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Jiang H, Liu J, Huang Q, Yang D. Effects of Short-Term Temperature Stress on Metabolic and Digestive Enzymes Activities of *Procambarus clarkii*. *Israeli Journal of Aquaculture - Bamidgeh*. 2024;76(4). doi:10.46989/001c.126179

Original Research Articles

Effects of Short-Term Temperature Stress on Metabolic and Digestive Enzymes Activities of *Procambarus clarkii*

Hanjun Jiang[1], Jiahui Liu[1], Qianqian Huang[1], Donghui Yang[1a]

[1] College of Fisheries, Xinyang Agriculture and Forestry University

Keywords: crayfish, low-temperature, high-temperature, enzymes activities

https://doi.org/10.46989/001c.126179

Israeli Journal of Aquaculture - Bamidgeh

Vol. 76, Issue 4, 2024

To investigate the effects of temperature stress on metabolic and digestive enzyme activities of *Procambarus clarkii*, the test species was transferred from 20°C (control group) to 10°C and 30°C, and samples were collected at 0, 3, 6, 12, 24, and 48h. The activities of pyruvate kinase (PK), hexokinase (HK) and hosphoenolpyruvate carboxykinase (PEPCK) in muscle and hepatopancreas of *Procambarus clarkii* was determined. The changes in α-amylase (α-AMY) and lipase (LPS) activity in intestinal and hepatopancreas were also examined. The results showed that the activities of digestive and metabolic enzymes in *Procambarus clarkii* were significantly affected by extreme water temperature (*P<0.05*). During the whole experimentation period, PK, HK, and PEPCK activities in the low-temperature group (10°C) were lower than those in the control group (20°C) (*P < 0.05*). Meanwhile, LPS and α-AMY activities in the low-temperature group (10°C) were also significantly lower than those in the control group (20°C) (*P < 0.05*). In the high-temperature group (30°C), the activities of PK, HK, and PEPCK metabolic enzymes in muscle and hepatopancreas decreased first. Then they increased, and their activity levels were significantly lower than those in the control group (*P<0.05*). At the same time, the activities of LPS and α-AMY digestive enzymes in the intestines and hepatopancreas were significantly lower than those in the control group(*P<0.05*). In summary, this study examined the impact of temperature stress on the metabolic and digestive enzyme activities in *Procambarus clarkii*, shedding light on its self-regulation mechanisms in response to temperature fluctuations. The findings provide a scientific foundation for understanding the species' adaptation to environmental changes.

INTRODUCTION

Procambarus clarkii, commonly known as crayfish, were introduced to China from Japan in the 1920s and were native to northern Mexico and Central America.[1] Because of its strong disease resistance, adaptability, fast reproduction rate, and rich nutrition, it is now widely distributed in almost all types of freshwater habitats in China, such as lakes and rice fields.[2] In China, the culture of *Procambarus clarkii* has developed rapidly in the past two decades and currently accounts for the largest proportion of commercially cultured freshwater crustaceans in China.[3] Nowadays, crayfish have become a culinary delicacy and are in great demand by people. Global crayfish production was reported to be 1.71 Mmt in 2018, and total crayfish production in China reached 2.89 × 10^6 tons in 2022.[4] Currently, with global temperatures rising, crayfish in China's typical rice-crayfish integrated systems are exposed to water temperatures of 35°C or higher during the summer.[5] Several challenges, including the adverse effects of temperature stress on crayfish, have hampered the development of crayfish farming.[3]

Crayfish can tolerate a wide range of temperatures; the optimal growth temperature is 21-27°C; when the water temperature is lower than 12°C, crayfish will stop feeding, and if the water temperature is higher than 30°C, they will burrow into the soil.[6] Studies have shown that temperature change can hamper the internal environment of aquatic organisms, resulting in DNA damage, cell apoptosis, and immune response.[7] Low temperatures can induce cold stress, leading to physiological and morphological damage in certain shrimp species.[8,9] When the temperature exceeds 33°C, high-temperature stress can cause metabolic disorders and immune disorders in crayfish, which may increase the death rate of crayfish.[10,11] In Hubei Province, China, the water temperature for raising crayfish varies significantly, reaching 31.4°C in July and dropping to 5.7°C in De-

a Corresponding author. Donghui Yang, E-mail: 13939712100@qq.com

cember.[5] Consequently, it is crucial to study the effects of temperature stress on the physiological indicators of crayfish.

The change in environmental temperature can affect the growth, metabolism, digestion, autoimmunity and development of aquatic animals.[3,12] The level of digestive enzyme activity represents the absorption and digestion level of nutrients in the body, and there is a mutually promoting interaction with food intake. There are few studies on the effects of temperature alteration on the metabolism and digestion of *Procambarus clarkii*. Therefore, this study was designed to examine the temperature change from 20°C (control) to a low-temperature group (10°C), and a high-temperature group (30°C). The activities of a series of metabolism-related enzymes and digestive enzymes in different tissues of crayfish were measured within 0, 3, 6, 12, 24, and 48 h under different temperature regimes. The effects of short-term temperature stress on the metabolism and digestion of *Procambarus clarkii* were explored to understand the physiological changes of this species under extreme temperature changes and to provide a theoretical basis for optimizing the management of *Procambarus clarkii* culture.

MATERIALS AND METHODS

FISH

In this experiment, crayfish were procured from Xiaolongshan Fishery Development Co., LTD., Luoshan County, Xinyang City. Crayfish as the experimental object was selected which was complete in body shape, similar in size, disease-free, vigorous, and weighed 16.17±1.68 g. The experimental crayfish were reared temporarily in a bucket of the indoor breeding system for three days before the stress test. During the three days of temporary maintenance, the water temperature was maintained at 20.0±1.0°C, and the pH was between 7.5 and 8.5. The crayfish stopped feeding during the experiment.

TEMPERATURE STRESS TEST

The crayfish were then divided into three groups to carry out the experiment. The first group was set as the control group (the water temperature was maintained at 20°C during the experiment), the second group was set as the low-temperature group (the water temperature was maintained at 10°C under the condition of low-temperature stress during the experiment), and the third group was set as the high-temperature group (the water temperature was maintained at 30°C under the condition of high-temperature stress during the experiment). At the same time, each group had three replicates, and each replicates cultured 10 crayfish. In the low-temperature group, ice packs were used to control the water temperature at 10.0±1.0°C, while in the high-temperature group, heating rods were used to control the water temperature at 30.0±1.0°C. During the experiment, other rearing conditions of all experimental groups remained the same, and the experiment lasted for 48 hours, during which continuous aeration was carried out to keep the experimental conditions at a stable level.

SAMPLE COLLECTION AND ENZYME ACTIVITY DETERMINATION

During the experiment, the three groups were randomly selected as samples at 0, 3, 6, 12, 24, and 48 h. Each group of crayfish was dissected, and the hepatopancreas, intestinal, muscle, and other tissues were removed and stored in a 1.5 mL centrifuge tube for refrigeration to determine enzyme activity. The activity of metabolic enzymes (hexokinase, pyruvate kinase, and phosphoenolpyruvate carboxykinase) in hepatopancreas, muscles, and digestive enzymes (α-amylase and lipase) in hepatopancreas and intestines of crayfish was determined using the commercially available kit (Jiangsu Enzyme Free Industry Co., LTD). The enzyme activity was calculated according to the fresh weight of the sample, and the unit definition was 1 nmol NADPH generated per g tissue per minute, which was defined as a unit of enzyme activity.

STATISTIC ANALYSIS OF DATA

The experimental results were expressed as mean±SD. SPSS 26.0 software was used for one-way ANOVA, and the minimum significant range method (LSD) was used for multiple comparisons. Significance analysis was performed using a T-test, and $P<0.05$ was considered significant.

RESULTS

EFFECT OF TEMPERATURE MUTATION ON PYRUVATE KINASE (PK) ACTIVITY IN HEPATOPANCREAS AND MUSCLE TISSUE OF CRAYFISH

The effect of temperature stress on pyruvate kinase (PK) in crayfish hepatopancreas is shown in (**Figure 1a**). In the hepatopancreas of the control group (20°C), the PK activity remained stable during the experiment. The activity of this enzyme increased significantly only at 12h and 48h ($P<0.05$). The PK activity in the 10°C-temperature group decreased first and then increased, and reached the lowest point at 6h and 12h, which was significantly lower than that in the control group ($P < 0.05$), and gradually increased at 24h, but the value was significantly lower than that in the control group ($P < 0.05$). At the same time, in the high-temperature treatment group (30°C), the PK activity in the hepatopancreas of crayfish was significantly lower than that in the control group ($P < 0.05$).

Meanwhile, we measured the PK activity in the muscles (**Figure 1b**). The results showed that PK activity decreased gradually in the low-temperature group and reached the lowest level at 48h. The PK activity in the 30°C-temperature group decreased first and then increased, and reached the lowest point at 12h, which was significantly lower than that in the control group ($P < 0.05$). It is worth noting that the PK activity significantly decreased at the same time point compared with the control group in the hepatopancreas of crayfish ($P < 0.05$). In muscle, except for 48h, there was

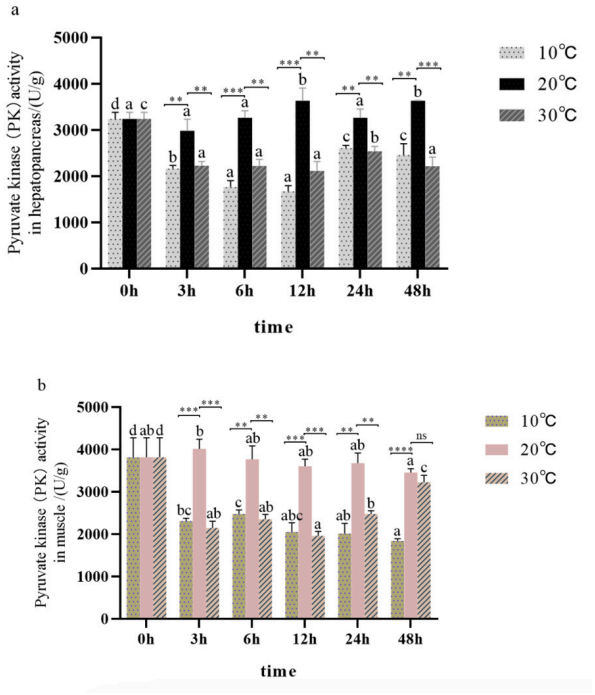

Figure 1. Effect of different temperatures on pyruvate kinase activity in hepatopancreas and muscle tissues of crayfish. * Indicates that there are significant differences between the data at the same sampling time point (*P* < *0.05*); Different lowercase letters indicate significant differences in data at different time points and 0h (*P* < *0.05*).

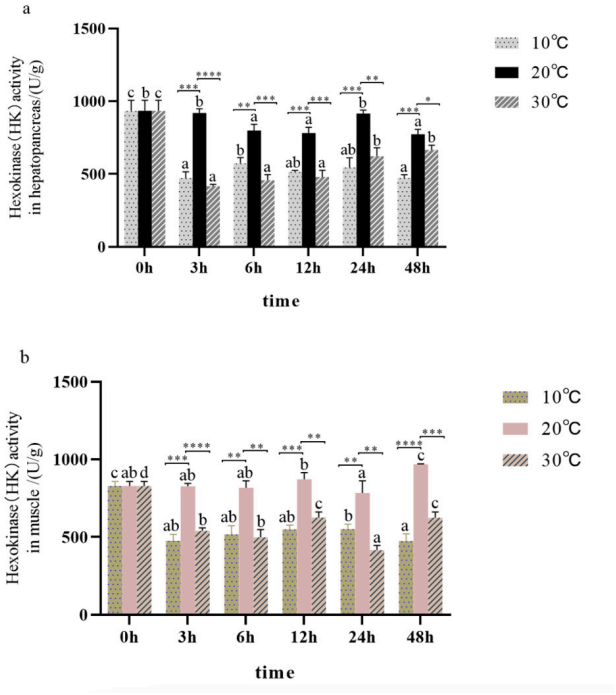

Figure 2. Effect of different temperatures on hexokinase (HK) activity in hepatopancreas and muscle tissues of crayfish. *Indicates that there are significant differences between the data at the same sampling time point (*P* < *0.05*); Different lowercase letters indicate significant differences in data at different time points and 0h (*P* < *0.05*).

a significant reduction in other experimental results compared with the control group (*P* < *0.05*). The above results indicated that the temperature stress inhibited the level of PK activity.

EFFECT OF TEMPERATURE ALTERATION ON HEXOKINASE (HK) ACTIVITY IN MUSCLE AND HEPATOPANCREAS TISSUE OF CRAYFISH

The effects of temperature mutation on hexokinase (HK) in crayfish's hepatopancreas and muscle tissue are shown in (**Figure 2**). In the hepatopancreas, the HK activity of the control group (20°C) decreased gradually during the experiment (**Figure 2a**). The activity of HK in the low-temperature group (10°C) showed a decreasing trend, and the data was significantly lower than that in the control group (*P* < *0.05*) and reached the peak value at 48h. In the high-temperature group (30°C), the activity of HK decreased first and then increased, reached the lowest peak value at 3h (*P* < *0.05*), and increased after 24h. In muscle, the activity of HK in the low-temperature group (10°C) was significantly lower than that in the control group and reached the lowest peak value at 48h (**Figure 2b**). The experimental data of the high-temperature group (30°C) was also significantly lower than that in the control group and reached the lowest peak value at 24 h.

EFFECT OF TEMPERATURE ALTERATION ON PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) ACTIVITY IN HEPATOPANCREAS AND MUSCLE TISSUE OF CRAYFISH

The effect of temperature mutation on phosphoenolpyruvate carboxykinase (PEPCK) activity in crayfish's hepatopancreas and muscle tissue is shown in (**Figure 3**). In hepatopancreas, PEPCK activity in the control group (20°C) remained stable during the test periods, and its level decreased at 6h and 12h (**Figure 3a**). PEPCK activity in the low-temperature group (10°C) was significantly lower than that in the control group and reached the lowest value at 48h (*P* < *0.05*). PEPCK activity in the high-temperature group (30°C) decreased first and then increased; it decreased to the lowest value at 6h (*P* < *0.05*) and increased gradually at 24h.

In muscle, PEPCK activity in the control group (20°C) increased during the experiment and reached the highest value at 48h (**Figure 3b**). At the same time, PEPCK activity in the low-temperature group (10°C) was significantly lower than that in the control group and reached the lowest value at 12h. PEPCK activity in the high-temperature group (30°C) decreased first and then increased, reaching the lowest value at 3h, and recovered to the initial level at 48h. The results showed that temperature mutation severely inhibited PEPCK activity expression in crayfish's hepatopancreas and muscle tissue.

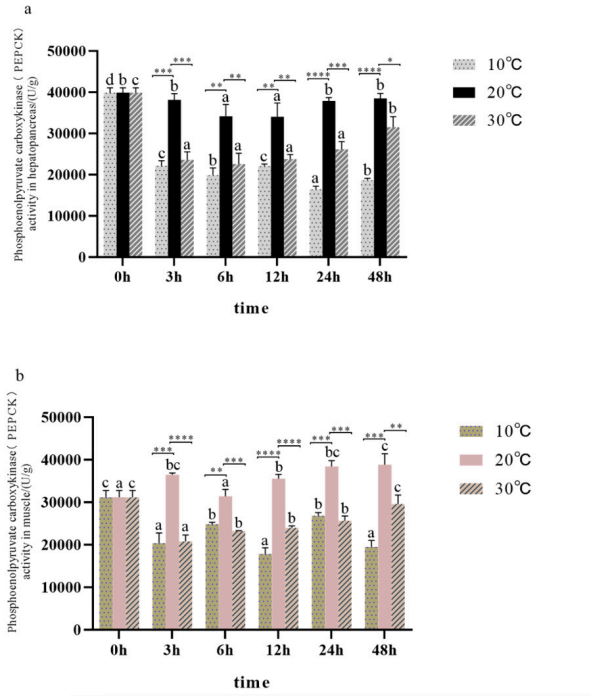

Figure 3. The effect of temperature mutation on phosphoenolpyruvate carboxykinase (PEPCK) activity in hepatopancreas and muscle tissue of crayfish. *Indicates that there are significant differences between the data at the same sampling time point (*P* < *0.05*); Different lowercase letters indicate significant differences in data at different time points and 0h (*P* < *0.05*).

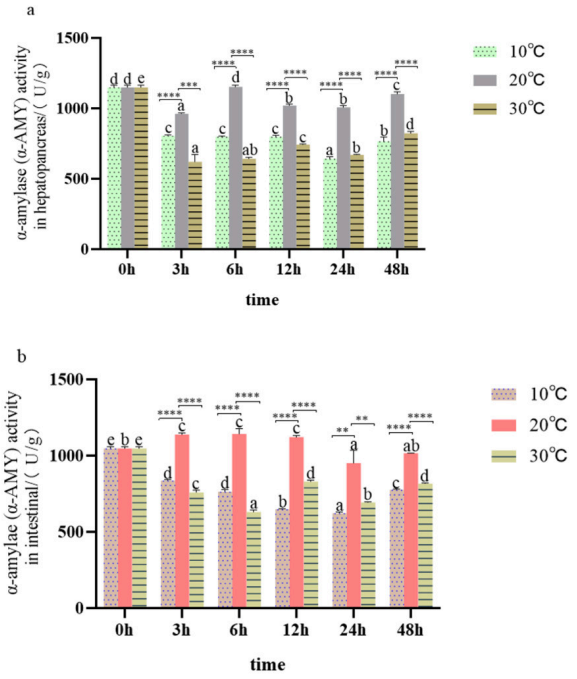

Figure 4. Effects of acute temperature changes on α-amylase (α-AMY) activity in the hepatopancreas and intestinal tissues of crayfish. *Indicates that there are significant differences between the data at the same sampling time point (*P* < *0.05*); Different lowercase letters indicate significant differences in data at different time points and 0h (*P* < *0.05*).

EFFECTS OF TEMPERATURE CHANGES ON A-AMYLASE (A-AMY) ACTIVITY IN HEPATOPANCREAS AND INTESTINAL TISSUES OF CRAYFISH

The effect of temperature alteration on α-amylase (α-AMY) activity in crayfish's intestinal and hepatopancreas tissue is shown in (**Figure 4**). In the control group (20°C), the α-AMY activity of hepatopancreas decreased during the experiment and remained at the initial level at 6h (**Figure 4a**). The α-AMY activity of hepatopancreas in the low-temperature group (10°C) was significantly lower than that in the control group during the experiment and decreased to the minimum at 24h. The α-AMY activity of hepatopancreas in the high-temperature group (30°C) decreased first and then increased, and the overall level was significantly lower than that in the control group, and it dropped to the minimum value at 3h.

In the intestine, the α-AMY activity of the control group (20°C) increased first and then decreased during the experiment (**Figure 4b**). In the low-temperature group (10°C), the α-AMY activity decreased first and then increased, and the overall level was significantly lower than that of the control group (*P* < *0.05*), and it dropped to the minimum value at 24h. The activity of α-AMY in the high-temperature group (30°C) decreased first and then increased, and the overall level was significantly lower than that in the control group (*P* < *0.05*) and reached the lowest value at 6h. It recovered at 12 and 48 h.

EFFECTS OF TEMPERATURE CHANGES ON LIPASE (LPS) ACTIVITY IN HEPATOPANCREAS AND INTESTINAL TISSUES OF CRAYFISH

The effect of temperature alteration on lipase (LPS) activity in crayfish's intestinal and hepatopancreas tissue is shown in (**Figure 5**). The LPS activity in the hepatopancreas of the control group (20°C) increased during the test period and peaked at 12h (**Figure 5a**). Compared with the control group, the LPS activity of hepatopancreas tissue in the low-temperature group (10°C) was significantly reduced during the test period and reached a minimum at 24h (*P* < *0.05*). The LPS activity in the high-temperature group (30°C) decreased first and then increased, which was significantly lower than that in the control group (*P* < *0.05*), and reached the lowest value at 6h, and gradually increased at 48h.

The LPS activity in the intestinal tissue of the control group (20°C) remained stable during the test period (0-24h), and the value decreased at 48h (**Figure 5b**). Compared with the control group, the LPS activity of the intestinal tissue in the low-temperature group (10°C) was significantly reduced during the test period and reached a minimum at 48h (*P* < *0.05*). The LPS activity in the high-temperature group (30°C) was also significantly lower than

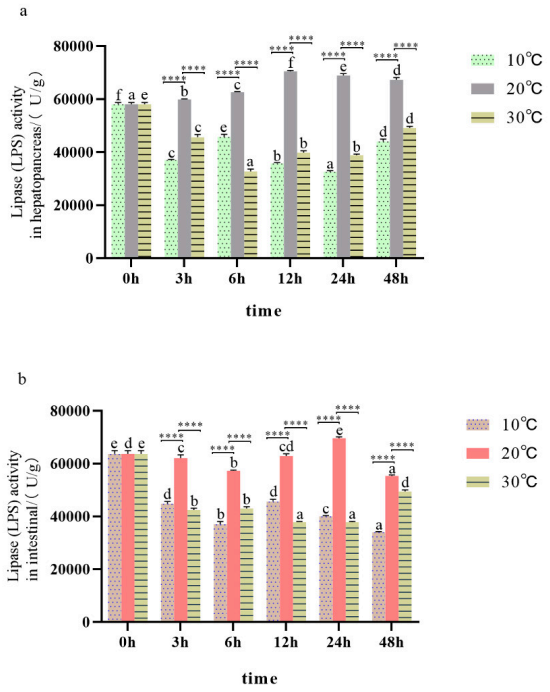

Figure 5. Effects of acute temperature changes on lipase (LPS) activity in hepatopancreas and intestinal tissues of crayfish. *Indicates that there are significant differences between the data at the same sampling time point (*P < 0.05*); Different lowercase letters indicate significant differences in data at different time points and 0h (*P < 0.05*).

that in the control group (*P < 0.05*) and decreased to the lowest level at 12 and 24h.

DISCUSSION

Animal energy metabolism mainly involves glycolysis, tricarboxylic acid cycle cyclic, oxidative phosphorylation, and other pathways. Hexokinase (HK) and pyruvate kinase (PK) are key rate-limiting glycolysis enzymes.[13] The higher the activity of HK and PK, the more vigorous the glycolysis process and the inhibition of the gluconeogenesis process. Phosphoenolpyruvate carboxylase (PEPCK) catalyzes the carboxylation of phosphoenolpyruvate (PEP), converting it to oxaloacetic acid (OAA). This enzyme plays an important role in the gluconeogenic pathway.[14] The results of this study showed that the HK and PK activity levels in hepatopancreas and muscle tissues of crayfish were significantly lower than those of the control group, indicating that the rate of glycolytic energy metabolism was at a downward trend at this temperature. At the same time, the activity levels of HK and PK in the hepatopancreas and muscle tissues of the high-temperature group (30°C) were significantly lower than those of the control group. In addition, we also tested PEPCK activity, and the results showed that PEPCK activity was significantly decreased in the high-temperature and low-temperature groups compared with that of the control group. These results suggest that temperature stress may inhibit the rate of gluconeogenic energy metabolic pathways in crayfish. In essence, gluconeogenesis and the energy pathway of glucose metabolism were severely affected in crayfish's hepatopancreas and muscle tissues under extreme water temperatures. Similarly, studies on *Apostichopus japonicus* reported that intestinal digestive and metabolic enzyme activities were very low in the high-temperature (21°C) and low-temperature (6°C) groups.[15]

Lipase (LPS) and α-amylase (α-AMY) are important digestive enzymes in crayfish. Digestive enzyme activity reflects the body's ability to digest nutrients and has a mutually promoting interaction with ingestion.[16] The results of this study showed that water temperature had a significant effect on the activity of digestive enzymes in crayfish. At 10°C, the activity levels of LPS and α-AMY in the intestinal and hepatopancreas of crayfish were lower than those in the control group. The results showed that the digestive ability of crayfish in the low-temperature group was decreased, and the digestive enzyme activity was also at a low level. Meanwhile, the activity levels of LPS and α-AMY in the hepatopancreas and intestinal tissues in the high-temperature group (30°C) were significantly lower than those in the control group. This may be due to excessive water temperature beyond the body's tolerance range, which causes metabolic disorders, thus inhibiting the secretion and activity of digestive enzymes. Relevant studies have shown that the activities of amylase, lipase, and pepsin of crayfish were significantly reduced under the high-temperature condition of 32-35°C.[17] In small yellow croaker, the activities of amylase and lipase increased first and then decreased when the water temperature increased from 27°C to 33°C.[18]

Changes in environmental temperatures often affect the feeding, metabolism, and digestive enzyme activities of crustaceans such as shrimp and crabs, which then affects their growth performance. *Procambarus clarkii*, a poikilothermic animal, is particularly responsive to changes in environmental temperature. Temperature stress caused changes in antioxidant enzymes in crustaceans such as *Procambarus clarkii*, *Marsupenaeus japonicus*, and *Scylla paramamosain*, adversely affecting the animal's performance.[3, 19,20] However, there are few reports on the effects of temperature changes on the nutritional metabolism of crayfish. Therefore, this experiment was conducted to study the effects of short-term extreme water temperature stress on digestion and metabolism of *Procambarus clarkii*, combined with the control group (20°C) and the temperatures significantly deviating from the suitable growth range (10°C, 30°C). The results showed that the enzyme activities of three metabolic enzymes (PK, HK, PEPCK) and two digestive enzymes (LPS, AMS) of crayfish were significantly lower than those of the control group (*P<0.05*). This may be due to the extreme water temperatures (10 °C, 30 °C) beyond the tolerance range of the crayfish, causing metabolic disorders, thus inhibiting the secretion and activity of digestive and metabolic enzymes. In short, extreme temperature changes can affect crayfish's metabolism and digestive enzyme activity, which in turn affects their growth performance. Therefore, controlling the appropriate water tem-

perature is an important factor for crayfish's normal growth and survival.

ACKNOWLEDGMENTS

This work was supported by the Key Scientific Research Projects of Colleges and Universities in Henan Province (24B240001); Henan rice and shrimp integrated planting (Huangchuan) industry Science and Technology Special Commissioner service group, Henan Province science and technology research project (232102321030)

AUTHORS' CONTRIBUTION

Conceptualization: Hanjun Jiang (Lead). Methodology: Hanjun Jiang (Equal), Jiahui Liu (Equal). Investigation: Hanjun Jiang (Equal), Jiahui Liu (Equal), Qianqian Huang (Equal). Writing – original draft: Hanjun Jiang (Lead). Writing – review & editing: Hanjun Jiang (Equal), Jiahui Liu (Equal). Formal Analysis: Jiahui Liu (Equal), Qianqian Huang (Equal). Funding acquisition: Jiahui Liu (Equal), Donghui Yang (Equal). Resources: Jiahui Liu (Equal), Donghui Yang (Equal). Supervision: Jiahui Liu (Equal), Donghui Yang (Equal).

COMPETING OF INTEREST – COPE

No competing interests were disclosed

ETHICAL CONDUCT APPROVAL – IACUC

In any case, we confirm that every effort has been made to alleviate the suffering of the test fish l crawfish, including the following details: All test fish were anesthetized before being sampled. We comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

Submitted: July 09, 2024 CST, Accepted: August 11, 2024 CST

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