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Hematological and Biochemical Responses of the Flowerhorn Fish to Hypoxia

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Abstract: Hematological and biochemical responses of the flowerhorn fish (*Amphilophus trimaculatus* x *Amphilophus citrinelllus* x *Vieja synspilum*) and their surviving strategies to hypoxia were investigated. Male and female flowerhorn fish were divided into five groups. Each group contained tree replications of five groups and was exposed to hypoxia by the substitution of nitrogen for oxygen for 12, 24 and 48 h, respectively. Blood sample was collected from the caudal vein and physiological-biochemical blood parameters analyzed. The results showed that hypoxia caused a significant increase in ventilation rate. Hematological parameters including red blood cell count, white blood cell count, hematocrit and hemoglobin concentration were significantly increased in fish exposed to hypoxia whereas mean corpuscular hemoglobin concentration remained the same. In addition, hypoxia caused significant increases in serum glucose, alanine amino-transferase, aspartate amino-transferase, creatine kinase and blood urea nitrogen. However, cholesterol and creatinine were significantly decreased. The effects of hypoxia on those parameters occurred in a time dependent manner. Changes in hematological parameters fully recovered after 1 week oxygen replenishment whereas biochemical parameters slowly returned to control levels. These suggest that acute hypoxia in the flowerhorn fish up to 48 h can affect physiology functions as indicated by changes in hematological and biological parameters. Alteration of most physiological functions can be restored by repayment of oxygen debt. The results also suggest that the flowerhorn fish coped with hypoxic condition by using both energy saving strategies and by attempting to increase the oxygen extraction capacity.

Key words: Flowerhorn, hypoxia, hematology, blood chemistry, fish, oxygen debt

INTRODUCTION

The flowerhorn is an economically valuable fish. The fish is a cross bred among *Amphilophus trimaculatus*, *Amphilophus citrinellles* and *Vieja synspilum* which originated from South America. However, it is becoming popular world wide because of its characteristics attractive to consumers. As a result of this, an intensive farming of the flowerhorn is increasing and economically important in several countries including Thailand where the flowerhorn is widely cultured.

It is well known that hypoxia is one of water pollutant problems caused by intensive fish culture. Many studies have demonstrated hematological and biological parameters in the assessment of fish health (Coles, 1986) and responses to hypoxia (Handy and Depledge, 1999). To date, very little is known on hematological and biological responses of the flowerhorn fish to hypoxia as well as the surviving strategies of these fish under hypoxic condition. Thus, the aims of the present study were to investigate the effects of hypoxia on hematological and biological parameters and to study surviving strategies under hypoxic condition in the flowerhorn fish. The results obtained will help to improve managements of the flowerhorn which is a species of increasing economic interest.

MATERIALS AND METHODS

Animal care: A total of 60 adult (100-150 g) the flowerhorn fish (*Amphilophus trimaculatus* x *Amphilophus citrinellus* x *Vieja synspilum*) of both sexes were purchased from Chatuchak market, Bangkok, Thailand. Experiments were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Thailand. Fish were randomly divided into 5 groups. Each group contained tree replications of 5 groups. Fish were maintained for 4 weeks in outdoor holding tanks with aerated well water (dissolved O_2 5.15±0.14 mg L^{-1}) at 28±0.18°C and fed with commercial food twice daily.

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Hypoxia exposure and recovery: Normoxic conditions were identified to those of the holding tank (dissolved O_2 5.15±0.14 mg L^{-1}). Hypoxic conditions were introduced by bubbling nitrogen directly into the water of the experimental tanks for 12, 24 and 48 h which caused dissolved O_2 level decreased from normoxia 5.15±0.14 to 1.36±0.04, 1.14±0.10 and 1.04±0.01 mg L^{-1}, respectively. At 12, 24 and 48 h hypoxia exposure, ventilation rates were measured. Fish from each experimental tank were captured and blood sampled. The water was then vigorously aerated and returned to normoxic levels (~5.12 mg L^{-1}) within 1 h and 45 min. Some fish from each experimental tank were captured at 12, 24 and 48 h after O_2 replenishment and blood sampled. The rest of the fish were allowed to recover. After 1 week, those fish were sacrificed and blood sampled.

Ventilation rate measurements: Ventilation rate was measured by counting the number of opercular beats >1 min (Wannamaker and Rice, 2000). Ventilation data were analyzed with linear regression to assess how ventilation rate varied with dissolved oxygen concentration (Wannamaker and Rice, 2000).

Blood sampling: The blood was collected at 12, 24 and 48 h to hypoxia and 1 week recovery. Fish were anesthetized with 0.005% 2-phenoxyethanol. Blood samples were taken by caudal puncture. Blood samples were divided into two aliquots. One aliquot coated with 0.1% ethylenediaminetetraacetic acid was stored at 4°C until used for analysis of hematological parameters (2 h). A second aliquot of blood sample was centrifuged at 3000 rpm for 10 min and serum was stored at -20°C until analysis of biochemical parameters.

Analysis of hematological and biochemical parameters: Hematological parameters were measured in duplicates and averaged for statistical use. Red Blood Cell count (RBC: $\times 10^6$ cells μL^{-1}) and White Blood Cell count (WBC: $\times 10^3$ cells μL^{-1}) were determined by hemocytometer methods (Stevens, 1977). Hematocrit (Ht: v/v ratio, %) was determined by microhematocrit method (Goldenfarb *et al*., 1971) and hemoglobin concentrations (Hb: g dL^{-1}) were determined by HC 510 analyzer (Hycel-Rennes, France) (Bentley *et al*., 1993; Buttarello *et al*., 1992). Mean corpuscular volume (MCV: μ cm^3 $cell^{-1}$), Mean Corpuscular Hemoglobin (MCH: pg $cell^{-1}$) and Mean Corpuscular Hemoglobin Concentration (MCHC: g L^{-1}) were calculated from RBC, Ht and Hb by standard formulas. Biochemical parameters were determined with commercially available Reflotron tests kits (Roche Diagnostics Corporation Indianapolis, Germany) including glucose (mg dL^{-1}), cholesterol (mg dL^{-1}), Creatine Kinase (CK: U L^{-1}) and aspartate aminotransferase (AST: U L^{-1}) according to the instruction of the manufacturers. Blood Urea Nitrogen (BUN: U L^{-1}), Creatinine (Cr) and alanine Aminotransferase (ALT: U L^{-1}) were determined with A15 BioSystems autoanalyzer (Roche Diagnostics Corporation indianapolis., Germany) based on Urease/kinetic and IFCC without pyr-P reaction, respectively.

Data presentation and statistical analysis: Data are expressed as mean±SE differences between means were assessed by one-way ANOVA followed by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Effects of hypoxia on mortality and behavioral changes: According to hypoxia, there was no mortality in any group. However, there was a marked reduction of feed intake in the flowerhorn fish with a decrease in dissolved O_2. Feed intake was reduced during 12 h hypoxia exposure and a lack of feed intake was observed during 24 and 48 h hypoxia exposure. Decreasing of dissolved O_2 also caused a decrease in locomotor activity. The lower the O_2 concentration, the less active the fish became. Approximately, 20% of the flowerhorn fish lost swimming coordination during exposure to 48 h hypoxia but all resumed normal swimming activity within 2 h after oxygen was retreated. After 1 week of O_2 replenishment, all of those behavioral changes could not be observed.

During 12 and 48 h of hypoxia exposure fish frequently floated near the surface of the water, expressing air breathing behavior. Opercula beat rate or ventilation rate was increased with time of exposure. A significant increase in ventilation rate with declining oxygen concentration was observed at each period of hypoxic exposure (12, 24 and 48 h) (Fig. 1a).

Exposure to hypoxia for 12, 24 and 28 h increased ventilation rates to 60.65±6.5, 69.07±5.81 and 74.67±3.40 beats min^{-1}, respectively as compared with normoxic condition (58.87±0.44 beats min^{-1}, 0 h) (Fig. 1b). Significant changes in ventilation rates were found after 24 h of hypoxia exposure (Fig. 1b). Recovery of ventilation rates to control levels was observed within 12 h after O_2 replenishment.

Effects of hypoxia on hematological parameters: RBC gradually and significantly increased from normoxic level of 1.46±0.04 ($\times 10^6$ cells μL^{-1}) with the time-course of hypoxia 12, 24 and 48 h to 1.68±0.04, 1.52±0.02 and 1.73±1.10 ($\times 10^6$ cells μL^{-1}), respectively (Fig. 2a). Ht in the

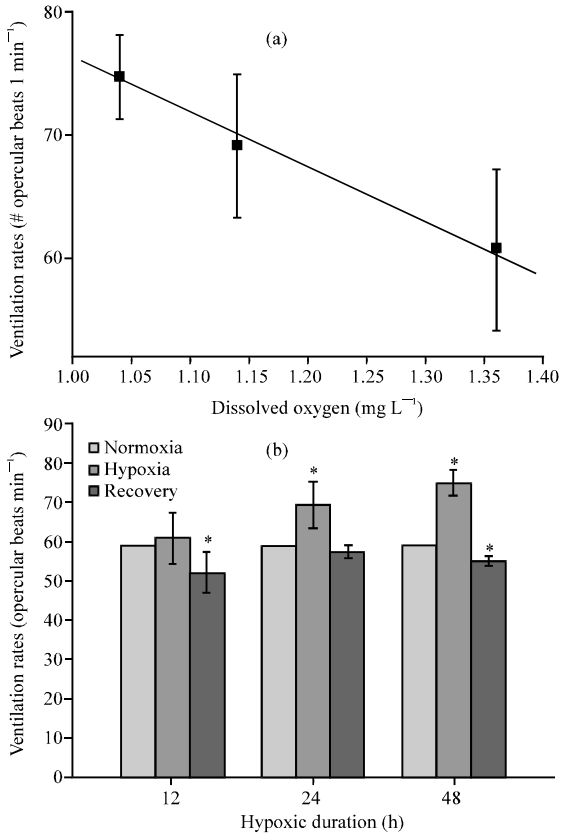

Fig. 1: Ventilation rates of flowerhorn fish at all dissolved oxygen level tested (a) and during normoxia, hypoxia, and 1 week recovery from hypoxia (b). Values are means±SE (n = 15 for each group). Within each group (hypoxia or recovery), significant differences from normoxia are indicated by an asterisk ($p \leq 0.05$)

blood showed a similar change to that of RBC with increase from normoxic level of 23.50±2.08 to 28.90±2.5, 29.40±2.33 and 32.70±0.85 (%), respectively (Fig. 2b). As with those above parameters, Hb had increased from normoxic level of 6.52±0.28 to 8.10±0.16, 7.54±0.04 and 8.33±0.01 g dL^{-1}, respectively (Fig. 2c). MCHC in hypoxic fish to MCHC in normoxic ones, did not show any marked change from the normoxic level (Fig. 2d). This was case for other hematology parameters, i.e., MCV and MCH. It is interesting to note that WBC increased dramatically to 2.65±0.27, 3.06±0.08 and 2.28±0.16 ($\times 10^6$ cells μL^{-1}) with 12, 24 and 48 h of hypoxia exposure, respectively (all compared to normoxic condition, 2.06±0.12$\times 10^6$ cells μL^{-1}) (Fig. 2e).

After 1 week of O_2 retreatment, all of those parameters mentioned above, except for WBC were not significantly different from the normoxic level.

Effects of hypoxia on biochemistry parameters: Plasma glucose level was significantly increased from normoxic level of 55.56±1.39 to 65.68±0.85 mg dL^{-1} after 12 h of hypoxic exposure (Fig. 3a-g). Increase in plasma glucose level gradually decreased to 62.54±0.51 and 52.21±1.8 mg dL^{-1} after 24 and 48 h of hypoxic exposure, respectively (Fig. 3a). Unlike plasma glucose level, plasma cholesterol level was significantly decreased from normoxic level of 175.22±1.68 to 153.00±8.54 mg dL^{-1} after 12 h of hypoxic exposure (Fig. 3b). Plasma cholesterol level reached lowest values after 24 h of hypoxic exposure (140.78±3.06 mg dL^{-1}) and then increased to 159.11±5.53 mg dL^{-1} after 48 h of hypoxic exposure (Fig. 3b). After 1 week of O_2 retreatment, plasma glucose levels returned to normoxic levels. Unlike plasma glucose levels and plasma cholesterol levels were slightly recovered but these did not reach normoxic levels.

Fish in hypoxic condition had significantly higher ALT and AST than the normoxic group (Fig. 3c and d). Both parameters gradually increased from normoxic level with the time-course of hypoxia. After 1 week of O_2 retreatment, ALT levels at 12 and 24 but not 48 h of hypoxic exposure returned to normoxic levels. Reversible changes of AST level was also found with 12 h of hypoxic exposure, however irreversible changes of AST levels were observed after 12 h of hypoxic exposure.

Fish in hypoxic condition had significantly higher BUN than the normoxic group (Fig. 3e). BUN gradually and significantly increased from normoxic level of 2.42±0.02 mg dL^{-1} with the time-course of hypoxia 12, 24 and 48 h to 2.60±0.19, 2.69±0.07 and 2.96±0.33 mg dL^{-1}, respectively (Fig. 3e). Unlike BUN, Cr was significantly decreased from normoxic level of 1.40±0.03 with the time-course of hypoxia (12, 24 and 48 h) to 0.87±0.09, 1.17±0.07 and 0.97±0.09 mg dL^{-1}, respectively (Fig. 3f). This caused the ratio of BUN to Cr elevated from normoxic level of 1.73 with the time-course of hypoxia (12, 24 and 48 h) to 2.99, 2.3 and 3.05, respectively (Fig. 4). It is interestingly to note that fish in hypoxic condition had significantly higher CK than the normoxic group (Fig. 3g). CK gradually and significantly increased from normoxic level of 714±56.11 U L^{-1} with the time-course of hypoxia 12, 24 and 48 h to 1425.32±52.48, 1762.67±101.75 and 1954.00±294.48 mg dL^{-1}, respectively (Fig. 3g). After 1 week of O_2 retreatment, BUN levels remained elevated. However, both Cr and CK levels slowly recovered.

Hypoxic recovery: The results have been clearly demonstrated that most of hematological and biochemical parameters fully recovered after 1 week of O_2 retreatment. However, some parameters could be approved within 12, 24 and 48 h of O_2 retreatment. For examples

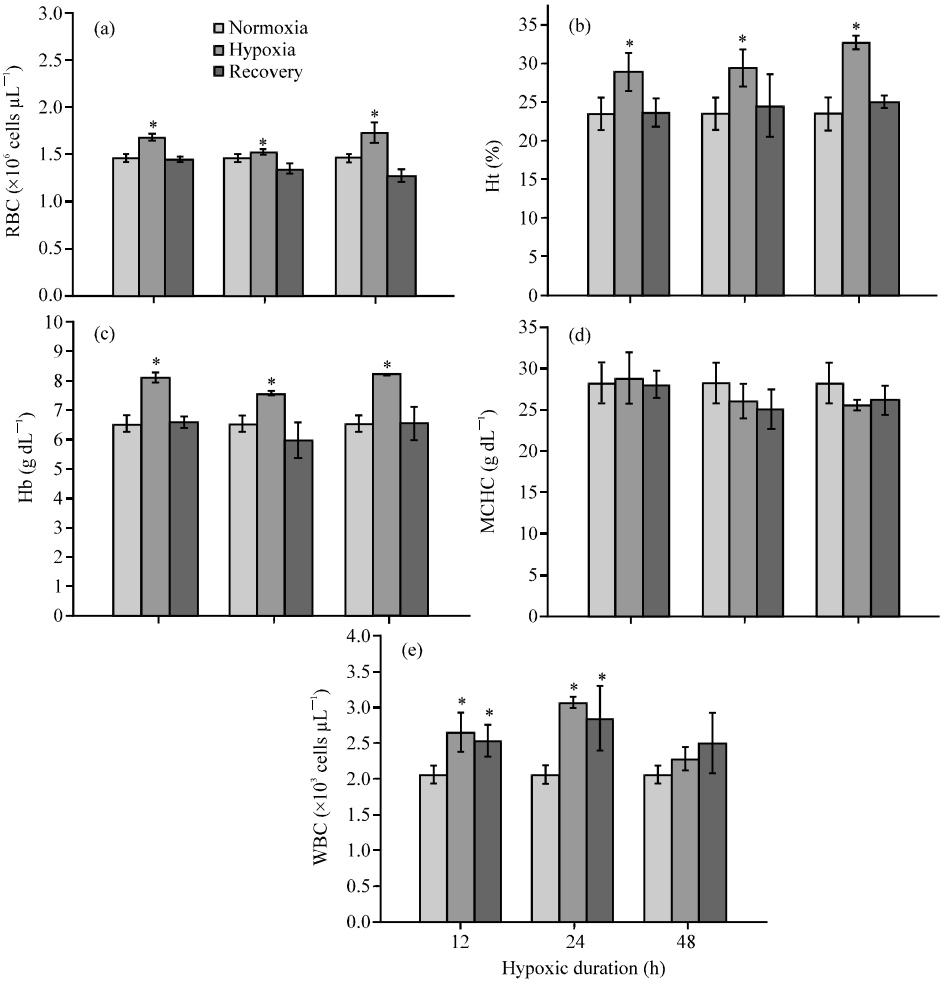

Fig. 2: Hematological indices of flower horn fish during hypoxia exposure and 1 week recovery from hypoxia. Values are means±SE (n = 15 for each group). Within each group (hypoxia or recovery), significant differences from normoxia are indicated by an asterisk ($p \leq 0.05$)

within 12 h, O_2 retreatment increased ventilation rates returned to normoxic levels (53.20±3.25). It was found that Ht (24.1±5.11%) fully recovered within 12 h after O_2 replenishment whereas other hematologic indices such as RBC (1.02±0.50×10^6 cells µL^{-1}), Hb (4.93±0.98 g dL^{-1}), MCHC (50.25±14.77 g dL^{-1}), MCV (351.27±221.79 g dL^{-1}) and MCH (50.25±14.77 g dL^{-1}) took longer period (<48 h) to recover. Thus, they were significantly lower than that of control levels. During 48 h of O_2 retreatment, it was interesting to note that WBC was still higher than that of control level.

Most of blood biochemical parameters could not fully recover within 1 week, except for BUN. These parameters slowly recovered (Fig. 3a-g).

Hematological and biochemical responses of the flowerhorn fish to hypoxia and their surviving strategies to hypoxia were investigated. The results showed that acute hypoxia up to 48 h significantly altered physiology functions as indicating by hematological and biochemical parameters. Altered hematological parameters recovered by repayment of O_2 debt within 1 week, except for WBC. However, most of biochemical parameters did not improve within 1 week of O_2 repayment. The results of these findings will be discussed.

It has been reported that mortality may result from exposure to hypoxia and tolerances differ among species and life stages (Dorfman and Westman, 1970; Sylvester *et al*., 1975; Burton *et al*., 1980; Coutant, 1985). In the present study, no mortality was found in the flowerhorn fish exposed to hypoxia from 12 up to 48 h. In addition, the results show that changes in most hematological and some biochemical parameters during hypoxia resumed after 1 week of O_2 repayment. Taken together, this indicated that the flowerhorn fish were

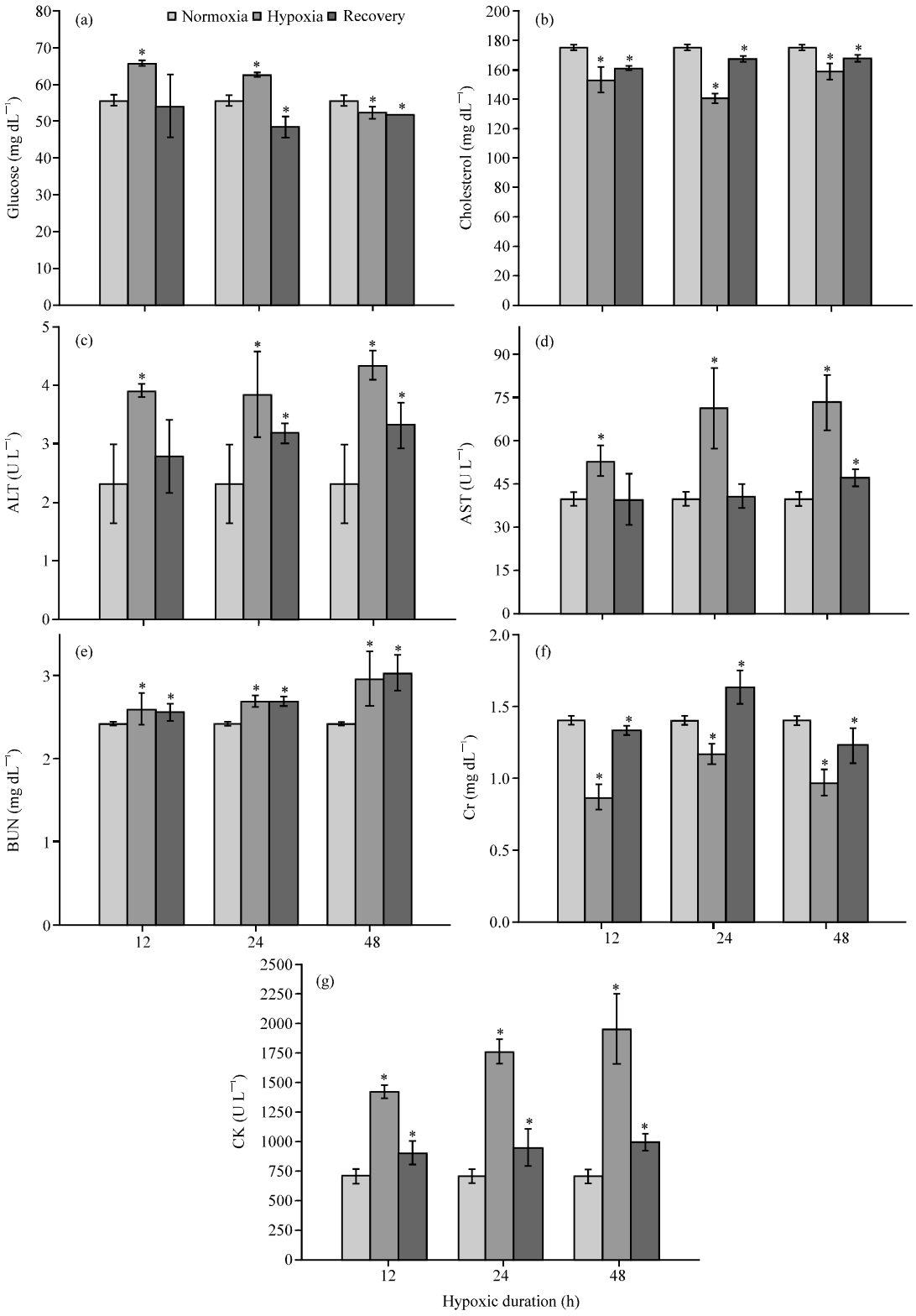

Fig. 3: Biochemical indices of flower horn fish during hypoxia exposure and 1 week recovery from hypoxia. Values are means±SE (n = 15 for each group). Within each group (hypoxia or recovery), significant differences from normoxia are indicated by an asterisk ($p \leq 0.05$)

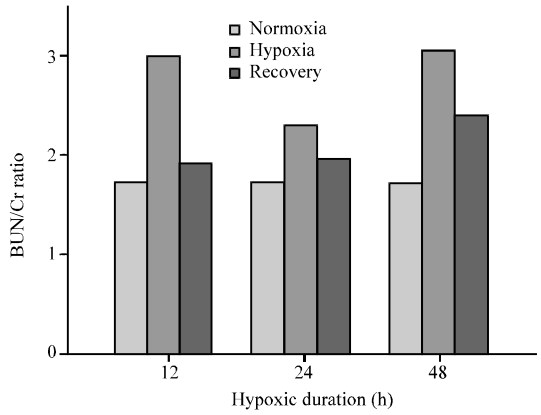

Fig. 4: The ratio of BUN to Cr of flower horn fish during hypoxia exposure and 1 week recovery from hypoxia

tolerant and well adapted in hypoxia condition. In the present study, we found that the flowerhorn fish, like other hypoxia/anoxia-adapted fish (Nilsson and Lutz, 1993), coped with hypoxic condition by using both energy saving strategies and by attempting to increase the oxygen extraction capacity. During hypoxia exposure, there was a marked reduction of feed intake. Similar responses have been previously reported in some other fish species (Brett, 1979; Brett and Blackburn, 1981; Pedersen, 1987; Pouliot and De La Noue, 1989; Van Dam and Pauly, 1995).

In hypoxia, the reduction of feed intake may lead to reducing energy demand and thereby, decreasing O_2 requirement (Van Dam and Pauly, 1995). In addition to reducing feed intake, lowering the locomotor activity was also found in this study. This is an obligate survival strategy in many hypoxia/hypoxic-adapted fish (Nilsson and Lutz, 1993). It is possible that to save energy, the flowerhorn fish exposed to hypoxia were less active than those under normoxia and this may have contributed to decreased rate of O_2 requirement.

Previous research demonstrated that ventilation rates increase with decreasing O_2 (Cech and Wohlschlag, 1973; Steffensen *et al*., 1982; Lallier *et al*., 1987; Pihl *et al*., 1991; Breitburg *et al*., 1994) but their was no previous information on ventilation rates of the flowerhorn fish. In the present study, the researchers found that ventilation rates increased <60-75% between 5 and 1 mg L^{-1} dissolved O_2. An increase in ventilation during hypoxia may explain the mechanisms behind patterns of hypoxia avoidance (Jones, 1952).

The physiological stress resulting from hypoxia is clearly reflected by hematological responses of the experimented flowerhorn fish. Classical primary hematological parameters such as RBC, Ht and Hb were increased while there was no change in MCHC. It is interesting to note that the number of WBC was significantly increased. Increases in the number of RBC which fish might compensate for poor oxygen uptake in hypoxic conditions may come from two mechanisms. The first mechanism is through erythropoiesis (Wepener *et al*., 1992). The second mechanism is via the release of RBC from the hemopoietic tissues by β-adrenergic action (Wepener *et al*., 1992). In the study, Ht value was significantly increased with hypoxia. The mechanisms whereby Ht value was increased in response to hypoxia could be due to either increased RBC volume as a result of osmotic changes or increased RBC numbers as a result of adrenergic-spleenic contraction in hypoxic conditions (Witters *et al*., 1990, 1991). In the present study, however the later is likely to be our explanation as no change in MCHC was observed. The present study showed a marked increase in Hb content with hypoxia. The elevated Hb can be due to increasing of RBC numbers. Increased WBC was observed in the periods of exposure to hypoxia and remained elevated at least 1 week after repayment of O_2. The exposure to hypoxia also increases the air-breathing frequency in the flowerhorn fish. This air-breathing behavior facilitates infections and stimulates immune responses (Moura *et al*., 1997).

As with hematological responses, biochemical responses can occur as a result of physiological stress due to hypoxia in the experimented flowerhorn fish. As a result of reduced feed intake, hyperglycemia can be observed after 12 h of hypoxia and remains elevated onward. This might indicate that substrate mobilization can keep up with glycolysis and that under prolonged hypoxic condition glycogen stores are still sufficient (Muusze *et al*., 1998). It is known that hypoxia can cause a decrease in cholesterol levels in fish due to adrenergic stimulation of lipolysis, phospholipid hydrolysis and inhibition of β-oxidation (Muusze *et al*., 1998). Decreased cholesterol levels could be due to the molecular genetic response to hypoxia as two genes previously associated with human metabolic disorders that affect cholesterol trafficking and degradation, the Niemann-Pick disease gene C and lysosomal acid lipase (cholesterol esterase) which were both up-regulated by hypoxia (Zhang *et al*., 2009).

ALT and AST activities are the most important enzymes relating to amino acid metabolism in the fish liver (Cowey and Walton, 1989). In the present study, acute hypoxia exposure may lead to inflammation of the liver as indicated by elevated ALT and AST levels. However, other organs such as the kidney and gills may also be affected (Coz-Rakovac *et al*., 2005). The increased ALT

and AST levels might occur via leakage of enzymes across damaged plasma membranes and/or increased synthesis of enzymes by the liver (Yang and Chen, 2003). Elevated BUN and Cr levels were observed in the present study. In fish, BUN is often used as an indicator of kidney dysfunction (Bernet *et al*., 2001). Increased concentrations of BUN occur as a result of renal lesions (Burtis and Ashwood, 1996). As with elevated ALT and AST levels, the increased BUN level might occur via inflammation of the liver damage leading to failure of producing ammonia from urea. Along with the liver damage, hypoxia-induced kidney impairment was also convinced as indicated by the elevated BUN level.

Increased concentrations of Cr, a waste product of the phosphorylation of ADP at the expense of the high-energy compound creatine phosphate, may reflect muscle and kidney dysfunction due to structural damage (Burtis and Ashwood, 1996). Elevation of CK is an indication of damage to muscle (Ishibashi *et al*., 2007). In the present study, the elevated CK level is consistent with Cr elevation which may occur as a result of muscle damage.

It was worthy to note that in the present study, most blood parameters was reversible, suggesting a possible trend of the flowerhorn fish to return to their normal status during a period of 1 week. However at the initial stage of recovery, swelling of RBC is likely to be occurred as indicated by the decreased MCHC. During a period of 1 week, however most of blood biochemical parameters gradually recovered except for BUN.

CONCLUSION

The present study provides a basis for understanding the link between hypoxia and hematological and biochemical responses in the flowerhorn fish. In the study, the flowerhorn fish exposed to hypoxia respond both with energy-saving strategies and with re-responses to maintain the supply of oxygen to the tissues. Understanding these links will help to improve managements of the fish.

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