Effects of Hypoxic Stress on Energy Metabolism in Red Sea Bream, Pagrus major-II

Response of Enzyme Activities and Metabolite Concentrations in Various Tissues of Red Sea Bream, Pagrus major, Subjected to Hypoxic Exposure*1

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Abstract: Juvenile red sea bream, Pagrus major, were exposed to hypoxia, a gentle decrease in partial pressure of oxygen (Po2) down to 26 mmHg, and subsequently maintained beneath this pressure, at 27.8°C. The increasing trend in respiratory frequency of the fish, caused by hypoxia, reached a maximum level at about 38 mmHg Po2 and thereafter decreased. The fish sank to the bottom around an hour after the Po2 fell below 26 mmHg, and after another half hour respiration was arrested. The hematocrit value and plasma glucose level were slowly increased by hypoxia up to the point at which the fish sank, although mean cellular hemoglobin content (MCHC) was decreased at the maximum level of respiratory frequency. Lactate dehydrogenase, creatine kinase activities, and lactic acid level in muscle, and hepatopancreas lactate concentration, were remarkably enlarged at the time the fish sank. Cytochrome c oxidase (CCO) activity in the brain significantly increased with increasing hypoxic load, though not in the gills. On the other hand, CCO activity in the hepatopancreas, kidney, and muscle markedly decreased at the maximum level of respiratory frequency, although its activity in these organs and the heart increased after the time at which the fish sank. These results suggest that metabolic depression in red sea bream in response to hypoxia temporarily occurs from the maximum level of respiratory frequency, though the metabolic function abreacts at around the time the fish sink.

Key words: Red sea bream; Hypoxic stress; Cytochrome c oxidase activity; Metabolite concentration

Marine fish reared by cage culture in bays endure a lot of stresses in the form of change in the environment, pathogens, inadequate food, and husbandry practices, etc.1). However, composite stress is difficult to assess and treat. We previously examined the effects of hypoxia on stress response and energy metabolism in the Japanese parrot fish, Oplegnathus fasciatus2), Nile tilapia, Oreochromis niloticus3), and red sea bream, Pagrus major1). Consequently, it was found that in Nile tilapia, which has a high tolerance for hypoxia, metabolic depression is associated with increased hypoxic load3). In Mozambique tilapia, Oreochromis mossambicus, flexible metabolic depression is demonstrated during hypoxia4) and/or anoxia5). Common carp, Cyprinus carpio, seem to respond to the anoxic condition by conversion of glycogen to lactic acid6). Goldfish, Carassius auratus, is able to survive under the anoxic condition for about 10 h at 20°C via the implementation of a modified anaerobic metabolism7). The survival methods these species of fish utilize in response to hypoxia and/or anoxia occurs via a diminution

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in oxygen consumption and without activation of anaerobic energy turnover\(^8\). However, data on metabolic depression in marine cultivated fish subjected to hypoxic exposure is scarce.

Our previous studies\(^1\) indicated that the stress response of release of cortisol and glucose in red sea bream occurred in response to the severe hypoxic condition. Additionally, the adenosine triphosphate concentration and the energy charge in the hepatopancreas, but not in the gill or brain, were considerably diminished in response to hypoxia. It was therefore suggested that the decreased energy status in the hepatopancreas coincident with stress response played an important role in overall metabolic depression and/or in energy consumption for gluconeogenesis. However, it is not clear whether the metabolic depression occurs in red sea bream subjected to hypoxic exposure. This study was undertaken to examine the response of enzyme activities and metabolite concentrations in various tissues of red sea bream exposed to hypoxia.

### Materials and Methods

**Fish and hypoxic exposure**

Juvenile red sea bream were obtained in 1997 from the Fisheries laboratory of Kinki University's Shirahama Experiment Station. Fish were fed daily with red sea bream formulated diet. Thirty-seven fish (body weight 45.1 ± 5.5g, body length 12.9 ± 0.6cm) were acclimated to two 500 l volume experimental tanks each for three days after pre-feeding at Kinki University Fish Nursery Center, Shirahama station. Rearing water was running, filtered sea water of 110-151 mmHg Pot at 28°C. Fish were not fed for 24 h before the initiation of the hypoxic experiment. A description of the experimental tank structure and methods of hypoxic exposure can be found in previous studies\(^1\). Partial pressure of oxygen in the water was decreased by degrees from 151 mmHg to 26 mmHg over two-and-a-half hours and subsequently sustained under 26 mmHg Pot for about 2 h. Fish activities were observed. Respiratory frequency, the number of fish that sank to the bottom of aquarium, and cumulative mortality of fish during hypoxia were recorded. Six to fourteen fish were sampled respectively at four points, as follows: normoxia at 151 mmHg Pot, maximum level of respiratory frequency at 38 mmHg Pot, upon sinking to the bottom of the aquarium approximately one hour after the Pot fell under 26 mmHg, and just before respiratory arrest around one-and-a-half hours after the Pot fell to less than 26 mmHg. Ten fish under the normoxic condition in an aquarium were sampled by using a hook and line just before the onset of the experiment\(^9\). The fish assessed at the other experimental points were sampled as soon as possible by using a small net inserted through the border of the cover on the tank. The hypoxic fish were easily captured by the net. Sampling methods of blood and tissues are described in our previous paper\(^5\).

### Analysis

The methods of measurement of hemoglobin and glucose and the extraction of an acid soluble fraction and the calculation for the mean cellular hemoglobin contents (MCHC) are described in previous reports\(^13\). Lactic acid in an acid-soluble fraction was measured by the reduction of nicotinamide adenine dinucleotide with lactate dehydrogenase, using the method of Hohorst\(^10\). For the analysis of enzyme activities, the remaining tissue was homogenized with 9 volumes of cold-distilled water using a glass homogenizer, and the homogenate was centrifuged at 8,000 × g for 10 min at 4°C. The resultant supernatant was used to analyze the cytochrome c oxidase (CCO; EC 1.9.3.1), aspartate aminotransferase (GOT; EC 3.6.1.1), alanine aminotransferase (GPT; EC 3.6.1.2), lactate dehydrogenase (LDH; EC 1.1.1.27), and creatine kinase (CK; EC 2.7.3.2), respectively. The assay procedure of CCO activity was the method of Cai and Adelman\(^11\), with some modification. GOT, GPT, LDH, and CK activities were determined with the Vision System following the modified methods of Bergmeyer \textit{et al.}\(^12\), Bergmeyer \textit{et al.}\(^12\), Amador and Wacker\(^13\), and Szasz \textit{et al.}\(^14\), respectively.
Statistics

The data were expressed as mean ± SD. To determine the difference among groups, data were subjected to Duncan's new multiple range test consequent to determining the significant differences among the means by a one-way ANOVA treatment ($p<0.05$). The Mann-Whitney U-test was also utilized for comparison among the medians in groups if the differences between the groups by the Kruskal-Wallis test subsequent to Bartlett's test were significant ($p<0.05$), respectively.

Results

Fish status under hypoxia

Respiratory frequency, percent of fish that sank to the bottom of the aquarium, and cumulative mortality of the red sea bream exposed to hypoxia are shown in Fig. 1. The respiratory frequency of fish tended to increase with increasing hypoxic load, and reached a maximum level at about 38 mmHg $P_0^2$. Subsequently, respiratory frequency showed a tendency to be decreased under hypoxia. The fish sank to the bottom approximately an hour after the $P_0^2$ fell under 26 mmHg, and fish respiration was arrested within the following half hour. Some fish sometimes picked oneself up and thrashed frantically after sinking until respiratory arrest.

Blood characteristics and plasma components

The hematocrit value increased linearly by hypoxia up to the time at which the fish sank under 26 mmHg $P_0^2$ (Fig. 2). The hemoglobin concentration in blood showed a tendency to be increased at the maximum level of respiratory frequency, although it showed almost no change after that point. Hence, MCHC was significantly decreased by hypoxia. Plasma glucose concentration significantly increased by hypoxic exposure and attained about three times the initial value.

Enzyme activities and metabolite concentrations

The activities of GOT and GPT in the hepatopancreas tended to rise slightly when the fish sank after the $P_0^2$ fell under 26 mmHg (Fig. 3). Lactate dehydrogenase activity in the hepatopancreas showed no remarkable change with increasing hypoxic load, whereas lactic acid concentration was augmented linearly from the
Changes in lactate dehydrogenase (LDH), aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) activities, and lactic acid content in hepatopancreas of red sea bream exposed to hypoxia. Each value represents the mean ± SD, n = 4 – 10. Refer to Figs. 1 and 2 for bias background and superscript letters, respectively.

As the fish sank, the CK activity in the dorsal ordinary muscle tended to increase, then decreased (Fig. 4). In addition, both LDH activity and lactic acid concentration in the muscle significantly increased when the fish sank, and lactate level was double its initial value. The LDH activity had a tendency to decrease thereafter.

As shown in Fig. 5, the CCO activities were higher in several organs in the following order: kidney > hepatopancreas > brain, heart > gill. However, CCO activity in the muscle was only around one-tenth the activity in the kidney of the normoxia fish. The CCO activity in the brain markedly elevated with increasing hypoxic load, and the activity in the gill showed no significant change. On the other hand, the CCO activity in the heart tended to decrease at the maximum level of respiratory frequency, and subsequently was significantly increased at just before respiratory arrest. Further, the activities in the hepatopancreas, kidney, and muscle remarkably
Metabolic Depression of Red Sea Bream in Hypoxia

decreased at the maximum level of respiratory frequency, and subsequently recovered from a state of low activity up to the point of respiratory arrest.

Discussion

In order to confirm the reproducibility of our results, fish status and blood characteristics were re-examined in this experiment, which employed fish of a size and rearing condition different from those of the previous experiment\(^1\). The change of respiratory frequency and fish status in red sea bream subjected to gradual hypoxia showed a modality similar to that found in previous studies of young red sea bream\(^1\), Japanese parrot fish\(^2\), and Nile tilapia\(^3\) (Fig. 1). However, as a result of differences in fish size and temperature, the point of the inflection point on the curve of respiratory frequency, the point at which fish sank, and the point of respiration arrest were slightly higher than the corresponding values previously found in young red sea bream\(^1\). In addition, some fish demonstrated bursts of frantic swimming immediately before respiratory arrest, which was not observed in Nile tilapia\(^3\).

Properties of blood such as the hematocrit value and hemoglobin concentration showed trends in this study that were similar to those in a previous study\(^1-3\). Namely, the hematocrit value increased linearly by hypoxia and the hemoglobin concentration showed a tendency to enlarge at the peak respiratory frequency, and thereafter showed almost no change (Fig. 2). Consequently, MCHC was significantly decreased by hypoxia, and an increase in hemocyte volume in relation to hypoxia may be induced at this time to increase the hemoglobin-oxygen affinity\(^19\). No significant differences in the response of respiratory frequency, behavior, or blood characteristics between young\(^1\) and juvenile red sea bream exposed to hypoxia at different temperatures were observed.

Plasma glucose concentration significantly increased from the maximum level of respiratory frequency up to the time at which the fish sank, and attained about three times the initial value (Fig. 2). The GOT and GPT activities tended to be elevated temporarily as the fish sank after the Po\(_2\) fell under 26 mmHg (Fig. 3). It has been shown that the cortisol increases hepatic gluconeogenesis enzyme activities, including those of GOT and GPT, in several fish\(^3,16,17\). The release response of cortisol and the gluconeogenesis via amino acid may occur around the time of elevation of glucose concentration, as previously described\(^1,3\).

On the other hand, lactic acid concentration in the hepatopancreas rapidly increased by hypoxia from the time at which the fish sank up to respiratory arrest (Fig. 3). It is conceivable that anaerobic glycolysis in the body was markedly activated to access an energy supply when the fish sank. Lactic acid concentration and LDH activity in muscle were also increased significantly, which finding was in agreement with lactate elevation in the hepatopancreas, although LDH activity-catalyzed pyruvic acid in the hepatopancreas showed no change (Figs. 3, 4). Sugita et al.\(^18\) researched the response of carbohydrate metabolism in carp to a short-time exercise and following resting, and indicated the presence of a Cori cycle between the muscle and the hepatopancreas. In the red sea bream exposed to hypoxia, the Cori cycle between the muscle and hepatopancreas is thought to present like that in carp.

Creatine kinase activity in the ordinary muscle had a tendency to be elevated in coincidence with the increase of LDH activity and lactic acid as the fish sank (Fig. 4). Increase of CK activity by utilization of phosphocreatine (Pcr) has been indicated in several fish\(^4,5\) subjected to hypoxia or anoxia. In particular, it has been suggested that Nile tilapia exposed to hypoxia sustained a high energy status in the muscle by utilizing Pcr and anaerobic glycolysis as they sank\(^9\). These results raise the possibility of use of Pcr in the muscle to sustain a high ATP level, which was shown until just before respiratory arrest our previous study\(^1\).

Cytochrome c oxidase activity is proportional to oxygen consumption in the tissue of many animals\(^19\). We previously reported that CCO activity in the brain, heart, muscle, and gill were
increased temporally, though this activity in the liver and kidney decreased from the maximum level of respiratory frequency in Nile tilapia\(^1\). In red sea bream, it has been shown that the oxygen consumption of whole body was significantly decreased by hypoxia\(^{20,21}\). In the present study, CCO activity in the brain, but not in the gill, significantly increased with increasing hypoxic load (Fig. 5). In contrast, CCO activity in the hepatopancreas, kidney, and muscle markedly decreased at the maximum level of respiratory frequency, without the acceleration of anaerobic respiration, though CCO activity in these regions increased from the time at which the fish sank (Figs. 3-5). The strategy of anoxia- and hypoxia-tolerant animals is to reduce oxidative demand or neuronal hypometabolism\(^{22}\). Recently, it was shown that the mitochondrial CCO serves as an oxygen sensor in hepatocytes\(^{23}\) and cardiac myocytes\(^{24}\), and that oxygen uptake in cells exposed to hypoxia decreased significantly owing to a remarkable reduction in the \(V_{\text{max}}\) of CCO. These results suggest that the red sea bream brain has preferential use of oxygen, and metabolic depression appears in the hepatopancreas, kidney, and ordinary muscle at around the maximum respiratory frequency for the brain and other tissues. However, the hepatopancreas, kidney, ordinary muscle, and even the heart showed an increase in CCO activities with the acceleration of anaerobic respiration from the time at which the fish sank. Concomitantly, some fish in the tank picked oneself up and swam spasmodically and thrashed. In a previous study on Nile tilapia, these behaviors and the increase of CCO activities in tissues were not observed from the time at which the fish sank up to the time of respiratory arrest\(^3\). Also, the effect of hypoxic stress on ATP and TA levels as well as energy charge in various tissues of red sea bream was far less than that in various tissues of Nile tilapia, as previously described\(^{12,13}\). These differences may depend on the species-specific adaptation strategies to hypoxia; a response of endurance or escape may be associated with the habit and/or habitat of a closed marsh and the open sea, respectively.

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Metabolic Depression of Red Sea Bream in Hypoxia


低酸素負荷に対するマダイ各種組織の酵素活性
および代謝物濃度の反応

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マダイ稚魚に、酸素分圧（PO2）を151から26 mmHgまで短時間30分かけて低下し、26 mmHg以下
で約2時間放置するストレスを27.8℃で負荷した。呼吸数は徐々に増加し、38 mmHg PO2前後で最大
値を示してその後に低下した。26 mmHg PO2以下では横転後に呼吸停止する魚が増えた。Ht 値およ
び血漿グルコース濃度はストレスの負荷に伴って増大し、MCHC は低下した。普通筋の CK、LDH
活性および乳酸含量、並びに肝臓臓の乳酸含量は横転時に増加した。鰭のシトクロム c オキシターゼ
（CCO）活性に顕著な変化はなかったが、脳のそれは低酸素負荷に伴って有意に増大した。一方、肝臓臓,
腎臓および普通筋の CCO 活性は、呼吸数最大時に激減したが、横転時以降には回復し、心臓の CCO
活性も同時期に増加した。これよりマダイは、呼吸数最大時以降に低酸素負荷に対する代謝抑制を示
すが、横転後には嫌気代謝の亢進とともに、その機能は解除されることが示唆された。