Comparative Biochemistry and Physiology, Part C 190 (2016) 1–8

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Effects of stocking density on antioxidant status, metabolism and immune response in juvenile turbot (*Scophthalmus maximus*)

Baoliang Liu [a,*,1], Rui Jia [b,1], Cen Han [c], Bin Huang [a,*], Ji-Lin Lei [a]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Qingdao 266071, China
[b] Wuxi Fisheries College, Nanjing Agricultural University, Wuxi 214081, China
[c] College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China

ARTICLE INFO

Article history:
Received 16 March 2016
Received in revised form 9 July 2016
Accepted 30 July 2016
Available online 03 August 2016

Keywords:
Scophthalmus maximus
Stocking density
Antioxidative enzymes
Metabolism
Gene expression

ABSTRACT

This study was designed to evaluate the physiological and immune responses of juvenile turbot to stocking density. Turbot (average weight 185.4 g) were reared for 120 days in a land based recirculating aquaculture system (RAS) under three stocking densities: low density (LD, ~9.3–26.1 kg/m^2, initial to final density), medium density (MD, ~13.6–38.2 kg/m^2) and high density (HD, ~19.1–52.3 kg/m^2). Fish were sampled at days 0, 40, 80 and 120 to obtain growth parameters and liver tissues. No significant difference was detected in growth, biochemical parameters and gene expression among the three densities until at the final sampling (day 120). At the end of this trial, fish reared in HD group showed lower specific growth rate (SGR) and mean weight than those reared in LD and MD groups. Similarly, oxidative stress and metabolism analyses represented that antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione (GSH)) and metabolic enzymes (glycerol-3-phosphate dehydrogenase (G3PDH) and glucose-6-phosphate dehydrogenase (G6PDH)) clearly reduced in the liver of turbot reared in HD group. The gene expression data showed that glutathione S-transferase (GST), cytochrome P450 1A (CYP1A), heat shock protein 70 (HSP 70) and metallothionein (MT) mRNA levels were significantly up-regulated, and lysozyme (LYS) and hepcidin (HAMP) mRNA levels were significantly down-regulated in HD group on day 120. Overall, our results indicate that overly high stocking density might block the activities of metabolic and antioxidant enzymes, and cause physiological stress and immunosuppression in turbot.

1. Introduction

Stocking density is considered one of the most important variables in intensive aquaculture influencing growth, welfare and health of farmed fish (Castillo-Vargasmachuca et al., 2012; Ellis et al., 2002; Ni et al., 2014). In commercial aquaculture, operation at higher stocking densities can reduce production costs, but high stocking density is a stressor, which induces chronic stress associated with deterioration in water quality, adverse social interactions or over-crowding, resulting in negative physiological and biochemical changes (Bolasina et al., 2006; Montero et al., 1999). A number of studies on cultured flatfish in which the effects of stocking density on growth performance is investigated have reported a growth reduction in higher-density treatments (Bolasina et al., 2006; Costas et al., 2008; Irwin et al., 1999). Thus, for a cost-effective production, it is necessary to find a balance between the maximum profit and the minimum incidence of physiological disorders and growth inhibition (Björnsson et al., 2012; Herrera et al., 2009).

Oxidative stress occurs when there is an imbalance between the activity of antioxidants and the generation of reactive oxygen species (ROS) (Lushchak, 2011). The antioxidants include antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and nonenzymatic antioxidant components such as glutathione (GSH) (Jia et al., 2013; Sayeed et al., 2003). In aquaculture, high density is a source of stress increasing the production of ROS (Braun et al., 2010; Vijayan et al., 1990). These ROS could damage cellular components and autoxidation function (Sayeed et al., 2003), result in oxidative injury and increase the incidence of diseases and deaths (Herrera et al., 2009; Iwama et al., 2011).

Like higher vertebrates, the immune system of teleost fish is composed of non-specific and specific immune system (Zapata et al., 2006). Non-specific defences are the most important defense mechanisms in fish. Many reports have shown the links of stress, depression of immune system, and disease (Magnadóttir, 2006). The effects of crowding stress on the immune parameters of few species of freshwater and marine fish have been documented (Li et al., 2006; Montero et al., 1999; Sadhu et al., 2014). High stocking density is a stressor that activates stress responses in fish, which caused alteration of immune-related enzymes, proteins or genes (Ni et al., 2014; Salas-Leiton et al., 2010; Vargas-Chacoff et al., 2014). Costas et al. (2013) reported that

* Corresponding authors at: Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 106 Nanjing Road, Qingdao 266071, China.
E-mail addresses: liubl@ysfri.ac.cn (B. Liu), huangbin@ysfri.ac.cn (B. Huang).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.cbpc.2016.07.007
1532-0456/

high stocking density reduced plasma lysozyme (LYS), alternative complement pathway (ACP) and peroxidase activities, suggesting some degree of immunosuppression in *Solea senegalensis*. Similar results were also reported in *Sparus aurata*, *Oncorhynchus mykiss* and *Oreochromis niloticus* reared in high density (Montero et al., 1999; Telli et al., 2014; Yarahmadi et al., 2014). Moreover, high density affected expression of genes involved in physiological stress such as cytochrome P450 1A (CYP1A), heat shock proteins (HSP70) and (HSP90), and innate immune system such as interleukin 1β (IL-1β), g-LYS and hepcidin (HAMP) (Ni et al., 2014; Salas-Leiton et al., 2010; Tapia-Paniagua et al., 2014). However, the information on the effects of stocking density on immune functions in turbot liver is very limited.

Turbot (*Scophthalmus maximus*) is a suitable species for aquaculture in Europe and China because of its considerable commercial value. This species has been intensively cultured in land-based farms including recirculation and flow-through systems (Irwin et al., 1999; Li et al., 2013). Initial ongrowing results indicated that this species could be adequately cultivated at high stocking densities (Aksungur et al., 2007; Baer et al., 2011). Nevertheless, there is little analysis regarding physiological and molecular responses caused by stocking density. Therefore, the purpose of this study aimed to evaluate the effects of stocking density on the antioxidative status, metabolism and expression of immune-related genes of turbot reared in a land-based RAS.

2. Material and methods

2.1. Experimental design and animals

The study took place in the farm of Shandong Oriental Ocean Sci-Tech Co., Ltd. (Shandong, China), and was operated in recirculating system supplied with high-quality well water, stabilized at 17–19 °C. The RAS consisted of ten 30-m^3 tanks connected to a biofilter and a mechanical filter. The oxygen content and water level (0.5–0.55 m in depth) in the fish tanks were monitored via the RAS computer. Make up water (16–18 °C) was pumped from a 20-m depth from the Laizhou Bay of China, mechanically filtered by two sand filters (5-μm filtration) and UV sterilized before entering the RAS units, and the water volume was ≤12% of the total system volume per day. The water residence time in the fish tanks was 1 h. The photoperiod was maintained at 12-hour light/12-hour dark using fluorescent light banks. Temperature, dissolved oxygen (DO), pH, and salinity in each tank varied slightly throughout the day, but in all cases remained at 18 ± 1.5 °C, 8 ± 1 mg/L, 7.9 ± 0.3, and 27.3 ± 3 g/L, respectively. Total ammonium nitrogen (TAN), nitrite and orthophosphates (PO_4-P) never exceeded 0.3 mg/L, 0.25 mg/L and 7.5 μM, respectively.

The experiments were performed in accordance with the guidelines on the care and use of animals for scientific purpose set by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Fishery Science. This study was specifically approved by the Committee on the Ethics of Animal Experiments of Yellow Sea Fisheries Research Institute at Chinese Academy of Fishery Science. All efforts were made to minimize the suffering of the animals. The turbot were reared in the RAS for 15 days to acclimatize to the experimental environment. A total of 20,400 fish (average individual weight 185.4 g) were reared for 120 days under three densities: low density (LD) with 1500 fish per tank (9.3 ± 0.11 kg/m^2 at initial density), medium density (MD) with 2200 fish per tank (13.6 ± 0.21 kg/m^2 at initial density), and high density (HD) with 3100 fish per tank (19.1 ± 0.38 kg/m^2 at initial density). Each density was tested in triplicate. No differences in weight and coefficient of variation (CV) for initial weight were found among the three densities. The turbot were fed a commercial-pellet diet (Ningbo Tech-Bank Co., LTD., Zhejiang, China), which contained 52% crude protein, 12% crude lipids, 16% crude ash, 3% crude fiber, 12% water, 5% Ca, 0.5% P, 2.3% lysine, and 3.8% sodium chloride. The fish were fed two times per day (08:00 and 20:00), and the daily feed rations (approximately 1.2% of fish weight/day) were adjusted based on observation of the feeding behavior and weight of fish. Fish were not fed 24 h before sampling (Arends et al., 1999).

2.2. Sampling

Growth was monitored by measuring weight and total length at 0, 40, 80 and 120 days during the trial. Total sample size represented 15% of the whole population in per tank (Garcia et al., 2013).

To perform the biochemical parameters determinations and genes expression analyses, 20 individuals of each tank were randomly removed at days 0, 40, 80 and 120, and killed by an overdose tricaine methane sulfonate (MS-222, 250 mg/L, Sigma, MO, USA). Liver was rapidly dissected, frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Growth parameters

Specific growth rate (SGR) was calculated from individual weights recorded on days 0, 40, 80 and 120. SGR (% day^{-1}) = 100 × (ln final weight − ln initial weight) / number of days. The corresponding stocking density (kg biomass / m^2) = number of individuals × average weight / surface.

2.4. Biochemical parameter assays

The liver sample was rinsed in ice-cold saline, and 0.1 g of each liver sample in 0.9 mL saline was ground in glass homogenizer tubes. The homogenate was centrifuged (4 °C, 3000 rpm, 10 min) to collect supernatant which was used to measure biochemical parameters.

Oxidative stress parameters, including SOD, GPx, CAT, GSH and malondialdehyde (MDA), in the liver tissues were measured using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China).

The activities of liver metabolic enzymes fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), hexokinase (HK, EC 2.7.1.11) and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) were determined using a Microplate Reader (Multiscan Go, Thermo Scientific, USA), using Software Scan 3.2 to Multiscango. Enzyme reaction rates were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by addition of homogenates (15 μL) at a pre-established protein concentration, omitting the substrate in control wells (final volume 275–295 μL) and allowing the reactions to proceed at 37 °C for pre-established times (Vargas-Chacoff et al., 2014). Protein concentration of liver homogenate was determined by the Bradford method, using bovine serum albumin as a standard (Bradford, 1976).

2.5. Total RNA isolation

The total RNA was isolated from liver tissues using a fast pure RNA kit (Dalian Takara, China) according to the manufacturer's instruction. The amount of RNA was measured using GeneQuant 1300 (GE Healthcare Biosciences, Piscataway, NJ) and its quality was checked on an agarose gel.

2.6. Quantification of genes expression levels

Total RNA (2 μg) from each sample was reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. The real-time quantitative PCR (qRT-PCR) was performed with an ABI PRISM 7500 Detection System (Applied Biosystems, USA). Each reaction mixture (20 μL) contained 4 μL of cDNA template (ten-fold diluted), 10 μL of SYBR® Premix Ex TaqTM (Perfect Real Time) (Takara Bio., China), 0.4 μL of ROXII, 0.4 μL of each primer (10 μmol/L) and 4.8 μL of ddH_2O.