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SHORT COMMUNICATION

Intra- and inter-specific variability of IGF-I expression in farmed cold and warm-water species in Iran

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Abstract Changes in the expression of liver insulin-like growth factor IGF-I mRNA in two growing cold-water fish species *Oncorhynchus mykiss* (one from locally produced and one from imported broodstock) and in two warm-water species, *Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix* were analyzed. Gene expression analysis in liver tissues by using real-time PCR revealed a significant difference in expression level of IGF-I between native and non-native rainbow trout ($P < 0.05$). IGF-I levels were found to have the following order: non-native rainbow trout> bighead carp> silver carp> native rainbow trout. Local strains of rainbow trout, bighead carp and silver carp exhibited stable IGF-I mRNA levels, at different stages of growth. The imported broodstock of rainbow trout exhibited increased IGF-I levels during the entire period studied, with a peak in the IGF-I mRNA levels of fingerlings. The results indicate intra- and inter-specific variability of IGF-I expression in farmed fish.

Keywords Fish growth . *Oncorhynchus mykiss* . *Hypophthalmichthys nobilis* . *Hypophthalmichthys molitrix* . IGF-I mRNA gene expression

Introduction

Oncorhynchus mykiss is a valuable and commercial species in some countries due to its specific biological and economic characteristics. Many strains of this species have been developed by breeders to improve growth, resistance to disease, survival, and the quality of meat (Gorjipoor et al. 2009). Many countries have the routine program of culturing their native stock of rainbow trout in which some non-native stocks could be potentially imported to boost the growth rate and higher production as claimed. Also carp is the biggest family of freshwater fish; *Hypophthalmichthys nobilis* (bighead carp) and *Hypophthalmichthys molitrix* (silver carp) are two important species of this family (Friedrich and Stepanowska 1999).

Insulin-like growth factors (IGF-I) is one group of hormones fundamentally involved in growth regulation (Dyer et al. 2004). IGF-I has been shown to promote growth in fishes (Chen et al. 2000; Degger et al. 2000) with studies indicating a significant and positive correlation between IGF-I and growth rates (Jones and Clemmons 1995; Larsen et al. 2001; Fox et al. 2006; De Santis and Jerry 2007). Nebo et al. (2017) showed how fasting in tilapia could induce an overexpression during the refeeding in IGF-I to being served for a compensatory growth. Jose Midhun et al. (2019) reported the expression of IGF-I gene was significantly boosted in the muscle tissue of *Oreochromis niloticus* when administrating with bacillus probiotic. The main source of its production is the liver and its synthesis and release in plasma is

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Table 1 Mean of total length, body weight for studied species (differences between subscripts indicate significantly different means with a, b and c in Duncan's test)

Fish species	Mean of body weight (g)	Mean of total length (cm)
Trout (Fingerling)	89.16± 6.64[a]	8.58± 1.02[a]
Trout (on-growing)	514 ± 19.60[b]	33.75 ± 0.98[b]
Trout (Growing)	690 ± 44.72[C]	38.14 ± 1.11[C]
Carp (Fingerling)	89.16± 17.5[a]	17.7± 0.61[a]
Carp (on-growing)	443.33 ± 31.41[b]	29.91 ± 2.49[b]
Carp (Growing)	913.33 ± 106.14[C]	37.58 ± 0.49[c]

influenced by the growth hormone, in other words, it has a liver source, and is created under the influence of growth hormone (Bjornsson 1997; Duan 1997; Reinecke et al. 1997). Besides growth, IGF-I has also been associated with other functions like fish metabolism, development, reproduction and osmoregulation in seawater (Reinecke et al. 2005).

IGF-I also plays a role in the growth and differentiation of immune cells (Smith 2010). In a study by Yada (2009), IGF-I and IGF-II improved the function of leukocytes in all rainbow trout and salmon and had significant effects on the production of superoxide in leukocytes in confrontation of disease in this fish.

Many studies have been conducted on the study of gene expression and colonization in various species and gene sequences have been recorded for many other commercial species in the Gene Bank (GenBank). In the lack of genetically studied the difference among such native and non-native cold-water species, and two warm-water species, the aim of the present study was to evaluate the effects of different body sizes on expression of IGF-I gene in liver of cold-water species (native and non-native *O. mykiss*) and warm-water species (*H. nobilis* and *H. molitrix*) in aquacultural purposes.

Materials and methods

Sample collection

Three samples of native and non-native *O. mykiss* in the three different body sizes (Table 1) cultured in similar raising conditions were purchased at the Allahyari's Fish Culture Center (Masal, Guilan, Iran). Three *H. nobilis* and *H. Molitrix* in the three different body sizes were provided from earthen ponds (Zobdeh Fish Culture Center, Jirdeh, Guilan, Iran) (Table 1).

At the beginning of study, some biometrical parameters such as total length (TL) and body weight (W) were measured. Fish were rapidly anaesthetized (Clove powder, 0.5 g/l) and killed by a blow to the head. Liver samples were taken in the pieces of 15-20 mg for each fish. Immediately after sampling, liver stored in nitrogen liquid until extraction of RNA. Then the entire RNA has been extracted according to the instructions of the company kit and its protocol.

RNA extraction and cDNA synthesis

Total RNA was extracted from fishes using by (Thermo Fisher Scientific, America). Total RNA was treated by DNAse to remove genomic DNA contaminating. The RNA quality was verified using RNA bands on a 1.5% agarose gel electrophoresis. cDNA synthesis performed by the Two Step kit called Thermo Fisher first strand synthesis kit according to manufacturer's protocol (Thermo Fisher Scientific, America). cDNA was used as template for PCR and real-time PCR. We need to look for genes that are housekeeping means present in all organisms and are the same in all and do not have highly specialized function and variability. 2m gene was chosen because of its features and presence in eukaryotic organisms, easy design and availability of primer (Table 2). The reaction mixtures for PCR amplification were consisted of 1 µl of first strand cDNA (2 µg), 10 µl of master mix (1x), 1 µl of Forward Primer (10 pmol/µl) and 1 µl Reverse Primer (10 pmol/µl), with 4 µl sterilized double water added to make up the volume distilled to 17 µl. Gradient PCR was used to find the right temperature for cDNA amplification with specific primers of IGF-1 and 2m-QPCR genes. The PCR condition was as follow: an initial denaturing step at 94 ˚C for 5 min, 35 cycles of denaturation

Table 2 Name and sequence of primers used

Primer name	Primer sequence (5'_ 3')	Gene	Product Length (bp)
IGFI-F	GGC ATT CCG TCT TC ATC AG	IGF-I	466
IGFI-R	CGG TAG TTC CTG TTG CCT GT		
2m- QPCR-Fß	AGC TGG GCA AGC CCA ACA CC	2m	127
2m- QPCR-Rß	TGG TGG AAG GCC AGG TCG CT		

Table 3 Times and temperature of 2m and IGF-1 genes PCR conditions

steps	(2m gene)		(IGF-1 gene)		Number of
	Temperature	Time	Temperature	Time	cycles
Initial denaturing	94 ˚C	5 min	94 ˚C	5 min	1
Annealing	61˚C	30 s	61˚C	30 s	35
Final extension	72˚C	5 min	72˚C	5 min	1

at 94 ˚C for 30s, annealing at 61˚C for 30s, extension at 72 ˚C for 30s and final extension at 72 ˚C for 5 min. Suitable temperature and concentration for cDNA amplification were confirmed by gel electrophoresis (Table 3).

Real-time PCR

Real-time PCR analyses were run in triplicate using the Real-time PCR system (96W Fast Real-Time PCR system, Roche, Germany) using PCR master Mix, SYBR Green (Fermentas) and Light Cycler system. The specific primers used in IGF-I in this step, 2m-QPCR (internal control gene), were used for gene reference in present study (Yarmohammadi et al. 2012) and then synthesized by the company Metabion (Germany). For the IGF-I gene from cross-species comparative alignments of sturgeon sequences available from gene bank by using the primer-blast program (https://blast.ncbi.nlm.nih.gov), a pair of primers for the gene and the best primer were selected based on the rate of yield for studying the gene expression. The set up conditions of the IGF-1 and 2m gene primers were exactly the same and the only difference was in the concentration of primers used for each reaction.

The PCR efficiency was calculated by using the following formula:

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

The relative changes in the expression of the IGF-I gene were calculated using the method of 2- ΔΔCt, 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). The stage with the lowest Ct was used as a calibrator to relative evaluation the gene expression of the target (De Santis et al. 2010).

Statistical analysis

Normalization of the expression level was done by performing 1-sample K-S, then the differences between groups analyzed by One-way ANOVA and Duncan's multiple range test. Pearson's correlation has been employed to see the relationship between variables. All analyses performed by SPSS 16 at $P < 0.05$.

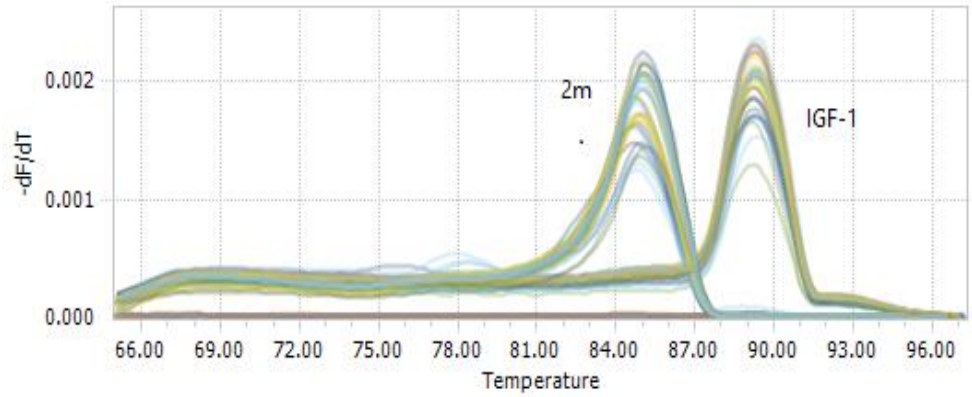

Fig. 1 Melting curve of *IGF-1* and *2m* genes

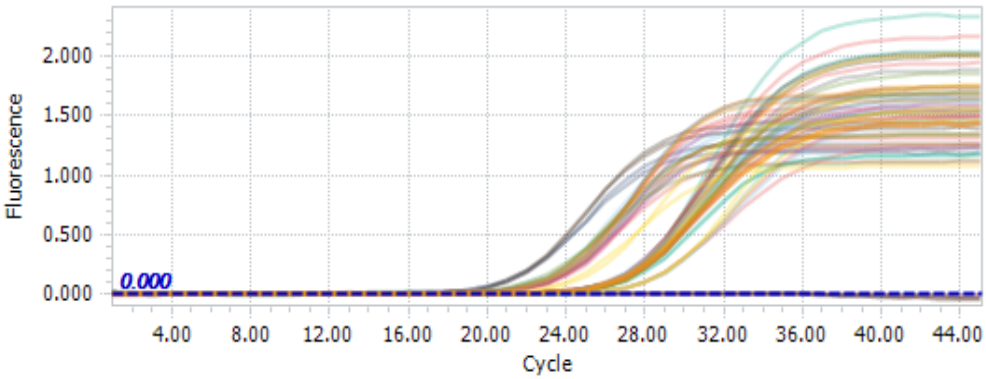

Fig. 2 IGF-1 and 2m gene amplification curves

Results

Evaluation of gene expression by Real-Time PCR

As mentioned, real-time PCR was used to study changes in gene expression. In this study, 2m gene was used as an internal control gene. Fig.s 1 and 2 show the fusion curve of these two genes in the Real-time PCR system. The presence of only one peak for each of the IGF-1 and 2m genes indicates the specific amplification of the target fragment and the absence of nonspecific products. Melting temperatures of IGF-1 and 2m are 90 and 85 ° C, respectively, according to the Fig. 1.

IGF-I gene expression

The results of present study showed that there was a significant difference in expression level of IGF-I between *O. mykiss* in two strains of native and non-native in different body sizes (F= 499.517, P=0.000) (Fig. 3). Furthermore, as an overall level of IGF-I gene expression in all species, we found it in this order: non-native rainbow trout> bighead carp> silver carp> native rainbow trout. According to Fig. 4, there was no significant difference in expression level of IGF-I between *H. nobilis* and *H. Molitrix* as warm water species in different body sizes (F= 1.648, P= 0.221).

There were no significant correlation between body weight (Fig. 5, r= -0.17, P= 0.31) and total length (Fig. 6, r= -0.29, P= 0.07) with relative expression level of IGF-I in all species studied in the present work. But a trend of slight negative relationship for both weight and total length is obvious with relative expression level of IGF-I in both Fig.s.

Discussion

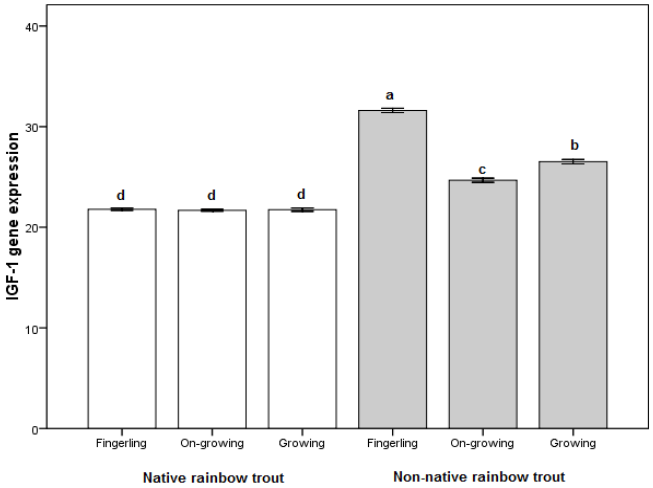

Fig. 3 Relative IGF-I gene expression in native and non-native *O. mykiss* liver tissue in different body sizes. Different letters shows there are significant differences among them ($P < 0.05$).

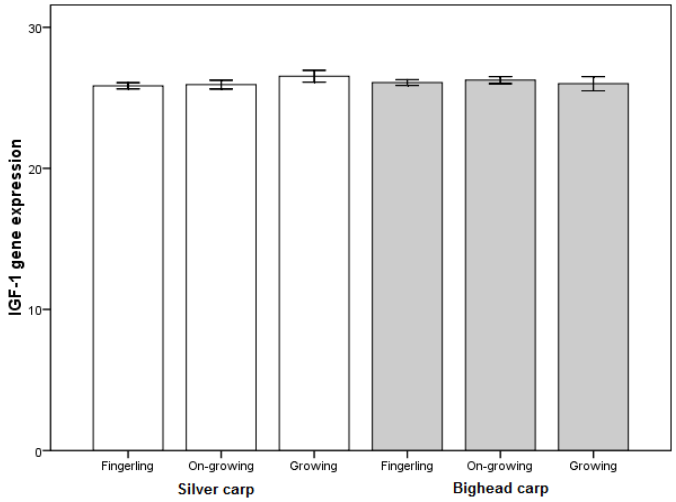

Fig. 4 Relative IGF-I gene expression between *H. nobilis* and *H. Molitrix* in the liver tissue in different body sizes

IGFs are potent mitogens for many different cell types and play important roles in growth and development (Vong et al. 2003). Alteration in the nutritional status, specifically fasting also affects the secretion rhythms of growth hormone (GH) and insulin-like growth factor-I (IGF-I) (Ayson et al. 2007). In fish species like salmon (Pierce et al. 2010) and tilapia (Pierce et al. 2011), GH is effective in triggering IGF-I expression at the hepatic level. In this study, it can be observed that size of body in three levels of fingerling, on-growing and growing stages had no significant difference on IGF-I mRNA gene expression in both warm-water species (Fig. 4). But non-native trout had larger amount of IGF-I mRNA expression with a significant difference rather than native trout (Fig. 3), whereas the fingerling size incorporated with the highest amount of IGF-I mRNA expression. Since several years, culturing the imported strains of rainbow trout including French, Norwegian, and American strains are routinely performed by delivering the eyed-egg stage to achieve the better growth rate than native strain which motivates the culturists for better production and higher profits. Gorjipoor et al. (2009) reported specific growth rate and other growth factors in the French larvae had been better than Iranian (native) and Danish larvae, which once again stressing on the importance of study of IGF-I mRNA expression corresponding to the growth rate of each strains together coincidently. In present study, both group of native and non-native trout raised under a similar culturing conditions and regarding to Gómez-Requeni et al. (2019) which demonstrated that different dietary regimes could alter

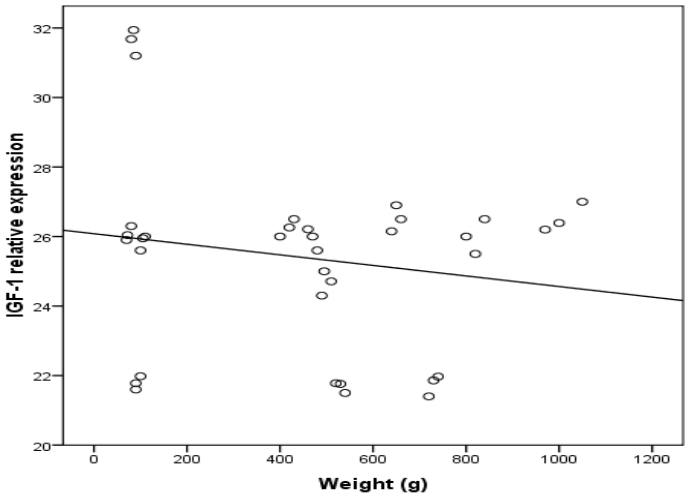

Fig. 5 Correlation between expression level of IGF-I and body weight in all species

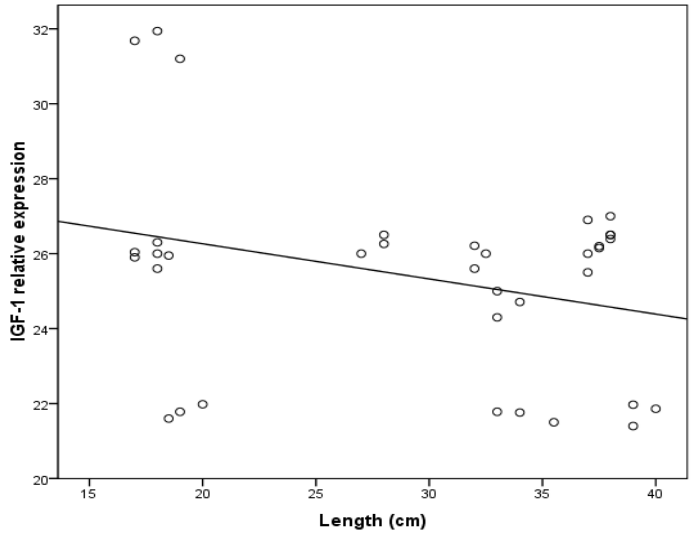

Fig. 6 Correlation between expression level of IGF-I and body total length in all species

the amount of IGF-I mRNA expression in pejerrey (*Odontesthes bonariensis*), the difference in IGF-I level of both trout could be attributed to their associated strains rather than culturing condition. Studies with transgenic rainbow trout have also shown that liver-specific gene expression patterns are more similar to those observed in faster growing domesticated rainbow trout, compared to slower growing wild-type fish (Tymchuk et al. 2009; Devlin et al. 2013).

In this study, there was no significant difference in expression level of IGF-I between two carps *H. nobilis* and *H. Molitrix* in different sizes ($P > 0.05$). In cultured systems, bighead carp shows a high growth potential and outperforms silver carp and grass carp in terms of net production (Woynaruvich 1968; Opuszynski 1981), but the level of IGF-I mRNA expression was relatively the same in both species. Indeed, many of these genes (GH, GHR2, GHR1, IGF2, and IGF1) are up-regulated in a variety of tissues (e.g., skeletal muscle, brain, and liver) from faster growing strains or individuals within several species including Nile tilapia *Oreochromis niloticus* (Huang et al. 2012), smooth tongued sole *Cynoglossus semilaevis* (Ma et al. 2001), and channel catfish *Ictalurus punctatus* (Peterson et al. 2004).

In present study, the IGF-I gene expression of liver in different fish are not significantly correlated to the fish size and species. IGFBP1 gene expression was up-regulated in the livers of small rainbow trout than bigger sizes (Kocmarek et al. 2014). However in our findings, expression level of IGF-I had a slight reverse correlation with weight and length that was in accordance with the previous works in other fish

species (Duan 1998; Wood et al. 2005), suggesting that IGF-I could be directly implicated in overall growth regulation and could be potentially investigated as a biomarker of growth in each species.

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Conflicts of Interest: The authors declare no conflict of interest.

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