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IGF-I gene expression in liver and white muscles confirming promotion effect of dietary NaCl on Growth indices of Giant sturgeon (*Huso huso)* juveniles

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Abstract

This study was conducted to evaluate the effects of different levels of NaCl used in the diet of farmed *Huso huso* juveniles based on gene expression of Insulin like growth factor (IGF), and IGF mRNA in the liver and muscle. The NaCl was added in 4 levels of 0 (control), 3, 6 and 9 percent to the basic diet. According to the results, significant changes in IGF 1-mRNA expression were observed in the treatments and control group (p<0.05). The data of IGF gene expressions showed that the highest levels were observed in the control group and in the 3% treatment. Moreover, in the tissue of white muscle, IGf mRNA gene expression showed highest IGF gene expression in the control group (6.4) and lowest expressions were in the 3% (0.7) and 6% (0.8) treatments. Results of growth indices including body weight, SGR, BWI, HSI and ADG showed they significantly increased in all treatments. Moreover, significant differences were observed among treatments and the control group (p<0.05). SGR rose from 1.41 in the control group to 1.58 in treatments 3% and 6% and the BWI increased from 68.88 in the control group to 98.28 in treatment 2. HSI increased from 3.64 g in the control group to 4.37 g in treatment 2. FCR showed significant difference in the control group (1.27) and in the treatment groups (1.14). Statistical analysis of FCR, SGR and IGF mRNA gene expression showed better growth indices in fish fed diets containing NaCl.

Keywords: Gene expression, *Huso huso,* IGF-I, NaCl, diet, growth.

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Introduction

The high capability of sturgeons as live fossils to adapt to unfavorable conditions of the environment has caused them to be alive from the dinosaur era to now (Duan, 1998). The Beluga sturgeon (*Huso huso*) is a very important species as the biggest fish of freshwaters (Falahatkar *et al.,* 2013). Also, it has the most precious caviar among sturgeons (Azari Takami, 2009). Regarding the high economic value of this species in producing valuable caviar and meat, it is important to consider the mechanisms that have affected its growth. Numerous authors have applied different compounds in the feeds of aquatics with the aim of investigating the effects of food additives on growth, food consumption, digestibility, changes in metabolic pathways, health factors and stress (Singh *et al*., 2008). NaCl is one of the essential minerals for animal life because it improves the taste of food and regulates body osmotic pressure. Nandeesha *et al.* (2000) reported that adding NaCl to the diet as a supplement can increase fish growth. Related research performed by Krumschnabel *et al.* (1993) and Tsintsadze (1991) on rainbow trout verified that euryhaline fishes prefer water with higher salinity and can grow better compared to freshwater fishes. This can be due to the natural need of endocrine fishes for salt to have better and normal conditions. So, if salinity increases, metabolic activities of growth will improve (Shen and Leatherland, 1978). Also experiments conducted by Gatlin *et al.* (1992), Salman and Eddy (1988), Eroldoğan, *et al.* (2004), and Appelbaum and Arockiaraj (2009) showed the positive effects of using NaCl in gilthead sea bream diets. Plisetskaya *et al.* (2002) investigated the effects of feeding conditions on levels of insulin in the blood of channel catfish. Fukada *et al.* (2012) considered IGF-I expression under different feeding conditions in the body of *Seriola quinqueradiata* and reported that feeding condition can be considered as the first physiological factor which determines insulin levels in plasma. Accordingly, it can be declared that diet conditions can control IGF-I mRNA gene expression in fishes (Wood *et al.,* 2005).

Different studies have determined IGF-I as a suitable growth index in teleost fish (Beckman, 2011). Some hormones such as thyroid hormones, and growth hormones, as well as IGFs are necessary to stimulate or regulate growth and can be regarded as probable growth indices (Beckman, 2011). IGF is produced primarily in the liver and its synthesis and release depends on growth hormones. Except for the liver, IGF-I can be synthesized and released in many tissues of fishes based on the type of tissue and for special receivers in the tissue under the effect of the autocrine paracrine mechanism (Duan, 1998; Mommsen, 1998). IGF-I contributes to the regulation of protein, lipid, and carbohydrate, and the metabolism of minerals in cells,

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differentiation and proliferation of cells and body growth (Moriyama *et al*., 2000). However the efficiency of endocrine specific factors as growth indices depends on the species and special conditions of the fish (such as diet and food composition, temperature, photoperiod, and stress). Although these conditions contribute to GH-IGF axis (Pitcha *et al*., 2008), the studies conducted on teleost fishes showed that there is a direct relation between the level of IGF-I in plasma and fish growth (Larsen *et al*., 2001; Fox *et al*., 2006; De Santis and Jerry, 2007). Considering that IGF-I has a key role to play in body growth; it should be measured as a very important biological marker of growth.

Few studies have been conducted on using NaCl in sturgeon diets. The purpose of this study was to compare the expression of IGF-I and 2m-QPCRβ genes to isolate the IGF-I gene sequence, determine the surface changes in muscle and liver tissues, and some fish growth indices in fish fed different percentages of NaCl and in the control group. The results of this research are expected to provide useful information for a better understanding of growth, nutritional status, and physiological properties of great sturgeon.

Material and method

During the present study, *Huso huso* juveniles were fed a diet including NaCl. Then IGF-I mRNA gene expression was investigated in liver tissue (as the main organ of IGF-I production in vertebrates) (Fukada *et al*., 2012) and white muscle of *Huso huso* (which construct a high percentage of the fish body and are affected by feeding conditions) (Beckman, 2011). Accordingly, 480 specimens of *Huso huso* with a mean weight of 173.80±0.49 g were stocked in 12 circular fiberglass tanks (2 m^3 each). Water exchange in each tank was carried out routinely with aerated well water at a rate of 0.2 L/s for all treatments. During the experiment, water temperature was 19.50±0.5 °C, dissolved oxygen concentration was recorded as 5.57±0.5 mg/l and pH fluctuated around 7.90±0.4 in all tanks measured using an American multi-meter Model HQ40d. In order to add salt to food and prepare the experimental diets (treatments), a special diet, GFSI (Grower Food of sturgeon I), containing 45 percent protein, 16 percent fat, and 5.3 percent fiber was bought from Fardaneh Company. The food was ground to a powder in a grinder (model Damicar Co, Tehran, Iran) for ten minutes, and then salt dissolved in 280 cc water was added to the food powder. For the control group, 280 cc of water without salt was added to the food powder. The salt water solution at the mentioned volumes was added to one kilogram of food, and mixed in a mixer (Pooya Notash Machinery Co, Mashhad). Then the mixed food was transferred to a meat grinder to form pellets which were then cut into a suitable size pellets for the fish mouth, and then placed in a dryer at 40°C for 24 h (Mohseni *et al*.,

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2008). Finally, the feed was stored in plastic bags at 4°C. The mentioned diet was prepared every 15 days in the International Sturgeon Research Institute. The feeding experiment was carried out for 12 weeks. At the end of the experiment, 16 samples of white muscle and liver of farmed *Huso huso* (from each replicate) were collected and kept in the original condition, and instantly transferred to the molecular laboratory of Islamic Azad University branch of Tonekabon.

RNA extraction and creation cDNA

Total RNA was extracted using a kit from Iran SiNaClon Company and following its protocol (SINACLON, Iran). RNA quality and cDNA production were evaluated according to Akbarzadeh *et al.* (2011). All of the muscle and liver samples were placed in a Tri-Reagent extraction buffer and digested completely using a Qiagen homogenizer. The total RNA from 16 samples including three treatment groups and one control group, each with two replicates (for both muscles and liver tissues) was extracted from the liver and muscle separately for each fish. The extracted RNA was placed in RNase free water and incubated at 55°C for 10 minutes in order to remove the entire DNA as an impurity. Finally, the quality of extracted RNA was evaluated using electrophoresis in Agarose gel (1.5%) with DNA SAFE STAIN (SiNaClon, Iran). At the end of the reaction, the extracted RNA samples were placed in a freezer at a temperature of-20°C to preserve their quality.

Primer design

In the present study, the β2m-QPCR primer was used for gene reference according to Yarmohammadi *et al.* (2012). The primers required for performing the qPCR of the IGF-I gene, based on protected regions of similar sequences of mRNA, were studied using the primer-blast program, and a pair of primers for each gene was selected as the best primer based on the rate of efficiency for studying the gene expression (Table 1). In other registered sturgeons in the gene bank including *Acipenser guldenestaedii* (GenBank no.DQ201138), *A. ruthenus* (GenBank no.DQ329352), *A. barii* (GenBank no. Fj428828) and *H. huso* (GenBank no.AB512770) the primer-blast method is used. In this study, the size of the product obtained from primers and the specific degree of action of primers were confirmed using agarose gel (1.5%) and gene sequencing.

Real-Time (quantitative) PCR or Qpcr tests:

The qPCR reaction was performed using a Light cycler (96W Fast Real-Time PCR system, Roche, Germany) and a Fermentase kit at 95°C for 10 minutes and 40 cycles to 30 seconds, according to standard instructions.

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Table 1: Conditions for primer preparation to isolate the gene sequence of IGF-I for *Huso huso*

Primer name	Primer sequence (5'-3')	Annealing T (°C)	Amplicon size (bp)
IPIGFI-F	GGC ATT CCG TCT TC ATC AG	58	466
IPIGFI-R	CGG TAG TTC CTG TTG CCT GT	58	466
2m- QPCR-F	AGC TGG GCA AGC CCA ACA CC	65	127
2m- QPCR-R	TGG TGG AAG GCC AGG TCG CT	65	127

The next step was reducing the temperature to 65°C for 20 seconds, and then increasing it to 74°C for 40 seconds. Finally, the sample was placed at this temperature for 7 minutes. All reactions were performed with two replicates. The expression levels of the gene (Ct) which were represented by the device indicated the number of cycles that the fluorescent signal detected gene variants. The standard curve was calculated based on different dilutions of cDNA (dilution from 0 to 10 and 1 to 2000). The PCR efficiency was calculated using the following formula (Radonic *et al*., 2004):

$$E\%=10^{\frac{1}{SLOPE}-1}\times 100$$

The relative changes in the expression of the IGF-I gene are calculated using the method of $2^{-\Delta\Delta Ct}$, which ΔΔCt is equal to ΔCt of the target gene minus ΔCt of the calibrator [ΔΔCt=ΔCt (Target gene)-ΔCt (calibrator)], ΔCt is the target gene equal to reference Ct gene. Ct (Target gene)-Ct (calibrator)=(Target gene) ΔCt and ΔCt of the calibrator is equal to ΔCt of the target gene for each sample minus ΔCt of the control sample (Livak and Schmittgen, 2001). Real Time PCR experiments were designed based on the requirements and MIQE manual published by Bustin *et al.* (2009).

The β2m gene was used as reference gene in sturgeons (*Huso huso* and *Acipenser stellatus*) (Wuertz *et al*., 2007). The normalization method was used based on the amount of nucleic acid ΔCq that was used by De Santis *et al*. (2010) and De Santis and Jerry (2011). This method is the modified form of the ΔΔCq method in which normalization occurs based on the entry of cDNA in the reaction (Fibly *et al.*, 2007).

In this method, precise evaluation of cDNA concentration was carried out after purification of reverse copy reaction. Therefore, related technical errors to differences in yield of reverse copy reaction were completely omitted. Purified supplement DNA did not inhibit the PCR reaction, thus the high yield of reaction (nearly 100%) and low variance of experiments (CV<1) were obtained. Considering the high precision of the ΔCq method in normalization of RT-QPCR experiments (De Santis *et al.*, 2010; De Santis and Jerry, 2011) and because no reference genes was found suitable for sturgeons, the above mentioned method was applied.

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Measurement of growth factor

Specific growth rate (SGR) and fast growth (logarithmic) during a period of time is often used to estimate growth rate increase (Bavcevic *et al.*, 2010). Also, other growth factors including food conversion rate (FCR), hepatosomatic index (HSI), body weight increase (BWI) and average daily growth (ADG), were evaluated in the different treatments and control groups. In order to determine growth rate, aside from measuring total weight and length of fish, growth indices were calculated using mathematical equations, based on available references (Bavcevic *et al.,* 2010).

Average Daily Growth:

$$ADG(g\ /\ fish\ /\ day) = \left[\frac{Wt - Wi}{Wi \times T}\right] \times 100$$

Body Weight Increase:

$$BWI = \left[\frac{Wt - Wi}{Wi}\right] \times 100$$

Specific Growth Rate:

$$SGR(day) = \left[\frac{LnWt - LnWi}{T}\right] \times 100$$

Food Conversion Ratio:

$$FCR = \frac{Food}{Wt - Wi}$$

Condition Factor:

$$K = \frac{Wt}{L^3} \times 100$$

Hepatosomatic Index:

$$HSI = \frac{WL}{WT} \times 100$$

Where W_i is the initial weight of fish, W_t is final weight, L is body length, WL is liver weight and T is duration of rearing period (Bavcevic *et al.,* 2010).

Statistical analysis

Normalization of growth parameters of the expression level between the samples (control and treatments) was done by Shapiro-Wilk and One way ANOVA was used to compare the means. After homogenizing groups, data was compared using Duncan's test. Gene expression data are reported as mean ± standard deviation (SD). IGF-I mRNA expression data were expressed as ratios. A Kolmogorov-Smirnov test was used to assess normality of distributions before one-way ANOVA. Post hoc multiple comparisons of means were performed using Duncan's multiple range test. All statistical analyses were undertaken using IBM SPSS Statistics version 20 software. Differences between treatments were considered significant at $p<0.05$.

Results

Gene expression

The results showed that primers are attached to the correct position of the gene; two separate 18S and 28S rRNA were shown clearly (Fig. 1) (the expression of the gene was normalized using a reference gene). The results of normalization showed significant differences in gene expression among treatments and the control group.

IGF mRNA gene expression in white muscle in T1, T2 and T3 decreased compared with the control group, but differences were not significant (Fig. 2).

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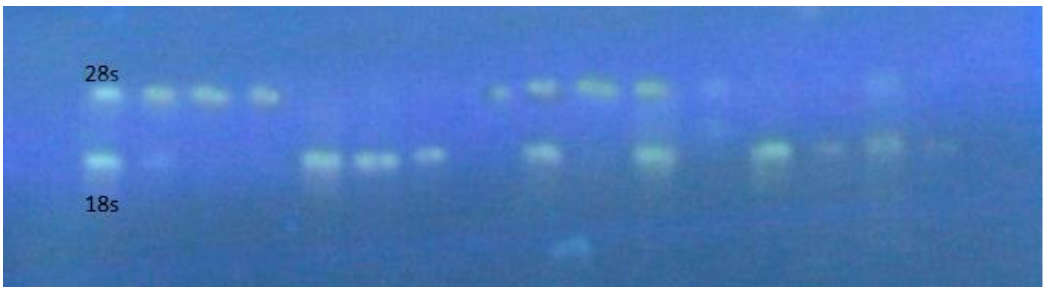


Figure 1: RNA quality assessment extracted from liver and muscle of *H. huso* on agarose gel(1.5%);in all samples two clear bands 18S and 28S of rRNA clearly observed (eight bands on the left are from liver and the others are from muscle).

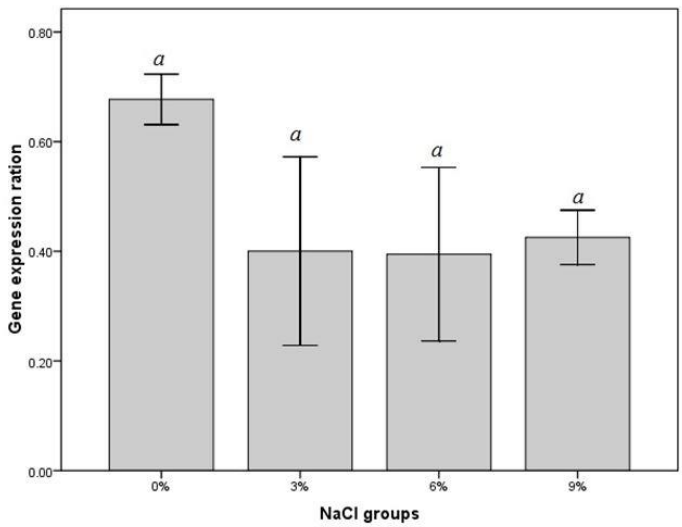


Figure 2: Relative IGF gene expression in *Huso huso* muscle tissue fed with different salt levels.

IGF mRNA gene expression in muscle showed no significant differences in treatments and control group (P>0.05) in that df between groups was 3, df within groups was 12, F = 1.247, and P = 0.336 (Fig. 2). However, there were significant differences in the level of gene expression in the liver of *H. huso* juveniles fed with different salt levels (df (between groups)=3, df (within groups)=10, F=6.36, P=0.010) (Fig. 3).

The results showed that during the experiment, the levels of IGF mRNA gene expression in liver samples decreased significantly (p<0.05) in all of three treatments compared to the control group.

Growth Index

According to the Shapiro-Wilk test, data related to weight in the first biometry showed a normal distribution (p>0.05). Moreover, based on Duncan's multivariate test and in order to compare the mean weights of young beluga in 3 phases of biometry, there were significant differences in treatments and control group (p<0.05).

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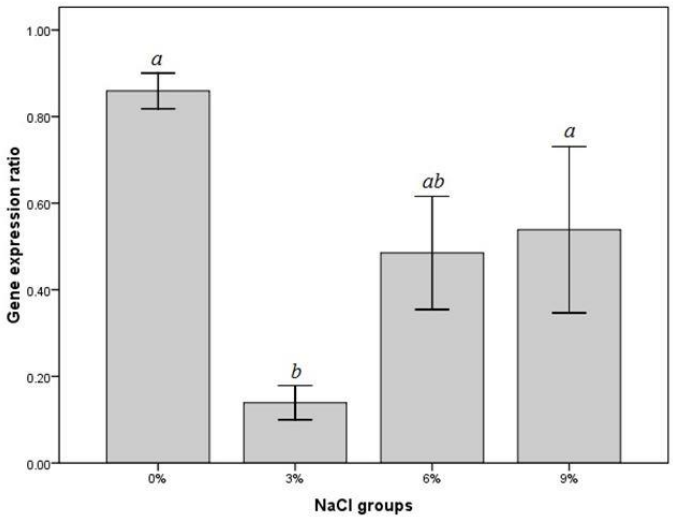


Figure 3: Relative IGF gene expression in *Huso huso* liver tissue fed with different salt levels.

The final weight increase of fingerlings in T1, T2, and T3 was significantly more than that in the control group (Table 2). Comparing two groups using Duncan's multivariate test, FCR in T1, T2, and T3 was significantly lower than that in the control group (P=0.041, df=3, F=3.559). The best FCR was observed in T1 and T3. Also significant differences were observed by comparing means of SGR (P=0.038, df=3, F=7.85), W_t (P=0.046, df=3, F=6.899) and ADG (P=0.038, df=3, F=7.85). Based on the results obtained by Duncan's multivariate test, the means of the mentioned items in T1, T2, T3 of *Huso huso* juveniles were significantly higher than those in the control group (Table 2).

Table 2: Growth parameters (mean ± SD) of *Huso huso* fed different levels of salt.

Parameters Group	W_i (g)	W_t (g)	BWI (%)	ADG (D/g)	SGR (%/day)	FCR	HSI (g/g)
Control	172.14±0.97	547.56±0.26 [b]	68.68±1.71 [b]	2.66± 0.02 [b]	1.41 ± 005.0 [b]	1.27± 0.002 [a]	3.16 ± 0.11
T1	173.80±0.49	640.36±27.1 [a]	91.16 ±11 [ab]	3.27± 0.14 [a]	1.58± 0.004 [a]	1.14± 0.05 [b]	3.76 ± 0.31
T2	173.87±0.03	633.63±30.02 [a]	98.62± 2.36 [a]	3.22± 0.14 [a]	1.58± 0.004 [a]	1.17 ± 0.05 [b]	4.37 ± 0.40
	173.87±0.03	633.63± 30.02 [a]	3.22± 0.14 [a]	1.58± 0.004 [a]	1.17 ± 0.05 [b]	4.37 ± 0.40	4.37 ± 0.40
T3	175.31±4.15	630.12±0.49 [a]	89.35± 1.98 [ab]	3.16± 0.07 [a]	1.56± 0.002 [a]	1.14± 0.001 [b]	3.24 ± 0.89

Discussion

Data analysis showed a significant decrease in IGF-I mRNA level in the liver and muscle of young beluga fed different levels of salt compared to the control group (p<0.05). The differences in the expression of this gene in the liver in T1, T2, and T3 were 4, 1.4 and 1.8 times less than that in the control group, respectively. Also, IGF mRNA gene expression in white muscle tissue didn't change dramatically in all treatments and in the control group (Fig. 2).

In the chemical synthesis of energy in the body and fat degradation, the role

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of the growth hormone (IGFI) is probably to break fats and regulate the secretion of enzymes that play a vital role in food digestion and absorption (Ayson *et al.*, 2007). The results of this study showed that hormones and food play an effective role in cell growth.

Considering that *Huso huso* is a euryhaline species, it may encounter a reduction of necessary ions which affect the functions of body enzymes and hormones. So, salt can be used as a mineral in rearing ponds to provide the metabolic needs of the fish. According to mentioned reports, in many cases, salt can affect growth factors in fishes in that, it can affect the metabolic activities of the host`s body (Gatlin *et al.*, 1992). Based on Nandeesha *et al.* (2000), many comments can be recorded for salt supplementation in the food of freshwater fishes to increase fish growth. The salt in the diet was diluted in water and the available ions may increase the absorption of amino acids (Gatlin *et al*., 1992).

Therefore, energy can be saved, because fish reared in freshwater may need more energy for sustainable ion and acid regulation, compared to marine fishes (Eroldogan *et al.*, 2004). The effects of salt on the promotion of growth have been recorded by many researchers. Salman and Eddy (1988), and Appelbaum and Arockiaraj (2009 and 2009) observed that rainbow trout which were fed a diet containing 12% salt, showed improved body growth. Also, the survival rate and FCR were increased which were compatible with the results of the recent study which found that SGR in all treatments increased significantly compared to the control. Moreover, the highest level of BWI percent was observed in T2. However HSI in the control group showed no significant difference with that in the treatments. The FCR assays showed that using salt in diets caused the FCR to decrease to 1.1 in all treatments which was significantly different compared with the control group (Table 2). This study shows that fishes fed a diet containing salt, have better growth compared to other fish. In a research carried out by Krumschnabel (1993) on rainbow trout in different salinities, it was shown that increasing salinity to 18 g/l, can increase growth and physiological activities of fishes. Also, a decrease in salinity to zero caused a reduction in growth (Krumschnabel and Lackner, 1993). In another research conducted by Tsintsadze, it was determined that an increase in water salinity led to a decrease in blood glucose indicating the need for more energy in fish for osmoregulation. So, if salinity increases, metabolic activities will improve (Shen and Leatherland, 1978).

A comparison of results of the aforementioned studies with the present research showed that the faster growth of fish can be due to the metabolic activity resulting from the new feeding condition. It was proved that IGF-I and IGF-II play an important role in cell proliferation and differentiation. They have biological effects on muscle growth, so as IGF-I causes differentiation in muscle cells, IGF-II

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can be considered as a stronger stimulator of cell proliferation (Rius-Francino *et al*., 2011: Seiliez *et al.*, 2011; Jiménez-Amilburu *et al*., 2013). The above mentioned results were compatible with the results of our study. According to the results, the IGF mRNA gene expression in liver significantly decreased in all treatments compared to the control during the experiment ($p<0.05$). In addition, the maximum gene expression was observed in the control group, while minimum gene expression was obtained in T1. Also, there were significant differences in gene expression in liver samples among other treatments (Fig. 3). Furthermore, in white muscle, the maximum IGF mRNA gene expression was observed in the control and the minimum occurred in T1 and T2, although the difference was not significant ($p>0.05$) (Fig. 2).

It is evident from the results obtained that by reducing IGF-I gene expression in the cells that received salt from the diet, NaCl can be a necessary mineral for the euryhaline aquatics reared in freshwater and it can supply the needed ions for *Huso huso* juveniles during the evolution of muscle cells. IGF and IGF-II are growth promoter polypeptides and they are originally produced in the liver by stimulation of the growth hormone. Their components contribute to evolution, growth, differentiation, and reproduction (Reinecke, 2010; Beckman, 2011). Recently, some studies have been conducted on the effects of IGFs on IGF-I and IGF-II gene expression of some fishes (Jeménez-Amilburu *et al*., 2013).

Further studies on structure and function of growth contributing genes like IGF-I, can certainly provide a better perspective on the role of these important molecules on the culture of valuable fishes such as sturgeons. Finally, studying IGF and obtaining more information on the physiological characteristics of IGF in sturgeons, may result in economical production in aquaculture that will be useful for the recent population growth. Wargelius *et al.* (2005) investigated the relation between the receptors of growth hormone and growth activity in Atlantic salmon. The changes in IGF-I gene expression and GH receptors showed that this hormone has a specific role in the growth of the spinal cord.

The statistical analysis of data related to the final biometry and experiments of growth gene expression showed better growth indices in treatments fed a salt supplement that indicate minimum IGF-I mRNA gene expression, minimum FCR, and maximum SGR compared to the control group which was indicative of better conditions. Finally, it seems that using salt in the diet of *Huso huso* juveniles can enhance growth ability, and make metabolism optimal that are the main purposes of aquaculture. Undoubtedly, further studies on the structure and function of growth contributing genes such as IGF-I can provide a better perspective on the role of these

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important molecules in the culture of valuable fishes like sturgeons. Finally, obtaining more information on the physiological characteristics of IGF in sturgeons can lead to economical production in aquaculture that will be useful for the present growing population.

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