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Effect of Toxicity of Chromium (VI) Stressors Alone and Combined to High Temperature on the Histopathological, Antioxidation, Immunity, and Energy Metabolism in Fish *Phoxinus lagowskii*

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Abstract: Fish in aquatic ecosystems are often impacted by environmental stressors like temperature fluctuations and exposure to heavy metals. Chromium (Cr^{6+}) is a known environmental pollutant that poses a threat to aquatic life. Various environmental factors, such as water temperature, have been found to affect the toxicity of dissolved chemicals in aquatic ecosystems. We investigated the toxicity of combinations of different concentrations of hexavalent chromium (Cr^{6+}) with high temperatures in fish. Hematological indices demonstrated changes in white blood cells (WBCs), hematocrit (HCT), red blood cells (RBCs), and hemoglobin (Hb) levels during the exposure. The qualitative and semi-quantitative analyses of different tissues confirmed that higher concentrations of Cr^{6+} caused more significant damage than lower concentrations, with evident alterations observed in circulatory and regressive aspects. Furthermore, brain acetylcholinesterase levels decreased in both single heavy metal exposure and combined exposure at a high temperature. The activity of antioxidant oxidase and immunological parameters increased in all treatment groups compared with the control group following long-term exposure. A significant and increased effect of Cr^{6+} in the high-temperature groups was observed on the evaluated biomarkers, suggesting a possible synergistic effect between Cr^{6+} and increased temperature. The integrated biomarker response (IBR) reported the highest level of stress at 10 mg/L Cr^{6+} combined with high temperature. The IBR analysis revealed that the highest activity of response enzymes, such as acid phosphatase (ACP), superoxide dismutase (SOD), and glutathione S-transferases (GST), was observed in the liver, whereas the gills displayed alkaline phosphatase (ALP), GST, and SOD activity, and the kidneys demonstrated SOD, ACP, and aspartate aminotransferase (AST) to be most active. Through histopathology, antioxidant enzymes, and metabolism- and immunity-related enzymes, we determined that high temperatures enhance the potential toxicity of Cr^{6+} in fish. We recommend conducting a thorough assessment of the impact of climate change, particularly temperature fluctuations, when studying the toxic effects of metal pollution, like chromium, in aquatic ecosystems.

Keywords: hexavalent chromium; fish toxicity; histopathology; antioxidant enzymes; metabolism- and immunity-related enzymes

Key Contribution: (1) High temperature combined with Cr is more damaging to fish than exposure to Cr alone. (2) Hematological indices demonstrated changes in white blood cells (WBCs), hematocrit (HCT), red blood cells (RBCs), and hemoglobin (Hb) levels during the exposure. (3) The qualitative and semi-quantitative tissue analyses confirmed that higher Cr^{6+} concentrations caused greater damage than lower concentrations. (4) The integrated biomarker response (IBR) reported the highest level of stress at 10 mg/L Cr^{6+} combined with high temperature.

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1. Introduction

The rapid economic development has resulted in an urgent environmental pollution concern [1], which is considerably evident in the proliferation and deposition of numerous heavy metals, which significantly impact the freshwater system [2,3]. Yang et al. (2018) identified chromium (Cr) as a priority heavy metal in China, particularly in agricultural regions, due to its common presence in the environment, and the main sources of chromium are anthropogenic activities such as mineral resources development, metal processing, smelting, and chemical production [4]. The concentration of Cr in aquatic environments exists primarily as trivalent chromium (Cr^{3+}) and hexavalent chromium (Cr^{6+}), from 3 μg/L to 40 mg/L [5]. Cr^{3+} is a crucial element involved in lipid, carbohydrate, and protein metabolism [6]. Upon entering cells, Cr^{6+} undergoes reduction by cellular reductants, leading to the production of reactive oxygen species (ROS) and ultimately resulting in oxidative stress damage, genotoxicity, and cytotoxicity in organisms [7,8]. Numerous studies have been conducted on its long-term exposure, which is harmful to aquatic animals, including the freshwater water flea *Daphnia magna*, *Channa punctatus*, and *Labeo rohita* [9–11]. Unfortunately, excessive application of Cr in several industries causes the discharge of untreated wastewater containing Cr^{6+} into water [9]. Furthermore, the concentration ranges of Cr in water and sediment samples in the rivers and lakes in the Heilongjiang Province were found to be 0.015 to 0.297 mg/L [12–14]. The wide distribution and high concentrations of Cr in aquatic environments have raised concerns regarding its safety in aquatic organisms.

The toxicity of metals, such as Cr, in aquatic systems is influenced by non-biological factors like high temperatures [15]. When exposed to high temperatures, the toxicity of chromium increases significantly in fish and other aquatic animals due to enhanced bioavailability [16]. Temperature, a susceptible factor for aquatic animals, affects fish by altering their physiological functions, such as growth, metabolism, reproduction, and the ability to regulate the stability of their internal environment in response to changes in the external environment [17]. A study found that the uptake rate and sensitivity of cadmium to water fleas *D. magna* increased as the temperature rose from 10 to 35 °C [18]. The combination of climate change and contaminant exposure could pose challenges for organisms at the limits of their physiological tolerance [19]. The interaction between heat stress and heavy metal (Cu) is known to exert a negative impact on *Oryzias melastigma* (10 and 32 °C) [19]. Wang et al. (2019) reported that low temperature at 12 °C and high temperature 20–24 °C can enhance the toxicity of chemicals to freshwater organisms, whereas these displayed the highest tolerance toward chemicals at their physiological optimal temperatures [20]. Such heat events are expected to occur more frequently in the future due to global climate change scenarios (IPCC, 2021). However, limited information is available on the specific toxic effects on fish and the physiological mechanisms of these contaminants on freshwater organisms during heat events.

Tissue histopathological investigation is a valuable method to assess the effects of environmental contaminants on the vital organs of fish under laboratory conditions [21]. The extent of pathological changes in different organs of fish is determined by the magnitude of the concentration and duration of exposure to pollutants [22]. Gills serve as the initial organs that are adversely affected in fish, which are highly susceptible to infection from water pollutants due to constant exposure, and respiratory problems serve as significant and early indicators of pollution exposure [23]. Additionally, fusion of lamellae, hyperplasia of epithelial cells, necrosis, cystic structures in the epithelium of secondary lamellae, and loss of secondary lamellae were observed in the gill tissue of heavy-metal-exposed *Oreochromis mossambicus*, *Mastacembelus armatus*, and *Anguilla* [23–26]. The liver is involved in the production of metabolism and is the primary organ for clearing toxic pollutants from the blood. The liver is easily damaged by the toxic effects of several pollutants; therefore, it is an important marker of aquatic pollution. In addition, kidneys and other organs are damaged by toxic effects. Temperature and exposure to contamination (inorganic and organic) may generate ROS, such as hydrogen peroxide, superoxide, and the hydroxyl radical, in

fish. Interestingly, dietary excessive Cr^{6+} did not decrease the oxidative stress or apoptosis in mud crabs, indicating a strong resistance to Cr^{6+} toxicity [27]. Thus, the response mechanisms of different species to Cr vary, and these data should be supplemented.

Amur minnow (*Phoxinus lagowskii*), a cold-water fish species and a common edible fish, is widely found in northeast China, Korea, and Russia [28]. The adverse effects of temperature increases on fish are well documented. However, the specific impact of a combination of high temperature and heavy metal stress has not yet been investigated in cold-water fish. In addition, *P. lagowskii* is an economically important freshwater fish which has been used as a sentinel species to evaluate the potential effects of environmental changes [29]. Although it is a highly suitable candidate species for the diversification of aquaculture due to its consumer preference and commercial properties, it has not received considerable research attention in China. Therefore, the effects of heavy metal Cr^{6+} stressors, alone and combined, and high temperature on *P. lagowskii* warrant investigation.

Biological detection is a valuable approach to assessing the concentrations of heavy metals in aquatic environments. It is crucial to carefully select organisms displaying significant effects [30]. Fish, as ectothermic organisms, are highly vulnerable to the impacts of climate change because of the effect of temperature on their metabolic rates and physiological functions [31]. To maintain fish species, their responses under exposure and metal accumulation in different fish body parts should be studied to suggest measures for the sustainable conservation of these cyprinids [32]. In the present study, we evaluated the long-term toxicity (30 days) of Cr alone and in combination with normal (18 °C) and high (25 °C) temperatures in *P. lagowskii*. To investigate the impact of Cr^{6+} exposure alone and in combination with heat on various parameters of *P. lagowskii* (histopathology, antioxidant activity, immune response, and energy metabolism), we examined the response mechanism of *P. lagowskii* to Cr^{6+} alone and Cr^{6+} at different temperature conditions.

2. Materials and Methods

2.1. Fish

A total of 360 healthy *P. lagowskii* (36.5 ± 3.32 g) were randomly allocated to 30 tanks (10 groups), resulting in 12 fish and 30 L of water in each tank. Dissolved oxygen, temperature, pH, and salinity (7.5 ± 0.5 mg/L, 18.0 ± 1.0 °C, 7.3 ± 0.2, and 28.0 ± 0.5, respectively) were monitored daily with a photoperiod of 14 h light/10 h dark. The bait rate was 2% of the body weight, which was fed twice daily for 4 weeks. Cr^{6+} solution was prepared from chromium trioxide (CAS: 1333–82–0, 99%, Macklin Biochemical Co., Ltd., Shanghai, China). This study was approved by the Ethics Committee of Harbin Normal University (HNUARIA2023009).

2.2. Experiment Design

All concentrations of Cr^{6+} were tested in triplicate, and the number of dead animals was recorded during 96 h of exposure to calculate the LC_{50} value using the Probit analysis method [33]. The following treatment protocol was used: Cr alone exposed with regular temperature, 18 °C (0 mg/L, C; 0.1 mg/L, T1; 1 mg/L, T2; 5.0 mg/L, T3; 10.0 mg/L, T4), and Cr^{6+} with high temperature at 25 °C (0 mg/L, CH; 0.1 mg/L, TH1; 1 mg/L, TH2; 5.0 mg/L, TH3; 10.0 mg/L, TH4) for 30 days. The following experimental groups were used opposite to the control group: unexposed to Cr; high temperature; Cr^{6+} exposure group: environmental concentration, 1/50th, 1/10th, and 1/5th of LC_{50}; and concurrent exposure to Cr^{6+} with high temperature (25 °C) (environmental concentration, 1/50th, 1/10th, and 1/5th of LC_{50}) groups. The Cr^{6+} and temperature were consistently monitored every other day during manual water exchange.

2.3. Blood Collection and Tissue Sampling

Fish were anesthetized using MS-222 (200 μg/mL, Green Hengxing, Beijing, China). After centrifuging the blood at 3000 revolutions per minute for 10 min, the non-hemolyzed plasma was stored in a deep freezer for subsequent biochemical analysis and evaluation of

hematological parameters, including red blood cell counts (RBC), white blood cell counts (WBC), hematocrit (Hct), hemoglobin (HGB), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and mean red blood cell hemoglobin content (MCH). Hematological indices were analyzed following the method described by [34]. One-third of the tissue was taken and fixed in Bouin solution, and one-third of the brain was extracted and kept at −20 °C to measure the biochemical indicators. One-third of the tissue was directly kept in liquid nitrogen and subsequently kept at −80 °C to extract the total RNA.

2.4. Serum Cortisol Concentrations

Serum cortisol concentrations were calculated using a custom enzyme-linked immunosorbent assay (ELISA) kit (Qiaodu Biotechnology, Shanghai, China) published for *Acanthopagrus schlegelii* and *Oncorhynchus mykiss* [35].

2.5. Histological Observation

The damage to the liver, gills, and kidney caused by Cr^{6+} alone and combined with high-temperature exposure was studied using hematoxylin and eosin (H&E) staining. Briefly, the tissues were immersed in 4% paraformaldehyde fixation fluid for 24 h. Then, the fixation fluid was replaced and the samples were fixed for at least 96 h. After fixation, the samples were dehydrated using a series of increasingly concentrated alcohol solutions (ranging from 75% to 100%) and cleared in xylene. Subsequently, the samples were embedded in paraffin and cut into sections with a microtome at a thickness of 5 µm. These sections were stained with hematoxylin and eosin (H&E). Once dried, the sections were observed under a light microscope (Imager A2, Zeiss, Gottingen, Germany) at a magnification of 400× and photographed using a digital camera.

2.6. Qualitative and Semi-Quantitative Tissue Analyses

Histological alterations were primarily studied using the previously published protocols [36,37]. Histopathological condition indices (I) for the liver, gills, and kidneys were adapted from previous studies [38,39]. Histopathological changes in the organs were categorized into five different patterns: circulatory, regressive, progressive, inflammatory, and neoplastic reaction patterns (Table S2). The importance of the observed alterations was defined using an "importance factor" (w) of 1, 2, or 3, indicating minimal, moderate, or severe pathological importance, respectively. Each alteration was also assigned a "score value" (a) from 1 to 6, reflecting the degree and extent of the alteration. Two indices, the reaction index of an organ (I org cat) and the organ index (I org), were calculated based on the importance factors and score values. These indices provide a measure of the significance of the lesions and the degree of damage, facilitating statistical evaluation. Data on reaction patterns and organ pathological indices for the livers and gills are presented as the mean ± standard error for each treatment.

2.7. Biochemical Parameters Determination

Frozen livers, gills, and kidneys were weighed and homogenized in a phosphate buffer (1:10, *w*/*v*). Several parameters, including the MDA content and SOD, CAT, GST, GPx, AST, ALT, ACP, ALP, and AchE activities in the liver, gills, and kidneys, were detected to analyze the effects of Cr^{6+} alone and combined with high temperature on oxidative stress in *P. lagowskii* using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Integrated Biomarker Response (IBR)

The integrated biomarker response (IBR), which is a stress index, was calculated using a previously established method which combines the results obtained from physiological and biochemical biomarkers [40,41]. To standardize the data, Y was calculated using the formula $Y = (X - m)/s$. Here, X represents the mean value of the biomarker for a specific

treatment, while m and s denote the overall mean and standard deviation of all data for that biomarker. To calculate the IBR, a score ($S = Y + |Min|$, $S \geq 0$ and $|Min|$ represents the absolute value for the minimum value for all calculated Y) for each biomarker was obtained. The difference between Yi and Y0 (control) was utilized to establish the biomarker deviation index (A). A was calculated for each biomarker in every exposed group to obtain an integrated multiple biomarker response. The IBRv2 for each group was subsequently calculated by summing the absolute values of A.

2.9. Data Analysis

All values were reported as means ± standard error (SE). The LD50 calculation was conducted using linear regression analysis. For the semi-quantitative assessment of tissues, one-way ANOVA was utilized to examine differences among treatments, with a subsequent Dunnett's test conducted if significant differences were observed in the initial analysis ($p < 0.05$) in order to identify significant differences in comparison to the control group. When assessing the variability of data in enzyme activities, Student's t-test was employed to test for significance between the control and exposed groups at the same time point, given that the data followed a normal distribution and exhibited homogeneity of variance. Furthermore, significance within the same group at different time points was evaluated using one-way ANOVA analysis, followed by Duncan's multiple interval test. A significance level of $p < 0.05$ was considered statistically significant (* < 0.05).

3. Results

3.1. Toxicity Tests

The lethal concentration (LC_{50}) of Cr and cumulative mortality of *P. lagowskii* at different concentrations at 96 h are presented in Table S1. The lethal concentration of Cr was 147.52 mg/L at 96 h. The 96 h LC_{50} concentration of Cr at 25 °C for *P. lagowskii* was 52.18 mg/L.

3.2. Histopathology of Liver, Gills, and Kidneys

3.2.1. Liver

As shown in Figure 1, the livers from the control fish did not display any histopathological changes after long-term exposure. The control group displayed typical liver tissue morphology, including compact liver parenchyma, clear hepatic cords, and centrally located nuclei within cells. Vacuolation foci were observed in the T2 group. In addition, structural changes such as diffuse vacuolation of hepatocytes and nuclear migration were observed in the T3 group, whereas cytoplasmic vacuolization was noted in the T4 group. No lesions were detected in the livers of the CH and TH1 fish. In addition, vacuolation foci were observed in the livers of the TH2 group. Cells in the TH3 group were surrounded by engorged capillaries and lymphocytic infiltrations. The livers in the TH4 group displayed bile stasis and focal necrosis in the biliary epithelium.

3.2.2. Gills

For gills, the unexposed (control) fish did not show pathological alterations in gill tissue (Figure 2). Tissues in the T1 group displayed slight histological alterations, such as edema. Histopathological lesions such as cellular hypertrophy and lamellar fusion were observed in the T2 group. Severe lesions were observed in the T3 group, including cellular hypertrophy, epithelial hyperplasia, curled gill lamella, and lamellar fusion. Severe lamellar fusion was observed in the gill tissues of fish in the T4 group. The histological changes in the gills were also noticed in the Cr^{6+}- and high-temperature-exposed fish, and are shown in Figure 2. Histological alterations such as lamellar curling fusion were significantly observed in the CH and TH1 groups of exposed fish. Gills in the TH3 group showed necrosis, lamellar fusion, and epithelial hyperplasia. Gill histopathological alterations, including necrosis, lamellar fusion, and lifting of lamellar, were observed in the TH4 group.

The most obvious degenerative alterations were hyperemia, epithelial hyperplasia, necrosis, and lamellar fusion.

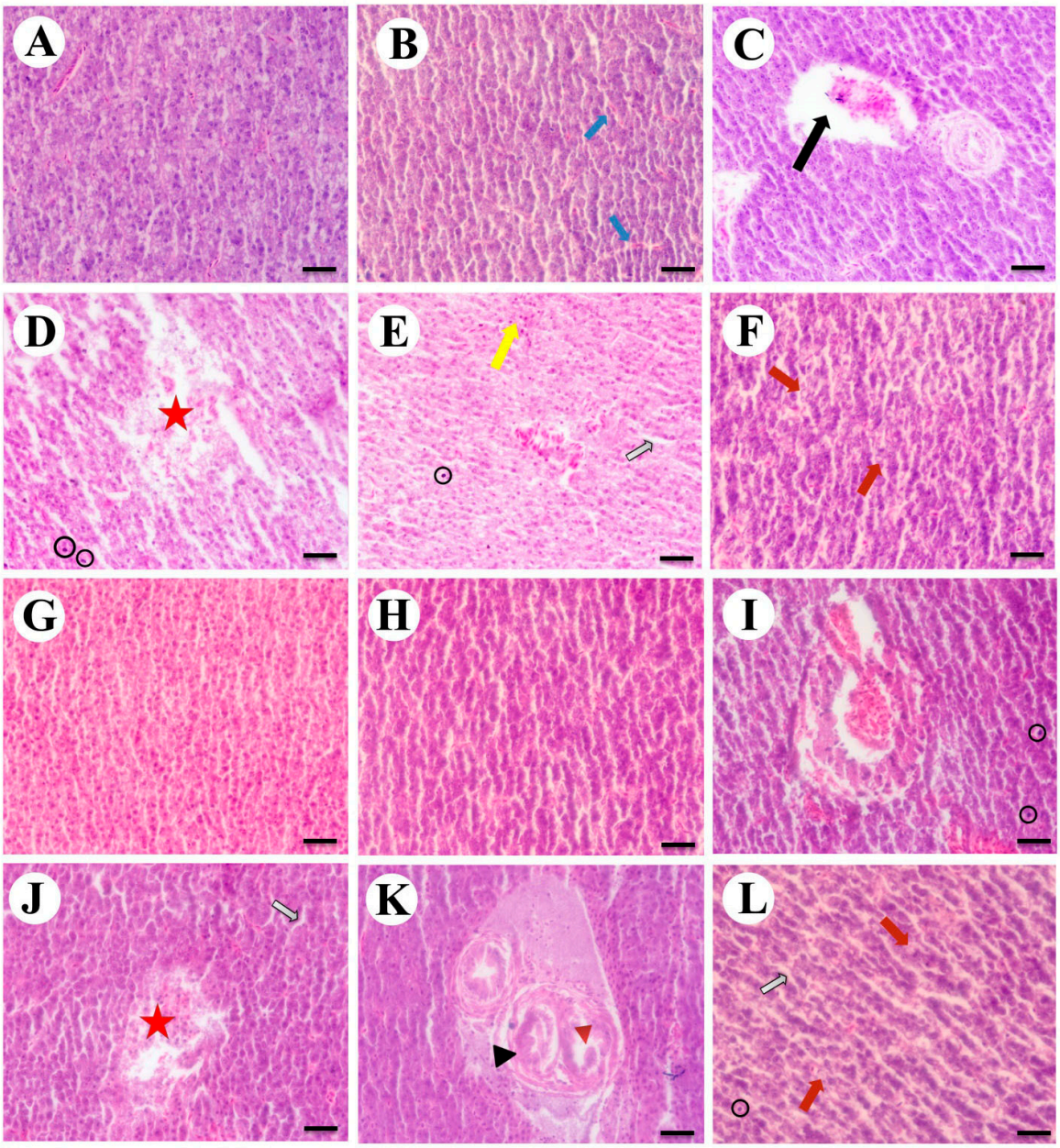

Figure 1. Histological changes in livers of *P. lagowskii* treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature for 4 weeks. (**A**) Control group showing normal structures of liver; (**B**) 0.1 mg/L Cr^{6+} group showing no changes in liver; (**C**) 1 mg/L Cr^{6+} group; (**D**) 5 mg/L Cr^{6+} group; (**E**) 5 mg/L Cr^{6+} group; (**F**) 10 mg/L Cr^{6+} group; (**G**) 25 °C group showing normal structures of liver; (**H**) 0.1 mg/L Cr^{6+}-25 °C group showing no changes in liver; (**I**) 1 mg/L Cr^{6+}-25 °C group; (**J**) 5 mg/L Cr^{6+}-25 °C group; (**K**) 5 mg/L Cr^{6+}-25 °C group; (**L**) 10 mg/L Cr^{6+}-25 °C group. Vacuolation foci are indicated by the black arrow. Diffuse vacuolations of hepatocytes are indicated by the red stars. Nuclear migrations are indicated by the yellow arrows. Cytoplasmic vacuolizations are indicated by the red arrows. Bile stasis and focal necrosis to the biliary epithelium are indicated by the red and black triangle, respectively. Hyperemia signs are shown by blue arrows. Pyknotic nuclei are shown in black circles. Increase of sinusoidal space shown by white arrows. Bars = 20 μm.

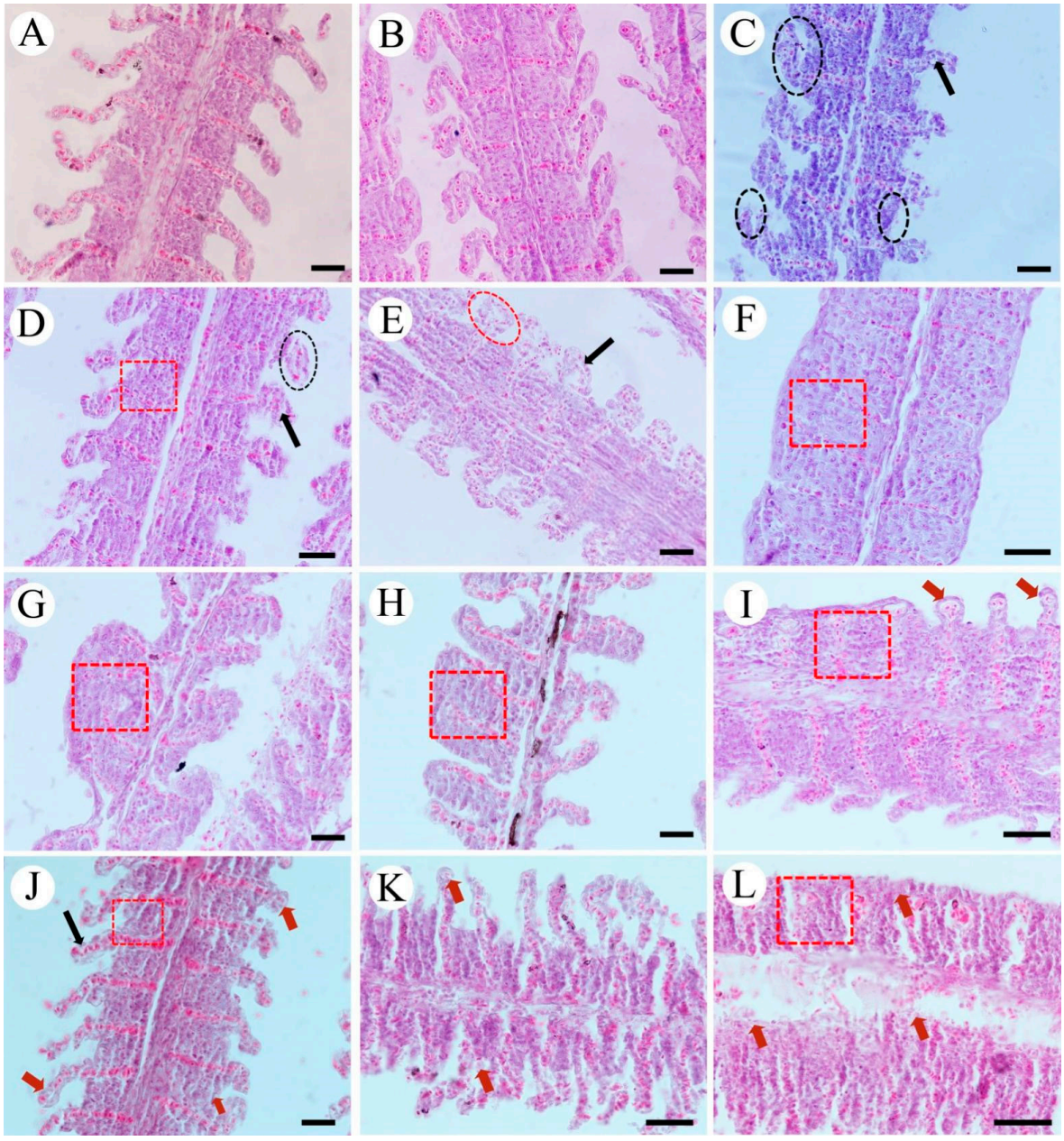

Figure 2. Histological changes in gills of *P. lagowskii* treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature for 4 weeks. (**A**) Control group showing normal structures of gills; (**B**) 0.1 mg/L Cr^{6+} group; (**C**) 1 mg/L Cr^{6+} group; (**D**) 5 mg/L Cr^{6+} group; (**E**) 5 mg/L Cr^{6+} group; (**F**) 10 mg/L Cr^{6+} group; (**G**) 25 °C group showing normal structures of liver; (**H**) 0.1 mg/L Cr^{6+}-25 °C group showing no changes in liver; (**I**) 1 mg/L Cr^{6+}-25 °C group; (**J**) 5 mg/L Cr^{6+}-25 °C group; (K) 10 mg/L Cr^{6+}-25 °C group; (**L**) 10 mg/L Cr^{6+}-25 °C group. Edema is indicated by the black circle. Lifting of lamellar is indicated by the black arrow. Lamellar curling is indicated by the red circle. Lamellar fusion is indicated by the red boxes. Necrosis is indicated by the red arrow. Bars = 10 μm.

3.2.3. Kidneys

The microscopic examination of kidney tissues (Figure 3) revealed normal histological images in the control group. Kidney tissues in T1 and T4 groups displayed normal histological structures. Histopathological lesions such as cystic tubules were observed in the T3 group. An increase in the tubule diameter was observed in the T4 group. Histopathological analysis demonstrated that fish in the TH2 group exhibited normal histological architec-

ture of the kidneys following exposure to 0.1 mg/L Cr^{6+} and high temperature. Hyaline droplet degeneration of the tubular epithelium and cystic tubule were observed in the TH3 group, whereas cystic tubules were found in the 10 mg/L Cr^{6+} and high-temperature co-exposure group.

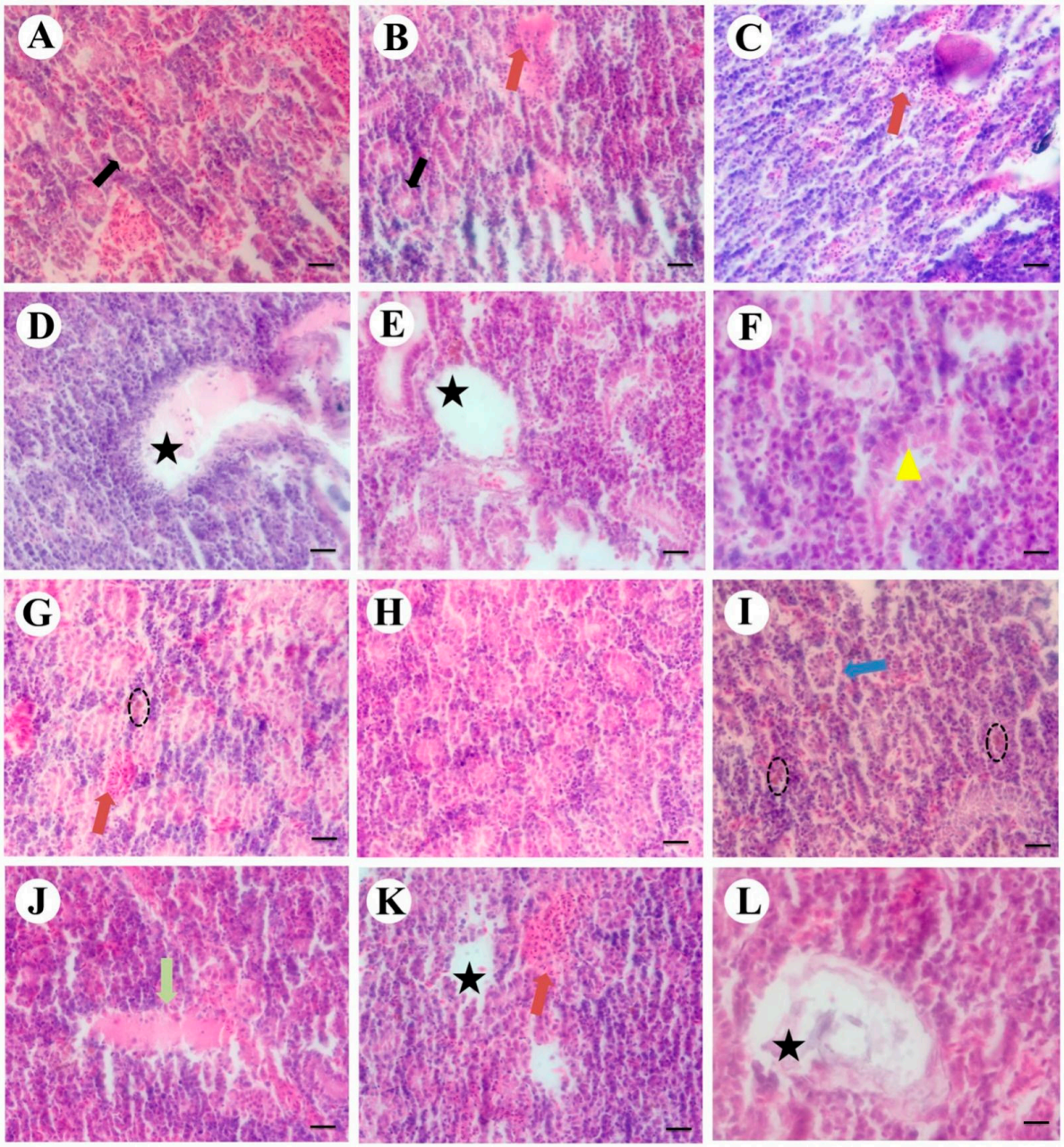

Figure 3. Histological changes in the kidneys in *P. lagowskii* treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature for 4 weeks. (**A**) Control group showing normal structures of liver; (**B**) 0.1 mg/L Cr^{6+} group showing no changes in liver; (**C**) 1 mg/L Cr^{6+} group; (**D**) 5 mg/L Cr^{6+} group; (**E**) 5 mg/L Cr^{6+} group; (**F**) 10 mg/L Cr^{6+} group; (**G**) 25 °C group showing normal structures of liver; (**H**) 0.1 mg/L Cr^{6+}-25 °C group showing no changes in liver; (**I**) 1 mg/L Cr^{6+}-25 °C group; (**J**) 5 mg/L Cr^{6+}-25 °C group; (**K**) 5 mg/L Cr^{6+}-25 °C group; (**L**) 10 mg/L Cr^{6+}-25 °C group. Increase in tubule diameter (ITD) is indicated by the yellow triangle. Cystic tubule is indicated by the black star. Hematopoietic tissues are indicated by the red arrow. Hyaline droplet degeneration of the tubular epithelium is indicated by the gray arrow. Bowman's space is indicated by the blue arrow. Some melanomacrophage centers were also observed, shown by the dashed circles. Bars = 20 μm.

3.2.4. Semi-Quantitative Evaluation of Tissues

Significant changes were observed in the liver for pathological categories in the T2, T3, and T4 groups (Figure 4), namely, circulatory ($F_{[4,10]} = 15.583$, $p < 0.001$), and also in the total pathological index ($F_{[4,10]} = 13.049$; $p < 0.001$). The obtained data ($F_{[4,10]} = 4.074$; $p = 0.033$) demonstrated significant regressive alterations following chronic exposure. A different response pattern, with predominance of circulatory, regressive, and inflammatory alterations, as well as circulatory and regressive changes, was observed following exposure to Cr^{6+} at high temperatures in the TH2, TH3, and TH4 groups compared with the control group. Significant changes were observed in the circulatory ($F_{[4,10]} = 56.833$; $p < 0.001$) and regressive ($F_{[4,10]} = 9.385$; $p = 0.002$) categories as well as the total ($F_{[4,10]} = 47.985$; $p < 0.001$). The total liver's pathological index was significantly higher for organisms exposed to the T2, T3, T4, TH2, TH3, and TH4 groups. There was a significant increase in the circulatory and total changes in TH3 and TH4 compared to fish in T3 and T4, respectively ($p < 0.05$).

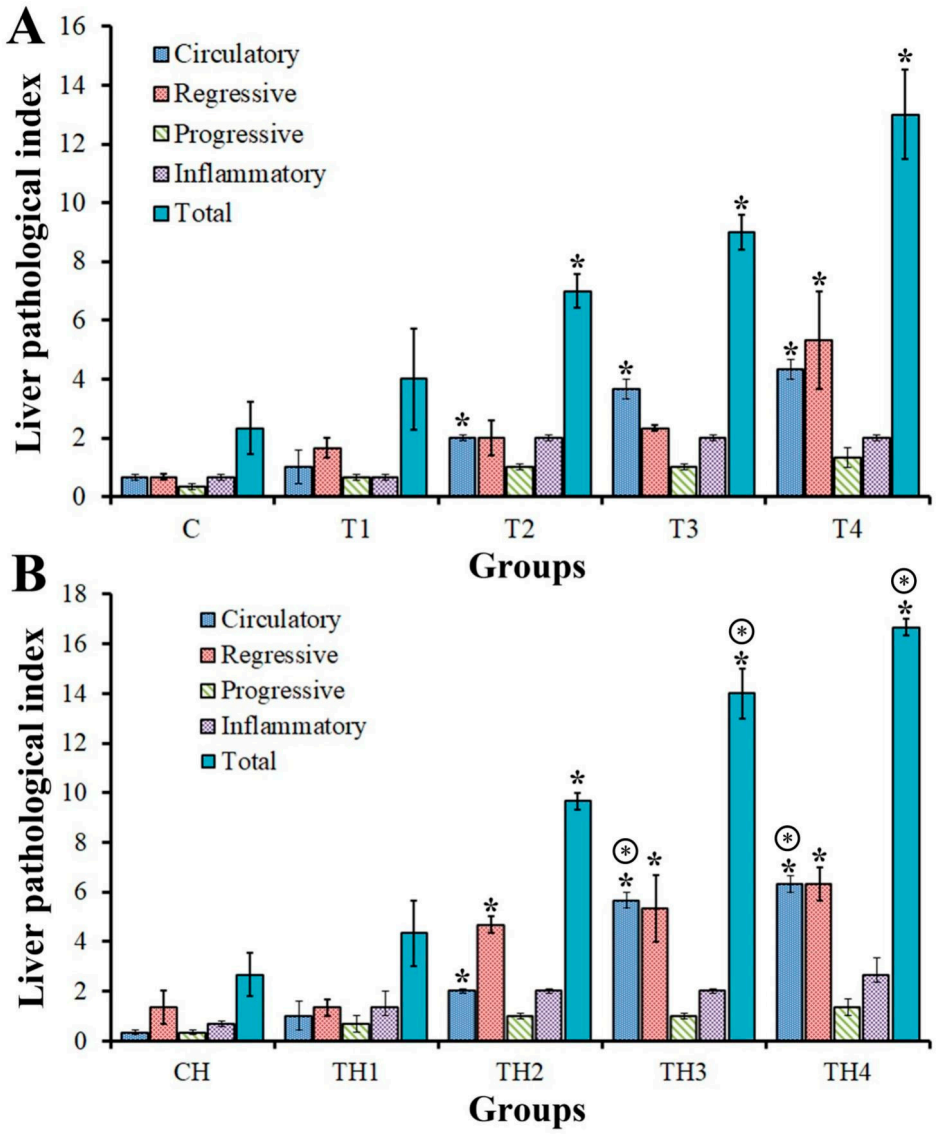

Figure 4. The total and categorical (circulatory, regressive, and progressive) pathological indices for livers after single Cr^{6+} and Cr^{6+} combined with exposure to high temperatures. (**A**) Single Cr^{6+} exposure. (**B**) Cr^{6+} exposure combined with high-temperature exposure. Data are expressed as mean values from each treatment ± standard error (SE). * represents categorical indices significantly different from the control group ($p < 0.05$). Encircled asterisks indicate significant differences compared to Cr^{6+} and relative Cr^{6+} exposures to high temperature ($p < 0.05$).

In the gills, no significant changes were observed for pathological categories (Figure 5). The total gill pathological index was significantly higher when exposed to T2, T3, and T4 groups ($F_{[4,10]} = 7.393$; $p = 0.005$). Significant changes in pathological indexes were observed in the TH2, TH3, and TH4 groups, including regressive ($F_{[4,10]} = 14.056$; $p < 0.001$) and total ($F_{[4,10]} = 13.114$; $p = 0.001$). There was a significant increase in the regressive and total changes in TH3 and TH4 compared to fish in T3 and T4, respectively ($p < 0.05$).

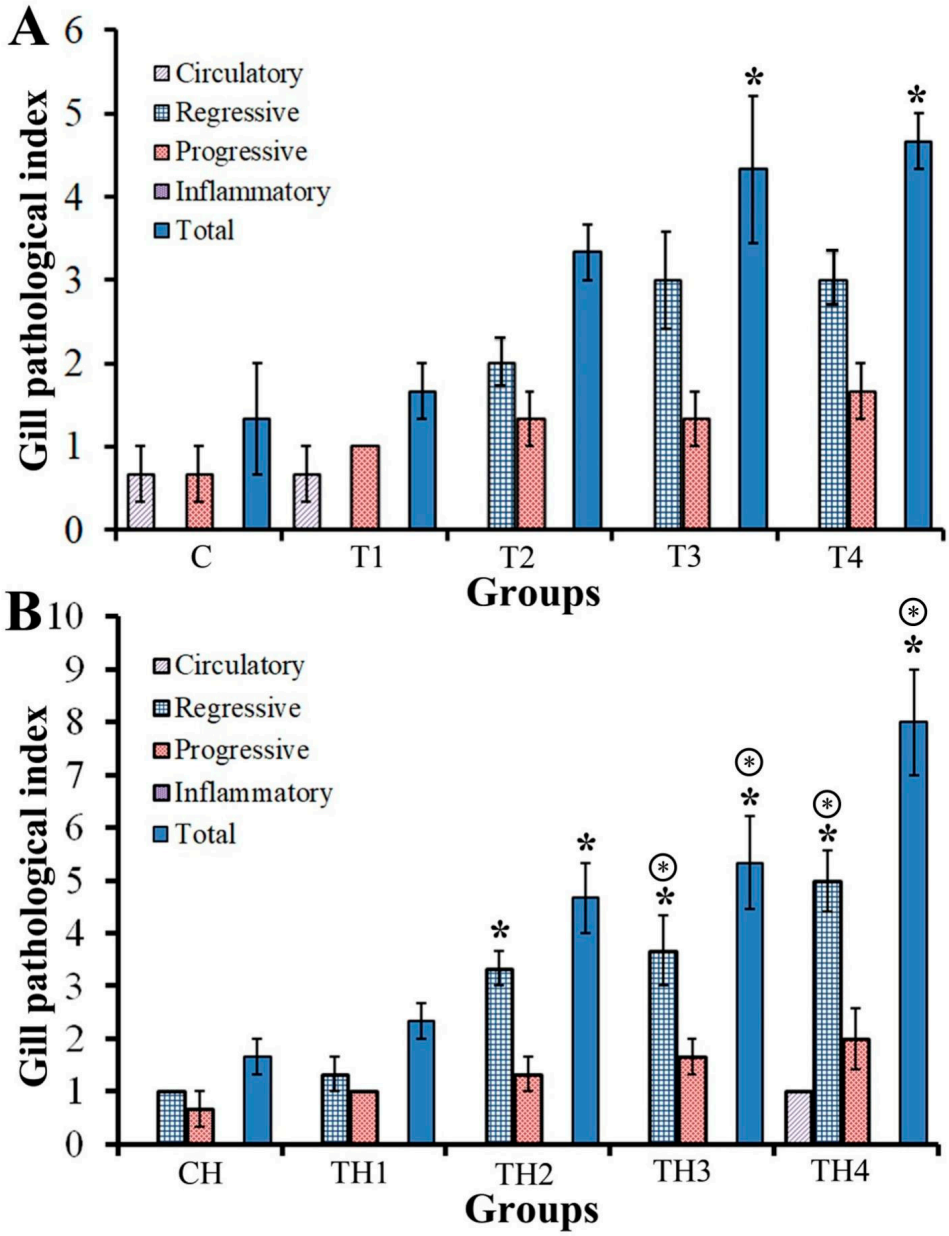

Figure 5. The total and categorical (circulatory, regressive, and progressive) pathological indices for gills after single Cr^{6+} and Cr^{6+} combined with exposures to high temperature. (**A**) Single Cr^{6+} exposure. (**B**) Cr^{6+} exposure combined with high-temperature exposure. Data are expressed as mean values from each treatment ± standard error (SE). * represents categorical indices significantly different from the control group ($p < 0.05$). Encircled asterisks indicate significant differences compared to Cr^{6+} and relative Cr^{6+} exposures to high temperature ($p < 0.05$).

In the kidneys, significant changes were also observed for pathological categories in the T3 and T4 groups (Figure 6), namely, circulatory ($F_{[4,10]} = 10.722$; $p < 0.001$), and also for the total pathological index ($F_{[4,10]} = 6.804$; $p = 0.007$). Regressive lesions were significantly

higher ($F_{[4,10]} = 10.722$; $p < 0.001$). A different pattern of response was also observed after Cr^{6+} with high-temperature exposure; circulatory and regressive changes were significantly higher in the TH3 and TH4 groups compared with the control group. A significant increase in regressive categories in the kidneys was also observed for the highest concentration tested (TH4; $F_{[4,10]} = 4.484$; $p = 0.025$). There was no significant change in the Cr^{6+} alone or Cr^{6+} with high-temperature exposure.

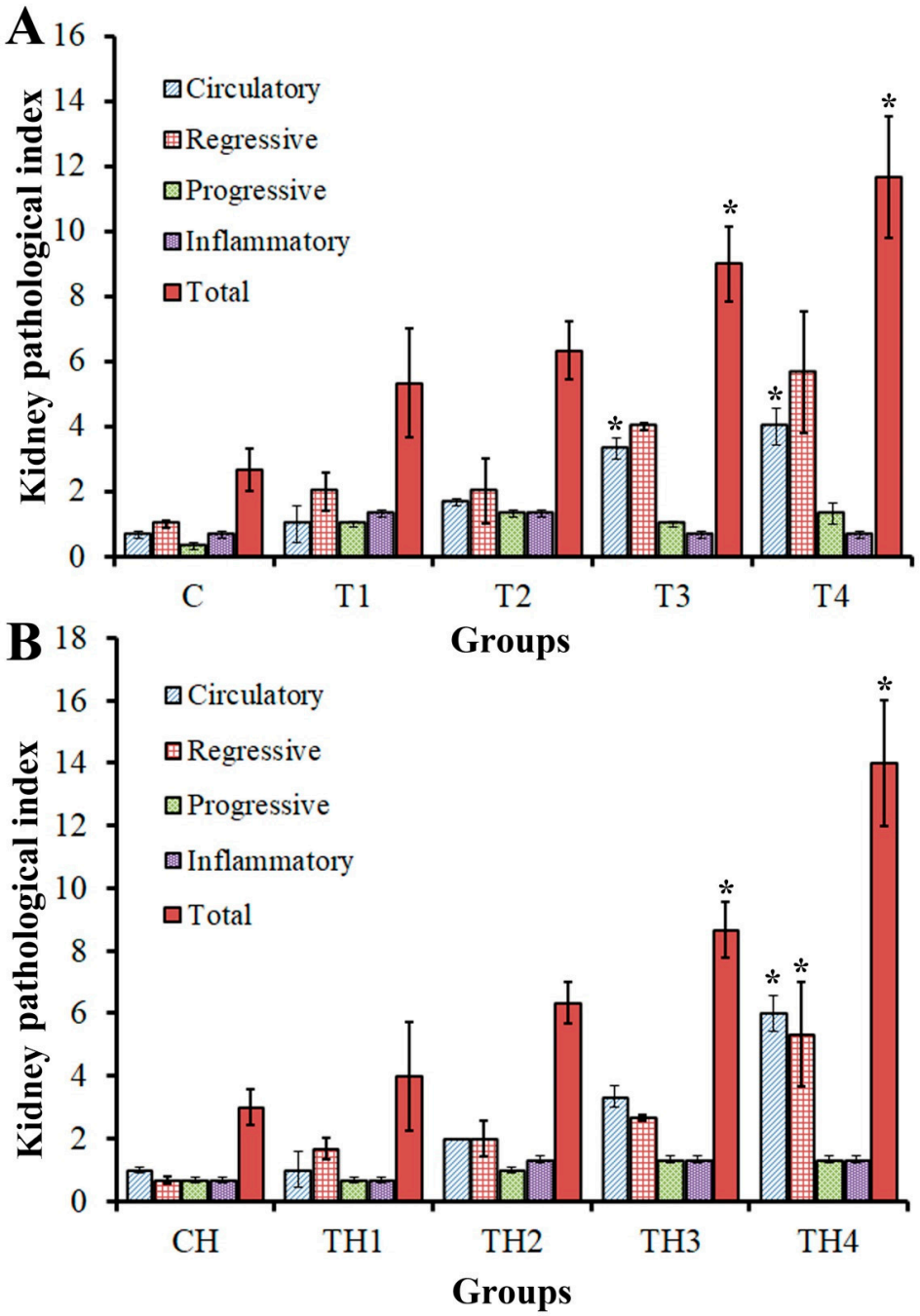

Figure 6. The total and categorical (circulatory, regressive, and progressive) pathological indices for kidney after single Cr^{6+} and Cr^{6+} combined with exposures to high temperature. (**A**) Single Cr^{6+} exposure. (**B**) Cr^{6+} exposure combined with high-temperature exposure. Data are expressed as mean values from each treatment ± standard error (SE). * represents categorical indices significantly different from the control group ($p < 0.05$).

3.3. Hematological Indices

When compared with the control, the number of WBCs was significantly increased in fish exposed to Cr^{6+} alone (Table 1). In Cr^{6+} with high-temperature exposure, the number of WBC increased in the TH4 group compared with the control group. Interestingly, fish in the CH, TH3, and TH4 groups displayed enhanced WBC numbers compared to the C, T3, and T4 groups, respectively. A significant increase in the HGB value was noted in fish exposed to single Cr^{6+} alone. The HGB value in the T4 group was reduced relative to the control group, although these did not differ from the control. In the Cr^{6+} with high-temperature exposure, the HGB value in the TH2 group was significantly lower than that in the CH group. No significant differences in MCHC, MCH, or MCV were observed in fish following long-term exposure to Cr^{6+} alone or Cr^{6+} with high temperature. The HCT value of *P. lagowskii* increased with a surge in the concentration, whereas it was significantly higher in the T4 group than in the control group. During exposure to Cr^{6+} with high-temperature exposure, fish in TH3 and TH4 groups displayed elevated HCT values compared to the T3 and T4 groups, respectively.

3.4. Serum Cortisol

We observed increased cortisol levels following exposure to a single heavy metal and combined high temperature. Cortisol in the T4 group was significantly higher than that in the C, T1, and T2 groups ($p < 0.05$, Figure 7). It was significantly higher in the TH3 group than in the CH, TH1, and TH2 groups ($p < 0.05$). In addition, the T3 group displayed significantly higher cortisol levels than the TH3 group ($p < 0.05$).

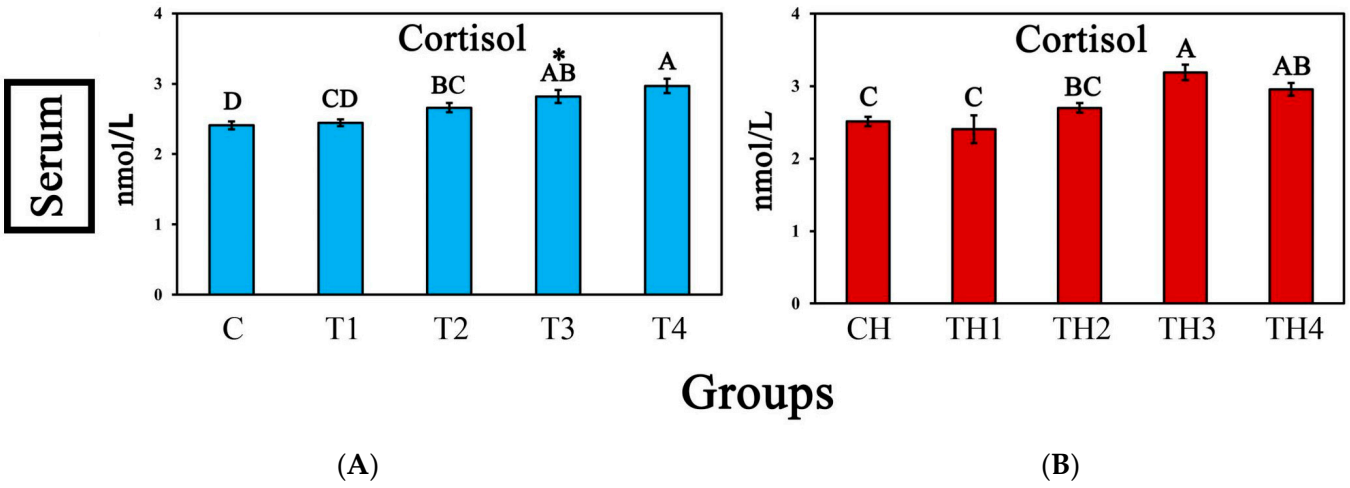

Figure 7. Cortisol levels in the serum in *P. lagowskii* treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature. (**A**) Single Cr^{6+} exposure. (**B**) Cr^{6+} exposure combined with high-temperature exposure. Distinct uppercase letters indicate differences among groups ($p < 0.05$). Asterisk indicates significant difference between Cr^{6+} and relative Cr^{6+} with high temperature.

3.5. Enzyme Activity

The activity of AchE in the T4 group was significantly lower than in the C and T1 groups ($p < 0.05$, Figure 8). The activity of AchE in heavy metal combined with the high-temperature treatment group was lower than that in the control group, and that in the TH3 group was significantly lower than that in the CH group ($p < 0.05$). In addition, AchE activity was significantly higher in the control, T1, and T3 groups, and in the high-temperature group compared to the normal-temperature group ($p < 0.05$).

Table 1. Hematological indices of *Phoxinus lagowskii* under the chromium alone and combined stressors to high temperature exposure. WBC—white blood cells; RBC—red blood cells; HGB—hemoglobin; MCHC—mean corpuscular hemoglobin cell; MCH—mean corpuscular hemoglobin; MCV—mean cell volume; HCT—hematocrit. Groups: 0 mg/L, C; 0.1 mg/L, T1; 1 mg/L, T2; 5.0 mg/L, T3; 10.0 mg/L, T4, and Cr^{6+} with high temperature at 25 °C (0 mg/L, CH; 0.1 mg/L, TH1; 1 mg/L, TH2; 5.0 mg/L, TH3; 10.0 mg/L, TH4. Differences were considered statistically significant at $p < 0.05$; significant effects are presented with different letters. Asterisks indicate significant differences compared to Cr^{6+} and relative Cr^{6+} exposures to high temperature.

Variables	**Groups**									
	C	**T1**	**T2**	**T3**	**T4**	**CH**	**TH1**	**TH2**	**TH3**	**TH4**
WBC	$6.83 \pm 0.87^{b*}$	19.63 ± 2.23^{a}	19.56 ± 2.22^{a}	$18.46 \pm 1.33^{a*}$	$19.3 \pm 4.07^{a*}$	13.82 ± 2.95^{b}	23.07 ± 0.5^{b}	22.22 ± 3.89^{b}	21.3 ± 3.96^{b}	32.91 ± 2.94^{a}
RBC	1.89 ± 0.4^{a}	1.41 ± 0.1^{a}	1.61 ± 0.21^{a}	1.27 ± 0.06^{a}	1.29 ± 0.11^{a}	1.18 ± 0.29^{a}	1.14 ± 0.17^{a}	1.02 ± 0.12^{a}	1.15 ± 0.18^{a}	1.32 ± 0.22^{a}
HGB	72.86 ± 5.34^{a}	61.6 ± 9.41^{ab}	62 ± 7.6^{ab}	48.29 ± 2.33^{b}	57.75 ± 7.2^{ab}	58.83 ± 3.49^{a}	56.5 ± 1.85^{a}	42.6 ± 4.34^{b}	50.8 ± 4.15^{ab}	47.8 ± 3.62^{ab}
MCHC	237.13 ± 4.89^{a}	246.5 ± 7.47^{a}	239 ± 2.07^{a}	235.44 ± 1.5^{a}	235.89 ± 3.39^{a}	241.67 ± 4.04^{a}	246.8 ± 15.27^{a}	243.14 ± 4.18^{a}	246.86 ± 6.96^{a}	239.57 ± 6.12^{a}
MCH	39.81 ± 0.67^{a}	38.53 ± 2.17^{a}	39.3 ± 0.49^{a}	37.93 ± 0.73^{a}	36.71 ± 0.89^{a}	37.28 ± 1.41^{a}	39.46 ± 2.54^{a}	35.41 ± 1.28^{a}	38.57 ± 0.64^{a}	36.66 ± 1.53^{a}
MCV	168.06 ± 2.75^{a}	162.14 ± 6.57^{b}	164.55 ± 1.63^{b}	160.97 ± 2.76^{b}	160.29 ± 2.21^{b}	157.73 ± 5.78^{b}	172.92 ± 3.39^{a}	149.17 ± 5.12^{b}	156.71 ± 3.82^{b}	152.71 ± 3.19^{b}
HCT	20.34 ± 2.55^{b}	21.58 ± 0.56^{ab}	23.4 ± 0.84^{ab}	$24.62 \pm 2.65^{ab*}$	$27.26 \pm 2.16^{a*}$	20.99 ± 1.91^{b}	20.88 ± 0.89^{b}	20.57 ± 1.37^{b}	29.59 ± 1.68^{ab}	32.76 ± 2.05^{a}

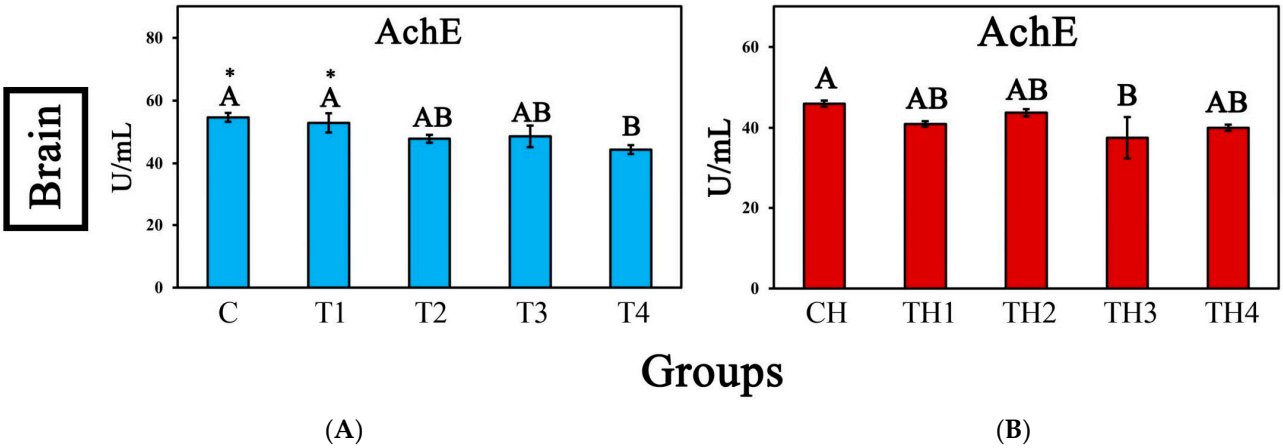

Figure 8. Brain AchE enzyme activities in *P. lagowskii* treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature. (**A**) Single Cr^{6+} exposure. (**B**) Cr^{6+} exposure combined with high-temperature exposure. Distinct uppercase letters indicate differences among groups ($p < 0.05$). Asterisk indicates significant difference between Cr^{6+} and relative Cr^{6+} with high temperature.

Compared with the control, significantly higher values of ACP, ALP, ALT, and AST activities were observed in the liver following exposure to Cr^{6+} with normal and high temperatures (Figure 9). In the Cr^{6+} exposure groups with normal and high temperatures, the ACP values in T3 and T4 fish were higher than in control fish. The ALP activity in the T4 group was significantly higher than that in the control group and T1 group during normal temperature exposure ($p < 0.05$). Similarly, the ALP activity was significantly higher in the TH4 group than in the control group and TH1 group during high-temperature exposure ($p < 0.05$). The ALP activity in the T3 group was significantly higher following exposure to Cr^{6+} with high-temperature exposure ($p < 0.05$). The ALP activity was significantly higher in the TH3 and TH4 groups than that in the control group ($p < 0.05$). In addition, significantly higher values of AST activity were noted in the T4, T1, T2, and T3 groups ($p < 0.05$). The AST activity in the TH4 group was significantly higher than that in the TH1, TH2, and TH3 groups ($p < 0.05$). After 10 mg L^{-1} Cr^{6+} treatment, the activity of ACP and ALP in the TH4 group was significantly higher than that in the T4 group ($p < 0.05$). The activity of ALT and AST in the Cr^{6+} with high-temperature exposure groups was higher than that in the Cr^{6+} with normal temperature groups.

The ACP and ALP activity in the gills in the T4 and TH4 groups was higher than that in the control group (Figure 9). In addition, no significant differences in the ALT activity were observed during the exposure to Cr^{6+} with normal temperature ($p > 0.05$). The activity of ALT in the TH4 group was higher than in the control and treated groups. The activity of AST in the T4 and TH4 groups was significantly higher than in other treatments and control groups ($p < 0.05$). The activity of ALT in the TH4 group was significantly higher than that in the T4 group. The activity of AST following exposure to Cr^{6+} and high temperature was significantly higher than that during exposure to Cr^{6+} with normal temperature ($p < 0.05$).

Compared with the control group, significantly higher ACP activity was observed in the kidneys of the T3 group (Figure 9). The ACP activity did not significantly differ between the Cr^{6+} exposure groups with normal and high temperatures ($p < 0.05$). No significant differences were observed in ALP activity in the Cr^{6+} normal exposure groups and the Cr^{6+} high-temperature exposure groups ($p > 0.05$). The activity of ALT in the T3 group was significantly lower than that in the T4 group, whereas that of AST was significantly higher in the T4 and TH4 groups than in the control group ($p < 0.05$). The activity of ACP in the CH group was significantly higher than in the C group ($p < 0.05$). The ALT activity in the TH3 group was significantly higher than that in the T3 group, and that in the TH4 group was significantly higher than that in the T4 group ($p < 0.05$). The activity of AST in the CH group was significantly higher than that in the C group, the activity of ALT in the TH3

group was significantly higher than that in the T3 group, and that in the TH4 group was significantly higher than that in the T4 group ($p < 0.05$).

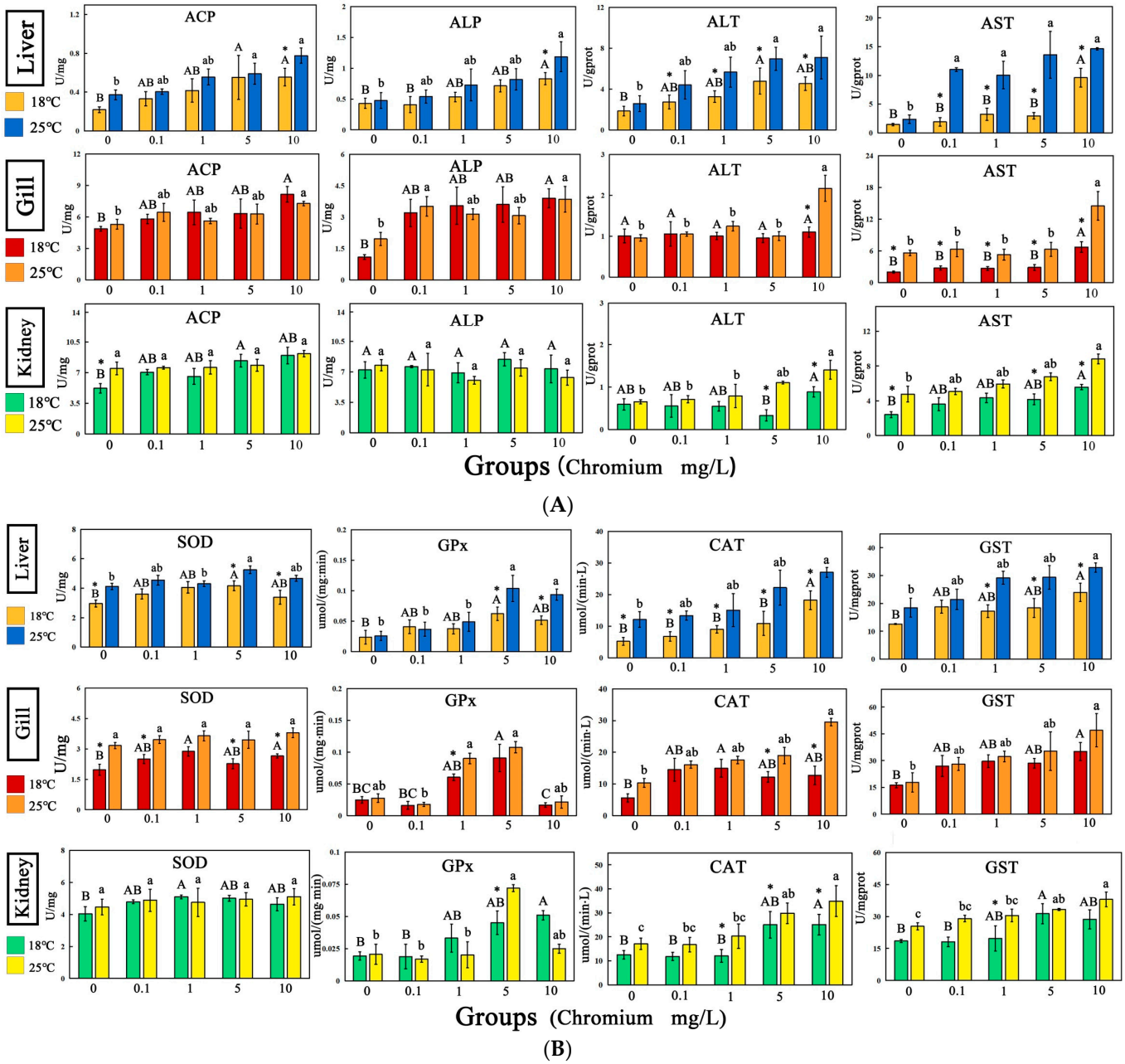

Figure 9. Enzyme activities of physiological and biochemical parameters in livers, gills, and kidneys treated with different combinations of Cr^{6+} alone and Cr^{6+}-high temperature. (**A**) Acid phosphatase (ACP), alkaline phosphatase (ALP), alanine amino-transaminase (ALT), and aspartate aminotransaminase (AST) in *P. lagowskii*. (**B**) Superoxide dismutase (SOD), catalase (CAT), glutathione S-transferases (GST), and glutathione peroxidase (GPx) in *P. lagowskii*. Bars with different letters indicate significant differences between Cr^{6+} alone or Cr^{6+}-high temperature groups ($p < 0.05$). * represents a significant difference between Cr^{6+} alone and Cr^{6+}-high temperature groups ($p < 0.05$).

The SOD activity in the liver was significantly higher in the T3 group compared with the control group and the T2 group (Figure 9). Similarly, the SOD activity in the TH3 group was significantly higher than in the control group and the TH2 group ($p < 0.05$). The GPx activity in the T3 group was significantly higher than in the control group, and both the TH3 and TH4 groups displayed significantly higher GPx activity compared to other

groups ($p < 0.05$). The CAT activity in the T4 group was significantly higher than that in the control and other groups ($p < 0.05$). Moreover, the TH4 group exhibited significantly higher CAT activity than the control group ($p < 0.05$). The GST activity in the T4 group was significantly higher than that in the C group, and the TH4 group exhibited significantly higher GST activity than the CH group ($p < 0.05$). In addition, the SOD activity in the CK group was significantly higher than in the control group, and the TH3 group displayed significantly higher SOD activity than the T3 group ($p < 0.05$). Furthermore, the TH4 group exhibited significantly higher SOD activity than the T4 group ($p < 0.05$). The GPx activity in the TH3 group was significantly higher than that in the T3 group, and the TH4 group showed significantly higher GPx activity than the T4 group ($p < 0.05$). The CAT activity following exposure to Cr^{6+} with high temperature was significantly higher than that following exposure to Cr^{6+} with normal temperature ($p < 0.05$). Moreover, GST activities in the TH2, TH3, and TH4 groups were significantly higher than in the T2, T3, and T4 groups ($p < 0.05$).

Compared with the control group, the SOD activity in the gills was significantly higher than that in T2 and T4 groups. No significant difference was noted in the SOD activity in the high-temperature group (Figure 9). The activity of the GPx enzyme in the T3 group was significantly higher than in the C, T1, and T4 groups, and significantly higher in the TH2 and TH3 groups than that in the TH1 group ($p < 0.05$). The CAT activity in the T2 and TH4 groups was significantly higher than in the control group ($p < 0.05$). The GST activity was significantly higher in the T4 and TH4 groups than in the control group ($p < 0.05$). The SOD activity in the CH group was significantly higher than in the C group, and that in the TH1 group was significantly higher compared to the T1 group ($p < 0.05$). Similarly, the GST activity in the TH3 group was significantly higher than in the T3 group, and that in the TH4 group was significantly higher than the T4 group ($p < 0.05$).

In the kidney, the SOD activity in the T2 group was significantly higher than in the control group ($p < 0.05$, Figure 9). However, no significant difference was noted in the SOD activity in the high-temperature group ($p > 0.05$). The GPx enzyme activity in the T4 group was significantly higher than that in the C and T1 groups, and the TH3 group displayed significantly higher GPx activity compared with the control TH1 and TH2 groups ($p < 0.05$). The CAT activity in the T4 group was significantly higher than that in the control, T1, and T2 groups, whereas the TH4 exhibited significantly higher CAT activity compared with the control and TH1 groups ($p < 0.05$). The GST activity in the T3 group was significantly higher than that in the C and T1 groups, whereas the TH4 group displayed significantly higher GST activity compared to the control, TH1, TH2, and TH3 groups ($p < 0.05$). The GPX activity in the TH3 group was significantly higher than that in the T3 group ($p < 0.05$). The CAT activity in the TH2 group was significantly higher than that in the T2 group, whereas the TH3 group exhibited significantly higher CAT activity compared to the T3 group ($p < 0.05$). Moreover, the TH4 group displayed significantly higher CAT activity compared to the T4 group ($p < 0.05$). The GST enzyme activity in the TH2 group was significantly higher than that in the T2 group ($p < 0.05$).

The IBR values and star plots for each concentration of Cr and Cr combined high temperature are depicted in Figure 10. The star plots demonstrate distinct trends for each biomarker. In addition, we observed that the IBR values in the liver and gills in the TH4 group were the highest. In spleen tissue, the highest IBR index was observed in the T3 group, followed by the TH3 and TH4 groups. These findings suggest that exposure to a combination of Cr^{6+} and a high temperature exerted more effects on fish compared to Cr^{6+} alone. Substantial ACP, SOD, and GST activities were observed in the liver, which is related to heightened immune response and oxidative stress. The ALP, GST, and SOD activities were considerably prominent in the gills, and these are also related to enhanced immune response and oxidative stress. Similarly, notable activities of SOD, ACP, and AST were observed in the kidneys, and these are related to elevated oxidative stress, metabolism, and immune response.

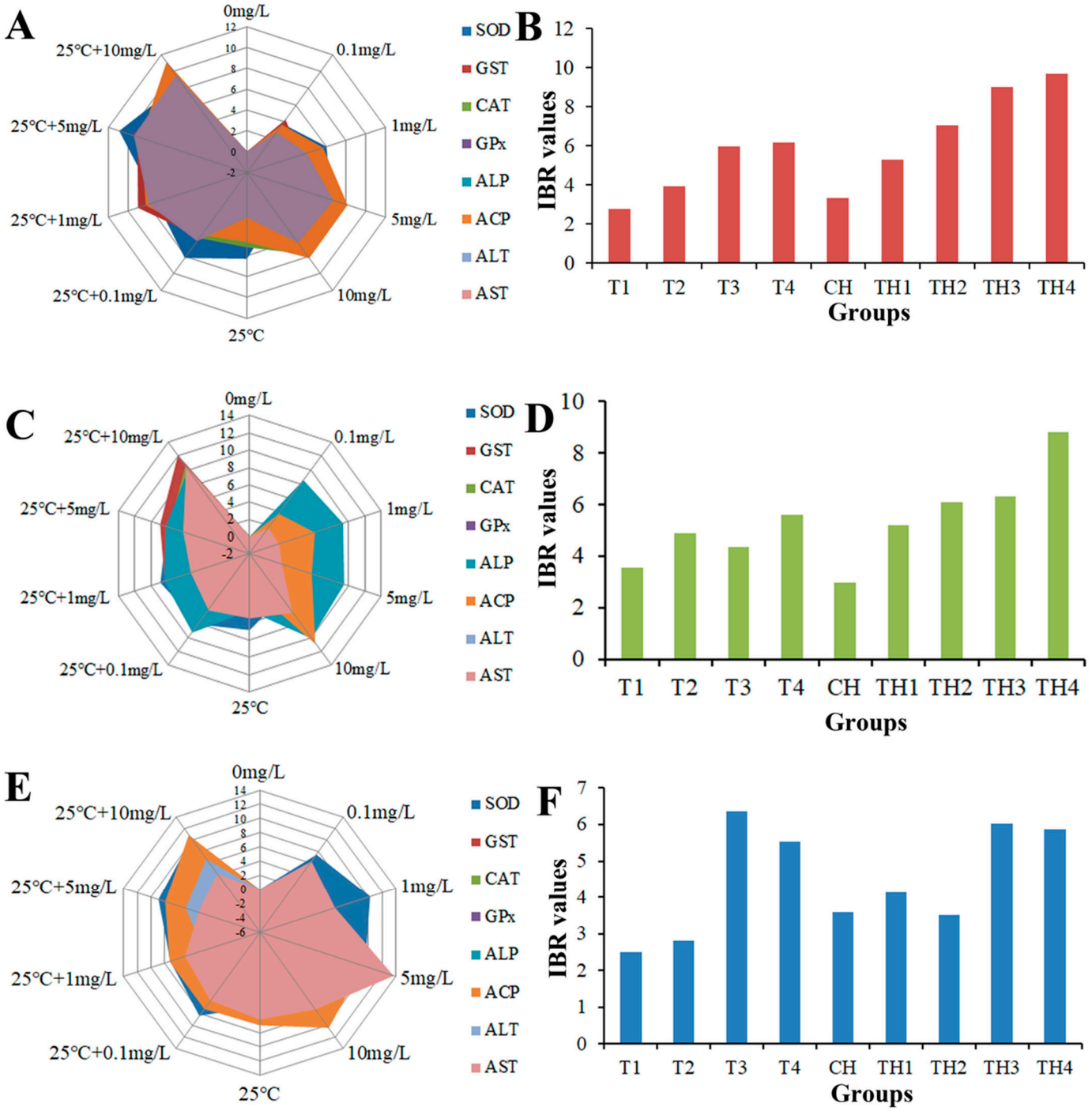

Figure 10. Radar plots of biomarker data in different groups and integrated biomarker response (IBR) index values for each group. (**A**,**B**) Liver. (**C**,**D**) Gill. (**E**,**F**) Kidney.

4. Discussion

Histopathology is a valuable tool in ecotoxicological research for the timely and accurate study of the effects of exposure to contaminants. In addition, it aids in the identification, description, and even quantification of lesions in specific vital organs of fish [42,43]. The liver is implicated in the storage, biotransformation, and excretion of xenobiotics, and tissue changes in the liver are an indicator of the overall health status of fish [44–46]. Similarly, gills can be studied to evaluate contamination in fish because they are directly and constantly exposed to contaminants in the water; gills exhibit rapid response times and are highly sensitive even to low concentrations of pollutants [47]. Kidneys play a crucial

role as excretory and osmoregulatory organs in fish, helping to maintain the water–salt balance [48]. Moreover, the kidneys of fish receive the largest proportion of postbranchial blood, indicating that renal lesions could potentially serve as reliable indicators of environmental pollution [49]. Thus, the liver, gills, and kidneys are suitable candidate organs to determine the effects of contaminants on fish and perform histological examinations, as they are highly responsive to exposure to xenobiotics. The tissue alterations observed in this study align with similar pathological responses documented in previous studies on Cr exposure. Numerous studies have reported that long-term Cr stress can lead to degenerative changes in fish; for instance, *Channa punctatus* chronically exposed to 1/10th of LC_{50} Cr^{6+} causes hypertrophy and necrosis in the liver and hepatocytes with massive vacuolation [50]. In addition, *Pangasianodon hypophthalmus* displays histopathologies in the liver, characterized by blood congestion, hemorrhage, melano-macrophage centers, vacuolation, and hepatocyte hypertrophy following long-term exposure to Cr^{6+} [51]. Our study confirmed that chromium contamination alone can cause damage to fish. As fish occupy the top position in the food pyramid, they are considered prime targets for the biomagnification of metals, potentially acting as transfer media to humans [52]. Therefore, this study is essential to increase awareness among the public and government officials, leading to necessary regulations. In addition, the present study reported generative changes in *P. lagowskii* following exposure to Cr^{6+} and high temperatures. Our results confirm that both regulatory and progressive categories were the most pronounced in the liver and kidneys. The histopathological changes observed following exposure to a combination of Cr and high temperature were stronger than those caused by Cr alone. This discovery aligns with the findings of Khan et al. (2006), elucidating the potential for climate-change-induced temperature elevation to heighten the susceptibility of aquatic organisms to heavy metal exposure [53]. Apart from such degenerative changes, extracellular histological alterations in the hepato-architecture, including diffuse vacuolation of hepatocytes, nuclear migration, engorged capillaries, bile stasis, and focal necrosis to the biliary epithelium, were reported. Cystic tubules, hyaline droplet degeneration of the tubular epithelium, and Bowman's capsules were found. Diffuse vacuolation of hepatocytes, engorged capillaries, bile stasis, and focal necrosis to the biliary epithelium in liver were found in *Oreochromis niloticus* exposed to Cr^{6+} alone [54]. Mishra et al. (2009) reported that in *Channa punctata* exposed to hexavalent Cr, hypertrophied epithelial cells of renal tubules and increased space expansion were evident within the Bowman's capsules in the kidney tissues of the fish [11].

Hematological alterations effectively reflect piscine health status, and biomarkers such as hemoglobin percentage and RBC and WBC counts are highly sensitive to toxic stress, including exposure to heavy metals [55,56]. A significant increase in WBC count, as well as decreases in RBC count and HGB value in *P. hypophthalmus* following exposure to Cr^{6+} for 30 days, were found [51]. Only a significant decrease in the HGB value was found in *Tilapia sparrmanii* exposed to Cr^{6+} chronically [54]. In addition, *Dicentrarchus labrax* exposed to extremely warm temperatures exhibited significant decreases in RBC count, Hb value, and Hct levels. However, an increased WBC count was noted following exposure to extreme warmth [57–59]. In the present study, the Hb value decreased significantly and the WBC count increased significantly, whereas the RBC count did not change during exposure to Cr^{6+} and high temperatures nor to Cr^{6+} alone. Heavy metal exposure can reduce the oxygen-binding capacity of Hb, thereby impacting the chemistry of RBCs and making them more susceptible to delivering oxygen to tissues [60,61]. Our study validated that fish exhibit increased vulnerability to physiological complications and exhibit elevated WBC levels when subjected to prolonged exposure to high temperatures with Cr^{6+}.

Cortisol functions to maintain homeostasis during stress by organizing energy sources [62]. It is commonly referred to as a stress hormone, and is released from the hypothalamic–pituitary–adrenal axis in response to exposure to heavy metals in fish [16]. Elevated cortisol levels demonstrate a bidirectional interaction between endocrine and immune systems, which is essential for maintaining life during distress. This interaction primarily causes the secretion of cortisol to regulate energy metabolism [62]. Therefore, increased cortisol

levels could be attributed to the activated collaboration between the immune system and the pituitary inter-renal axis in eliminating heavy metals [63].

Exposure to a different combination of Cr^{6+}-high temperatures and Cr^{6+} alone induced oxidative stress in *P. lagowskii*. Chromium (VI) undergoes intracellular reduction by cellular reductants, causing the formation of reactive intermediates such as Cr (V), Cr (IV), and eventually Cr (III), which is a more stable form [64]. These reductants reduce Cr (VI) and consequently promote the generation of ROS through a Fenton-like redox cycling mechanism. In addition, they can potentially disrupt cell mitochondria [65]. Compared with the control group, exposure to Cr^{6+} alone remarkably enhanced the activities of SOD, CAT, GPx, and GST, suggesting that these enzymes provide a protective function and are indicators of oxidative stress during Cr^{6+} exposure. A similar result was reported in *Anabas testudineus* under exposure to Cr^{6+} alone [15]. Compared to exposure Cr^{6+} alone, exposure to low concentrations of Cr^{6+} combined with high temperatures remarkably enhanced the activities of SOD, CAT, GPx, and GST, implying that high-temperature conditions can enhance the chronic toxicity of Cr.

The activities of several enzymes, including AST, ALT, ALP, and ACP, in the liver, gills, and kidneys were estimated to determine the extent of toxicity induced by Cr^{6+}-high temperatures and Cr $^{6+}$ exposure. Compared with the control, their activities were significantly higher. A similar observation was reported by Mohamed et al. (2020) in *Oreochromis nilotic* livers following a long exposure to Cr^{6+} [54], which could be attributed to liver cell damage and subsequent leakage of enzymes from these damaged cells [66]. Kumar et al. (2022) demonstrated that combined Cr, pH, and temperature in fish caused heightened ALT and AST activities [15]. The liver in fish is known to enhance immunity and facilitate the degradation of toxic substances [67]. Liver enzymes, that is, AST and ALT, are transaminase enzymes involved in protein and amino acid metabolism, and are frequently used as biomarkers of health status in vertebrate animals [68,69]. Hence, the activities of ALT, AST, ALP, and ACP are important parameters to mirror the degree of liver pathological damage. The AST functions as a catalyst in transferring the amino group from aspartic acid to be used as biomarkers, causing the formation of oxaloacetic acid and glutamic acid [24]. Similarly, ALT catalyzes the transfer of the amino group from alanine to α-ketoglutaric acid, leading to the formation of pyruvic acid and glutamic acid [24]. In addition, AST and ALT are links between carbohydrate and protein metabolism [70]. Cr binds to the free radical of amino acid, which is involved in cellular glucose metabolism and glutathione production [15]. ACP and ALP participate in a series of physiological metabolic responses; they not only prevent pollutants from entering the body, but also participate in the immune systems of aquatic animals [71,72]. Our study revealed that even low levels of chromium can lead to higher levels of ACP, ALP, ALT, and AST in the liver, indicating potential liver damage. The permissible limit for chromium in surface water is 0.1 mg/L and 0.05 mg/L for drinking water, as stated by the World Health Organization [73] (WHO, 2011). Industries such as electroplating, textile and dye manufacturing, metal plating, and battery production are sources of chromium contamination, with levels ranging from 0.5 mg/L to 270 mg/L [74]. Therefore, the impact of low concentrations ($\leq$0.1 mg/L) of chromium on aquatic organisms warrants further investigation, and efforts should be made to address chromium removal in industrial wastewater. Ramish et al. (2024) found that, in rivers with cadmium concentrations ranging from 0.02 to 0.07 mg/L, cadmium levels in the edible parts of fish were measured between 0.1 to 0.4 mg/kg, suggesting that excessive consumption of chromium-contaminated fish could be detrimental to health and present a significant public health concern [75]. These findings lay the groundwork for deeper insights into the potential impacts of chromium and for enhancing the study of ecotoxicology and risk assessment related to this metal. We found that Cr^{6+}-high temperature exposure significantly increased the ACP and ALP activities. Guo et al. (2021) suggested that increased ACP activity revealed that the immune response prevented negative physiological changes in the fish hepatopancreas [76]. Altogether, changes in

immune parameters demonstrated that Cr^{6+}-high temperature exposure disturbed the immune system and induced immune toxicity in *P. lagowskii*.

Acetylcholinesterase (AChE) is widely recognized as a valuable neuro-biochemical parameter for assessing environmental stress [77]. Our findings indicate that AChE was significantly reduced in response to both individual heavy metal stress and combined high temperature compared with the control group. This aligns with a study on *Tor putitora*, which also displayed decreased levels of AChE following the bioaccumulation of heavy metals [62]. Similarly, the activity of AChE in *Oreochromis mossambicus* decreased after exposure to silver nanoparticles [78]. In addition, Gobi et al. (2018) reported a notable decline in non-enzymatic antioxidant activity, oxidative stress, and non-specific immune responses in freshwater fish species *O. mossambicus* and *O. niloticus* following exposure to selenium and copper [79]. AChE functions as a potential neurotransmitter in the central nervous system; however, its functioning can be altered or reduced following exposure of aquatic organisms to toxic metals and chemicals [78].

IBR is a potential tool to assess fish susceptibility to toxicants using multiple biomarker responses [80,81]. Enzymes including ACP, SOD, and GST in the liver; ALP, GST, and SOD in the gills; and SOD, ACP, and AST in the kidneys, displayed the highest response among these enzymes. Consequently, these enzymes are appropriate for the IBR index. The results of the present study suggest that, under varied exposures, enzymes display higher IBR values in the TH4 group, consisting of exposed liver and gill tissue of fish, and in the T4 and TH3 groups, comprising exposed spleen tissue of fish. Our results confirm that biomarkers, including ACP and AST, are sensitive in all tissues.

5. Conclusions

Long-term exposure to low concentrations of chromium alone can also cause damage to fish. The impact of global warming and heavy metal stress on fish, particularly a combination of heavy metals and high temperatures, has gained increasing attention. Our results reveal that prolonged exposure to heavy metals, in combination with high temperature, causes significant changes in fish histology and tissue lesions. Furthermore, the highest tissue damage was observed following exposure to a high concentration of Cr or in combination with high temperature. In addition, long-term exposure to heavy metals and combined heat stress caused a notable increase in the activities of oxidative stress, metabolic, and immune-related enzymes, particularly ACP, ALP, and SOD, in the liver, gills, and kidneys of fish, respectively. However, changes in the enzyme activity varied across different tissues. The IBR results indicated that these enzymes could serve as biomarkers for pesticide pollution in aquatic environments. In addition, the SOD results demonstrated that oxidative stress occurred following exposure to a combined high temperature of 0.1 mg/L Cr. Overall, this study serves as a warning regarding the detrimental effects of a combination of Cr and high temperature on the environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9050168/s1, Table S1. Lethal concentration of chromium alone and combined stressors for different exposure in *P. lagowskii*. Table S2. Histopathological alterations of liver, gill and kidney.

Author Contributions: T.H.: methodology, data curation, writing—original draft. C.Y.: methodology, investigation, formal analysis. Z.N.: methodology, data curation, validation. T.L.: methodology, data curation, validation. W.M.: supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Ethics Committee of Harbin Normal University (approval code: HNUARIA2023009; Approval date: 23 April 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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