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Effect of seawater pH on selected blood biochemical parameters of juvenile turbot *Scophthalmus maximus* (Linnaeus, 1758)

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ABSTRACT

The effect of seawater pH on blood physiology in juvenile turbot *Scophthalmus maximus* (Linnaeus, 1758) reared in recirculating aquaculture systems (RAS) was examined. *S. maximus* (19.89±0.25 g) were exposed to six pH levels at 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. Haemoglobin content (HBC) at acidic conditions was higher compared to that under alkaline conditions. The frequency of total nuclear anomalies (FTNA) was high at pH levels below 7.3 or above 8.3. The relationship between FTNA and pH was well described using a quadratic equation. Alkaline phosphatase activity was significantly declined at pH above 8.3, while total superoxide dismutase activity (T-SOD) remained virtually unchanged among all treatments. This study demonstrated that HBC and FTNA can be used as reliable biomarkers to evaluate the effect of pH in aquatic ectotherms from physiological standpoints. Our findings suggest that environmental pH in RAS ranging from 6.8 to 7.8 is appropriate for juvenile turbot aquaculture.

Keywords: Biomarkers, Blood physiology, pH, Recirculating aquaculture systems, *Scophthalmus maximus*

Introduction

Water pH is a vital parameter for survival and optimum growth of aquatic ectotherms. Decrease in pH can impact fishes in numerous ways by affecting aerobic activity (Munday *et al*., 2009), individual growth (Shuangyao *et al*., 2018) and overall survivability (Munday *et al*., 2009). Similarly, increased pH can also influence ectotherms, disturbing the acid-base balance of the organisms (Miles *et al*., 2007). Despite knowing the potential impacts of shifting pH in ectotherms, a few studies have been conducted on the pH threshold of species (Baldisserotto, 2011; Shuangyao *et al*., 2018). In general, fish are relatively tolerant to mild increases or decreases of water pH, where cortisol and prolactin have been shown to be critical in maintaining the ionic and acid-base balance (Kwong *et al*., 2014). Interestingly, some freshwater fish for instance, *Paratrygon aiereba* may grow faster in slightly acidic water (Alabaster and Lloyd, 2013).

Fish blood biochemical parameters such as haemoglobin content (HBC) (Mousavi and Yousefian, 2012), micronuclei frequency (Sanchez-Galan *et al*., 1998; Valskiene *et al*., 2018), alkaline phosphatase (AKP) (Sheikhzadeh *et al*., 2017) and total superoxide dismutase (T-SOD) (Rahimnejad *et al*., 2017) have been broadly used to interpret the effect of chemical intoxication, environmental factors and diets on immunity of marine fish. Fish haemoglobin (Hb) functions in fish respiratory metabolism (Pan *et al*., 2017) and can be categorised into Class I (Borza *et al*., 2009), II (Weber and de Wilde, 1976) and III Hbs (Ikeda-Saito *et al*., 1983). Among the three categories, Class I and III Hbs in marine fish are sensitive to environmental changes such as pH (Ikeda-Saito *et al*., 1983; Borza *et al*., 2009). It has been demonstrated that marine fish may adapt to environmental pressures such as hypoxia and long-term temperature changes by altering the Hb isoforms (Borza *et al*., 2009). Total nuclear anomalies (FTNA%) can be used to evaluate integrated response to the complex mixture of aquatic pollutants, which may result in genomic alterations in fish (Castro *et al*., 2018). It has been suggested that the micronuclei frequency in fish can be applied as a biomarker tool for environmental threat (Sanchez-Galan *et al*., 1998). Nuclear anomalies in marine fish are elucidated, based on micronuclei and other nuclear abnormalities, such as nuclear buds, bi-nucleated and fragmented-apoptotic cells (Barsiene *et al*., 2006). Micronuclei in marine fish are produced from chromosomes and may generate delayed cell division due to lack of centromere, damage in centromere or defect in cytokinesis (Heddle *et al*., 1991). Serum antioxidant enzymes such as AKP and T-SOD have been demonstrated as the first line of the immune

system against oxidative stress (Sheikhzadeh *et al*., 2017). Besides, alkaline phosphatase as a polyfunctional enzyme has physiologic functions in the immune system and cellular oxygen-carrying system by catalysing the transfer of phosphate groups (Sheikhzadeh *et al*., 2017). Superoxide dismutase catalyses the breakdown of superoxide anion and the reactive oxygen species (ROS) into molecular oxygen and less reactive hydrogen peroxide species to defend against oxidative cell damage (Rahimnejad *et al*., 2017). Previous research on marine shellfish suggested that acidification impacts physiological condition and function of haemocytes in *Mytilus edulis*, however, acidification has no influence on measured immuno-surveillance parameters such as the superoxide anion level (Bibby *et al*., 2008). To date, whether seawater pH influences marine fish physiology through oxidative stress and/or antioxidant responses is still not known.

In recirculating aquaculture systems (RAS), environmental pH tends to decline as bacteria produce acids and fish respiration generates carbon dioxide (Losordo *et al.,* 1998). Turbot, *Scophthalmus maximus* (Linnaeus, 1758), is one of the major cultured fish in RAS in Europe (do Prado *et al*., 2018) and China (Shuangyao *et al*., 2018). The effect of pH on growth performance and histology of turbot juveniles has been reported (Wang *et al*., 2013a, b; Shuangyao *et al*., 2018). However, effects of pH on blood biochemical parameters in turbots have not yet been studied. The present work aimed to generate baseline information on selected haematological parameters in juvenile *S. maximus* exposed to different pH levels and to determine the optimum pH for aquaculture of turbot in RAS based on haematology.

Materials and methods

Experimental fish

Juvenile *S. maximus* were obtained from Tianzheng Co. Ltd. (Dalian, China) (Shuangyao *et al*., 2018). Juveniles were blotted dry with filter-paper to remove excess moisture and weighed individually to the nearest 0.01 g using an electronic weighing scale (Shuangyao *et al*., 2018). Fish with similar weight (19.89±0.25 g) were randomly distributed into 18 continuously aerated 200 l polyethylene tanks (25 turbots per tank) at six different pH treatments *viz.,* 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. Each pH treatment was assigned to tanks in triplicate. Turbots were first acclimated at respective pH levels for 10 days at the Key Laboratory of Mariculture and Stock Enhancement in North China's Sea, Ministry of Agriculture, P. R. China, with a photoperiod of 12 h light - 12 h darkness. Seawater pH was measured with a Hanna Instruments 8314 pH meter (USA). Turbots were fed twice daily (8:00 and 16:00 hrs) at 3% ration level with a formulated fish diet bought from Tianzheng Co. Ltd. (54.98% crude protein, 19.16% total lipid, 14.40% ash and 6.41% moisture and energy content: 20.87 kJ g^{-1} dry matter) (Shuangyao *et al*., 2018).

Experimental system

The experimental system used in the present study has been described in Shuangyao *et al*. (2018). Briefly, a titration system with a valve was designed to keep pH stable. The titration system was operated by gravity flow and the acid or alkaline solution bottle was filled with 500 ml 0.5 M H_2SO_4 or 500 ml 1 M NaOH. The drop rate of the solution was approximately 1.5 ml min^{-1} in order to maintain stable pH in different treatments. A valve was used to minimise 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 ppt) was maintained at 6.0±0.5 mg l^{-1}, temperature at 18.0±0.5°C, un-ionised ammonia level at >0.06 mg l^{-1}, total seawater hardness of 6000.4±6.2 $CaCO_3$ mg l^{-1} and total alkalinity of 100.1±3.9 $CaCO_3$ mg l^{-1} (Shuangyao *et al*., 2018).

Parameters measurement

The experimental duration was 56 days. During the experimental period, faeces and uneaten feed were siphoned out of the tank about 1 h after feeding. As a result, nearly 20% of the seawater was siphoned out from the tank and was replaced daily with new seawater at same pH levels. At the end of the experiment, turbots were subjected to 24 h starvation, followed by anesthetisation with 200 mg l^{-1} MS-222. Five individuals from each tank (3 x 5 individuals per treatment) were randomly captured for blood collection. Blood was sampled from caudal vein using 1 ml heparinised disposable syringes. Blood samples from the same treatment with a total volume of 1 ml were immediately transferred into a 1.5 ml Eppendorf tube and centrifuged at 3500 rpm, at 4°C for 10 min. The supernatant fluid was thereafter collected as the serum and stored at -80°C for further haematological analyses.

Haemoglobin content (g l^{-1}) was determined by a Spotchem™ EZ SP-4430 system (ARKRAY, Amstelveen, The Netherlands) at 540 nm using the cyanomethaemoglobin method (Blaxhall and Daisley, 1973). Total serum protein content was determined using an autoanalyser (Max Mat Hycel), based on the principle of the biuret reaction. Bovine serum albumin was used as a standard and data were expressed in mg ml^{-1} (Coeurdacier *et al*., 2011). A drop of blood collected from the caudal vein was directly smeared on clean slides and air-dried. Smears were subsequently fixed in methanol for 10 min and stained with 10% Giemsa solution for 8 min. The frequency of total nuclear anomalies (%) was evaluated

at a degree of 500 cells per fish by scoring at 1000 × magnification using an Olympus microscope (BX51) with a digital camera DP70 (Olympus, Tokyo, Japan). Only cells with intact cellular and nuclear membrane were scored. Round or ovoid non-refractory particles with colour and structure similar to chromatin, with a diameter of 1/3 to 1/20 of the main nucleus and clearly detached from the main nucleus were interpreted as micronuclei (Barsiene *et al*., 2006).

Alkaline phosphatase activity (U l^{-1}) was determined by the colourimetric assay using AKP Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). One unit of activity is defined as the amount of AKP required to transform 1 mg of phenol in the reactive substrate at 37°C (Hu *et al*., 2015). Total superoxide dismutase activity (U ml^{-1}) was spectrophotochemically measured by the ferricytochrome *c* method at 505 nm and 37°C using xanthine/xanthine oxidase as the source of superoxide radicals. One unit (U) represents 50% of inhibition by T-SOD of nitric ion production in this condition (Hu *et al*., 2015).

Statistical analysis

All treatments were assigned using a completely randomised design. Statistical analyses were performed using Origin software (OriginPro 2018b, Originlab, Massachusetts, USA). Data were presented as mean±standard deviation (SD). Statistical analyses were performed by one-way ANOVA. Duncan's multiple range tests were applied to identify the differences between pH treatments when significant differences were indicated at $p>0.05$.

Results and discussion

Effects of pH on HBC and FTNA in juvenile turbot

The average HBC in juvenile turbot declined at higher pH treatments (Fig. 1). With the increase of pH, HBC increased significantly ($p<0.05$) from the pH 6.3 treatment to pH 6.8, followed by a significant decrease ($p<0.05$) from pH 6.8 to 7.8 (Fig. 1). Thereafter, the HBC remained stable ($p>0.05$) (Fig. 1). The FTNA peaked at pH 6.3 (11%) and reached the lowest point at pH 7.8 (4.4%) (Fig. 2). The relationship between FTNA and pH was well described using the quadratic equation, $FTNA = 3.7835 \times pH^2 - 58.631 \times pH + 231.519$, $R^2 = 0.969$ (Fig. 2). From this quadratic equation, the lowest FTNA recorded was 4.38% when the pH was 7.75 (Fig. 2).

Detailed knowledge of haematological features is particularly important to assess the optimal condition in RAS (Pan *et al*., 2017). The results of this study revealed that HBC in turbot was higher in acidic conditions in comparison to alkaline conditions. One possible explanation could be that Hb content of juvenile turbots at low pH levels exhibited the Root effect, as a result, Hb at low pH failed to be fully oxygenated. The incomplete oxygenation led to higher concentrations of unbound Hb at low pH levels than that at high pH levels (Pelster, 2001). Another explanation for the higher HBC in acidic conditions could be that alkaline seawater exerted only a slight effect on Hb conformation and on the stability of HBC (Kim *et al*., 2005).

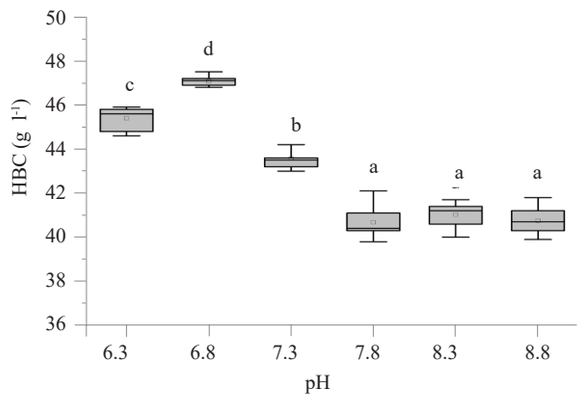

Fig. 1. Effect of pH on haemoglobin content (HBC, g l^{-1}) of juvenile *S. maximus*. All data represent Mean±SD (n=3). Data with different letters indicate significant differences ($p<0.05$).

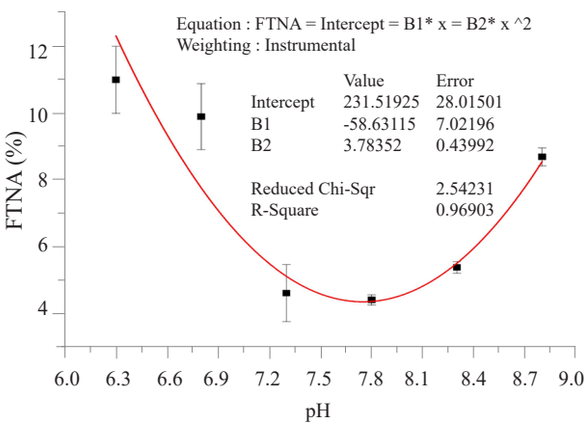

Fig. 2. Effect of pH on the frequency of total nuclear anomalies (FTNA%) of juvenile *S. maximus*. All data represent Mean±SD (n=3). Data with different letters indicate significant differences ($p<0.05$).

The current study, for the first time, demonstrated that FTNA in juvenile turbots was high at a particular range of pH (below 7.3 or above 8.3), indicating that both higher and lower pH levels generated unfavourable impacts on turbots by altering the shape of red blood cells. It has been shown that fish subjected to toxic conditions exhibited

a higher micronuclei frequency (Sanchez-Galan *et al*., 1998; Valskiene *et al*., 2018). In addition, the application of the micronuclei frequency parameter has been shown to be a precise biomarker for fish exposed to environmental threat (Sanchez-Galan *et al*., 1998). Furthermore, the current research showed that turbots exposed to acidic seawater generated more severe nuclear anomalies than those exposed to alkaline pH conditions, indicating that juvenile turbots have a higher tolerance to alkaline conditions compared with acidic conditions.

Effects of pH on AKP and T-SOD in juvenile turbot

There were no differences ($p>0.05$) of AKP activity between pH 6.3 and 7.8 or between pH 8.3 and 8.8 (Fig. 3). However, the AKP activity in treatments of pH 7.3 and 7.8 was significantly higher ($p<0.05$) than the activity in treatments of pH 8.3 and 8.8 (Fig. 3). There were no differences ($p>0.05$) of T-SOD activity among treatments (Fig. 4). The activity of AKP in the serum of turbot in the present work was significantly lower at higher pH levels compared to the lower pH levels, indicating that juvenile turbots under higher pH required more AKP to maintain the regular transfer of phosphate groups (Sheikhzadeh *et al*., 2017). In contrast, the activity of T-SOD in the serum was comparable ($p>0.05$) among treatments, although there was a tendency to increase at higher pH levels. The change in the activity of serum T-SOD in the present work was in agreement with previous research on the change of T-SOD in the liver of juvenile turbots (Shuangyao *et al*., 2018). It has been demonstrated that not all parameters related to immunity in aquatic ectotherms present the same alteration when the water pH changes (Bibby *et al*., 2008). Therefore, the current study suggested that it would not be reliable to examine whether the change of pH will affect the performance of aquatic ectotherms merely based on examining immune parameters.

Based on the results of the present study, we suggest that HBC and FTNA, instead of AKP and T-SOD, are more suitable indicators to determine whether the change of pH will affect the performance of juvenile turbots in RAS. In addition, we concluded that a pH scope of 6.8 to 7.8 is appropriate for juvenile *S. maximus* culture in RAS based on haematological parameters. The range of pH levels selected was in accordance with our previous studies on growth and histology (Wang *et al*., 2013a, b; Shuangyao *et al*., 2018). This study lays foundation for future work on the effect of pH in fish species reared in recirculating aquaculture systems. Moreover, further work may also be conducted to investigate metabolic measurements (oxygen consumption rate) to better understand the influence of pH on aquacultured species from physiological standpoints (Wang *et al.,* 2019a, b).

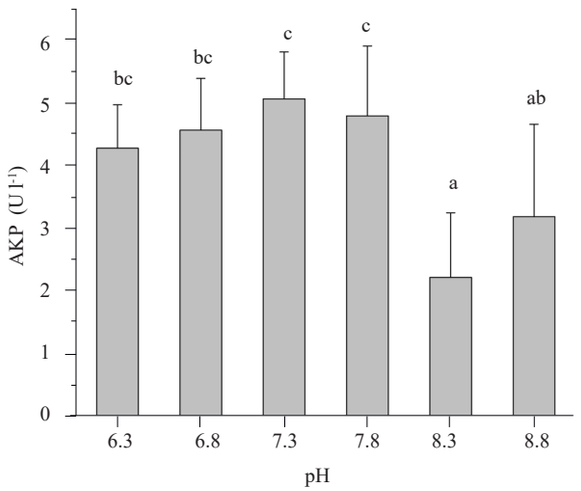

Fig. 3. Effect of pH on alkaline phosphatase activity (AKP, U l^{-1}) of juvenile *S. maximus.* All data represent Mean±SD (n=3). Data with different letters indicate significant differences ($p<0.05$).

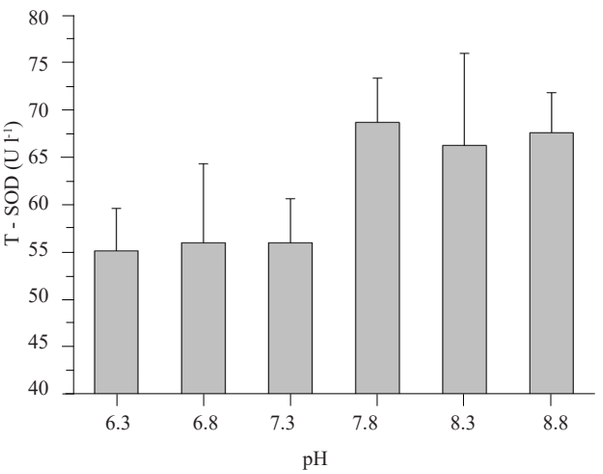

Fig. 4. Effects of pH on total superoxide dismutase activity (T-SOD, U ml^{-1}) of juvenile *S. maximus.* All data represent Mean±SD (n=3) ($p<0.05$)

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