Serum cortisol concentrations change in tiger grouper, *Epinephelus fuscoguttatus* in response to water temperature and salinity stress

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Received: March 2015 Accepted: June 2016

Abstract

The present exposition was designed to evaluate blood serum changes in tiger grouper, *Epinephelus fuscoguttatus* in response to changes in water temperature and salinity. Uniformly sized fingerlings were randomly distributed into different groups at fifteen fish per tank, in two replicates. Each group represented experimental fishes subjected to water temperatures of 18, 24, 28 and 30°C (control), and salinity of 10, 20 and 30 (control) ppt respectively. Replicate groups of fishes in each tank were exposed to these physiological stressors for 4 and 36 hours. At the end of experiment, blood samples were collected via caudal vein. The collected blood was centrifuged to obtain serum, andanalyzed for cortisol and glucose concentrations, using ELISA method. Results showed that decrease (to 28, 24 and 18°C) in water temperature from the control (30°C) and salinity from 30 ppt to 20 and 10 ppt for 4 and 36 hours influenced changes in the physical appearances (skin coloration) and behaviors (swimming vigor, opercula movement and schooling) of fish, to suggest responses to stress. Further, analyses of serum cortisol revealed consistently higher concentrations at the lower temperatures tested (to 28, 24 and 18°C), than the optimum tolerated by the species. Interestingly, fish maintained at 28°C for 36 hours contained lower serum cortisol concentrations compared to control groups. Meanwhile, the increment of the serum cortisol concentration occurs at decreased water salinity from 30 ppt to 20 and 10 ppt for 4 and 36 hours. It is concluded that: 1) water temperature and salinity are important physiological stressors; 2) sudden alteration of these factors leads to stress, and should therefore be avoided or minimized; 3) results of the present trial suggest that the blood serum factors are reliable parameters for evaluating the level of stress in fish.

Keywords: *Epinephelus fuscoguttatus*, Temperature and salinity change, Cortisol concentration, Behavior, Stress

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Introduction

Variable water parameters like salinity, temperature, and dissolved oxygen concentration play crucial roles in aquaculture (Wedemeyer, 1996; Little 2002; Martínez-Porchas *et al*., 2009), as they influence the immune response in fish (Dominguez *et al*., 2005). The extent to which these parameters change could affect reared aquatic animals, leading to diseases and mortalities. This is as fish especially, face more challenge of coping the stress caused by the rapid temperature fluctuations in the environment compared to other reared animals, because sudden or drastic changes in water temperature stress and weaken them to the point that promotes latent bacterial infections to occur or worsen (Barton, 2002; Dominguez *et al*., 2005). Stress was defined by Schreck *et al.* (2001) as a cascade of physiological and biochemical events that occur when an organism tries to re-establish homeostasis, as a consequence of perceived threats. Quick response of animals to stress is reported to be adaptive, allowing the organism to react in counteracting such perceived threats; although chronic stress is maladaptive, negatively affecting growth, reproduction and the immune status (Maule *et al*., 1989; Pickering, 1992; Schreck *et al*., 2001). Generally, the higher susceptibility of fish to stress than other farmed animals is largely due to their greater interaction with and dependence on their immediate environment (Barton, 2002).

Among farmed fish, tiger grouper, *E. fuscoguttatus* a species found growing in coral or rocky reefs, is a more desirable species to culture with respect to a number of factors including; tolerance to fluctuations in particulates, salinity, water temperature, pH, and over-crowding. This is because the species is found living in a wide range of salinities (11-41ppt), in both warm and temperate climates. Similarly, grouper tolerates water temperature as low as 22ºC, and has also been shown to survive at 30ºC in the tropics (Wedemeyer, 1996; Little, 2002; Martínez-Porchas *et al*., 2009). The aquaculture of grouper has recently attracted global attention, as it is increasingly becoming famous for its excellent taste among food lovers, due to its organoleptic qualities. Interestingly, the economic value of the species has skyrocketed due to rising market demand, especially in south-east Asian countries like Malaysia, Hong Kong and Singapore (Sadovy *et al*., 2003; Pierre *et al*., 2008). This may explain the expansion in the aquaculture of the species in Asia, as increasingly worldwide fish remains among the reliable sources of animal protein.

Unfortunately in tropical countries such as Malaysia, the east coast of the peninsula experiences heavy rainfalls during the northeast monsoon season. Within this season, aquatic parameters such as temperature, salinity and dissolved oxygen usually change rapidly, and affect the grouper farms.

Therefore, fish farmers endure challenges of maintaining steady supply of products to the market due to changes in the environmental (physical, chemical and biological) components that are fatal to fish. Consequently, studies investigating the effects on immune responses of fish due to fluctuations in these environmental parameters are necessary, to enable farmers understand how cultured fish cope with changes under such challenging situations (Bisset, 1948; Bowden, 2008). Moreover, to meet increasing demands for fish, it is important that farmers focus attention on rearing species (such as groupers) with fast growth potentials, good reproductive capacity, as well as high resistance to stress and diseases occasioned by well-developed immune system (Dominguez *et al*., 2004).

The principal objective of the current exposition was therefore, to evaluate the physiological responses of tiger grouper, *E. fuscoguttatus* to the changes in water temperature and salinity as the environmental stressors. To achieve this, changes in the fish serum cortisol concentrations were monitored following alterations in water temperature and salinity. The study is preliminary to a novel approach aiming to improve the natural health of tiger grouper in order to produce fish with fast growth potentials, good reproductive capacity and, highly resistant to stress and diseases.

Materials and methods

Human and animal rights, and ethical considerations

All animals used in the present experiment were treated humanely, according to approved procedures and guidelines of the University Putra Malaysia animal ethics committee guidelines. The number of animals, and procedure used in the present experimentation were approved by the committee.

Fish husbandry

One hundred and fifty (150) tiger grouper *E. fuscoguttatus* fingerlings, with initial mean weight and length measuring 8.65 ± 0.15g and 7.00 ± 0.5 cm, respectively were bought from a commercial hatchery in Terengganu, Malaysia. The fish were transported in continuously aerated poly-ethylene bags to the research facility at the Laboratory of Marine Biotechnology, Institute of Bioscience (IBS), Universiti Putra Malaysia. Prior to use in the current experiment, the fish were acclimated in a 5000 L fiber-glass holding tank containing filtered sea water at salinity and temperature levels of ~30 ppt and 30°C, respectively for 2 weeks. During the acclimation period, fish were fed standard commercial feed (Love Larvae, Japan) containing about 40% crude protein, at the rate of 5% biomass, offered twice daily (Son *et al.*, 2009; Tovar-Ramírez *et al.*, 2010). Water parameters were checked (YSI 556: Handheld Multi-Probe Meter, USA) on a daily routine and recorded

as 6.53 ± 0.41 mg L^{-1}, and 8.21 ± 0.32 for dissolved oxygen and pH, respectively. These were noted to meet the optimal living conditions required by the species.

Inducing physiological stress and blood sampling

During the experimental procedure, fish were transferred from the holding tanks into $2m^3$ culture aquarium tanks (1m × 2m × 1m) containing filtered seawater, and connected in a Recirculation Aquaculture System (RAS). For the effect of water temperature changes, one hundred and twenty (120) uniformly sized fingerlings were randomly distributed into four groups (A, B, C and D) at fifteen fish per tank, in duplicate. Groups A, B and C represented experimental groups with water temperatures regulated to induce heat stress at 18, 24, and 28°C, respectively. Fish in group D (i.e. with water temperature maintained at 30°C or optimal temperature for tiger groupers) served as the control. Each tank was fitted with a thermometer, and the desired water temperature was maintained constant during the experiment in an air-conditioned room, using a water heater fitted with thermostat. Fish were exposed to the physiological stressor (assigned experimental temperatures) in two incubation sets; i.e. one set for 4 hours and the other for 36 hours, and their physical behaviors were observed. At the end of the exposure period, 3 fish were randomly removed per tank for blood sample collection. Meanwhile, for the effect of water salinity changes, ninety (90) uniformly sized fingerlings were randomly distributed into three groups (A, B, and C) at fifteen fish per tank, in duplicate. Salinity of water in the tank was adjusted to 10 (Groups A) and 20 (Groups B**)** parts per thousand (ppt) by diluting seawater with dechlorinated municipal water, respectively. Fish in group C (i.e. with water salinity maintained at 30 ppt or optimal salinity for tiger groupers) served as the control. Fish were exposed to the physiological stressor in two incubation sets; i.e. one set for 4 hours and the other for 36 hours, and their physical behaviors were observed. At the end of the exposure period, 3 fish were randomly removed per tank for blood sample collection. (Refer to Fig. 1 for schematic representation of the experimental procedure).

To collect blood samples, the selected fish per tank were immediately anesthetized with 2-phenoxyethanol. Blood sample was collected from each fish separately, with needle and syringe via the caudal vein into capillary tubes. Serum was obtained by centrifuging the collected blood samples at 9600 x *g*, for 5 minutes, and stored at 4ºC until analyzed for cortisol and glucose concentrations, which was carried out within an hour of serum collection.

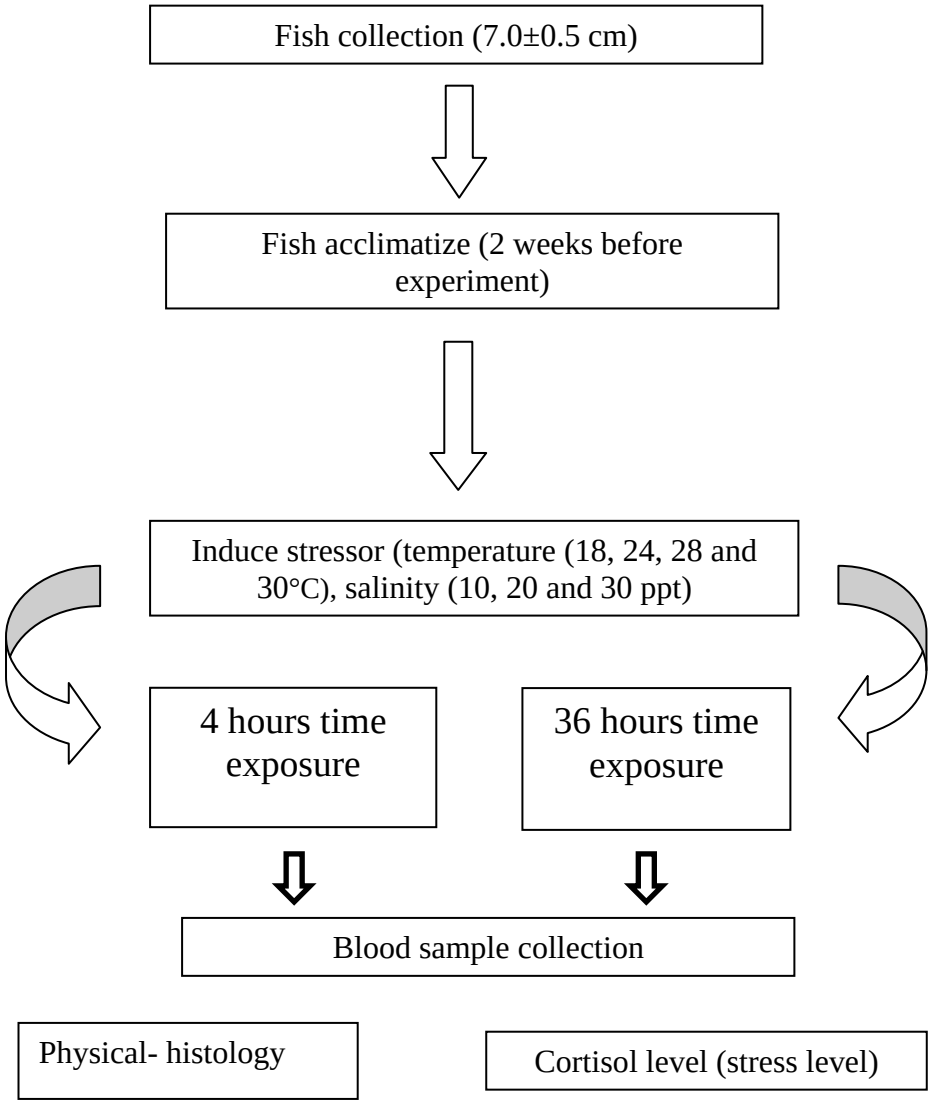

Figure 1: Schematic diagram summarizing the experimental procedure in Tiger Grouper *Epinephelus fuscoguttatus*; from maintenance of fish during exposure to physiological stressor (water temperature and salinity changes) for 4 and 36 hours, to collection of blood samples for analysis.

Measurement of serum cortisol and glucose concentrations

Concentration of cortisol in experimental fish serum was measured using an enzyme-linked immunosorbent assay (ELISA) method, according to slightly modified procedure utilized by Engvall and Perlmann (1971). Briefly, 10 µL of serum was immediately diluted with 190 µL of distilled water in a correlate-EIA kit (Assay Designs, Ann Arbor, MI, USA) and incubated at 37°C for 1 h. At the end of the incubation time, a stop solution was added to the diluted serum, and the optical density was measured at 405 nm, using a tunable microplate reader (VERSAmax, Sunnyvale, CA, USA).

Serum glucose concentration was measured by using a pipette to inject 10 µL of serum into a VITROS DT60II chemical analyzer (Kodak, New York, USA). All experiments were repeated three times.

Results

Effects of changes in water temperature and salinity on physical behavior of fish

Survival of fish during the present experimental procedure was 100%. In the control group (subjected to water temperature 30°C and salinity 30 ppt), the fish displayed normal body color, calm swimming activity at the bottom of the tanks, and social (schooling) behavior. However, signs of stress became noticeable in the behaviors and physical appearances of the fish in the aquaria with water maintained at different temperatures than the control (i.e. 18, 24 or 28°C) and different salinities (i.e 10 or 20 ppt).

Physical activities of fish in terms of body movement during swimming became vigorous, including rapid increase in opercula movements. Fish body color also became slightly darker, although the animals continued swimming in schools during the 4 hours exposure to different water temperatures and salinities. Prolonged exposure (36 hours) of experimental fish to different temperatures and salinity however, led to generalized melanosis as the body of fish became distinctly darker in coloration. Fish also abandoned social interaction in terms of schooling, as groups of fish chose to swim as separate individuals. Furthermore during 36 hours exposure, fish started to swimming near the water surface increasingly gasping, as evidenced by elevated opercula movements. Later, swimming and other forms of body movement, and physical activities of the groupers significantly decreased, as they seemed to be adjusted to the new water temperatures and salinity.

Effects of water temperature changes on serum cortisol concentration

The effects of changes in water temperature on stress response in tiger grouper were monitored in the present trial through changes in the serum cortisol concentrations. Fig. 2 illustrates effects on fish serum cortisol concentrations following changes in water temperature from 30°C to 18, 24 and 28°C, respectively when exposed for 4 and 36 hours. Interestingly, results show that the serum cortisol concentration of fish maintained in the control tank (30°C) was 0.208 ng mL^{-1} when exposed for 4 hours, but slightly decreased to 0.202 ng mL^{-1} when the exposure time was increased to 36 hours. Serum cortisol levels in grouper varied when the fish were kept in water maintained at 18, 24 and 28°C, respectively. The mean serum cortisol concentration of fish held at 18, 24 and 28 °C after 4 hours of exposure were measured to be 0.506, 0.406 and 0.394 ng mL^{-1}, respectively.

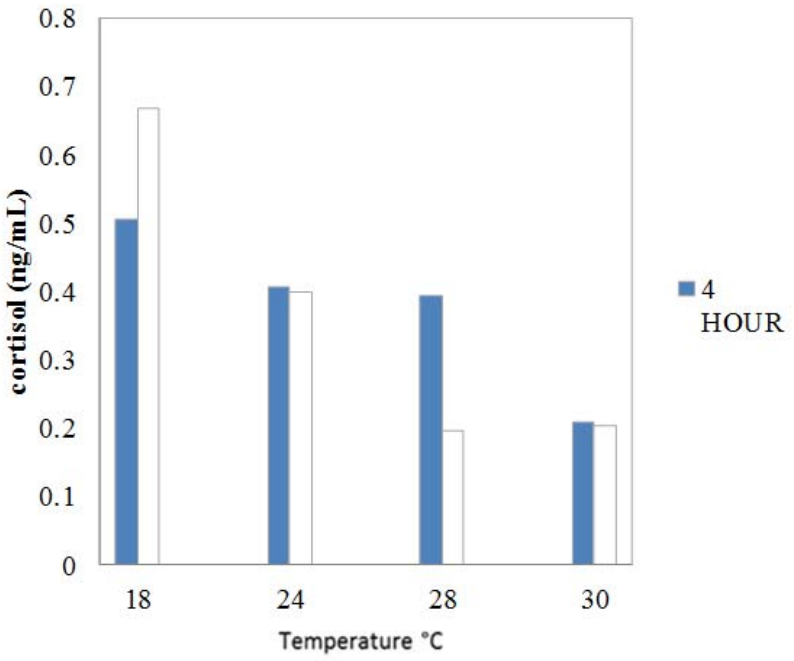

Figure 2: Effect of temperature changes on serum cortisol concentration in Tiger Grouper, *Epinephelus fuscoguttatus* monitored over 4 and 36 hour periods.

Subsequently, serum cortisol levels changed when the exposure time was increased to 36 hours, as the mean serum cortisol concentration at 18, 24 and 28°C were recorded as 0.668, 0.398 and 0.195 ng mL^{-1}, respectively. In other words, increase in exposure time from 4 to 36 hours led to decrease in the serum cortisol levels in fish maintained in water at 24 and 30°C, respectively. However for fish kept at 18°C, the increase in exposure time from 4 to 36 hours led the fish to increase serum cortisol concentration.

Effects of water salinity changes on serum cortisol concentration

The effect of changes in water salinity on serum cortisol concentration in grouper is shown in Fig. 3. The change in water salinity gives an impact on serum cortisol concentration in grouper. The water salinity of the tank was changed from 30 (control) to 10 ppt for 4 and 36 hours exposure time intervals in order to determine its effect on physiological stress in grouper. It was found that the serum cortisol concentration was lowest (0.208 ng mL^{-1}) in fish maintained at the control salinity. The serum cortisol concentration kept increasing as the water salinity decreased. The mean serum cortisol concentration of fish held at 10 and 20 ppt after 4 hours of exposure were measured to be 0.954 (the highest level) and 0.37 ng mL^{-1}, respectively.

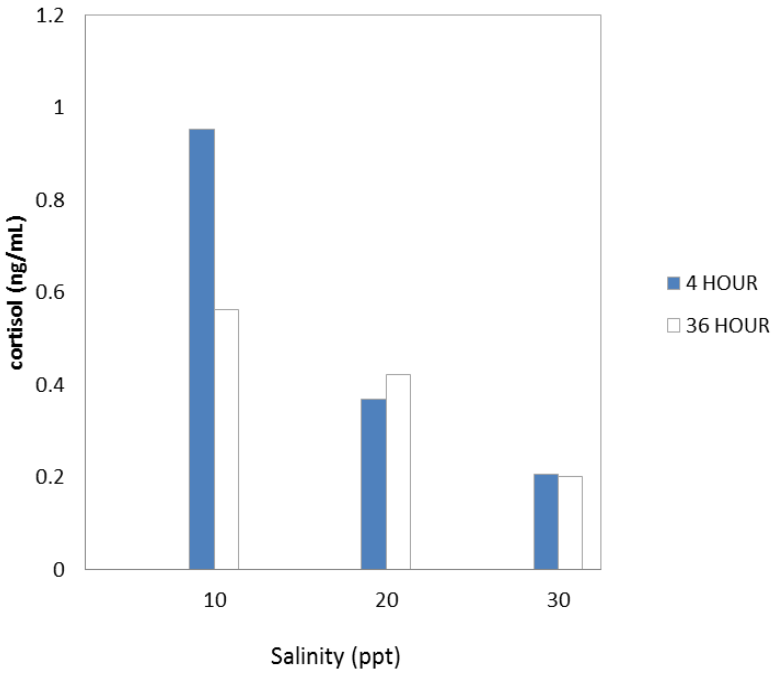

Figure 3: Effect of salinity changes on serum cortisol concentration in Tiger Grouper, *Epinephelus fuscoguttatus* monitored over 4 and 36 hour periods.

Meanwhile, almost similar trend of results noted in serum cortisol concentration when fish were exposed to the physiological stressor for 4 hours was also repeated when the exposure time was increased to 36 hours as shown in Fig. 3. The increment of the serum cortisol concentration occurs at low water salinity after fish were exposed to the physiological stressor for 36 hours. The serum cortisol concentration in fish maintained at the control salinity was 0.202 ng mL^{-1}. The serum cortisol concentration were recorded as 0.422 ng mL^{-1} and 0.564 ng mL^{-1} (the highest level) when the fish were held at decreased water salinity of 20 and 10 ppt, respectively.

Discussion

According to Little (2002), changes in physical behaviors and activities of fish are common indicators of environmental stressors, as changes in the environment may accelerate metabolic rates. For example, sudden acceleration in metabolism translates into increase in fish requirement for oxygen, which could simultaneously affect the supply of oxygen because of its low solubility in water (Dominguez *et al*., 2005). Therefore, environmental stressors can sometimes manifest in behavioral changes in farm animals, as they struggle to cope with such changes. In this regard, changees in water temperature and salinity are of

environmental factors that causes stress in cultured fish (Arends *et al*., 1999).

Generally, the steroid hormone cortisol has wide acceptance as the indicator of choice for stress measurement in fish, as it was noted to increase after exposure to physical stressors (Barton and Iwama, 1991; Wendelaar-Bonga, 1997; Barton, 2002). In order to determine the stress level of individual fish therefore, circulating cortisol levels in the blood are usually measured (Redding *et al*., 1984; Montero *et al*., 1999). Many researchers demonstrated interest to evaluate change in water temperature as a stressor to fish, and monitoring its effects on blood chemistry including; serum cortisol (Pottinger *et al*., 2003; Haukenes *et al*., 2008) and glucose concentrations (Barton *et al*., 2002; David *et al*., 2005). This is because stress is reported to elevate serum cortisol and glucose levels (Haukenes *et al*., 2008). According to Barton (2002) and Martinez-Porchas *et al*. (2009), testing for the steroid hormone cortisol offers a good signal, and is widely accepted as an indicator of stress in many fish species. Vijayan and Moon (1995) on the other hand, opined that when compared to cortisol level, blood glucose concentration ultimately vary as an indicator of stress due to a variety of causes.

From basic fish physiology, it is commonly accepted that water temperature is important, and affects the rate at which physiological processes occur (Dominguez *et al*., 2004). In particular, the body temperature of fish is essentially the temperature of the surrounding water (Fry, 1967) so that their entire behavior and physiology are influenced and limited by environmental temperature. In most fish, blood cortisol level promptly attains its highest concentration immediately after the organism is stressed, and returns to basal levels after some time when homeostasis is re-established, in accordance with the results of Iwama *et al*. (2004). Consistent with these observations, results recorded in the current trial revealed that the level of stress of the experimental fish increased when the water temperature was decreased from the optimal level, evident in the increased serum cortisol concentrations. These changes likely led to physiological changes on the short term (4 hours). When prolonged, the physiological changes likely led to metabolic imbalances, such as increase in protein breakdown that further increased demands on the body, leading to biochemical exhaustion (which manifested in abandonment of social schooling behavior, gasping, vigorous opercula movements and slow body movements during swimming).

Similar to results of the present trial, Le Morvan *et al*. (1998) found that in carp, plasma cortisol level suddenly increased following abrupt decrease in water temperature from 20 to 12 °C for 2 h. Apart from water temperature; other stressors also affect blood cortisol concentrations. For example, cortisol

concentration in red drum also increased rapidly during some handling procedures, but returned to basal level within 48 hours (Robertson *et al*., 1987). Common dentex (*Dentex dentex*) increased its blood glucose and cortisol levels immediately after handling and then returned to the basal level after 8 hours (Morales *et al*., 2005). Carp (*Cyprinus carpio*) showed increased plasma cortisol concentrations when retained in anglers' keep nets but the levels returned to basal within 4 hours (Pottinger, 1998).

A few studies on the changes in water salinity towards a few fish species also have been reported including rainbow trout (Yada *et al*., 2001), tilapia (Yada *et al*., 2002, and Breves *et al*. 2010) and sturgeon, (Zhao *et al.*, 2011). In this study, the effect of water salinity on the serum cortisol concentration in tiger grouper, *E. fuscoguttatus*, was assessed for fish held at 10 and 20 ppt salinities at 30 °C where the augmentation of the serum cortisol concentration occurs at low water salinity. The present results seem to be in divergence with results in grouper *Epinephelus malabaricus* in which serum cortisol levels increased in the fish transferred to high salinity (Tsui *et al*., 2012). The variance in these outcomes might be partially due to difference in the salinity tolerance range between the two species, as well as their osmoregulatory capacity and the environment condition to which the fish were adapted. In addition, there are possibilities that size of the fish also contribute to the difference (Dominguez *et al*., 2005). In our experiments, we used fish with body weights 8.65 ± 0.15g and length measuring 7.00 ± 0.5 cm, while the fish weighing 46.37 ± 5.10g and length 14.87 ± 1.84 cm were used by the other researchers (Tsui *et al*., 2012).

With prolonged enervation of the endocrine system however, Barton *et al*. (2005) and Fast *et al.* (2008) all recorded that some fish show weak increases in blood cortisol concentrations. This may likely be caused by exhaustion of the endocrine system as a result of prolonged hyperactivity (Hontela *et al.*, 1992) or due to the organism becoming adapted to the stressor condition. For example, Vijayan and Leatherland (1990) and Mommsen *et al.* (1999) mentioned that when an organism experiences suboptimal conditions for a considerable period of time, the release of cortisol decreases because the interregnal tissue of the stressed animal becomes less sensitive to the action of ACTH or other pituitary hormones. Based on these observations, it is clear that blood cortisol level is a good signal to evaluate for acute stress in fish (Martínez-Porchas *et al.*, 2009).

Stress hormones activate a number of metabolic pathways that result in alterations in blood chemistry and haematology (Barton and Iwama, 1991; Randall and Perry, 1992; Vijayan *et al*., 1994; Mommsen *et al.*, 1999; Barton *et al.*, 2002) and plays major roles in the bioenergetics of animals, principal of

which involves its transformation to chemical energy (ATP), which may in turn be expressed as mechanical energy (Lucas, 1996) such as vigorous swimming, opercula movement and gasping. Under suboptimal or stressful conditions (influenced internally or externally) the chromaffin cells release catecholamine hormones; adrenaline and noradrenaline into the blood circulation (Reid *et al*., 1998).

In conclusion, tiger grouper exposed to water temperatures and salinity lower than the optimal exhibited physiological changes, as serum cortisol concentrations changed when the fish were subjected to water temperature changes from 30°C to 28, 24 and 18°C and salinity from 30 ppt to 20 and 10 ppt. The present study highlights that serum cortisol levels change in fish transferred from optimal to lower temperatures and salinity. In addition, changes in serum cortisol concentrations occur in the monitored species regardless of short (4 h) or long term (36 h) exposure and that parameter used are good signals useful for assessing physiological stress in fish, as demonstrated in the present trial.

Acknowledgments

This work was funded by the Research University Grant Scheme (RUGS) (Grant No. 9199880). The authors also graciously appreciate the contributions of Mr. Zainan Ariffin in the set-up and maintenance of the experimental tanks during the feeding trial, as well as the facilities provided for the study by the Laboratory of Marine Biotechnology, Institute of Bioscience, University Putra Malaysia.

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