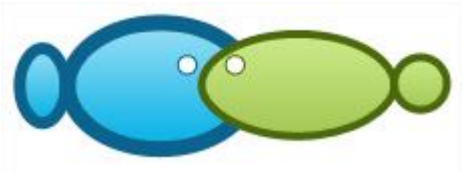

High water temperature impairs physiological responses in red hybrid tilapia: effects on cortisol and its regulation

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Abstract. For many aquatic species, any changes in water temperature affect their survival. This study was performed to investigate the effect of high water temperature on physiological responses in red hybrid tilapia. Male red hybrid tilapia were gradually acclimated and exposed to 31°C for 14 days. Samples of plasma were obtained from these heat-stressed (31°C) fish at the same time as another group of non-stressed (28°C) fish at day 1, 7 and 14. Plasma cortisol, 11β-hydroxylase, calcium, sodium, magnesium and potassium concentrations were measured in both groups. Total protein plasma and osmolality were also determined. Red hybrid tilapia exposed to 31°C display significant differences in cortisol and 11β-hydroxylase levels while displaying altered plasma ionic compositions. Based on these findings, thermal limitations may adversely impact the physiological responses of red hybrid tilapia thus directly affect its aquaculture production. This is particularly important given the predicted changes in water temperature due to global climate change.

Key Words: red hybrid tilapia, water temperature, Cortisol, 11β-hydroxylase, ionic compositions.

Introduction. Aquaculture plays a significant role in socio-economic impact of many countries by driven more job vacancies, income and food necessities. However, this sector is also vulnerable to environmental challenge. For instance, the rise of water temperature due to global warming is considered as one of the major influences on the total aquaculture production (Idris et al 2014; Hamdan et al 2015). Any significant changes in water temperature can be either detrimental to aquatic animals; risking them to extinction (Pörtner & Farrel 2008; Pörtner & Peck 2010) or adaptive (Radoslav et al 2013). However, studies on the impacts of global warming on aquaculture production are still limited although loss of aquaculture production due to changes of water temperature has been reported worldwide. For instance, Aphunu & Nwabeze (2012) have found negative effect of global warming on aquaculture development and production in Nigeria. Meanwhile, a group of researchers reported that extreme hot weather had caused stress and mortalities of *Oreochromis niloticus* in Brazil (Mian et al 2009), Indonesia (Anshary et al 2014) and Thailand (Suanyuk et al 2008; Rodkhum et al 2011; Pimolrat et al 2013). The influence of high temperature on the aquaculture industries has also been reported in Malaysia (Hayrol et al 2013). In a survey conducted among the aquaculture farmers in Sarawak, Malaysia, found climate change significantly associated with the loss of economic productivity (Hamdan et al 2015). For instance, there were reported deaths of farmed red hybrid tilapia (*Oreochromis* sp.) in floating cage culture at Kenyir Lake, Terengganu, Malaysia associated with high water temperature (Siti-Zahrah et al 2008; Najiah et al 2012).

Cortisol is one of the stress-associated hormone in fish, produced by the anterior part of the kidney i.e. the interrenal body, homologous to mammalian adrenal cortex (Iwama et al 1999). Cortisol has both glucocorticoid and mineralcorticoid actions in teleost (Wendelaar Bonga 1997; Mommsen et al 1999). Concurrently, this hormone has

been extensively studied in fish in relation with the occurrence of various stressful conditions (Pankhurst 2011). The magnitude of the stress response is frequently described as the increase in cortisol over the basal cortisol level after the stress treatment (Barton 2000). Cortisol has been found to produce a number of physiological effects; such as stimulation of Na-K ATPase in the plasma membrane of the gill chloride cells (Dang et al 2000). Furthermore, cortisol also plays an important role in the regulation of plasma ionic composition (McCormick 2011), oxidative stress (Dorval et al 2003) and immune system (Aluru & Vijayan 2009). In teleost, cortisol value is normally reported within the range of 0 to 150 ng mL^{-1}, depending on species, sex and reproduction status (Barton 2002; Milla et al 2009). Biosynthesis of cortisol however, depends on the catalyzation of 11ß-hydroxylase, a cytochrome enzyme that hydrolyzes 11-deoxycortisol into cortisol (Applebaum et al 2010; Uno et al 2012). This enzyme also catalysed 11-deoxycorticosterone (DOC) into corticosterone. In fish, this enzyme has been mostly observed in the testes of which also participate in spermatogenesis (Liu et al 2000; Kusakabe et al 2002). However, the signalling pathways leading to cortisol production associated with thermal stress response in teleost is poorly understood.

This study aims to investigate the effect of sublethal thermal stress on cortisol and 11-ß-hydroxylase levels in relation to ionic and osmoregulation in red hybrid tilapia. This study may help us to understand the impact of elevated water temperature on the physiological response in red hybrid tilapia which is beneficial for the aquaculture management of red hybrid tilapia.

Material and Method

Experimental species and acclimation. This study was conducted from October 2014 to October 2016. Male red hybrid tilapia, *Oreochromis* sp. of 400-600 g body weight and 20-30 cm standard length were sampled from the cage culture at Kg Jenang, Marang, Kuala Terengganu (05° 07' 905 N and 103° 12.231' E) in the eastern coastal region of Peninsular Malaysia. At the sampling time, the physiochemical parameters of the water at the cage culture were recorded by using YSI 556 MPS (USA) with range between 26-28°C, 3.5-5 ppm, 6.8-7.3, for temperature, dissolved oxygen and pH, respectively. Healthy fish sampled from Kg Jenang, Marang were brought back to the lab at School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu and acclimatized in the fiberglass (holding tanks) equipped with recirculating aquaculture system (RAS) for 2 weeks. Fish were fed at 5.0% of their body weight (BW) twice per day to satiation (2.5% at 09:00 h and 2.5% at 16:30 h) with commercial pelleted feed (Star Feedmills Sdn Bhd, Malaysia).

Experimental design. Experiments were conducted in a RAS of 500 L/tank, equipped with thermoregulator. Thirty healthy fish were separated equally into thermal treatment (heat-stressed) and control (non-stressed) group (n = 15 each group). Experiments were run in triplicates. Individual fish body weight and length were determined prior to the experiments. In stressed group, the water temperature was gradually increased at 1°C every 8h (Beitinger et al 2000) from 28 till 31°C (Hazza et al 2014) and later maintained for 2 weeks to 31°C (Meeuwig et al 2004). In non-stressed group, the temperature was maintained at 28°C. Blood and testes were sampled on days 1, 7 and 14 for both heat-stressed and non-stressed groups. Individual fish was anesthetized with NIKA Transmore (0.1 mL L^{-1}) prior to blood collection and later euthanized humanely. Approximately 2 mL of blood was collected from the gills near the gills arch by using 23 G needle, and divided into 2 vacutainer tubes. Plasma samples were obtained by centrifuging the whole blood collected in vacutainer heparinised tube (2,000 rpm) at 4°C for 20 min while serum were collected by allowing the whole blood in non-heparinised vacutainer tube to clot at room temperature prior to centrifugation (10,000 rpm, 4°C, 10 min) (Falk et al 1996). The obtained supernatant for each plasma and serum were transferred into a separate clean 1.5 mL eppendorf tube, stored at -20°C for subsequent quantification analyses.

Water quality. Water quality parameters (water temperature, pH and dissolved oxygen) were also measured and recorded using YSI 556 MPS (USA) concurrently with blood and tissue sampling. Data for dissolved oxygen were translated into oxygen saturation levels following Bergheim et al (2006). The experiment was repeated three times using different batches of fish.

Cortisol level. Plasma cortisol level was determined using a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) quantification kit (Enzo Life Sciences, Inc., NY, USA). Thawed plasma and standards were pipetted into a 96-well microplate. All solutions were prepared according to the manufacturer's instruction. The assay validity for fish cortisol has been reported to be comparable to Cayman Cortisol Assay Kit (Thompson et al 2014). Cortisol standards were used to generate the standard curve. Plasma cortisol levels were measured at optical density of 405 nm using Halo MPR-96 Visible Microplate Reader (Dynamica GmBH, UK) by comparing with a range of standard concentrations. The measurements were performed in triplicate, being expressed as ng mL^{-1}.

11β-hydroxylase. The fish was dissected on ice and testes were removed and thoroughly washed several times with saline to remove any residual and stored at -20^{o}C until use. Testicular 11-ß-hydroxylase activity was determined using 11-ß-hydroxylase assay kit (USCN Life Science SED546Ra). The validity of this assay kit has been reported by Stachon et al (2014). Absorbance of 11-ß-hydroxylase was measured at 340 nm at room temperature using 96-well plate reader (Halo MPR-96, Dynamica GmBH, UK). The enzyme activity was calculated based on plotted linear graph of standard curve and expressed as ng mL^{-1}.

Osmolality, total protein and ionic compositions. Plasma osmolality (mOsm kg^{-1}) was determined using a freezing point depression osmometer model 3320 (Advanced Instruments, USA). The total plasma protein was determined by Bradford assay (Bradford 1976). Approximately, 200 µL of the respective plasma were diluted with 0.8 mL of distilled water and gently mixed with 5 mL of ready-made Bradford Reagent (Sigma B6916). After the incubation at room temperature for 5 min, the absorbance of solution was measured using a Shimadzu UVmini-1240 spectrophotometer at 595 nm (Shimadzu, USA). Bovine serum albumin (BSA) (Thermo scientific, USA) was used as standard for standard curve preparation. The protein concentration was calculated based on the plotted linear graph of standard curve and expressed as g/100 mL. Ionic composition of plasma (Na^{+}, Ca^{2+}, K^{+}, Mg^{2+}) was determined by ion chromatography (Metrohm, USA) provided by Central Laboratory, Universiti Malaysia Terengganu. Standard solutions were made with analytical grade reagents (Merck) dissolved in deionized water. Standard curve of each ion was obtained and calculated using 4 different concentrations and expressed as mmol L^{-1}.

Data analysis. All data collected were computed for mean±SEM (standard error mean). Normality was determined by using Shapiro-Wilk Test. The difference in mean among exposure period for each non-stressed and heat-stressed groups were analysed by one-way analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test. Data were also subjected to Student's T test to compare the mean between non-stressed and heat-stressed groups for each day of exposure. Statistical analyses were performed using IBM SPSS Statistics version 20.0, and the level of significance for all tests was set at $p < 0.05$.

Results. Water quality parameters are given in Table 1. Results show that pH was steadily increased in heat-stressed group while oxygen saturation levels were gradually decreased. However, there was no significant difference ($p > 0.05$) among various day of exposure and between groups with respect to pH and oxygen saturation levels.

Table 1

Effects of thermal stress on pH and dissolved oxygen

Day	pH		Oxygen saturation, %	
	28^oC	31^oC	28^oC	31^oC
1	6.93±0.12	7.05±0.24	72.10±0.14	70.10±0.14
7	7.01±0.10	7.19±0.10	70.80±0.08	69.00±0.14
14	6.95±0.20	7.22±0.11	71.65±0.06	67.10±0.06

Values are presented as mean±SEM.

Cortisol level. Mean cortisol levels in plasma samples from non-stressed and heat-stressed groups for days 1, 7 and 14 are presented in Figure 1. The mean plasma cortisol (ng mL^{-1}) levels for heat-stressed groups at day 1, 7 and 14 were 9.2±0.2, 9.7±0.3, 9.1±0.3 pg m mL^{-1}, respectively. Our results showed that the cortisol levels in heat-stressed group steadily increased as the duration of exposure increased as at day 1 and 7 but slightly decreased on day 14. Day 7 showed significantly higher ($p < 0.05$) cortisol level compared to day 1 and 14. No significant difference was found in plasma cortisol levels between days 1 and 14 ($p > 0.05$). Whilst, no differences in cortisol levels existed among non-stressed group at various day of exposure with 8.0±0.1, 8.2±0.0, 8.2±0.1 ng mL^{-1}. For each day of exposure, the cortisol level in heat-stressed groups were significantly higher ($p < 0.05$) when compared to non- stressed groups.

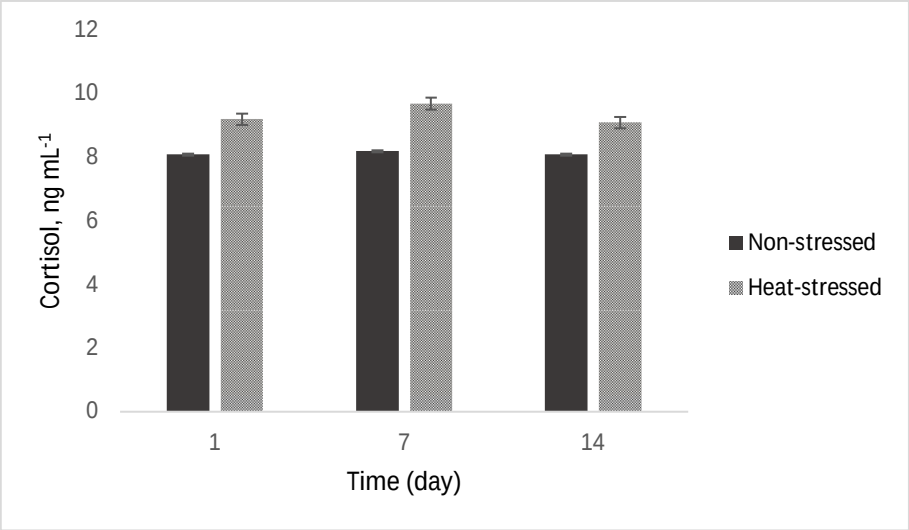

Figure 1. Plasma cortisol levels (ng mL^{-1}) in red hybrid tilapia of heat-stressed and non-stressed group (mean±SEM); n = 15.

11β-hydroxylase activity. The mean 11-ß-hydroxylase levels in the testis of male red hybrid tilapia *Oreochromis* sp. (Figure 2) for days 1, 7 and 14 were 0.73±0.05, 0.73±0.06 and 2.03±0.15 ng mL^{-1}, respectively, indicating a gradual increase with the increased of duration. The day-14 enzyme level was significantly higher ($p < 0.05$) than days 7 and 1. However, there was no significant difference between days 1 and 7 ($p > 0.05$). In non-stressed group, no significant differences were seen at day 1, 7 and 14 with 0.61±0.00, 0.60±0.02 and 0.64±0.01, respectively. For each day of exposure, the 11-ß-hydroxylase levels in heat-stressed groups at day 14 was significantly higher ($p < 0.05$) when compared to non-stressed group.

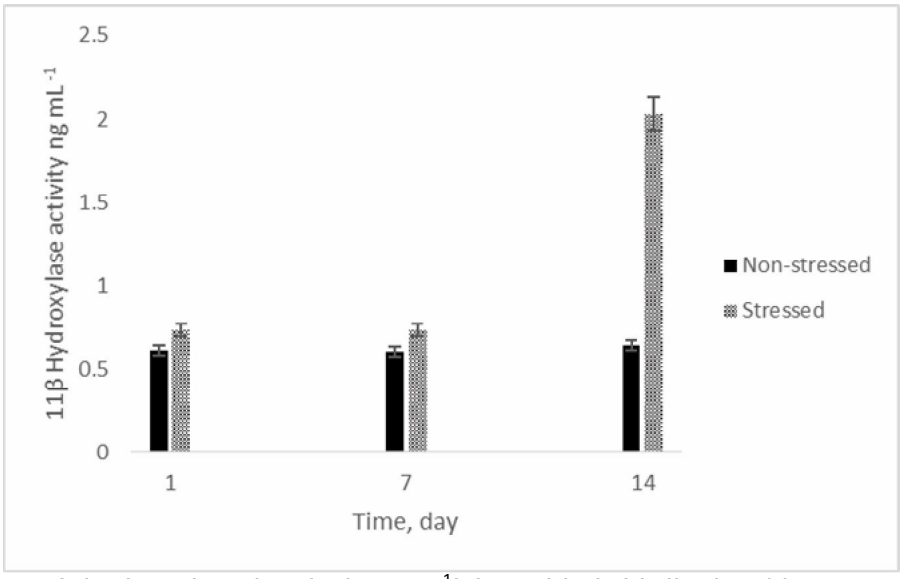

Figure 2. 11-ß-hydroxylase levels (ng mL^{-1}) in red hybrid tilapia of heat-stressed and non-stressed group (mean±SEM); n = 15.

Osmolality, total protein and ionic compositions. The mean plasma osmolality levels (mOsm kg^{-1}) were 306.10±5.31, 314.80±6.47 and 334.90±8.78 on days 1, 7 and 14 (Figure 3), respectively, indicating a gradual increase with the increment of exposure duration. The day-14 osmolality was significantly higher ($p < 0.05$) compared to day 7 and 1. In non-stressed group, only a small variation were seen at different day of exposure ($p > 0.05$). At day 14 the difference in osmolality was seen between non-stressed and heat-stressed group ($p < 0.05$).

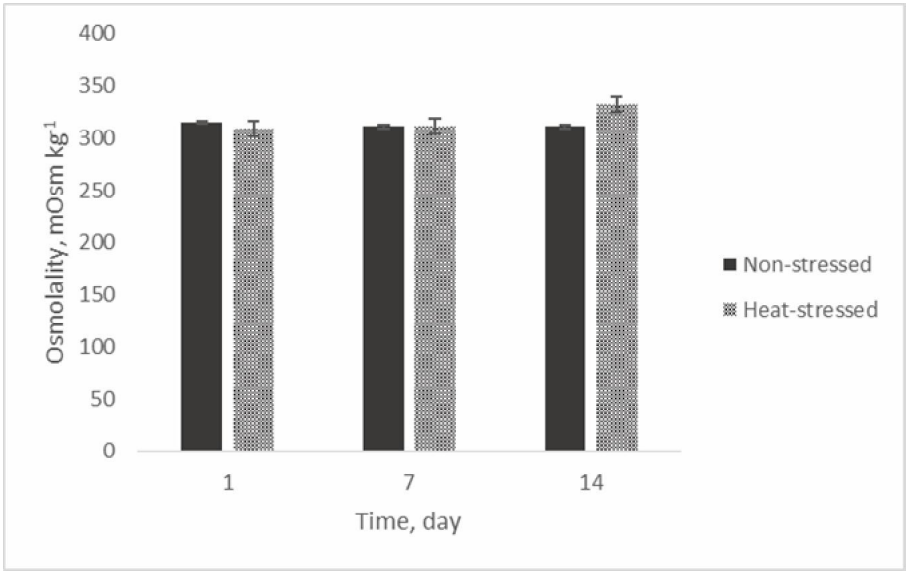

Figure 3. Plasma osmolality levels (mOsm kg^{-1}) in red hybrid tilapia of heat-stressed and non-stressed group (mean±SEM); n = 15.

The mean total protein levels (g/100 mL) in the plasma samples of heat-stressed group on days 1, 7 and 14 were 35.5±0.3, 34.2±0.2 and 33.2±0.3 (Figure 4), respectively, indicating a slight decrease with the increased duration of exposure. However, the differences were not significant ($p > 0.05$). The mean total protein levels in the plasma samples of non-stressed group on days 1, 7 and 14 were 34.7±0.1, 34.3±0.2 and 33.8±0.1, respectively (Figure 4). When compared between heat-stressed and non-stressed group for each day of exposure, however, no significant difference ($p > 0.05$) was seen.

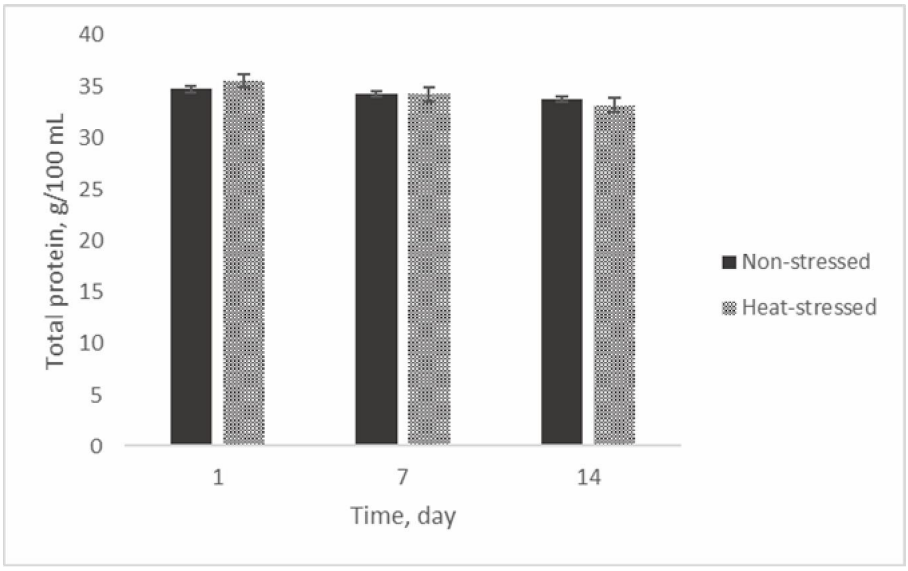

Figure 4. Total protein levels (g/100 mL) in red hybrid tilapia of heat-stressed and non-stressed group (mean±SEM); n = 15.

The mean plasma ionic composition (Na^+, Ca^{2+}, K^+, Mg^{2+}) of heat-stressed group on days 1, 7 and 14 are presented in Table 2. Na^+ decreased from 123.67±6.93 on day 1 to 117.50±3.56 and 116.78±3.22 mmol L^{-1} on days 7 and 14, respectively, with no significant difference among days 14 and 1, and 7 ($p < 0.05$). Ca^{2+} on days 1, 7 and 14 were 4.78±0.38, 5.96±0.41, and 9.09±0.75 mmol L^{-1}, respectively, indicating an increment with the increased duration with a significant difference found on day 14 ($p < 0.05$). K^+ levels in the plasma were 24.96±1.64, 24.20±1.08, and 29.67±2.11 mmol L^{-1} on days 1, 7 and 14 with no significant difference among days 1, 7 and 14 ($p < 0.05$). Mg^{2+} levels on days 1, 7 and 14 were 1.44±0.08, 1.38±0.07, and 1.69±0.04 mmol L^{-1}, respectively with no significant difference among days 1, 7 and 14 ($p < 0.05$). In non-stressed group, only a slight variation were seen at different day of exposure ($p > 0.05$). When comparison were made on the ion levels between heat-stressed and non-stressed group, Na^+, Ca^{2+} and Mg^{2+} were significantly lower ($p < 0.05$) than those of non-stressed group while K^+ were 6 to 8 folds higher ($p < 0.05$) in heat-stressed group compared to non-stressed group.

Table 2

Plasma ion levels (mmol L^{-1}) in red hybrid tilapia of heat-stressed and non-stressed group

Ion	*Group*	*Day*		
		1	*7*	*14*
Na^+	Heat-stressed	123.67±6.93^a	117.50±3.56^a	116.78±3.22^a
	Non-stressed	140.15±3.42^b	138.56±4.41^b	145.12±2.03^b
Ca^{2+}	Heat-stressed	4.78±0.38^a	5.96±0.41^a	9.09±0.75^b
	Non-stressed	12.56±2.22^b	10.85±2.0^b	13.93±3.08^c
K^+	Heat-stressed	24.96±1.64^a	24.20±1.08^a	29.67±2.11^a
	Non-stressed	3.80±3.21^b	4.25±1.17^b	4.13±0.51^b
Mg^{2+}	Heat-stressed	1.44±0.08^a	1.38±0.07^a	1.69±0.04^a
	Non-stressed	2.58±0.05^b	2.84±0.13^b	2.41±0.20^b

Data shown are means±SE. Mean with the same letters for each ion within column or rows are not significantly different ($p > 0.05$).

Discussion. Global increase in aquatic temperature has emerged as one of the main problem to the aquaculture farmers worldwide. In aquatic environment, any changes in water temperature will affect oxygen solubility thus altering the aquatic animal's physiological functions due to capacity-limitation of thermal tolerances (Abele & Puntarulo 2004; Chown et al 2004). This situation poses a serious negative impact to socio-

economic perspective because aquaculture sector is practically important in global food production to meet the rapid growth in human population (Hamdan et al 2015).

Thermal limitation influences the abundance and distribution of species at various levels of biological organisations (for review see Pörtner et al 2007). Intraspecies differences of thermal limits slightly vary across size and age (Peck et al 2013; Motani & Wainwright 2015; Clark et al 2016). Majority of tropical fish species are stenothermal i.e. with narrow thermal tolerance windows of which will limit their persistence. As such, these warm water fishes are vulnerable to rising of water temperature. As temperatures rises above their optimal limit, their survival rate will be off-set due to alteration in the metabolic activity (Simčič et al 2015). For freshwater fish species, however, the ability to adjust their thermal sensitivity is limited. Therefore, understanding the mechanism underlying the physiological responses in freshwater fishes is key to predicting the effects of climate change on fish population (Pörtner & Farrel 2008). In this study, aside from temperature, other water quality parameters such as pH and the oxygen saturation during trial period were found within the acceptable range (Wedemeyer 1996; Boyde 2012) for both heat-stressed and non-stressed group.

Cortisol is a hormone that is associated with stress. This stress hormone regulates many physiological processes (McCormick 2001; Pankhurst 2001; Evans 2002). Cortisol synthesis is regulated by 11ß-hydroxylase, thus the level of either the hormone or its associated enzyme can therefore be used as an important indicator of any physiological disturbances in fish (Applebaum et al 2010). A great majority of studies has focussed on the cloning and expression of fish testicular 11ß-hydroxylase associated with spermatogenesis (Cavaco et al 1997; D'Cotta et al 2001; Wang & Orban 2007) but with limited data on its regulation of cortisol synthesis in fish *per se*. Our study showed that fish exposed to 31^{o}C secreted higher cortisol levels compared to non-stressed group (control) which suggest the fish were stressed and cortisol levels were enhanced in relation to the duration of stress. Plasma cortisol also shows a strong link with temperature in brook trout, *Salvelinus fontinalis* (Chadwick et al 2015). High cortisol levels were also reported in *O. niloticus* acclimated to 38 and 40°C during 10-min short term exposure as observed by Delaney et al (2005). However, a slight decrease of cortisol level on day 14 compared to day 1 and 7 in heat-stressed group in the present study might also suggest that the fish were adapting to the thermal stress despite significantly higher activity of 11ß-hydroxylase, a mitochondrial enzyme, one of the superfamily member of cytochrome P450. This highly conserved P450 enzyme occurs in 2 isoforms: i) P450-scc or side chain cleavage or CYP11A which is involved in the conversion of progesterone to cortisol in the adrenal cortex and; ii) P450-11beta or CYP11B which catalyzes the conversion from testosterone to 11ß-hydroxytestosterone (precursor of 11-ketotestosterone, 11-KT); a major androgen in fish that promotes spermatogenesis. On the other hand, the expression of CYP11B1, which is involved in the synthesis of 11-KT, was strongly associated with morphological differentiation of the testis (Blasco et al 2010). As such histological examination of the testes may be needed to determine the involvement of 11ß-hydroxylase on testicular differentiation. It is also probable that the increased levels of 11ß-hydroxylase may indicate that it is being produced at an accelerated rate (Mornet et al 1989) triggered by adrenocorticotropin hormone (ACTH) although it has been reported that circulating ACTH marginally elevate the cortisol levels in tilapia (Balm et al 1994). In mammals, Stachon et al (2014) found that 11ß-hydroxylase was significantly increased in older rats compared with the younger rats which imply that 11ß-hydroxylase activity is also associated with age.

Stress hormones are known to influence homeostasis in aquatic ectotherms (Sardella et al 2008). Cortisol may increase the number of water channels thus increase the water permeability and uptake in animals. Our results indicate that cortisol has reduced the plasma ionic concentration except for potassium where it has been secreted in plasma tremendously. Other previous studies have shown different relationships between acclimation temperature and plasma resting cortisol levels; for instance in channel catfish, *Ictalurus punctatus*, where the cortisol level decreases with increasing acclimation temperature (Strange 1980).

Changes in the value of blood parameters have been recognized as possible biomarkers which provide vital information on the fish health status and its environmental condition (Tavares-Dias & Moraes 2007; Sturrock et al 2013). The concentrations of plasma ions are indicators of the homeostasis ability in a fish and their levels are usually stable (Burton 1986). The data on ionic compositions in tilapia is reported elsewhere (Hrubec et al 2000). Any changes in plasma ion concentrations may be an adaptation to another condition or may be caused by a malfunction of the osmoregulatory mechanisms. In this study, plasma sodium, potassium, calcium and magnesium concentrations were all influenced by temperature. However, despite large changes in plasma ion compositions, no significant impact on plasma osmolality was seen. Plasma osmolality typically ranges between 260-330 mOsm kg^{-1} in freshwater teleosts (Sampaio & Bianchini 2002). Other studies found that cortisol-mineralcorticoid receptor (cortisol-MR) expression is higher in brain of fish compared to osmoregulatory organs (Sakamoto et al 2016) while other studies indicate a small role of cortisol-MR signalling in osmoregulatory mechanism in comparison to cortisol-glucocorticoid receptor (cortisol-GR) (Takahashi & Sakamoto 2013). In acute stress, the recovery period of ion homeostasis in exercise stress of rainbow trout, *Oncorhynchus mykiss* was completed within 12 h and in confinement stress within 24 h (Postlethwaite & McDonald 1995). Therefore, it is probable that red hybrid tilapia was unable to maintain the plasma ion homeostasis at 31^{o}C as early as 24 hours. Clearly, extensive studies are needed to investigate the impact of elevated temperature on osmoregulatory mechanisms in red hybrid tilapia particularly on the importance of physiological routes of the decrease and increase of plasma ionic compositions.

Total plasma protein of the whole blood is considered vital to the haematological value of fish. In fish, thermal stress is commonly associated with enhanced expression of heat shock proteins (Hsps). Many reports the induction of Hsps in different cells or tissues of fish under stressful conditions (Iwama et al 1999; Sørensen et al 2003; Oksala et al 2014). Fish, unlike mammals, have only one cortisol receptor (Mommsen et al 1999), and the association between cortisol and Hsp and the effects of stress on this association has been demonstrated recently (Basu et al 2001, 2003). Although we do not investigate the effect of heat stress on Hsp levels, we found that the levels of plasma total protein in red hybrid tilapia of heat-stressed group were similar to non-stressed group, thus suggesting that Hsp levels may not be affected at 31^{o}C. It is also possible that the production of Hsp may only be triggered at certain point of thermal tolerance, however, need further investigation.

In conclusion, our data suggest the importance of water temperature changes induced-physiological responses in red hybrid tilapia should not be underestimated. With the threat of aquatic warming scenarios, detailed knowledge on the mechanisms underlying these physiological changes in fish natural populations may be crucial for the management of these global aquaculture resources of freshwater fish species.

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