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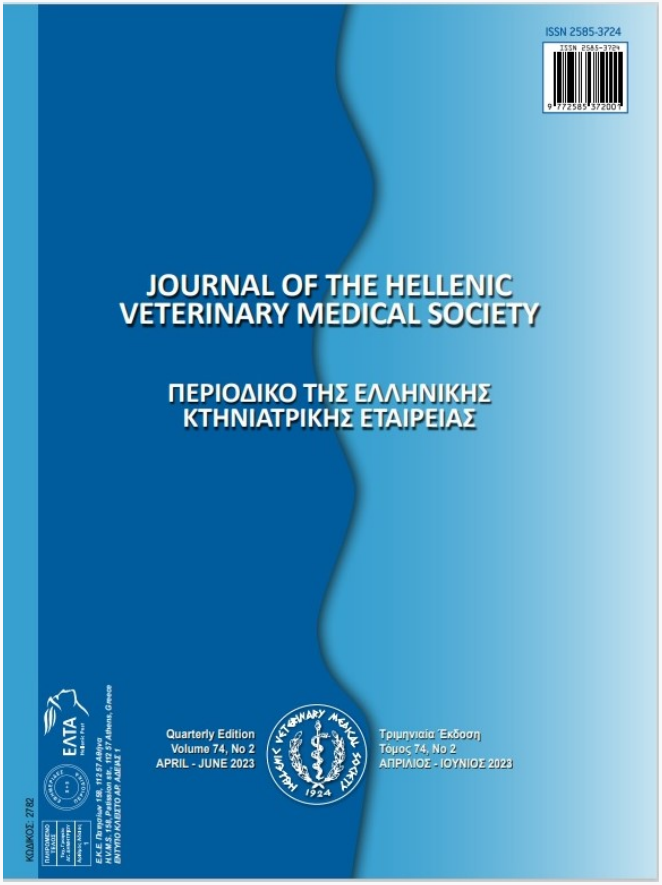

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Effects of age and seasonal temperatures on cortisol levels and GHR, IGF-I, and IGF-II expressions in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT: Cellular growth and organ development are regulated by the growth hormone (GH)/insulin-like growth factor (IGF) endocrine axis with regard to alternating environmental conditions and age. The objective of the current research is a) to identify growth factor genes in the muscles and liver of rainbow trout at various seasonal temperatures and in different age ranges and b) determine serum cortisol hormone levels, a stress marker. Serum cortisol levels did not differ between the trout groups with respect to temperature and age. The highest serum glucose concentrations were observed in adult trout in summer ($p<0.001$). The mean GH concentration in juvenile and adult trout was found to be higher in the summer season than in the winter season ($p<0.001$). No difference was detected in the liver GHR mRNA levels of juvenile and adult trout in winter, while IGF-I and IGF-II mRNA levels in the liver and muscle tissues were higher in juvenile trout compared to adults. Among trout of different ages, GHR, IGF-I, and IGF-II mRNA levels in the liver in summer were revealed to be higher in juvenile trout in comparison with adults. Muscle IGF-I mRNA levels in summer were found to be higher in adult trout than in juveniles. Liver and muscle IGF-II mRNA levels in juvenile trout increased in winter compared to summer. While GHR and IGF-II mRNA levels in the liver tissue of adult trout were higher in winter compared to summer, IGF-I mRNA levels were higher in summer. GHR, IGF-I, and IGF-II mRNA levels in the muscle tissue of adult trout were found to be higher in summer in comparison with winter. In conclusion, the expression of the GHR, IGF-I, and IGF-II genes in rainbow trout was affected by changing seasonal temperatures.

Keywords: Seasonal temperature; age; cortisol; growth hormone; insulin-like growth factor

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INTRODUCTION

The biology of fish is affected by diverse endogenous and exogenous factors, including seasonal temperature, age, dissolved oxygen, and salinity, controlling their vital activities. Seasonal temperature affects endocrine hormone activities, growth efficiency, reproduction and immune ability of fish, whereas water temperatures exceeding the threshold value lead to fish deaths. Moreover, temperature alters the physiological functions related to the stress response of fish. First, the physiological stress response in fish involves the release of stress hormones, e.g., cortisol. An increase in plasma glucose levels usually occurs after elevated plasma cortisol levels in fish. It is possible to utilize biochemical parameters, e.g., plasma cortisol and glucose levels, as general stress indicators in fish (Canosa *et al.,* 2007; Akhtar *et al.,* 2013; Fürtbauer and Heistermann, 2016).

Environmental signals, e.g., photoperiod, temperature, and food integrated with neuroendocrine responses to the genetic code, primarily forming the structure of the organism, regulate growth. Insulin-like growth factors (IGFs) represent the main components of multiple processes that control metabolism and growth (Le Roith *et al.,* 2001). Various correlations between IGFs and growth are induced by acute temperature alterations. However, adaptation to permanent differences in environmental conditions usually leads to concordant relationships. In general, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) can be utilized in an effective way as growth indices for fish in distinguishing between fish with various physiological statuses and discriminating and classifying differences among habitats (Beckman, 2011). It is a known fact that the growth-promoting impacts of growth hormone (GH) in ectotherms, such as teleost fish, are mediated by IGF-I through the autocrine/paracrine pathway (Reinecke, 2010). Furthermore, as a direct effect, GH directly initiates the intracellular signaling pathway by binding to the growth hormone receptor (GHR) in target cells (Dehkhoda *et al.,* 2018).

Today, producing rainbow trout, among the primary cultured species, is the major source for Turkish aquaculture with an increasing trend. Therefore, the optimum temperature requirements of rainbow trout of different ages grown naturally in aquaculture should be determined, and the highest production efficiency should be obtained in the shortest time and with the least loss. Since the growth rate of fish represents the main factor in aquaculture, the impacts of temperature, nutrient conditions, and age on the gene expression of GHR and IGFs have significant potential in planning the optimization of rainbow trout health and production. Therefore, the objective of our research is to reveal whether the growth-promoting impact of temperature can be associated with an alteration in IGF-I, GF-II, and GHR gene expressions in juvenile and adult rainbow trout.

MATERIALS AND METHODS

Animals and experimental design

Juvenile and adult rainbow trout weighing between 50 and 1000 g were obtained from a private farm (Çine, Aydın, Turkey) in the winter and summer seasons. Juvenile and adult trout were fed in ponds at the same time, daily with *ad libitum* food intake. During the sample collection, water temperatures were measured as 18 °C in winter (mid-January) and 27 °C in summer (mid-July). The trout were divided into four groups as winter juvenile trouts (50-100 g, n = 10), winter adult trouts (500-1000 g; n = 10), summer juvenile trouts (50-100 g, n = 10), and summer adult trouts (500-1000 g, n = 10). Approval for our research was received from the Experimental Research Ethics Committee of Aydın Adnan Menderes University (Ethical Council Number: 654583101/2014/140).

During blood collection from the tail vein, the fish were anesthetized with quinaldine at a 1/20,000 mg/L concentration. After anesthesia, 2-2.5 ml of blood was collected into sterile tubes by entering the tail vein of the fish with an injector. The blood collection was completed in 45 seconds at the latest. Then, the fish was dissected, and specimens of white skeletal muscles from the body's dorsal part and of the liver were removed. The obtained tissues were frozen immediately in liquid nitrogen and kept at a temperature of – 80 °C until the analysis was conducted.

Biochemical measurements

Serum was acquired by centrifuging a whole blood sample at 10,000 × g for a period of 5 min. After centrifugation, the measurement of serum glucose levels (mg/dl) was performed by the colorimetric measurement of glucose oxidase enzyme activity (Tietz, 1994). COR levels (μM) and GH levels (pg/ml) were determined by utilizing an ELISA kit (Bioassay Technology Laboratory, China) and an ELISA kit (Cusabıo, China), respectively.

RNA isolation from tissues and cDNA synthesis

The extraction of total RNA was carried out from liver and muscle tissues with a commercial RNA isolation kit (Roche, Germany). The NanoDrop 1000 spectrophotometer (Thermo Scientific, San Jose, USA) was utilized to confirm the concentration and purity of RNA for every sample. RNA samples were kept at a temperature of – 80 °C until their use.

Following RNA isolation, cDNA synthesis was performed with a cDNA commercial kit by utilizing the LightCycler Nano Real-Time PCR System (Roche, Germany). For cDNA synthesis, 1 µg of total RNA, 1 µl of hexamer primer, 1 µl of oligo primer (dT), and 2 µl of dH_2O were added to PCR tubes. To complete the total volume to 13 µL, 9 µl of total RNA was added to the reaction mix. The incubation of the reaction mix was performed at a temperature of 65 °C for a period of 10 min, and 4 µl of the reaction buffer, 0.5 µl of RNase inhibitor, 2 µl of dNTP, 0.5 µl of transcriptor reverse transcriptase were added to the reaction mix. Then cDNA was amplified at a temperature of 55 °C for a period of 60 min, following which it was heated at a temperature of 85 °C for a period of 5 min.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative real-time PCR (RT-PCR) was carried out with a LightCycler 480 SYBR Green I master kit for the GHR, IGF-I, and IGF-II genes (Roche, Germany). The synthesis of all primers was performed by Genmar (Izmir/Turkey), and their sequences are presented in Table 1. Thermal cycling conditions were determined as initial activation at a temperature of 95 °C for 10 min, followed by 45 PCR cycles at 95 °C for 5 s, 60 °C for 10 s, 72 °C for 15 s, and a final extension at 60 °C for 20 s. Systematic monitoring of a melting curve was carried out at the end of every run with the objective of confirming the specificity of the amplification reaction. The cycle threshold was measured for every sample, and relative mRNA abundance was calculated according to that of the β-actin reference gene. The comparative CT method was employed for the purpose of estimating the relative mRNA expression. The $2^{-\Delta\Delta CT}$ method was utilized with the aim of determining the relative mRNA expression (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was carried out using the SPSS 22.0 (SPSS Inc., Chicago, IL, USA) package program. The Shapiro-Wilk test was used to test all obtained data in serum for normality, statistical evaluations among the groups were made by one-way ANOVA analysis, and Tukey's test was employed as a post hoc test. Differences between means were accepted as significant at the $p<0.001$ (95%) probability level. The Mann-Whitney U test was carried out with the objective of testing the significance of pairwise differences in gene expressions, and an overall 5% type-1 error level was employed for inferring statistical significance ($p<0.05$).

RESULTS AND DISCUSSION

Serum glucose, cortisol, and GH levels

Table 2 contains serum glucose, cortisol, and GH levels related to age factor and temperature changes. Our study determined that trout was insensitive to seasonal temperature changes and serum cortisol hormone levels did not change between the groups (F: 0.84; $p>0.001$). The reason for this may be that the cortisol hormone secreted in the organism is released from the gills to the ambient water after reaching the target tissues. The results were in line with the studies by Pankhurst and King (2010) and Zarejabad *et al.* (2010), indicating that serum cortisol levels did not differ statistically between trout at low and high temperatures.

Table 1. Sequences of PCR primers for detecting the three target genes (GHR, IGF-I, and IGF-II) and the reference gene (β-actin) in rainbow trout by real-time PCR.

Primer name	Sequence (5′–3′)	Amplicon (bp)	NCBI Accession no.
IGF-I	F: GTGACATTGCCTGCATCTTATC	863bp	NM001124696
	R: CTGGAAGAAATGACCGCTAGA		
IGF-II	F: ACTCTTGCGCTCTCTCTTTCAT	1148bp	NM001124697
	R: CCGCTAAGGATCCACCTAAAT		
GHR	F: TTCGAGGCAGGAGAATCATCA	2822bp	NM001124731
	R: CCCACTAAAGAGTCCCGATTTC		
β-*actin*	F: GATCTGGCATCACACCTTCTA	1958bp	NM001124235
	R: TCTTCTCCCTGTTGGCTTTG		

F=forward primer; R=reverse primer

The maximum serum glucose concentrations were observed in adult trout in summer (F: 3.18; p<0.001). It was determined that the stress response to temperature changes occurred in adult trout. There may be two reasons for this increase. First, the rising seasonal temperature increased the nutritional requirement of adult trout depending on body size and, thus, changed the enzyme activities playing a role in carbohydrate, lipid, and protein metabolism. Second, glycogen stores in muscle and liver tissues may have decreased, and the released glucose may have increased circulating levels to meet the metabolic energy needs at high temperatures (Vargas *et al.,* 2009). However, serum glucose levels decreased in juvenile trout in winter. This may be due to the slowing of metabolism in relation to reduced food intake at low temperatures.

It is known that GH regulates growth in fish at different water temperatures (Sloat and Reeves, 2014; Panıcz *et al.,* 2015). Previous research has reported that serum GH levels increase at high temperatures (Figueroa *et al.,* 2009; Saera *et al.,* 2007). In our research, the mean GH concentrations in juvenile and adult trout were found to be higher in summer than in winter (F: 3.60; p<0.001). This may have originated from elevated metabolic activity at high temperatures. It is also thought that elevated metabolic activity at high summer temperatures increases pituitary secretion and thus increases circulating GH secretion. Shrimpton *et al.* (2000) stated that juvenile salmon had very low plasma GH levels in spring compared to adult salmon. Our study found no statistical difference between the GH levels of juvenile and adult trout at similar seasonal temperatures.

GHR, IGF-I, and IGF-II mRNA expression levels in the liver and muscle tissues of rainbow trout with seasonal temperature changes

Statistical analysis demonstrated that GHR, IGF-I, and IGF-II gene expressions varied significantly between various seasons (Figures 1, 2, 3). In our research, the GHR, IGF-I, and IGF-II gene expressions in adult trout exhibited temperature sensitivity and seasonal variation. The expression of GHR in the liver of adult trout was found to be significantly higher in winter compared to summer (2.34-fold), whereas it increased by 5.90-fold in the muscle tissue in summer (p<0.05) (Figure 1). Despite high GH levels in adult trout in summer, liver GHR mRNA levels increased in winter. This may be due to the inhibition of receptor activation as a result of the induction of intracellular inhibitors or the prevention of receptor activation by high GH levels. In our study, liver and muscle IGF-I mRNA expressions in adult trout were detected to be higher in summer in comparison with winter (2.85-fold and 3.40-fold, respectively) (p<0.05) (Figure 2). It was determined that high serum GH levels in adult trout in summer increased IGF-I gene expression in the liver tissue by an indirect effect. Additionally, it was revealed that increased IGF-I activity in the liver tissue regulated muscle IGF-I expression through the autocrine/paractin effect. Likewise, studies have reported that the IGF-I gene is expressed in the liver and muscle tissues and is among the main stimulators of somatic growth (Hevrøy *et al.,* 2013; Jiménez-Amilburu *et al.,* 2013; Huang *et al.,*2016; Bildik *et al.,* 2019). The IGF-II gene expression in the muscle tissue of adult trout increased by 3.26-fold in summer compared to winter (p<0.05) (Figure 3). Increased muscle IGF-I and IGF-II mRNA levels in parallel with increased muscle GHR mRNA levels at high temperatures revealed a tissue-specific muscle response. These findings showed that the seasonal temperature increase positively affected the GH/IGF axis in adult trout.

In juvenile trout, liver GHR mRNA expression was 3.52-fold higher in summer than in winter. However, it increased by 5.6-fold in the muscle tissue in winter (p<0.05) (Figure 1). Increased serum GH levels in juvenile trout in summer caused an increase in GHR expression levels in the liver through a direct effect.

Table 2. Serum glucose, cortisol, and GH levels of adult and juvenile rainbow trout at different seasonal temperatures.

	Winter Adult Trouts (n = 10)	Winter Juvenile Trouts (n = 10)	Summer Adult Trouts (n = 10)	Summer Juvenile Trouts (n = 10)
Glucose (mg/dl)	35.8 ± 4.6^{ab}	23.32 ± 1.4^{b}	43.7 ± 6.1^{a}	33.2 ± 4.3^{ab}
Cortisol (ng/ml)	23.4 ± 3.4	23.04 ± 2.8	24.1 ± 2.9	29.8 ± 4.1
GH (pg/ml)	1086.5 ± 54.1^{b}	1054.3 ± 55.8^{b}	1465.1 ± 151.4^{a}	1629.7 ± 281.4^{a}

Values are mean ± standard deviation (x ± SEM). Different lowercase letters ($^{a, b}$) refer to a significant difference in the groups ($p < 0.001$).

Likewise, Saera-Vila *et al.* (2007) reported the highest liver GHR mRNA expression in young gilt-head breams in the summer season with water temperature in the range of 20-30 ˚C. In our research, the IGF-II gene expression in the muscle tissues of juvenile trout increased by 3.0-fold in winter compared to summer ($p<0.05$) (Figure 3). Increased muscle GHR mRNA levels as a result of adaptation to low temperatures increased IGF-II mRNA levels in the muscle tissue in the winter season, independent of the autocrine/paracrine pathway of liver IGF-I. Although IGF-I is the main factor for promoting growth in mammals, IGF-II represents the main growth factor that regulates early development (Dai *et al.,* 2015). Our study showed that IGF-II might regulate the development and growth in the muscles of juvenile trout at low temperatures.

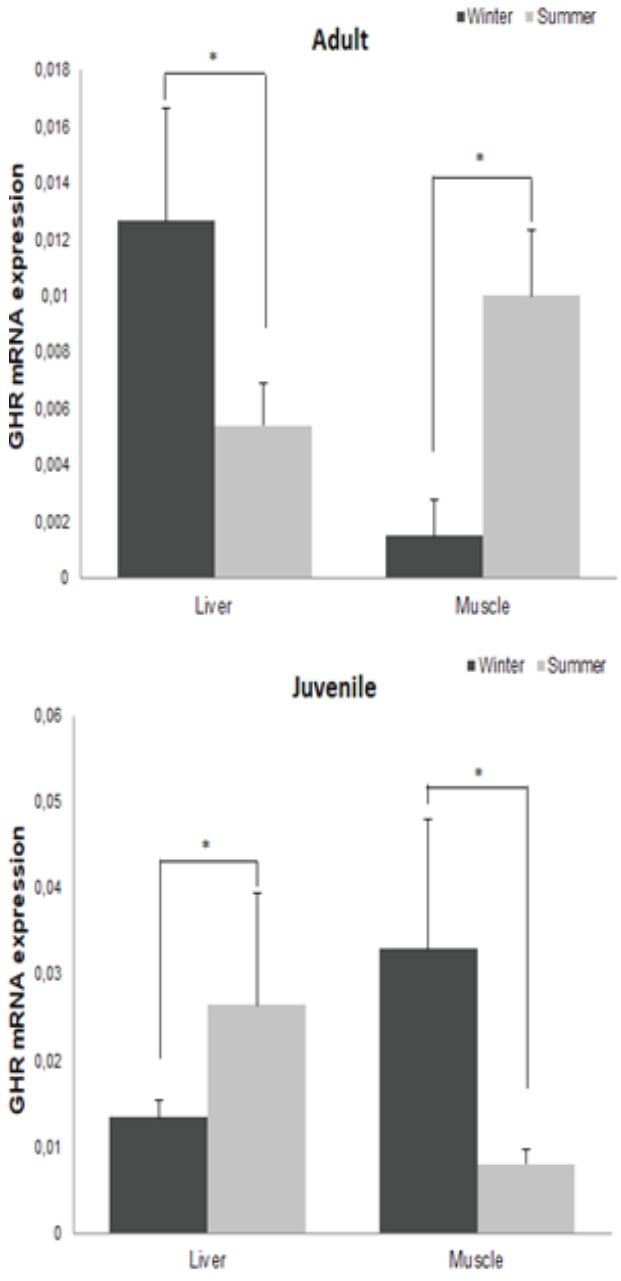

Figure 1. GHR mRNA expression levels in the muscles and liver of adult and juvenile rainbow trout at different seasonal temperatures. * $p<0.05$

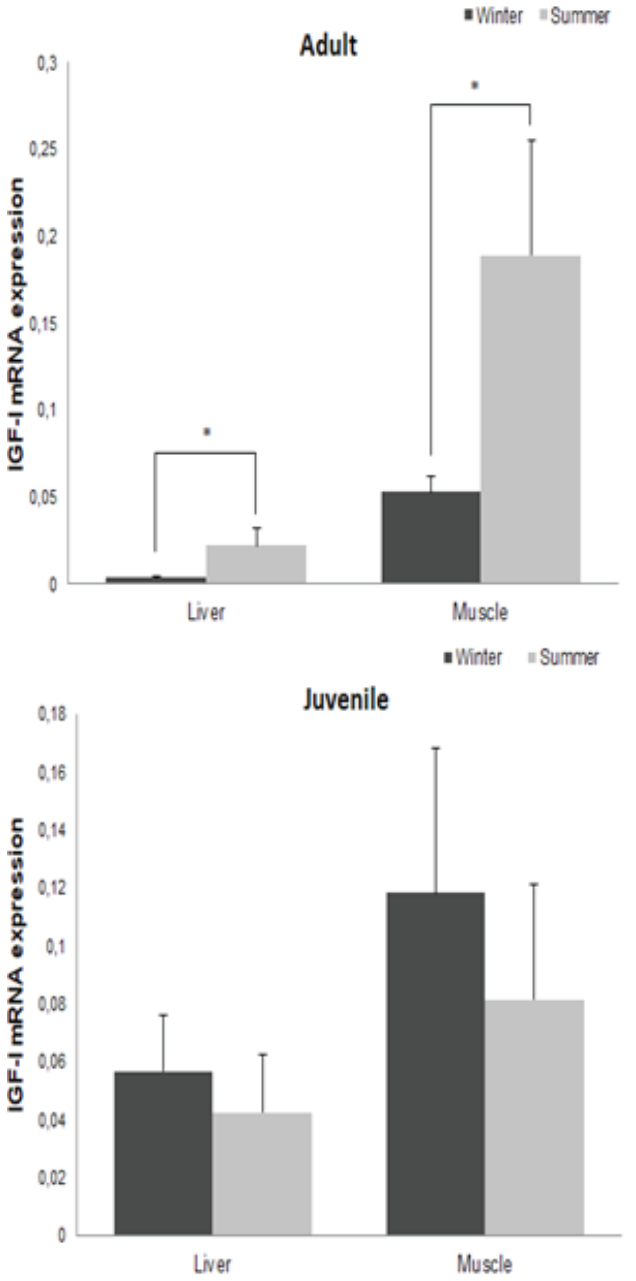

Figure 2. IGF-I mRNA expression levels in the muscles and liver of adult and juvenile rainbow trout at different seasonal temperatures. * $p<0.05$

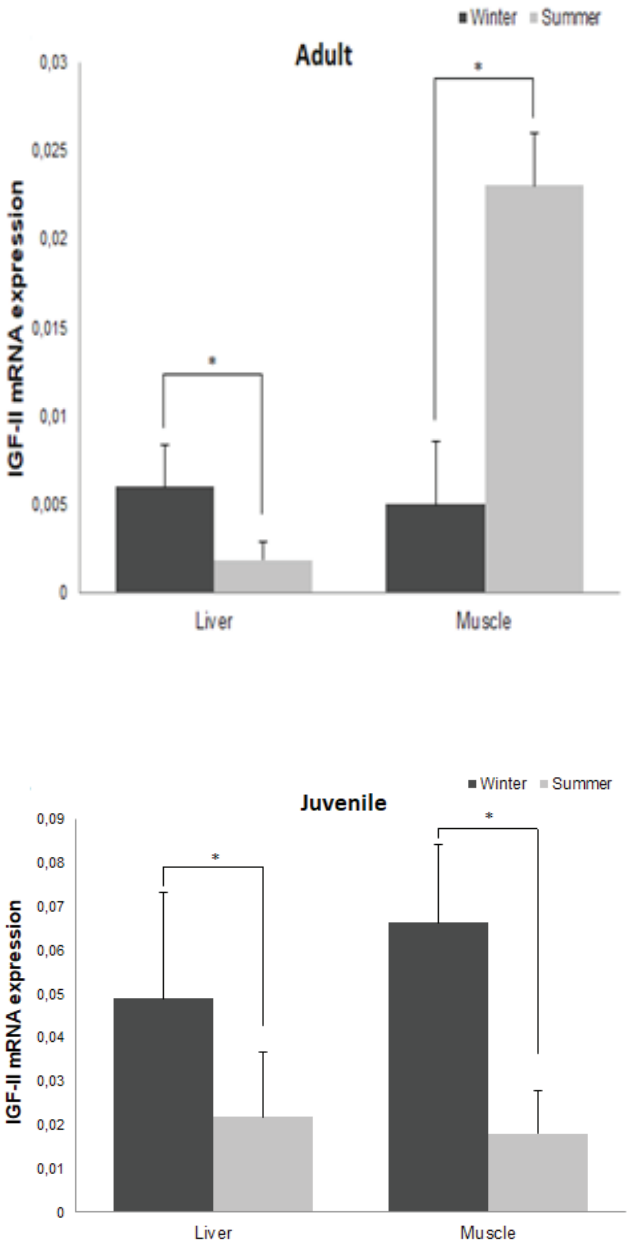

Figure 3. IGF-II mRNA expression levels in the muscles and liver of adult and juvenile rainbow trout at different seasonal temperatures. * p<0.05

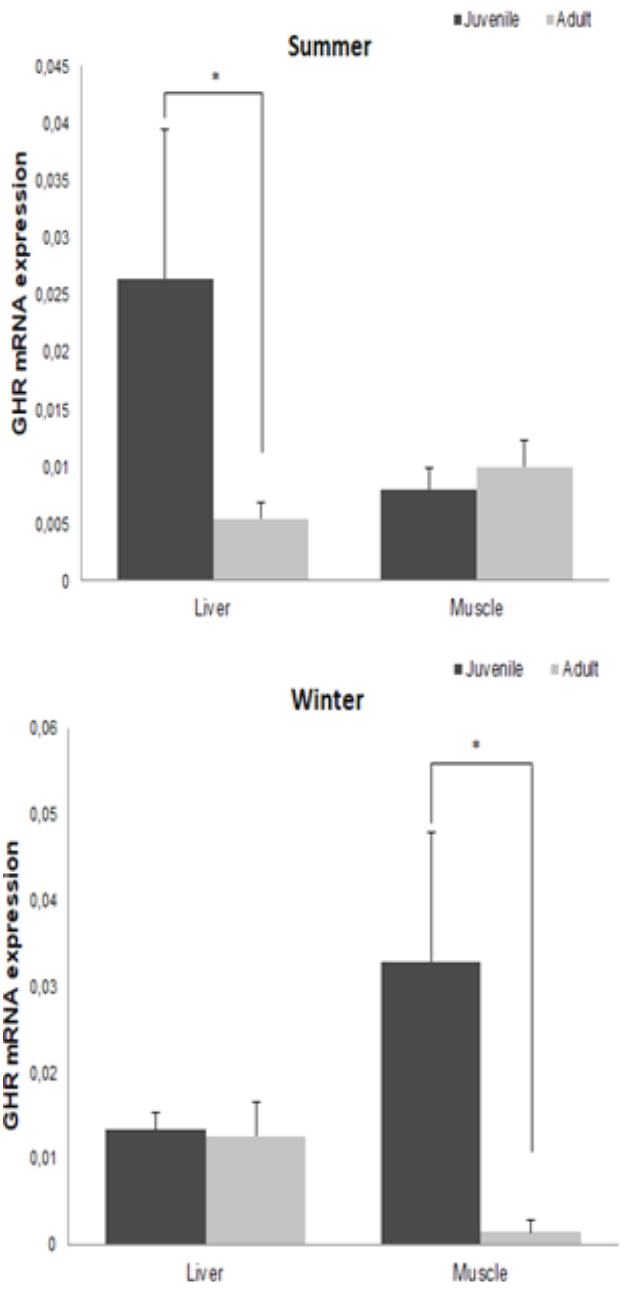

Figure 4. GHR mRNA expression levels in the muscles and liver of rainbow trout of different ages in summer and winter. * p<0.05

GHR, IGF-I, and IGF-II mRNA expression levels in the liver and muscle tissues of rainbow trout of different ages

GHR, IGF-I, and IGF-II gene expressions indicated statistical differences between different age groups at the same seasonal temperatures. In our study, muscle GHR expression in winter was higher in juvenile trout than in adults (approximately 11.36-fold, p<0.05). However, liver GHR expression levels did not vary between the age groups (Figure 4). Furthermore, serum GH levels were positively correlated with liver GHR expression levels. Especially GHR gene expression in the liver tissue was suppressed by the negative feedback mechanism of increased liver IGF-I expression. In winter, the transcription of the IGF-I gene in the liver and muscle tissues was higher in juvenile trout than in adults (16.0-fold and 2.50-fold, respectively, p<0.05) (Figure 5). Liver IGF-1 activity may have increased muscle IGF-I levels through the autocrine/paracrine effect. Similarly, IGF-II expression in the liver and muscle tissues was higher in juvenile trout compared to adults in winter

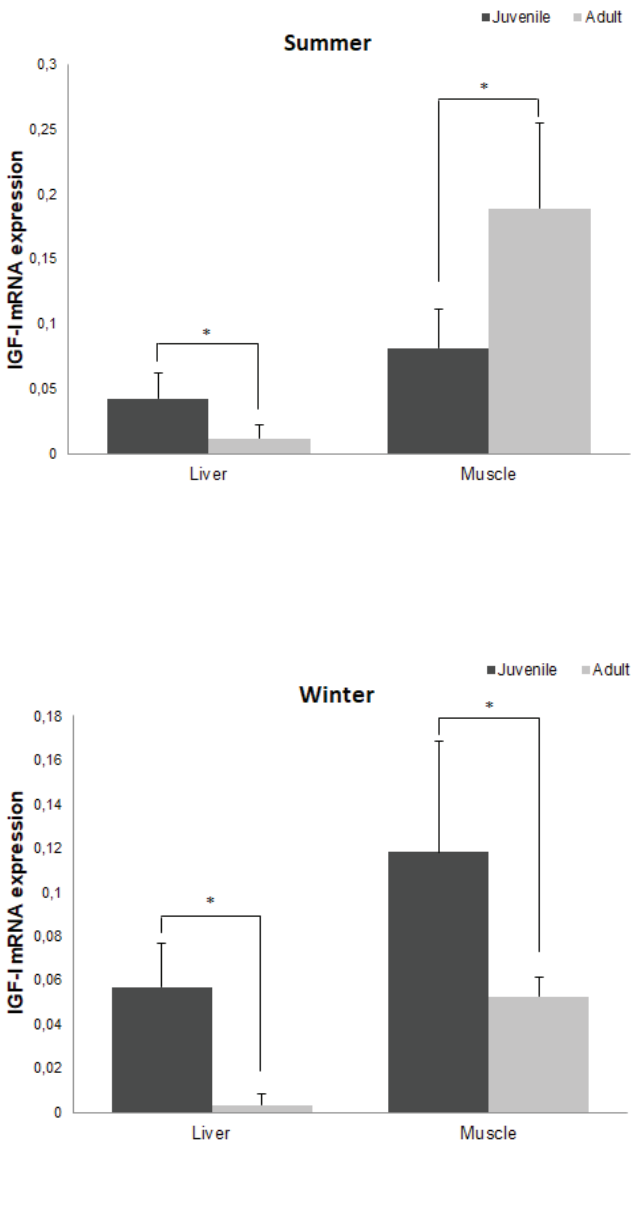

Figure 5. IGF-I mRNA expression levels in the muscles and liver of rainbow trout of different ages in summer and winter. * p<0.05

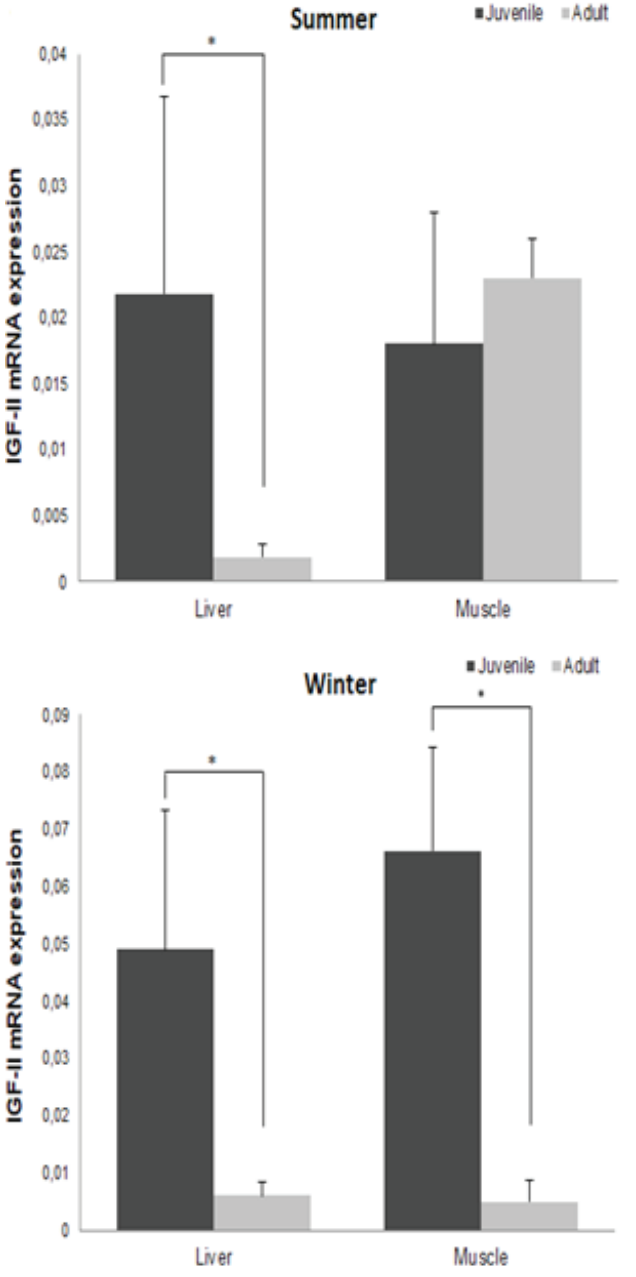

Figure 6. IGF-II mRNA expression levels in the muscles and liver of rainbow trout of different ages in summer and winter. * p<0.05

(approximately 9.0-fold, p<0.05) (Figure 6). This provides evidence that endocrine IGF-II has an effect on IGF-I in juvenile trout at low temperatures in winter. The findings of our study showed that juvenile trout were more successful in adapting to low temperatures.

Liver GHR, IGF-I, and IGF-II mRNA expressions in summer were higher in juvenile trout compared to adults (6.87-fold, 3.23-fold, and 14.63-fold, respectively, p<0.05) (Figures 4, 5, 6). Increased serum GH levels and the hepatic transcriptional activity of the GHR gene in summer increased liver IGF-I and IGF-II expression levels in juveniles. Likewise, the studies conducted on juvenile trout (Gabillard *et al.,* 2005), young mirror carps (Huang et al., 2016), young gilt-head breams (Saera-Vila *et al.,* 2007), and young hybrid striped basses (Davis and Peterson, 2006) reported that liver IGF-I mRNA amounts and plasma IGF levels were elevated in parallel with an increase in temperature. The above-mentioned findings showed that liver IGF-I and IGF-II

gene activity could regulate growth and development in juvenile trout by the autocrine/paracrine effect. Muscle IGF-I mRNA expression was revealed to be higher in adult trout than in juveniles by 2.32-fold in summer ($p<0.05$). It is thought that increased muscle IGF-I mRNA levels may cause hyperplasia and hypertrophy in the muscle tissue of adult trout.

CONCLUSION

The induction of the GH and IGF gene expressions takes place in response to a number of stress stimuli, including environmental stress factors. In conclusion, the way temperature acclimation in trout of different ages influenced the regulation of the GH and IGF multigene family was demonstrated. Our study showed that the GH/IGF axis was positively impacted by the seasonal temperature increase in adult trout. Furthermore, juvenile trout were found to be more successful than adults in adapting to temperature changes. Our work could be regarded as a pioneer and comparison point for further studies in fish farming in Turkey.

CONFLICT OF INTEREST

The authors declare no competing interests.

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