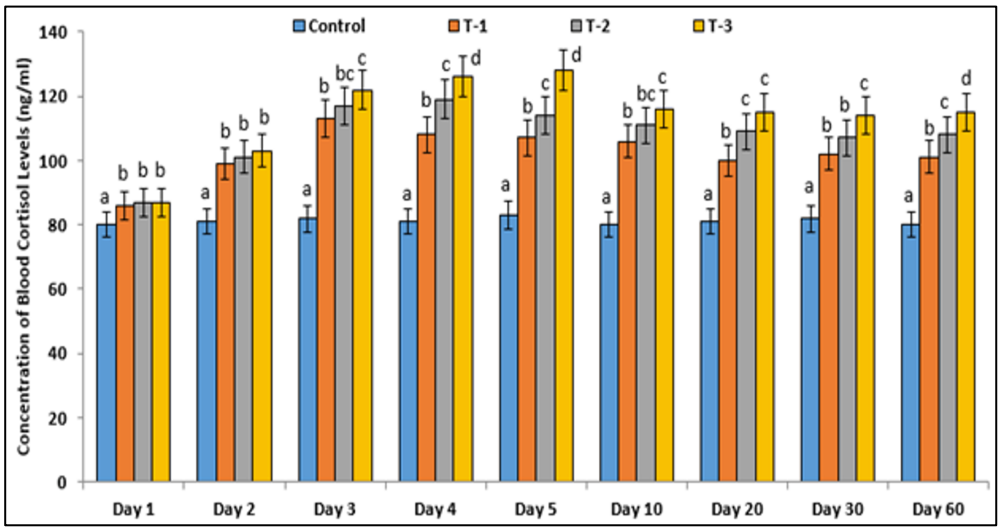

Figure 5: Changes in the number of blood cortisol levels for experimental Rohu (*Labeo rohita*) across the sampling times.

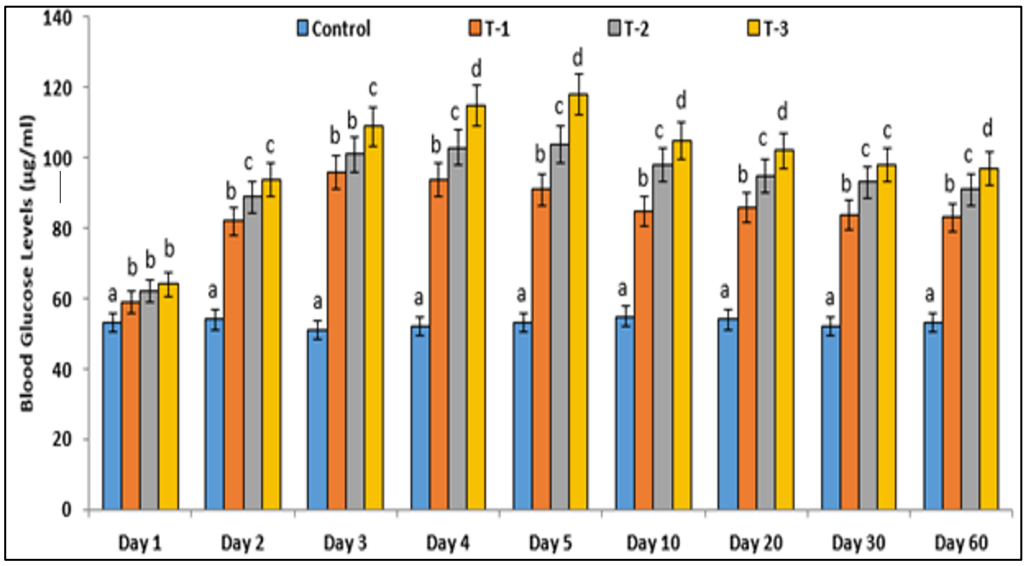

Figure 6: Changes in blood glucose levels of experimental Rohu (*Labeo rohita*) across the sampling times.

Changes in gill structure

Any kind of change in gill lamellae and gill filaments could not be found in the control group but huge changes were found in the treatment group (Figure 7). A slightly damaged and ruptured gill filament was found in T1 (.5mgL) carbofuran. Heavy load of mucus/slime cells, damaged gill filament, and ruptured gill lamellae were found in T2 (1mg/L) carbofuran. Heavy load of mucus/slime cells, damaged gill filament, and severely ruptured gill lamellae were found in T3 (2mg/L) carbofuran.

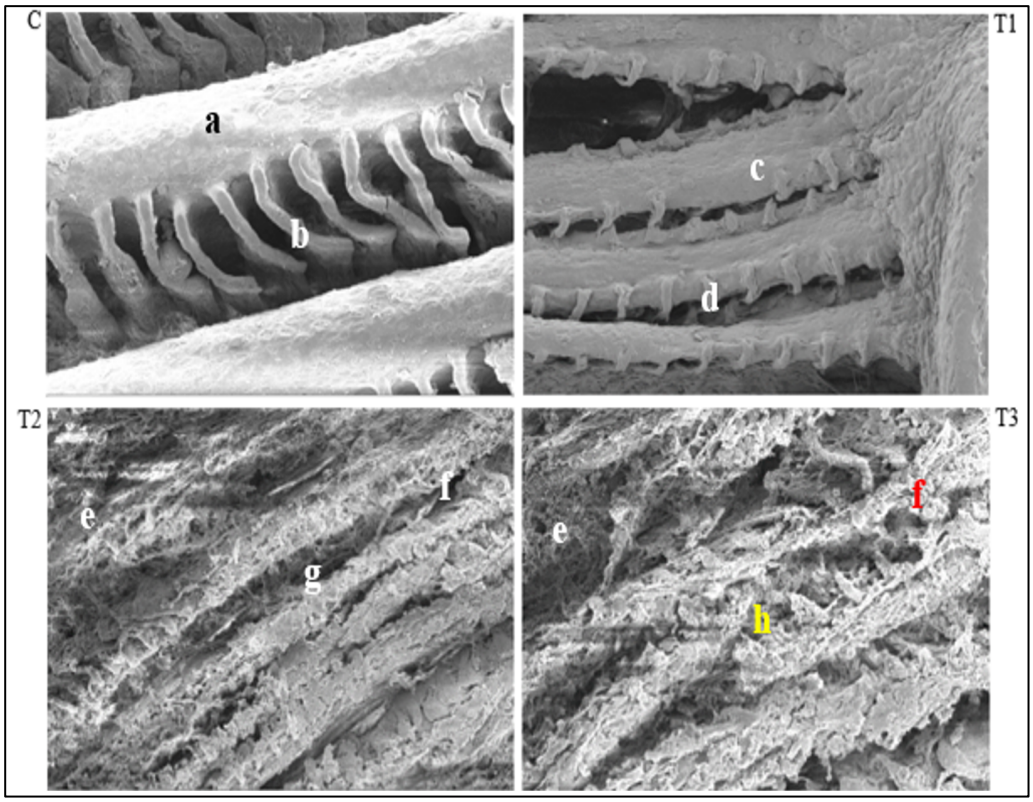

Figure 7: Gill ultra-structure of Rohu (*Labeo rohita*) through scanning electron microscopy (SEM): a) Normal structure of gill filament in control condition, b) Normal structure of gill lamellae in control condition, c) slightly damaged gill filament in T1 (0.5 mg/L Carbofuran),

d) slightly ruptured gill filament in T1, e) heavy load of mucus/slime cells in T2 (1 mg/L Carbofuran) and T3 (2 mg/L Carbofuran), f) damaged gill filament in T2 and T3, g) ruptured gill lamellae in T2 and h) severely ruptured gill lamellae in T3. Images were taken at 1000x magnification covering 20µm area.

Discussion

Significantly higher growth and survival performance of Rohu ($P < 0.05$) at control condition over the treatments (Table 1 and Figure 1) indicate adverse effects of carbofuran on growth and survivability. Significant differences ($P < 0.05$) in growth and survival performance among the treatments (T1 – T3) indicate different carbofuran doses differentially affected experimental Rohu. Any type of environmental stressor adversely affects the growth, wellbeing and survivability of fish with severity depends on the intensity of stress (Sabbir et al., 2010; Moshtaghi et al., 2018; Rahi et al., 2018). Therefore, carbofuran dose specific significant differences in growth and survivability were observed among the experimental Rohu in this study.

The O_2 consumption is an important indicator for the physiological status of an organism that directly influences the entire biological functions (Mushigeri & David, 2002; Aziz et al., 2018; Rahi et al., 2021b). Fishes tend to show higher rates of O_2 consumption under stressful conditions to counterbalance the imposed stress, therefore, any deviation from the standard rate of O_2 consumption is an indicative of the intensity of stress (Foss et. al., 2003; Islam et al., 2011; Lai, 2017; Rahi et al., 2021c). Significantly higher O_2 consumption rates in T1 – T3 (Figure 2) compared to the control indicate higher levels of imposed stress due to carbofuran treatment on treatment groups of Rohu. The highest level of O_2 consumption at T3, followed by T2 and T1 indicate carbofuran dose specific strength of stress on experimental Rohu.

Counting the number of blood cells is an important indicator for evaluating the overall health and immunity status of a fish (Shirangi et al., 2016; Rahi et al., 2017; De et al., 2019; Rahi et al., 2023). Higher blood cells count indicate better immunity and health status while lower counts indicate poor health condition (Li et al., 2014; Seibel et al., 2021). Under stressful conditions, blood cell lysis occurs causing reductions in total blood cell counts while also increase different types of blood cell deformity (Islam et al., 2014; Chowdhury et al., 2023; Mou et al., 2024) Significantly higher number of blood cell counts (Figure 3) and lower deformity types (Figure 4 and Table 2) in the control group compared to the treatments (T1 – T3) clearly support the findings of earlier studies.

Blood cortisol and blood glucose levels are also two important additional parameters that indicate the actual intensity of stress on fish (Islam et al., 2015; De et al., 2019; Mridul, 2024). Different fish species have a specific range of glucose and cortisol level for optimum functions; any increase from this range indicates the spectrum of stress (Seibel et al., 2021; Zeehad et al., 2024). In the current study, Rohu *(Labeo rohita*) exposed to carbofuran treatments showed higher levels of glucose and cortisol compared to the control (Figures 5 and 6) indicate higher levels of imposed stress.

Gills are the primary route of entry for any pollutant in fish that pose degenerative changes in the exposed tissues of fish (Winkalar et al., 2007; Akram et al., 2023; Muhammad et al., 2023). Sub-lethal concentrations of different pollutants are known to damage the filaments as well as primary and secondary gill lamellae of fish (Schreck et al. 2001; Saravanan et al., 2011; Aziz et al., 2017; Aliko et al., 2018; Somero 2020). Experimental fishes (*Labeo rohita*) exposed to

carbofuran treatments affected the gill tissue structure remarkably (Figure 7) with moderate to severe damage depending on the dose of pesticide (T1 – T3). This damage in the gill region of *L. rohita* makes them extremely vulnerable to pathogenic infections, difficulties in O_2 transportation through the blood stream.

Conclusion

The present study investigated the effects of carbofuran on Rohu *(Labeo rohita*) to infer how it affects different biological aspects of this important fish species. Results of this study demonstrate that different carbofuran doses adversely affected the growth, survival rate, O_2 consumption, blood parameters and gill ultra-structure of Rohu. Blood glucose and cortisol levels were increased with carbofuran treatments while remaining other parameters showed declining trends. Higher levels of blood and cortisol levels have an inverse relationship with fish growth. Results also indicate a minor dose of pesticide can impose a negative effect on fish both in the wild and farming conditions. Therefore, appropriate measures must be taken to restrict the use of pesticides for agricultural activities (by controlling the indiscriminate use and also ensure the use of biodegradable pesticides).

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Ethical approval

The authors confirm that animal ethics clearance was approved by the "Animal Ethics Committe" of Khulna University (Ref. No.: KUAEC-2021/09/21).

Informed consent

Not available

Data availability statement

The authors confirm that they do not have any data to share. All of the generated from the experiments conducted under this study are presented in the form of tables and figures.

Conflicts of interest

The authors confirm that there is no conflict of interests for publishing this study.

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Ayesha Akter MITU: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing original draft.
Wasim AKRAM: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing original draft.
Md. Monirul Islam MRIDUL: Conceptualization, Investigation, Methodology, Software, Validation, Review and editing.
Md. Shariar Kabir ZEEHAD: Data curation, Formal analysis, Methodology, Validation, Visualization.

Md. Lifat RAHI: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Review and editing.
"All authors have read and agreed to the published version of the manuscript."

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