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Temperature-Dependent Biochemical Alterations in *Oreochromis niloticus* exposed to Pendimethalin and Fluometuron

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ABSTRACT

Pesticides contamination and global warming are significant environmental challenges that threaten aquatic ecosystems. Aquatic organisms are often exposed to pesticide mixtures rather than individual compounds, leading to complex toxicological interactions that may enhance adverse effects. Additionally, temperature increase due to climate change can influence pesticide persistence and toxicity. This study aimed to investigate the combined biochemical effects of pendimethalin and fluometuron mixtures under different temperature conditions in the blood of *Oreochromis niloticus*. Fish were exposed to environmentally relevant low (0.1 ppb) and high concentrations (1 ppb) of herbicide mixtures for 96 hours at 22^{0}C and 28^{0}C. Results demonstrated significant interactions between pesticide concentration and temperature for all measured parameters. Cortisol level increased at 22^{0}C in both pesticide-exposed groups compared to control, while it decreased at 28^{0}C. Estradiol and testosterone levels were reduced 28^{0}C in both exposure concentrations. Thyroid hormones (triiodothyronine (T_3) and thyroxine (T_4)) were reduced in the high concentration group at both temperatures. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities were decreased across both pesticide concentrations at both temperatures, except in the low concentration group at 28^{0}C. Ion levels were also reduced in the high pesticide concentration at 28^{0}C. Principal component analysis (PCA) confirmed that the most pronounced biochemical alterations were observed in the high concentration group at 28^{0}C, reflecting the synergistic effect of both stressors. These findings suggest that elevated temperature exacerbates the toxic effect of pesticide mixtures, emphasizing the need to examine interactive stressors to better predict the impacts of climate change on non-target organisms.

Keywords: Aquatic toxicology, Biochemical biomarkers, Fish, Global warming, Pesticide mixtures.

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Introduction

Global warming, which significantly alters the Earth's climate through rising temperatures, represents one of the significant challenges in today's. Simultaneously, pesticide toxicity poses another serious threat to life on Earth, with profound implications for both environmental and public health [1]. A key question is how global warming, as a thermal stressor, and environmental pollution, as a chemical stressor interact to impact ecosystems. Some studies have reported the interactive effects of rising temperatures and pesticides on biota [2-3]. Research indicates that elevated temperatures enhance the uptake of pesticides and lead to alterations in the stress response and maintenance of homeostasis in organisms [4]. Researchers also predict an increase in pesticide usage in the coming decades due to the accelerated degradation of pesticides and the proliferation of pest species as a consequence of global warming [5] . Pesticides can also reach surface waters through agricultural runoff and spray drift, affecting non-target organisms such as fish. In addition to the widespread and indiscriminate use of pesticides in agriculture, unregulated domestic applications may also impact non-target organisms [6].

Pendimethalin is a dinitroaniline herbicide widely used to control weeds in both aquaculture and agriculture. It has a molecular weight of 281.3 g/mol, high lipophilicity (log KOW: 5.4 for pH:6.5 at 20^{0}C), and low water solubility (0.33 mg/L at 20^{0}C), which reflect a strong tendency to adsorb to sediments and may bioaccumulate in aquatic organisms [7-8]. Pendimethalin is environmentally persistent, with a hydrolysis half-life exceeding 365 days and photolysis half-lives reported approximately 21 days under continuous exposure [8]. Although its Groundwater Ubiquity Score (GUS) index indicates low leaching potential; environmental monitoring has detected pendimethalin concentrations of up to 0.9 µg/L in groundwater and ranging from 0.01 µg/L to 17.6 µg/L in surface water [9]. Notably, a recent study in Spain waters reported a mean concentration of 0.255±0.148 µg/L, exceeding the upper limit for human drinking water set by the European Union [10]. Pendimethalin has also been classified as a potential human carcinogen by the United States Environmental Protection Agency, increasing further concern about its environmental impact [11-12]. In aquatic organisms, reported effects of pendimethalin include erythrocyte abnormalities in *Cyprinus carpio*, a reduction in protein content in tissues such as the testes and ovaries of spotted snakehead (*Channa punctata*), reproductive impairment through decreased testosterone levels, and oxidative stress in walking catfish (*Clarias batrachus*) due to altered antioxidant defense enzymes [10-12]. Fluometuron is a phenylurea herbicide widely used to control broadleaf weeds and grasses in agricultural crops [13]. It has a molecular weight of 232.2 g/mol, moderate lipophilicity (log KOW: 2.3) and relatively high-water solubility (110 mg/L at 20^{0}C), reflecting greater mobility in aquatic environments [14]. Its GUS suggests a moderate to high leaching potential, raising concerns about contamination of water resources. Fluometuron is considered environmentally persistent, with a photolysis half-life of approximately 3 days in water and its hydrolysis half-life varies with pH, remaining stable at neutral pH. Reported concentrations of fluometuron range from 6.42-18.36 µg/L in surface waters of Spain, and from 1.95 to 317.6 µg/L in surface waters of North Greece [14-15]. Acute toxicity test report 96-hour LC_{50} values of 170 mg/L for carp, 55 mg/L for catfish and 30 mg/L for rainbow trout [16]. Although fluometuron is classified as slightly toxic to aquatic organisms according to U.S. Environmental Protection Agency, fish may still exhibit high sensitivity to fluometuron through various mechanisms. Previous studies have demonstrated that phenylurea herbicides can disrupt endocrine function, including anti-androgenic effects in *Tilapia nilotica* and cause neurotoxic responses in zebra fish [17-20].

Selected pesticides, pendimethalin and fluometuron, are extensively used in Cukurova region, Southern Anatolia, predominantly for pest control in cotton agriculture. Previous studies have primarily focused on evaluating the individual effects of these pesticides on non-target organisms like fish. However, environmentally realistic assessments that consider multiple stressors simultaneously, such as pesticide mixtures combined with elevated temperatures, remain scarce in the current literature. Addressing this gap is necessary for understanding ecological impacts under realistic environmental scenarios and for improving environmental risk assessment. Therefore, this study aimed to investigate how elevated temperatures might influence the toxicity of pesticide mixtures. We hypothesized that higher temperature could increase the toxicity of pesticide mixture in fish. To test this hypothesis, we analyzed biochemical parameters in *O. niloticus* exposed to environmentally relevant concentrations of pendimethalin and fluometuron mixture at 22^{0}C and 28^{0}C for 96 hours. Specifically, we assessed reproductive hormone levels (cortisol, estradiol, testosterone), thyroid hormones (T_3 and T_4), liver enzyme activities (ALT and AST) and ion concentrations (Na^+, K^+, Cl^-) indicative of kidney function.

Materials and Methods

Animals

O. niloticus (33.67±1.57 g, 11.47±0.95 cm) samples were obtained from the Cukurova University Fish Culture Farm. Fish were acclimated for one month in 140 L glass aquaria filled with dechlorinated and aerated tap water. Water quality parameters were maintained as follows: temperature 20±2^{0}C, pH 8.01±0.73, dissolved oxygen 7.40±0.22 mg/L, alkalinity 261 mg/L $CaCO_3$, and total hardness 285 mg/L $CaCO_3$. A 12:12 h light-dark photoperiod was used throughout. Water was renewed daily by transferring the fish to freshly prepared aquaria, in accordance with standard protocols (APHA, AWWA, WPCF, 1998). Fish were fed ad libitum once daily with commercial pellets (Pınar, İzmir, Türkiye), and uneaten feed was removed to prevent water quality deterioration. All fish used in the experiments were maintained under identical laboratory conditions. All procedures were conducted in accordance with the Helsinki Declaration and approved by the local ethics committee (31.10.2023; 8).

Experimental Design

In this study, the commercial formulations of pendimethalin [(N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine), Stomp®, BASF, 450 g/L] and fluometuron [(1,1-dimethyl-3-(3-(trifluoromethyl)phenyl)urea), Cottonex®, ADAMA, 500 g/L], were used. Environmentally relevant, sublethal nominal concentrations were selected based on levels previously reported in surface waters [15,21]. Fish were exposed to these pesticides under semi-static conditions, in which test solutions were daily renewed by transferring fish into freshly prepared pesticide solutions to compensate for loss. Water temperature in the aquarium was maintained by an in-line chiller and a submerged heater set to 22^{0}C and 28 ^{0}C. Fish were not acclimated to the higher test temperature prior to pesticide exposure. No mortality was observed in any treatment groups during the exposure period. The experimental groups were arranged as follows:

<u>Control</u>: Fish maintained in pesticide-free tap water at 22^{0}C and 28^{0}C (n=6 per group).

<u>Low concentration pesticide mixture</u>: Fish exposed to a mixture of 0.1 ppb pendimethalin + 0.1 ppb fluometuron at 22^{0}C and 28^{0}C for 96 hours.

<u>High concentration pesticide mixture</u>: Fish exposed to a mixture of 1 ppb pendimethalin + 1 ppb fluometuron mixture at 22^{0}C and 28^{0}C for 96 hours.

The experiments were combined from three replicated independent experiments.

Blood Collection and Analysis

At the end of the experiment, blood samples were collected from the caudal vein of each fish. Plasma was separated by centrifuging the blood samples at 4000 rpm for 15 minutes. Biochemical parameters, including reproductive hormone levels (cortisol, estradiol and testosterone), thyroid hormones (T_3 and T_4), liver enzyme activities (ALT and AST) and ion concentrations (Na^+, K^+ and Cl^-) were performed using a Roche Cobas Integra 800 autoanalyzer. All analyses were performed on the same day that the blood samples were collected.

Statistics

A Two-Way Analysis of Variance (ANOVA) was performed to assess the effects of pesticide concentration and temperature, as well as their interaction, on the measured biochemical parameters. Significant differences between experimental groups were determined using the Student Newman-Keuls (SNK) post hoc test, with statistical significance set at P≤0.05. In addition, PCA analysis was conducted using standardized biochemical data to explore group separation patterns and to identify the variables contributing to pesticide-induced toxicity. All statistical analyses were conducted by GraphPad Prism 9.0 software. Data are presented as mean±standard deviation (SD).

Results and Discussion

Hormones

A Two-Way ANOVA conducted for all hormones revealed significant main effects for both concentration (P ≤ 0.0001) and temperature (P ≤ 0.0001), as well as for their interaction (P ≤ 0.0001). The SNK post-hoc test for individual group comparisons indicated that both pesticide exposures significantly increased cortisol levels at 22^{0}C compared to the control group, while both low and high concentration exposures decreased cortisol levels at 28^{0}C relative to the control group. No significant differences were observed between the two control groups (Table 1). The primary stress response in fish includes endocrinological changes, such as elevated corticosteroid levels. Cortisol, a glucocorticoid hormone secreted by the adrenal glands, is widely recognized as a significant stress-related hormone in fish and is commonly used to evaluate the effects of various stressors, including xenobiotics, temperature, and photoperiod [22]. Cortisol also plays a key role in ion regulation and energy metabolism. In our study, elevated cortisol levels at 22^{0}C were observed in response to both pesticide mixtures. Similarly, significant increases in cortisol levels have been reported in various fish species, including *O. mossambicus* exposed to chlorpyrifos, *Chanos chanos* exposed to endosulfan, *Labeo rohita* exposed to deltamethrin, and *Carassius auratus* exposed to a combination of pendimethalin and linuron [23-25]. In contrast, we found that elevated temperatures led to a reduction in cortisol levels in tilapia exposed to both pesticide mixtures for 96 hours. Few studies in the literature have demonstrated increased cortisol levels in response to elevated temperature [26]. However, a tendency for decreased cortisol levels in goldfish after 16 days of pesticide exposure at 32^{0}C has also been reported [27]. This result suggests that thermal stress may disrupt the normal functioning of the hypothalamic-pituitary-adrenal axis in fish, altering the induction of a stress response to the pesticides.

Table 1. The results of hormone levels in *O. niloticus* exposed to pendimethalin and fluometuron mixtures at 22^{0}C and 28^{0}C for 96 hours.

Cortisol (ug/dL)	Control	Low Concentration	High Concentration
22 ^{0}C	9.01±0.19 ^{abx}	10.20±0.60 ^{aby}	14.12±0.79 ^{az}
28 ^{0}C	9.68±0.35 ^{abw}	1.91±0.16 ^{bt}	8.59±0.38 ^{abv}
Estradiol (pmol/L)			
22 ^{0}C	2012±2.16 ^{ax}	1113±3.59 ^{ay}	2217±6.27 ^{az}
28 ^{0}C	5527±4.65 ^{bw}	854.70±5.47 ^{bt}	1476±5.31 ^{bv}
Testosterone(ng/dL)			
22 ^{0}C	216.6±2.66 ^{ax}	206.10±4.19 ^{ay}	170.60±1.71 ^{az}
28 ^{0}C	614.9±4.14 ^{bw}	ND	245.90±4.62 ^{bv}
T_3 (pg/mL)			
22 ^{0}C	9.33±0.06 ^{ax}	9.22±0.08 ^{ax}	7.56±0.65 ^{az}
28 ^{0}C	11.61±0.75 ^{bw}	ND	2.55±0.09 ^{bv}
T_4 (ng/dL)			
22 ^{0}C	0.31±0.01^{ax}	0.47±0.00 ^{aby}	0.27±0.00 ^{az}
28 ^{0}C	0.48±0.01 ^{abw}	0.60±0.01 ^{bt}	0.38±0.00 ^{bv}

Different letters (x, y, z, w, t, v) in each row indicate statistically significant differences between concentrations and a-b letters in each column indicate statistically significant differences between temperatures (P≤0.05). ND: Not Determined.

According to the post-hoc test, a reduction in estradiol levels was observed in the low concentration group at 22^{0}C compared to the control, while estradiol levels increased in the high concentration group. Additionally, both low and high concentrations decreased estradiol levels at 28^{0}C compared to the control group. On the other hand, estradiol levels were higher in the 28^{0}C control group than in the 22^{0}C control group. For testosterone levels, the SNK post-hoc test revealed that both pesticide mixtures at 22^{0}C and high concentration exposure at 28^{0}C reduced testosterone levels compared to their respective control groups. However, testosterone levels were found to be higher in the 28^{0}C control group than in the 22^{0}C control group. Testosterone levels at the 28^{0}C low concentration group were not detected due to being outside the reference range. Similar findings have been reported in previous studies. For instance, decreased testosterone levels were observed in *C. batrachus* exposed to pendimethalin, in Nile tilapia exposed to the phenylurea herbicide diuron, and in tilapia exposed to pesticide mixtures, which also showed decreased estradiol and testosterone levels. Lower levels of estradiol and testosterone have also been observed in fish species exposed to temperatures higher than optimal [28]. The timing of spawning in several fish species is directly influenced by temperature and photoperiod. Temperature plays a significant role in gonadal development, gametogenesis, gamete quality, and embryo development [29]. Studies have shown that high temperatures can modify gonadotropin-releasing hormone secretion and gonadal steroidogenesis in fish [30]. In the present study, a possible explanation for the decreased steroid hormone levels in fish is that the pesticide mixture and elevated temperatures alter the gonadal biosynthetic capacity through the suppression of gonadotropin secretion.

The SNK post-hoc test revealed that T_3 levels significantly decreased in the high concentration exposure for both temperatures compared to their respective controls (Table 1). No significant differences were observed between the low concentration exposure at 22^{0}C and its control group. Additionally, T_3 levels at the 28^{0}C low concentration group were not detected due to out of reference range. For T_4, the SNK post-hoc test indicated that T_4 levels increased in the low concentration group at both temperatures compared to the controls, whereas T_4 levels decreased in the high concentration group at both temperatures. T_3 and T_4 levels were also found to be higher in the 28^{0}C control group than in the 22^{0}C control group. Thyroid hormones play a crucial role in brain development, growth, energy metabolism, immunity, and reproduction in fish. Pesticide exposure and temperature can affect thyroid function in fish. For example, high temperatures have been shown to increase the T_4 degradation rate to T_3 in trout, and combined pesticide exposure has been shown to alter circulating thyroid hormone levels in zebrafish, as well as decrease thyroid hormone levels in humans exposed to pesticides [31-33]. In this study, we found that T_3 and T_4 levels were more affected by both high concentration of pesticide mixtures and high temperature exposure compared to the optimal temperature condition. These findings suggest that changes in thyroid hormone levels may significantly impact fish health by altering energy metabolism, immunity, and reproduction.

Enzymes

For ALT and AST levels, a Two-Way ANOVA revealed significant main effects for both concentration ($P \leq 0.0001$) and temperature ($P \leq 0.0001$), as well as for their interaction ($P \leq 0.0001$). The SNK post-hoc test for individual group comparisons showed that ALT levels decreased in both pesticide concentrations at 22^{0}C and in the high concentration group at 28^{0}C compared to their respective controls, whereas ALT levels increased in the low concentration group at 28^{0}C compared to the control (Table 2). On the other hand, AST levels significantly decreased in both low and high concentration exposures at both temperatures compared to their controls. ALT and AST are primarily found in the liver and play key roles in transamination, which involves amino acid synthesis and the deamination pathway. These processes enable interconversion between carbohydrate and protein metabolism, especially during stress when organisms experience high energy demands [34]. The liver, as the main organ for detoxification, is highly sensitive to stress, making these enzymes important biomarkers in toxicological studies for evaluating fish health [35]. In this study, decreased levels of ALT and AST enzymes were observed in all experimental groups, except for the low concentration pesticide mixture at 28^{0}C. Both enzymes were most affected under the combined conditions of high concentration and high temperature, with a 43% decrease in ALT and a 30% decrease in AST. The literature presents conflicting results regarding ALT and AST levels in fish exposed to stressors. Some studies have reported increased ALT and AST levels in catfish, carp, and tilapia exposed to pesticides, as well as in spotted seabass and rainbow trout subjected to thermal stress [35-38]. Conversely, other studies have shown decreased levels of these enzymes in fish exposed to pesticides [35-39]. In this study, the observed changes suggest that liver damage caused by pesticide toxicity likely alters the circulating levels of ALT and AST enzymes, which are critical indicators of hepatic function and fish health.

Table 2. The results of liver enzymes levels in *O. niloticus* exposed to pendimethalin and fluometuron mixtures at 22^{0}C and 28^{0}C for 96 hours.

ALT (U/L)	Control	Low Concentration	High Concentration
22 ^{0}C	43.30±1.74 ^{ax}	31.08±0.45 ^{bwy}	37.39±0.47 ^{az}
28 ^{0}C	32.24±0.34 ^{bwy}	40.03±0.17 ^{bct}	18.09±0.31 ^{bcv}
AST (U/L)			
22 ^{0}C	347.9±1.53 ^{ax}	254±3.90 ^{ay}	329.6±1.79 ^{az}
28 ^{0}C	423.3±2.05 ^{bw}	416.9±1.49 ^{bt}	292.5±3.46 ^{bv}

Different letters (x, y, z, w, t, v) in each row indicate statistically significant differences between concentrations and a-c letters in each column indicate statistically significant differences between temperatures (P≤0.05).

Ions

For ion levels, a Two-Way ANOVA revealed significant main effects for concentration ($P \leq 0.0001$) and temperature ($P \leq 0.0001$), as well as their interaction ($P \leq 0.0001$). The SNK post-hoc test for individual group comparisons showed that Na^+ levels increased in the low concentration group at 28^{0}C compared to the control, while they decreased in the high concentration group (Table 3). However, no significant differences were detected between groups at 22^{0}C. Potassium levels significantly decreased in the low concentration group at 22^{0}C and in both concentration groups at 28^{0}C compared to their respective controls, whereas they increased in the high concentration group at 22^{0}C. Chloride levels increased in the low concentration group at 28^{0}C compared to the control but decreased in the high concentration group. No significant differences in Cl^- levels were observed between groups at 22^{0}C. Additionally, Na^+ and Cl^- levels were lower in the 28^{0}C control group than in the 22^{0}C control group, while K^+ levels were higher in the 28^{0}C control group than in the 22^{0}C control group. Ion levels are widely recognized as bioindicators used to assess the homeostatic mechanisms of organisms. Na^+, K^+, and Cl^- levels play critical roles in osmoregulation and homeostasis in fish [37]. Specifically, Na^+ and K^+ are predominant ions that regulate acid-base balance, thereby maintaining ionic sufficiency for tissue function [37]. K^+ also plays a significant role in the nervous, secretory, and digestive systems [39]. Previous studies have demonstrated that pesticide exposure and temperature changes can alter ion levels in fish [38]. In our study, Na^+ and Cl^- levels were not significantly affected by pesticide exposure at 22^{0}C. However, significant changes in ion levels were observed in fish exposed to both pesticide concentrations at 28^{0}C. These findings suggest that temperature significantly influences pesticide toxicity on the ion regulation, leading to abnormalities in ion levels and disrupting osmoregulatory mechanisms in fish.

Table 3. The results of ion levels in *O. niloticus* exposed to pendimethalin and fluometuron mixtures at 22^{0}C and 28^{0}C for 96 hours

Na^+ (mmol/L)	Control	Low Concentration	High Concentration
22 ^{0}C	154.80±2.98 ^{abx}	155.80±3.30 ^{ax}	151.00±2.94 ^{abx}
28 ^{0}C	135.00±2.58 ^{bw}	148.00±2.16 ^{abt}	125.30±3.86 ^{bv}
K^+ (mmol/L)			
22 ^{0}C	5.45±0.21 ^{ax}	4.71±0.12 ^{ay}	6.23±0.32 ^{abz}
28 ^{0}C	7.90±0.15 ^{bw}	6.97±0.12 ^{bt}	6.49±0.12 ^{abv}
Cl^- (mmol/L)			
22 ^{0}C	137.70±1.12 ^{abx}	139.50±2.51 ^{abx}	135.90±2.52 ^{abx}
28 ^{0}C	122.00±2.16 ^{bw}	135.50±2.67 ^{abt}	114.00±2.96 ^{bv}

Different letters (x, y, z, w, t, v) in each row indicate statistically significant differences between concentrations and a-c letters in each column indicate statistically significant differences between temperatures ($P \leq 0.05$).

To integrate the biochemical alterations and understanding of the overall patterns of group separation, a PCA test was performed. The first two principal components explained a total of 66.1% of the variance in the dataset, with PC1 accounting for 37.9% and PC2 for 28.2%. The control group at 22^{0}C clustered tightly, indicating physiological homeostasis under optimal conditions, whereas control group at 28^{0}C exhibits clear shift along PC1, characterized by increased ions and sex hormones, suggesting that temperature alone may induce endocrine and ionic parameter changes. PCA results clearly showed that the high concentration mixture group at 28^{0}C showed the most notable separation, indicating a synergistic effect of pesticide exposure and elevated temperature, likely leading to disruption in biochemical parameters. Interestingly, the low concentration exposure group at 28^{0}C clustered unexpectedly close to the control group at 22^{0}C rather than other groups at 28^{0}C. This pattern may reflect adaptive mechanisms, compensatory physiological responses or individual variation. On the other hand, low and high concentration groups at 22^{0}C showed intermediate separation, indicating concentration-dependent but thermally moderated biochemical changes. These findings demonstrate that temperature is a critical modulator of concentration-dependent pesticide toxicity and may disrupt biochemical parameters (Figure 1).

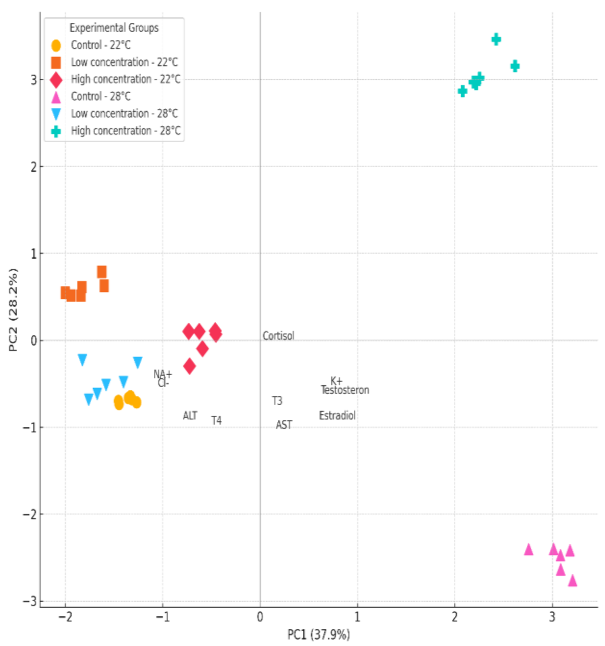

Figure 1. Principal component analysis (PCA) of biochemical alterations in *O. niloticus* exposed to pendimethalin and fluometuron mixtures at 22^{0}C and 28^{0}C for 96 hours.

Conclusion

In summary, elevated temperatures exacerbated the toxicity of pendimethalin and fluometuron mixtures in *O. niloticus*. The interactive effects of acute exposure to multiple stressors disrupted endocrine function, detoxification mechanisms, and osmoregulatory processes. These findings were further supported by PCA, which confirmed clear group separation and identified important key biochemical markers contributing to concentration and temperature-dependent stress effects. This highlights the importance of assessing multiple environmental stressors, particularly under climate change conditions. Therefore, it is important to evaluate the effects of multiple stressors in non-target organisms are affected by ongoing global warming to take precautions.

Limitations

Although this study provides important insights into the acute effects of pesticide mixtures and elevated temperature on blood biochemical parameters in *O. niloticus*, several limitations should be considered. First, the current findings reflect acute exposure outcomes and may not fully represent chronic or long-term physiological responses. Second, the study investigated only two temperature conditions; however, natural aquatic environments experience a broader range of thermal fluctuations, which may affect pesticide toxicity in different ways. Finally, although the selected biochemical parameters offer fast and practical indicators of environmental impact, additional analyses such as molecular and/or histological assessments could provide a more detailed understanding of the underlying mechanisms of pesticide toxicity. Future research should consider chronic, long-term, or seasonal exposure across broader temperature gradients and include additional analyses to enhance environmental risk assessment.

Conflict of Interest

The authors declare no conflicts of interest.

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Ethical Approval

The experimental protocol was conducted in compliance with the Helsinki Declaration and approved by the ethics committee of the Cukurova University according to the guidelines for the care and use of animals in laboratory research (31.10.2023; 8).

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