Aquaculture 498 (2019) 271–278

Contents lists available at ScienceDirect

Aquaculture

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

Effect of stocking density on growth performance, plasma biochemistry and muscle gene expression in rainbow trout (*Oncorhynchus mykiss*)

Saeed Zahedi[a,*], Arash Akbarzadeh[a], Jalil Mehrzad[b], Ahmad Noori[a], Mohammad Harsij[c]

[a] Department of Fisheries, University of Hormozgan, Bandar Abbas, Iran
[b] Department of Microbiology and Immunology, University of Tehran, Tehran, Iran
[c] Department of Fisheries, University of Gonbad-e Kavus, Gonbad-e Kavus, Iran

ARTICLE INFO

Keywords:
Stocking density
Muscle
Myosin heavy chain
Myostatin
Follistatin
Oncorhynchus mykiss

ABSTRACT

The present study investigated in young-of-the-year rainbow trout, the effects of stocking density on growth parameters, plasma biochemistry and the expression of muscle stress- and growth-related genes including heat shock protein 70 (*HSP70*), insulin-like growth factor-1(*IGF-1*), myosin heavy chain (*MHC*), myostatin-1a (*MSTN-1a*) and follistatin (*FST*). Animals were exposed to three different stocking densities: 12, 24 and 44 kg/m^3, termed as low (LD), moderate (MD) and high (HD) density treatments, respectively, in an open culture system for 60 days. Also, on day 40, the culture density of a group of fish were reduced from 44 to 12 kg/m^3 (reduced density; RD treatment) for a further period of 20 days. The results showed significant changes in growth parameters, so that the final weight and condition factor were significantly decreased and feed conversion ratio was increased in HD compared to LD treatments. On the other hand, no significant alterations were found in fin, hepatosomatic and viscerosomatic indices as well as plasma glucose, total protein, triglyceride, cholesterol and cortisol contents among different experimental groups. The expression of *HSP70* was upregulated in HD compared to LD on days 20, 40 and 60, being the expression of *MHC* decreased and *MSTN-1a* increased significantly on day 60 only. The expression of *IGF-1* and *FST* did not show any significant variations among treatments. After 20 days of RD treatment, no significant changes were observed in growth parameters between RD and HD, being final weight and condition factor lower and feed conversion ratio higher than LD values. In addition, the expression of *HSP70* and *MSTN-1a* was significantly downregulated, and *MHC* was upregulated in RD groups compared to HD ones. In conclusion, increase of stocking density, reduced the growth and changed the expression of stress- and growth-related genes in muscle. Finally, the 20-day period of RD treatment could not recover the fish to LD conditions and a longer treatment period or, alternatively, experience of adjusted stocking densities would probably be required for a full recovery.

1. Introduction

In the recent century, rainbow trout farming industry is considered an important sector of worldwide growing aquaculture industry (FAO, 2016). One of the most important challenges in this industry is reaching the production systems and approaches that satisfy sustainability of the industry, fish welfare, public health, environmental issues and farmer income (North et al., 2006b; Iwama, 2007; Baldwin, 2011). The first priority of trout farmers is improving growth performance and subsequently production and therefore, there is an inherent enthusiasm to increase biomass per unit of volume (North et al., 2006a), known as stocking density (SD). The installation and operation of trout farm require a high capital investment per unit volume that could be compensated by increasing the productivity per unit of area (North et al., 2006a; Naderi et al., 2017b).

High SD has been shown as a chronic stressor that produces a wide variety of effects on physiology and welfare status of cultured fish manifested as the primary, secondary and tertiary stress responses. The primary response in stressed fish involves neuro-hormonal stimulation, resulting in an increase in catecholamine and corticosteroid secretions (Foo and Lam, 1993). Secondary responses include the disturbance of hydromineral balance, and increases in cardiac output, oxygen uptake and mobilization of energy substrates to cover higher energetic demand (Wendelaar Bonga, 1997). Tertiary stress response involves changes in growth, reduced feed intake, impaired immunity and health, reproduction, or even survival of individuals and/or populations (Ellis et al., 2002; Suárez et al., 2015; Yarahmadi et al., 2016; Naderi et al., 2017b).

* Corresponding author at: Department of Fisheries, P.O. Box: 3995, University of Hormozgan, Bandar Abbas, Iran.
E-mail address: szahedit@gmail.com (S. Zahedi).

https://doi.org/10.1016/j.aquaculture.2018.07.044
Received 24 January 2018; Received in revised form 4 July 2018; Accepted 25 July 2018
Available online 27 July 2018

On the other hand, several studies has rejected the stressful nature of SD, and have concluded that rainbow trout can safely tolerate high SD (Baker and Ayles, 1990; Kebus et al., 1992). In this sense, discrepancy of results in literature could be due to non-standardized and diverging experimental conditions like type of production system, tank size and shape, loading densities, hydraulic retention time, time of sampling, selected biomarkers etc., all of which might contribute to the complexity of the subject.

Under this premise, the study of molecular biomarkers could help to a better understanding of subcellular events and pathways and thus, assessment of stress and welfare conditions (Gornati et al., 2004). Muscle tissue is the main commercial portion of fish, being considered as the first target in growth studies as well as the final goal of aquaculture (Videler, 2011). However, to our knowledge the effects of SDs on the muscle growth genes of rainbow trout have not been reported.

There are some molecules that can exert regulatory effects on muscle turnover. In this sense, heat shock proteins (HSPs) are a family of highly conserved cellular proteins present in all organisms acting as chaperones. Among them, heat shock protein 70 (HSP70) is an important molecule which mediates different cellular actions like repair and degradation of altered or denatured proteins (Basu et al., 2002). Iwama et al. (2004), suggested its role preventing muscle damage or atrophy in response to environmental stressors.

There are other molecules that promote muscle synthesis, and could be used as relevant indicators. For example, insulin-like growth factor (IGF) system acts as a potent positive key regulator of muscle growth, and IGF-1 could be considered as an important growth index in fish (Montserrat et al., 2007). In muscle tissue, IGFs directly stimulate cell proliferation, differentiation and hypertrophy and, furthermore, inhibit its atrophy (Castillo et al., 2006; Duan et al., 2010).

In this line, myosin is the most abundant protein and the most important structural component of striated muscle consisting of two myosin heavy chain (MHC) subunits and four light chain ones. Myosin is a ubiquitous motor protein and an ideal indicator for growth studies, and its mRNA levels are known as a possible marker of trout muscle growth (Overturf and Hardy, 2001).

By other hand, myostatin (MSTN/growth differentiation factor-8 or GDF-8), was already considered as a negative regulator of fish muscle growth. Recently it has also been accepted as a general inhibitor of cell proliferation and cell growth modulating tissue mass (Gabillard et al., 2013). Myostatin-1 (myostatin-a and myostatin-b) predominates in skeletal muscle, brain, testes, spleen and eye (Garikipati et al., 2006). MSTN treatment in trout induces the atrophy of myotubes (Seiliez et al., 2013), and glucocorticoids (like cortisol) have been documented to downregulate fish MSTN (Rodgers et al., 2003).

Regarding to previous, follistatin (FST), best known as an activin inhibitor, has been suggested as one of the most efficacious antagonists of MSTN (Rebhan and Funkenstein, 2008; Schneyer et al., 2008). FST acts as a binding protein to MSTN, and thereby can regulate its availability. FST plays a role in muscle growth and its overexpression in trout is positively correlated with such growth (Medeiros et al., 2009).

Therefore, all of IGF-1, MHC, MSTN-1a and FST act as muscle growth regulators and to date no studies have addressed white muscle *MHC*, *MSTN* and *FST* expressions at mRNA level in rainbow trout under conditions that can affect fish growth such as chronic stress.

We hypothesize that muscle growth is linked to muscle growth-related genes, and decreased somatic growth under stress could be due to changes in the expression of these regulatory molecules. The first aim of the present study was to evaluate the effects of different SD levels (low or LD, medium or MD and high or HD) on growth performance and plasma biochemical parameters of rainbow trout as well as the possible changes in white muscle stress- and growth-related gene expression during a period of 60-day experimentation. Furthermore, the recovery to lower SD values in fish previously stressed by high density could trigger a compensatory response on growth and regulatory factors. To our knowledge, there are no studies reporting the molecular mechanisms involved in growth affectation by chronic stress in rainbow trout. In this sense, this study can provide useful information concerning expression of regulatory factors of muscle growth in cultured animals under conditions in which welfare can be affected.

2. Materials and methods

2.1. Fish and experimental conditions

The experiments were conducted in accordance with the Animal Ethics Guidelines of Hormozgan University. The all-female rainbow trout (*Oncorhynchus mykiss*, Walbaum) eyed eggs were purchased from Aqualande Co. (France) in June 2016 and incubated in Sadaf 2 trout propagation center (Kashmar, Khorasan Razavi, Iran). The juveniles were reared at outdoor raceways until young sub-adult fish (270 ± 8 g mean body weight; BW). Then, they were transferred to Sadaf 1 trout propagation and culture center in February 2017 (Torbat-e Heydariyeh, Khorasan Razavi, Iran) and distributed in indoor concrete octagonal tanks under density near 12 kg/m^3. A 4-week adaptation period was adopted before the experimental procedure. After adaptation, the fish (404.5 ± 10 g mean BW, 33.1 ± 0.1 cm mean body length) were randomly distributed into experimental concrete octagonal tanks (2500-L effective value, diameter/depth ratio of 3:1, 0.7-m depth, clockwise water rotation) at three stocking densities (SD) in triplicates as follow: low density (LD = 12 kg/m^3 close to average SD of small trout farms and ponds), moderate density (MD = 24 kg/m^3 close to average SD of raceways) and high density (HD = 40 and consequently, 44 kg/m^3, close to upper limit of most of circular and polygonal tanks with aeration). The selected densities were within the range of SDs that rainbow trout usually experience in systems with aeration (without oxygenation). In order to maintain the desired SDs, the required number of fish were removed weekly.

Octagonal tanks were continuously supplied with untreated aerated water directly from a spring (temperature 13.5 ± 0.2 °C, pH 7.2 ± 0.1, electrical conductivity (EC) 700 ± 10 μmos/cm) at a constant flow rate (1 L min^{-1} kg^{-1} fish) to maintain the same loading density. The measured water quality parameters within experimental tanks were as follow: temperature using thermometer (daily basis), dissolved oxygen (DO) using oximeter (daily basis, YSI 550, YSI inc. USA), pH using a multi-meter (daily basis, AZ instrument, Taiwan), ammonia using a test kit (weekly basis, Hanna instruments, USA) and EC using a multi-meter (biweekly basis, AZ instrument, Taiwan). The average water temperature, DO, pH, NH_3 and EC were 14 + 0.3 °C, 7.7 ± 0.5 mg/L, 7.2 ± 0.1, < 0.002 mg/L and 690 ± 15 μmos/cm.

During adaptation and experimental periods, the fish were fed 1.3% biomass twice daily (at 09:00 and 17:00) under ambient lighting in accordance with manufacturer's tables (Skretting Aquaculture, Italy, 42% crude protein, 24% crude fat, 3% crude fiber and 6% ash; 19 MJ/kg of feed).

2.2. Sampling procedure

Experiment started in March 2017 where the fish stocked in mentioned SDs for two months (March to May 2017). Also, on day 40, the culture density of a group of fish were reduced from 44 to 12 kg/m^3 (reduced density; RD treatment), and the fish were reared for a further period of 20 days. Samplings were made every 20 days up to 60 days. Fish were starved for 24 h prior to the start of each sampling, and six fish from each treatment (two per tank) were randomly dip-netted and anaesthetized in a bath of clove powder solution (200 ppm), weighted, and measured. Blood samples were drawn from the caudal vessel of each fish using syringes and placed in tubes that were kept on ice. The blood was then centrifuged for 10 min at the 1500 rpm. The obtained plasma were stored at −80 °C, and then used for biochemical assays. The fish liver, viscera and left-hand white muscle were collected and weighted for biometric determinations. Muscle samples from the dorsal

white musculature was snap-frozen in liquid nitrogen and stored at −80 °C until total RNA was isolated.

2.3. Growth parameters

At the end of experiment, all fish were weighted (BW), and total and fork length (TL and FL) as well as fin lengths including pectoral, ventral, anal and dorsal were measured. The fin length was measured from the base to the outside edge of each fin at its longest point by means of a caliper (North et al., 2006b). The growth and feeding parameters were calculated as follows:

Mortality rate: MR% = (final number of fish/initial number of fish) × 100, specific growth rate: SGR = ((ln final BW – ln initial BW)/days) × 100, Fulton's condition factor: CF = (BW/FL^3) × 100, feed conversion ratio: FCR = (feed intake/(final BW – initial BW)), fin index: FI = (fin length (cm)/FL (cm)) × 100 (Bosakowski and Wagner, 1994), hepatosomatic index: HSI = (liver weight (g)/BW (g)) × 100 and viscerosomatic index: VSI = (viscera weight (g)/BW (g)) × 100.

2.4. Analytical methods

2.4.1. Cortisol and metabolites in plasma

Glucose (GLU), total protein (TP), triglyceride (TG) and cholesterol (TCH) levels in plasma were estimated using an automatic analyzer (Hitachi 7600–110, Hitachi Co., Tokyo, Japan). Serum cortisol were measured in duplicate for each sample using an ELISA kit (Diagnostics Biochem Canada Inc., Ontario, Canada).

2.4.2. qPCR analysis

Total RNA was isolated from 50 mg of frozen muscle tissues using NucleoSpin RNA kit (NucleoSpin, Machery-Nagel) according to manufacturer's instruction. To eliminate possible genomic DNA contamination, DNase I treatment was used (NucleoSpin). The integrity of RNA was evaluated using 1.5% agarose gel electrophoresis, and RNA quantity was determined by a NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All RNA samples had 260/280 ratios of 1.8–2.0 and 260/230 values of 2.0–2.2. For cDNA synthesis, 2 μg of the extracted total RNA as template was reverse transcribed to cDNA using 200 U of Superscript III reverse transcriptase (Thermo scientific, USA), oligo (dT) primers and random hexamers according to the manufacturer's protocol. All cDNA was stored at −20 °C until use. Primers for each gene were designed according to the reported sequences for rainbow trout genes (HSP70, IGF-1, MHC, MSTN-1a and FST) in GeneBank database (NCBI) using primer 3 software (v. 0.4.0) (http://bioinfo.ut.ee/primer3/), and were further validated by Primer-BLAST. The specificity and size of the obtained amplicons were checked on 1.5% agarose gel and in melting curves after each qPCR experiment. Primers were synthesized by Macrogen incorporation (Seoul, South Korea, Table 1). Quantitative PCR was conducted using a Master Mix (5 × HOT FIREPol® EvaGreen® qPCR Mix Plus, no ROX; Solis BioDyne Inc.) and a Rotor-Gene Q System (Qiagen, Germany). A qPCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles consisting of a 20 s of denaturation at 95 °C, 20 s of annealing at 60 °C and finally, 20 s of extension at 72 °C. Fluorescence readings were performed at the end of each cycle. The mRNA expression of target genes relative to the reference gene (β-actin) was calculated by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001); where $\Delta\Delta CT = (Ct_{\text{target gene in treatment}} - Ct_{\text{reference gene in treatment}}) - (Ct_{\text{target gene in control}} - Ct_{\text{calibrator gene in control}})$. The LD group was considered as control in the formula.

2.5. Statistical analyses

Statistical analyses of data were carried out using the SPSS statistical package program (ver. 17.0, IBM, New York, NY, USA). Biochemical and gene expression data were expressed as means ± standard error (SEM). For statistical analysis among groups on days 20, 40 and 60, one-way ANOVA was used to detect the differences ($p < 0.05$). When a significant difference was found, Duncan's multiple range test was used.

3. Results

3.1. Mortality and growth performance

There was no mortality in LD group, but there were a 1.5 and 2.5% mortality in MD and HD, respectively. According to growth performance (Table 2), there were no significant differences between LD and MD in final BW, but it was reduced significantly at the HD treatments ($p < 0.05$). HD weight decreased as 7.34% and 6.24% compared to LD and MD weight, respectively. No differences were observed in final TL and FL as well as SGR among treatments. In contrast, CF in both MD and HD treatments were significantly decreased, and FCR was significantly increased in HD only, compared to LD ($p < 0.05$). FI of pectoral, pelvic, anal and dorsal fins (Data were not shown) as well as HSI and VSI (Table 3) showed no significant difference among tested SD groups. Moreover, there were no mortality in RD treatments during the 20-day period. There were no significant changes at all measured growth parameters in RD compared to HD ones, but final BW and CF values were still significantly lower than LD ($p < 0.05$, Table 2). Also, FCR values were meaningfully higher in RD compared to LD groups ($p < 0.05$, Table 2). Likewise, the RD treatments did not have any major difference in final TL and FL, SGR, FI, HSI and VSI (Tables 2 and 3).

3.2. Plasma cortisol and metabolites

As shown in Table 4, SD had no significant effect on the plasma levels of GLU, TP, TG and TCH. Also, plasma cortisol showed no significant differences among SDs during course of the study (Fig. 1). In addition, all the measured biochemical factors of RD treatments showed no significant difference compared to other groups ($p > 0.05$, Table 4). Moreover, the levels of cortisol decreased in RD compared to HD and, furthermore, were higher than LD values, but these changes were not meaningful (Fig. 1).

Table 1
The primers used for real-time PCR.

Gene symbol	Forward (5'→ 3')	Reverse (5'→ 3')	Genbank accession no
HSP70	GAACAAATCCTTCAACCCAGAG	TTGAAGTAGGCAGGGACTGTG	NM_001124228.1
IGF-1	TGTGTCTCCTGTACCCACACC	GCCTCTCTCTCCACACACAAAC	NM_001124696.1
MHC	CCAGCACATGTCTAAGTTCAGG	TCTGAGCTTGTTGACCTGAGTC	Z48794.1
MSTN-1a	GAAGTCACCACCGTGTTTCTG	TTGTTTCACGTCGATACTTTGC	NM_001124282.1
FST	GCAAGATTAACAGGAGAAGCAAG	ACCCGTCTTTGTATGTCTTTCC	NM_001160483.1
β-ACT	CTTGCAGCCTTAAGTCTTGGTC	GGCACCCTAATCACCTCTGAC	NM_001124235.1

Forward and reverse primer sequences for rainbow trout HSP70, IGF-1, MHC, MSTN-1a, FST and β-ACT genes are shown.

Table 2
Growth performance and related indices in young-of-the-year rainbow trout reared under different stocking density and subsequently recovered.

	Initial weight(g)	Final weight(g)	Initial TL (cm)	Final TL (cm)	Initial FL (cm)	Final FL (cm)	SGR	CF	FCR
LD	406.78 ± 6.21	685.48 ± 12.21[a]	33.08 ± 0.22	38.17 ± 0.25	31.99 ± 0.16	37.61 ± 0.21	0.87 ± 0.04	1.28 ± 0.01[a]	1.01[b]
MD	402.56 ± 4.07	677.44 ± 10.51 [a]	32.82 ± 0.09	38.26 ± 0.22	31.85 ± 0.09	37.81 ± 0.24	0.87 ± 0.03	1.24 ± 0.01[b]	1.03[b]
HD	404.52 ± 3.61	635.15 ± 7.61[b]	33.43 ± 0.11	38.41 ± 0.25	32.63 ± 0.15	37.21 ± 0.27	0.76 ± 0.03	1.23 ± 0.02[b]	1.15[a]
RD	556 ± 4.41	602.11 ± 17.11[b]	36.42 ± 0.41	38.22 ± 0.13	35.41 ± 0.31	37.15 ± 0.13	–	1.20 ± 0.02[b]	1.16[a]

Data are presented as mean ± SEM. Data was analyzed through one-way ANOVA besides Duncan comparisons. Data with different superscript letters (a, b) in the same column mean significant differences among experimental groups ($p < 0.05$). LD: low density, MD: medium density, HD: high density, RD: reduced density, TL: total length, FL: fork length, SGR: specific growth rate, CF: condition factor, FCR: feed conversion ratio.

3.3. Gene expression

The effect of SD on selected muscle stress- and growth-related genes was investigated at different days of sampling using real-time RT-PCR. The muscle HSP70 was upregulated in HD compared to LD at all days of sampling, and compared to MD on day 60 only ($p < 0.05$). But this difference was significant for MD compared to LD at the last sampling ($p < 0.05$, Fig. 2A) The HSP70 expression was 3.6, 2.4 and 6.2-fold higher in HD compared to LD on days 20, 40 and 60, respectively. Muscle IGF-1 mRNA expression remained unchanged among SDs. It increased in HD compared to LD on the first and third samplings but these differences were not statistically significant. No significant alterations were observed in MHC mRNA expression between LD and MD treatments. But, MHC expression was significantly decreased 58.3% in HD treatments compared to that of LD at the end of sampling ($p < 0.05$, Fig. 2C). White muscle MSTN-1a mRNA levels showed 1.08, 1.74 and 2.61-fold increase in HD compared to those of LD on days 20, 40 and 60, respectively, but these increases were significant only on day 60 ($p < 0.05$). No significant changes were observed in MSTN-1a expression between MD and HD treatments (Fig. 2D). Although the obtained qPCR results showed a slight increase in FST gene expression in MD and HD treatments compared to LD ones, they were not statistically significant (Fig. 2E). After 20-day reduction of density from 44 to 12 kg/m^3, the HSP70 and MSTN-1a expressions were downregulated in RD compared to HD groups ($p < 0.05$, Fig. 2A and D). In contrast, RD treatment caused significant increases in MHC expression compared to HD groups ($p < 0.05$, Fig. 2C). Finally, only HSP70 gene expressions were significantly higher in RD compared to LD groups ($p < 0.05$).

4. Discussion

Our results showed that the high density (HD) negatively affected the growth parameters (FW, CF and FCR) of young-of-the-year rainbow trout, but none of the growth parameters (except CF) differed significantly between LD and MD groups. Growth reduction is generally considered a good indicator of chronic stress (Valenzuela et al., 2017). In the present study, we selected the young sub-adult trout during their growing period because: 1) rainbow trout has the high BW increase (4–6 g/per day) during this age, and thus, growth differences are more evident, 2) rainbow trout stocked in ponds and tanks, especially in one-layer culture/single output table farms, usually experience high SD during final steps of their culture period (North et al., 2006a) and 3) the majority of stocking density (SD) studies in rainbow trout has focused on juveniles and younger fish, but the stress response to SD may be different by age (Bagley et al., 1994). Likewise, the study was carried on muscle. Skeletal muscle represents most of the body mass as lateral body musculature of trout comprises near 60% of their BW and the rate of protein accumulation in skeletal muscle largely determines growth rate (Johnston, 1999; Johnston et al., 2011).

Previous SD studies on rainbow trout have reported an affected growth in animals (Ellis et al., 2002; Naderi et al., 2017b). In our trial, trout strictly fed 1.3% BW with extruded food, and they consumed all the provided feed. Therefore, the amount of food consumed was not a limiting factor during the experiment. Although, it was observed that LD and to some extent, MD fish had higher appetite, and they could intake higher food amounts if it was provided. In contrast, the HD treatments, could not intake excess food, and sometimes, the extruded feed remained on the water level for a few minutes, until it was ultimately consumed. The reduction of appetite and satiety levels in HD may be an inherent/endogenous characteristic of fish rooted in, for example, nervous or neuroendocrine system, in a way that fish in crowded conditions is not as comfortable as non-crowded conditions for feed intake. It has been known that serotonin is implicated in the inhibition of food intake in vertebrates by reducing the activity of genes stimulating food intake (Mechaly et al., 2017). The elevated levels of brain seratonergic activity of rainbow trout under high SD has been improved (Laursen et al., 2013). In this regard, we hypothesize that less feeding energy was used for weight gain with increasing SD. This is in line with our findings, in which fish FW and CF were lower and fish were more emaciated in HD treatment. Therefore, higher energetic requirements of fish to cope with HD stress could cause reduced growth in trout (Wendelaar Bonga, 1997).

We found no significant alterations in all tested plasma biochemical factors among fish under different SD on days of sampling. It is worth noting, the first sampling took place at day 20, and we did not measure the changes in biochemical parameters at the onset of exposure to high SD to avoid the additional effects of handling as a stressor during first hours/days. It is known that changes in plasma biochemical factors usually take place during first hours/days of stress response and the changes usually return to their basal levels as time elapsed (Zahedi et al., 2013, 2014). Glucose and cortisol are usually increased during the first hours/days after exposure to stressful conditions, and

Table 3
Biometric indices in young-of-the-year rainbow trout reared under different stocking density and subsequently recovered.

	HSI (%)			VSI (%)		
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60
LD	1.06 ± 0.04	1.16 ± 0.1	0.99 ± 0.04	9.07 ± 0.62	8.8 ± 0.44	8.1 ± 0.35
MD	1.15 ± 0.08	1.1 ± 0.05	1.17 ± 0.07	9.6 ± 0.56	8.2 ± 0.42	9 ± 0.3
HD	1.14 ± 0.06	1.22 ± 0.05	1.03 ± 0.05	8.9 ± 0.35	9.09 ± 0.38	8.3 ± 0.36
RD	–	–	1.07 ± 0.06	–	–	8.3 ± 0.44

Data are presented as mean ± SEM ($n = 6$). Data was analyzed through one-way ANOVA besides Duncan comparisons. LD: low density, MD: medium density, HD: high density, RD: reduced density. HSI: hepatosomatic index, VSI: viscerosomatic index.

Table 4
Changes in plasma metabolites of young-of-the-year rainbow trout reared under different stocking density and subsequently recovered.

	LD			MD			HD			RD	
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60	Day 60
Glucose (mg/dL)	50.1 ± 2	60.17 ± 6.3	55.4 ± 3.8	60 ± 7.4	60.3 ± 4.9	55 ± 3.2	38.8 ± 7.8	59.3 ± 2.1	54.2 ± 5.1	59.5 ± 13.2
Total protein (g/dL)	4.2 ± 0.27	4.5 ± 0.2	4.7 ± 0.3	4.1 ± 0.08	4.6 ± 0.1	5.5 ± 0.1	5.03 ± 0.3	4.6 ± 0.1	4.6 ± 0.2	4.7 ± 0.3
triglyceride (mg/dL)	423.8 ± 71.9	282.3 ± 30.6	322.6 ± 34.5	338.3 ± 44.8	338.3 ± 50.5	261.2 ± 35.4	437.2 ± 33.8	409 ± 38.4	266.5 ± 26.2	346 ± 35.6
cholesterol (mg/dL)	385.8 ± 25.5	361.7 ± 47.6	394.8 ± 38.9	435.5 ± 35.4	324.3 ± 23.3	482.3 ± 71.1	363.3 ± 26.6	326 ± 17.4	363.3 ± 26.6	453.8 ± 25.2

Data are presented as mean ± SEM. (n = 5–6). Data were analyzed through one-way ANOVA besides Duncan comparisons. LD: low density, MD: medium density, HD: high density, RD: reduced density.

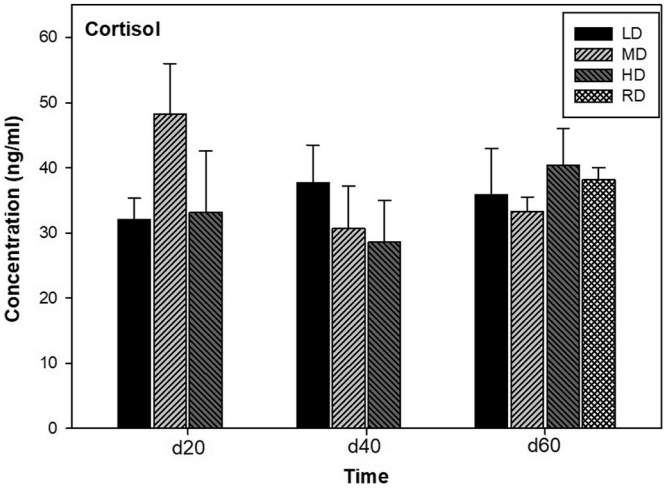

Fig. 1. Plasma cortisol concentrations (ng/mL) of young-of-the-year rainbow trout reared under different stocking density. LD (12 kg/m^3), MD (24 kg/m^3), HD (44 kg/m^3) and RD (reduced density from 44 to 12 kg/m^3) treatments on days 20, 40, 60 (mean ± SEM, n = 5–6).

subsequently decrease because of homeostatic mechanisms and acclimation of hypothalamus-pituitary-interrenal axis (Zahedi et al., 2013; Naderi et al., 2017b). Our results indicated that the levels of blood cortisol could not be considered as a good indicator during the long-term high SD experience. It has been already known that blood cortisol is not a reliable biomarker for chronic stress response especially during long-term exposures (Kebus et al., 1992; Ellis et al., 2002; Trenzado et al., 2007; Naderi et al., 2017b, 2017c; Rebl et al., 2017). Similarly, plasma total proteins (TP), triglycerides (TG) and cholesterol (TCH) levels did not change during the 60-day exposure to HD. TP has widely been used in SD investigations. Previous authors reported decreased plasma TP (Suárez et al., 2015) or unchanged values (Yarahmadi et al., 2016; Naderi et al., 2017a) in rainbow trout exposed to higher densities. Furthermore, Suárez et al. (2015) detected lower levels of TG and TCH in rainbow trout reared at higher SD. In contrast, and in line with the current findings, SD had no effects on serum TG and TCH in Atlantic salmon (Liu et al., 2017).

The muscle transcripts of HSP70 were significantly increased in HD compared to LD at all sampling times. HSPs are induced by different stressors, and they protect the proteins inside the cells (Iwama et al., 2004). High SD can significantly induce the expression HSP70 at the mRNA (Gornati et al., 2004) and protein levels (Küçükbay et al., 2009; Naderi et al., 2017c). It is known that the upregulation of HSP70 is related with muscle protein repair and protection, and myosin heavy chain fiber type changes, as well (Locke et al., 1991; Kilgore et al., 1998). Our results suggested that HD is not totally safe for young-of-the-year rainbow trout and may partially cause stressful conditions and subsequent changes in the expression of certain molecules that may further result in manifestation of tertiary stress responses.

The results of the present study showed that the expression of IGF-1 did not show a significant trend in muscle kept at HD (up to 44 kg/m^3). Fasting and prolonged stress can result in decreased growth and IGF-1 expression (Montserrat et al., 2007), and environmental and physical stressors affect growth functions and GH/IGF-1 axis (Pickering et al., 1991; Pickering, 1993). Valenzuela et al. (2017) showed that, in fine flounder (*Paralichthys adspersus*), SD directly affects muscle growth and downregulates the GH/IGF system.

The expression of MHC gene was decreased in HD compared to LD at the last sampling. This may be related to the higher protein degradation/catabolic mechanisms in higher culture density. Accordingly, Valenzuela et al. (2017) showed that crowding stress in fine flounder (*Paralichthys adspersus*) triggers a concomitant activation of the ubiquitin-proteasome system and apoptosis, followed by the activation of

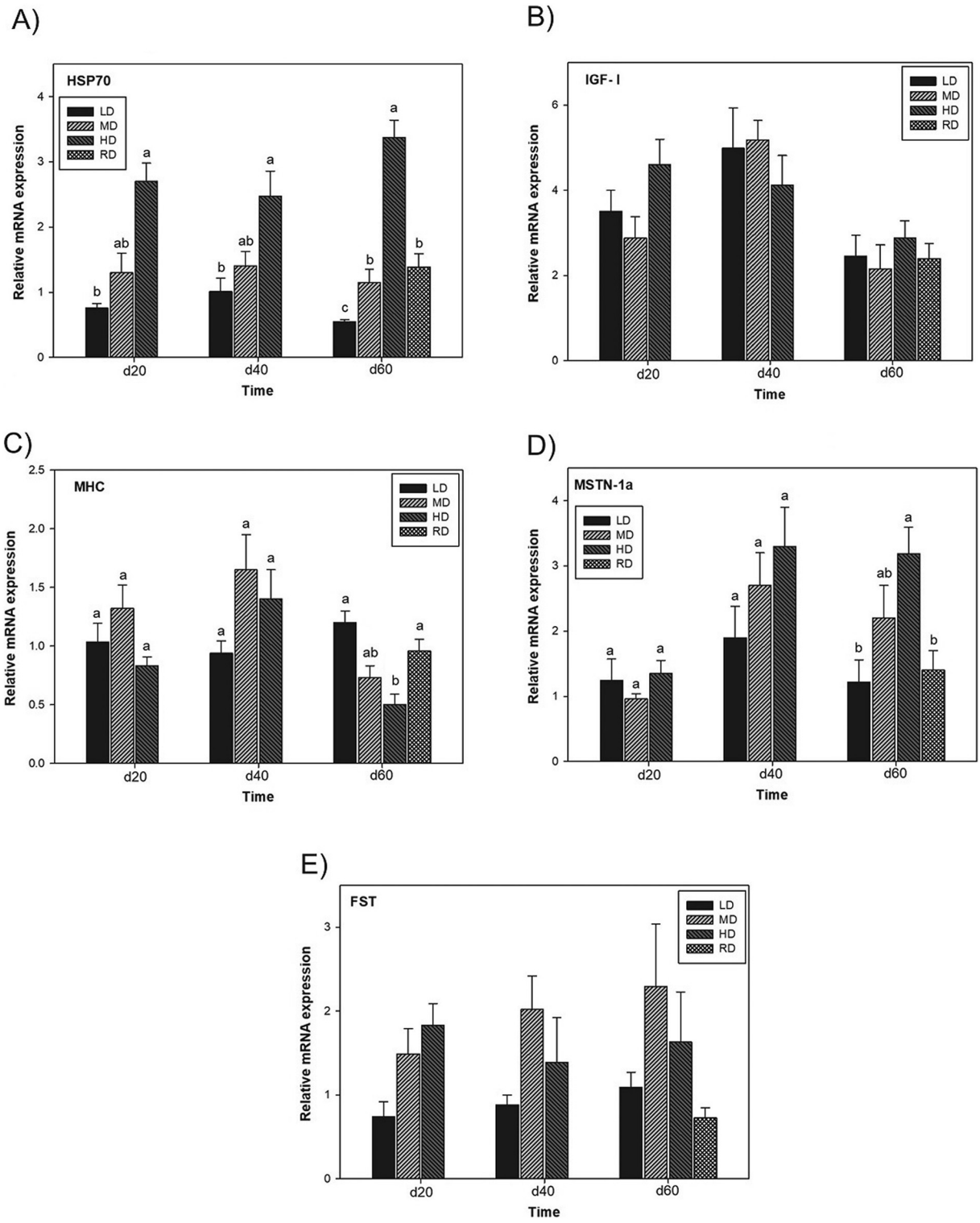

Fig. 2. Gene expressions in white muscle of young-of-the-year rainbow trout reared under different stocking density. Significant differences among treatments in each sampling day were determined by ANOVA one-way besides Duncan comparisons. Different letters (a, b and c) mean significant differences among groups in a same time ($p < 0.05$). LD (12 kg/m^3), MD (24 kg/m^3), HD (44 kg/m^3) and RD (reduced density from 44 to 12 kg/m^3) treatments on days 20, 40, 60 (mean ± SEM, n = 5–6). A (HSP70, heat shock protein 70), B (IGF-1, insulin-like growth factor-1), C (MHC, myosin heavy chain), D (MSTN-1a, myostatin 1a) and E (FST, follistatin).

autophagy.

The presented findings revealed significant increases in muscular MSTN-1a transcripts in HD compared to LD (day 60). Contrary to the findings of the present study, high SD is reported to have no effect on muscle and brain MSTN expression in zebra fish *Danio rerio* (Helterline et al., 2007). Interestingly, the increase of MSTN-1a mRNA levels was consistent with trout BW decrease, probably as a result of decreased muscle protein synthesis.

Our results suggested that FST expression was unchanged during the course of the study. FST and FST-related proteins have been shown to

be potent antagonists of MSTN (Rebhan and Funkenstein, 2008). In a study on transgenic rainbow trout, overexpressing FST, Medeiros et al. (2009) showed double muscling resulted from inactivation of MSTN through the binding of increased protein levels of FST. On this point, more studies are required to determine the SD effects on FST, MSTN and other muscle regulator factors.

The growth parameters were unchanged in reduced density (RD) treatments compared to HD ones; but final BW, CF and FCR were still different compared to LD (Table 2). Therefore, the 20-day recovery period did not cause a full recovery in RD growth parameters to reach the LD ones. The results of present RD treatments were against our previous experiences and observations in the large-scale trout culture where the reduction of SD to lower values has positive and compensatory effects on rainbow trout growth, and it can be employed as a culture strategy by farmers specially in young and sub-adult stages of rainbow trout (> 300–2000 g). With these results, we speculate that those observations in large-scale culture might be related to water quality improvement as a result of decreased fish density not SD per se since water quality is a critical factor in growth of fish under high SD (Ellis et al., 2002). The results of the present study can be explained by the selected reduced density (12 Kg/m^3) that could have exposed the fish to a dramatic decrease. Thus, this new conditions might increase either social interactions or activity in RD groups. For future studies, we propose a lower reduction of density for RD treatments in order to better understanding of physiological effects on fish.

HSP70 expression decreased in RD compared to HD but it was still higher than LD suggesting that the duration of RD (20 days) was not long enough for complete recovery and, probably, more recovery time was required. Here, IGF-1 expression decreased in RD compared to HD but it was not significant. IGF-1 recovery after a period of stress has been documented in some studies. Chauvigné et al. (2003) showed that fast myotomal muscle IGF-I and IGF-II mRNA levels in rainbow trout significantly increased after 4 and 34 days of re-feeding, respectively. In addition, MHC increased in RD compared to HD but there was no significant difference between RD and LD. Increased expression of cytoskeleton and myosin mRNA transcripts was observed in rainbow trout after 7–36 days of re-feeding, demonstrating that a recovery or repair period from starvation was needed prior to the growth resumption (Rescan et al., 2007). White muscle MSTN-1a mRNA expression decreased in RD compared to HD, and it could reach to LD values. Finally, FST changes remained unchanged during RD compared to both HD and LD. According to this parameters, it is difficult to compare results with previous data since literature is lacked.

5. Conclusion

In conclusion, the 60-day treatment of increased SD level to HD caused chronic crowding stress and affected the growth parameters in young-of-the-year rainbow trout. Consonant to the observed growth alterations, the expression of stress- and growth-related genes suggested that chronic stress of SD up to 44 kg/m^3 in an open system without oxygenation is not totally safe. Furthermore, the reduction of SD from HD to LD caused some recovery in gene expression levels in fish, but did not result in a compensatory growth. It is likely that 20-day RD might not be long enough to cause complete recovery, or a pronounced decrease in SD could have caused this observation. More investigations are required to study the specific mechanisms and signaling pathways during SD reduction.

Competing interests

All authors declare that they have no competing interest.

Acknowledgments

We would like to thank Hormozgan University for supporting this work under student research grant contract (8, 1393/3/21). We thank Dr. Mohsen Navari, Dr. Behzad Mohammadi, Mr. Shahvali, and Mrs. Aboolfathi for their useful comments. We also express our deep sense of gratitude to Sadaf 1 and 2 staff for their help during the course of this work.

References

Bagley, M.J., Bentley, B., Gall, G.A., 1994. A genetic evaluation of the influence of stocking density on the early growth of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 121 (4), 313–326.
Baker, R.F., Ayles, G.B., 1990. The effects of varying density and loading level on the growth of Arctic charr (*Salvelinus alpinus* L.) and rainbow trout (*Oncorhynchus mykiss*). World Aquacult. 21 (1), 58–62.
Baldwin, L., 2011. The effects of stocking density on fish welfare. Plymouth Stud. Sci. 4 (1), 372–383.
Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295 (2), 173–183.
Bosakowski, T., Wagner, E.J., 1994. Assessment of fin erosion by comparison of relative fin length in hatchery and wild trout in Utah. Can. J. Fish. Aquat. Sci. 51 (3), 636–641.
Castillo, J., Ammendrup-Johnsen, I., Codina, I., Navarro, I., Gutiérrez, J., 2006. IGF-I and insulin receptor signal transduction in trout muscle cells. Am. J. Phys. Regul. Integr. Comp. Phys. 290 (6), 1683–1690.
Chauvigné, F., Gabillard, J.C., Weil, C., Rescan, P.Y., 2003. Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. Gen. Comp. Endocrinol. 132 (2), 209–215.
Duan, C., Ren, H., Gao, S., 2010. Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins: roles in skeletal muscle growth and differentiation. Gen. Comp. Endocrinol. 167 (3), 344–351.
Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. J. Fish Biol. 61 (3), 493–531.
FAO, 2016. The State of World Fisheries and Aquaculture. FAO, Rome, Italy.
Foo, J.T.W., Lam, T.J., 1993. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in tilapia, *Oreochromis mossambicus*. Aquaculture 115, 145–158.
Gabillard, J.C., Biga, P.R., Rescan, P.Y., Seiliez, I., 2013. Revisiting the paradigm of myostatin in vertebrates: insights from fishes. Gen. Comp. Endocrinol. 194, 45–54.
Garikipati, D.K., Gahr, S.A., Rodgers, B.D., 2006. Identification, characterization, and quantitative expression analysis of rainbow trout myostatin-1a and myostatin-1b genes. J. Endocrinol. 190 (3), 879–888.
Gornati, R., Papis, E., Rimoldi, S., Terova, G., Saroglia, M., Bernardini, G., 2004. Rearing density influences the expression of stress-related genes in sea bass (*Dicentrarchus labrax*, L). Gene 34, 111–118.
Helterline, D.L., Garikipati, D., Stenkamp, D.L., Rodgers, B.D., 2007. Embryonic and tissue-specific regulation of myostatin-1 and-2 gene expression in zebrafish. Gen. Comp. Endocrinol. 151 (1), 90–97.
Iwama, G.K., 2007. The welfare of fish. Dis. Aquat. Org. 75, 155–158.
Iwama, G.K., Afonso, L.O.B., Todgham, A., Ackerman, P., Nakano, K., 2004. Are hsps suitable for indicating stressed states in fish? J. Exp. Biol. 207, 15–19.
Johnston, I.A., 1999. Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177 (1), 99–115.
Johnston, I.A., Bower, N.I., Macqueen, D.J., 2011. Growth and the regulation of myotomal muscle mass in teleost fish. J. Exp. Biol. 214 (10), 1617–1628.
Kebus, M.J., Collins, M.T., Brownfield, M.S., Amundson, C.H., Kayes, T.B., Malison, J.A., 1992. Effects of rearing density on the stress response and growth of rainbow trout. J. Aquat. Anim. Health 4 (1), 1–6.
Kilgore, J.L., Musch, T.I., Ross, C.R., 1998. Physical activity, muscle, and the HSP70 response. Can. J. Appl. Physiol. 23 (3), 245–260.
Küçükbay, F.Z., Yazlak, H., Karaca, I., Sahin, N., Tuzcu, M., Cakmak, M.N., Sahin, K., 2009. The effects of dietary organic or inorganic selenium in rainbow trout (*Oncorhynchus mykiss*) under crowding conditions. Aquac. Nutr. 15 (6), 569–576.
Laursen, D.C., Silva, P.I., Larsen, B.K., Höglund, E., 2013. High oxygen consumption rates and scale loss indicate elevated aggressive behaviour at low rearing density, while elevated brain serotonergic activity suggests chronic stress at high rearing densities in farmed rainbow trout. Physiol. Behav. 122, 147–154.
Liu, B., Liu, Y., Sun, G., 2017. Effects of stocking density on growth performance and welfare-related physiological parameters of Atlantic salmon *Salmo salar* L. in recirculating aquaculture system. Aquac. Res. 48 (5), 2133–2144.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25 (4), 402–408.
Locke, M.A.R.I.U.S., Noble, E.G., Atkinson, B.G., 1991. Inducible isoform of HSP70 is constitutively expressed in a muscle fiber type specific pattern. Am. J. Phys. Cell Physiol. 261 (5), C774–C779.
Mechaly, A.S., Richardson, E., Rinkwitz, S., 2017. Activity of etv5a and etv5b genes in the hypothalamus of fasted zebrafish is influenced by serotonin. Gen. Comp. Endocrinol. 246, 233–240.
Medeiros, E.F., Phelps, M.P., Fuentes, F.D., Bradley, T.M., 2009. Overexpression of follistatin in trout stimulates increased muscling. Am. J. Phys. Regul. Integr. Comp. Phys. 297 (1), R235–R242.
Montserrat, N., Gabillard, J.C., Capilla, E., Navarro, M.I., Gutiérrez, J., 2007. Role of

insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 150 (3), 462–472.
Naderi, M., Keyvanshokooh, S., Salati, A.P., Ghaedi, A., 2017a. Effects of chronic high stocking density on liver proteome of rainbow trout (*Oncorhynchus mykiss*). Fish Physiol. Biochem. 1-13.
Naderi, M., Keyvanshokooh, S., Salati, A.P., Ghaedi, A., 2017b. Effects of dietary vitamin E and selenium nanoparticles supplementation on acute stress responses in rainbow trout (*Oncorhynchus mykiss*) previously subjected to chronic stress. Aquaculture 473, 215–222.
Naderi, M., Keyvanshokooh, S., Salati, A.P., Ghaedi, A., 2017c. Proteomic analysis of liver tissue from rainbow trout (*Oncorhynchus mykiss*) under high rearing density after administration of dietary vitamin E and selenium nanoparticles. Comp. Biochem. Physiol. Genom. Proteom. 22, 10–19.
North, B.P., Ellis, T., Turnbull, J.F., Davis, J., Bromage, N.R., 2006a. Stocking density practices of commercial UK rainbow trout farms. Aquaculture 259 (1), 260–267.
North, B.P., Turnbull, J.F., Ellis, T., Porter, M.J., Migaud, H., Bron, J., Bromage, N.R., 2006b. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 255 (1), 466–479.
Overturf, K., Hardy, R.W., 2001. Myosin expression levels in trout muscle: a new method for monitoring specific growth rates for rainbow trout *Oncorhynchus mykiss* (Walbaum) on varied planes of nutrition. Aquac. Res. 32 (4), 315–322.
Pickering, A.D., 1993. Growth and stress in fish production. Aquaculture 111 (1), 51–63.
Pickering, A.D., Pottinger, T.G., Sumpter, J.P., Carragher, J.F., Le Bail, P.Y., 1991. Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83 (1), 86–93.
Rebhan, Y., Funkenstein, B., 2008. Inhibition of fish myostatin activity by recombinant fish follistatin and myostatin prodomain: potential implications for enhancing muscle growth in farmed fish. Aquaculture 284 (1), 231–238.
Rebl, A., Zebunke, M., Borchel, A., Bochert, R., Verleih, M., Goldammer, T., 2017. Microarray-predicted marker genes and molecular pathways indicating crowding stress in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 473, 355–365.
Rescan, P.Y., Montfort, J., Rallière, C., Le Cam, A., Esquerré, D., Hugot, K., 2007. Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. BMC Genomics 8 (1), 438.
Rodgers, B.D., Weber, G.M., Kelley, K.M., Levine, M.A., 2003. Prolonged fasting and cortisol reduce myostatin mRNA levels in tilapia larvae; short-term fasting elevates. Am. J. Phys. Regul. Integr. Comp. Phys. 284, R1277–R1286.
Schneyer, A.L., Sidis, Y., Gulati, A., Sun, J.L., Keutmann, H., Krasney, P.A., 2008. Differential antagonism of activin, myostatin and growth and differentiation factor 11 by wild-type and mutant follistatin. Endocrinology 149 (9), 4589–4595.
Seiliez, I., Taty, G.C.T., Bugeon, J., Dias, K., Sabin, N., Gabillard, J.C., 2013. Myostatin induces atrophy of trout myotubes through inhibiting the TORC1 signaling and promoting Ubiquitin–Proteasome and Autophagy-Lysosome degradative pathways. Gen. Comp. Endocrinol. 186, 9–15.
Suárez, M.D., Trenzado, C.E., García-Gallego, M., Furné, M., García-Mesa, S., Domezain, A., Alba, I., Sanz, A., 2015. Interaction of dietary energy levels and culture density on growth performance and metabolic and oxidative status of rainbow trout (*Oncorhynchus mykiss*). Aquac. Eng. 67, 59–66.
Trenzado, C.E., de la Higuera, M., Morales, A.E., 2007. Influence of dietary vitamins E and C and HUFA on rainbow trout (*Oncorhynchus mykiss*) performance under crowding conditions. Aquaculture 263 (1), 249–258.
Valenzuela, C.A., Zuloaga, R., Mercado, L., Einarsdottir, I.E., Bjornsson, B.T., Valdes, J.A., Molina, A., 2017. Chronic stress inhibits growth and induces proteolytic mechanisms through two different non-overlapping pathways in the skeletal muscle of a teleost fish. Am. J. Phys. Regul. Integr. Comp. Phys. 314 (1), 102–113.
Videler, J.J., 2011. An opinion paper: emphasis on white muscle development and growth to improve farmed fish flesh quality. Fish Physiol. Biochem. 37 (2), 337–343.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.
Yarahmadi, P., Miandare, H.K., Fayaz, S., Caipang, C.M.A., 2016. Increased stocking density causes changes in expression of selected stress-and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 48, 43–53.
Zahedi, S., Mirvaghefi, A., Rafati, M., Mehrpoosh, M., 2013. Cadmium accumulation and biochemical parameters in juvenile Persian sturgeon, *Acipenser persicus*, upon sub-lethal cadmium exposure. Comp. Clin. Pathol. 22 (5), 805–813.
Zahedi, S., Mirvaghefi, A., Rafati, M., Rafiee, G., Mojazi Amiri, B., Hedayati, M., Makhdoomi, C., Zarei Dangesaraki, M., 2014. The effect of sub-lethal exposure to copper and the time course of recovery in clean water on biochemical changes in juvenile fish (*Acipenser persicus*). Mar. Freshw. Behav. Physiol. 47 (4), 253–264.