

The effects of a sudden salinity change on cortisol, glucose, lactate, and osmolality levels in grouper *Epinephelus malabaricus*

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Abstract Grouper *Epinephelus malabaricus* (weighing 46.37 ± 5.10 g) previously maintained in 24‰ seawater were transferred to 14, 19, 24 (control), 29, and 34‰ seawater. Serum cortisol, glucose, lactate, and osmolality levels were measured at 7 time points during 240 min. Serum cortisol and glucose levels of fish transferred to 29 and 34‰ seawater significantly increased to the highest after 10 and 20 min, respectively. No significant differences in serum cortisol and glucose levels were observed for the fish after 30 min among all treatments. Serum lactate level of fish transferred to 14, 19, 29, and 34‰ seawater was significantly lower than that of the control fish after 10–30 min. However, no significant differences in serum lactate were observed 60 min among five treatments. The serum osmolality of the fish following 240-min transfer increased directly with salinity, whereas the osmoregulatory capacity value (medium osmolality–plasma osmolality) of the fish following 240-min transfer was inversely related to salinity. It is concluded that grouper showed strong osmoregulation in 14–34‰ seawater. Serum cortisol

and glucose levels of fish transferred to 29 and 34‰ seawater increased rapidly in 10–30 min, indicating an early stress response.

Keywords *Epinephelus malabaricus* · Salinity · Cortisol · Glucose · Lactate · Osmolality

Introduction

Groupers are widely cultured in cages and ponds in the Indo-Pacific region and have high market potential (Chen and Tsai 1994; Millamena 2002, Jiang 2010). Malabar grouper *Epinephelus malabaricus* inhabits in various environments like rocky, mud, estuary, and coral reef areas in the tropical and subtropical regions. Salinity is one of the most important environmental factors affecting metabolism, oxygen consumption, ammonia excretion, growth, and survival of fish. Survival and growth of *E. tauvina* reared at 25‰ salinity were significantly higher than those of fish reared at other salinities (Akatsu et al. 1983). Culture of grouper is likely to encounter sudden change in salinity due to heavy rainfall and continuous sunny day. In addition, fish farmers are likely to decrease the salinity level because they think that lowering the salinity may increase the growth of grouper.

Cortisol, glucose, and lactate levels are general stress indicators in fish (Santos and Pacheco 1996; Pacheco and Santos 2001). Fish sampled at contaminated sites had an impaired capacity to increase their plasma

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cortisol levels in response to stress (McMaster et al. 1994; Hontela et al. 1997). An elevated blood cortisol level results in gluconeogenesis, and cortisol is implicated in osmoregulatory process (Mommsen et al. 1999).

Serum cortisol and glucose levels of tilapia *Oreochromis mossambicus* increased steadily following 4-h acclimation to freshwater, 10 and 30‰ (Cataldi et al. 2005). Groupers encounter stress condition due to handling, transportation, and change in salinity level (Estudillo and Duray 2003). However, little is known about changes in cortisol, glucose, lactate, and osmolality levels, and their roles in energy metabolism and osmoregulation in grouper. This study investigated physiological changes in *E. malabaricus* subjected to sudden changes in salinity. Serum cortisol, glucose, lactate, and osmolality levels of *E. malabaricus* subjected to salinity change were examined.

Materials and methods

Experimental fish

Male and female Malabar grouper *E. malabaricus* (mean initial weight, 46.37 ± 5.10 g and mean initial length, 14.87 ± 1.84 cm) from a private farm in Pingtung, Taiwan, were shipped to the laboratory and held in tanks containing 34‰ seawater at water temperatures of 22–24°C for 2 weeks. The fish were fed twice a day with a commercial grouper diet (Shye Yih Feeding CO., Ltd.) based on 5% of body weight. Seawater pumped from the Keelung coast adjacent to the National Taiwan Ocean University was filtered through a sand and gravel filter tower and aerated for 3 days using air-lifting. The salinity was then adjusted 1–2‰ per day with dechlorinated municipal water until a salinity of 24‰ was reached. The fish were reared for 1 week. During the acclimation period, the fish were fed artificial feed manufactured by Full-Rich Feed (Pingtung, Taiwan) every day, but were not fed 12 h prior to the experiment. During the experimental period, the water temperature, dissolved oxygen, and pH were maintained at $22.3 \pm 0.56^\circ\text{C}$, 6.53 ± 0.41 mg l⁻¹, and 8.21 ± 0.32 , respectively.

Sampling and analytical procedures

There were 5 test solutions, 14, 19, 24, 29, and 34‰ seawater and 7 exposure times, 0, 10, 20, 30, 60, 120,

and 240 min. There were 210 fishes were sampled from the holding tank and individually released into a plastic basket (60 × 30 × 42 cm) containing 24‰ seawater for 3 days. Fish were then suddenly transferred to tank containing 200 l of each test solution. Each tank was aerated with an air stone. Six replicates were used for each test solution and sampling time. The fish were sampled 0, 10, 20, 30, 60, 120, and 240 min after being transferred, and blood samples were taken by inserting a syringe (25G × 2.54 cm) into the caudal vessel. After coagulation, serum samples were obtained by centrifugation at 2,500 rpm for 10 min (HERMLE Z233MK-2, Hamburg, Germany) and stored at -80°C for subsequent analysis.

Cortisol level was measured using an enzyme-linked immunosorbent assay (ELISA) method (Engvall and Perlmann 1971). Briefly, 10 µl serum was immediately diluted with 190 µl of distilled water in a correlate-EIA kit (Assay Designs, Ann Arbor, MI, USA). After 1 h of incubation at 37°C, serum was added with stop solution, and the optical density was measured at 405 nm using a tunable microplate reader (VERSAmax, Sunnyvale, CA, USA). Glucose and lactate levels were measured by injecting 10 µl serum with a DT pipette into a VITROS DT60II chemical analyzer (Kodak, New York, USA). Serum osmolality and medium osmolality (MO) were measured by injecting 20 µl sample with a disposable tip, then putting the sampler into a micro-osmometer (model 3MO plus, Advanced Instruments, Norwood, MA, USA). The osmoregulatory capacity was calculated based on the difference between the medium osmolality (MO) and serum osmolality (PO).

Statistical analysis

All data on serum cortisol, glucose, lactate, osmolality, and osmoregulatory capacity levels were subjected to a one-way analysis of variance (ANOVA) using the SAS software system v. 8.1 (SAS Institute, Cary, NC, USA) to determine the sample variance. If significant differences were indicated at the 0.05 level, then Duncan's multiple range test was used to separate the different means. Linear relationships between ambient salinity, time period, serum cortisol, glucose, lactate, osmolality, and the osmoregulatory capacity levels were tested with the general linear regression model procedure and stepwise procedure in v. 8.1 of the SAS computer software system. Statistical significance was determined at a level of $p < 0.05$.

Results

No fish died during the salinity transfer. In the control fish, serum cortisol, glucose, lactate, and osmolality levels were $16.305 \pm 2.963 \text{ nmol l}^{-1}$, $1.257 \pm 0.057 \text{ mmol l}^{-1}$, $1.875 \pm 0.320 \text{ mmol l}^{-1}$, and $310.0 \pm 26.5 \text{ mOsm kg}^{-1}$, respectively.

Serum cortisol level of fish under salinity change

Serum cortisol of fish 10 min after being transferred to 29 and 34‰ seawater increased significantly to 84.246 and $97.465 \text{ nmol l}^{-1}$, respectively. Thereafter, serum cortisol level decreased with time. No significant difference in the cortisol level was observed among fish 120 min after being transferred to all test solutions. The mean serum cortisol level of fish transferred to 14 and 19‰ seawater was 16.114 and $15.464 \text{ nmol l}^{-1}$, respectively. No significant difference in serum cortisol level was observed between the control fish and the fish transferred to 14 and 19‰ seawater (Fig. 1).

Serum glucose level under salinity change

Serum glucose increased directly with salinity and exposure time. Serum glucose levels of fish 20 min after being transferred to 29 and 34‰ seawater increased significantly from 1.26 to 1.80 and

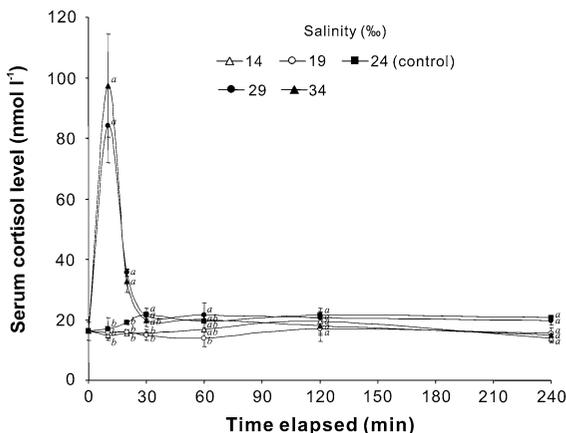


Fig. 1 Time-course changes in serum cortisol levels (nmol l^{-1}) of *Epinephelus malabaricus* under different salinity stresses. The comparisons are made with unstressed fish sampled at each time. The curved lines indicate the mean values and the standard error ($n = 6$). Data with different letters differ significantly ($p < 0.05$) among different salinities

2.77 mmol l^{-1} , respectively. Serum glucose level of fish 20 min after being transferred to 34‰ seawater was significantly higher than that of the control fish and of the fish transferred to other test solutions. After 20 min, serum glucose level decreased with increasing exposure time in the fish transferred to 29 and 34‰ seawater. No significant difference in serum glucose level was observed among the fish 60 min after being transferred to all test solutions. Serum glucose level of fish transferred to 14, 19, and 24‰ seawater showed no significant differences to that of control fish. Mean glucose level of fish transferred to 14, 19, and 24‰ seawater was 1.308 ± 0.045 , 1.263 ± 0.026 , and $1.322 \pm 0.071 \text{ mmol l}^{-1}$, respectively (Fig. 2a).

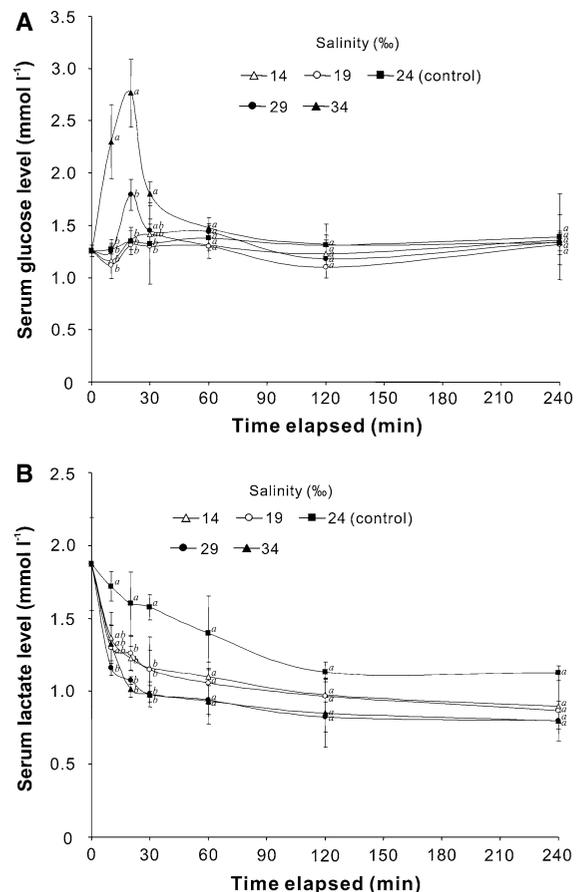


Fig. 2 Time-course changes in serum glucose (mmol l^{-1}) (a) and serum lactate levels (mmol l^{-1}) (b) of *Epinephelus malabaricus* under different salinity stresses. The comparisons are made with unstressed fish sampled at each time. The curved lines indicate the mean values and the standard error ($n = 6$). See Fig. 1 for statistical information

Serum lactate level under salinity change

Serum lactate level of fish 30 min after being transferred to 14, 19, 29, and 34‰ seawater were significantly lower than that of control fish. Although serum lactate level of fish transferred to 29 and 34‰ seawater were lower than that of fish transferred to 14 and 19‰ seawater, no significant differences among these groups were found at any time period. No significant differences in serum lactate levels were observed among the fish 60 min after being transferred to all test solutions. Serum lactate levels of fish transferred to 29 and 34‰ seawater were significantly lower than those of fish transferred to 14 and 19‰ seawater (Fig. 2b).

Serum osmolality level of fish under salinity change

Serum osmolality of control fish was 310.0 mOsm kg⁻¹. No significant difference in serum osmolality was observed in the fish 60 min after being transferred to any test solution. However, serum osmolality directly increased with salinity during the first 60 min. Serum osmolality of fish 240 min after being transferred to 14, 19, 24 (control), 29, and 34‰ seawater was 246.7, 260.0, 296.7, 317.5, and 346.7 mOsm kg⁻¹, respectively (Fig. 3a).

Medium osmolality was 376, 504, 646, 790, and 921 mOsm kg⁻¹ for the 14, 19, 24, 29, and 34‰ seawater, respectively. Osmoregulatory capacity of fish 240 min after being transferred to 14, 19, 24, 29, and 34‰ seawater was 129.3, 244.0, 349.3, 472.5, and 574.3 mOsm kg⁻¹, respectively (Fig. 3b). The linear relationship between the medium osmolality (X) and the differential value (medium osmolality–plasma osmolality, Y) was as follows:

$$Y = 0.8127X - 172.24 \quad (R^2 = 0.9993).$$

Discussion

Cortisol levels between 20.549 and 102.747 nmol l⁻¹ are considered normal physiological levels in teleosts (McDonald and Milligan 1997). In the present study, serum cortisol level was maintained at a low level of 16.305 nmol l⁻¹ in the control fish.

Plasma cortisol level of fish increased during capture stress within 5–6 min, whereas glucose and

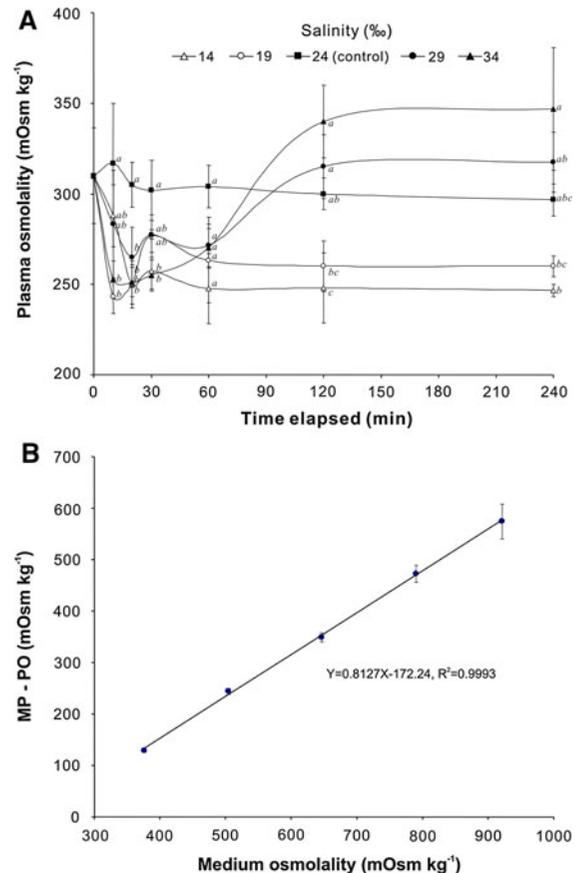


Fig. 3 Time-course changes in plasma osmolality levels (mOsm kg⁻¹) of *Epinephelus malabaricus* under different salinity stresses. The comparisons are made with unstressed fish sampled at each time (a). Differences in the medium osmolality (MO) and plasma osmolality (PO) (mOsm kg⁻¹) of *E. malabaricus* 240 min after different salinity shocks (b). The curved lines indicate the mean values and the standard error ($n = 6$). See Fig. 1 for statistical information

lactate levels did not increase (Grutter and Pankhurst 2000). Fish under stress exhibit characteristic acute increases in plasma levels of catecholamines including adrenaline and noradrenaline and increased level of corticosteroid and cortisol (Sumpter 1997). The feedback in response to increases in catecholamine and corticosteroid levels is generally an increase in the plasma level of glucose generated by the glucose-mobilizing effects of both classes of hormone (Barton and Iwama 1991; Wendelar Bonga 1997). Plasma cortisol, glucose, and lactate levels increased in the common carp *Cyprinus carpio* after 20 min of cold shock (Tanck et al. 2000). Plasma cortisol, glucose, and lactate levels of channel catfish *Ictalurus*

punctatus significantly increased after exposure to high unionized ammonia and oxygen depletion (Small 2004). The present study indicated that the serum cortisol level increased in the fish subjected to high salinity stress. Serum cortisol level directly increased with salinity during the first 20 min after transfer. A similar result was found in arctic charr *Salvelinus alpinus* contaminated by polychlorinated biphenyls (Jorgensen et al. 2002).

Plasma cortisol is directly related to environmental stress. Plasma cortisol of *O. mossambicus* fry reared in seawater was 55.8 ng ml^{-1} , which is 2.44-fold higher than that of fry reared in freshwater (Ron et al. 1995). Serum cortisol and glucose levels of tilapia *O. mossambicus* acclimated to fresh water, iso-osmotic water (10‰), and seawater (30‰) consistently increased after 4 h (Pottinger and Carrick 1999). In the present study, serum cortisol level of the fish 10 min after being transferred to 29 and 34‰ seawater significantly increased to 84.246 and 97.465 nmol l^{-1} , which is significantly higher than the initial value (16.305 nmol l^{-1}). Furthermore, no significant difference in serum cortisol was observed for the fish transferred to 14 and 19‰ seawater. Serum cortisol level of fish decreased 10 min after being transferred, and serum cortisol returned to its initial level 30 min after transfer. Similar results occurred with serum glucose. Serum glucose increased in the fish transferred to 29 and 34‰ seawater, whereas serum glucose decreased with exposure time 20 min after transfer. These findings indicate that fish under high salinity stress exhibit physiological responses. In contrary, the absence of serum cortisol and glucose changes indicated that no stress responses occurred in the fish after being transferred to 14 and 19‰ seawater.

The secondary responses that occur in energy metabolism include changes in blood glucose and lactate levels (Wendelar Bonga 1997). Acute hyperglycemia occurred for the fish following 20 min to high salinity stress together with an increase in the serum cortisol level. In the present study, the fish following a high-salinity transfer were hyperglycemic. The highest serum glucose level occurred at 20 min after being transferred to 34‰ seawater. This response lagged 10 min behind the cortisol level. This result demonstrates the physiological relationship between cortisol and glucose after salinity stress. No significant differences in serum glucose level was observed among the fish transferred to low salinity and high

salinity and control fish after 60 min. The hyperglycemic level of fish subjected to a high salinity transfer increased. Serum lactate did not increase significantly during the experiment. However, the results showed that serum lactate decreased with increasing time. Lactate is the major product of anaerobic glycolysis in multicellular organisms (Richards et al. 2009; Omlin and Weber 2010). The fact that the glucose level had returned to a normal after 30 min indicates that the fish did not undergo anaerobic glycolysis. Stress may cause change in serum lactate. Even if lactate decreased slightly, no statistically significant difference remained after 60 min. Serum lactate level of groupers transferred to high salinity was lower than the grouper transferred to low salinity. It is considered due to short exposure. Laiz-Carrión et al. (2002) reported that decrease in lactate level is commonly occurred in chronic conditions.

Stress-induced elevation of plasma cortisol is a known indicator of the primary stress response, whereas plasma glucose and lactate levels are indicators of the secondary stress response (Barton and Iwama 1991; Wendelar Bonga 1997). The temporal patterns of seawater acclimation in rainbow trout *O. mykiss* showed two different phases: initial adaptation (critical phase) and a subsequent stabilization phase (Bath and Eddy 1979). The initial phase lasts approximately 4 days and includes rapid physiological changes, whereas the stabilization phase includes slower physiological changes and results in a new equilibrium with seawater within 8–10 days. In the present study, *E. malabaricus* adjusted its blood osmolality during the first 20 min, and blood osmolality became constant after 2 h.

Cortisol plays an important role in adaptation to hyperosmotic environments. Cortisol proliferates in gill chloride cells and activates $\text{Na}^+ - \text{K}^+$ ATPase activity (Seidelin and Madsen 1997). It is known that stress-induced hormonal responses, such as elevated levels of plasma cortisol and catecholamines, produce osmotic imbalances in fish following exposure to hypertonic and hypotonic environments (Mazeaud and Mazeaud 1981; Schreck 1981; Pickering and Pottinger 1995). Cortisol, a stress hormone, was implicated in osmoregulatory processes (Mommsen et al. 1999). In the present study, serum cortisol and glucose levels changed during the first 60 min. The osmolality began to change from 60 min and became constant after 120 min.

The difference between plasma and medium osmolality increased with ambient salinity. Grouper is a strong osmoregulator and can be cultured in various salinity levels. The good capability in osmoregulation may also explain increases in plasma cortisol and glucose at the beginning of the transfer. Zhao et al. (2011) transferred Chinese sturgeon *Acipenser sinensis* juveniles directly from freshwater to 5, 10, 15, 20, and 25‰ brackish water for 192 h. At a salinity of 5‰, serum osmolality did not significantly differ to that of control sturgeon. However, serum osmolality of fish following transfer from 10 to 20‰ brackish water increased significantly with the ambient salinity. Meanwhile, serum osmolality reached a peak at 12 h for the fish following 24-h transfer to 10 and 15 from 20‰ brackish water. Breves et al. (2010) studied tilapia *O. mossambicus* acclimated in freshwater and measured the plasma osmolality of the fish following acute salinity challenges in brackish water (17‰). The plasma osmolality increased above 450 mOsm kg⁻¹ 2 h after an acute change in salinity resulting from a transfer from freshwater to brackish water. A change in the ambient salinity causes stress in fishes. Both freshwater and marine fish can tolerate environmental salinity changes. The difference in tolerance between species depends on their osmoregulatory ability and the environment to which they are acclimated.

Cortisol of *O. mossambicus* increased rapidly by 3 h after the transfer and remained elevated for 3 days (Kammerer et al. 2010). Plasma osmolality was elevated at 6–8 h. In the present study, cortisol and glucose levels were significantly elevated within 30 min for the grouper transferred to high salinity. Furthermore, serum osmolality elevated at 2 h after transfer. This result indicated that the osmoregulatory ability of the grouper is more efficient than that of the tilapia.

Grouper transferred to high salinity exhibited physiological changes. Serum cortisol and glucose levels increased even in the short term for the fish subjected to sudden transfers, but no significant difference was observed for the fish transferred to lower salinities. The present study indicated that serum cortisol and glucose levels increased in the fish transferred to high salinity. In addition, serum cortisol and glucose levels significantly increased 10 and 20 min after transfer. These results indicate that the fish exhibited different responses to ambient salinity stress in relation to salinity changes. To adapt to acute

changes in ambient salinity, the grouper increased serum cortisol and glucose within 30 min to stabilize the plasma osmolality. Serum osmolality had stabilized by 2 h after an acute salinity change.

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