Progress in Biological Sciences
Vol. 5, Number 1, Winter / Spring 2015/51-60

Transcription of growth hormone mRNA as a molecular marker of egg quality in Siberian sturgeon (*Acipenser baerii*)

Received: December 4, 2014; Accepted: February 14, 2015

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ABSTRACT

The Siberian sturgeon (*Acipenser baerii*) is an economically important species. Sturgeon stock assessment during fingerling production is considered to be one of the most difficult phases of hatchery rearing. The present study investigated the expression of growth hormone (GH) mRNA in unfertilized eggs, fertilized eggs, eyed eggs (2 day before hatching), non-viable eggs and newly-hatched Siberian sturgeon. Knowledge about the expression of GH during egg and embryo development can help determine the quality of the eggs. This can help predict larval viability and, to some extent, directly determine the growth and survival of young fish. Little is known about the effect of this hormone on egg quality in sturgeon. The present study analyzed relative GH mRNA expression using the $2^{-\Delta\Delta CT}$ method. Ribosomal protein L6 (RPL6) transcripts were used as the housekeeping gene for normalization of GH mRNA transcription. The highest levels of GH mRNA were found in eyed eggs and the lowest levels were detected in non-viable eggs ($P< 0.05$). These findings suggest GH mRNA as a potential marker for egg quality in Siberian sturgeon.

Keywords: egg quality, gene expression, growth hormone, siberian sturgeon.

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Introduction

Recent studies have shown that the vertebrate egg contains a good representation of the different classes of maternal hormones and growth factors that are vital to egg development (1, 2, 3) and influence egg quality (4). Several studies have examined the expression of maternal origin hormones such as thyroid and steroid hormones (5) in egg fish, but there is less information on the presence of other hormones or growth hormone in fish eggs. Recent studies have found growth hormone (GH) and GH mRNA in oocytes and suggest that GH is a possible marker for oocyte maturation and early cell cleavage of the embryo (6, 7, 8). Hormone and mRNA transcripts may be transferred to oocytes prior to their separation ovarian tissue; thus, GH may play a role in the complex epigenetic process that regulates the transition from maternal (ooplasmic) to embryo genome control (6, 7, 8). Furthermore, mRNA transcript encoding for GH, prolactin (PRL), somatolactin (SL), insulin-like growth factors (IGFs) (9-15) and receptors for hormones and growth factors (12,15-17) have been found in early embryonic stages of several fish species. The presence of GH, PRL and SL and factors related to them, such as IGF, in the first stages of development may indicate that these growth-related proteins are important to the early development of fish.

The Siberian sturgeon (*Acipenser baerii*) is a member of the family *Acipenseridae*. In recent years, over-fishing, loss of spawning grounds and pollution have caused a marked decline in the valuable wild stocks of this economically important species (18). Aquaculture of sturgeon can help stem the decline in wild populations (18). The Siberian sturgeon is the main species for sturgeon aquaculture in Iran, Moldova, the Czech Republic, Hungary, Germany, France, Chile, China, and Russia. Sturgeon fingerling production is considered to be one of the most difficult phases of hatchery rearing.

The present study determined the gene expression levels GH mRNA during the early development of the Siberian sturgeon (*Acipenser baerii*). The results of this study can improve understanding of expression of GH mRNA during egg and embryo development in most ancient osteichthye groups. This knowledge would improve the genetic quality and boost production in fish farms by providing basic molecular tools for egg quality determination.

Materials and Methods

Biological material

All samples were obtained by artificial spawning of Siberian sturgeon at the International Sturgeon Research Institute in the city of Rasht in Iran in 2012. After insemination, a 1-h silt treatment was conducted to eliminate egg adhesiveness (19). After adhesion removal, the fertilized eggs were transferred into Zoak incubators equipped with a running freshwater system. The average temperature was 13.5 ± 0.5, dissolved oxygen was 8.1 ± 0.5 and pH was 7.5 ± 0.3 during all experiments, which were carried out in a 12h:12 h (light:dark) photoperiod.

The unfertilized eggs, non-viable eggs (poor-quality eggs collected 2 days before hatching), fertilized eggs, eyed eggs (collected 2 days before hatching) and newly-hatched larvae of Siberian sturgeon were collected as specimens. Eggs that remained deposited at the bottom of the incubator were considered to be of good quality. Eggs that floated to the surface were considered to be of poor quality. Microscopic examination of

the eggs floating on the surface, herein referred to as non-viable eggs, showed that they did not exhibit development similar to eggs that remained at the bottom of the incubators. The samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction and RT-PCR

Total RNA was extracted from pooled tissue samples (50 mg) of each sample using BIOZOL Reagent (Bioflux-Bioer, China) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (20). The quantity of RNA was measured (NanoDrop ND-1000, USA) by measurement of optical density at 260 nm and the quality of RNA was determined using ethidium bromide staining of ribosomal RNA bands on 1% agarose gel (Fig. 1). The total RNA was treated using DNase to remove any DNA contamination and the first strand of cDNA was synthesized from 5 µg of total RNA using BioRT cDNA Synthesis Kit (Hangzhou Bioer Technology, China). The polymerase chain reaction (PCR) reaction mixtures comprised 3 µl of first strand cDNA, 1.5 µl of dNTP (10 mM), 0.5 µl of each primer (10 pm), 2 µl $MgCl_2$ (50 mM), 2.5 µl 10 reaction buffer, 0.3 µl *Taq* polymerase (5u /µl) in double sterilized water added to produce a volume of 25 µl. The following steps were carried out to determine the PCR temperature profile: initial denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 30 s and final extension at 61°C for 5 min. The PCR products were loaded onto 1.5% agarose gel and visualized by staining with ethidium bromide.

Primer design

Ribosomal protein L6 (RPL6) transcripts were used as the housekeeping gene for normalization of data, as shown in Table 1 (21). The GH primer was designed based on conserved regions of the partial sequences of *A. baerii* cDNA-GH (Gen Bank access number # Fj428829.1). The primer GH was designed using Oligo software (Oligo V5, Molecular Biology Insights; CO, USA). The specificity and size of the amplicons obtained from the primer pairs (RPL6-GH) was verified on 1.5% agarose gel (Fig. 2).

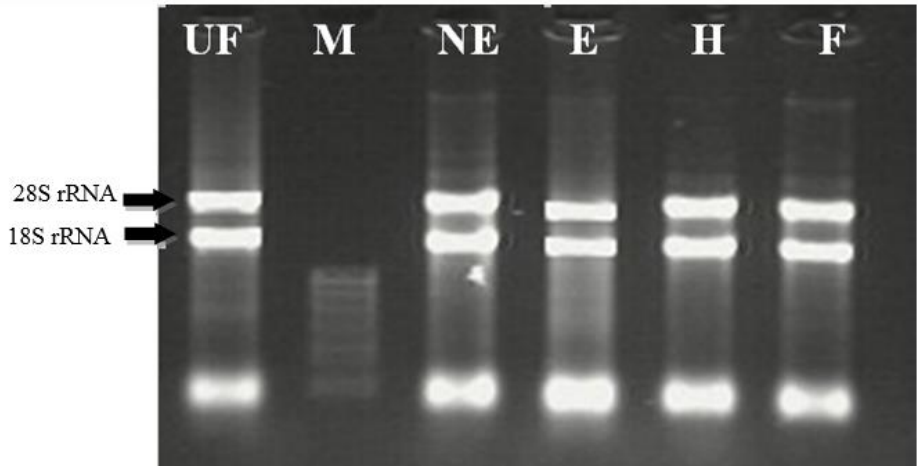

Figure 1. Confirmation of RNA quality. The quality of RNA samples was confirmed by electrophoresis on 1% agarose gel and stained with ethidium bromide. In each sample, distinct bands for *18S* and *28S* rRNA indicate the integrity of the RNA extracted. Samples are extracted from (left to right): unfertilized eggs (UF), markers (M), non-viable eggs (NE), eyed eggs (E), newly hatched larvae (H) and fertilized eggs (F).

Table 1. Source and sequences of primers

Gene	Sequence	Source	Expected size
RPL6-qPCR-F	GTGGTCAAACTCCGCAAGA	Akbarzadeh et al., 2011	149
RPL6-qPCR-R	GCCAGTAAGGAGGATGAGGA		
GH-real-time-F	TTTCCCTGGCTCTCATCCAGTC	Gene Bank # Fj428829.1	159
GH-real-time-R	TCCAAAACCTCCTTCACCGAGA		

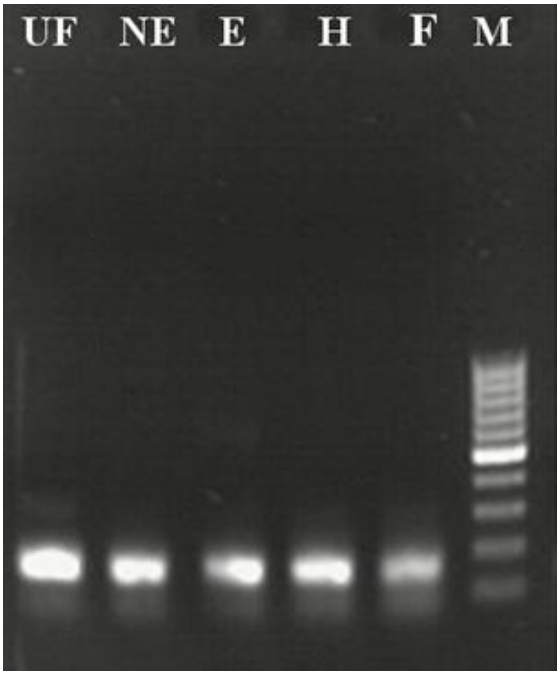

Figure 2. Amplified PCR products of first-strand cDNA synthesized with RPL6. The amplified products were electrophoresed on 1.5% agarose gel. Samples represent products from (left to right): unfertilized eggs (UF), non-viable eggs (NE), eyed eggs (E), newly hatched larvae (H), fertilized eggs (F) and markers (M).

Quantitative real-time PCR

Standard curves were generated from a dilution series of pooled cDNA (4 serial dilutions from 1 to 1/1000) for each primer pair to estimate efficiency. The PCR efficiencies were calculated and utilized for PCR correction for each primer pair (22). Standard curves showed $R^2 > 0.98$ and the corresponding real-time PCR efficiencies were 0.99 for GH and 0.96 for RPL6 (Fig. 3). Real-time PCR analysis was run using the CFX96 Real-time PCR system (Bio-Rad, USA). First-strand cDNA was diluted (1/1; v/v) and used as templates for RT-qPCR analysis. Reactions (12 µl total volume) containing 3 µl diluted template, 0.18 of each primer (10 Pmol) and SYBR Green qPCR Super Mix as instructed by the BioEasy SYBR Green I Real-Time PCR kit (Hangzhou Bioer Technology; China). Analysis was run in triplicate using the following cycling parameters: 94°C for 2 min; 94°C for 10 s; 40 cycles of 60°C for 30 s; 52°C for 15 s and; 72°C for 30 s. Conditions were similar for all PCR assays. No signal was observed for all primer pairs in reactions run without template control and when the samples were run without RT controls to test for genomic DNA contamination.

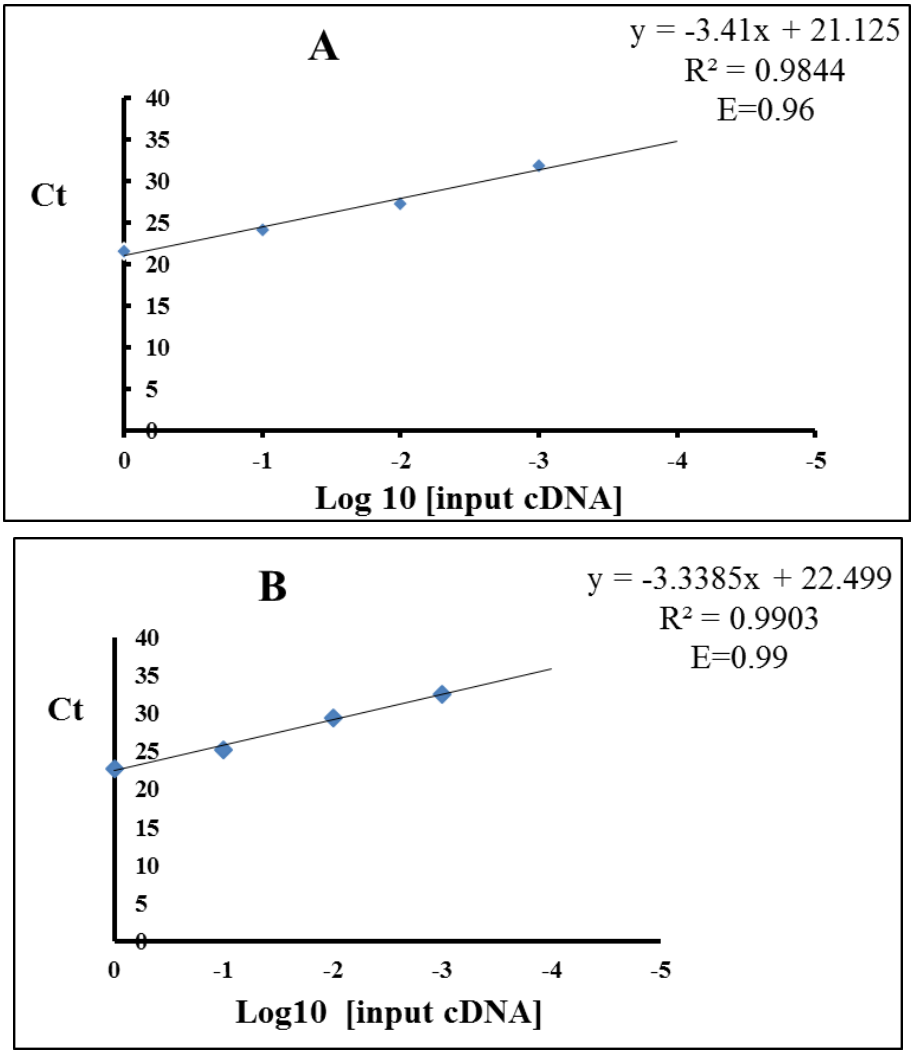

Figure 3. Standard curves for cDNA concentration vs. Ct. The standard curve for primer pair RPL6 (A) and primer pair GH (B). The cDNA concentrations are shown as serial dilutions from (1 to 1/10000). Trendline equations, real-time PCR efficiencies (E) and correlation coefficients (R^2) are shown for each primer pair.

Statistical analysis

Relative GH mRNA expression was analyzed using the $2^{-\Delta\Delta CT}$ method (23). Of the egg and embryo samples, the sample with the lowest Ct value was chosen as the calibrator sample to measure differential mRNA expression of a target gene. Where important, the data was log10-transformed subject to normality and homogeneity of variance testing. Differences in GH mRNA expression between samples were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc analysis for multiple comparisons. For all statistical tests, $P<0.05$ was considered to be significant.

Results

Real-time quantitative PCR was used to study GH transcription in the samples of Siberian sturgeon. Figure 4 shows the relative difference in GH mRNA levels between samples. There were significant differences between the GH transcription levels of different samples of Siberian sturgeon. In comparison, no significant difference was observed with the levels of RPL6 mRNA (Fig. 2).

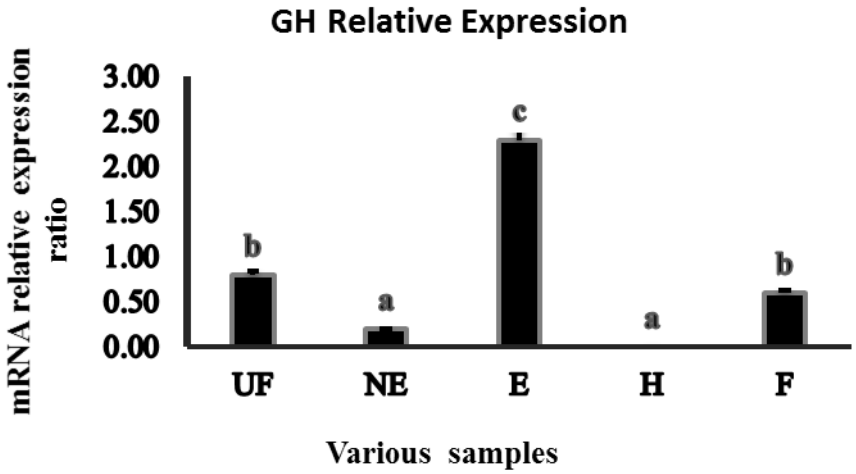

Figure 4. GH gene transcription levels. Total RNA was reverse-transcribed and used for quantitative real-time PCR. The relative amount of Siberian sturgeon mRNA GH was normalized to the level of RPL6 from each RNA sample. Relative mRNA expression was analyzed using the $2^{-\Delta\Delta CT}$ method. The data are shown as mean ± SEM (n = 6). The statistical significance of differences of the normalized mRNA GH data between groups was analyzed using one-way ANOVA followed by Tukey's test. Different letters indicate significantly different values at $p < 0.05$. Abbreviations: unfertilized eggs (UF); non-viable eggs (NE); eyed eggs (E); newly hatched larvae (H) and fertilized eggs (F).

The results confirm that GH mRNA was detected in all sample eggs and embryos, but not in hatched larvae. The highest levels of GH mRNA were found in eyed eggs. No significant change in transcriptional level was found between unfertilized eggs and fertilized eggs. The level of GH mRNA expression was higher in unfertilized and fertilized eggs than in non-viable eggs. There were no statistically significant differences in GH mRNA between non-viable eggs and newly-hatched larvae. The lowest level of GH mRNA expression was detected in non-viable eggs.

Discussion

The present study examined the presence of GH mRNA transcript in unfertilized eggs, non-viable eggs, eyed eggs, newly-hatched larvae and fertilized eggs of Siberian sturgeon using quantitative real-time PCR. Recent studies have shown that vertebrate eggs contain a good representation of different classes of maternal hormones and growth factors. In this study, GH was detected in fertilized and unfertilized eggs of Siberian sturgeon. The expression of GH in oocytes seems to occur in a wide range of vertebrates as in ovine (24), monkey (25), humans (26), cow (27) and rat (28). GH mRNA expression has been detected in fertilized and unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) (15), orange-spotted grouper (*Epinephelus coioides*) (29) and alligator gar (*Atractosteus spatula*) (3), but was not detected in unfertilized and fertilized eggs of the Japanese eel (*Anguilla japonica*) (30).

The GH mRNA in unfertilized eggs suggests its presence in maternal gametes. Transcripts detected in fertilized eggs may be due to the maternal GH messages from unfertilized eggs. Low levels of GH mRNA expression were found in non-viable eggs .The presence of GH in non-viable and viable eggs of the Siberian sturgeon and in other fish species suggests that this hormone plays an important role in egg development.

In this investigation of Siberian sturgeon,

the highest level of GH mRNA expression was detected in eyed eggs. Several studies have indicated the expression of members of the growth hormone family (PRL and SL) and IGF in embryos of several fish species, including PRL in tilapia (9), PRL and SL mRNA in rainbow trout (15), and IGF I and II in rainbow trout (13). Recent studies have shown that GH mRNA is present at the embryo stage in some fish. For example, GH mRNA has been found to express in embryos of tilapia (9), rainbow trout (15), European sturgeon (*Beluga, Huso huso*) and Persian sturgeon (*Acipenser persicus*) (31), Chinese sturgeon (*Acipenser sinensis*) (1) and milkfish (*Chanos chanos*) (32).

The presence of GH mRNA and other hormones in the embryos of fish may indicate that these hormones are essential to embryonic development in major fishes. Moreover, reports indicate that GH mRNA was not detected in orange-spotted grouper (*Epinephelus coioides*) (29) and gilthead sea bream (2) embryos. These reports suggested that GH mRNA was unimportant for growth in these types of fish. It has been reported that the pituitary gland of sturgeon is formed before hatching (33). This likely increases the expression of GH mRNA in the embryonic stages of Siberian sturgeon and can be attributed to the formation of the pituitary gland and the endocrine action of the pituitary.

In the present study, GH mRNA was not detected in newly-hatched larvae of Siberian sturgeon. This is consistent with similar observations for orange-spotted grouper (29) and Japanese eel (30) and is inconsistent with observations of Persian sturgeon and Beluga (31), Chinese sturgeon (1) and milkfish (32). The absence of GH in newly-hatched larvae of Siberian sturgeon does not indicate low efficiency in reverse transcription or poor quality of RNA from these samples, because RPL6 was used as a reference gene for all samples and showed no apparent difference in its expression between samples. The reason for the absence of GH mRNA in newly-hatched larvae may be related to the absence of endogenous production by the larvae.

The findings demonstrated the presence of GH in the unfertilized eggs, non-viable eggs, eyed eggs and fertilized eggs of Siberian sturgeon and suggest that GH may play a key role in development of their eggs. Moreover, the expression of GH mRNA was higher in unfertilized eggs, fertilized eggs and eyed eggs than in non-viable eggs, suggesting a role for GH in egg quality. These findings indicate that GH mRNA may affect egg quality in Siberian sturgeon, their ability to produce viable larvae and, to some extent, may directly determine growth and survival of young fish. These results suggest that GH mRNA may generate a potential marker for egg quality in the Siberian sturgeon.

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