Cloning of FSHβ, LHβ, and glycoprotein α subunits from the Russian Sturgeon (Acipenser gueldenstaedtii), β-subunit mRNA expression, gonad development, and steroid levels in immature fish

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Abstract

The Russian sturgeon, Acipenser gueldenstaedtii, is a late-maturing Acipenseriformes. To elucidate the role of FSH and LH in its reproduction, we cloned its glycoprotein α-subunit (GPα) and gonadotropin β-subunits (FSHβ and LHβ) using 5′ and 3′ RACE-PCR. The nucleotide sequences of the Russian sturgeon (st) GPα, FSHβ, and LHβ are 345, 384, and 411 bp long, encoding peptides of 91, 115, and 114 amino acids, respectively. The deduced amino acid sequence of each mature subunit showed high similarity with those of other teleosts. Sequence analysis showed that stFSHβ is more similar to higher vertebrate FSHβs (35–37%) than to higher vertebrate LHβs (26–30%). The next objective of this work was to compare the development of sturgeon gonads at the very first stages of their growth with the expression of their gonadotropins. Sturgeons at ages 1, 2, 3 or 4 years were sacrificed. The expression of their gonadotropin β-subunits was determined using quantitative real-time PCR, and their gonads were examined histologically, followed by a determination of the plasma levels of estradiol in females and 11-ketotestosterone (11-KT) in males. The expression levels of stFSHβ subunit was found to be higher in fish at 3 and 4 years of age than in 1-year olds. mRNA levels of stLHβ were higher than those of stFSHβ in both genders. Moreover mRNA levels of stFSHβ detected in females were significantly higher than those found in males. Even at age 4 years, all female Russian sturgeons tested contained gonads at the pre-vitellogenic stage, with small oocytes and very low levels of estradiol in the plasma. However, among the males, at ages 3 and 4 years, we found testes that contained spermatids and spermatozoa. Those males were found to have significantly high GSI (gonadosomatic index; gonadal weight as a percentage of BW) levels, stLHβ expression and 11-KT levels.
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Keywords: Gonadotropin; Estradiol; 11-Ketotestosterone; Real-time PCR; Sturgeon; Gene expression; Puberty

1. Introduction

Sturgeons (st) are one of the most ancient groups of Osteichthyes. They are found along the coasts of the Atlantic and Pacific oceans, in the Mediterranean and Black seas, and in many rivers, lakes, and inland seas (Dettlaff et al., 1993). The decline in sturgeon populations in their native habitats, mainly the Caspian Sea, due to over-fishing for meat and production of caviar, destruction of their spawning grounds, and pollution of the water, has led to their introduction into aquaculture. White sturgeon (Acipenser transmontanus) and the Russian sturgeon, Acipenser gueldenstaedtii, are the most important species in the sturgeon family. Despite the fact that they carry positive genetic, resistance to diseases, and have been caught as a valuable sport fish.
Siberian sturgeon (*Acipenser baeri*) are the leading species which have been adapted to aquaculture, and as such they are the most investigated sturgeons with regard to culturing aspects. A severe problem in sturgeon management is slow and asynchronous ovarian maturation. While most males mature at the age of 4 years, females reach first sexual maturity between 6 and 12 years and have biennial ovarian cycles (Doroshov et al., 1997).

Gonadotropins (GtHs) are glycoproteins (GPs) consisting of two noncovalently bound subunits, α and β, which have been intensively studied, especially in fish adapted to aquaculture conditions [reviewed by Yaron et al. (2003)]. As in mammals, both GtHs are heterodimeric GPs, sharing the same α-subunit and distinct β-subunits, the latter conferring their biological specificity. cDNA sequences encoding GtH subunits have been isolated and characterized from more than 19 fish species, representing seven teleostean orders [reviewed by Yaron et al. (2000)]. Recently, Querat et al. (2001) established that the duality of GtHs occurred at the time of the chondrichthyans’ emergence, prior to the split between actinopterygians and sarcopterygians.

Moberg et al. (1991) showed the existence of two GtHs in the white sturgeon (*A. transmontanus*) termed stGtH-I and stGtH-II. Pituitary and plasma concentrations of stGtH-I were found to be higher than those of stGtH-II during vitellogenesis and the early stages of spermatogenesis. Conversely, pituitary and plasma concentrations of stGtH-II were greater than those of stGtH-I during ovulation and spermiation. A gonadotropin-releasing hormone analog (GnRHa) was effective in stimulating the release of both GtHs in mature males and pre-ovulatory females, with a maximal response occurring in the spring. Collectively, the data support the view that sturgeons possess a dual GtH system controlling reproduction (Moberg et al., 1995). Querat et al. (2000) cloned the β-subunits of stGtH-I and stGtH-II of *A. baeri* and, based on their phylogenetic tree, suggested that they should be termed FSH and LH, respectively.

Although the sturgeon is a well-studied fish and there are many articles describing its steroid levels during advanced stages of oocyte maturation and spawning, very little is known about the first stages of puberty in this species.

Due to its high production rates and caviar quality, the Russian sturgeon (*A. gueldenstaedtii*), originally from the Caspian Sea, was chosen to enrich the variety of commercial fish species in Israel. As a prerequisite for a study of its reproduction, we cloned the genes encoding the α- and β-subunits of the GtHs and analyzed the expression of the β-subunits in immature male and female sturgeons grown under local aquaculture conditions. In addition, we characterized the plasma steroid levels of the sturgeons during their first 4 years in culture.

2. Materials and methods

2.1. Fish and sampling procedure

Russian sturgeons (*A. gueldenstaedtii*) originated in the Caspian Sea were brought from Russia and reared at “Dan Fish Farms” (Upper Galilee, Israel; 31°30’N, 34°45’E), under aquaculture conditions, from eggs. Fish were maintained in 250- to 500-m³ concrete tanks at ambient temperature (22–24 °C) and photoperiod, and were fed twice a day with 4 mm pelleted feed (trout feed; Zemach Feed Mills, Zemach, Israel; containing 50% protein and 18% fat), at 0.5–1% of their biomass, depending on the season. One-, two-, three-, and four-year-old sturgeons were sampled in August, when water temperature was 22 ± 0.5 °C. Each fish was anesthetized in a clove oil bath (0.25 mg/L), weight and length were recorded, and blood was taken from the caudal vasculature into heparinized syringes. After centrifugation, the plasma was stored at −20 °C until processing. The pituitary gland was removed and stored in RNA Later buffer (Ambion, Austin, TX). The gonads were removed, weighed and a portion taken for histology. Total RNA was extracted from freshly excised pituitaries of sturgeon females and males from each age group (1, 2, 3, and 4 years) by means of the RNeasy total RNA kit (Qiagen, Alameda, CA), according to manufacturer’s recommendations.

2.2. cDNA cloning of sturgeon GtHs

Total RNA was extracted from four pituitaries of sturgeon, freshly excised, by means of RNeasy total RNA kit (Qiagen, Alameda, CA), according to manufacturer’s recommendations. First strand cDNA was synthesized from 2 µg of the total RNA by Superscript System (Gibco). The second-strand cDNA were synthesized for 3' and 5' rapid amplification of cDNA ends (RACE)-PCR using a RACE kit (Roche Applied Science, Mannheim, Germany). Gene-specific primers for the 3' RACE were designed according to the sequences of relative genes of *A. baeri* (Accession numbers in GenBank—GPz: AJ310342; LHβ: AJ251656; FSHβ: AJ251658). Gene-specific primers: P9 (Table 1) for the GPz subunit, P1 (Table 1) for the FSHβ subunit, and P5 (Table 1) for the LHβ subunit, were used for 3' RACE, along with the primer provided in the kit. PCR was carried out in a volume of 50 µL using 2.5 U of Taq polymerase (Promega, Madison, WI), 5× buffer (Promega), 1.5 mM MgCl2, dNTPs (0.2 mM final concentration of each nucleotide), 100 pmol of each primer, and 4 µL of cDNA for the β-subunits or 2 µL for the GPz. Cycling parameters were: 3 min denaturation at 94 °C, followed by 40 cycles of 1 min denaturation at 94 °C, 2 min annealing at 52 °C and 3 min extension at 72 °C, for FSHβ; or followed by 34 cycles
of 1 min denaturation at 94 °C, 1 min annealing at 52 °C and 1 min extension at 72 °C for LHβ; or followed by 10 cycles of 15 s denaturation at 94 °C, 30 s annealing at 52 °C and 40 s extension at 72 °C for the Gpα subunit. All PCRs were terminated with an additional extension at 72 °C for 7 min. For the 5′ RACE-PCR, the gene-specific reverse primers were designed according to the sequences of the cDNA cloned in the 3′ RACE (P10, P2, and P6 for Gpα, FSHβ, and LHβ, respectively; Table 1) and were used with the primer provided in the kit. PCRs were as already described. The cycling parameters were 3 min denaturation at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 54 °C and 1 min extension at 72 °C for FSHβ; or followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C for LHβ; or followed by 10 cycles of 30 s denaturation at 94 °C, 40 s annealing at 30 °C, and 50 s extension at 72 °C, for the Gpα subunit. All PCRs were terminated with an additional extension at 72 °C for 7 min. The resulting amplified DNA was ligated into a pGEM-T Vector System (Promega), following the manufacturer’s protocol. PCR amplification was performed with P3 and P4 (Table 1) for stFSHβ, P7 and P8 for stLHβ, and P11 and P12 for Gpα as primers to obtain the full-length cDNA. Ligations, transformations, and plasmid preparations were according to Sambrook et al. (1989). The sequencing was carried out at the Center for Genomic Technologies of the Hebrew University. At least three independent clones were sequenced in each case.

2.3. Sequence assembly and analysis

The sequences of each cDNA were assembled using the GAP4 software package (Bonfield et al., 1995). Sequence analysis, molecular weight, and isoelectric point calculations were carried out by Wisconsin Package 10.0, Genetics Computer Group (GCG). The position of the signal-peptide cleavage site was determined using the SignalP V1.1 program (Nielsen et al., 1997). Multiple sequence alignments and cluster analysis by the neighbor-joining method were performed using the ClustalX program (Higgins and Sharp, 1989).

2.4. Histological analysis

The gonad samples were fixed in Bouin and subsequently processed for light microscopy. Paraffin sections of 6 μm were stained with hematoxylin and eosin. The terminology used for the sturgeon hybrid, the bester (Amiri et al., 1996a,b), was adopted for our descriptions.

Mean oocyte diameter was calculated for each fish after measuring five of the largest oocytes present in a histological section. Only oocytes sectioned through the nucleus were measured. Since ovarian development in the sturgeon is very uniform, only several sections per fish (n = 6) needed to be examined.

The presence and amounts of spermatogonia, spermatocytes, spermatids, and spermatozoa in the testes were studied semiquantitatively and randomly over the entire surface of a mid-longitudinal section of the testes. Testes were classified into the following categories: immature (or young) testes, containing no germ cells (stage A); testes containing a small number of spermatogonia (stage B); and mature testes, showing active spermatogenesis with spermatocytes and spermatids (stage C).

2.5. Real-time PCR

To compare the mRNA levels of the β-subunits of sturgeon FSH and LH, the relative abundance of their mRNA was normalized to the amount of an
endogenous reference, the 18S subunit of rRNA, by the comparative threshold cycle (C_{T}) method, according to Levavi-Sivan et al. (2004b). The relative amount of each \( \beta \)-subunit’s mRNA was calculated by the formula \( 2^{-\Delta \Delta C_{T}} \), where \( \Delta C_{T} \) corresponds to the difference between the \( C_{T} \) measured for stFSH\( b \) or stLH\( b \), and that measured for 18S rRNA. To validate this method, serial dilutions were prepared from a pituitary cDNA sample (0.5, 0.1, 0.02, 0.01, and 0.005), and the efficiencies of each \( \beta \)-subunit and 18S ribosomal RNA amplifications were compared by plotting \( \Delta C_{T} \) versus log (template), according to the method of Muller et al. (2002). Linear regressions of the plots showed the following \( R^{2} \) values and slopes, respectively: 0.976 and −3.00 for 18S rRNA; 0.999 and −3.096 for stFSH\( b \); and 0.992 and −2.80 for stLH\( b \).

Total RNA was prepared from individual pituitaries using Trizol (Invitrogen, Carlsbad, CA) and each sample was reverse-transcribed at 57 °C using Reverse-iT 1st Strand Synthesis Kit (ABgene, Surrey, UK) and random hexamers, according to the manufacturer’s protocols.

Gene-specific primers used for the real-time PCR were designed using Primer3 Software. The primers used for stFSH\( b \) amplified a 170-bp product and corresponded to nt 139–158 and 328–309 (P13 and P14, respectively, Table 1, Accession No. AY519657). Primers for stLH\( b \) (P7 and P15, Table 1, Accession No. AY333426) amplified a 190-bp product. Primers for 18S rRNA (P16 and P17, Table 1, Accession No. AY188400) amplified a 180-bp product. The PCR mixture consisted of 5 \( \mu l \) of dilute cDNA sample, 0.75 pmol of each primer, and 7.5 \( \mu l \) of Mastermix for Syber Green I (ABgene) in a final volume of 15 \( \mu l \). Amplification was carried out in a RotorGene 3000 Sequence Detection System (Corbett Research, Sydney, Australia) under the following conditions; for 18S rRNA: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 20 s, and then a final extension at 72 °C for 20 s; for stFSH\( b \): initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 62 °C for 20 s, and extension at 72 °C for 15 s; for stLH\( b \): initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 64 °C for 20 s, and extension at 72 °C for 15 s. Amplification of stFSH\( b \), stLH\( b \) and 18S rRNA cDNAs was performed simultaneously in separate tubes and in duplicate, and the results were analyzed with the Q-Gene software (BioTechniques Software Library at: www.BioTechniques.com). Dissociation-curve analysis was run after each real-time experiment to ensure that there was only one product. To control for false positives, a reverse-transcriptase negative control was run for each template and primer pair.

### 2.6. ELISA for steroids

Estradiol and 11-ketotestosterone (11-KT) were determined by enzyme-linked immunosorbent assay (ELISA), according to Cuisset et al. (1994); Nash et al. (2000) and Levavi-Sivan et al. (2004a), using acetycholinesesterase as a label. The anti-11-KT was donated by Dr. D.E. Kime (Sheffield, UK) and was previously described in Cuisset et al. (1994). The anti-estradiol was described in Levavi-Zermansky and Yaron (1986). All samples were analyzed in duplicate, and for each ELISA plate, a separate standard curve was run. The lower limits of detection were 0.93 and 0.50 pg/ml for 11-KT and estradiol, respectively. The intra- and inter-assay coefficients of variance were less than 7 and 11%, respectively. Steroid levels in the sturgeon plasma, determined by ELISA, were validated by verifying that serial dilutions were parallel to the standard curve.

### 2.7. Statistical analysis

Data are presented as means ± SEM. The significance of the differences between group means of hormone or mRNA levels was determined by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) test using the Graph-Pad Prism software (GraphPad, San Diego, CA) with the level of significance in different groups set at \( p < 0.05 \).

### 3. Results

#### 3.1. cDNA cloning and sequence analysis of the stFSH\( b \), stLH\( b \), and GP\( z \) subunits

The full-length cDNA of stFSH\( b \) was compiled from the data obtained by the 5’ and 3’ RACE, and the nucleotide and deduced amino acid sequences are shown in Fig. 1 (EMBL Accession No. AY519657). The cDNA was 1080-bp long and had an open reading frame of 384 bp, beginning with the first ATG codon at position 37 and ending with the stop codon at position 421. A putative polyadenylation signal, ATTAAA, was recognized 25 bp upstream of the poly(A) tail. The position of the signal-peptide cleavage site was predicted at position 15, yielding a signal peptide of 14 amino acids and a mature peptide of 114 amino acids. The calculated \( M_{r} \) of stFSH\( b \) polypeptide chain is 12173.61 and the polypeptide has an isoelectric point of 4.25. Two putative N-linked glycosylation sites were found at positions 12 and 29 from the N-terminus of the putative mature peptide.

The full-length cDNA of stLH\( b \) was compiled from the data obtained by the 5’ and 3’ RACE, and the nucleotide and deduced amino acid sequences are shown in Fig. 2 (EMBL Accession No. AY333426). The cDNA
was 599-bp long and had an open reading frame of 411 bp, beginning with the first ATG codon at position 70 and ending with the stop codon at position 481. A putative polyadenylation signal, ATAAA, was recognized 16 bp upstream of the poly(A) tail. The position of the signal-peptide cleavage site was predicted at position 23, yielding a signal peptide of 22 amino acids and a mature peptide of 115 amino acids. The calculated Mr of stLHβ polypeptide chain is 14812.18 and the polypeptide has an isoelectric point of 4.85. One putative N-linked glycosylation site was found at position 8 from the N-terminus of the putative mature peptide.

The full-length cDNA of the stGP α-subunit was compiled from the data obtained by the 5′ and 3′ RACE, and the nucleotide and deduced amino acid sequences are shown in Fig. 3 (EMBL Accession No. AY519658). The cDNA was 655-bp long and had an open reading frame of 345 bp, beginning with the first ATG codon at position 57 bp and ending with the stop codon at position 402 bp. A putative polyadenylation signal was found at position 16 of the cDNA sequence.
signal, ATAAA, was recognized 21 bp upstream of the poly(A) tail. The position of the signal-peptide cleavage site was predicted at aa 25, yielding a signal peptide of 24 amino acids and a mature peptide of 91 amino acids. The calculated Mr of the stGPa polypeptide chain is 10502.1 and the polypeptide has an isoelectric point of 8.84. Two putative N-linked glycosylation sites were found at positions 55 and 77 from the N-terminus of the putative mature peptide.

3.2. Comparison of stFSHb, stLHb, and stGPa

The deduced amino acid sequences of the Russian sturgeon's FSHb, LHb, and GPb subunits were compared with homologous subunits from a number of other fish species (Fig. 4, Table 2). Gaps marked by dashes are introduced to maximize the alignments of the cysteine residues between the subunits (Fig. 4). The highest degree of identity was found with A. baeri (91, 98, and 100% identity for stGPa, stLHb, and stFSHb, respectively), followed by the subunits of Anguilliformes (48–72%), and cyprinids (45–72%). The percent identities of the sturgeon β-subunits were 40–59% with those of the Perciformes and 42–59% with those of the salmonid species. The lowest level of identity was observed with Fundulus heteroclitus (53, 46, and 39% for stGPb, stLHb, and stFSHb, respectively). The identity with other percomorph fishes was in the range of 40–47% for stFSHb, 46–59% for stLHb, and 54–62% for the stGPb subunits. In both stFSHb and stLHb subunits, the positions of all 12 cysteines are conserved, and so are the N-glycosylation sites. The identity between stFSHb and stLHb is very low (43%), and can be attributed to the 12 conserved cysteines and other conserved regions.

3.3. Expression of stFSHb and stLHb in males and females

Real-time quantitative PCR, which enables the specific and sensitive detection of transcripts, was used to study the expression of the GtH β-subunits at different ages and in different genders. mRNA levels of stFSHb were significantly higher in females than in males (Fig. 5A), while those of stLHb were similar in both genders (Fig. 5B). mRNA levels of stFSHb in females were significantly lower in their first year of life, increased dramatically during the second year, and then did not change significantly until the fourth year. The mRNA levels of stLHb did not increase significantly until the age of 4 (Fig. 5). In both genders, the levels of stLHb were significantly higher than those of stFSHb.

3.4. Gonad development and steroid levels

No significant difference was found during the first three years between males and females in respect to their weight. However, the female's weight was significantly higher than that of the males after 4 years of growth (6.8 kg ± 1.53 and 4.23 kg ± 1.0 for females and males, respectively). In this study, we used four fish groups at the ages of 1 year (n = 19), 2 years (n = 14), 3 years (n = 19), and 4 years (n = 18). Histological analysis and steroid-level determinations were performed on all fish. Fish from the age of 2 years on had differentiated gonads, enabling estradiol determination in females and 11-KT levels in males. Levels of both steroids were determined in all younger (1-year-old) fish.

Histological analysis showed that most females, irrespective of their age, were at the pre-vitellogenic stage,
Fig. 4. Alignment of the amino acid sequences of the FSHβ, LHβ, and glycoprotein α (GPα) subunits of sturgeon and other actinopterygian species. The putative N-linked glycosylation sites are boxed. The sequences were extracted from the GenEMBL and Swiss-Prot databases, or taken from published articles.

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according to the classification of Van Eenennaam and Doroshov (1998) for the Atlantic sturgeon and that of Amiri et al. (1996a,b) for hybrid sturgeon. Gonadal histology of representative 4-year-old female Russian sturgeons is shown in Fig. 6. The females sampled in this study had oocytes ranging from 50 to 460 μm in diameter. Fig. 6B shows an oocyte at the perinucleolar stage—nucleoli are distributed as granules surrounding the germinal vesicle and only a single cortical vesicle can be seen at the bottom. Vitellogenic granules were not detected. The oocytes have a thin vitelline envelope (Fig. 6). A low correlation ($r^2 = 0.06$; $n = 30$) was found between estradiol levels and GSI (gonadosomatic index; gonadal weight as a percentage of BW), and between estradiol levels and oocyte diameter ($r^2 = 0.01$; $n = 30$), indicating that the females were at the pre-vitellogenic phase.

Histological analysis of 1-year-old fish showed that sex in most of them was still undifferentiated. At the age of 2 years, 33% of the females contained oocytes with a diameter <100 μm, while 67% contained oocytes with diameters between 100 and 140 μm. At the age of 3 years, all females contained oocytes with a diameter of 150–180 μm. However, at the age of 4 years, only 21% of the females contained oocytes with a diameter <100 μm, whereas 50% contained oocytes of 100–180 μm and 28% already contained oocytes >180 μm. Estradiol levels were very low in all females at all ages (Table 3).

Males differentiated faster than females. As early as the first year, we found three differentiating males (out of 25 fish). Most of the males (19 out of 25 sampled) were still immature with testicular lobes containing spermatogonia. However, some of the males, aged 3 and 4 years, had already developed gonads containing spermatozoa in some of the lobules.
The presence and amounts of spermatogonia, spermatocytes, spermatids, and spermatozoa in the testes were studied semi-quantitatively and randomly over the entire surface of a mid-longitudinal testis section. Testes were classified into the following three categories: quiescent testes containing no germ cells apart from primary spermatogonia and the fat pad (stage A, Fig. 6C; stage 1 according to the classification of Van Eenennaam and Doroshov (1998)); testes containing small numbers of spermatogonia (stage B, Fig. 6D; stage 3 according to the classification of Van Eenennaam and Doroshov (1998)); and mid-spermatogenesis testes where the majority of the cysts contain active spermatogenesis with spermatocytes and spermatids as well (stage C, Fig. 6E; stage 4 according to the classification of Van Eenennaam and Doroshov (1998)). The appearance of meiotic (from stage A to stage B) as well as post-meiotic germ cells (transition from stage B to stage C) was accompanied by a significant increase not only in GSI values, but also in 11-KT levels (Fig. 7). When the males were grouped according to their developmental stage of spermatogenesis, a high correlation was found, not only with their GSI values, but also with their plasma 11-KT levels (Fig. 7).

Table 3

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>E2 (ng/ml ± SEM)</th>
<th>n</th>
<th>11-KT (ng/ml ± SEM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.239 ± 0.034a</td>
<td>19</td>
<td>0.283 ± 0.05a</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>0.41 ± 0.07a</td>
<td>9</td>
<td>5.22 ± 2.14b</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>0.72 ± 0.06a</td>
<td>8</td>
<td>5.92 ± 0.91b</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>0.48 ± 0.11a</td>
<td>13</td>
<td>5.52 ± 1.60b</td>
<td>5</td>
</tr>
</tbody>
</table>

At the first year E2 and 11KT were measured in all the fish. Means marked by different letters differ significantly (p < 0.05).
4. Discussion

This paper describes the molecular cloning and sequence analysis of three GtH subunits, stGPα, stFSHβ, and stLHβ, from the Russian sturgeon (A. guledenstaedti). Molecular characterization of the GtH cDNAs allowed the development of a quantitative real-time PCR and the investigation of changes in pituitary GtH mRNA levels during the early stages of puberty in male and female sturgeons.

The sturgeon is a bony fish belonging to the ancient super order Chondrostei in the class Osteichthyes, and it was somewhat surprising, to find that the sequences of the GtH β-subunits were grouped together with those of the dogfish (Scyliorhinus canicula) belonging to another class of fish, the Elasmobranchii, and also with those of frogs and higher tetrapods rather than to other osteichthyns such as the more evolved Teleostei. Similar results have been reported in a comparison of these subunit sequences in A. baeri (Querat et al., 2000).

The Russian sturgeon, like other Acipenseriformes, is a tetraploid with 250 chromosomes (2n) (Fontana, 1994). Despite the tetraploidy of its genome, only one type of clone was cloned from each of the GtH subunits in this study. This suggests only one gene copy for each subunit, a conclusion which should be verified via Southern blot analysis. A comparison of the deduced amino acid sequence of stFSHβ with those of other GP hormone β-subunits revealed two peculiar characteristics which differed from the situation in teleosts where the N-terminus exhibits an unexpected divergence at sites of N-glycosylation and cysteine (Yaron et al., 2001). The common pattern of 12 cysteines and two N-linked glycosylation sites characterizing the tetrapod β-subunits of FSH is conserved in the Russian sturgeon. Hormone glycosylation is important for receptor-mediated activation of adenylyl cyclase upstream of G-protein activation (Arey et al., 1997; Beitins and Padmanabhan, 1991).

In the α-subunit, the positions of all 10 cysteines and the two putative N-linked glycosylation sites of the sturgeon are completely conserved relative to other fish and mammalian species. It also appears that the region from amino acids 33 to 66 is highly conserved, consisting of two paired adjacent cysteines and the first putative N-linked glycosylation site. This region is suggested to be involved, in both human (Xia et al., 1994) and red seabream (Gen et al., 2000), in the processes of subunit assembly and/or receptor binding. stGPα mRNA contains a non-consensus polyadenylation signal (AT-TAAA), which has been found to be the most frequent variant of the AATAAA signal (Sheets et al., 1990). The same signal motif has also been found in the striped bass α-subunit mRNA (Hassin et al., 1995), in both coho salmon α-subunits (Dickey and Swanson, 2000), and in the tilapia GPα (Gur et al., 2001). The duality of the α-subunit has been suggested to emerge from a duplication of the entire genome in some of the species (Kobayashi et al., 1997). However, although the Russian sturgeon is tetraploid (Fontana, 1994), only a single α-subunit was cloned in this study.

Among GtH subunits, the sequences of the GPα subunit show the highest degree of conservation across vertebrates (53–91%). Typically, sequence alignment revealed a lower degree of amino acid identity between the FSHβ (39–51%) subunits for every species, as compared with the amino acid identity among the fish LHβ subunits (49–68%). This may indicate a more rapid diversification of FSHβ than LHβ during evolution, as has been suggested previously (Querat et al., 2000, 2001; Yaron et al., 2003).

In the present study, stFSHβ mRNA levels were very low in fish at early stages of testicular development, increased at the more advanced stages of spermatogenesis, and appeared to fall in fish with even more advanced stages of spermatogenesis. stLHβ mRNA levels did not change significantly during the first 4 years of life. In contrast, stFSHβ mRNA levels were low during the first year, but increased thereafter and remained high in 4-year-old females. mRNA levels of stLHβ did not change significantly during the course of the experiment. Nevertheless, the increase in the stFSHβ mRNA in females was not accompanied by either an increase in estradiol levels or any sign of vitellogenesis in the ovary. The expression patterns of stFSHβ and stLHβ have been found to differ at different stages of the reproductive cycle in many fish from different groups: salmonids (Swanson et al., 1991), blue gourami (Jackson et al., 1999), tilapia (Yaron et al., 2001), goldfish (Sohn et al., 2001), and seabream (Elizur et al., 1996). Pituitary and plasma
concentrations of FSH in white sturgeon were found to be higher than those of LH during vitellogenesis and early stages of spermatogenesis. Conversely, pituitary and plasma concentrations of LH were higher than those of FSH during ovulation and spermiation (Moberg et al., 1995).

The present study shows sexual dimorphism in FSHβ gene expression in immature (2- to 4-year-old) Russian sturgeon, whereby the mRNA levels of FSHβ were significantly higher in females than in males. A somewhat similar situation has been reported in goldfish, where the increase in FSHβ transcripts in males during the breeding season is less pronounced than in females. The non-synchronous pattern of expression of the two β-subunits in male goldfish was explained by the constantly high GSI in the mature-stage testis throughout the year with only a gradual increase in androgen levels during the spawning season (Sohn et al., 1999).

Sexual dimorphism in GtH subunit mRNA levels has been described in seabreams as well. In both gillhead seabream (Sparus aurata; Elizur et al., 1996) and red seabream (Pagrus major; Gen et al., 2000), FSHβ mRNA levels were higher in males than in females, whereas those of LHβ were similar in both genders. However, a comparison with the present study would not be valid because the investigated seabreams were adult fish during the spawning season whereas the fish in the present study were still far from maturity.

In contrast to the situation in salmonids and eels, stLHβ mRNA is already detectable in juvenile sturgeon of both genders. The presence of LHβ transcripts in the pituitary of immature fish has been reported in another late-maturing fish, the black carp (Mylopharyngodon piceus), more than 4 years before puberty (Gur et al., 2000), and in the common carp as well (Kandel-Kfir et al., 2002). Although this suggests a different gonado-tropic function during early stages of development, further research is needed to confirm this notion; it is possible that in addition to its recognized role during final oocyte maturation, LH has other endocrine functions in juveniles. High levels of LHβ have also been found in juvenile rainbow trout (Oncorhynchus mykiss; Naito et al., 1991), striped bass (Morone saxatilis; Hassin et al., 1999), seabream (Gen et al., 2001), and recently, in the primitive catfish (Vischer et al., 2003). Moreover, in salmon and trout, LH has been found in the pituitaries of juvenile fish (Ito et al., 1993; Naito et al., 1991; Suzuki et al., 1988). In this context, it should be noted that previous studies have indicated the presence of two types of receptor for GtH in different teleosts (Oba et al., 2001). It has also been shown that FSH receptor interacts with both of the homologous LH and FSH, whereas LH receptor interacts specifically with LH in catfish (Vischer and Bogerd, 2003) and salmon (Miwa et al., 1994). The mechanisms underlying gender-specific patterns of FSHβ gene expression in the pituitary of male and female sturgeon are still unclear. However, the expression of GtH subunit genes is regulated by various endocrine factors, including hypothalamic releasing hormones, gonadal steroids and peptides that can contribute to these changes [reviewed by Yaron et al. (2001, 2003)].

Gametogenesis in Russian sturgeon is generally similar to that in other sturgeon species. Sturgeons are gonochoristic, and intersex is rare. Sturgeon females display group-synchronous oocyte development, with distinct clutches of vitellogenic and pre-vitellogenic follicles. In the hybrid sturgeon, bester, seasonal changes in estradiol, testosterone and vitellogenin levels were well correlated with the progress of oogenesis, and oocytes less than 0.6 mm in diameter were at pre-vitellogenic stages (Amiri et al., 1996b). Females white sturgeon, at late vitellogenesis stage have oocytes with a diameter of 3.33 ± 0.05;mm (Linares-Casenave et al., 2003) and estradiol level of 2–4 ng/ml. This is in agreement with the present study where we found that all of the females had pre-vitellogenic oocytes with a diameter of 0.05–0.45 mm, and low levels of estradiol. Low levels of estradiol was also found in juvenile fish of another late-maturing fish species, the black carp (Mylopharyngodon piceus) (Gur et al., 2000).

Male and female Russian sturgeon have distinctly different rates of sexual maturation. Spermatogenesis occurs rapidly, as suggested by the testis histology, 11-KT levels and GSI values. Some of the four-year-old males already have cysts, most of which contain spermatocytes and spermatids. Reproductive development in females is slower: at the age of 4 years, most of the females sampled in this study were still pre-vitellogenic. In other sturgeons as well, males mature faster and at a younger age than females; in the Atlantic sturgeon, all young sturgeon caught in the river that had sexually differentiated gonads were males (Van Eenennaam and Doroshov, 1998). In Actipenser persicus, sampled in their natural habitats, mature males were significantly younger than their female counterparts (Safi et al., 1999).

We found a dramatic increase in the level of 11-KT in males between the first and second year (Table 3). This can be attributed to either sex differentiation or to the onset of spermatogenesis. In male trout, the male-specific expression of P450c11 (11β-hydroxylase; a key enzyme catalyzing the synthesis of 11-oxygenated androgens) is directed to the involvement of 11-oxygenated androgens in testicular differentiation (Govoroun et al., 2001; Liu et al., 2000). When GtH is secreted from the pituitary, spermatogonial mitosis switches from stem-cell renewal to proliferation toward meiosis. It appears that GtH does not act directly on germ cells, but rather through the gonadal biosynthesis of 11-KT, which is a major androgen in teleosts (Miura et al., 1991a,b). In various teleosts, this steroid has been shown to be synthesized in the testis following GtH
stimulation, and high levels have been detected in the serum during spermatogenesis [reviewed by Miura and Miura (2003)]. 11-KT has been found crucial for spermatogenesis in the Japanese eel (Miura et al., 1991a,b), goldfish (Kobayashi et al., 1991), and Japanese huchen (Amer et al., 2001). These findings indicate that 11-KT is one of the factors involved in the initiation of spermatogonial proliferation toward meiosis. In the hybrid sturgeon, 11-KT levels have also been found to be higher during late spermatogenesis and pre-spermiation and lower at the degeneration stage (Amiri et al., 1999).

In conclusion, we show that the expression levels of both GtH β-subunits are higher in 3- and 4-year-old fish than in 1-year-olds. mRNA levels of stLHβ are higher than those of stFSHβ in both genders. Moreover, mRNA levels of stFSHβ in females are significantly higher than those found in males. We also show that male and female Russian sturgeon have distinctly different rates of sexual maturation. While all females exhibited gonads at the pre-vitellogenic stage, with small oocytes and very low levels of estradiol, 3- and 4-year-old males had testes with spermatids and spermatooza. Those males were found to have significantly high GSI levels, stLHβ expression and 11-KT levels.

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References


