



## Full length article

Effect of flow velocity on the growth, stress and immune responses of turbot (*Scophthalmus maximus*) in recirculating aquaculture systemsXian Li<sup>a,b,c</sup>, Liqin Ji<sup>a</sup>, Lele Wu<sup>a</sup>, Xiaolong Gao<sup>a</sup>, Xueqin Li<sup>a</sup>, Jun Li<sup>a</sup>, Ying Liu<sup>b,d,\*</sup><sup>a</sup> Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China<sup>b</sup> Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266235, China<sup>c</sup> Center for Ocean Mega-Science, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao, 266071, China<sup>d</sup> School of Marine Science and Environmental Engineering, Dalian Ocean University, Dalian, 116023, China

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## ABSTRACT

Land-based recirculating aquaculture systems (RAS) are widely utilized for turbot (*Scophthalmus maximus*) culture. Flow velocity in the tank is essential to maintain water quality, conservation of energy and fish welfare. However, little is known about how turbot respond to different velocities in the long term. In this study, water quality was kept constant, allowing the effect of flow velocity on the feeding intake, growth, plasma biochemical indexes, innate (non-specific) immunity and immune-related stress gene expressions in the skin to be examined in isolation in RAS. Turbot (average body length 20.10 cm) were reared for 60 days in RAS under three velocities, 0.06 m s<sup>-1</sup>, 0.18 m s<sup>-1</sup>, and 0.36 m s<sup>-1</sup>, corresponding to approximately 0.3 body length per second (bl s<sup>-1</sup>), 0.9 bl s<sup>-1</sup> and 1.8 bl s<sup>-1</sup>, respectively. The results showed that at velocities of 0.36 m s<sup>-1</sup> (1.8 bl s<sup>-1</sup>), juvenile turbot were subject to stress accompanied by a reduced growth rate. A velocity of 0.36 m s<sup>-1</sup> was also found to significantly reduce SOD and GSH activity, and the concentration of total protein in plasma, while concentrations of urea nitrogen (BUN) and total bilirubin (TBIL) increased. There was an up-regulation of cathepsin D and lysozyme (LZM) in the skin at the highest velocity, implying the activation of stress and immune responses. At the medium velocity of 0.18 m s<sup>-1</sup> (0.9 bl s<sup>-1</sup>), turbot increased their feed intake, obtained an elevated special growth rate (SGR), and exhibited significantly higher AKP and ACP activity in plasma. Overall, the results suggest that excessively high velocities are a stressor for turbot inducing an immune response in the skin, which is sensitive to environmental changes. A velocity of approximately 0.9 bl s<sup>-1</sup> is suggested to promote growth and obtain better innate immunity of cultured turbot.

## 1. Introduction

Turbot (*Scophthalmus maximus*) is a marine fish of high commercial value. It is the most important cultured flatfish in Europe and Asia. In China, turbot culture represents a large proportion of land-based tank-cultured fish, in particular in recirculating aquaculture systems, and the annual production of turbot accounted for more than 80% of total global aquaculture output over the last decade [1].

Land-based recirculating aquaculture systems (RAS) have grown in importance in the global aquaculture industry, as they consume less water per kilogram of fish produced, ensure stable water conditions and result in lower levels of pollution in the aquatic environment [2]. Several marine flatfish species including Japanese flounder (*Paralichthys olivaceus*), starry flounder (*Platichthys stellatus*), half-smooth tongue sole (*Cynoglossus semilaevis* Günther), and turbot have been successfully cultured in RAS, even at stocking densities up to 20 kg m<sup>-2</sup>

[1–4]. In contrast to swimming fish, the majority of flatfish spend most of their time on the bottom, and as a result are more sensitive to water quality at the bottom of tanks. The flow structure in rearing tanks plays an essential role in removing uneaten feed and feces from the tank, circulating dissolved oxygen, and maintaining water quality [5–7]. A moderate velocity in tanks should benefit water quality, while conserving energy.

In the wild, fish are sensitive to heterogeneous flow velocities, and may use this to their advantage by selecting regions with an optimal flow velocity thus reducing the energy expenditure required for station holding while maximizing energy gain through feeding opportunities [8]. Several studies have been carried out in recent years to determine optimal velocities for maintaining fish health and water quality in aquaculture systems [9–14]. In relation to flatfish, a positive relationship between growth and flow velocity has been reported for juvenile Californian halibut (*Paralichthys californicus*) [11], Japanese flounder

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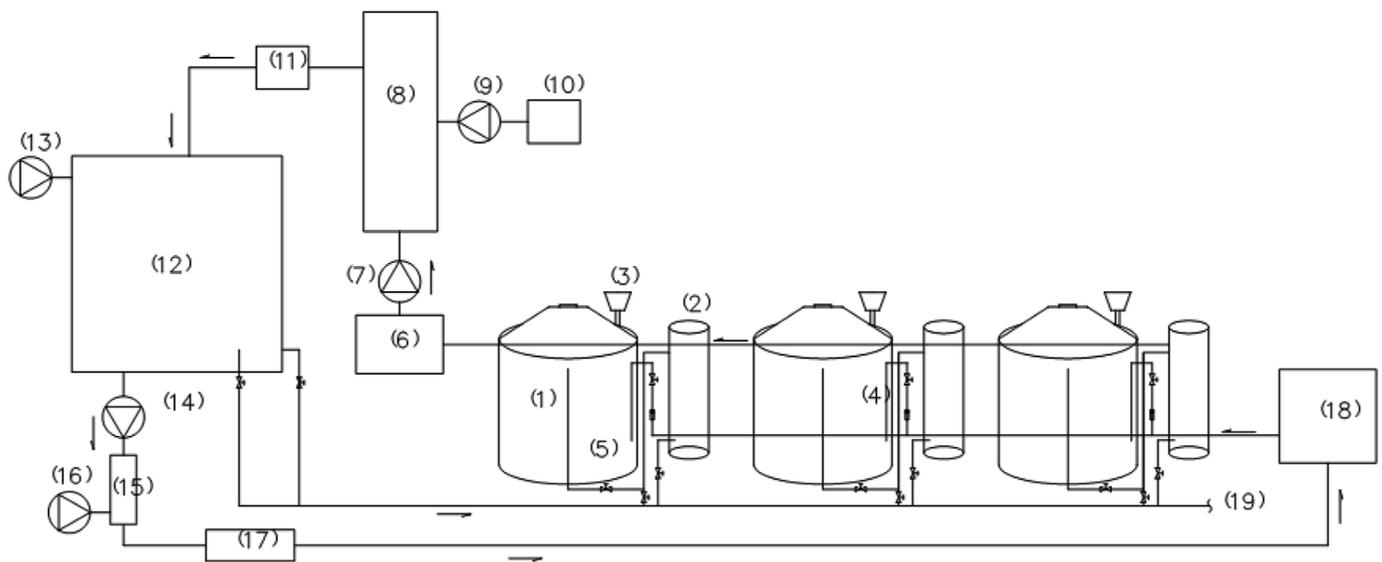


Fig. 1. The structure of the water-loop in the experimental recirculating aquaculture system.

(*Paralichthys olivaceus*) [13], and summer flounder (*Paralichthys dentatus*) [14]. Studies carried out by Schram et al. [15] and Sun et al. [16] have shown that increased flow rate resulted in a higher specific growth rate (SGR) and better welfare of juvenile turbot, explained by differences in water quality, in particular levels of carbon dioxide and unionized ammonia nitrogen. However, little is known about the influence of flow velocities on the performance of juvenile turbot under similar water quality conditions. Furthermore, there have only been limited studies on the effect of water velocity on the growth and physiology of turbot over the long term. Almansa et al. [17] examined the distribution of a turbot population in a tank under different water velocity treatments and found that velocities between 0.33 and 0.46 body length per second ( $\text{bl s}^{-1}$ ) promoted a homogenous turbot distribution.

Once the velocity exceeds the tolerance levels of fish, stress occurs. Fish and other organisms have developed several mechanisms to withstand stress, which include physiological regulations and biochemical and cellular specializations [18]. In their body, there is a continuous balance between production and elimination providing certain steady-state reactive oxygen species (ROS) level, and several stresses would disturb the dynamic equilibrium leading to enhanced ROS level [19]. Antioxidant defenses, including enzyme systems, low-molecular-weight compounds, and some proteins act to remove ROS [20]. Superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) have often been used as biomarkers of the oxidative stress in fish [19,20]. It has been demonstrated that the increase of flow rate significantly increases SOD activity in the serum of turbot, along with the promotion of water quality [16].

Many studies have reported a link between stress and immune system depression [15–17]. The immune system of teleost fish is composed of innate and adaptive components, with the innate immune system representing one of the most important defense mechanisms in fish. The innate immune system can be divided into physical barriers, cellular and humoral components, while humoral parameters include growth inhibitors, several lytic enzymes and components of the complement pathways, agglutinins and precipitins, natural antibodies, cytokines, chemokines and antibacterial peptides [21,22]. The skin of teleost fish and the mucus are the primary defense barriers, which are metabolically active and able to rapidly adapt to a wide variety of stressors [23–26]. Mucins are glycoproteins with a high molecular weight and contain one or more protein domains with sites of extensive O-glycan attachment [27]. They constitute an important part of the mucosal defense system in fish [28]. Lysozyme (LZM) is one of the important defense molecules of the fish innate immune system which is

present in mucus, lymphoid tissue, plasma and other body fluids possess. It displays lytic activity against bacteria and could activate the complement system and phagocytes [29]. Cathepsins are lysosomal endoproteolytic aspartic proteinases, which play an important role in protein degradation in lysosomes and are involved in a wide range of physiological processes in mammals and innate immune responses in fish [30]. It has reported that unsuitable flow velocity is a stressor, which produced structural alterations in the skin epithelium and an increase of skin immune-related gene expressions of Atlantic Salmon, including genes in the cathepsin and mucin family [31].

In the present study, we attempted to quantify the effect of flow velocity enhanced by the pump in the tanks on the performance of turbot including feed intake, growth, oxidative stress and immune responses in isolation in RAS, manipulated with the same hydraulic retention time of the cultured tanks. The results of this research would provide practical suggestions for turbot culture, particularly in RAS.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments with animals were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of the Institute of Oceanology, Chinese Academy of Sciences. The field studies did not involve in any endangered or protected species.

### 2.2. Experimental recirculating aquaculture systems

Two experimental RAS (Fig. 1), each including three 370-L rearing tanks, (diameter 0.80 m, height 0.70 m, and water volume  $0.35 \text{ m}^3$ ) used in the current study located in the institute of oceanology, Chinese academy of sciences (Qingdao, China). The three rearing tanks in each system were randomly assigned to one of three different experimental groups. There was a single water-treatment loop in the system, where water flows from the rearing tank, passes through uneaten feed and feces trappers, flows into a mechanical filter, is pumped into a protein skimmer which is injected with ozone on a daily basis, and then runs through a  $\text{CO}_2$  stripper and biofilter. The water is then subject to aeration, UV disinfection and temperature adjustment, before it returns to the rearing tank. The renewal rates of these two systems were equal and less than 10% per day.

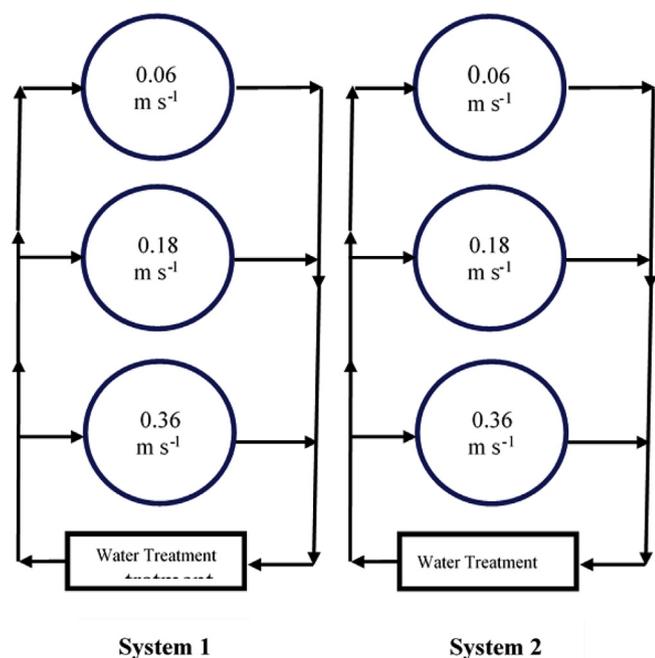


Fig. 2. Distribution of different velocities in two experimental recirculating aquaculture systems.

### 2.3. Fish stock

Juvenile turbot were provided by a commercial turbot farm (Shangdong Oriental Ocean Sci-Tech Co., Ltd., China). Fish ( $n = 200$ ) were randomly divided into the two RAS and allowed to acclimatize to the experimental conditions for two weeks. During the acclimatization period, juvenile turbot were fed 1.5% of their initial body weight per day by auto feeders. Dissolved oxygen (DO) at the outlet of the tanks was maintained at levels above  $7 \text{ mg L}^{-1}$ , water temperature was maintained at  $16.00 \pm 1.00 \text{ }^\circ\text{C}$ , and fish were subject to a natural photoperiod regime.

### 2.4. Experimental design and velocity adjustment

A velocity enhancer (Wuxi Pump Engineering company, Wuxi, China) was fixed closed to the lower end of the inlet pipe, using suckers, in each tank. Holes (diameter 0.50 cm) were drilled every 10 cm along the outlet pipes of the velocity enhancers. The outlet pipe was attached parallel to the inlet pipe of the tank to promote the water flow. The average water velocity in each treatment (Fig. 2) was  $0.06 \text{ m s}^{-1}$ ,  $0.18 \text{ m s}^{-1}$ , and  $0.36 \text{ m s}^{-1}$ , corresponding to approximately  $0.3 \text{ bl s}^{-1}$ ,  $0.9 \text{ bl s}^{-1}$  and  $1.8 \text{ bl s}^{-1}$ , respectively. The water velocity in each tank was recorded using an electronic flow meter (LS1206B, Nanshui Co., Ltd., China) and adjusted every two days. The hydraulic retention time of water in all fish tanks was 2 h.

#### 2.4.1. Experimental fish and feed

At the start of the experiment, all fish were mixed and a total of 150 similar, healthy fish were selected and randomly placed into the six culture tanks. The initial weight of the fish was  $162.00 \pm 8.11 \text{ g}$  (total length  $20.10 \pm 1.12 \text{ cm}$ ) and stocking density was  $8.10 \pm 0.04 \text{ kg m}^{-2}$  separately. During the 60-day experimental period, fish were fed twice daily (8:00 a.m. and 6:00 p.m.), by hand, at the feeding station. Feeding was stopped when several pellets left on the bottom of the tank. Twenty minutes after feeding, any uneaten feed was collected from the uneaten feed and feces trappers and the feed intake was calculated. Feed intake per fish per day was calculated every two days, and expressed as  $\text{g}/(\text{fish}\cdot\text{day})$ , for each treatment.

#### 2.4.2. Water quality monitoring

During the experiment, DO levels at the outlet of the tanks was maintained above  $7 \text{ mg L}^{-1}$ , water temperature was  $18.00 \pm 1.00 \text{ }^\circ\text{C}$ , and water salinity was  $31.00 \pm 0.80\%$ . Given the influence of velocity on water quality, DO, pH, and  $\text{CO}_2$  concentration were measured and recorded daily (YSI-556MPS, Yellow Springs Instruments Inc., Ohio, USA and  $\text{CO}_2$  Analyzer, OxyGuard, Birkerød, Denmark). Water parameters (total ammonia nitrogen: TAN, nitrite:  $\text{NO}_2^- \text{-N}$ , total suspended solids: TSS, and chemical oxygen demand: COD) at the outlet of each tank were measured every three days following the methods provided in sea water quality standards of the state environmental protection administration of China [16].

#### 2.5. Fish sampling and analysis protocol

At the start and end of the experiment, fish were starved for 24 h and the weight of each individual was recorded (under a low level of anesthesia, tricaine mesylate,  $20 \text{ mg L}^{-1}$ ), to determine growth performance. At the final sampling, five fish from each tank were randomly selected for plasma sample collection. Fish were anaesthetized with  $40 \text{ mg L}^{-1}$  tricaine mesylate, and blood was collected from the caudal vein using sterilized syringes with heparin. Once collected, samples were kept on ice. Plasma was obtained from the samples through centrifugation at  $4000g$  at  $4 \text{ }^\circ\text{C}$  for 10 min and samples were stored at  $-80 \text{ }^\circ\text{C}$  until analysis. After blood samples were taken, skin samples for gene expression analyses were collected. The skin was cleaned twice using 75% ethanol before taking a  $1 \text{ cm}^2$  sample from the middle of the body surface above lateral line on the eye side of each fish. Skin samples were frozen directly in liquid nitrogen and transferred to  $-80 \text{ }^\circ\text{C}$  for storage.

##### 2.5.1. Growth parameters

Growth parameters were calculated as follows [16]:

$$\text{Feed conversion ratio (FCR)} = \text{FI}/(\text{B}_f - \text{B}_i);$$

And,

$$\text{SGR (\% per day)} = (\ln W_t - \ln W_i)/t \times 100\%.$$

Where FI is the amount of feed intake,  $\text{B}_f$  is final biomass,  $\text{B}_i$  is initial biomass,  $W_t$  is average weight of turbot at the end of the experimental period,  $W_i$  is the average weight of turbot at the start of the experimental period, and  $t$  represents the day of the experimental period.

##### 2.5.2. Innate immunity and biochemical parameters in plasma

Activities of SOD, CAT, GSH, LZM, AKP and ACP in the plasma were measured using detection kits (Nanjing Jiancheng, Nanjing, China). SOD activity was determined using water soluble tetrazolium salt as a superoxide detector according to the method of Peskin and Winterbourn [32]. CAT activity was assayed by measuring the rate of decrease in  $\text{H}_2\text{O}_2$  absorbance as described by Aebi [33]. The GSH content and the activity of LZM were estimated as described by Jia et al. [34]. The activity of alkaline phosphatase (AKP) and acid phosphatase (ACP) were measured as described previously using detection kits (Nanjing Jiancheng, Nanjing, China) [35]. The concentrations of total protein (TP), glucose (Glu), Triglyceride (TG), total cholesterol (TCH), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), urea nitrogen (BUN), and total bilirubin (TBIL) were analyzed using enzymatic methods and assay kits (Nanjing Jiancheng, Nanjing, China).

##### 2.5.3. Gene expression in skin

Total RNA was isolated from skin samples using the TRIzol reagent (Invitrogen, USA) and treated with the TURBO DNA-free™ kit (Invitrogen) to remove genomic DNA. The concentration and integrity of total RNA were assessed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA). A sample of  $1 \mu\text{g}$  RNA was used as the template for the synthesis of cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). cDNA was diluted 10-

**Table 1**  
Forward and reverse primers for qPCR.

Target gene	Accession number	Forward (5'-3') Reverse(5'-3')
<i>Cathepsin D</i>	EU077233.1	GCCCGTCATCACCTTCAACC AAGCCAACCTCTGTCGTATCCC
<i>Cathepsin B</i>	KY593337.1	CTTCACCCTCACCTTCTCCTCC TGCTCCCACTACCAATCCTC
<i>Cathepsin L</i>	KY593341.1	CATGCTGCCATCGTTCCA GTCCTGACCTTTGTCTGTGCC
<i>Lysozyme</i>	AJ250732.1	GCAATGGGATGAGCAATTACAG CGTTGGAGGTGGCGCTCT
<i>Mucin-2</i>	KU238186.1	CTGGACGCTGGGAATGTGC AAGTCGCTTGTATTGTTGGTGC
Reference gene 18s rRNA	EF126038.1	ATGGCGTCTTAGTTGGTG CTCAATCTCGTGTGGCTGAA

fold for Quantitative real-time PCR (qPCR).

qPCR was used to detect the expression of five differentially expressed genes including cathepsin D, cathepsin B, cathepsin L, lysozyme and mucin-2 (Table 1). Oligonucleotide primers were designed as shown in Table 1 qPCR was performed using an ABI 7500 Fast instrument and 7500 software v2.0.1 (Applied Biosystems, USA). The PCR mixture contained 2  $\mu$ L diluted cDNA, 10  $\mu$ L 2  $\times$  SYBR Green PCR Mix (Takara, Dalian, China), 4  $\mu$ M of each gene-specific primer, 0.4  $\mu$ L ROX Reference Dye and 6.8  $\mu$ L distilled water in a final volume of 20  $\mu$ L. Cycling parameters were: 95  $^{\circ}$ C for 30 s, followed by 40 cycles at 95  $^{\circ}$ C for 5 s, 30 s at specific annealing temperature, followed by a melt curve stage after the cycling stage. The specificity of qPCR was analyzed by agarose gel and melting curve analysis. The expression levels of target genes were calculated using the  $2^{-\Delta\Delta C_t}$  method as described in Ref. [36].

## 2.6. Statistical analysis

Water quality, growth and biochemical parameters in the plasma were expressed as means  $\pm$  SD. Gene expression data (relative fold changes) were represented as mean values  $\pm$  SE. All statistical analyses were performed with SPSS 19.0 software. All the indexes were compared at each velocity treatment using one-way ANOVA and Tukey's test. Significance was set at the 0.05 level.

## 3. Results

### 3.1. Water quality

Given the increase of weight of fish and feed intake over time, the experimental period was divided into three stages, days 0–20, days 21–40, and days 41–60. TAN, NO<sub>2</sub><sup>-</sup>-N, COD, TSS, CO<sub>2</sub>, DO and pH concentrations recorded at each of these time periods are presented in Table 2. Both pH and DO levels were found to decrease with increasing levels of fish feed ( $p > 0.05$ ). COD concentrations were slightly lower ( $p > 0.05$ ) at the 0–20 day stage with increasing velocity, while the opposite was the case for CO<sub>2</sub> and DO ( $p > 0.05$ ).

In both the 21–40 and 41–60 day periods, TSS decreased ( $p > 0.05$ ) with increased velocities due to the movement of uneaten feed and feces to the central drain pipe facilitated by increased water movement. CO<sub>2</sub> and DO levels were found to increase slightly ( $p > 0.05$ ) with increasing velocity. No significant differences in water quality parameters among the three velocity levels were found during the entire periods.

### 3.2. Feed intake and growth

As shown in Fig. 3, the differences in feed intake between the different treatments were apparent ( $p < 0.05$ ) from day 8. There was a

**Table 2**

Water quality parameters at the outlets of rearing tanks with different velocities during the three time periods of the experiment.

Period	Parameters (mg L <sup>-1</sup> )	0.06 m s <sup>-1</sup>	0.18 m s <sup>-1</sup>	0.36 m s <sup>-1</sup>
0–20 day	TAN	0.33 $\pm$ 0.04	0.33 $\pm$ 0.03	0.32 $\pm$ 0.01
	NO <sub>2</sub> -N	0.13 $\pm$ 0.06	0.13 $\pm$ 0.06	0.13 $\pm$ 0.05
	COD	2.18 $\pm$ 0.56	1.97 $\pm$ 0.38	1.88 $\pm$ 0.33
	TSS	50.33 $\pm$ 9.60	43.67 $\pm$ 5.70	58.99 $\pm$ 16.98
	CO <sub>2</sub>	2.50 $\pm$ 0.12	2.52 $\pm$ 0.15	2.55 $\pm$ 0.18
	DO	7.09 $\pm$ 0.32	7.12 $\pm$ 0.33	7.22 $\pm$ 0.28
21–40 day	pH	8.20 $\pm$ 0.13	8.21 $\pm$ 0.12	8.22 $\pm$ 0.12
	TAN	0.29 $\pm$ 0.05	0.29 $\pm$ 0.06	0.31 $\pm$ 0.01
	NO <sub>2</sub> -N	0.14 $\pm$ 0.06	0.14 $\pm$ 0.06	0.15 $\pm$ 0.06
	COD	2.19 $\pm$ 0.30	2.11 $\pm$ 0.31	2.01 $\pm$ 0.37
	TSS	42.00 $\pm$ 12.66	35.33 $\pm$ 9.31	37.00 $\pm$ 9.38
	CO <sub>2</sub>	3.12 $\pm$ 0.22	3.25 $\pm$ 0.31	3.30 $\pm$ 0.25
41–60 day	DO	6.71 $\pm$ 0.12	6.74 $\pm$ 0.14	6.88 $\pm$ 0.11
	pH	7.89 $\pm$ 0.33	7.89 $\pm$ 0.33	7.91 $\pm$ 0.34
	TAN	0.49 $\pm$ 0.11	0.48 $\pm$ 0.08	0.50 $\pm$ 0.08
	NO <sub>2</sub> -N	0.14 $\pm$ 0.03	0.15 $\pm$ 0.02	0.15 $\pm$ 0.03
	COD	1.42 $\pm$ 0.45	1.46 $\pm$ 0.55	1.35 $\pm$ 0.59
	TSS	64.99 $\pm$ 16.26	41.11 $\pm$ 13.16	45.00 $\pm$ 9.60
	CO <sub>2</sub>	4.15 $\pm$ 0.52	4.52 $\pm$ 0.38	4.54 $\pm$ 0.55
	DO	6.31 $\pm$ 0.17	6.31 $\pm$ 0.16	6.51 $\pm$ 0.15
	pH	7.55 $\pm$ 0.10	7.51 $\pm$ 0.09	7.55 $\pm$ 0.09

significant increase ( $p < 0.05$ ) in feed intake at velocities of 0.18 and 0.36 m s<sup>-1</sup> when compared with the low velocity (0.06 m s<sup>-1</sup>). Feed intake was found to be significantly higher ( $p < 0.05$ ) on days 8, 14, 18, 28, 34, 38, 52, 54, and 56 at the highest velocity level (0.36 m s<sup>-1</sup>), in comparison to the medium level of velocity (0.18 m s<sup>-1</sup>).

In relation to growth, the relatively elevated SGR and decreased FCR were recorded at 0.18 m s<sup>-1</sup> (Table 3). Lowest SGR was found at the lowest velocity (0.06 m s<sup>-1</sup>), while highest FCR was recorded for the highest level of velocity (0.36 m s<sup>-1</sup>).

### 3.3. Oxidative defense and innate immunity parameters in plasma

The results showed that water velocity had a significant effect on SOD, GSH, AKP and ACP activities (Table 4). SOD and GSH activity significantly decreased ( $p < 0.05$ ) with increasing water velocities up to 0.36 m s<sup>-1</sup> and there was no significant difference between the treatments of 0.06 and 0.18 m s<sup>-1</sup>. AKP and ACP activity was significantly higher ( $p < 0.05$ ) at 0.18 m s<sup>-1</sup>, compared with the other two velocity treatments. No significant differences ( $p > 0.05$ ) in AKP and ACP activity were detected between the 0.06 m s<sup>-1</sup> and 0.36 m s<sup>-1</sup> velocity treatments. Furthermore, there were no significant differences ( $p > 0.05$ ) in CAT and LZM activity among the three treatment groups.

### 3.4. Biochemical parameters in plasma

Plasma protein concentrations were found to be significantly lower ( $p < 0.05$ ) at the highest velocity (0.36 m s<sup>-1</sup>) (Table 5). No significant differences found between velocities of 0.06 and 0.18, or 0.06 and 0.36 m s<sup>-1</sup>.

BUN and TBil levels were found to be significantly higher ( $p < 0.05$ ) at the highest velocity (0.36 m s<sup>-1</sup>). There were no significant differences in the concentration of Glu, TCH, TG, HDL, LDL, and LDH among the three treatments.

### 3.5. Gene expression in skin tissue

qPCR was carried out on five genes to investigate whether different water velocity levels caused transcriptional changes in skin. qPCR analysis showed higher transcription of investigated genes with the increasing water velocity overall (Fig. 4). As shown in Fig. 4A and D, there was a significant up-regulation ( $p < 0.05$ ) in the transcription of

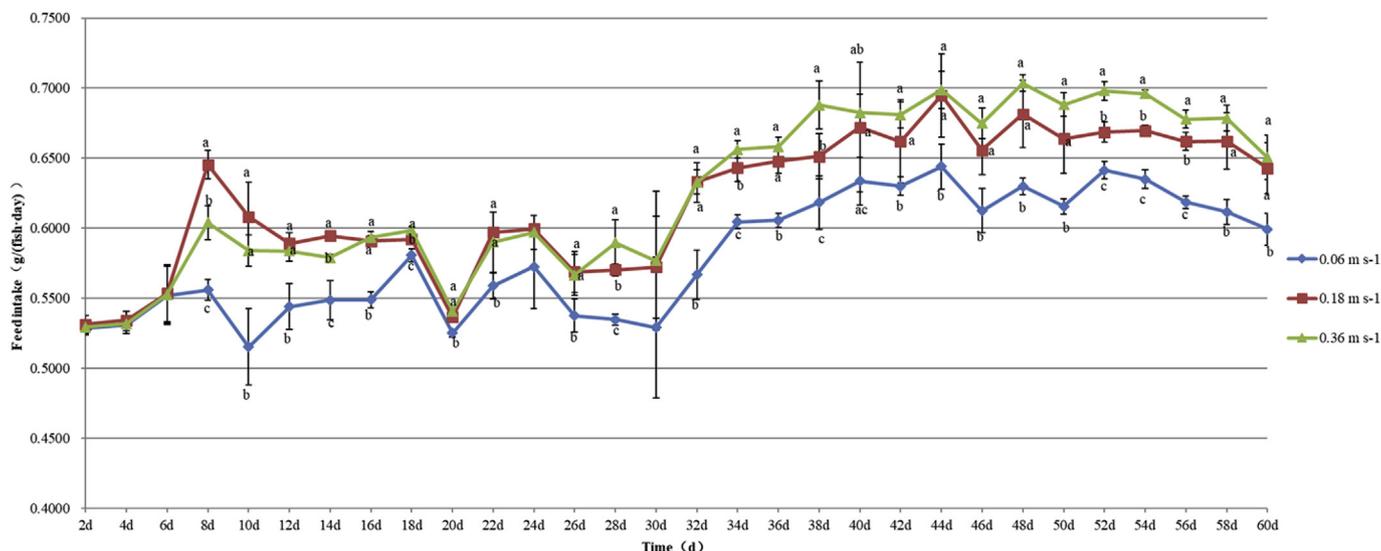


Fig. 3. Daily feed intake of turbot in each of the three different velocity treatments during the experimental period.

Table 3

Growth parameters of turbot in rearing tanks with three different velocity treatments.

Water velocity	Tank	SGR	FCR
0.06 m s <sup>-1</sup>	1	0.865%	1.34
	2	0.788%	1.40
0.18 m s <sup>-1</sup>	1	0.952%	1.17
	2	0.897%	1.24
0.36 m s <sup>-1</sup>	1	0.830%	1.54
	2	0.852%	1.63

Table 4

Oxidative defense and innate immunity parameters in plasma at the three different velocity treatments.

Parameters	Velocity		
	0.06 m s <sup>-1</sup>	0.18 m s <sup>-1</sup>	0.36 m s <sup>-1</sup>
SOD (U ml <sup>-1</sup> )	107.42 ± 9.82 <sup>a</sup>	102.20 ± 11.14 <sup>ab</sup>	92.69 ± 13.74 <sup>b</sup>
CAT (U ml <sup>-1</sup> )	0.98 ± 0.11	0.94 ± 0.19	0.91 ± 0.12
GSH (μmol L <sup>-1</sup> )	44.62 ± 7.49 <sup>a</sup>	36.63 ± 8.94 <sup>a</sup>	27.35 ± 5.64 <sup>b</sup>
LZM (U ml <sup>-1</sup> )	93.53 ± 7.37	98.24 ± 6.60	94.71 ± 6.13
AKP(U L <sup>-1</sup> )	128.52 ± 10.71 <sup>a</sup>	159.94 ± 11.23 <sup>b</sup>	134.94 ± 9.99 <sup>a</sup>
ACP (U L <sup>-1</sup> )	140.66 ± 13.57 <sup>a</sup>	170.65 ± 9.28 <sup>b</sup>	132.66 ± 16.42 <sup>a</sup>

Table 5

Biochemical parameters in plasma at the three different velocity treatments.

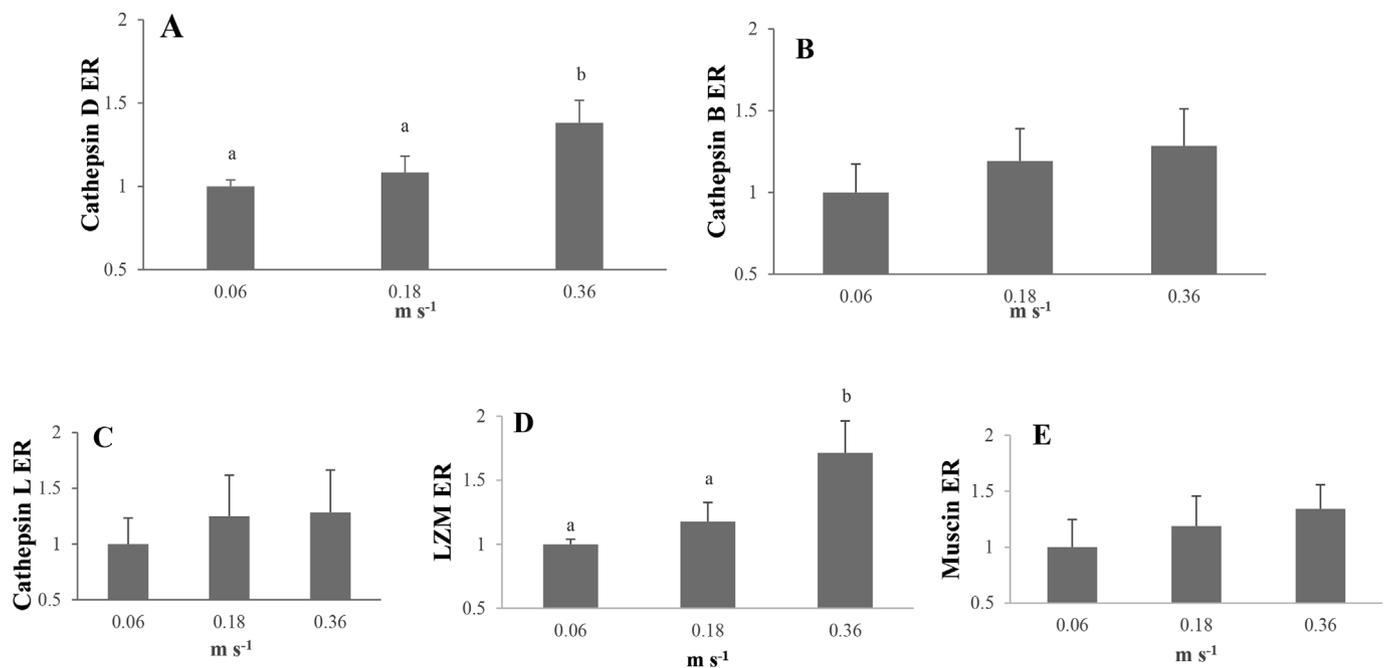
Parameters	Velocity		
	0.06 m s <sup>-1</sup>	0.18 m s <sup>-1</sup>	0.36 m s <sup>-1</sup>
Protein (mg mL <sup>-1</sup> )	41.36 ± 5.39 <sup>ab</sup>	45.16 ± 5.07 <sup>a</sup>	37.04 ± 5.08 <sup>b</sup>
GLU (mmol L <sup>-1</sup> )	1.91 ± 0.37	2.17 ± 0.31	2.07 ± 0.36
TCH(mmol L <sup>-1</sup> )	3.95 ± 0.80	3.45 ± 0.56	3.56 ± 0.75
TG(mmol L <sup>-1</sup> )	2.11 ± 0.52	2.10 ± 0.53	1.95 ± 0.61
HDL(mmol L <sup>-1</sup> )	1.63 ± 0.19	1.61 ± 0.27	1.53 ± 0.21
LDL(mmol L <sup>-1</sup> )	0.74 ± 0.29	0.65 ± 0.19	0.75 ± 0.22
LDH (U L <sup>-1</sup> )	8.38 ± 0.78	8.76 ± 0.38	8.10 ± 0.75
BUN(mmol L <sup>-1</sup> )	3.64 ± 0.84 <sup>a</sup>	3.88 ± 0.53 <sup>a</sup>	4.62 ± 0.78 <sup>b</sup>
TBil (μmol L <sup>-1</sup> )	3.33 ± 0.81 <sup>a</sup>	3.61 ± 0.72 <sup>ab</sup>	4.39 ± 0.63 <sup>b</sup>

cathepsin D and LZM at 0.36 m s<sup>-1</sup> compared to the other treatment groups. No significant differences were found in the expression of cathepsin B, cathepsin L and mucin among the three treatments.

#### 4. Discussion

In general, there were two strategies to enhance the water velocity of the circle tanks in RAS, either by increasing the flow rate of systems corresponding with the promotion of the water quality and huger consumption of the energy, or by the pump of velocity enhancer in the tanks. Previous studies examining the impact of water flow rates on the performance of turbot reported that heterogeneous growth was attributed to differences in water quality, particularly in RAS [15,16]. To our knowledge, there is no published research examining the impact of water velocity on turbot welfare. In the current study, the flow velocity was enhanced by the pump in the tanks and no significant differences in water quality parameters (TAN, NO<sub>2</sub><sup>-</sup>-N, COD, TSS, CO<sub>2</sub>, DO and pH) were found among the three different velocity treatments. All parameters were within the recommended values given for turbot in RAS [34,37,38]. This meant that the study that aim of investigating the impact of water velocity alone on fish physiology was possible. Slight differences (*p* > 0.05) in TSS, DO and CO<sub>2</sub> were detected among the three treatments, due to differences in feed intake and the capacity of the water to remove feces to the central drain between the three treatments. There were no fish mortalities during the experimental period. It was established that velocities between 0.33 and 0.46 bl s<sup>-1</sup> promoted a homogenous distribution of turbot (means: 22 cm). However, when given a choice, fish avoided swimming against water flows over 0.58 bl s<sup>-1</sup>. At velocities > 0.98 bl s<sup>-1</sup>, turbot no longer appeared to be able to maintain their position [17]. In the current study, all fish were able to maintain their position on the tank bottom at each of the three velocity treatments and avoided being carried by the current to the central area. Fish kept at 0.36 m s<sup>-1</sup> (equal to 1.8 bl s<sup>-1</sup>) showed a constant waving of the posterior parts of dorsal fin and anal fin.

In the current study, the experimental fish were fed to satiation. At the end of the experimental period, turbot reared at 0.18 m s<sup>-1</sup> (0.9 bl s<sup>-1</sup>) exhibited a promoted SGR than those reared at 0.06 and 0.36 m s<sup>-1</sup>. This is in keeping with the findings of Merino et al. [11] and Ogata et al. [13], who obtained similar results with Japanese flounder and juvenile Californian halibut. The authors suggested that the optimum water velocity for growth occurred at about 1.0 bl s<sup>-1</sup> (9–16 cm bl fish) and the highest feed conversion rate was at a higher velocity of 1.5 bl s<sup>-1</sup>. Fish with higher activity levels must consume more to provide the energy required to sustain swimming activity, and moderate exercise improves growth performance [39]. Some species of flatfish were unable to utilize the feed required for growth as efficiently at lower and higher velocities, compared with fish at the intermediate



**Fig. 4.** Effects of water velocity on selected genes analyzed with qPCR. Expression ratio (ER) of genes relative to the highest velocity group ( $0.36 \text{ m s}^{-1}$ ) as measured in skin; A) cathepsin D, B) cathepsin B, C) cathepsin L, D) lysozyme, and E) mucin. Error bars indicate the standard error from the mean. Different letters denote significant differences between densities ( $p < 0.05$ ).

velocity [11,14]. In the current study, the highest velocity ( $0.36 \text{ m s}^{-1}$ ) increased fish feed intake but failed to promote growth.

Stress increases the intracellular formation of ROS which can attack the antioxidant defense system, leading to the loss of antioxidants, such as SOD, CAT and GSH [40]. In the present study, SOD and GSH levels in the plasma were remarkably lower in fish exposed to velocities of  $0.36 \text{ m s}^{-1}$ . These low levels were considered to be a response to the stress of the excessively high velocity and may reflect the limited ability of the antioxidant systems in turbot to completely remove harmful superoxide radicals, possibly further reducing fish welfare. Similar responses were found when turbot were under stress due to stocking density [41]. In some cases, the concentrations of the antioxidant component were elevated when impairment of the immune system occurred [34]. These differential effects were explained by differences in overall glucocorticoid sensitivity or receptivity of the immune response being affected [42]. AKP and ACP have been demonstrated to play an important role in disease defense and could improve the phagocytic activity of macrophages. A significant elevation of plasma AKP and ACP activity is a marker of the promotion on innate immunity [43]. The results of the current study revealed that a velocity of  $0.18 \text{ m s}^{-1}$  could improve the innate immune response of juvenile turbot.

Several studies have established that blood biochemical parameters are important tools which indicate physiological stress responses and the general health of fish under environmental changes. Bilirubin is a degradation product formed as a result of heme catabolism in the liver. High levels of bilirubin can result in liver dysfunction, haemolysis or blood disorders [44]. Fornaroli et al. [45] reported a significant increase in bilirubin of juvenile Amur sturgeon (*Acipenser schrenckii*) under high stocking densities. In the present study, the elevation of TBil levels indicated that turbot may have been stressed at the high velocity of  $0.36 \text{ m s}^{-1}$ . It is clear that when the swimming speed of fish is higher than optimal, swimming becomes unsustainable and stressful, and the ensuing anaerobic metabolism will create an oxygen debt [46]. But there were no differences found in levels of plasma glucose, TCH, TG, HDL, and LDH among three velocity treatments. Total plasma protein is a measurable humoral component of the nonspecific defense mechanism, and elevated levels may imply better health status in fish [47].

Total protein in the plasma of turbot decreased significantly at  $0.36 \text{ m s}^{-1}$ , while turbot in the  $0.18 \text{ m s}^{-1}$  treatment had the highest concentration of total protein in plasma. The final products of protein in fish are inosine, BUN, uric acid,  $\text{NH}_3$  and a large proportion of nitrogenous waste. In freshwater teleost, most nitrogenous waste ( $\text{NH}_3/\text{NH}_4^+$ ) is discharged via the gills, while in marine teleost, nitrogenous waste is discharged in the urine [48]. In mammals, kidney damage is clinically associated with elevated BUN levels, due to decreased excretion of urea in the urine [49]. An increase of plasma BUN in this study in the  $0.36 \text{ m s}^{-1}$  treatment indicated stress in turbot as well.

Fish have a unique skin barrier that prevents their body from being directly exposed to pollutants and stressors. Mucins, cathepsins and LZM were chosen as markers of the innate immunity. In fish skin, a significant increase in the transcription of cathepsin D was detected at  $0.36 \text{ m s}^{-1}$ . Cathepsin D is a lysosomal endoproteolytic aspartic proteinase, which plays a role in the activation of proteins destined for secretion and the processing of antigens for presentation to the immune system in fish [50]. Sveen et al. [31] reported up-regulated expressions of Cathepsins in the skin of Atlantic salmon when fish were stressed due to high stocking densities and reduced specific water flow. A similar increase was also found in the mRNA expression of LZM at  $0.36 \text{ m s}^{-1}$  in the current study. Jia et al. [41] reported that the mRNA levels of LZM were down-regulated in the skin with decreased LZM activity in the mucus when turbot were stressed by excessively high stocking densities and explained as an immunodepression due to chronic stress. In the current study, the up-regulation of cathepsin D and LZM indicates stress due to high velocities, possibly resulting in active cathepsin and LZM synthesis concerning the performance of fish in growth, oxidative stress responses and biochemical parameters in plasma. Besides the mucin gene was chosen as a marker for mucous cell activity and mucus production in fish skin [31]. While no differences were found among the three treatment groups, it seemed that cathepsin D and LZM genes in fish skin was sensitive to water velocity stress and are potential markers of immune response to stress caused by high water velocity in turbot.

## 5. Conclusion

This is the first study examining the effects of water velocity on growth, feed intake, oxidative stress and innate immune responses in the plasma and skin of turbot in RAS. In the current study, no significant differences in water quality parameters were found among the three different velocity treatments. However, when flow velocity reached  $0.36 \text{ m s}^{-1}$  ( $1.8 \text{ bl s}^{-1}$ ), juvenile turbot were subjected to stress, resulting in a reduction of growth and feed intake, a significant reduction of SOD and GSH activity, and a significant increase in the concentrations of TBil and BUN in the plasma. There was a clear up-regulation in the transcription of cathepsin D and LZM in fish skin at the velocity of  $0.36 \text{ m s}^{-1}$ . The medium velocity level of  $0.18 \text{ m s}^{-1}$  ( $0.9 \text{ bl s}^{-1}$ ), resulted in an increased feed intake, promoted SGR, and significantly higher levels of AKP and ACP activity in the plasma. From the results of this study, a velocity of around  $0.9 \text{ bl s}^{-1}$  is recommended for turbot culture. In addition, velocity enhancer pumps are recommended to promote the water flow in RAS once the water quality is at optimal levels for aquatic animals, rather than increasing the hydraulic retention time of systems, given the considerations of energy saving and animal health.

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