Short-time Transport Stress Affected Biochemical Parameters in *Takifugu Rubripes*

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**Abstract.** Transport manipulation is a routine practice during the culture of fishes. Stress responses will occur during transport. In order to assess the impact of short-time transport on the health of juvenile *Takifugu rubripes*, a 30-minute simulated transportation was carried out. Result showed that Lactate and LDH in serum, muscle and liver of test fish respond rapidly after transport. Serum antioxidative and immune parameters, liver function parameters also changed significantly after transport. Most parameters recovered at 168 hour after transport. In conclusion, at least 168 hours recovery time is recommended for *Takifugu rubripes* suffered 30 minutes transport stress.

1. Introduction

Transport is an often occurred manipulation during fish culture. During transport, fish are exposed to many stressors including high density induced crowding, variation of dissolved oxygen, water temperature, and ammonia nitrogen. These stressors act on fish may evoke primary responses, such as increases in corticosteroid and catecholamine hormones, secondary responses such as metabolic changes, immune function changes, and tertiary responses such as modified behavioral patterns [1]. The changes of these physiological and biochemical parameters will damage the health status of fish. An extreme example is that transport stress will easily induce mass mortality of *Coilia nasus*, an economic migration fish between Yangtze River and estuary [2]. Furthermore, fish body surface, which act as physical barrier against infection, could easily be scratched during transport. The changes in body parameters and damage of body surface make fish more susceptible to adventitious infection. In addition, fish under transport may secrete more mucus as a stress reaction. This will worsen water quality and impair the overall health of fish.

Pufferfish *Takifugu rubripes*, which contains high content of essential amino acids, poly-unsaturated fatty acids and docosahexaenoic acid [3], is widely cultured and consumed in China, Korea and Japan. Before transport, pufferfish is usually packed into plastic bags with cold water and pure oxygen. This will relieve violent collision and bite elicited by transport, and will ensure high transport survival. However, people pay no attention to the health status of pufferfish subjected to transport. At present, evaluation of transport stress on health status of pufferfish is not found. In this paper, we simulated a short-time overland transport of pufferfish, assayed biochemical parameters during recovery to evaluate transport stress on its health status.
2. Materials and Methods

2.1. Fish and Acclimation
Juvenile pufferfish (100-150 g) were obtained in a cement tank (6.0 m × 6.0 m × 1.5 m) from Tianjin Haisheng Aquaculture Co. Ltd. (Binhai New Area, Tianjin, China). Pufferfish were acclimated in two cubic net cages (1 m³) for seven days, with 84 individuals in each net cage. The two net cages were fixed in a cement tank, from which pufferfish were obtained. Pufferfish were fed twice a day. Seawater temperature was 21 °C, salinity was 20, dissolved oxygen was above 8 mg/L, ammonia nitrogen was below 0.01 mg/L, and nitrite was below 0.02 mg/L.

2.2. Simulated Transport
Two plastic tanks with 20 L seawater each were put on a cart with a portable blower and air diffusers. Seawater temperature in plastic tank was cooled to 17 °C with ice bag in advance. The plastic tanks were covered with sunshade net to avoid sunshine and disturbance. After fasted for 12 h, 84 pufferfish from one net cage were equally distributed into two plastic tanks (20 L). The cart was pulled to simulate overland transport for 30 min. Then, pufferfish were put back into the net cage immediately. During the simulated transport, air was pumped into the seawater continually and water temperature was monitored every 10 min. The 84 pufferfish in another net cage, fasted for 12 h likewise but not subjected to transport, served as control.

2.3. Sampling and Biochemical Parameters Assay
Pufferfish were sampled at 0 h, 8 h, 12 h, 24 h, 48 h, 96 h, and 168 h after 30 min simulated overland transport. Twelve transport fish and 12 control fish were promptly caught by a hand-held net from the two net cages at each sampling. Fish were euthanized by overdose of tricaine methanesulfonate (MS-222; Hangzhou Animal Medicine, Inc., China), then weighed. Blood was took from caudal vein. A 100 μL of blood was mixed with 20 μL 8% heparin to assay oxidative radical production. The rest blood was distributed in centrifuge tubes and stored at 4 °C overnight for serum separation. Dorsal muscle and liver were excised after blood sampling. All samples were stored at -20 °C for further use. Serum of four fish were pooled and make three iterations, then stored at -20 °C until further use. Dorsal muscle and liver of three fish were excised after blood sampling, and stored at -20 °C for further use. The remaining eight fish were returned to the company.

Blood oxidative radical production was assayed spectrophotometrically by means of nitroblue tetrazolium (NBT) reduction, according to Siwicki et al. [4] with some modifications. A stock solution of NBT was freshly prepared by dissolving 0.2 g NBT into 100 mL sterile saline water. The NBT working solution was prepared by equal volume mixing NBT stock solution with 1 mg/mL glucose (1 mg glucose dissolved into sterile 0.15 M phosphate buffer solution, pH 7.2). Fifty microliters of blood was added to 50 μL of NBT working solution in a centrifuge tube and incubated at 28 °C for 30 min. The centrifuge tube was shake every ten minute. Then, a 75 μL sample was taken from this mixture, added to 1.5 mL of N, N-dimethyl formamide (DMF;Amresco,America) to dissolve formed formazan crystals, and centrifuged at 1000 rpm for 15 min. The supernatant was determined in a spectrophotometer (UVmini-1240, Shimadzu, Japan) at 620 nm, with DMF as blank.

Catalase (CAT), Total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), malondialdehyde (MDA), lysozyme (LZM), glucose (GLU), total glutathione (T-GSH), reduced glutathione (GSH), aspartate amino transferase (AST), and alanine aminotransferase (ALT) in serum were assayed. Lactate, lactate dehydrogenase (LDH) in serum, muscle and liver were determined. These parameters were assayed with commercial kits manufactured by Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

2.4. Water Quality Monitoring
Water temperature, dissolved oxygen (DO), pH, salinity were monitored every day using YSI 556 m (Yellow Spring Instrument Co., Yellow Spring, Ohio). Ammonia nitrogen and nitrite were measured using commercial kits (Sunpu Biochemistry Co., Beijing, China).
2.5. Statistical Analysis
Data were subjected to independent-sample $T$ test with SPSS 16.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Water Quality
Before and after the simulated transport, water quality in the plastic tanks was monitored. Water temperature was 17-16 °C, DO was 8.5-8.2 mg/mL, pH was 8.5-8.4, salinity was 20, ammonia nitrogen was below 0.01 mg/mL, nitrite was below 0.01 mg/mL. After the simulated transport, pufferfish were transferred into net cage. Water quality in the net cages were as following: temperature 21 °C, DO 8.5 mg/mL, pH 8.5, salinity 20, ammonia nitrogen < 0.01 mg/mL, nitrite < 0.01 mg/mL.

3.2. Lactate and LDH in Serum, Muscle and Liver after Transport
Serum lactate level increased immediately after transport, maintained high level in 96 hours, and declined to control level at 168 h (Figure 1a). Serum LDH activity was significantly lower than control at 0 h, 8 h after transport, then increase at 12 h and 48 h, and get back to control level at 168 h (Figure 1b). Muscle lactate maintained significant higher level than control in 168 hours (Figure 1c), while muscle LDH fluctuated, increased at 0 h, 8 h, decreased at 12 h, 48 h, and then increased at 168 h (Figure 1d). Liver lactate content in transport group was near control group in 24 hours, then increased significantly at 48 h, and decreased to control level at 168 h (Figure 1e). Liver LDH activity in transport group elevated significantly after transport, reaching peak value at 12 h, then declined to control level after 96 h (Figure 1f).
Figure 1. Variation of serum lactate (a), serum LDH (b), muscle lactate (c), muscle LDH (d), liver lactate (e), and liver LDH (f) in Takifugu rubripes after 30 minutes transport. Data between transport and control group were subjected to independent-sample T test. * indicates significant difference ($P<0.05$), ** indicates extremely significant difference ($P<0.05$). Data are means ± SD, n=3.

3.3. Antioxidative and Immune Parameters after Transport

Serum T-AOC in transport group was statistically higher after transport, reaching peak value, 4-fold of control, at 12 h (Figure 2a). Afterward, serum T-AOC decreased to control level.

Serum T-SOD in transport group was significantly lower than control at 8 h and 168 h, while there was no statistically difference at other sampling time (Figure 2b).

Serum CAT in transport group was statistically higher than control group at 8 h, 12 h, and 96 h after transport, while no statistically difference was observed at other sampling time (Figure 2c).

Figure 2d showed that serum MDA in transport group was significantly higher, 2.3-fold of control, at 0 h post-transport, then descended hastily at 8 h. Although serum MDA in transport group fluctuated and significant differences were observed at 24 h, 48 h, 96 h, it increased to control level at 168 h.

Figure 2e and Figure 2f showed than serum T-GSH and GSH varied greatly after transport. Serum T-GSH significantly increased at 0 h, decreased at 8 h, then increase to peak value at 24 h, and decreased rapidly to control level at 48 h. Serum GSH in transport group rose to top value at 8 h, then decreased gradually, returned to control level at 96 h.

Figure 3a showed that serum LZM in transport group varied statistically significant at 0 h and 8 h, notably higher at 12 h and 24 h, then decreased to the lowest value at 96 h, and returned to control level at 168 h.

Blood oxidative radical production dropped significantly after transport, reached the lowest value at 12 h, and rose to control level at 168 h (Figure 3b).

Serum glucose elevated significantly from 8 h post-transport, reaching peak value at 48 h, and declined to control level at 168 h (Figure 3c).

3.4. Liver Function Parameters after Transport

Serum aspartate aminotransferase (AST) was notably elevated at 0 h, 12 h, and decreased to control level at 24 h (Figure 4a). However, serum alanine aminotransferase (ALT) was notably higher than control group at 12 h, 48 h, and 168 h (Figure 4b).
Figure 2. Variation of serum T-AOC (a), T-SOD (b), CAT (c), MDA (d), T-GSH (e), and GSH (f) in *Takifugu rubripes* after 30 minutes transport. Data between transport and control group were subjected to independent-sample T test. * indicates significant difference ($P<0.05$), ** indicates extremely significant difference ($P<0.05$). Data are means ± SD, n=3.
Figure 3. Variation of serum lysozyme (a), blood oxidative radicals production (b), serum glucose (c) in *Takifugu rubripes* after 30 minutes transport. Data between transport and control group were subjected to independent-sample *T* test. * indicates significant difference (*P*<0.05), ** indicates extremely significant difference (*P*<0.05). Data are means ± SD, *n*=3.
Figure 4. Variation of serum AST (a) and ALT (b) in Takifugu rubripes after 30 minutes transport. Data between transport and control group were subjected to independent-sample T test. * indicates significant difference ($P<0.05$), ** indicates extremely significant difference ($P<0.05$). Data are means ± SD, n=3.

4. Discussion

Prior works have documented that stressors will evoke physiological responses and impair the health of fish [1, 5]. These findings are very important reference for fish farmers to ensure the health of fishes during their culture process. In order to reduce transport induced mortality and ensure fish health, it is very important to reduce stress and its detrimental effects, provide favorable environment and enough recovery time for transported fishes. However, no related report about transport stress on pufferfish was found to date. The main purpose of this paper was to test the survival and find out suitable recovery time for juvenile pufferfish suffered 30 min transport stress. One hundred percent of survival rate was achieved during and after transport. As most parameters were recovered in 168 hours, it is the recommended recovery time for juvenile pufferfish suffered 30 min transport.

However, transport stress induced agonistic behaviors, physiological responses, survival, and recovery time varied greatly among different fish species. Coilia nasus is very sensitive to collision and transport. Almost a hundred mortality if it suffered these stress [2]. Eleutheronema tetractylum suffered from 6 hours transport stress result in 27.5% cumulative mortality [6]. By testing the antioxidative and immune parameters, Cynoglossus semilaevis fry suffered from 11 hours practical transport, need at least 96 hours for recovery without death [7]. Hybrid grouper (Epinephelus fuscoguttatus ♀ × E. lanceolatus ♂) subadult suffered from 30 min transport stress, need 168 hours for recovery without death [8]. The survival and recovery time of this paper are in agreement with hybrid grouper mentioned above.

In this paper, serum lactate increased rapidly while serum LDH first decreased then increased significantly after transport. Lactate and LDH in muscle and liver increased simultaneously after transport. These results indicate that lactate and LDH are sensitive transport stress indicators for T. rubripes. With contrary to pufferfish, serum LDH of hybrid grouper elevated after 30-minute transport, and decreased to control level at 96 h [8]. In view of high level lactate and its long duration in serum, muscle and liver of transport pufferfish in this paper, it’s preferable to take some measures to relieve the detrimental effect of lactate. Augmenting dissolve oxygen of aquaculture water may contribute to
fish blood oxygen concentration, consequently, contribute to the oxidation of lactate into pyruvic acid by LDH. Pyruvic acid is catalyzed irreversibly into acetyl-CoA by pyruvate dehydrogenase complex. As pantothenic acid is component of coenzyme A (CoA), thiamine pyrophosphate (TPP), lipoic acid, CoA, flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NAD⁺) are five cofactors of pyruvate dehydrogenase complex. So oral administration or bathing with pantothenic acid, thiamine, riboflavin and nicotinamide could contribute to the catalyzing of pyruvic acid. Further experiment should be carried out to test this hypothesis.

As a product of lipid peroxide, serum MDA level increased significantly at 0 h after transport. This indicated that 30 minutes transport induced oxidative stress in pufferfish. GSH can reduce lipid peroxide products to unsaturated fatty acid. High level of GSH and low level of MDA in transport group at 8h, 12h indicated that GSH exerted its reduction property. As ascorbic acid can reduce oxidized glutathione (GSSG) into GSH, hence contribute body to cope with oxidative stress.

We used NBT reduction to detect oxidative radical production of blood cells. Result in this paper showed that fish subjected to transport produced less oxidative radicals. This indicated that transport could induce high level of antioxidant in blood cells. Further experiment need to be carried out to assay antioxidant level in blood cells to verify this hypothesis.

The high level of serum AST and ALT in transport group indicated that transport damaged liver function of pufferfish. High level of serum glucose in transport group implied that more energy was aroused to cope with transport stress.

In conclusion, 30-minute transport stress induces significant changes of lactate, LDH, antioxidative and immune parameters, and liver function of pufferfish. At least 168-hour recovery time was recommended.

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6. References
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