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PROOF
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An Evaluation of the Effect of Salinities on Oxygen Consumption and Wellbeing in the Hybrid Grouper *Epinephelus fuscoguttatus* × *E. lanceolatus*

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Abstract

The oxygen consumption and wellbeing of hybrid grouper *Epinephelus fuscoguttatus* × *E. lanceolatus* (TG × GG) juveniles were evaluated after 60 days of being exposed to different salinities (10, 15, 20, 25, and 30 psu) in triplicate (20 fish / tank; 55.0±1.0 g body weight; 12.0±0.5 cm total length). The fish were fed daily with commercial pellet (42% protein, 9% ash, and 11% lipid).Results showed that the oxygen consumption rate (OCR)was significantly lower at15 psu but higher at 25 and 30 psu, indicating the high metabolic rate of fish in high salinities. The fish's wellbeing was monitored through growth and blood parameters. The fish cultured at 15 psu grew better in terms of final body weight, specific growth rate (SGR), feed efficiency (FE), and condition factor (CF) as compared with the fish at other salinities. Blood hematologic parameters were significantly higher in 15 psu but lower in 25 and 30 psu, whereas blood biochemical parameters were significantly lower in 15 psu but higher in 25 and 30 psu compared with other salinities. Overall, the TG × GG hybrid grouper showed the best OCR and wellbeing when cultured in 15 psu salinity. The findings will be useful in further studies on the management of TG × GG hybrid grouper.

Introduction

Variable water parameters, such as temperature, dissolved oxygen (DO), and salinity concentration, play critical roles in aquaculture (Fazio, Marafioti, Arfuso, Piccione, & Faggio, 2013) because they affect the growth parameters in fish. A change in these parameters could affect cultured aquatic animals, leading to diseases and mortalities. Sudden or drastic unfavorable changes in water parameters, such as salinity, weaken the animals to the point of acquiring bacterial infections (Chu, Nurhamizah, Dee'ana, & Sufian, 2016). The response of animals to these unfavorable environmental conditions includes negative effects on their growth, respiration, and blood biochemical parameters (Toledo, Caberoy, & Quinitio, 2004).

TG × GG hybrid grouper is a farmed fish species that live in a wide range of salinities (10–40 psu) in both warm and temperate climates and thus are more suitable for culture than purebred species with regard to a number of factors, including tolerance to fluctuations, salinity, water temperature, pH, and stock density (Kidder, Petersen, & Preston, 2006). The aquaculture of grouper has recently attracted global attention and is increasingly becoming popular for its excellent taste among food lovers due to its great meat quality (Shapawi, Mustafa, & Ng, 2011). Interestingly, the economic value of this species has increased because of its faster growth rate compared with its parent species. This phenomenon has attracted the attention of fish culturists especially in Southeast Asian countries such as Hong Kong, Singapore, and Malaysia (Rahimnejad *et al.,* 2015). This event may explain the

expansion in the aquaculture of the species in Asia because fish remains one of the reliable sources of animal protein worldwide. Unfortunately, in tropical countries such as Malaysia, the east coast of the peninsula experiences heavy rainfalls during the northeast monsoon season. Water parameters, such as salinity, rapidly vary and affect grouper farms during this season. Therefore, fish farmers face difficulty in maintaining a steady supply of products to the market due to the physical, chemical, and biological components of the environment that are fatal to fish. Studies on the effects on the oxygen consumption and blood parameters of fish due to fluctuations in environmental parameters are necessary to enable farmers to manage these cultured fish under such challenging situations (Kidder *et al.,* 2006). To meet the increasing demand for fish, farmers must focus on rearing species, such as hybrid groupers, which exhibit fast growth, good reproductive capacity, and high resistance to environmental stressors and diseases due to their well-developed immune system (Rahimnejad *et al.,* 2015). This study aims to determine the effect of salinity on the oxygen consumption and wellbeing (growth, hematologic, and biochemical parameters) of TG × GG hybrid grouper juvenile subjected to different water salinities (10, 15, 20, 15, and 30 psu).

Materials and Methods

Fish Acclimation and Experimental Design

TG × GG hybrid grouper juvenile were transported from a local hatchery in Banting, Selangor, Malaysia (2°'0" N, 101°'0" E) and placed in a laboratory of the Marine Science Laboratory in Universiti Kebangsaan Malaysia (UKM). Acclimation was conducted in six stocking tanks (1.96 m × 1.02 m × 0.61 m, 1200 L in size and capacity) in recirculation systems supplied with UV sterilization, biological filters, and temperature controllers (26 °C). The tanks were fixed in the light and darkness for 12 h each. The fish were fed twice a day (9:00 and 17:00 h) with commercial diet (Star feed: Marine 9982/84, CP Group, Malaysia: 42% protein, 9% ash, 11% lipid) to apparent satiation, which was determined as the point when the fish stopped actively feeding and pellets remained at the bottom of the tanks for more than 2 min (De, Ghaffar, Bakar, & Das 2016). After two weeks of acclimation, 300 fish (body wet weight: 50.5±2.0 g; total length: 10.0±0.5 cm) were randomly stocked in 15 tanks (200 L). The fish were kept under the same condition as during the acclimatization period and were tested in five experimental salinities (10, 15, 20, 25, and 30 psu) with triplicates of 20 fish per tank. Experimental salinities were achieved by a daily increase of 2 psu using dechlorinated tap water mixed with natural sea water. Salinity levels were measured using refractometer (S-10E; Atago Co., Ltd, Washington, DC, USA). Water quality parameters, including pH (pH meter, Quimis), dissolved oxygen (DO), and water temperature (oximeter, YSI) were maintained at pH 7.8±0.3, 6.5±1.0 mg l^{-1}, and 26.0±0.2°C, respectively, and were measured daily during the 60-day experimental period.

Oxygen Consumption

Oxygen consumption was measured following the procedures described by Sun, Huang, Cao, Wang, and Tan (2010) at the end of the 60-day experimental period. The respiratory chamber used (tubular Plexiglass length: 25.5 cm; diameter: 15 cm; volume: 4.3 L) was placed in a glass aquarium water bath at a desired acclimation salinity. Oxygen partial pressure (pO_2) was measured using a radiometer oxygen electrode (SA7-530-200) connected to a four-channel Fire Sting oxygen analyzer logger software version 3.0. Respirometry experiment consisted of a 60-minute cycle (Das *et al.,* 2018). An oxygen solubility table was used to convert pO_2 values to oxygen concentration in ml O_2 L^{-1}. The oxygen consumption rate (OCR, ml O_2 L^{-1} h^{-1}.) was calculated using the equation of Sun et al. (2010) as follows:

$OCR = 60\ [C(t_f\text{-}t_i)] \times (V_r\text{-}V_a)/(t_f\text{–}t_i)]$, where $C(t_f\text{-}t_i)$ is the consumption of total oxygen value in the water at time t, t_f is the finish time (min) of the measurement period, $t_i$is beginning time of the measurement period, V_r is the volume of the respirometer, and V_a is the volume of the animal.

Growth Performance

The fish were anesthetized using α-methyl quinolone (Transmore, Nika Trading) (0.22 ml L^{-1} in 3 L of sea water) for 10 min. Body weight was measured every 10 days using an electronic balance. Growth parameters calculated were according to the formula (Pérez-Casanova, Lall, & Gamperl, 2009) as follows:

Specific growth rate, *SGR = [(ln Wf–ln Wi)/T] x 100*, where *Wf* is the fish body weight at the beginning of the period and *Wi* is the fish body weight at the end of the period;

Feed efficiency, *FE* = (*BG*/*F*), where *BG* is the body weight gain (g) and *F* is the feed given (g); and condition factor, *CF* = (*BW*/*L3*), where *BW* is the fish body weight (g) and *L* is the fish length (cm).

Blood Collection and Analysis

The fish (n = 9 fish / group) were randomly collected from each salinity group at the end of the experimental period and anesthetized in α-methyl quinolone (Transmore, Nika Trading) (0.22 ml L−1 in 3 L of sea water) for the analysis of the hematological and biochemical responses. Blood was immediately collected from the caudal vein with a heparinized 2 mL disposable syringe. Hematological indices, such as

hemoglobin (Hb) and hematocrit (Hct) value levels were evaluated. Hb concentration was measured spectrophotometrically using cyanmethemoglobin method (Akrami *et al.,* 2015). Hct was centrifuged at 3,500 rpm for 10 min in standard heparinized microhematocrit capillary tubes, and the percentage of the packed cell volume was calculated (Barros, Lim, & Klesius, 2002). Red blood cell (RBC) and white blood cell (WBC) concentration was analyzed using diluted blood samples by adding Dacie's fluid for RBC and Turk's solution for WBC. The cells were counted manually using a Neubauer hemocytometer (Akrami *et al.,* 2015). Mean cell hemoglobin (MCH), mean cell volume (MCV), and mean cell hemoglobin concentration (MCHC) were calculated according to the formula (Douglass and Jane, 2010) as follows:

$$MCH\ (pg\ cell^{-1}) = Hb\ gdL^{-1} / RBC\ (\times 10^6\ \mu L) \times 10$$
$$MCV\ (fL) = Hct\ \% / RBC\ (\times 10^6\ \mu L) \times 10$$
$$MCHC\ (g\ L^{-1}) = Hb\ (gL^{-1}) / Hct\ (\%).$$

For biochemical analysis, the collected blood samples were centrifuged (12,000 rpm, 5 min) to separate the plasma, which was stored at −70 °C for the following analysis of total protein (TP), albumin (ALB), globulin (GLO), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) (Dacie and Lewis, 2001). A kit slide was used for the analysis by using automatic biochemical analyzer (FUJI DRI-CHEM 3500i; Fujifilm Co., Japan).

Data Analysis

Data were expressed as means with the standard deviation (SD) of the means and analyzed using the SPSS 16.0 for Windows software based on ANOVA multiple range test. Significance was assigned to differences at 0.05.

Results and Discussion

The initial body weight was similar among treatments (P>0.05) (Table 1). Nevertheless, the measured final body weight, SGR, FE, and CF were significantly affected by water salinity. The final body weight, SGR, and FE decreased with increasing salinity and were significantly higher in high salinity (25 and 30 psu) than in low salinity (10–20 psu) (P<0.05) (Table 1). Meanwhile, CF was significantly high in high salinities (Table 1).

The results show that OCR differs among salinities (P<0.05) (Figure 1). However, mean value OCR was

Table 1. Growth parameters in TG × GG hybrid grouper juvenile maintained in different salinities for 60 days

Salinity	Initial body weight (g)	Final body weight (g)	SGR (g/day)	FE (gain/g dry feed)	CF
10 psu	50.5±1.0^a	51.0±0.5^a	52.0±1.0^a	51.2±1.5^a	52.0±0.5^a
15 psu	57.3±0.11^a	58.0±0.23^a	57.8±0.26^a	55.8±0.12^b	55.6±0.14^b
20 psu	0.21±0.02^a	0.21±0.05^a	0.18±0.01^a	0.14±0.02^b	0.11±0.03^b
25 psu	2.13±0.07^a	2.40±0.34^a	2.32±0.25^a	1.84±0.21^b	1.64±0.25^b
30 psu	1.82±0.06^a	1.71±0.15^a	1.79±0.08^a	1.16±0.05^b	1.21±0.07^b

SGR: Specific growth rate, FE: feed efficiency, CF: condition factor. Mean values ± SD are presented for each parameter. Significant differences within the diets are indicated by different letters (P<0.05).

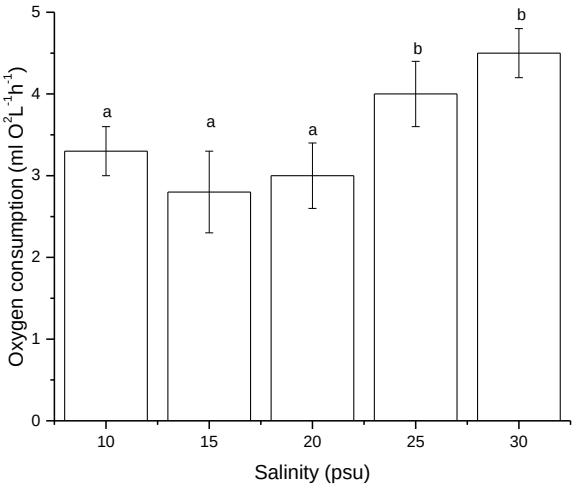

Figure 1. Whole-body oxygen consumption in TG × GG hybrid grouperjuvenile (n = 9) after acclimation to different salinities for 60 days. Data are expressed as mean ± SD. Different letters indicate significant differences (*P*<0.05) between salinity groups at the same sample time point.

significantly the highest in the 30 psu salinity group (4.5±0.05 mL O_2 L^{-1} h^{-1}), whereas the lowest was observed in 15 psu (2.8±0.5 ml O_2 L^{-1} h^{-1}) (Figure 1). Similarly, this pattern could be observed in hematological parameters of hybrid grouper in different salinities.

In hematological indices, RBC, Hb, Hct, MCV, MCH, MCHC, and WBC values changed within each salinity group (Table 2). The hybrid grouper exposed to 15 psu had significantly higher (P<0.05) RBC (2.2±0.3 ×10^6 µL), Hb (60.0±15.1 g L^{-1}), Hct (30.0±2.1%), MCV (183.5±15.8 fL), MCH (28.1±4.7 pg $cell^{-1}$), and MCHC (165.3±25.8 g L^{-1}) but lower WBC (8.7±2.3 ×10^3 µL) than the other salinity groups (Table 2). The significantly lowest (P<0.05) value was observed in 30 psu salinity group with RBC (1.6±0.1 × 10^6 µL), Hb (28.7±14.0 g L^{-1}), Hct (24.5±2.1%), MCV (126.3±21.0 fL), MCH (14.0±5.4 pg $cell^{-1}$), MCHC (92.6±24.5 g L^{-1}) and high WBC (12.9±2.1 × 10^3 µL) (Table 2).

The average TP was significantly different in all groups with the highest in 15 psu (3.9±0.1 g dL^{-1}) and the lowest in 30 psu (3.0±0.2 g dL^{-1}) (Table 3). In addition, the ALB level in 15 psu (0.7±0.1 g dL^{-1}) was significantly the highest among the groups (P<0.05). The ALB levels (0.3±0.1 g dL^{-1}) decreased significantly in the 30 psu salinity group (P<0.05). The GLO levels in 25 and 30 psu salinity groups were also decreased significantly (P<0.05) compared with those in the 20, 15, and 10 psu salinity groups. By contrast, the plasma levels of ALP (863.2±188.2 U L^{-1}) and ALT (506.7±101.8 U L^{-1}) were relatively high in the 30 psu group, whereas the lowest value (ALP 442.3±166.2 U L^{-1}, ALT 240.8±107.0 U L^{-1}) was observed in the 15 psu group (Table 3). No significant difference was observed in both 10–20 psu groups and 25–30 psu groups.

This study aimed to determine the effect of different salinity ranges (10, 15, 20, 25, and 30 psu) on the TG × GG hybrid grouper juvenile. The final body weight, SGR, and FE of the TG×GG hybrid grouper decreased with increasing salinities. This finding agrees with the growth performance reported for the TG × GG hybrid grouper (Noor, Das, Cob, & Ghaffar, 2018), *E. coiodes* x *E. lanceolatus* (Sutthinon, Thongprajukaew, Saekhow, & Ketmanee,2015); flounder *Paralichthys orbignyanus* (Sampaio & Bianchini, 2002) and turbot *Scophthalmus maximus* (Imsland, Foss, Gunnarsson, & Berntssen, 2001), where increasing salinity leads to poor growth parameters (SGR and FE). Significantly increased body weight, SGR, and FE were also observed at salinities of 10−20 psu. Thus, this range of salinities might be near a physiological salinity optimum for this species. The range of salinities is near to that of the hybrid grouper's parent species, the giant grouper *E. lanceolatus* whose optimum physiological salinity is 15–20 psu (Singhabun & Kummee, 2015). High salinity may trigger stress, lower the feed intake, and consequently result in poor growth of the fish because osmoregulation and ionic regulation must be achieved in the TG×GG hybrid grouper (Handeland *et al.,* 2014). In this study, CF increased with decreasing salinity. The CF of a fish is an indicator of the general fish condition and reflects physical and biological circumstances, fluctuations in the interaction among feeding conditions, and physiological factors (Odedeyi, Fagbenro, Bello-Olusoji, & Adebayo, 2017). Data obtained on CF can provide information on the specific condition under which organisms are developing (Araneda, Pérez, & Gasca-Leyva,2008). CF in this study is greater than the value indicating the wellbeing of fishes in different salinities. However, the values of CF were significantly high in low salinities (10 – 20 psu), suggesting that fish cultured in low salinities were healthier than those cultured in high salinities (25 – 30 psu). These findings are in agreement with the study of Garcia and Chapman (2015), who recorded the CF value of the goliath grouper *E. quinquefasciatus* in the range of 1.6–2.4 when cultured in 10 psu compared with the CF value of 1.5 – 1.6 in 20 psu.

The OCR values were significantly higher in 25 and 30 psu than those in 15 and 20 psu. OCR values are related to the respiration rate, which is used to measure the response to stress in fishes (Boeuf & Payan, 2001). OCR normally determines the metabolic status of fish subjected to different environmental parameters (Sampaio & Bianchini, 2002). This parameter value was significantly high in high salinity (25 and 30 psu) and low in low salinity (10, 15, and 20 psu). The response of TG × GG hybrid grouper juvenile in the tested range of salinities corresponded with the marine euryhaline fish, the OCR of which is high in high salinities (Boeuf & Payan, 2001; Varsamos, Nebel, & Charmantier, 2005). High OCR is related to faster gill

Table 2. Haematologic parameters for TG×GG hybrid grouper juvenile

Salinity	RBC (×10^6 µL)	Hb (g L^{-1})	Hct (%)	MCV (fL)	MCH (pg $cell^{-1}$)	MCHC (g L^{-1})	WBC (×10^3 µL)
10 psu	1.9±0.1^a	54.0±10.3^a	29.1±2.0^a	174.8±23.5^a	27.5±5.8^a	147.7±20.5^a	9.5±1.2^a
15 psu	2.2±0.3^a	60.0±15.1^a	30.0±2.1^a	183.5±15.8^a	28.1±4.7^a	165.3±25.8^a	8.7±2.3^a
20 psu	2.1±0.2^a	55.0±10.4^a	29.5±1.8^a	178.0±20.6^a	26.8±5.2^a	160.6±22.3^a	9.2±1.5^a
25 psu	1.6±0.1^b	30.5±11.5^b	24.8±2.2^b	130.2±16.2^b	15.8±4.3^b	95.2±25.8^b	12.8±2.0^b
30 psu	1.6±0.1^b	28.7±14.0^b	24.5±2.1^b	126.3±21.0^b	14.0±5.4^b	92.6±24.5^b	12.9±2.1^b

RBC: Red blood cell, HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, WBC: white blood cell. Mean values ± SD are presented for each parameter. Significant differences within the diets are indicated by different letters (P<0.05).

Table 3. Plasma biochemical parameters for TG × GG hybrid grouper juvenile

Salinity	TP (g dL^{-1})	ALB (g dL^{-1})	GLO(g dL^{-1})	ALP (U L^{-1})	ALT (U L^{-1})
10 psu	3.6±0.2[a]	0.6±0.2[a]	3.40±0.1[a]	483.0±200.5[a]	280.1±100.5[a]
15 psu	3.9±0.1[a]	0.7±0.1[a]	3.5±0.2[a]	442.3±166.2[a]	240.8±107.0[a]
20 psu	3.8±0.2[a]	0.6±0.2[a]	3.4±0.3[a]	467.5±156.7[a]	255.4±104.8[a]
25 psu	3.0±0.2[b]	0.3±0.1[b]	2.8±0.2[b]	855.5±153.3[b]	500.5±103.3[b]
30 psu	3.0±0.1[b]	0.3±0.1[b]	2.7±0.3[b]	863.2±188.2[b]	506.7±101.8[b]

TP: Total protein, ALB: albumin, GLO: globulin, ALP: alkaline phosphate, ALT: alanine transaminase. Mean values ± SD are presented for each parameter. Significant differences within the diets are indicated by different letters (P<0.05).

opening, which indicates that the fish is in stressful conditions (Varsamos *et al.,* 2005). Jensen, Madsen, & Kristiansen (1998) presented that this phenomenon may represent an adaptive mechanism for fish to reserve energy by maintaining a low OCR when coping in a wide salinity concentration until the optimum salinity was reached. Low OCR leads to high growth parameters as observed in the fish cultured in low salinities (10 – 20 psu).

Hematological and biochemical values, such as anemic condition and stress status (Peres, Santos, & Oliva-Teles, 2013) supply a reliable range for evaluating the fish's health. RBC characteristics (Hb and Hct content) display the efficiency of oxygen transport from respiratory systems to tissues (Cook, Wells, & Herbert, 2011; Nespolo & Rosenmann, 2002). RBC together with the Hb and Hct values in hybrid grouper TG × GG was relatively low in high salinities (25 and 30 psu), suggesting that the fish has an anemic condition. Meanwhile, the MCV, MCH, and MCHC values of healthy fish ranged at 170 – 350 fL, 26 – 100 pg $cell^{-1}$, and 145 – 200 g L^{-1}, respectively (Hrubec, Cardinal, & Smith 2000). However, these values were lower than the average in the hybrid grouper juveniles reared in high salinity; this result is probably due to the size of the erythrocytes (Knowles, Hrubes, Smith, & Bakal, 2006). Furthermore, these values correspond with the fish reared in high salinity, which have high OCR. Researchers found that fish with high levels of WBC can effectively fight infections due to the direct correlation between WBC counts and immune responses (Davis, Maney, & Maerz, 2008). The WBC value in TG × GG hybrid grouper was significantly lower in 25 and 30 psu than those in the 10 – 20 psu. Similarly, a high WBC was reported in mullet, *Mugil cephalus*, reared in high salinity (>25 psu) (Fazio *et al.,* 2013); the authors suggest that fish have poor growth performance under stressful conditions.

TP level is commonly used to diagnose fish health and disease, and its typically range is from ~4 g / dL to ~7 g / dL in fish (Yanagisawa & Hashimoto, 1984). When fish are under stress, the primary response increases the plasma cortisol, and the protein level increases as a secondary response (Davidson, Davie, Young, & Fowler, 2000). Abdelkahlek, Ghazy, and Abdel-Daim (2015) observed low levels of TP in fish under stress. In the present study, low TP level was observed in hybrid grouper reared in high salinity conditions (25 and 30 psu). The low concentrations of TP in fish is probably due to the structural liver alterations and impaired control of fluid balance, which may lead to the failure of protein synthesis due to stress (e.g., salinity) (Bernet, Schmidt, Wahli, & Burkhardt-Holm (2001). In addition, a correlation between TP with ALB and GLO is expected because ALB and GLO are parts of TP. The ALB level in the 15 psu salinity group was significantly higher than that in the 25 and 30 psu groups (P<0.05). The main function of ALB is to regulate the osmotic pressure of the blood and to transport some of the exogenous chemicals (e.g., hormones) (Rummer, Wang, Steffensen, & Randall, 2014). The GLO level in 25 and 30 psu salinity groups was decreased significantly compared with that in 10, 15, and 20 psu groups (P<0.05). Hasheesh, Marie, Abbas, Eshak, and Zahran (2011) reported the effect of salinity changes on the blood parameters of the Nile Tilapia *Oreochromis niloticus* with decreasing GLO values with increasing salinity. Similarly, in the rainbow trout, *Oncorhynchus mykiss*, the GLO levels were decreased with an increase in salinity (Banaee, Sureda, Mirvaghefi, & Ahmadi, 2011).

Blood AST and ALT levels are indicators of liver function in the liver and spleen cells as aminotransferase; their activation is elevated upon exposure to stress from water salinity changes, which indicates damage to liver cells (Chien, Pan, & Hunter, 2003). Similar results were observed in this study, in which the levels of both AST and ALT increased in response to increasing salinity (25 and 30 psu). Prolonged exposure to high water salinity resulted in cell damage, which intensifies as a result of exposure to high salinity. AST and ALT activities are related with liver malfunction, stress, and harmful environmental exposure and are characterized by a wide variation among fish species (Di Marco *et al.,* 2011). ALT is the optimum serum indicator for the diagnosis of liver damage (Ahmdifar, Akrami, Ghelichi, & Zarejabad, 2011). ALT activity is high in stressed fish (Molina *et al.,* 2005). This finding is in agreement with our results, where the fish were apparently in a stressful condition at high levels of salinity (25 and 30 psu) because their ALT level was significantly high.

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