Livestock Science 141 (2011) 69–75

Contents lists available at ScienceDirect

Livestock Science

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

Increasing stocking density causes inhibition of metabolic–antioxidant enzymes and elevates mRNA levels of heat shock protein 70 in rainbow trout

Ercüment Aksakal [a,*], Deniz Ekinci [b], Orhan Erdoğan [c,d], Şükrü Beydemir [e,d], Zuhal Alım [e], Saltuk Buğrahan Ceyhun [f,d]

[a] Atatürk University, Agriculture Faculty, Department of Agricultural Biotechnology, 25240, Erzurum, Turkey
[b] Ondokuz Mayıs University, Agriculture Faculty, Department of Agricultural Biotechnology, 55200, Samsun, Turkey
[c] Atatürk University, Science Faculty, Department of Molecular Biology and Genetics, 25240, Erzurum, Turkey
[d] Atatürk University, Biotechnology Application and Research Center, 25240, Erzurum, Turkey
[e] Atatürk University, Science Faculty, Department of Chemistry, 25240, Erzurum, Turkey
[f] Atatürk University, Hınıs Vocational Training School, 25600, Erzurum, Turkey

ARTICLE INFO

Article history:
Received 21 February 2011
Received in revised form 3 July 2011
Accepted 12 July 2011

Keywords:
Stocking density
Rainbow trout
Pentose phosphate pathway
Gene expression
Heat shock protein

ABSTRACT

The objective of this study was to determine the effects of stocking density on the activity of glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and glutathione reductase (GR) enzymes in liver, muscle, gill and kidney tissues of rainbow trout. Fish were reared at different stocking densities (15 kg/m^3, 20 kg/m^3, 25 kg/m^3 and 30 kg/m^3). After adaptation period of 30 days, the experiment was carried out for two months. Stocking density of the control group was 15 kg/m^3. Increasing stocking density caused inhibition in the activities of the enzymes except for kidney G6PD and 6PGD. Activity of both pentose phosphate pathway enzymes unexpectedly increased only in kidney whereas inhibition was observed in other tissues. Since the most powerful and gradual attenuation was observed in muscle tissue for all enzymes, we performed quantitative Real Time PCR to examine the expression of heat shock protein (Hsp70) gene in muscle in order to understand whether the decrease in enzyme activities is associated with stress. The mRNA expression data showed that Hsp70 expression levels significantly elevated at 25 kg/m^3 and 30 kg/m^3 stocking densities. Overall results indicate that increasing stocking density blocks the activity of metabolic and antioxidant enzymes and causes considerable stress in rainbow trout. The most susceptible tissue to increasing stocking density was observed to be the muscle.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Interest in the welfare of intensively farmed fish has increased worldwide recently. As an area of particular concern, stocking density has been emphasized and the effects of stocking density are known to be complex and appear to comprise of numerous interacting and case specific factors (Ashley, 2007). Studies made on these factors are important to improve welfare. It is known that stocking density, diet, feeding technique and management procedures may affect welfare. Generally, stocking density can be defined as the weight of fish per unit volume or per unit volume in unit time of water flow through the holding environment (Ashley, 2007; Ellis, 2001). So, stocking density has a substantial importance in welfare in the fish farming including rainbow trout. Rainbow trout is one of the most cultured fish species in aquacultural industry around the world. Therefore, there are many studies with effects of density on production and physiological parameters of some aquacultures including rainbow trout.

* Corresponding author. Tel.: +90 442 2311064; fax: +90 442 2360958.
E-mail address: ercuaksakal@gmail.com (E. Aksakal).

1871-1413/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.livsci.2011.07.006

For instance, Montero et al. (1999) investigated the effect of high stocking density on one of the most important marine fish species for Mediterranean aquaculture, gilthead seabream (*Sparus aurata*). They found that stocking density affected growth, biochemical composition, immune status and hematology. In addition, North et al. (2006) examined the impact of stocking density on the welfare of rainbow trout. They stocked juvenile rainbow trout in triplicate in 1.82 m^3 flow-through tanks at densities of 10, 40, and 80 kg m^{-3} over a 9-month period. As a result, it was demonstrated that stocking density did not significantly affect growth or mortality, but it had a significant effect on fin condition. Besides, there are some studies in literature on fish growth and survival associated with effects of stocking density (Björnsson, 1994; Holm et al., 1990; Sodeberg and Meade, 1987). Also, some other articles report about some alterations of fish behavior, metabolism etc. connected with high stocking density (Alanärä and Brännäs, 1996; Vijayan et al., 1990). As mentioned above, environmental conditions including stocking density influence genetic components, some stress factors, enzyme activities and oxygen consumption etc. of living beings including fish metabolism. Especially, some factors are important as a target and they have critical function for living metabolism from metal toxicity to density (Björnsson, 1994; Ekinci et al., 2007, 2011; Holm et al., 1990; Sodeberg and Meade, 1987). Among these factors, stocking density is well known as biological stress factor in aquaculture.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49) is the first enzyme in the pentose phosphate pathway. The principal physiological function of pentose phosphate pathway is the production of NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis (Erdogan et al., 2004; Kuo et al., 2000). In addition, NADPH also participates in cell-membrane protection and cell detoxification from xenobiotics through the glutathione reductase-peroxidase system and the mixed-function oxidases (Barroso et al., 1999; Díez-Fernández et al., 1996; Frank, 2005).

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44) is the third enzyme of the pentose phosphate metabolic pathway, catalyzing the conversion of 6-PGA (6-phosphogluconate) to D-riboluse-5-phosphate in the presence of $NADP^+$. The reaction, catalyzed by 6PGD, yields NADPH, which protects the cell against oxidant agents by producing reduced GSH (Bianchi et al., 2001; Lehninger et al., 2000) and a step in the pentose phosphate pathway of glucose metabolism. For this reason, 6PGD contributes to antioxidant protection (Kozar et al., 2000; Srivastava and Beutler, 1989).

Glutathione reductase (glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7) is a key enzyme in glutathione metabolism (Coban et al., 2007; Senturk et al., 2008a, 2008b). This enzyme is essential for reduction of glutathione disulfide (GSSG) to the reduced form glutathione (GSH), necessary for protection of cells against oxidative stress (Senturk et al., 2009a, 2009b). In addition, recent results suggest that GSH is essential for cell proliferation (Poot et al., 1995; Rendón et al., 2004), and plays a role in the regulation of apoptosis (Van den Dobbelsteen et al., 1996). Alternatively, high GSSG concentrations inhibit a number of important enzyme systems, including protein synthesis (Deneke and Fanburg, 1989).

It has been suggested that the heat shock proteins might serve as biomarkers of stress and environmental insult in aquatic organisms (Sanders, 1993). These proteins are classified on molecular weight with the predominant classes being Hsp100, Hsp90, Hsp70, Hsp60 and the small HSPs (MW <40 kDa). The heat shock proteins were discovered upon induction by hyperthermia and act to confer protection against the cytotoxic consequences of protein denaturation. Denaturing conditions such as exposure of cells to hypoxia, ethanol, heavy metals and pesticides also induce synthesis of HSPs (Dorts et al., 2009; Nover, 1991; Lee and Corry, 1998; Lewis et al., 1999; Lindquist and Craig, 1988). Several HSPs exhibit readily detectable constitutive levels that increase in concentration in response to exposure to stressors. These constitutive species are essential cellular elements associated with protein folding and transport, receptor binding and proteolysis (Zarate and Bradley, 2003).

Since there are not enough investigations on the effects of stocking density on various parameters of living systems and we have not found any relation between stocking density and pentose phosphate pathway enzymes G6PD, 6PGD and antioxidant enzyme GR, we aimed in this study to examine whether increasing stocking density causes negative results on these enzymes and to see if the expression of stress related protein Hsp70 elevates upon increased stocking density.

2. Materials and methods

2.1. Materials

TRIzol reagent for total RNA isolation and ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis were purchased from Invitrogene Co, Carlsbad, CA, USA. FastStart TaqMan Probe Master used for Real time applications was purchased from Applied Biosystems, Carlsbad, CA, USA. Chemicals used for enzyme activity measurements were purchased from Sigma-Aldrich, Munich, Germany or Merck, Darmstadt, Germany. All other chemicals were of analytical grade and obtained from either Sigma-Aldrich or Merck.

2.2. Fish husbandry and experimental design

Rainbow trouts were obtained from the fisheries department of the agricultural faculty at Atatürk University in Erzurum and weighed of 130 ± 20 g. They were fed twice a day with a commercial pelleted trout feed (at 1% body weight). Trout feed was purchased from Pınar Yem Company, İzmir, Turkey. Fish were fed a commercial pellet diet with 49.4% protein, 18.2% fat, 94.3% dry matter, and 9.8% ash at a daily ratio of 1% of their body weight mass during the study. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association.

Prior to the experiment, fish were kept in 1 × 1.2 m (diameter-deep) fiber-glass tanks for one month. The average water temperature was 10 °C during the experiments. Aeration was provided along the experiments. The water quality parameters were measured as $O_2 = 8.6$ ppm, pH = 7.7, $SO_4^{-2} = 0.33$ mg/l, PO_4^{-3} = trace, $NO_3^- = 3.45$ mg/l, NO_2^- = trace, and conductivity = 230 μs/cm. Throughout the experiments, three tanks were used as controls (15 kg/m^3) while other three experimental groups (three tanks for each group)

Table 1
Primer and probe sets for rainbow trout Hsp70 and β-actin control.

Primers and probes		Sequence (5′–3′)	GenBank accession number
β-actin	Forward	TGGCCGTACCACCGGTAT	AF254414
	Reverse	GCAGAGCGTAGTCCTCGTAGATG	
	Probe	^{FAM}-CTCCGGTGACGGCGTGACCC-^{TAMRA}	
Hsp70	Forward	TCAAGAGGAAACACAAGAAGGA	AB062281
	Reverse	TGGTGATGGAGGTGTAGAAGTC	
	Probe	^{FAM}-TCAGCCAGAACAAGCGGGCT-^{TAMRA}	

were 20, 25, 30 kg/m^3. After 2 months, five animals from each tank were randomly sampled, immediately stunned and sacrificed. Tissue samples were transferred into liquid nitrogen and stored at −80 °C until analysis.

2.3. Enzyme assays

2.3.1. Preparation of the homogenate

Tissue samples were washed three times with 50 mM Tris–HCl + 0.1 M Na_2SO_4 (pH 8.0), and each was homogenized by liquid nitrogen, transferred to the same buffer, and centrifuged at 4 °C, 15,000 *g* for 60 min. Supernatant was used in further studies (Le Trang et al., 1983).

2.3.2. Determination of enzyme activities

Glutathione reductase activity was measured spectrophotometrically at 25 °C by the modified method of Carlberg and Mannervik (1975). One enzyme unit was defined as the amount that oxidizes 1 μmol NADPH per minute under the assay conditions. The activity buffer contained: 50 mM NaH_2PO_4, 5 mM EDTA, pH: 7.8. GSSG concentration was 20 μM.

Glucose-6-phosphate dehydogenase activity was measured by Beutler's method (Beutler, 1971). One enzyme unit was defined as the enzyme amount reducing 1 μmol $NADP^+$ per 1 min. The activity buffer contained: 50 mM NaH_2PO_4, 5 mM EDTA, pH: 7.8.

6-Phosphogluconate dehydrogenase enzymatic activity was measured by Beutler's method (Beutler, 1971). One enzyme unit was defined as the enzyme amount reducing 1 μmol $NADP^+$ per 1 min. The activity buffer contained: 50 mM NaH_2PO_4, 5 mM EDTA, pH: 7.8.

2.3.3. Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976), with bovine serum albumin as a standard.

2.4. Real-time PCR assay

Total RNA was isolated from frozen muscle tissues of both control and increased stocking density groups using TRIzol reagent. RNA was treated with DNase in order to avoid genomic contamination. RNA concentrations and quality were verified by means of spectrophotometer (OD at 260 nm) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at −20 °C until use.

Primers and TaqMan probe were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using rainbow trout Hsp70 (GenBank accession number AB062281) and BLASTed to ensure correct mRNA sequences. β-Actin was used as the reference gene (GenBank accession number AF254414) (Johansen and Overturf, 2005). For target and reference genes, two primers and an internal, fluorescent labeled TaqMan probe 5′-end, reporter dye FAM (6-carboxyflourescein), 3′-end, quencher dye TAMRA (6-carboxytetramethylr-hodamine) were used. The primer and probe sequences can be found in Table 1.

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The PCR was carried out in a reaction volume of 50 μl containing template DNA, 900 nM of forward and reverse primers, 250 nM TaqMan probe, and 25 μl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. The following thermal cycling conditions were used: 50 °C for 2 min for activation of optical AmpErase UNG enzyme, 95 °C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min.

The relative expression levels were calculated using the modified comparative CT method (Pfaffl, 2001). The PCR efficiencies were calculated from standard curves using the formula $E = 10^{[-1/\text{slope}]}$ where E is the efficiency and slope is the slope of the standard curve. The β-actin gene was used as the internal reference. Efficiencies were calculated to be 1.97 and 1.88 for β-actin and Hsp70, respectively.

2.5. Statistical analysis

All measurements were triplicated. The statistical analysis was performed using SPSS (version 17.0) software. Data were presented as mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) after checking for normality. The significant means were compared by Duncan's multiple range tests at $p<0.05$.

3. Results

3.1. G6PD activity

In liver tissue, the only significant decrease in G6PD activity was observed at 30 kg/m^3. Increasing stocking density caused a significant and gradual decrease in muscle tissue although

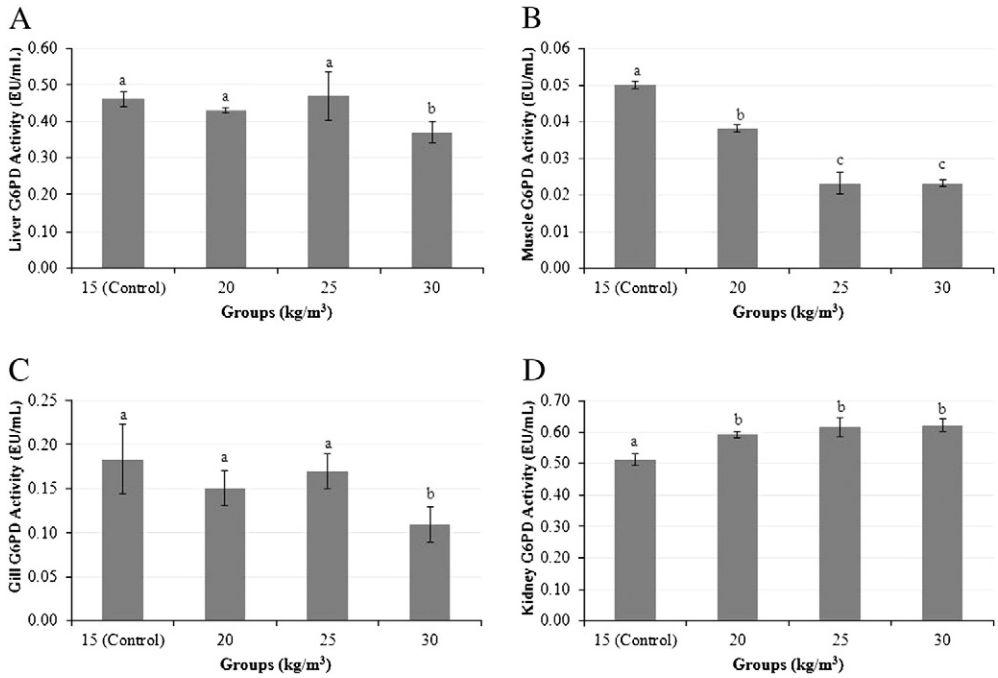


Fig. 1. Stocking density dependent alterations in G6PD activity (A: liver; B: muscle; C: gill; D: kidney) (a, b, c, and d show statistical difference at $p<0.05$).

there was no significant difference between 25 kg/m^3 and 30 kg/m^3. Gill G6PD enzyme activity behaved very similar to liver G6PD such that the only significant decrease was observed in 30 kg/m^3. As for kidney, surprisingly, 20 kg/m^3 stocking density caused a significant elevation compared to control group, but the G6PD activity remained stable for 25 kg/m^3 and 30 kg/m^3 afterwards (Fig. 1).

3.2. 6PGD activity

Liver 6PGD activity did not change significantly between control group and 20 kg/m^3. However, 25 kg/m^3 and 30 kg/m^3 stocking density caused a significant decrease. Similar to G6PD, muscle 6PGD activity significantly and gradually decreased upon increased stocking density. Gill

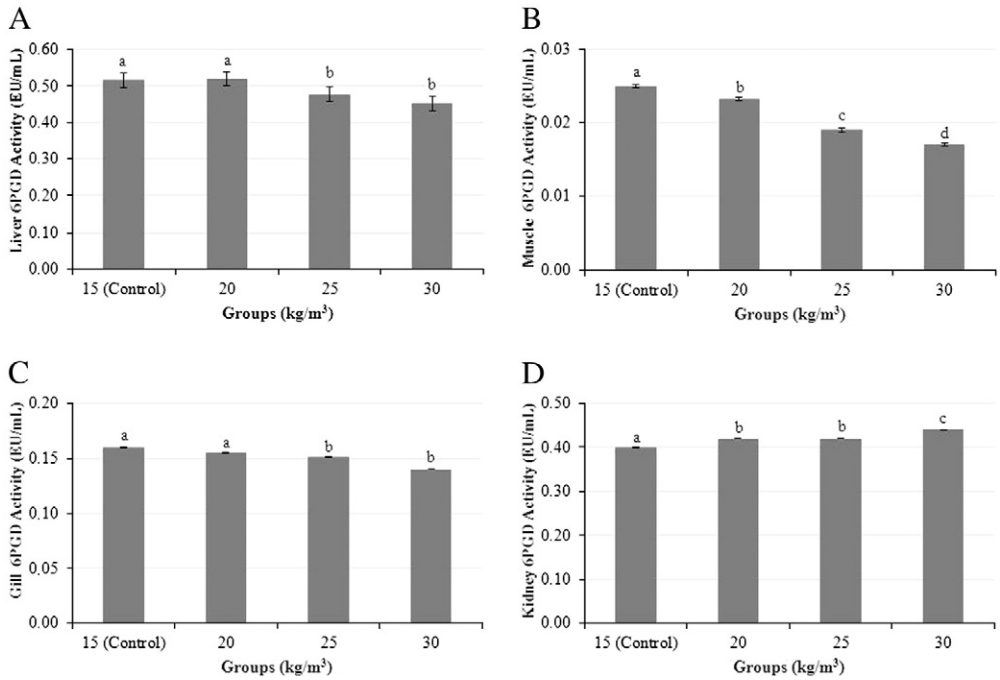


Fig. 2. Stocking density dependent alterations in 6PGD activity (A: liver; B: muscle; C: gill; D: kidney) (a, b, c, and d show statistical difference at $p<0.05$).

6PGD activity was not significantly different from the control group in 20 kg/m^3. However, 25 kg/m^3 and 30 kg/m^3 stocking density significantly inhibited the enzyme. Kidney enzyme activity increased with increasing stocking density similar to G6PD although no significant difference was observed between 20 kg/m^3 and 25 kg/m^3 (Fig. 2).

3.3. GR activity

Liver GR enzyme activity of 20 kg/m^3 was not significantly different from the control group, but 25 kg/m^3 and 30 kg/m^3 stocking density caused significant inhibition. Very similar to G6PD and 6PGD, muscle GR activity significantly and gradually decreased upon increased stocking density. The gill enzyme activity also decreased significantly and gradually upon increased stocking density, but no significant difference was detected between 25 kg/m^3 and 30 kg/m^3. Kidney GR activity was not inhibited significantly by 20 kg/m^3, but 25 kg/m^3 and 30 kg/m^3 caused significant decrease (Fig. 3).

3.4. Hsp70 expression

mRNA levels of Hsp70 increased gradually with stocking densities in muscle for 25 kg/m^3 and 30 kg/m^3, but no significant increase was observed for 20 kg/m^3 (Fig. 4).

4. Discussion

Fish must be provided with optimal environmental conditions in order to achieve potential growth and profit in fish farms. Any deviation from these conditions can result in decreased performance and is clear to affect the profitability of the aquaculture industry. Because high stocking density can contribute to reduced performance due to a number of factors, the effect of stocking density on the welfare is the key factor in fish production. In this context, there are a lot of studies showing that high stocking density produces a wide variety of effects on cultured fish populations, such as alterations in physiological parameters and poor feed utilization, results in mortality and poor growth, and inhibits some vital enzymes.

Li et al. (2006) investigated the effect stocking density on phenoloxidase, superoxide dismutase, and peroxidase Chinese shrimp (*Fenneropenaeus chinensis*). According to their results, phenoloxidase activity increased with stocking density. On the contrary, superoxide dismutase activity did not show any changes with the stocking density. No significant alterations were observed in peroxidase activity upon different stocking densities.

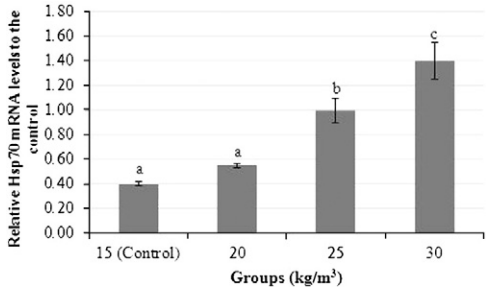


Fig. 4. Relative mRNA levels of Hsp70 to β-actin in *Oncorhynchus mykiss* muscle upon increasing stock density (a, b, and c show statistical difference at $p<0.05$).

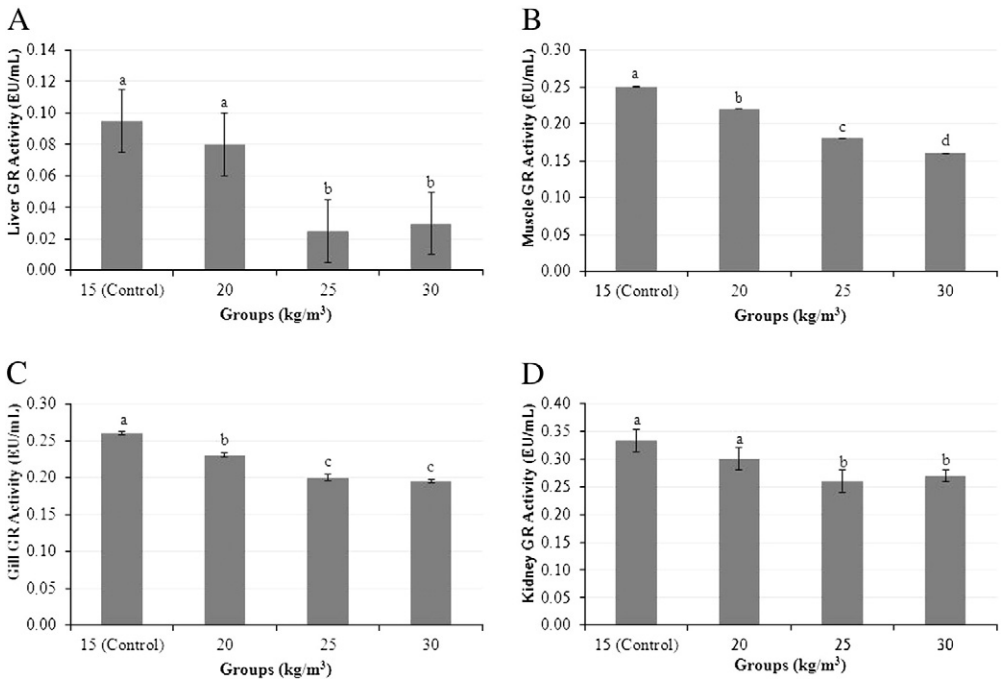


Fig. 3. Stocking density dependent alterations in GR activity (A: liver; B: muscle; C: gill; D: kidney) (a, b, c, and d show statistical difference at $p<0.05$).

4.1. Influence of increasing stocking density on G6PD activity

There are some studies in literature about the effects of various parameters on G6PD enzyme activity (Sahin et al., 2010). For example, inhibitory effects of some pesticides, namely cypermethrin, propoxur and deltamethrin on rainbow trout erythrocyte G6PD were investigated. Results of the study revealed that the pesticides significantly inhibited the enzyme activity (Senturk et al., 2009c). The influence of deltamethrin on G6PD from rainbow trout muscle, liver, gill and kidney was also investigated. The study showed that deltamethrin significantly inhibits the enzyme at low concentrations (Ceyhun et al., 2010). However, no reports could be found about the effect of stocking density on the activity of G6PD enzyme from different tissues of rainbow trout. Thus, we analyzed the alterations in G6PD activity upon increased stocking density. We observed that G6PD activity is strongly influenced by increasing stocking density. However, unexpected increase was evidenced for the kidney enzyme. The reason of this increase merits further investigations at this moment. Nevertheless, it is possible to propose that the kidney increases the activity of these enzymes to produce erythrocytes since increasing density causes a reduce in oxygen amount. Also, NADPH production increases at the same time. Thus, from overall findings, we can say that increasing stocking density attenuates G6PD activity in rainbow trout.

4.2. Influence of increasing stocking density on 6PGD activity

Tranulis et al. (1991) reported the effects of starvation and temperature variations on 6PGD from rainbow trout liver. Jürss et al. (1987) also showed the effects of temperature, food deprivation and salinity on rainbow trout 6PGD. Deltamethrin was shown to inhibit 6PGD from rainbow trout tissues at very low concentrations (Ceyhun et al., 2010). Since there are not enough investigations on rainbow trout 6PGD enzyme, we aimed to see whether it is affected by stocking density. We have seen that increased stocking density inhibits the activity except for an increase for the kidney enzyme which is very similar to G6PD.

4.3. Influence of increasing stocking density on GR activity

Studies regarding the influences of various factors on the activity of fish GR have gained great attention over the past years due to the important antioxidant capacity of the enzyme. For instance, Tekman et al. (2008) showed that metal ions inhibit rainbow trout liver GR at very low concentrations. Ceyhun et al. (2010) also reported that pesticides affect the fish GR enzymes at low dosages. Because of the importance of the enzyme in fish antioxidant metabolism, we wanted to investigate the effects of stocking density on GR. The decrease in GR activity might cause very important results and inhibitors of this enzyme must be well defined. Overall findings of our study indicated that increasing stocking density causes inhibition in GR activity which might lead to production of oxidative stress. To support this information, we analyzed the alterations in the expression of stress related protein Hsp70.

4.4. Influence of increasing stocking density on Hsp70 expression

Elevated levels of various heat shock proteins have been measured in tissues of fish exposed to environmental contaminants, such as heavy metals (Duffy et al., 1999; Williams et al., 1996), industrial effluents (Janz et al., 1997; Vijayan et al., 1998), pesticides (Bagchi et al., 1996; Hassanein et al., 1999; Sanders, 1993), and polycyclic aromatic hydrocarbons (Vijayan et al., 1997, 1998). We aimed in the current study to analyze the stress induced by increasing stocking density. Thus, we checked the expression levels of the heat shock protein 70 upon increased stocking density. Since the most powerful and gradual attenuation was observed in muscle tissue for all enzymes, we performed quantitative Real Time PCR for the expression of Hsp70 in muscle in order to understand whether the decrease in enzyme activities is associated with stress. The Hsp70 mRNA levels significantly increased by increased stocking density for 25 kg/m^3 and 30 kg/m^3, but no significant increase was observed for 20 kg/m^3. Therefore, we suggest that increasing stocking density causes considerable stress for rainbow trout.

5. Conclusion

Variation of the stocking density can be a source of stress for fish. The stocking density is directly related to animal comfort and to fish culture productivity, and can be a determining factor in the economic return on production. The ideal density is one that does not cause substantial reduction in growth rates or environmental quality. Specially, stress has vital effects both on welfare and productivity in farmed fish and it has been linked to reduction in growth, abnormal behavior and immuno-depression. Results of our study show that increasing stocking density attenuates the activity of important metabolic and antioxidant enzymes and induces the expression of stress related protein Hsp70 in rainbow trout. Thus, increasing stocking density might cause undesirable results for aquaculture and fish welfare by disrupting the physiological balance.

Acknowledgment

Research support was provided by Atatürk University Scientific Research Project (Project Number: BAP-2007/103). Therefore, the authors would like to acknowledge Atatürk University for supporting this project.

References

Alanärä, A., Brännäs, E., 1996. Dominance-feeding behaviour in Arctic charr and rainbow trout: the effect of stocking density. J. Fish Biol. 48, 242–254.

Ashley, P.J., 2007. Fish welfare: current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199–235.

Bagchi, D., Bhattacharya, G., Stohs, S.J., 1996. *In vitro* and *in vivo* induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. Toxicology 112, 57–66.

Barroso, J.B., Peragón, J., García-Salguero, L., De la Higuera, M., Lupiáñez, J.A., 1999. Variations in the kinetic behaviour of the NADPH-production systems in different tissues of the trout when fed on an amino-acid-based diet at different frequencies. Int. J. Biochem. Cell Biol. 31, 277–290.

Beutler, E., 1971. Red Cell Metabolism Manual of Biochemical Methods. Academic Press, London. pp. 68–70.

Bianchi, D., Bertrant, O., Haupt, K., Coello, N., 2001. Effect of gluconic acid as a secondary carbon source on non-growing L-lysine producer cells of *Corynebacterium glutamicum*. Purification and properties of 6-phosphogluconate dehydrogenase. Enzyme Microbiol. Technol. 28, 754–759.

Björnsson, B., 1994. Effects of stocking density on growth rate of halibut (*Hippoglossus hippoglossus* L.) reared in large circular tanks for three years. Aquaculture 123, 259–270.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Ceyhun, S.B., Senturk, M., Ekinci, D., Erdogan, O., Ciltas, A., Kocaman, M., 2010. Deltamethrin attenuates antioxidant defense system and induces expression of heat shock protein 70 in rainbow trout. Comp. Biochem. Physiol. C 152, 215–223.

Coban, T.A., Senturk, M., Ciftci, M., Kufrevioglu, O.I., 2007. Effects of some metal ions on human erythrocyte glutathione reductase: an in vitro study. Protein Pept. Lett. 14, 1027–1030.

Deneke, S.M., Fanburg, B.L., 1989. Regulation of cellular glutathione. Am. J. Physiol. 257, 163–173.

Díez-Fernández, C., Sanz, N., Cascales, M., 1996. Changes in glucose-6-phosphate dehydrogenase and malic enzyme gene expression in acute hepatic injury induced by thiochetamide. Biochem. Pharmacol. 51, 1159–1163.

Dorts, J., Silvestre, F., Tu, H.T., Tyberghein, A.E., 2009. Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. Environ. Toxicol. Pharmacol. 28, 302–310.

Duffy, L.K., Scofield, E., Rodgers, T., Patton, M., Bowyer, R.T., 1999. Comparative baseline levels of mercury, Hsp70 and Hsp60 in subsistence fish from the Yukon–Kuskokwim delta region of Alaska. Comp. Biochem. Physiol. C 124, 181–186.

Ekinci, D., Beydemir, S., Alim, Z., 2007. Some drugs inhibit in vitro hydratase and esterase activities of human carbonic anhydrase-I and II. Pharmacol. Rep. 59, 580–587.

Ekinci, D., Ceyhun, S.B., Senturk, M., Erdem, D., Kufrevioglu, O.I., Supuran, C.T., 2011. Characterization and anions inhibition studies of an α-carbonic anhydrase from the teleost fish *Dicentrarchus labrax*. Bioorg. Med. Chem. 19, 744–748.

Ellis, T., 2001. What is stocking density. Trout News CEFAS 32, 35–37.

Erdogan, O., Ciftci, M., Ciltas, A.K., Hisar, O., 2004. Inhibition effects of some antibiotics on the activity of glucose 6-phosphate dehydrogenase enzyme from rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) erythrocytes. Turk. J. Vet. Anim. Sci. 28, 675–681.

Frank, J.E., 2005. Diagnosis and management of G6PD deficiency. Am. Fam. Physician 72, 1277–1282.

Hassanein, H.M.A., Banhawy, M.A., Soliman, F.M., Abdel-Rehim, S.A., Muller, W.E.G., Schroder, H.C., 1999. Induction of Hsp70 by the herbicide oxyfluoren (goal) in the Egyptian Nile fish, *Oreochromis niloticus*. Arch. Environ. Contam. Toxicol. 37, 78–84.

Holm, J.C., Refstie, T., Bo, S., 1990. The effect of fish density and feeding regimes on individual growth rate and mortality in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 89, 225–232.

Janz, D.M., McMaster, M.E., Munkittrick, K.R., Van Der Kraak, G., 1997. Elevated ovarian follicular apoptosis and heat shock protein 70 expression in white sucker exposed to bleached kraft pulp mill effluent. Toxicol. Appl. Pharmacol. 147, 391–398.

Johansen, K.A., Overturf, K., 2005. Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar. Biotechnol. 7, 576–587.

Jürss, K., Bittorf, Th., Wacke, R., 1987. Effects of temperature, food deprivation and salinity on growth, RNA/DNA ratio and certain enzyme activities in rainbow trout (*Salmo gairdneri* R). Comp. Biochem. Physiol. B 87, 241–253.

Kozar, R.A., Weibel, C.J., Cipolla, J., Klein, A.J., Haber, M.M., Abedin, M.Z., Trooskin, S.Z., 2000. Antioxidant enzymes are induced during recovery from acute lung injury. Crit. Care Med. 28, 2486–2491.

Kuo, W., Lin, J., Tang, T.K., 2000. Human glucose-6-phosphate dehydrogenase (G6PD) gene transforms NIH 3t3 cells and induces tumors in nude mice. Int. J. Cancer. 85, 857–864.

Le Trang, N., Bhargava, K.K., Cerami, A., 1983. Purification of glutathione reductase from gerbil liver in two steps. Anal. Biochem. 133, 94–99.

Lee, Y.J., Corry, P.M., 1998. Metabolic oxidative stress-induced Hsp70 gene expression is mediated through SAPK pathway. J. Biol. Chem. 273, 29857–29863.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2000. Principles of Biochemistry, 2nd ed. Worth Publishers Inc, New York, pp. 558–560.

Lewis, S., Handy, R.D., Cordi, B., Billinghurst, Z., Depledge, M.H., 1999. Stress proteins (Hsp's): methods of detection and their use as an environmental biomarker. Ecotoxicology 8, 351–368.

Li, Y., Li, J., Wang, Q., 2006. The effects of dissolved oxygen concentration and stocking density on growth and non-specific immunity factors in Chinese shrimp, *Fenneropenaeus chinensis*. Aquaculture 256, 608–616.

Lindquist, S., Craig, E.A., 1988. The heat shock proteins. Annu. Rev. Genet. 22, 631–677.

Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M., 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol. Biochem. 20, 53–60.

North, B.P., Turnbull, J.F., Ellis, T., Porter, M.J., Migaud, H., Bron, J., Bromage, N.R., 2006. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 255, 466–479.

Nover, L., 1991. Heat Shock Response. 2000 Corporate Blvd., N.W. CRC Press, Florida, pp. 140–205.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res. 29, 2003–2007.

Poot, M., Teubert, H., Rabinovitch, P.S., Kavanagh, T.J., 1995. De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. J. Cell. Physiol. 163, 555–560.

Rendón, J.L., Arenal, I.P., Flores, A.G., Uribe, A., Plancarte, A., Hernández, G.M., 2004. Purification, characterization and kinetic properties of the multifunctional thioredoxin-glutathione reductase from *Taenia crassiceps* metacestode (cysticerci). Mol. Biochem. Parasitol. 133, 61–69.

Sahin, A., Senturk, M., Ciftci, M., Varoglu, E., Kufrevioglu, O.I., 2010. The effects of chemical and radioactive properties of Tl-201 on human erythrocyte glucose 6-phosphate dehydrogenase activity. Nucl. Med. Biol. 37, 389–394.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49–75.

Senturk, M., Kufrevioglu, O.I., Ciftci, M., 2008a. Effects of some antibiotics on human erythrocyte glutathione reductase: an in vitro study. J. Enzyme. Inhib. Med. Chem. 23, 144–148.

Senturk, M., Gülçin, I., Ciftci, M., Küfrevioğlu, O.I., 2008b. Dantrolene inhibits human erythrocyte glutathione reductase. Biol. Pharm. Bull. 31, 2036–2039.

Senturk, M., Talaz, O., Ekinci, D., Cavdar, H., Küfrevioğlu, O.I., 2009a. In vitro inhibition of human erythrocyte glutathione reductase by some new organic nitrates. Bioorg. Med. Chem. Lett. 19, 3661–3663.

Senturk, M., Irfan Kufrevioglu, O., Ciftci, M., 2009b. Effects of some analgesic anaesthetic drugs on human erythrocyte glutathione reductase: an in vitro study. J. Enzyme. Inhib. Med. Chem. 24, 420–424.

Senturk, M., Ceyhun, S.B., Erdogan, O., Kufrevioglu, O.I., 2009c. In vitro and in vivo effects of some pesticides on glucose-6-phosphate dehydrogenase enzyme activity from rainbow trout (*Oncorhynchus mykiss*) erythrocytes. Pestic. Biochem. Phys. 95, 95–99.

Sodeberg, R.W., Meade, J.W., 1987. Effects of rearing density on growth, survival, and fin condition of Atlantic salmon. Progr. Fish-Cult. 49, 280–283.

Srivastava, S.K., Beutler, E., 1989. Glutathione metabolism of the erythrocyte. The enzymic cleavage of glutathione-haemoglobin preparations by glutathione reductase. Biochem. J. 119, 353–357.

Tekman, B., Ozdemir, H., Senturk, M., Ciftci, M., 2008. Purification and characterization of glutathione reductase from rainbow trout (*Oncorhynchus mykiss*) liver and inhibition effects of metal ions on enzyme activity. Comp. Biochem. Physiol. C 148, 117–121.

Tranulis, M.A., Christophersen, B., Blom, A.K., Borrebaek, B., 1991. Glucose dehydrogenase, glucose-6-phosphate dehydrogenase and hexokinase in liver of rainbow trout (*Salmo gairdneri*). Effects of starvation and temperature variations. Comp. Biochem. Physiol. B 99, 687–691.

Van den Dobbelsteen, D.J., Nobel, C.S.I., Schlegel, J., Cotgreave, I.A., Orrenius, S., Slater, A.F., 1996. Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. J. Biol. Chem. 271, 15420–15427.

Vijayan, M.M., Ballantyne, J.S., Leatherland, J.F., 1990. High stocking density alters the energy metabolism of brook charr, *Salvelinus fontinalis*. Aquaculture 88, 371–381.

Vijayan, M.M., Pereira, C., Forsyth, R.B., Kennedy, C.J., Iwama, G.K., 1997. Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation enzymes in rainbow trout treated with b-naphthoflavone. Life Sci. 61, 117–127.

Vijayan, M.M., Pereira, C., Kruzynski, G., Iwama, G.K., 1998. Sublethal concentrations of contaminant induce the expression of hepatic heat shock protein 70 in 2 salmonids. Aquat. Toxicol. 40, 101–108.

Williams, J.H., Farag, A.M., Stansbury, M.A., Young, P.A., Bergman, H.L., Petersen, N.S., 1996. Accumulation of Hsp70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. Environ. Toxicol. Chem. 15, 1324–1328.

Zarate, Z., Bradley, T.M., 2003. Heat shock proteins are not sensitive indicators of hatchery stress in salmon. Aquaculture 223, 175–187.