World Journal of Fish and Marine Sciences 6 (1): 30-39, 2014
ISSN 2078-4589

DOI: 10.5829/idosi.wjfms.2014.06.01.74162

Effect of Hypoxia on Some Plasma Hormones, Metabolite and Electrolyte Concentrations of Pigface Bream Fish; *Lethrinus lentjan*

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Abstract: The aim of this study was to determine experimentally the physiological and biochemical effects of low oxygen on Pigface bream; *Lethrinus lentjan*. Two levels of dissolved oxygen (1 and 2 ml/l) exposure for 3, 6, 24 and 48 hours were selected for hypoxia exposure in this study. Blood sample analyses were carried out to examine the effects of hypoxia on electrolytes, some catecholamines, cortisol, triiodothyronine, thyroxine and metabolites. Comparative studies were also done to examine the effects of gender and body size during normoxia and hypoxia exposure. Pigface bream were fairly tolerant to hypoxia exposure, reduced their activity and increased their ventilation. Na^+ and Cl^-, Mg^{2+} did not show any significant variation among time of exposure or treatment (1 and 2 ml/l DO). The results showed a significant increase of plasma adrenaline, triiodothyonine and thyroxine concentrations, while plasma cortisol,noradrenaline and glucose concentrations showed a significant decrease. Some fish died during exposure to 1ml/l DO for 48 hours (18% of the females and 50% of the males). Those that died were significantly larger than the survivors. Thus, it could be concluded that smaller fish and females had different strategies for tolerating hypoxia exposure than larger fish and males.

Key words: Fish · Hypoxia · Plasma hormone · Metabolite and Electrolyte

INTRODUCTION

Hypoxia is defined as the level of dissolved oxygen of less than 2.8 mg O_2/l equivalent to 2 ml O_2/l or 91.4 mm [1]. Hypoxia can be a natural phenomenon caused by vertical stratification such as formation of haloclines and thermoclines. More often hypoxia is due to excessive anthropogenic input of nutrients and organic matters into water bodies with poor circulation [2, 3]. Nowadays, hypoxia or anoxia (no oxygen) can affect thousands of km² of marine waters. It has been commonly reported for waters around North and South America, Africa, Europe, India, South-east Asia, Australia, Japan and China [4]. Major ecological problems, including mass mortality of pelagic fish and marine animals, declines in benthic animal populations and declines in fisheries production are common in many parts of the world [5, 6]. In some marine systems with extremely limited water exchange and excessive anthropogenic inputs of nutrients, benthic water has become permanently hypoxic/anoxic [7]. In the last few decades, an increase in nutrient levels is clearly evident in coastal waters all over the world. Such an increase is primarily attributed to intensive farming, application of fertilizers, deforestation and discharge of domestic waste waters.

Coastal areas play a major role in the economy of the Kingdom of Saudi Arabia as they support fisheries and related commercial activities. Seasonal development of dissolved oxygen deficits (hypoxia) represents an acute system-level perturbation to fishery sustainability in coastal ecosystems around the globe at large, including Kingdom of Saudi Arabia. The level at which most animals species are found to be affected is 2 ml/l (2.8 mg/l) [1]. Anthropogenically induced hypoxia is an accelerating problem in both freshwater and marine coastal systems [8, 9]. Hypoxia is one physicochemical factor that can depress the immune capacity [10, 11], increase mortality of young fish, reduced growth rates and alter distribution and behavior of fishes [12, 13] as well as change the relative importance of organisms and pathways of carbon flow within food webs. Hypoxia and anoxia can lead to large reductions in the abundance, diversity and harvest of fishes within the affected waters. Negative effects of hypoxia on fish, habitat and food webs potentially make

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both fish populations and entire systems more susceptible to additional anthropogenic and natural stressors.

Numerous studies have been carried out on physiological and biochemical responses of aquatic animals to hypoxia, especially on fish [2, 7, 13]. In general terms, aquatic animals respond to hypoxia by first attempting to maintain oxygen delivery, then by conserving energy expenditure and reducing energy turnover and finally by enhancing energetic efficiency of those metabolic processes that remain and derive energy from anaerobic sources [14 - 16]. Maintaining oxygen delivery may be achieved by increasing water flow over gills and enhancing gill diffusion capacity by increasing the number of perfused gill lamellae [17, 18]. The second strategy for survival under hypoxia is to conserve energy expenditure by metabolic depression. This may be mediated through a reduction in general metabolism, down regulation of protein synthesis, or the down regulation and/or modification of certain regulatory enzymes in the anaerobic and aerobic pathways [16]. Some fish may regulate their energy production in anaerobiosis through the covalent modification of certain regulatory enzymes in glycolysis.

Fishes exhibit a range of tactics to counteract aquatic hypoxia. As the oxygen decreases the fish usually respond by escaping to other environments, but if escape from the hypoxic stress is not possible (Flight or Fight) a variety of physiological mechanisms can be involved to compensate for low oxygen availability [19] Short term physiological responses, include increases in the frequency and/or stroke volume of gill ventilation [20], regulation of the proportion of metabolic depression [21]. Many typical biochemical responses to hypoxia are reported in fishes including glycogen bulk enhancement particularly in the liver [22]. Reduction in the metabolic rate is another biochemical response. It is often reported and usually named metabolic depression [22]. While suppressed metabolic rate during hypoxic episodes can decrease oxygen demand in the short-term, activity must be restored eventually to allow feeding, reproduction and escape from predators [8]. The link between hypoxia and fish responses combines behavioral and physiological strategies that can mitigate the effects of exposure. In order to understand the sublethal consequences of hypoxia, it is important to determine what dissolved oxygen (DO) levels fish avoid and their physiological and biochemical responses, such as acclimation and metabolic depression.

The aim of this study was to study the effect of hypoxia exposure on hormones, metabolites and electrolyte parameters of Pigface bream*; Lethrinus lentjan*. The behavior and mortality of Pigface bream during hypoxia exposure were reported. The effect of gender and body size on these parameters during hypoxia were also studied.

MATERIALS AND METHODS

Experimental Fish: Pigface bream weighing 245.9±8.6g and with a total length of 23.5±1.4cm were caught by trap. Immediately after being caugh from Jeddah port, fish were placed in a seawater circulation pool on the fishing boat until reaching the acclimation tanks on the beach. The fish were kept in 400 L recirculating seawater fiberglass tanks, which were located at the Faculty of Marine Sciences- King Abdulaziz University. The range of water temperature in the tanks was 24-28°C and salinity was 35-36psu. The fish were kept in these tanks for two weeks for acclimation before experimentation and fed commercially-prepared fish pellets once a day [23]. Before hypoxic exposure, the fish were kept undisturbed in small experimental tanks, for at least 24 hours with constantly recirculating aerated seawater filtered over activated charcoal at a temperature 25.9±0.1°C, salinity 35.8±0.8psu, ammonia 2.01±0.6µm/l, nitrite 0.23±0.04µm/l and phosphate 0.5±0.01µm/l and exposed to a 12 h D: 12 h L photoperiod. Feeding was stopped 24 h before experimentation [24].

Experimental Design: A continuous flow system was set up to provide the desired constant level of ambient dissolved oxygen throughout the entire experimental period in the experimental tanks. Essentially, the system consisted of two seawater reservoirs: an overhead 600 L tank and a fully aerated 400 L fish acclimation tank connected to a 400 L water circulation tank. The circulation tank was connected to the eight experimental tanks. Each fish was kept individually in a 45 L experimental tank. Seawater was pumped from the circulation tank to each of the experimental tanks at a flow rate of 1.5 L min^{-1} circulating through a biological filter.

Each experimental tank was a closed system modified to adjust the oxygen content of the water. Each experimental tank was a glass tank (width = 30 cm; length = 60 cm, depth = 40 cm; 45 L volume) provided with a pair of ports, which served as the inlet (from the

circulation tank) and outlet (drain) for water. The sides of the experimental tanks were covered with black paper to minimize disturbances to the fish.

The experimental setup designed for hypoxic exposure consisted of the main unit Aqua Traul Oxygen Monitoring and Control System (Dryden Aqua, Scotland, UK.), Oxygen probes (Dryden Aqua, Scotland, U.K.). Each Aqua Traul could be fitted with 8 oxygen probes. The Aqua Traul system had 8 control relay outputs (for monitoring and control applications) connected to solenoid valves to regulate the oxygen and nitrogen input to 4 experimental tanks. Oxygen and nitrogen were purged in the tanks by flexible tygon tubing (ID 4mm, OD 1.2 cm) attached to ceramic gas diffusers (Dryden Aqua, Scotland, U.K.) placed inside the tanks.

The supply of oxygen and nitrogen from the respective cylinders was adjusted manually according to the requirement. The time required to bring the DO level in the system to the desired level was about 10-20 minutes. Water samples (250 ml) from the experimental and holding tanks were collected using Niskin sample bottles for water quality criteria measurements.

Hypoxic Exposure Protocol: Two different levels of hypoxia, 2.0±0.9 (2.8 mg/l) (25% saturation) and 1.1±0.2 ml L^{-1} O_2 (1.4 mg/l: 12.5% saturation) were selected for the present study. After exposure to these dissolved oxygen levels, a terminal blood sample was taken at the end of 3, 6, 24 and 48 hours [25]. The same number of control fish as the experimental fish were maintained at 7.9±0 ml L^{-1} O_2 (100% saturation). Feeding was suspended 48 h before experimentation [26].

Pigface bream were netted at the end of the desired exposure from the experimental holding tanks and stunned by a blow on the head. After blood sampling, the weight of fish (g) and length (cm) were determined. The behavior of fish during hypoxia exposure and mortality were monitored and reported every two to four hours. After fish death, the gender was determined and the weight and body length of each fish were measured.

Plasma Electrolytes Analysis: Whole blood was allowed to clot at room temperature for 30 min and centrifuged at 11000 rpm for 5 min to obtain plasma [27]. All plasma samples were immediately frozen at-80 °C. The Beckman Synchron CX7 System (CX3 module) was used to determine plasma lactate, glucose, electrolytes, cortisol, Thyroxine (T_4) and Tri-iodothyronine (T_3) in plasma in this study. The CX7 system determined sodium and chloride by measuring electrolyte ion activity in solution.

Plasma Glucose, Lactate, Cortisol, Triiodothyronine (T_3) and Thyroxine (T_4) Analysis: The activity of plasma lactate dehydrogenase (LDH) was determined by the enzymatic reaction utilizing lactate dehydrogenase with conversion to pyruvate and production of peroxide as described by Bergemyer [28]. The concentration of plasma glucose was measured according to Trinder [29]. Cortisol was tested by stimulating cortisol production in a coarse head kidney homogenate with ACTH in vitro, as described by Levesque *et al.*[30]. T3 and T4 were measured with radioimmunoassay kits as described in Levesque *et al.* [30].

Catecholamines Analysis: The plasma catecholamines (adrenaline, noradrenaline and dopamine) were determined by High Performance Liquid Chromatography (HPLC) with electrochemical detection.

Statistical Analysis: The data are presented as means ± SEM. One way ANOVA was used to compare between means. Significance was accepted when $P<0.05$. Analysis was carried out using SAS (Version 8) program.

RESULTS

Behavior of Hypoxia-exposed Fish: During hypoxia exposure, the surviving fish reduced their routine locomotor activity. After a certain time of hypoxia exposure, approximately 20 hours, the fish lay almost immobile at the bottom and appeared to increase their ventilation rate. The surviving fish remained quiet until the end of the experiment. The nonsurviving fish, swam up in the water column and showed burst swimming movement. After that, the fish lost their balance and sank to the bottom. Afterwards the fish remained inactive at the bottom until they died.

Fish Mortality: No deaths occurred in fish exposed for 3, 6, 24 hours to 1 and 2ml/l DO and fish exposed to 2 ml/l DO for 48 hours. All the deaths occurred in fish exposed to 1 ml/l DO for 48 hours. From 29 fish exposed to 1ml/l DO for 48 hours, 12 were males and 17 were females. Six males died (50%) while only 3 females died (18%) from the total number of females and males exposed to 1 ml/l DO for 48 hours. The mean body weight (305±32.1g) and body length (24.7±1.0cm) of surviving fish were significantly ($P<0.05$) smaller than the body weight (425.1±33.1g) and body length (28.7±0.8cm) of fish that died (Figure 1). Three fish died after 26 hours, one fish died after 28 hours, two fish died after 30 hours, two fish died after 36 hours and one fish died after 40 hours.

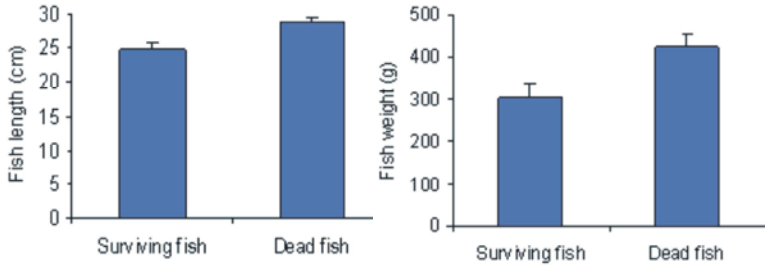

Fig 1: Body weight and body length of surviving and dying *L. lentjan* during hypoxia exposure. Data represents mean ± SEM.

Table 1: The electrolytes [sodium (Na+), chloride (Cl-), magnesium (Mg2+) and calcium (Ca2+)] of normoxia (N), Control (Cont) and hypoxia exposed L. lentjan to 1 and 2 ml/l DO for 3, 6, 24 and 48 hours.

Ions	N	Cont(3 h)	3h2m/l	3h1ml/l	Cont(6 h)	6 h2ml/l	6 h1ml/l	Cont(24 h)	24 h2ml/l	24 h1ml/l	Cont(48 h)	48 h2ml/l	48 h1ml/l
Na+	150.1± 2.7(10)	178.7± 2.8(16)	175.1± 2.4(12)	171.1± 3.1(10)	174.1± 1.7(19)	169.4± 2.5(9)	177.8± 4.4(10)	173.7± 2.6(20)	176.1± 3.6(9)	178.1± 2.6(10)	170.8± 3.8(19)	173.8± 5.0(10)	172.4± 3.7(10)
Cl-	137.9± 2.5(10)	161.9± 3.1(16)	154.4± 2.0(12)	156.1± 2.4(10)	154.6± 2.7(20)	148.4± 2.0(9)	151.0± 1.3(10)	149.7± 2.1(20)	158.0± 3.9(10)	147.1± 1.3(10)	150.0± 1.2(19)	153.7± 1.64(10)	154.3± 3.59(10)
Mg2+	0.26± 0.03(13)	0.18± 0.01(16)	0.14± 0.01(12)	0.20± 0.04(10)	0.10± 0.01(19)	0.11± 0.01(9)	0.10± 0.01(8)	0.10± 0.02(18)	0.20± 0.05(10)	0.08± 0.02(9)	0.16± 0.01(18)	0.16± 0.02(10)	0.15± 0.02(10)
Ca2+	0.66± 0.07(10)	0.61± 0.03(12)	0.61± 0.05(9)	0.62± 0.02(6)	Nm	Nm	nm	0.58± 0.15(2)	0.48± 0.16(3)	Nm	0.48± 0.02(4)	0.60± 0.09(3)	0.60± 0.09(3)

Data represents mean ± SEM, (n) number of fish. nm: no measurements

Plasma Electrolytes: Table (1) shows the electrolyte concentrations in normoxia, control and hypoxia exposed fish at different levels of DO (treatment: 1ml/l and 2ml/l DO) and different time of exposure (3, 6, 24 and 48 hours).

Plasma sodium (Na^+) concentrations ranged from 150.1mmol/l in normoxic fish to 178.7 mmol/l in control fish at 3 hours. There were no trends of increasing or decreasing plasma Na^+ concentrations in fish when exposed to hypoxia. Chloride (Cl^-) concentrations were highest in control fish compared to that of hypoxia exposed fish. However the variation was not significant. Magnesium (Mg^{2+}) concentrations showed fluctuations in their levels. As for Na^+ and Cl^-, Mg^{2+} did not show any significant variation among time of exposure or treatment (1 and 2 ml/l DO). The Calcium (Ca^{2+}) concentrations were not examined in control fish and fish exposed to 1 and 2 ml/l DO for 6 hours and 1 ml/l DO for 24 hours because not enough plasma were available for analysis. The plasma Ca^{2+} concentrations were higher in fish exposed to 1 and 2 ml/l DO for 48 hours than their controls. However this level was not higher than that of normoxic fish and control and hypoxia exposed fish for 3 hours.

The plasma electrolyte (Na^+, Cl^-, Mg^{2+} and Ca^{2+}) concentrations did not show any significant effect of time, treatment, gender, body weight or body length, interaction of time and treatment, interaction of treatment and gender, interaction of time and gender and interaction of time, treatment and gender during hypoxia exposure (P>0.05). However, only Ca^{2+} concentration was significantly affected by body weight, treatment and interaction of time and treatment (P<0.05). Plasma Ca^{2+} concentrations increased with increasing body weight.

Plasma Hormones: Table (2) show the normoxia, control and hypoxia fish data of *L. lentjan* at different time of exposure (3, 6, 24 and 48 hours) and treatment (1 and 2 ml/l DO) and their statistical analyses.

Noradrenaline (NA) concentrations were affected significantly by treatment, interaction of treatment and gender and interaction of treatment, time and gender during hypoxia exposure (P<0.05). The NA concentrations of fish exposed to 1 ml/l DO were significantly lower than that of fish exposed to 2 ml/l DO. The NA concentrations of females exposed to 2 ml/l DO were significantly higher than that of males exposed to 1 ml/l DO and than those of control females (P<0.05). The interaction of treatment, gender and time significantly affected the NA concentration of fish during hypoxia exposure (P<0.05). The details of the treatment, time and gender interaction affect in NA concentrations of fish are presented in Table 2. On the other hand, body size, time, gender, interaction of time and gender and interaction of time and treatment did not significantly affect NA concentrations (P>0.05).

Adrenaline (A) concentrations were significantly affected by body size, treatment, interaction of time and treatment and interaction of time and gender (P<0.05) during hypoxia exposure compared with control fish. The A concentrations decreased with increasing

Table 2: The concentrations of hormones in normoxia (N), control (Cont) and hypoxia-exposed L. lentjan at different time of exposure (3, 6, 24 and 48 hours (h)) and treatment (1 and 2 ml/l DO).

Hormones	N	Cont(3h)	3h2ml/l	3h1ml/l	Cont(6h)	6h2ml/l	6h1ml/l	Cont(24h)	24h2ml/l	24h1ml/l	Cont(48h)	48h2ml/l	48h1ml/l
Noradrenaline	29.1±	36.0±	61.0±	35.0±	28.9±	27.5±	22.8±	33.2±	27.6±	26.7±	30.5±	50.8±	27.9±
(nmol/l)	3.5(10)	8.5(16)	18.3(9)	5.0(7)	5.0(15)	9.2(10)	4.5(10)	5.7(15)	4.3(12)	7.9(10)	4.3(16)	8.3(10)	3.1(12)
Adrenaline	53.1±	49.2±	52.6±	46.7±	33.6±	31.2±	49.5±	29.1±	37.0±	22.0±	20.8±	43.4±	27.6±
(nmol/l)	8.7(5)	14.2(13)	6.1(7)	4.5(7)	4.2(15)	9.5(10)	13.6(9)	4.1(14)	6.7(12)	3.2(11)	2.6(13)	10.7(10)	4.8(13)
Dopamine	2.5±	2.5±	3.1±	1.8±	2.5±	1.2±	2.2±	5.6±	5.3±	2.0±	9.2±	12.5±	10.2±
(nmol/l)	0.5(9)	0.4(14)	0.2(9)	0.1(8)	0.7(8)	0.3(10)	0.3(6)	1.9(12)	1.8(8)	0.3(10)	1.4(13)	3.1(8)	2.7(7)
Cortisol	113.0±	92.7±	136.6±	134.8±	113.4±	55.0±	146.3±	173.4±	164.0±	209.1±	58.9±	151.4±	182.9±
(nmol/l)	51.4(5)	26.1(17)	58.6(6)	51.0(8)	38.1(9)	14.4(5)	73.9(4)	27.3(16)	36.9(10)	67.6(7)	17.0(17)	36.1(10)	51.3(10)
T3(pmol/l)	13.3±	10.2±	11.4±	9.3±	28.1±	44.7±	38.1±	24.0±	25.3±	28.0±	15.2±	30.3±	19.7±
	2.6(10)	1.8(19)	2.7(10)	3.7(9)	2.6(8)	0.22(2)	6.0(2)	4.1(11)	4.1(7)	8.0(4)	3.5(15)	4.9(5)	4.3(9)
T4(pmol/l)	54.2±	32.7±	31.1±	21.9±	23.5±	36.7±	28.6±	21.2±	21.0±	26.6±	26.0±	31.6±	33.8±
	5.6(5)	5.1(18)	7.1(8)	7.1(8)	5.4(6)	7.3(3)	7.1(6)	3.6(16)	5.9(7)	3.6(8)	4.8(14)	4.8(10)	7.8(9)

Data represents means ± SEM, (n) number of fish.

Table 3: The concentrations of glucose and lactate in normoxia (N), control (Cont) and hypoxia exposed L. lentjan at different time of exposure (3, 6, 24 and 48 hours (h)) and treatment (1 and 2 ml/l DO).

Parameters	N	Cont(3h)	3h2ml/l	3h(1ml/l)	Cont(6h)	6h2ml/l	6h1ml/l	Cont(24h)	24h(2ml/l)	24h(1ml/l)	Cont(48h)	48h2ml/l	48h1ml/l
Glucosemmol/l	3.1±	3.5±	3.8±	3.3±	2.6±	2.0±	2.0±	2.6±	2.4±	2.0±	2.4±	2.5±	2.7±
	0.4(9)	0.3(15)	1.0(11)	0.3(10)	0.1(20)	0.2(9)	0.1(10)	0.2(20)	0.3(10)	0.2(10)	0.1(19)	0.2(10)	0.1(10)
Lactatemmol/l	0.7±	0.8±	0.8±	0.6±	0.5±	0.3±	0.8±	0.4±	0.7±	0.3±	0.5±	0.5±	0.5±
	0.1(10)	0.1(16)	0.2(9)	0.2(10)	0.1(20)	0.1(9)	0.1(10)	0.1(20)	0.1(10)	0.1(9)	0.1(16)	0.1(10)	0.1(10)

Data represents mean ± SEM, (n) number of fish.

body size. The A concentrations of fish exposed to 2 ml/l DO were significantly higher than that of fish exposed to 1 ml/l DO and control fish. The A concentrations in females exposed to 2 ml/l DO were significantly higher than that of males exposed to 1 and 2 ml/l DO and lower than control males and females. Also the A concentrations of females exposed to 3 hours were higher than that of males exposed for the same time and males and females exposed for 6, 24 and 48 hours. The time, gender, interaction of treatment and gender and interaction of time, treatment and gender significantly affected the A concentration (P<0.001). The A concentrations of fish exposed for 3 hours were significantly higher than that of fish exposed to 6, 24 and 48 hours. The A concentrations of females were higher than that of males.

Dopamine (DA) concentrations were highly significantly affected by time (P<0.001) and were significantly affected by body size (P<0.05) during hypoxia exposure compared with control fish. The larger fish had lower plasma DA concentrations. The plasma DA concentrations of fish exposed for 48 hours were higher than that of fish exposed for 3, 6 and 24 hours. The DA concentrations were not affected significantly by treatment, gender, interaction of treatment and time, interactions of treatment and gender, interaction of time and gender and interaction of time, treatment and gender (P>0.05). The changes in DA concentrations did not show any clear trend during hypoxia exposure.

Cortisol (Cort) concentrations were significant affected by time (P<0.05) during hypoxia exposure compared with control fish. Within 3 hours of hypoxia exposure, the Cort concentrations were higher in fish exposed to 1 and 2 ml/l DO than control fish. The levels of Cort were three-fold higher in fish exposed to 1 and 2 ml/l DO for 48 hour compared to control fish. On the other hand, body size, treatment, gender, interaction of time and gender and interaction of treatment and gender did not affect Cort concentrations significantly.

Triiodothyronine (T_3) concentrations were affected significantly by treatment and interaction of treatment and gender (P<0.05) and highly significantly affected by body size (P<0.001) during hypoxia exposure. The T_3 concentrations were lower in larger fish during hypoxia exposure. The T_3 concentrations of fish exposed to hypoxia were higher than control fish at all times except for fish exposed to 1ml/l DO for 3 hours which were lower than their control group. The time, gender and interactions between time and gender did not affect plasma T_3 concentrations significantly (P>0.05) during hypoxia exposure.

Thyroxine (T_4) concentrations were higher in fish exposed to 1 and 2 ml/l DO for 48 hours and to 1 ml/l DO for 24 hours than control fish (Table 2). However, plasma T_4 concentrations were not affected significantly by time, treatment, gender, body size, interaction of time and treatment and interaction of time and gender.

Plasma Metabolites: The glucose concentrations of fish exposed to1 and 2ml/l DO for 6 hours and to 1ml/l DO for 24 hours were lower than control fish (Table 3). However, the glucose concentrations did not significantly vary between control and hypoxia-exposed *L. lentjan* due to time, treatment, interaction of time and treatment, body weight or body length, interaction of treatment and gender and interaction of time, treatment and gender ($P>0.05$). The interaction of time and gender only showed significant differences ($P<0.05$). The glucose concentrations of females exposed for 3 hours were significantly higher than that of females exposed for 24 and 48 hours.

The lactate concentrations of hypoxia-exposed fish did not vary significantly compared to control fish at all times and levels of exposure nor were there any significant interactions among them ($P>0.05$). Also, no significant affect of body weight and length or gender on plasma lactate concentrations were found during hypoxia exposure ($P>0.05$).

DISCUSSION

Fish Behavior: Hyperventilation was the initial response of *L. lentjan* to hypoxia exposure. Environmental hypoxia elicits hyperventilation in a variety of species [6, 13, 31, 32]. Increased ventilation minimizes hypoxia stress both by increasing oxygen uptake and by enhancing convective conditions for CO_2 removal [32]. The reduction of surviving *L. lentjan* activity was also reported in other species such as *Solea solea* as the strategy of fish to save energy [33]. The other fish had a different strategy before death ensued. They tried to swim up in the water column with burst swimming movement until they lost their balance and sank to the bottom. Van Raaij *et al.* [34] reported that the strenuous avoidance behavior with burst activity of nonsurviving *Oncorhynchus mykiss* were because of five-fold catecholamines elevation. The inability to escape from the hypoxic environment and the large impact of burst-type activity on whole body physiology results in mortality. The physiological and biochemical parameters were not examined for non-surviving *L. lentjan*. However, the mortality of these fish could relate to the elevation and/or changes in these parameters. The surviving fish remained quiet until the end of experiments. This behavior could be the reason for the few or no changes in physiological and biochemical parameters of this group of fish.

Fish Mortality: In the present study, no fish died at the two levels (1 and 2 ml/l) of DO at all time of hypoxia exposure 3, 6, 24 and fish exposed to 2 ml/l DO for 48 hours. However, 31% of the fish exposed to 1ml/l DO for 48 hours died. Woo and Wu [17] studied the affect of 0.7 ml/l DO for 7 hours of hypoxia exposure in black seabream *Mylio macrocephalus*. They found that no fish died. Also, Woo and Wu [17] studied the same species at low level of DO (0.4ml/l) for 7 hours of hypoxia exposure. They reported fish mortality occurring within a few hours. There were some reported fish mortalities at higher level of water DO and shorter time of exposure than this study in certain species such as *Oncorhynchus mykiss* and *Gadus morhua* [34, 35]. Together with the results of the above studies, it appears that *L. lentjan* were also tolerant to hypoxia exposure. The fish that died were significantly larger in size than that of surviving fish. Indeed, it has been documented within several fish species that smaller individuals are more tolerant of hypoxia than larger individuals [13, 36]. Thus, *L. lentjan* smaller individuals were also more tolerant of hypoxia than larger individuals. In females, 18% of the fish died whilst 50% of the males died after exposure to 1ml/l DO during 48 hours. Overli, Sorensen and Nilsson [31] reported that male fish were more aggressive than females. Timmermanand Chapman [37] studied the effect of hypoxia on behavioral and physiological responses of *Poecilia latipinna*. They concluded that females were more tolerant to hypoxia than males. Sandoval and Matt [38] studied the effect of hypoxia in endocrinology and metabolites of human. They suggested that women more sensitive to hypoxia than men. The females *L. lentjan* were more tolerant to hypoxia than males. Therefore, it could be that females fish are more tolerant to hypoxia than males.

Plasma Electrolytes: The plasma electrolytes (Na^+, Cl^-, Mg^{2+} and Ca^{2+}) concentrations did not show any significant trend of decreasing or increasing during hypoxia exposure. The treatment and interaction of time and treatment significantly affected Ca^2 concentration. This could be due to the few number of samples (2 to 4 samples per treatment for 24 and 48 hours of control and hypoxia exposure were examined) examined for Ca^{2+} concentration. Woo and Wu [17] studied the effect of hypoxia on plasma levels of Ca^{2+} and Na^+ of Black seabream, *Mylio macrocephalus*. They found that Ca^{2+} levels were unchanged while Na^+ increased. Arends *et al.*[39] studied the effect of air exposure and confinement on plasma Na^+, Ca^{2+} and Mg^{2+} of Gilthead seabream,

Sparus aurata. They reported a slight increase in Na^+, a doubling of Mg^{2+} concentrations and no effect on Ca^{2+} levels during stress. Rotlant *et al*. [40] also studied the effect of handling and confinement on plasma Na^+ and Cl^- concentrations of *Sparus aurata*. They reported an increase in plasma Na^+ and Cl^- concentration for 4 hours then a return to their initial levels after 24 hours. Woo and Wu and Soivio *et al*.[17, 41] only reported an increase in plasma Na^+ concentrations and Kakuta *et al*.[42] reported an increase in plasma of Mg^{2+} concentrations throughout hypoxia exposure. All other available data suggest that hypoxia does not affect levels of ions. This study also indicated that *L. lentjan* plasma ions (Na^+, Cl^-, Mg^{2+} and Ca^{2+}) were not affected by hypoxia. The little or no significant variations of plasma ions concentrations could be related to selective gain of ions that enter the fish from the hyperionic environment [13, 42].

The plasma electrolytes concentrations of *L. lentjan* were not affected significantly by gender and body weight or body length during hypoxia exposure. However, plasma Ca^{2+} concentrations were significantly affected by body weight. However, this may relate to the low n-values for the plasma Ca^{2+} measurements.

Plasma Hormones: The catecholamines (Noradrenaline (NA), Adrenaline (A) and Dopamine (DA)) levels fluctuated with no certain trend of increase or decrease during hypoxia exposure of *L. lentjan*. The levels of catecholamines were also not affected by hypoxia stress in other different species [6, 43, 44]. This has been attributed to the hypoxia not being sufficiently severe, because the reduction in blood PO_2 was not enough to stimulate catecholamine release. The reduction of blood PO_2 during hypoxia exposure should be more than half their initial level to cause elevation in plasma catecholamines hormones [43, 45]. The levels of A were significantly higher in females than that of males during hypoxia exposure. While NA and DA did not vary significantly between genders during hypoxia exposure. Mazeaud *et al*. [46] found that NA was higher in males whereas A did not vary between the genders during stress. This variation between this study and Mazeaud *et al*. [46] study could be due to species variation. On the other hand, the A and DA concentrations were affected significantly by body size. The plasma A and DA levels were lower in larger fish. Whereas plasma NA was not affected by body size in *L. lentjan*. The A and DA levels of normoxic *L. lentjan* are not affected by body size or gender. This indicates that A levels in *L. lentjan* was related to body size and gender during hypoxia stress and that the DA levels relate to body size.

Cortisol (Cort) concentrations were three-fold higher in fish exposed to 1 and 2ml/ DO for 48 hours compared to their controls. The elevations of plasma cortisol due to different stress exposure were reported for different species of seabream [39, 40, 47, 48]. Gfell, Kloas and Hanke [49] demonstrated that the head kidney of *Cyprinus carpio* is influenced by the parasympathetic system acting on interrenal cells, by showing that acetylcholine stimulated cortisol secretion *in vitro*. The parasympathetic activation was also reported to elevate plasma cortisol in seabream *Sparus aurata* [39]. The parasympathetic activation could also be the reason of increasing plasma cortisol concentrations in *L. lentjan* during hypoxia exposure. The gender of the fish can affect the plasma cortisol levels during stress [46, 50]. No affect of gender on plasma cortisol levels of *L. lentjan* during hypoxia exposure were seen in this study. This variation could be related to species variation. Also body size did not affect the plasma cortisol levels of *L. lentjan*.

Plasma triiodothyronine (T_3) concentrations, in general, were significantly higher in hypoxia exposure groups than their controls. According to Wu *et al.* [51] the levels of plasma T_3 were reduced in *Cyprinus carpio* when exposed to hypoxia. However, the plasma T_3 concentrations were reported to be elevated after other stress exposure in different species of fish [52]. Waring *et al*. [52] suggested that the elevation of plasma T_3 concentrations during stress were related to increasing hepatic monodiodination of T_4 into T_3. The plasma T_3 concentrations were affected significantly by body size during hypoxia exposure of *L. lentjan*. Larger fish had lower plasma T_3. Gender did not affect the T_3 levels of *L. lentjan* during hypoxia exposure. Wu *et al.* [51] reported variation between genders in plasma T_3 concentrations during hypoxia stress in *Cyprinus carpio*. However, the level (0.7ml/l DO) and period of hypoxia exposure (8 weeks) were different in the [51] study compared to this study (1 and 2ml/l DO for 48 hours).

Plasma thyroxine (T_4) concentrations were not affected significantly during hypoxia exposure of *L. lentjan*. However, these result as also reported in other studies [53, 54]. Plasma T_4 was also reported to decrease or increase according to the level and period of stress exposure [55, 56]. There were no affects of body size or gender in T_4 concentrations of *L. lentjan* during hypoxia exposure.

Plasma Metabolites: The concentrations of plasma glucose and lactate were not affected significantly by hypoxia in *L. lentjan*. It could be related to decrease in metabolic rates due to decreased activity [14, 16, 22]. The lactate levels were not affected during hypoxia exposure show that the tissues of *S. aurata* had enough oxygen to prevent anaerobiosis [41]. There were no significant affects of gender, body weight or body length on glucose and lactate concentrations of *L. lentjan* during hypoxia exposure. However, these factors did not affect the concentrations of glucose and lactate in normoxic fish. This indicated that gender and body size did not affect the levels of glucose and lactate of hypoxia exposed *L. lentjan*.

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