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# Effect of enviromental temperature on heat shock proteins (HSP30, HSP70, HSP90) and IGF-I mRNA expression in *Sparus aurata*

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**Abstract**

Ambient temperature is one of the most important environmental factors affecting physiological mechanisms and biochemical reactions of living organisms. Thus the effect of ambient temperature on HSPs and IGF-I gene expression levels in the liver and muscle tissues of *Sparus aurata* were investigated in this research. The levels of HSPs, and IGF-I gene expression of the liver and muscle of *Sparus aurata* were analyzed in by qRT-PCR. The experiment was done in July (27 ˚C) and January (18˚C). HSP70 mRNA relative expression levels in the muscle on January were significantly higher than July (approximately 1.7 fold), whereas HSP30 gene expression in the liver on July was increased by 2.0 fold ($p$<0.05). Transcription of other heat shock proteins and IGF-I were not affected by water temperature changing. The HSP findings of the research show that these proteins are important and sensitive in the average adaptation.

**Keywords**: *Sparus aurata*, Temperature, IGF-I, HSPs, Gene expression

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## Introduction

The biology of fish is affected by various environmental stressors such as water temperature, dissolved oxygen, salinity which control their existence (vital activities), the aquatic environment and the poikilotherm's inability to control its temperature. Therefore, fish are an available model to study the results of thermic stress in the whole organism for both short and long time scales. Temperature is an important factor changing their biogeographic circulation during evolutionary time. At that, daily and seasonal temperature variations have a significant influence on the living of individual fish (Kelly *et al.,* 2000; Basu *et al*., 2002). Regulation of gene expression constitutes the main component of the aquatic organisms` response to stress factors such as temperature. Stress proteins are collectively the only one of the molecular mechanisms that fish utilized to tolerate stress, and these proteins have pleiotropic effects, connecting with multiple systems in various ways regulated by the endocrine system (Kayhan and Atasayar, 2010). HSPs play important physiological roles in cellular stress responces and a have critical function in the development of thermotolerance, are involved in many regulatory pathways, and protection from stress-induced cellular damages. The major heat shock genes in fish do not contain introns and so the mRNA can be directly translated into new proteins within minutes of exposure to stress (Iwaws *et al*., 1998; Kregel, 2002). As a result, fishes are suggested an alternate and ideal model system to research the physiological, ecological, and evolutionary genomics of heat shock proteins (Basu *et al*., 2002).

Growth is regulated by the integration of environmental signals as food, photoperiod and temperature with neuroendocrine answers to the genetic code that essentially establish the organism's structure. The insulin-like growth factors (IGFs) are fundamental elements of multiple processes controlling both growth and metabolism (Le Roith *et al.*, 2001). Acute changes in temperature and salinity induce different relations between IGF1 and growth but adaptation to permanent differences in environmental condition generally result in concordant relations. Generally, by distinguishing between fish in different physiological status and discriminating and classifying differences among habitat one may be effectively used IGF1 as a growth index for fishes (Beckman, 2011). There is good evidence showing that IGF-I played a major role in mediated pathways the growth-supporting effects of GH in ectotherms like teleost fish. Most studies of IGF-I in fish have focalized on identification of the fish IGF, measuring changes in IGF-I in blood and tissue IGF-I expression in response to varying nutritional conditions and season, and detecting control of IGF-I production by growth hormone and other endocrine factors, such as thyroid hormone ( Schmid *et al*., 2003; Carnevali *et al*,. 2005; Reinecke *et al*., 2005). We therefore investigated HSP30, HSP70, HSP90 and IGF-I gene expression response to temperature changing in the liver and white muscle of adult *Sparus aurata*.

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## Materials and methods

Ten adult *S. aurata*, weighing about 700-800 g, were obtained in January (water temperature, 18˚C) and July (water temperature, 27˚C) from the commercial fish farm in Turkey (6° 39' 33.2820" N and 29° 7' 34.8492" E). The fish were dissected, and samples of the liver and white skeletal muscles from the dorsal side of the fish were removed. After removing, the tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C until being analyzed.

*RNA Isolation from tissues and cDNA synthesis*:

Total RNA was extracted from liver and muscle using commercial RNA isolation kit Roche, Germany Cat No. 11667157001). One ml of TriPure isolation reagent per 50 to 100 mg was added onto the tissue sample. Samples were homogenized in Qiagen Tissuelys LTE (Germany) using steel beats at 4500 rpm for 45 seconds. The cooled samples incubated 5 minutes at room temperature. Chloroform was added to 200 µl of each homogenized tissue, waited 5 minutes. Then, samples centrifuged at 12000 g for 20 min at 4˚C and then 500 µl isopropanol added to 500 µl supernatant which containing RNA, incubated 10 min. Supernatants were centrifuged at 12000 g at 4˚C for 10 minutes, after removal the upper supernatant, 1 ml ethyl alcohol 75% was added to residue. Later, they were centrifuged at 7500 g at 4 ˚C for 5 minutes; supernatants were eliminated, removed ethanol at 57˚C. Supernatants were re-suspended by sterile DNase/RNase free water, samples were stored at -80˚C until used. RNA concentrations were determined by optical density measurement at 260 and 280 nm. Purity assessed by the A260/280 nm ratio (1,733).

Following RNA isolation, cDNA synthesis was performed using Light Cycler Nano Real Time PCR for cDNA commercial kit (Roche Cat. No: 04896866001). For complementary DNA synthesis, 1 µg of total RNA from each sample was added PCR into tubes containing 2 µl random hexamer primer and 6 µl $dH_2O$. The reaction mix was incubated at 65˚C for 15 min. After that, 4 µl reaction buffer, 0,5µl RNaz inhibitor, 2 µl dNTP, 0,5 µl Transcriptor Reverse transcriptase was added to reaction mix. First- strand cDNA synthesis was allowed to proceed at 55˚C for 30 min, after which time the reaction was incubated at 85˚C for 5 min.

Real-time RT-PCR analysis were performed using SYBR green qPCR Master Mix (Roche, Fast Start Essential DNA Green Master), according to the manufacturer's procedure using a Light Cycler Nano Real Time PCR. All primers were synthesized by Genmar (Izmir/Turkey) and had the following sequences (Table 1).

**Table 1: Forward and reverse primer sequences of *Sparus aurata* β-actin, IGF-I, HSP30, HSP70 and HSP90 genes are shown.**

| Gene | Forward (5′→3′) | Reverse (5′→3′) | GenBank No |
|---|---|---|---|
| β-actin | CGACGGACAGGTCATCACCA | AGAAGCATTTGCGGTGGACG | AF384096.1 |
| IGF-I | AGTGCGATGTGCTGTATC | CAGCTCACAGCTTTGGAAG- | EF563837.1 |
| HSP30 | CCTCAGCCTGGACACTCAG | CTGCCCACCTGTTTGACTG | GU060312.1 |
| HSP70 | AATGTTCTGCGCATCATCAA | CCAACCTTTTTGTCCAATCC | EU805481.1 |
| HSP90 | GACCACCCCATTGTCGAG | TCACAGCCTTGTCGTTCTTG | DQ012949.1 |

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β-actin (Santos *et al*., 1997) was also amplified in each assay as a control for using equal amounts of RNA in the RT-PCR reaction (Table 2). Relative gene expression was calculated using a $\Delta\Delta C_t$ method (Haimes and Kelley, 2010).

**Table 2: Real Time PCR condition**

| Temperature (ºC) | Ramp rate ( ºC $s^{-1}$) | Time (sec) |
|---|---|---|
| | Hold | |
| 95 | 5 | 600 |
| | Amplification (45 cycle) | |
| 95 | 5 | 10 |
| 55 | 4 | 10 |
| 72 | 5 | 16 |
| | Melting | |
| 60 | 4 | 20 |
| 95 | 0,1 | 20 |
| | Hold | |
| 40 | 5 | 40 |

*Statistical analysis*

Statistical analysis of the data was performed with a SPSS 15.0 software. Because the absence of normal distribution in normality test done, the significance between two means was determined by the non-parametric Mann– Whitney's U-test. Descriptive statistics are given as median with lower (25th percentile) and upper (75th percentile) quartile.

## Results

Expression of IGF-1 and HSPs mRNA in the liver and white skelatal muscle of *S.au*rata determined by RT-PCR. The relative mRNA expression levels of genes to β-Actin (housekeeping gene) is given in Fig. 1 a,b,c,d. When tissue samples taken from fish, the water temperature was measured 27 ˚C in July and 18˚C in January. All sample/primer combination showed down regulation of gene expression to reference gene. Median values for July and June of HSP30, HSP70, HSP90 and IGF-I were statistically compared. Statistical analysis revealed a significant variation between different seasons for HSP70 and HSP30 genes expression. HSP70 mRNA relative expression levels in the muscle on January were significantly higher than July (approximately 1.7 fold) (Fig. 1b), whereas HSP30 gene expression in the liver on July was increased by 2.0 fold ($p$<0.05) (Fig. 1a). However, in the liver and muscle, temperature changes did not affect transcription of IGF-I and HSP90 genes ($p$>0,05) (Fig. 1c,d). The expression of HSP70 and HSP 30 genes showed temperature sensitivity and seasonal variation.

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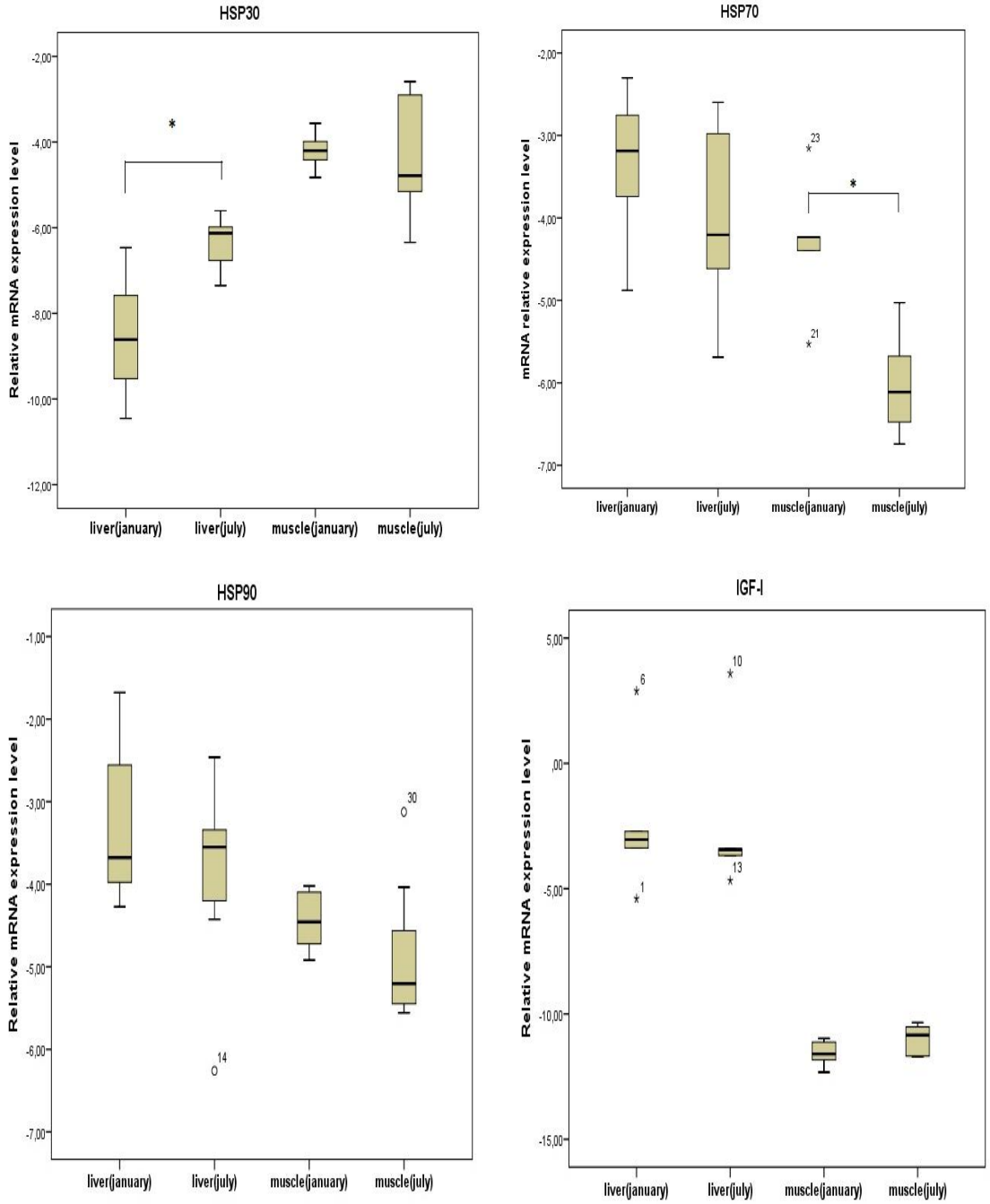



**Figure 1: HSP30 (a), HSP70 (b),HSP90 (c) and IGF-I(d) relative mRNA expression level in muscle and liver of *Sparus aurata* on January and July.**

## Discussion

Physiological processes, metabolism, growth and life of Ectothermic vertebrates such as fishes are under the influence of the ambient temperature. At the tolerance limits, every species has the most suitable temperature changing during its lifetime depending on its volume for its metabolism, feed consumption, and growth. Metabolic rate and growth of many temperate species slow down to adapt to long-term low temperature. In contrast, temperatures rising above the upper tolerance limit may cause more

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rapid hazardous effects (Kullgren, 2011). GH-IGF-I system in fish and water products is accepted as a good indicator in terms of determining the growth performance and nutritional status. It was observed that growth and seasonal changes altered the expressions of hypophyseal hormones (Bhandari *et al*., 2003).

Gabillard *et al*. (2003) fed trout adlibitum for a period of 10 weeks in the environment of 8, 12 and 16˚C. The amounts of the liver mRNA and plasma IGF levels increased in parallel with the increase in temperature. A significant correlation between liver mRNA expression and plasma IGF was found. The muscle tissue findings in the researchers' study showed that the autocrine/paracrine effect of IGF-I and IGF-II was not an important regulator in the growth stimulation with the effect of temperature.

Growth regulation in the adult Atlantic salmon was investigated in seawater at 13, 15, 17 and 19˚C for 45 days. While no change was observed in the plasma IGF-I concentrations, IGFBP mRNA expressions increased in the fish raised at 17˚C and decreased in the fish raised at 19˚C. Muscle IGF-I mRNA expression decreased in the fish raised at 15, 17 and 19˚C, but no change was detected in the liver IGF-I mRNA levels. The researchers argued that for Atlantic Salmon with a big muscle mass to adapt to long-term temperature exposure, decreased IGF-I and IGF-II mRNA levels mediated, in addition to GH-related lipolytic effects, and water temperature above 13˚C affected the growth of fish negatively (Hevrøy *et al*., 2013). In the young mirror carps fed for 60 days at 18, 23 and 28˚C water temperature, the IGF-I mRNA expression in the brain and liver, body weight and specific growth rate increased in linear with the temperature rises; on the contrary,hepatosomatic index and viscerosomatic index decreased. A positive correlation between the IGF-I mRNA levels and body weight and specific growth rate was found. Saera-Vila *et al.* (2007) determined that the liver IGF-I and GHR mRNA expression in the young gilt-head breams (*S. aurata*) in summer months when water temperature was 20-30˚C was maximum. In contrast, IDF-II and GHRI were too much expressed in the muscle and fatty tissue during the winter months. Davis and Peterson (2006) found the plasma IGF levels of fish living at 25 and 30˚C to be higher in young hybrid striped basses which were kept at 5, 10, 15, 20, 25 and 30˚C for 5 days. Researchers noted that the fish kept at low temperatures consumed much more feed; they argued that the experimental findings could be a result of the temperature and feed interaction.

In this study, the IGF-I gene expression in muscle and the liver tissues of gilt-head breams did not show a significant change in January and July. In the studies carried out on trouts (Gabillard *et al*,. 2003), young gilt-head breams (Saera-Vila *et al*., 2007), young mirror carps (Duan, 1997; Huang *et al.*, 2015) young hybrid striped basses (Davis and Peterson, 2006), it was observed that liver IGF-I mRNA amounts and plasma IGF levels increased in parallel with the temperature rises. These studies, in which IGF mRNA expressions increased with the temperature, are experimental studies, and young fish were used as the experimental group. No change

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in the liver mRNA expression with the temperature was determined in the adult Atlantic salmon (Hevrøy *et al*., 2013), however, it was found out that muscle IGF-I mRNA synthesis decreased. The liver findings of the current study are similar to the results of Hevrøy *et al*. (2013), however, different from the experimental studies, tissues of gilt-head breams living in the natural culture medium were used in this study. For this reason, the study results bear importance, as it is considered that IGF-I levels may be important in the adaptation of the fish to the environment they live in.

Almost every organism has a cellular mechanism to get protected from the harmful effect of heat stress through a special protein class, molecular chaperons or heat shock proteins (HSPs). Heat shock proteins (HSP) are a defence mechanism of organisms against stress preserved at a high rate in all organisms, which is induced by factors such as thermal shock like hyperthermia, UV, heavy metals, oxidants, oxidative stress, osmotic stress, temperature, exercise, metabolic change, ageing, extreme density of the population, aggression-competition between other fish species and individuals of the same species, and microorganisms (Iwaws *et al.*, 1998). Madeira *et al.* (2013) found that fish species gave different responses to the increase in the water temperature, and while the increase in temperature created oxidative stress in some species, it did not create a cause of any stress in others, and there was a positive correlation between lipid peroxidation and HSP 70. While HSP 70 genes were expressed in the trout gonad fibroblast (RTG-2) cells which were applied in vitro thermal shock (28˚C, 3 hours), no change was observed in HSP90 (Ojima *et al*., 2005). Similarly, it was argued that HSP70 expression could play a key role in the adaptation to the environment in the study carried out in vivo and on the hepatocytes isolated from *Mugil cephalus* (Padmini and Usha Rani, 2008). In another study, no change was observed in the muscle, liver, heart, and gill tissue HSP70 and HSP60 proteins of the trouts which were applied cold shocking at 1˚C for 1 hour after having been kept at 17˚C for 6 weeks (Washburn *et al*., 2002). HSP 70 expression decreased in the liver and red muscle tissue of the basses which were not given food for 2 weeks, and it increased in the 3-week hunger. While HSP 90 increased during the 2-week hunger, no change was observed during the 3-week hunger (Antonopoulou *et al*., 2013). In a study in which the effect of summer and winter seasons (13-35˚C) on the HSP 70 levels of the liver, muscle and gill tissues of gilt-head breams and common soles living in the Kuwait gulf was investigated, while no difference was found between the fish species; contrary to the expectations, it was observed that the liver and muscle HSP70 levels increased in the winter. It was argued that the adverse finding between the HSP levels and temperature could be a result of the genetic adaptation of fish to the hot climate conditions of the region (Beg *et al*., 2010). It was determined that the muscle and liver HSP 70 gene expression of *H. antarcticus* fish which were applied acute thermal shock (2 hours) upregulated for ~3 .4 times at 10˚C; ~5.97 times at 15˚C (Clark *et al.,* 2008). HSP70 in blood, muscle, brain, liver, heart and brain tissues of the trouts kept at 25˚C

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for 3 hours, and HSP30 increased in all tissues except blood (Currie *et al.,* 2000). The increase in water temperature in *S. aurata* stimulated the HSP 70 and HSP 90 protein synthesis in the liver, blood, and muscles (Feidantsis *et al.,* 2012). In the study, the water temperature in the summer season when the tissue samples of the fish were taken was measured as 27°C, and as 18°C in winter. HSP70 from the thermal shock proteins was expressed 1.7 times more in the low water temperature when compared to the high temperature in muscle tissue; HSP30 gene in the liver tissue was expressed 2 times more in the summer season compared to the winter. The transcription of other thermal shock proteins was not affected by the change in the water temperature. Contrary to the research findings in which short-term thermal shock was applied (Currie *et al.*, 2000; Feidantsis *et al.,* 2012), the expression of HSP 70 gene in muscle tissue increased during winter as it did in the results of Beg *et al*. (2010). The HSP findings of the research show that these proteins may be important in the average adaptation.

Induction of transcription of genes belonging to the HSPs and IGF-I occurs as a response to several stress stimuli such as environmental stress factors. Even the minimum temperature fluctuations in the environmental habitat of the aquatic organism can affect biochemical reactions and physiological processes at the molecular level. The results from this study demonstrate how temperature acclimation in fish affects the regulation of the HSPs multigene family. These proteins are important and sensitive to adapt in its natural habitat.

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