Iranian Journal of Fisheries Sciences 17(4) 675-689 2018
DOI:10.22092/ijfs.2018.116814

Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*

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Received: October 2015 Accepted: December 2016

Abstract

This study aimed to elucidate the influence of environment pH on survival, growth, energy allocation and oxidative damage of juvenile *Scophthalmus maximus* (19.89±0.25 g). Six pH treatments (6.3±0.2, 6.8±0.2, 7.3±0.2, 7.8±0.2, 8.3±0.2, 8.8±0.2) lasting for eight weeks were included. Measurements of survival (SR), feed conversion ratio (FCR), specific growth rate (SGR), weight gain rate (WGR), energy allocation, liver superoxide dismutase activity (SOD), catalase activity (CAT), and malondialdehyde concentration (MDA) were done. Results show that SR did not vary from pH 6.3 to 7.8, but reduced then significantly (p<0.05); FCR raised remarkably (p<0.05) while SGR and WGR decreased pronouncedly (p<0.05). Most of the food energy was used in metabolism, followed by growth, feces loss and nitrogenous excretion. Energy deposited for growth showed a decreasing tendency when pH raised; while for metabolism showed a reverse trend. SOD showed insignificant difference from pH 6.3 to 7.8, but the activities then elevated obviously (p<0.05) though a slight decrease was found from pH 8.3 to 8.8 (p>0.05). CAT kept stable between pH 6.3 and 7.3, then sharply increased (p<0.05), but no significant differences were found between pH 8.3 and 8.8 (p>0.05). Subsequent decrease in MDA was found from pH 6.3 to 8.3 then the concentration smoothly increased. Overall, our results indicate that a pH in the range of 6.8 to 7.8 is recommended in the growth environment in cultivation of juvenile turbot.

Keywords: Juvenile turbot (*Scophthalmus maximus*), Water pH, Survival, Growth, Energy allocation, Oxidative damage

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[DOR: 20.1001.1.15622916.2018.17.4.2.6]

Introduction

Water pH is an important factor affecting the metabolism of fish. Fish grow slowly when exposed to very acidic or alkaline pHs (Townsend and Baldisserotto, 2001; Parra and Baldisserotto, 2007). Previous studies indicate that reduced sea water pH can affect developmental (Havenhand *et al*., 2008; Kurihara, 2008; Ellis *et al*., 2009), metabolic (Munday *et al.*, 2009), and behavioral processes (Munday *et al*., 2009) of some marine fish species. Likewise, an increase in pH may disturb acid-base balance, ammonia excretion and ion loss over the gills (Wood, 2001; Perry and Gilmour, 2006; Kwong and Perry, 2013; Ghanbari *et al*., 2014). However, most of the studies are aimed at effects of very acidic or alkaline pH on fish, few studies exposing species to slightly acidic or alkaline waters (Baldisserotto, 2011). In general, fishes appear to be relatively tolerant to mild increases or decreases in pH (Dockray *et al*., 1998; Ishimatsu *et al*., 2008), as prolactin and cortisol play key roles when fish are exposed to acidic conditions (Kwong *et al*., 2014). Some species even grow better in slightly acidic water (Alabaster and Lloyd, 1982; Morgan *et al*., 2001; Munday *et al*., 2009).

Analyses of energy metabolism provide a useful integrative view of stress effects on physiological performance of a variety of marine species (Lannig *et al.*, 2010). Ocean acidification affected energy metabolism in shellfish and the deviation of environmental factors from the evolutionary optimum for a given species might lead to deleterious impacts on energy homeostasis (Lannig *et al*., 2010).

Oxidative stress is defined as an unbalanced state between pro-oxidants and antioxidants, resulting in elevated production of reactive oxygen species (ROS) and free radicals (Metcalfe and Alonso- Alvarez, 2010), as well as an increase in cell membrane lipid peroxidation (malondialdehyde–MDA) (Bar-Or *et al*., 2015). In fact, superoxide dismutase, catalase, and malondialdehyde are always employed as oxidative stress markers to assess redox status (Chan *et al*., 2015). The SOD-CAT system represents the first line of defense against oxidative stress (Kádár *et al*., 2006), and MDA content is often regarded as an end-product of lipid peroxidation (Oruc *et al*., 2004). As to fresh water fish species, the SOD activity of liver will increase when fish are exposed to low pH conditions (Ma *et al*., 2001). As to marine shellfish, ocean acidification may impact the physiological condition and functionality of the hemocytes (Bibby *et al.*, 2008). However, whether environmental pH influences marine fish through oxidative stress and/or antioxidant responses has not yet been clarified.

In recirculating aquaculture systems (RAS), pH tends to decline as bacteria produce acids and fish respiration generates carbon dioxide (Losordo *et al*., 1998). Turbot, *Scophthalmus maximus*, is an important flatfish with ecological and economic importance being cultured in RAS. Several studies have been done on the effects of

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stocking density, dissolved oxygen, salinity and temperature on the growth performance of *S. maximus* (Irwin *et al*., 1999; Imsland *et al*., 2001; Pichavant *et al.*, 2001). The influence of water pH on biochemical composition and gill microstructure of turbot has been illustrated as well (Wang *et al*., 2013a, b). However, no work has yet examined the influence of slightly acidic or alkaline pH on survival, growth, energy allocation or oxidative stress of *S. maximus*.

The aim of this study is to 1) understand how the growth performance and energy budget parameters of juvenile *S. maximus* are affected by water pH; 2) quantify the physiological changes of oxidative stress parameters at various environmental pH; 3) determine the optimum range of seawater pH for aquaculture of juvenile turbot in recirculation aquaculture systems.

Materials and methods

Animals and acclimation

Juvenile turbot were obtained from Tianzheng Co. Ltd. (Dalian, China). The fish were acclimated at water pH 8.1 for 10 days at the Key Laboratory of Mariculture and Stock Enhancement in North China's Sea, Ministry of Agriculture, P. R. China. During acclimation, the type and concentration of food was identical to that used in the experiments. Feeding was discontinued 24 h before the experiment to reduce stress during fish translocation.

Experimental design and set-up

Turbot juveniles were blotted dry with filter-paper to remove excess moisture and weighed individually to the nearest 0.01 g using an electronic balance, then the juveniles with similar weight (19.89±0.25 g) were randomly distributed in 18 continuously aerated 200 L polyethylene tanks (25 fish per tank). Five juveniles from each tank were sampled (15 individuals per pH treatment) and dried in an oven at 65 °C to constant weight, then stored at −20 °C for estimating the initial energy content. The remaining 20 fish per tank were used as the final stocking density per tank. The six levels of pH treatment were 6.3±0.2, 6.8±0.2, 7.3±0.2, 7.8±0.2, 8.3±0.2 and 8.8±0.2, respectively. Water pH was measured with a Hanna Instruments 8314 pH meter (USA). A titration system with a valve was used to keep pH stable day and night. The titration system was operated by gravity flow and the acid or alkaline solution bottle was filled with 500 mL 0.5 mol L^{-1} H_2SO_4 or 1 mol L^{-1} NaOH. The drip rate of the solution was approximately 1.5 mL min^{-1} in order to maintain the pH levels in the various tanks. A valve was used to minimize changes in the drip rate due to decreasing head pressure as the solution level dropped. During acclimation and the experiment, the dissolved oxygen in seawater (salinity 31 ‰) was maintained above 6.0 mg L^{-1}, temperature at 18.0±0.5 °C, un-ionized ammonia level was less than 0.06 mg L^{-1}, total water hardness of 6000.4±6.2 $CaCO_3$ mg L^{-1} and total alkalinity of 100.1±3.9 $CaCO_3$ mg L^{-1}. Photoperiod was 12 h light–12 h darkness，and fish were fed twice daily (8:00 and 16:00) with a formulated fish

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diet (54.98 % crude protein, 19.16 % crude lipid, 14.40 % ash and 6.41 % moisture; energy content: 20.87 KJ g^{-1} dry matter) at 3 % of body weight per day. The food was weighed to the nearest 0.01 g using the electronic balance. Each pH level was assigned to the tanks in triplicate. During the course of the experiment, the feces and uneaten feed were collected using a siphon tube about an hour after feeding stopped. Therefore, nearly 20 % of the water was siphoned and was daily replaced with new water of similar pH. The salt of the uneaten feed and feces absorbed from the seawater was rinsed away with distilled water, then the uneaten feed and feces were separated, dried at 50 °C for 48 h until completely drying, then weighed to the nearest 0.01 g using the electronic balance (Ackerman *et al*., 2015). Hence, the daily food consumption as well as the total food consumed during the experiment could be precisely calculated.

Sample collection for analysis

This experiment was conducted for eight weeks. At the end of the experiment, fish experienced a 24 h food deprivation and then they were anesthetized with 200 mg L^{-1} MS-222 (Boylan *et al*., 2015). After being anesthetized, five individual fish in each tank were weighed and used to calculate the feed conversion ratio, specific growth rate and weight gain rate. Five juveniles per tank were randomly captured for estimating the final energy content. After being rinsed in distilled water, these 5 fish were dried at 50 °C for 48 h until completely drying, then weighed to the nearest 0.01 g using the electronic balance (Ackerman *et al*., 2015). We captured another five individuals randomly for extraction of the livers to calculate the oxidative stress. The livers were rinsed in distilled water before homogenization. All procedures were conducted at 0 °C, unless otherwise stated (Ma *et al*., 2015).

Calculation for survival ratio and growth parameters

The survival ratio (SR), feed conversion ratio (FCR), specific growth rate (SGR), and weight gain rate (WGR) were calculated as follows according to Yu *et al*. (2016):

$SR= [(F_2-F_1)/T] \times 100\%$

$FCR= W/(W_2-W_1)$

$SGR= [(\ln W_2-\ln W_1)/T] \times 100\%$

$WGR= [(W_2-W_1)/ W_1] \times 100\%$

Where F_1 and F_2 stands for the fish individuals per tank at the beginning and at time T, respectively; W_1 and W_2 are the fresh weight (blotted with paper towels) of one turbot at the beginning and at time T, respectively; T means the duration of the experiment, i.e., 56 days; W stands for the total food (dry weight) consumed in 56 days.

The estimation of energy budget

The energy contents of turbot bodies, feed and feces were measured by an HDC 6000 oxygen bomb calorimeter (Hunan, China). The energy budget was calculated as C=F+U+G+R (Warren and Davis, 1967; Cui *et al*., 1996). Where, C (KJ $fish^{-1}$) represents the energy value of food consumed per fish,

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calculated by total feed intake (g fish^{-1}) multiplies energy content in the diet (KJ g^{-1}) (Santos *et al*., 2013); F, energy lost in feces; U, energy lost through nitrogenous excretion; G, energy retained as growth; R represents total metabolism. The value of R was calculated as in the following equation: R= C-G-F-U. The estimation of U was based on the nitrogen budget equation: $C_N= F_N+G_N+U_N$; $U=(C_N-F_N-G_N) \times 24.8$, where C_N is the nitrogen consumed from food; F_N, the nitrogen expelled by feces; G_N, the nitrogen deposited in growth, U_N, the nitrogen deposited in excretion (Cui *et al.*, 1991); 24.8, the energy constant in excreted nitrogen per gram (KJ g $^{-1}$) (Cui *et al.*, 1992). The nitrogen contents were determined by the Kjeldahl method (Bremner and Mulvaney, 1982).

Hepatic oxidative stress parameters

Frozen livers were homogenized individually (10 % w/v) in ice-cold extraction buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) with several strokes using a Teflon pestle (099CK4424, Glas-Col, TerreHaute, USA). The homogenate was centrifuged at 12,000 *g* for 20min under refrigeration and obtained supernatants were stored at −80 °C for the oxidative stress parameters assay. The activities of antioxidant enzymes and lipid-peroxidation level were calculated with the commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Superoxide dismutase (SOD) activity was spectrophotochemically measured by the ferricytochrome *c* method at 505 nm and 37 ℃ using xanthine/xanthine oxidase as the source of superoxide radicals (Zuo *et al.*, 2013). Catalase (CAT) activity was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm and 37 ℃ according to Aebi (1984). Lipid-peroxidation levels were determined by quantifying the concentration of thiobarbituric-acid-reacting substances (TBARS), expressed as the malondialdehyde (MDA) concentration, nmol mg tissue^{-1} (Suárez *et al.*, 2015).

Statistical analysis

All treatments were assigned using a completely randomized design. Statistical analyses were performed using Excel and SPSS 16.0 statistical software. Data are presented as mean ± standard deviation. Statistical analyses were performed by one-way ANOVA, and Duncan's multiple range tests were applied to identify the differences between conditions when significant differences were indicated at the 0.05 level.

Results

Survival rate

With increasing environmental pH, the survival rate had a decreased tendency. SR had no significant differences when water pH ranged from 6.3 to 7.8, but the ratio reduced significantly from pH 8.3 to 8.8 (p<0.05) (Fig. 1).

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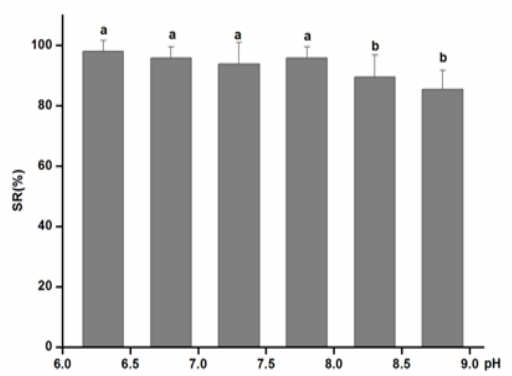


Fig. 1. Effects of water pH on survival rate (SR %) of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p<0.05).

Growth indices

With environmental pH rising, FCR elevated. The significantly high value was observed at pH 8.8 (p<0.05), followed by pH 8.3; and the lowest value was observed at pH 6.3 (p< 0.05), followed by pH 6.8 (Fig. 2).

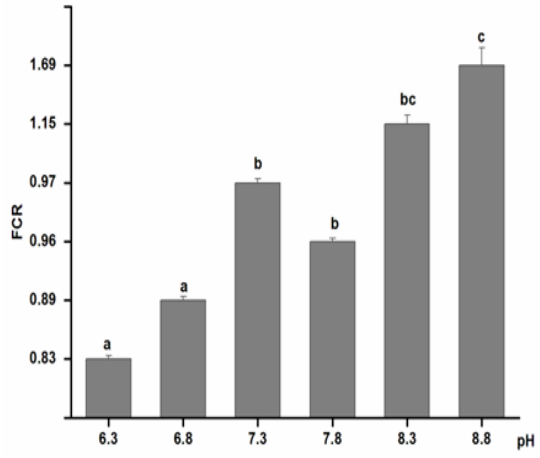


Figure 2: Effects of water pH on feed conversion ratio (FCR) of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p<0.05).

SGR decreased as water pH increasing. The highest value was observed at pH 6.3 (p<0.05), and the lowest value was observed at pH 8.8 (p<0.05), no obvious differences were observed from pH 6.8 to pH 7.8 (p>0.05) (Fig. 3).

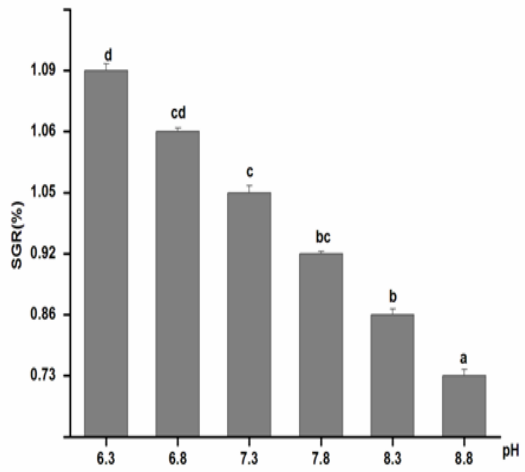


Figure 3: Effects of water pH on specific growth rate (SGR %) of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p< 0.05).

With pH increasing, WGR depressed continuously. The pronouncedly high value was observed at pH 6.3 (p< 0.05), followed by pH 6.8 and 7.3, and the lowest value was observed at pH 8.8 (p<0.05) (Fig. 4).

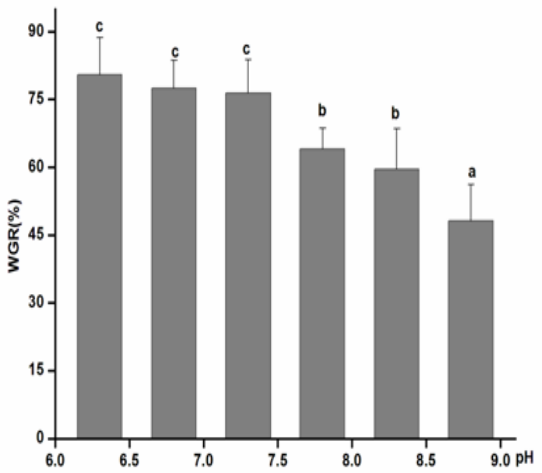


Figure 4: Effects of water pH on weight gain rate (WGR %) of juvenile *Scophthalmus maximus*. Values are mean (±SD). Data with different letters indicate significant differences (p<0.05).

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Energy budget

The highest energy consumed from food was obtained at pH 7.3 (5.91±0.21 KJ fish^{-1}), and the lowest was obtained at pH 8.8 (4.44±0.16 KJ fish^{-1}). With the increase of water pH, the energy deposited for feces and excretion showed variation. The energy deposited for growth decreased, while for metabolism increased. R accounted for the largest proportion of consumed energy at all experimental water pH levels, followed by G, F and U. With respect to R, turbot at pH 8.3 deposited more energy for metabolism (73.85±0.77 %) than in the other pH groups. Regarding G, the juveniles at pH 6.3 spent more energy on growth (32.01±4.36 %) than at the other pH treatments. We also adopted a formula proposed by Cui and Liu (1990), i.e., 100A= 60R+40G. Where, A is the energy assimilated by the fish, and calculated as A= C-F-U. Based on this formula, the energy deposited for growth ranged from 21.51±2.72 % to 37.48±5.11 %, and had a decreasing trend with the rise of pH. The energy used for metabolism showed a reverse tendency, and the proportion varied from 62.52±5.72 % to 76.69±0.64 % (Table 1).

Table 1: Energy budgets [1] of juvenile *Scophthalmus maximus* at different pH levels.

pH	6.3	6.8	7.3	7.8	8.3	8.8
C KJ fish^{-1}	4.92±0.09^{b}	5.37±0.05^{c}	5.91±0.21^{d}	4.73±0.01^{b}	5.17±0.11^{c}	4.44±0.16^{a}
As a percentage of C (%)						
F	2.72±0.06^{b}	2.97±0.15^{c}	2.05±0.21^{a}	2.59±0.05^{b}	1.83±0.03^{a}	2.99±0.15^{c}
U	0.18±0.01^{a}	1.52±0.26^{b}	2.68±0.28^{c}	1.84±0.13^{b}	4.09±0.10^{d}	1.71±0.28^{b}
G	32.01±4.36^{d}	26.94±2.13^{bc}	28.11±2.56^{c}	26.08±1.11^{b}	20.23±2.56^{a}	22.21±2.93^{a}
R	65.09±5.95^{a}	68.57±1.17^{a}	67.16±0.96^{a}	69.48±1.05^{ab}	73.85±0.77^{b}	73.09±0.68^{b}
As a percentage of A (%)						
R	62.52±5.72^{a}	71.79±1.12^{b}	70.49±0.92^{b}	72.71±0.09^{b}	78.49±0.73^{c}	76.69±0.64^{bc}
G	37.48±5.11^{d}	28.21±2.23^{bc}	29.51±2.69^{c}	27.29±1.16^{b}	21.51±2.72^{a}	23.31±3.08^{a}

[1] Energy budgets is calculated as C=F+U+G+R (Warren and Davis, 1967; Cui *et al*., 1996), or A=C-F-U (Cui and Liu., 1990), where C (KJ fish^{-1}) represents the energy value of food consumed per fish, calculated as total feed intake (g fish^{-1}) multiplies energy content in the diet (KJ g^{-1}) (Santos *et al.*, 2013); F, energy lost in feces; U, energy lost through nitrogenous excretion; G, energy retained as growth; R, energy for metabolism; A, assimilated energy. Values are Mean (±SD). Data with different letters indicate significant differences (p<0.05).

Hepatic oxidative stress parameters

The activities of SOD in liver had no pronounced differences when environmental pH ranged from 6.3 to 7.8 (p>0.05), but the activities then elevated remarkably (p<0.05) though a slight decrease was found from pH 8.3 to 8.8 (p>0.05). The highest SOD activity was obtained at pH 8.3 (p<0.05) and the lowest value was obtained at pH 6.8 (p<0.05) (Fig. 5).

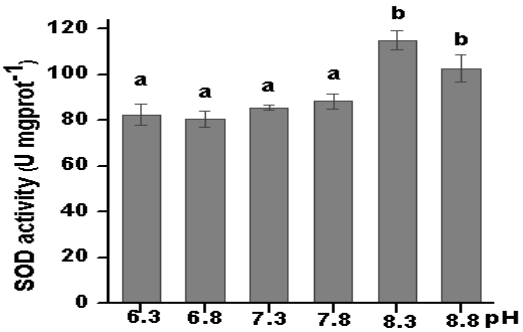


Figure 5: Effects of water pH on activity of superoxide dismutase (SOD) in the liver of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p<0.05).

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Liver CAT kept stable between pH 6.3 and 7.3, then the activity sharply increased (p<0.05), followed by slight reduction from pH 8.3 to 8.8 (p>0.05) (Fig. 6).

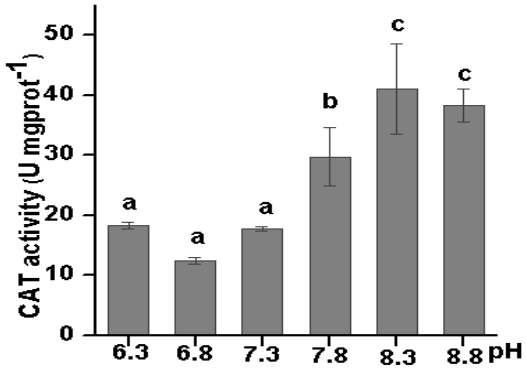


Figure 6: Effects of water pH on activity of catalase (CAT) in the liver of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p<0.05).

No significant differences were observed in MDA concentrations neither from pH 6.3 to pH 7.8 (p>0.05), nor between pH 8.3 and pH 8.8 (p>0.05), but the concentrations between group pH 6.3 and pH 7.8 are higher than pH 8.3 and pH 8.8 groups (Fig. 7).

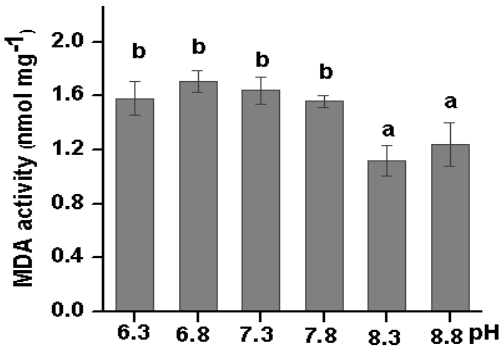


Figure 7: Effects of water pH on malondialdehyde concentration (MDA) in the liver of juvenile *Scophthalmus maximus*. Values are mean (± SD). Data with different letters indicate significant differences (p<0.05).

Discussion

The influence of water acidification on fish growth has been studied (Wolfe *et al*., 2013; Heuer and Grosell, 2014), and it is likely that the influence of pH on fish growth varies according to fish species, life stage, and water quality. Several studies showed higher fish growth rates at lower pH values (Sochasky, 1981; Dockray *et al.*, 1998). However, much work was focused on the influence of very acidic or alkaline pH on fish growth, few studies researched the effect of slight increase or decrease of water pH on teleosts (Baldisserotto, 2011), especially for marine fish, because of the technical difficulty of maintaining seawater pH stable at different levels (Thompson and Bonnar, 1931; Clarkson *et al*., 2015).

Our results revealed that the survival of juvenile turbot had no significant differences when water pH ranged from 6.3 to 7.8, but the survival reduced from 7.8 to 8.8 significantly. The data suggest alkaline environment has negative effects to juvenile turbot which lead to obvious decrease in growth. With increasing environmental pH, the feed conversion ratio increased, while the specific growth rate and the weight gain rate decreased. This result is due to the fact that fish has physiological adaptations to cope with lower environmental pHs (Altshuller and Linthurst, 1983). When exposed to acidic environment, some physiological responses, such as H^+-ATPase for acid secretion, prolactin and cortisol will increase to promote Na^+ intake, thus contributing to ionic and acid–base

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homeostasis (Kwong *et al.*, 2014). Also, slightly acidic environment may stimulate appetites and dietary intake by the fish and results in better growth (Morgan *et al.*, 2001; Munday *et al*., 2009). Many fishes are ammoniotelic (IP and Chew, 2010), a large proportion of the ammonia produced in fish originates from the catabolism of amino acids (French *et al*., 1981). Previous research has demonstrated that a large proportion of excreted ammonia remains as un-ionized form (NH_3) in high pH conditions, and NH_3 has the ability to enter into the plasma and tissues, generating a negative influence on fish health (Wilkie and Wood, 1996). Once un-ionized ammonia levels increase in surrounding environment, the diffusion of NH_3 from the plasma to water reduces and plasma NH_3 increases, resulting in reducing growth and productivity of fish (Tomasso *et al*., 1980; Frances *et al*., 2000). Acidic water, on contrary, has the ability to change NH_3 into the ionized form (NH_4^+), and NH_4^+ is considered less toxic (Wood, 2001; Miron *et al*., 2008). Therefore, marine fish exposed in acidic water may have a better growth than in high alkaline environment.

The energy, lost through nitrogenous excretion, occupied 0.18 %-4.09 % of the consumed food in the present research. Similar result has been obtained by Jobling (1994) that energy lost through nitrogenous excretion of teleosts comprised 1.2 % to 12 %. Our study revealed that the energy deposited for growth and metabolism occupied higher scales of the energy intake than the energy lost in feces and through nitrogenous excretion. Moreover, the energy allocated for growth is less than the energy spent for metabolism. To accommodate the complicated circumstances of ocean, marine fish need to spend more energy in respiration than in growth (Cui and Liu., 1990). Previous studies have shown that sub-optimal conditions create metabolic energy demands might exceed energy supplied from food and/or accrue in somatic energy resources (Guppy and Withers, 1999). Therefore, high proportions of energy deposited for metabolism in high alkaline environment indicated that turbot juveniles were experiencing deleterious conditions (Wang *et al*., 2013a, b).

Superoxide dismutase and catalase in liver have been used as oxidative damage biomarkers (Datta *et al*., 2013). Higher contents of SOD and CAT in liver found at higher pH levels in our study indicated that turbot in high pH environment were suffered with an increase of $O_2^{\cdot -}$ production (Zhang *et al*., 2004). SOD catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, CAT subsequently converts H_2O_2 to H_2O, both SOD and CAT protect cells from the toxicity of H_2O_2 (He *et al*., 2014). In order to maintain the dynamic balance between pro-oxidants and antioxidants, as well as to reduce the negative effects of ROS, a large number of antioxidant molecules and enzymes are produced (Martinez-Alvarez *et al*., 2002). Consequently, the higher level of SOD and CAT in alkaline environment became a possible reason for the depressed MDA concentration found in our research.

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This study shows that water pH plays a key role on survival, growth performance, energy budget, and oxidative stress of turbot. When environmental pH ranges from 6.8 to 7.8, turbot gain more weight, grow faster, and allocate more energy into growth. Therefore, we recommend a pH range from 6.8 to 7.8 to optimize the growth of turbot. This conclusion has significant implications for a cost-efficient optimization of recirculating aquaculture systems for maximizing the commercial productivity of juvenile *Scophthalmus maximus*.

Acknowledgments

The authors wish to thank Prof. Jianguang Qin and Prof. Bernardo Baldisserotto for helpful discussions. This work was supported by the Marine public welfare projects of China (Contract: 2012418025).

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