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RESEARCH PAPER

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Physiological and Energy Metabolism Responses of Chinese Loach *Paramisgurnus dabryanus* (Dabry de Thiersant, 1872) to Waterless Preservation during Transport

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Abstract

To investigate waterless stress during transport from aquacultures to sale points on the physiological and energy-metabolism properties of *Paramisgurnus dabryanus,* to improve for improving traditional preservation methods, the levels of glucose, cortisol, triglyceride, and cholesterol in serum and the activities of hexokinase (HK), pyruvate kinase (PK), lactic dehydrogenase (LDH), and succinate dehydrogenase (SDH) in muscle were determined. Chinese loaches were subjected to water, less-water, and no-water conditions for 8h. Less-water and no-water stress significantly increased cortisol concentration in serum, while triglycerides and total cholesterol levels slightly decreased. In the initial stages of stress, glucose dropped dramatically and then recovered gradually, eventually exceeding that in the control group. The activities of HK, PK, and LDH increased and then decreased. While HK and PK activities were significantly higher at the end than at the beginning of the stress, LDH activity returned to its pre-stress level. Under less-water and no-water stresses, SDH activity first decreased and then increased. Overall, there were no significant differences between less-water and no-water groups in the measured parameters, except SDH, at the end of the experiment, and variations in all parameters were similar. Waterless preservation conditions resulted in acute stress, acute hyperglycemia, and energetic compensation.

Introduction

Fish freshness is one of the main concerns of aquaculture as a source of high quality food for human consumption. Although aquaculture production systems typically operate over smaller geographical areas than many terrestrial animal production systems, it is often necessary to transport live fish long distances from aquatic breeding farms to sale points. “Less-water” and “no-water” preservation are common low-cost, water-conserving methods used to transport and store live fish. These methods often involve maintaining the fish being out of water for longer than 6 h, making them vulnerable to stress, disease, and death (Paterson *et al*., 2005; Ridgway *et al*., 2006). However, successful transportation can be achieved by reducing stresses, such as handling and transport densities. Stress responses are divided into three categories: primary, secondary, and tertiary (Wendelaar-Bonga, 1997). Primary stress responses include an increase in the circulating levels of catecholamine and cortisol. Secondary stress responses follow from primary responses and include an increase in energy mobilization, characterized mainly by hyperglycemia and increase in circulating lipids, which provide the energy necessary to cope with stress.

Other secondary stress responses are related to the hydromineral balance and cardiovascular, respiratory, and immune functions (Barton, 2002). Primary and secondary responses can instigate tertiary stress responses that affect fish whole body performance, such as growth, reproduction, and survival (Schreck, 2010).

Research on stress responses and their related physiological changes has proven useful in modifying aquaculture techniques to reduce stress in fish preservation and transport. According to Zeng, Chen, and Shen (2014), under waterless preservation, a 1°C h^{-1} temperature drop was conducive for preserving the activity of lactate dehydrogenase and cold acclimation likely contributed for retaining aerobic and anaerobic metabolism as well as for decreasing the damage of blood oxidation. Mi, Qian, and Mao (2012) found that under waterless preservation and transportation, the combination of eugenol and low temperature affected physiological parameters of crucian carp (*Carassius auratus*), but muscle quality was not influenced. Different fish species show different responses to stresses; thus, an assessment of the stress response to waterless preservation is necessary for each species that is exposed to this stress during transportation.

Chinese loach (*Paramisgurnus dabryanus*) is a common, freshwater, omnivorous fish, mainly distributed in Asia (Cho, Kim, Kim, & Nam, 2012). Due to its taste and flavor, high nutritional value, rapid growth rate, and increased tolerance to stress environments, Chinese loach has become a favorite food and one of the most commercially important cultured species in East Asia, especially in China and Korea, with a gradually increasing market demand in recent years (Xia, Zhao, Du, Zhi, & Chang, 2011; You, Zhao, Liu, & Regenstein, 2011; Zhang *et al.*, 2015a, 2015b). It is very similar to *Misgurnus anguillicaudatus*, both in appearance and ecology. It is typically an air breathing species, and, using its posterior intestine as an accessory respiratory organ, *P. dabryanus* can survive in wet mud for extended drought periods (Zhang *et al*., 2016; Zhang, Zhang, Wang, Gu, & Fan, 2017). Much of the literature on the molecular biology and physiological stress responses of *P. dabryanus* is based on experiments conducted in artificial culture conditions (Hao, Ling, & Hong, 2014; Li, Ling, Ge, Ye, & Han, 2015; Li., He, Wang, Chen, & Chang, 2017). However, few detailed reports have considered the physiological and energy metabolism responses of *P. dabryanus* to waterless preservation conditions. The present study investigated the trends in the physiological and energy metabolic parameters of *P. dabryanus* under waterless preservation transport conditions (i.e., low temperature and without water). Our results provide insight on the physiological characteristics of *P. dabryanus* under waterless preservation conditions and a scientific basis for choosing appropriate transportation methods and improving waterless preservation or transportation techniques for live aquatic products.

Materials and Methods

Fish and Acclimation

Five hundred healthy *P. dabryanus* (mean weight: 38.20±3.70 g) were obtained from Tianjin Hongteng Aquaculture Technology Development Co., Ltd (Tianjin, China). The fish were acclimated in 5 fiberglass tanks (7000 L) filled with freshwater, each containing 100 individuals. Each tank was provided with continuous aeration and a water flow-through system (flow rate 500 $L \cdot h^{-1}$, dissolved oxygen >5 mg L^{-1}, NH_3 concentration 0.2–0.3 mg/L, H_2S concentration <0.01 mg/L, and pH 7.60–8.00).

Experimental Procedures

After 5 days of acclimation, individuals of similar body size were fasted for 24 h and then randomly divided into 3 groups (5 tanks per group, 30 individuals per tank): control group (30 fish per tank with 40 L water), less-water group (weight of fish equaled the weight of the water, 30 fish per tank with 1.2 L water), no-water group (1 tank with 30 fish). The tanks (length × width × height: 30 cm×40 cm× 40 cm) were placed into a low-temperature incubator with ventilating system and temperature control system including a refrigerating and heat-up machine. Temperature was maintained at 8.00±0.56°C. 5 individuals per group were sampled at 0, 1, 2, 4, and 8 h post-treatment. Throughout the experiment, fish survival rate was 100% in the three groups (Table 1).

Sample Collection and Preparation

Following Gao *et al.* (2017) and Zeng *et al.* (2014), fish were anesthetized using MS-222 (0.05 g in 10 L water), and venous blood and muscle tissue samples were taken from each fish within the control, less-water, and no-water groups. Blood was collected from the

Table 1. Survival rate of *Paramisgurnus dabryanus* after less-water and no-water preservation

	Group	0h	1h	2h	4h	8h
Survival rate (%)	Control	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
	Less-Water	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
	No-Water	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0

Values are means ± S.D..

caudal vasculature using a 1-mL disposable syringe and kept in a 1.5-mL centrifuge tube for 2 h at about 4°C before centrifugation (Universal 320R, Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany) for 10 min at 3,000 rpm (Di Marco *et al.*, 2011). The serum collected from each tube was kept at -80°C (Thermo Scientific Forma 702, USA) until cortisol, glucose, triglycerides, and total cholesterol analyses. Muscle tissues were cut into 0.1-0.3-g pieces, homogenized in 9 volume saline (4°C, 0.86%), and centrifuged for 10 min at 3000 rpmunder 4°C (Gao *et al*., 2017). The supernatants were collected and stored at -80°C until measurements of hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), and succinate dehydrogenase (SDH) activities.

Sample Analysis

Serum cortisol was determined using a Fish Cortisol ELISA (enzyme-linked immunoassay) Kit (R & D Systems, Minneapolis, USA), according to the manufacturer's instructions. Serum glucose was determined using a Glucose Assay Kit (Rongsheng Biological and Pharmaceutical Co. Ltd., Shanghai, China), according to the manufacturer's instructions. Triglycerides, total cholesterol, and the activities of HK, PK, LDH, and SDH were measured via colorimetric methods using the Triglycerides Assay Kit, Total Cholesterol Assay Kit, Hexokinase Assay Kit, Pyruvate Kinase Assay Kit, Lacate Dehydrogenase Assay Kit and Succinate Dehydrogenase Assay Kit (Jiancheng Biological Engineering Institute, Nanjing, China), respectively, following according to the manufacturer's instructions. Total protein content was determined using the Coomassie Brilliant Blue G250 dye binding method (Bradford, 1976).

Statistical Analysis

Data are expressed as means ± standard deviations (S.D.). Two-way analysis of variance

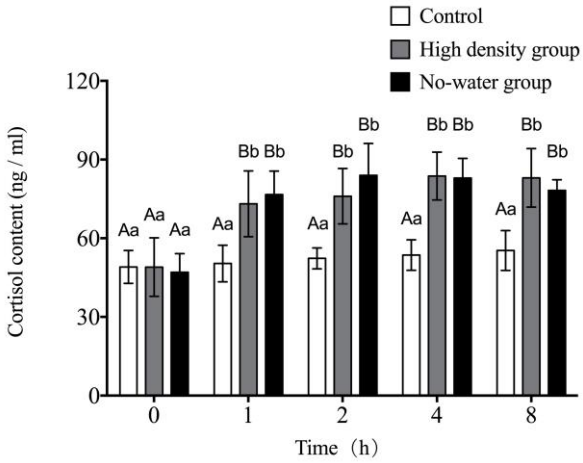

Figure 1. Variation in cortisol concentrations in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

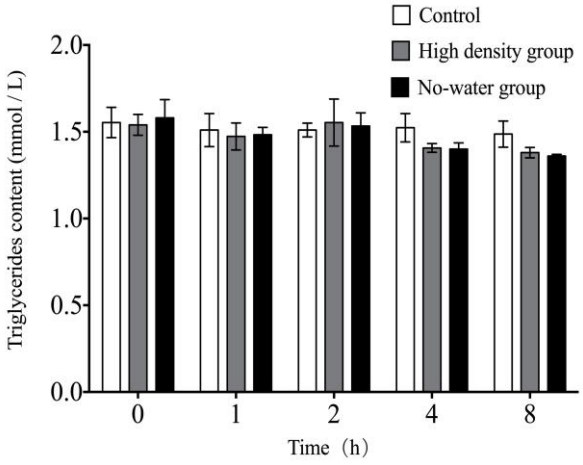

Figure 2. Variation in triglyceride concentrations in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

(ANOVA) and Duncan's multiple comparison tests were conducted in SPSS 21.0 (IBM, New York, USA), considering P<0.05 as statistically significant.

Results

Effect of Waterless Preservation on Cortisol

Serum cortisol concentrations increased significantly (P<0.01) following less-water and no-water stress treatments (Figure 1). There was no significant difference between less-water and no-water groups at each time point (P>0.08). The two-way ANOVA showed that water conditions, exposure time, and the interaction between the two factors had significant effects on cortisol concentrations (P<0.045).

Effect of Waterless Preservation On Triglycerides and Total Cholesterol

Figure 2 shows that after 1–2 h, triglyceride concentrations in the less-water and no-water groups were similar to that of the control group. As exposure time increased, triglyceride concentrations declined, reaching the minimum levels after 8 h. At 4–8 h, triglyceride concentrations in both treatment groups were not significantly lower than in the control group (P>0.06). Total cholesterol concentrations decreased in both less-water and no-water groups, and although recovering slightly after 8 h, they were still lower than before the experiment (Figure 3). The two-way ANOVA showed that only exposure time significantly affected triglyceride and total cholesterol concentrations (P<0.02).

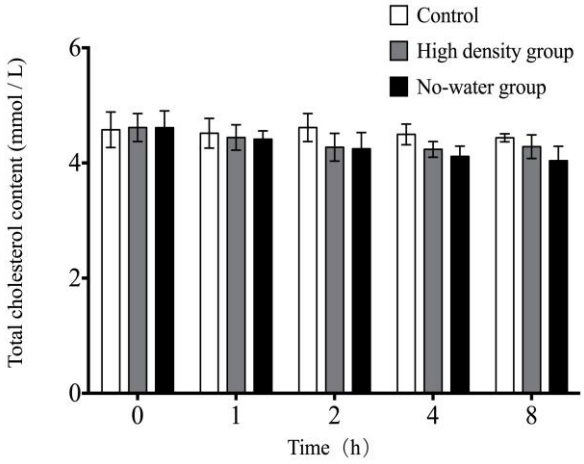

Figure 3. Variation in total cholesterol concentrations in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

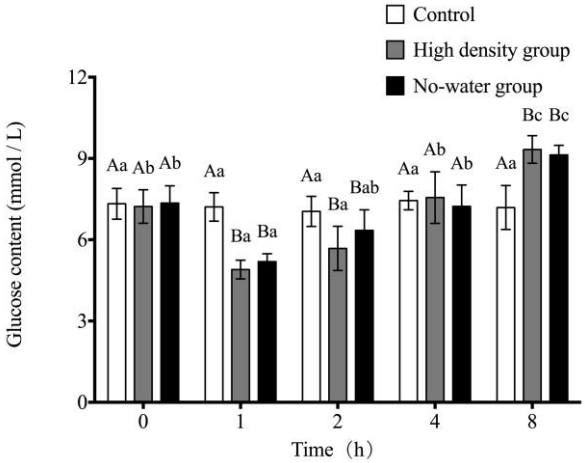

Figure 4. Variation in glucose concentrations in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

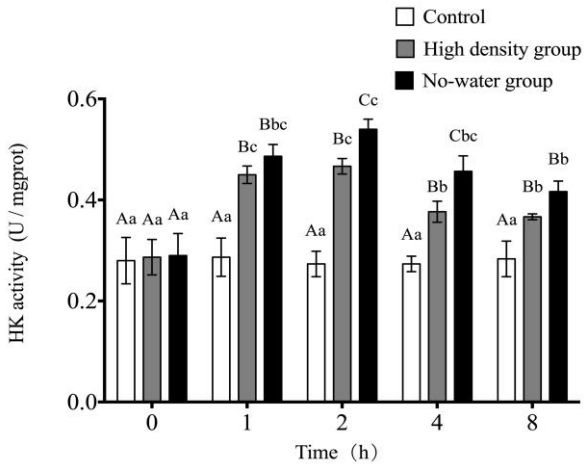

Figure 5. Variation in HK activity in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

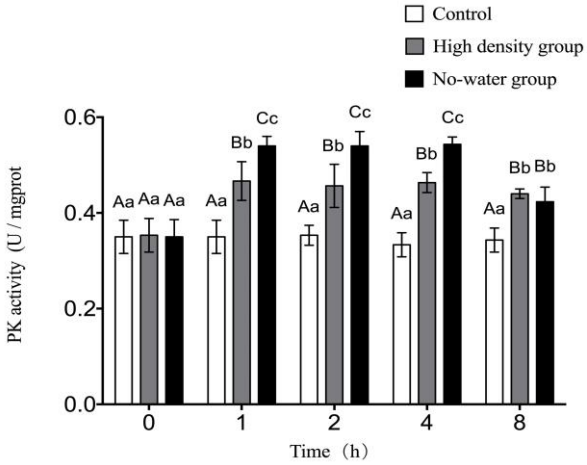

Figure 6. Variation in PK activity in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

Effect of Waterless Preservation on Glucose and Glucose Metabolism Enzymes

Serum glucose levels exhibited similar patterns in less-water and no-water groups, dropping dramatically within the first hour and then recovering gradually (Figure 4). At 4 h, the glucose levels were identical in treated as control groups, but at 8 h they significantly exceeded the levels of the control group (P=0.01). Water conditions, exposure time, and the interaction between the two factors significantly affected glucose levels (P<0.007).

A change in the trend of glucose-metabolism enzymes was observed under waterless stress in Figures 5-8. The activity of HK in the less-water and no-water groups increased significantly at 1 h and 2 h, and then decreased slightly during 4–8 h; however, it was significantly higher than before the experiment (P<0.04). In the no-water group, HK activity was significantly higher than in the less-water group at 2 h and 4 h (P<0.01). The activity of PK showed a similar trend to HK activity; however, PK activity was high at 1–4 h, and then declined at 8 h, although it was always significantly higher than before the experiment (P=0.04). During 1–4 h, the PK activity in the no-water group was significantly higher than in the less-water group (P<0.05), but at 8 h the PK activity was not significantly different (P=0.19) between these groups. The activity of LDH increased dramatically during 1–2 h in the less-water and no-water groups, and then declined to pre-stress levels, while the activity of SDH showed the opposite trend: it dropped dramatically during 1–2 h, and then increased slightly at 4 h and 8 h, being always significantly lower than before the experiment (P<0.04). The two-way ANOVA showed that water conditions, exposure time, and the interaction between the two factors significantly affected the activities of HK, PK, LDH, and SDH (P<0.01).

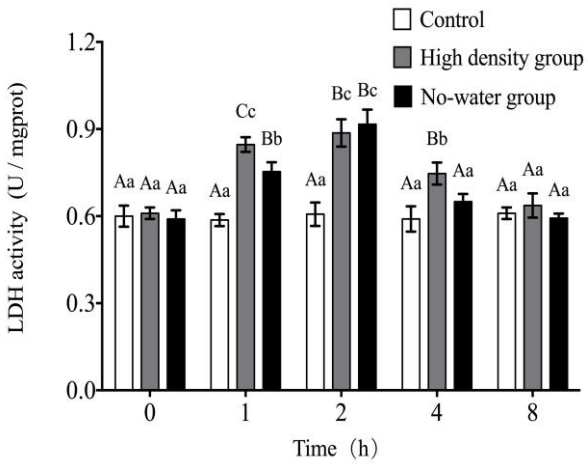

Figure 7. Variation in LDH activity in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

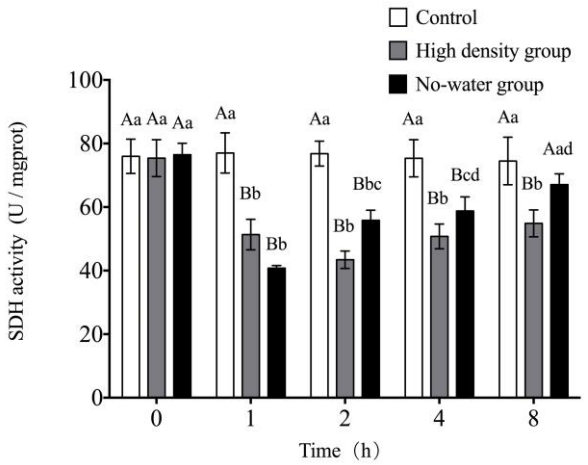

Figure 8. Variation in SDH activity in *Paramisgurnus dabryanus* after less-water and no-water preservation. Values are means ± S.D. Different lowercase letters indicate significant differences among sampling times per treatment group; different capital letters indicate significant differences among treatment groups at the same sampling time.

Discussion

Cortisol is an important stress hormone that is elevated by environmental stimuli such as tagging, crowding, and temperature (Sulikowski, Fairchild, Rennels, Howell, & Tsang, 2005; Sulikowski, Fairchild, Rennels, Howell, & Tsang, 2006; Zeng *et al.*, 2014;). Cortisol has been used to evaluate stress levels associated with waterless preservation transport in other species. For example, Mi *et al.* (2012) found that low temperature and waterless preservation conditions significantly increased cortisol concentration in Crucian carp (*C. auratus*). Gomes *et al.* (2003) found that increased mortality and higher concentrations of plasma cortisol were associated with higher transport densities in *Colossoma macropomum*. According to Congleton, LaVoie, Schreck and Davis (2000), cortisol level was higher in *Oncorhynchus tshawytscha* when transport densities were at their maximum. In the present study, serum cortisol concentrations significantly and persistently increased within 4 h and then began to decrease slightly in the less-water and no-water groups. These results indicate that the stress response of *P. dabryanus* to waterless conditions was significant under acute stress.

Cholesterol and triglycerides are important for supplying and storing energy, and to indicate lipometabolism status (Shi *et al.*, 2010). According to Wedemeyer and McLeay (1970), stress can induce hypercholesterol effects in teleosts, and several previous studies (Hoseini, Hosseini, & Nodeh, 2011; Hoseini, Hedayati, & Ghelichpour, 2014; Hoseini, Lluis, Abolhasani, & Rajabiesterabadi, 2016), also detected that cholesterol and triglycerides decreased immediately after exposure to stress, suggesting an initial arrest in lipid energy supply. In the present study, cholesterol and triglycerides showed a slight decrease after exposure to water stress, although this decrease

was not significant in relation to that observed in the control group, suggesting that lipometabolism was not significantly affected by water stress. In addition, the significant decrease in glucose level shown initially suggested that in both less-water and no-water conditions, the main energy source was glucose, not lipids. Ellsaesser and Clem (1987) also found that acute stress influenced glycometabolism, but not lipometabolism.

Energy metabolism could reflect not only the internal physiological condition of organisms, but also the effects of external factors. Waterless conditions and temperature are two of the most important environmental factors (Sulikowski *et al.*, 2006; Samaras, Papandroulakis, Costari, & Pavlidis. ,2016). In general, body temperature of aquatic animals fluctuates with ambient temperature, with concomitant variation in their metabolic rate (Tian, Dong, Wang, & Wu, 2006). Wang, Zhu and Xu (2005) reported that when organisms experienced increased demand for oxygen or internal hypoxia, they increased the energy available for consumption by producing more energy.

Glucose is the major substrate for energy production. Exposure to subzero temperature stress produced hyperglycemia accompanied by an increase in glycogen phosphorylase activity, but no change in glucose-6-phosphatase activity (Benziger & Umminger, 1973). The cooling process increases blood glucose via gluconeogenesis, while the heating process increases blood glucose via glycogenolysis (Seibert, 1985). Under optimal conditions, the relative balance of blood glucose depends upon two pathways: glycolysis and its reverse pathway-gluconeogenesis (Metón, Fernández, & Baanante 2003). Conversely, under stress conditions, blood glucose concentration notably changes (Hoseini *et al*., 2016; Samaras *et al*., 2016). Many stress factors rapidly elevate blood glucose levels in fish (Connors, Schneider, Genoway, & Barraclough,1978; Woo, 1990; Kuo & Hiesh, 2006). Enzymes HK and PK are crucial in the glycolysis process and have an important role in maintaining hemolymph or blood glucose levels. While HK cooperates with glucose transporters within the cell membrane to improve the utilization of exogenous glucose (Allert, Ernest, Poliszcak, Opperdoes, & Michels, 1991; Sangiao-Alvarellosa *et al*., 2006), PK catalyzes pyruvate and releases ATP during this reaction. In fact, PK is a rate-limiting enzyme of glycolysis (Lemos, Salomon, Gomes, Phan & Buchholz 2003). In the present study, glucose levels decreased dramatically after stress, but HK and PK activities increased significantly, suggesting that fish used more energy to respond to the stress. Because normal energy production could not meet the increased energy demand, glycolysis was upgraded to glucose oxidative phosphorylation to compensate for the high energy consumption due to stress. As more glucose was utilized for energy production, glucose concentrations declined and glycolysis was enhanced. According to Mommsen, Vijayan, and Moon (1999), at the onset of stress, fish glucose is supplied by glycogenolysis, but when glycogen is depleted, fish maintain glucose levels via corticosteroid-induced gluconeogenesis. According to Seibert (1985), the cooling process also increases glucose via gluconeogenesis. In the present study, soon (1h) after waterless preservation stress began, serum cortisol reached a high level while glucose reached a minimum; as cortisol increased, glucose recovered. This indicated that gluconeogenesis was repressed at the early period of waterless preservation or that there was a regulation lag. However, under prolonged stress, the extra consumption of glucose was replenished by re-activating gluconeogenesis, which allowed recovering glucose levels and increasing them to levels higher than before the stress. Due to glycolysis and gluconeogenesis re-balancing, energetic homeostasis was maintained, aiding in the self-regulation of internal metabolism in fish (Hochachka & Lutz, 2001).

The cytoplasmic enzyme LDH is a key link between glycolysis and the tricarboxylic acid cycle (TCA). Under aerobic conditions, pyruvate is oxidized to CO_2, H_2O, and ATP, while under anaerobic conditions LDH reduces pyruvate to L-lactate releasing a small amount of ATP. Thus, LDH is a useful indicator of organisms' capability for anaerobic metabolism (Zietara, Gronczewska, Stachowiak, & Skorkowski, 1996).

As an important component of the mitochondrial inner membrane, SDH is not only a constituent of succinate-Q-reductase, but also plays a key role in the TCA cycle. Its activity influences oxidative phosphorylation thereby reflecting the level of aerobic metabolism (Wang, Zou, & Zhang, 2005). In the present study, LDH activity increased first and then recovered, which was opposite to SDH activity. In the early stage of thermal stress, aerobic respiration was weakened, leading to an insufficient energy supply. To increase energy production, anaerobic respiration was enhanced and lactate accumulated in the body as a result. Because, in the present study, LDH, a key enzyme of anaerobic metabolism, and SDH, a key enzyme of aerobic metabolism, returned to their before-stress levels, fish might adapt to less-water or no-water conditions after a short time. The recovery of aerobic respiration is likely related with the intestinal airbreathing ability of *P. dabryanus*. At the same time, more pyruvate molecules were released into the TCA cycle to increase energy production. Following stress, anaerobic metabolic enzymes were active at a higher level, implying that fish adjusted metabolic processes to counteract the effects of stress.

Conclusion

In the present study, *P. dabryanus* enhanced the activity of its metabolic enzymes to increase metabolism and replenish energy in response to waterless preservation, which resulted in acute stress,

acute hyperglycemia, and energetic compensation. The less-water and no-water groups showed similar changes in physiology and energetic compensation. Further studies on the quality and biochemical properties of fish muscle and on changes in physiological parameters during the recovery period are needed for developing optimum preservation and transport conditions.

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