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Changes of vitellogenin and Lipase in captive Sterlet sturgeon *Acipenser ruthenus* females during previtellogenesis to early atresia

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Abstract Plasma chemistry, lipid metabolism and vitellogenin gene expression of captive Sterlet sturgeon *Acipenser ruthenus* were studied in different maturity stages. A total of 32 fish were sampled, and maturity stages were identified on the basis of histological criteria and direct observation. Females were classified to four groups: previtellogenic, vitellogenic, post-vitellogenic, and atresia. Blood, gonad and liver tissue samples were taken through non-lethal biopsy. Our results showed that plasma levels of glucose, cholesterol, triacylglycerol, high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein, calcium, phosphorus, alkaline phosphatase activity, albumin and total protein increased during ovarian development and were highest at post-vitellogenic stage. The lowest amounts in atresia stage demonstrate that lipid and energy imbalance was related to reabsorption and digestion of the yolk. These results suggested that the VLDL was the main plasma lipoprotein component of Sterlet. We determined that lipoprotein lipase and hepatic lipase activity increased during vitellogenesis process which suggested the role of lipase enzymes in regulating blood lipid metabolism. RT-PCR analysis indicates that Vitellogenin (VTG) mRNA could be detected both in livers and ovaries of female Sterlet. Throughout the study, the expression level of VTG gene showed an increase both in ovaries and in livers reaching its peak at late vitellogenesis stage. This strongly indicated a relation between VTG mRNA and ovarian development.

Keywords Sturgeon · Lipid · Lipoprotein · VTG mRNA · Biochemical · Oogenesis

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Introduction

The formation, development and maturation of the female fish ovum (oogenesis) is a dynamic and complex physiological process that can be subdivided normally into three successive main stages; previtellogenesis, vitellogenesis, and oocyte maturation (Lubzens et al. 2010). The vast majority of oocyte growth occurs during vitellogenesis, as estrogen produced by the follicle cells stimulates hepatic

vitellogenin synthesis and secretion into the systemic circulation (Wallace 1985). Following vitellogenesis, post-vitellogenic follicles must be competent to undergo both maturational and ovulatory processes to respond to the gonadotropin surge, resume meiosis, and release viable ova (Breton et al. 2012). However, oocyte maturation competence and germinal vesicle breakdown sometimes fail, resulting in ovary regression or atresia, a process by which ovarian follicles degenerate and subsequently are resorbed by the ovary (Linares-Casenave et al. 2002).

Oocyte growth, particularly in oviparous species, is characterized by the intense deposition of products, e.g., RNAs, proteins, lipids, vitamins, and hormones (Babin et al. 2007). Oocytes store large amounts of yolk material and oil (neutral lipids) in the cytoplasm during oogenesis. In fish, the main yolk precursor is a glycolipophosphoprotein, vitellogenin (VTG). This molecule is synthesized in the liver under estrogenic induction and transported via the bloodstream to the ovary, where it is then selectively internalized into the oocytes (Wallace 1985). VTG is thought to have played a pivotal role in the intracellular and extracellular transfer of lipids and liposoluble substances (Babin et al. 1999), aside from their importance as the major transporter of polar lipids, especially phospholipids into oocytes (Lund et al. 2000). The highly negative charge of phosvitin allows VTG to ionically bind calcium and deliver many of the basic building blocks, e.g., amino acids, lipids, inorganic phosphates, and calcium for the growing oocyte (Patino and Sullivan 2002). The increase in plasma protein, calcium, magnesium, and phosphoprotein content has been described previously for sturgeons as indicators to predict vitellogenin levels indirectly (Linares-Casenave et al. 2003; Akhavan et al. 2015). Beside this, partial or full-length VTG cDNAs have been cloned in different fish species (NCBI database) providing potential for a wide application of the VTG transcripts for the measurement of VTG. Measurement of VTG mRNA has been successfully performed for quantifying the VTG values and has proved to be equally as effective in this capacity as VTG protein (Schmid et al. 2002). Molecular characterization of the cDNA of the gene encoding the VTG has received some prior study in sturgeon. The VTG of white sturgeon *Acipenser trasmontanus* was previously investigated by Kroll (1990), and also one VTG transcript has been characterized in the same species by Bidwell et al. (1991) and Bidwell and Carlson (1995). VTG mRNA expression was used as a marker in juvenile Chinese sturgeon *Acipenser sinensis* treated with 17β-estradiol and 4-nonylphenol (Zhang et al. 2005).

The oocytes of fish also accumulate high amounts of neutral lipid (Wiegand 1996), which consists of triacylglycerol (TAG), wax esters, and steryl esters. It has been suggested transport of neutral lipids is done by lipoproteins from adipose tissue to the ovary (Leger et al. 1981). Lipoproteins can be classified into several classes on the basis of their specific density, and these classes are very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Babin and Vernier 1989). Among the lipoproteins, VLDL which is mainly TAG could play an important role in transport of lipids to the ovary (Ando and Matsuzaki 1996) and also fish have HDL-dominant lipoprotein profile, and therefore, the majority of plasma cholesterol is transported in association with HDL (Henderson and Tocher 1987).

The TAG from VLDL is cleaved by a specific enzyme, lipoprotein lipase (LPL), which is considered a key enzyme in whole-body lipid metabolism and balance, and the extra-hepatic rate-limiting enzyme in the hydrolysis of circulating TAG during ovarian development (Ibanez et al. 2008; Ma et al. 2011).

In comparison with lengthy gonadal cycle in the large migratory species, Sterlet *Acipenser ruthenus*, the smallest freshwater sturgeon species is an ideal model to study the reproductive physiology of sturgeon due to earliest maturation (Holčik 1989). Given the importance of the vitellogenin, lipoproteins, and lipase in regulation of lipid and oocyte growth, this study set out to quantify the blood lipid profile, lipase enzymes activity, and dynamics of expression pattern of VTG mRNA in Sterlet sturgeon throughout ovarian development to assess the relative contribution of these parameters in ovarian development.

Materials and methods

Fish and rearing condition

The Sterlet stocks were obtained originally as larva from Hungary in 2004, transported by airplane to Iran, and reared at the Shahid Dr. Beheshti Sturgeon Fish Propagating and Rearing Complex, Rasht, Guilan. The

animals examined in the present study were produced by artificial propagation from the Hungarian broodstocks in 2010–2011. Larval rearing was carried out in circular concrete tanks (VNIRO type, 3 m diameter, 17 cm depth), fed with newly hatched artemia, and then reared for 4–5 years in special flow-through rectangular concrete tanks (25 × 3 × 0.6 m) under natural seasonal fluctuations of photoperiod and temperature (ranged from 5 ± 2.2 °C in June to 25 ± 2.4 in August, dissolved oxygen > 6 mg L^{-1} and pH 6.8–7.5). Fish were fed by 0.5–3 % body weight per day with a commercial trout pelleted diet (36 % crude protein, 14 % crude fat, 10 % ash, 11 % moisture) throughout cultivation according to their nutritional requirements, seasonal temperature, and fish size.

Isolation of ovarian follicles and sampling procedure

Fish were deprived of food one day before sampling procedure. One hundred fish from the 4-year-old stock of Sterlet sturgeon were randomly captured and deeply anesthetized in clove powder extract bath individually (400 mg L^{-1}, Ghiasi et al. 2014). Then each fish transferred to the special table for sampling. Biopsy and histological procedures were used to determine the sex and stage of gonadal development. The biopsy of gonads was done according to Chebanov and Galich (2011) by a special steel probe (diameter 3–4 mm groove length 3–6 cm) which was inserted between the rows of abdominal scutes between the third and forth scutes from the end at an acute angle to the body axis to a depth of 5 cm. At probe torsion, some gonadal tissue was left in the cut edge. After sampling, sex identification was done by visual examination of ovary and testis (Falahatkar et al. 2011). To consider different maturity stages, eight females of comparable developmental stage and size were selected for each of four gonadal development stages ($n = 32$ fish among a total of 100; for more information see histological observation and gonadal stage determination section). Blood sampling (2 mL) was taken by a heparinized syringe from the caudal vasculature, spun at 1600 g, 10 min for extracting the plasma, and stored at −70 °C until assay. Non-lethal liver and ovary biopsies were carried out by cutting a small portion of liver and ovary. To this regard, the 2- to 3-cm incision was made approximately five ventral scutes anterior from pelvic fin and midway between the ventral midline and scutes (Falahatkar et al. 2013). The liver tissue and a portion of ovarian tissue were cut using a scalpel and tissue forceps and quickly immersed in RNA*later*® solution (Life Technologies, GmbH, Darmstadt, Germany) for overnight at 4 °C and then stored at −20 °C until RNA extraction. Beside this, a small portion of gonad samples were immediately preserved in 10 % neutral buffered formalin and sturgeon ringer solution (Dettlaff et al. 1993) for further morphological and histological assessment. The incision was closed with two simple sutures (Chapman and Park 2005). The entire biopsy procedure takes 3 min. All procedures involving fish care and manipulation were carried out in accordance to the regulations of National Health and Medical Research Council, Australia, on the care and use of animals for scientific purposes.

Histological observation and gonadal stage determination

Formalin-preserved gonadal tissues were dehydrated in different percent of alcohol by automatic linear tissue processor (Scilab, OPTI-WAX, Yorkshire, UK) and embedded in paraffin, sectioned at 6 μm with a Leica microtome (Leica RM 2125RT, Nussloch, Germany) and subsequently stained with hematoxylin and eosin. Finally, histological slides were examined by light microscopy (Olympus BX51, Japan). The developmental stages of ovaries were classified into 4 stages according to general appearance (Bruch et al. 2001) and histological terminology (Amiri et al. 1996; Linares-Casenave et al. 2002) as follows: (1) Previtellogenic (visible whitish eggs, perinucleolus stage), (2) Vitellogenic (bright yellow appearance, yolk stage), (3) Final oocyte maturation (large dark ovaries, Migratory nucleus stage), and (4) Atretic follicles (reabsorbing oocytes).

Plasma analyses

The concentration of glucose, triacylglycerol (TAG), cholesterol (CHOL), HDL, calcium (CA), phosphorus (P), alkaline phosphatase activity (ALP), albumin (ALB), globulin (GLB), and total protein (TP) were measured with enzymatic colorimetric procedure (according to the manufacturer's instructions) using an autoanalyzer (Mindray, BS-200, Nanshan, China)

and standard analysis kits from Pars Azmoon (Karaj, Iran). In brief, plasma glucose concentrations were measured by using glucose oxidase (Falahatkar et al. 2009). TAG and CHOL concentrations were measured at 20–25 °C, 340 nm; minimum detectable limit 5 mg dL^{-1} by an enzymatic method based on GPO-PAP and CHOD-PAP, respectively (Rifai et al. 1999). Plasma HDL was measured by use of polyethylene glycol-cholesterol esterase according to the color intensity at 600 nm (Sharpe and MacLatchy 2007). CA concentrations were measured by colorimetry (612 nm, 20–25 °C; MDL = 15 mg dL^{-1}) in the presence of methylthymol blue. The color intensity in this procedure was proportional to the concentration of CA (Gindler and King 1972). P was determined according to Henry et al. (1974) at 546 nm, 37 °C; MDL = 2 mg dL^{-1}. ALP activity was measured with the Deutsche Gesellschaft fur Klinische Chemie (DGKC) method at 405 nm (Rick 1990). TP were measured by using the standard Biuret method at 540 nm (Doumas et al. 1981). ALB were detected by the bromocresol green binding method, and also the GLB values were calculated by subtracting ALB from TP (Kumar et al. 2005).

Plasma LDL and VLDL cholesterol concentrations were calculated based on total cholesterol, HDL cholesterol, and TG measurements according to the Friedewald equations (Friedewald et al. 1972).

The plasma lipoprotein lipase (LPL) and hepatic lipase (HL) activity were measured throughout different maturity stages. These assays employed the quantitative sandwich enzyme immunoassay technique using available kits (Cusabio, Wuhan, China). LPL and HL were measured in a duplicate run with an inter-assay coefficient of variation of <10 % and intra-assay precision of <8 %. Samples and standards were pipetted into specific antibody-coated LPL tubes, and assay procedure was carried out according to the kit protocol. The color intensity develops in proportion to the amount of LPL and HL and is measured with Elisa reader (Mindray, MR-96, Shenzhen, China). The minimum detection limit was 7.81 pg ml^{-1} and 0.039 U ml^{-1} for LPL and HL assays, respectively.

RNA isolation

Total RNAs from the liver and oocyte samples were extracted using RNA Isolation Kit (Qiagen, Limburg, the Netherlands) according to the manufacturer's instructions and perform a DNase treatment. The quality and concentration of total RNA were determined at 260 nm, and impurities were assessed by the OD 260 nm/OD 280 nm ratio using Picodrop P200 system (Alpha Biotech Ltd., Glasgow, UK). Besides this, RNA quality has been tested by verifying the presence of 28S:18S rRNA ratio fractions after running for 80 min (80 volts) in a 1 % agarose gel and followed by staining with ethidium bromide. First-strand cDNA was synthesized from 1 μg of total RNA preparations using the protocol of a reverse transcriptase kit (Thermo Scientific, Bremen, Germany) in a total volume of 20-μl reaction.

Primer design

Four cDNA sequences of VTG from *Acipenser baerii* (GenBank: HQ007054.1), *Acipenser persicus* (GenBank: JX244892.1), *Acipenser transmontanus* (GenBank: U00455.1), and *Acipenser sinensis* (GenBank: AJ745099.1) were aligned by CLC main Workbench (CLC, Denmark). Also, for β-actin, six cDNA sequences from *A. baerii* (GenBank: JX027376.1), *Acipenser oxyrhynchus* (GenBank: HQ439361.1), *A. transmontanus* (GenBank: FJ205611.1), *A. sinensis* (GenBank: AJ745100.1), and *Acipenser schrenckii* (GenBank: AY649619.1) were used for alignment. Primers for VTG and β-actin were designed from conserved regions by Primer3web software (Ver. 4.0).

Real-time polymerase chain reaction

Amplification and detection of specific products were performed using Step One Real-Time PCR System (Life Technologies, Carlsbad, California, USA), SYBR® Green I PCR Master Mix kit (Life Technologies, Carlsbad, California, USA), and specific primers. For all expression assays, the program was set to 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C with collection of fluorescent data. Finally, melting curves were determined by denaturing PCR products for 15 s at 95 °C followed by a ramp down to 60 °C for 1 min followed by a gradual 0.3 °C/s climb to 95 °C continuously recording the fluorescence. The sizes of all PCR products were verified by inspection of the dissociation curve and by gel electrophoresis. In this study, β-actin was employed as a non-regulated reference gene, and it was found to be stably expressed. The stability of β-actin was analyzed by

the ΔCT method (Livak and Schmittgen 2001, Schmittgen and Livak 2008). Then VTG mRNA data results were normalized to β-actin as an internal control. The difference between Ct value of the reference gene and the target gene was calculated for each mRNA by taking the mean Ct of duplicate reactions and subtracting the mean Ct of duplicate reactions for the reference RNA measured on an aliquot from the same RT reaction (ΔCt = Ct target gene − Ct reference gene). All samples were then normalized to the ΔCt value of a calibrator sample to obtain a ΔΔCt value (ΔCt target – ΔCt calibrator). In order to evaluate the differential mRNA expression of the target gene, the sample at VTG stage was chosen as the calibrator sample. The relative quantification of mRNA expression was calculated using 2 − ΔΔCt method (Livak and Schmittgen 2001).

Statistical analyses

All data have been expressed as mean ± standard error. All statistical procedures were carried out using SPSS software (SPSS 13.0, Chicago, IL) at a 95 % significance level. Normality of data and homogeneity of variances were analyzed by the Kolmogorov–Smirnov test and Levene's test, respectively. After that, one-way analysis of variance (ANOVA) was performed and followed by Turkey's post hoc test to determine significant differences throughout different maturity stages.

Results

Weight changes and oocyte development

No infection or mortality was seen during the liver and ovarian biopsy procedures. All wounds and spots were healed after 1 month. In this study, classification of Sterlet was done by micro- and macroscopic appearance of the advanced oocytes on the basis of oocyte diameter, number of lipid droplets and amount of yolk into four groups: previtellogenic (PV, oocyte diameter 105.5 ± 34.4 μm), vitellogenic (VTG, 283.1 ± 84.3 μm), post-vitellogenic (POV, 825.3 ± 122.7 μm), and atresia oocytes (Fig. 1).

Sterlet at VTG and POV stages experienced one gametogenic cycle previously, evidenced by pigment clusters of previously resorbed ovarian follicles embedded in ovarian tissue (5 years). On the contrary, female at PV stage showed no evidence of a prior maturation cycle and were presumed to be virgin spawners (4 years).

On the basis of total weight and length results, no significant difference was found in VTG (985 ± 238.1 g, 63 ± 4.2 cm), POV (998.3 ± 148.4 g, 63.4 ± 4.6 cm), and atretic (936 ± 121.4 g, 60.4 ± 4.3 cm) stages of female sterlet. However, the mean weight and length of fish in VTG and POV stage were significantly higher than PV (424 ± 163.4 g and 48.4 ± 4.1 cm) (Table 1).

Plasma metabolites

Changes in plasma metabolites were undertaken on the female sterlet throughout ovarian development are presented in Table 2. Glucose concentrations in plasma showed increasing trends and has reached its peak at the POV (64.9 ± 18.3 mg dL^{-1}), which was significantly different from atretic ovarian samples ($F = 4.228$, $df = 3$, $P = 0.015$). Plasma CHOL, TAG and HDL showed the same pattern. Plasma levels of CHOL, TAG and HDL increased notably throughout ovarian development and reaching to the maximum levels at POV females. Moreover, significant difference was found among POV with PV and atretic females in terms of CHOL ($F = 6.192$, $df = 3$, $P = 0.003$), TAG ($F = 6.735$, $df = 3$, $P = 0.002$) and HDL levels ($F = 8.189$, $df = 3$, $P = 0.001$). Although the same tendency was observed in LDL and VLDL plasma concentrations. However, these levels were significantly varied depending on maturity stages. LDL and VLDL concentrations in plasma showed increasing trends and reached a maximum at POV samples (43.7 ± 17.4 and 52.4 ± 13.7 mg dL^{-1}, respectively), which was significantly different from that measured in samples from other maturity stages ($F = 7.597$, $df = 3$, $P = 0.001$ and $F = 16.685$, $df = 3$, $P = 0.001$ respectively). A significant elevation in mean plasma CA levels occurred during gonad development and showed significant difference at VTG and POV fish with PV and atretic females ($F = 13.578$, $df = 3$, $P = 0.001$). Changes in plasma P and ALP levels also matched with other biochemical parameters and showed significant difference between VTG and POV with PV fish ($F = 6.474$, $df = 3$, $P = 0.003$ for P and $F = 4.454$, $df = 3$, $P = 0.014$ for ALP). The variations in albumin levels also matched with other metabolites indices, again peaking at POV which was

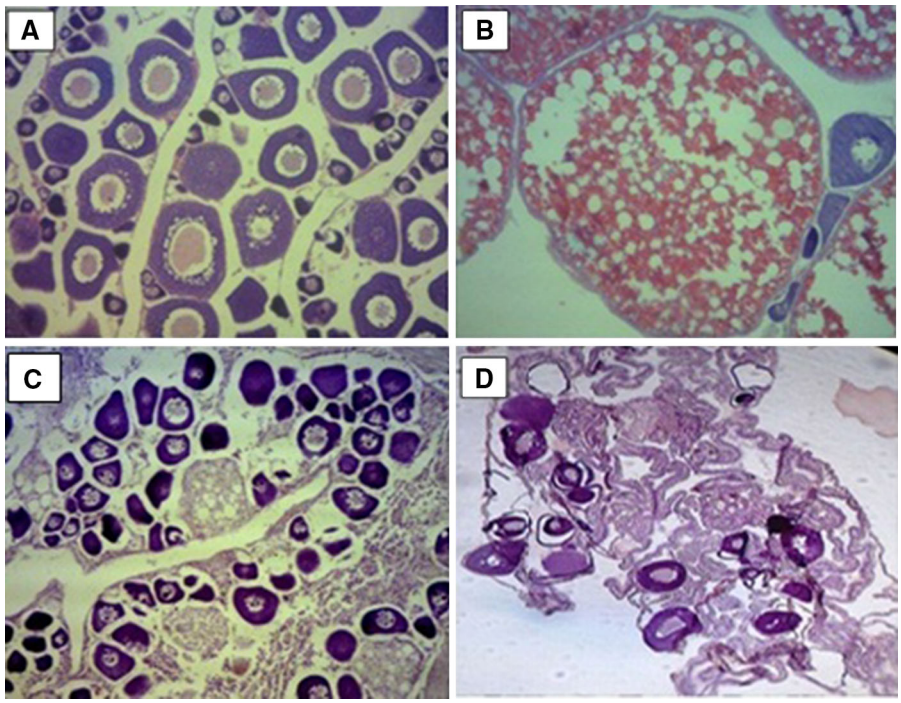

Fig. 1 The panels show the histological sections of sterlet sturgeon gonads stained with hematoxylin & eosin at different developmental stages. *PV* Previtellogenic (**a**), *VTG* Vitellogenic (**b**), *POV* Post vitelogenic (**c**) and Atresia: Absorbic follicoles (**d**)

Table 1 Forward (F) and reverse (R) sequences of primers used in quantitative real-time PCR

Gene	Primer name	Sequence (5′–3′)	Product length (bp)
VTG	VTG-F	GATGGATATTGGCATGGCTTA	208
	VTG-R	AAGCTTCTGCCTTGCTTCAG	
β-actin	β-actin-F	GGTATCCTGACCCTGAAGTA	89
	β-actin-R	TTGTAGAAGGTGTGATGCCAG	

significantly higher than PV and atretic samples (F = 6.049, df = 3, P = 0.003). The effect of maturation status on plasma total protein was noticeable. PV female sterlet showed significantly lower amounts in comparison with other developmental stages (F = 14.443, df = 3, P = 0.001). The values of GLB showed an upward trend, and then decreased, but there was no significant difference among different developmental stages.

Lipoprotein lipase and hepatic lipase activities

Plasma LPL and HL activities are shown in Table 2. LPL and HL plasma activity in VTG stage were slightly higher than PV females, but differences were not significant. The LPL activity of POV female is significantly higher than PV and atretic sterlet (Table 2, F = 5.504, df = 3, P = 0.006), while HL plasma activity showed significant difference between POV sterlet and other maturity stages, and also it is found between atretic and PV females (Table 2, F = 35.396, df = 3, P = 0.001).

Expression levels of VTG during gonadal development

Expression profiles of VTG in liver and ovarian tissues of female sterlet throughout ovarian development were analyzed by real-time quantitative PCR. Two hundred bp products were observed in the both liver

Table 2 Biochemical indices in sterlet sturgeon *Acipenser ruthenus* females at different maturity stages

	Previtellogenic	Vitellogenic	Post-vitellogenic	Artesia
Glucose (mg dL^{-1})	49 ± 5.3^{ab}	60.3 ± 14.4^{ab}	64.9 ± 18.3^{a}	40 ± 4.4^{b}
Cholesterol (mg dL^{-1})	49.4 ± 1.3^{b}	66.1 ± 17.3^{ab}	97.3 ± 37.9^{a}	52.3 ± 2.3^{b}
Triacylglycerol (mg dL^{-1})	80.5 ± 9.5^{b}	124.3 ± 42.9^{ab}	178.2 ± 60.5^{a}	101 ± 9.6^{b}
High-density lipoprotein (mg dL^{-1})	15 ± 1.4^{b}	19.7 ± 7.1^{ab}	27.9 ± 7.6^{a}	14.3 ± 3.2^{b}
Low-density lipoprotein (mg dL^{-1})	18.4 ± 1.7^{b}	24.8 ± 12.7^{b}	43.7 ± 17.4^{a}	17.8 ± 2.2^{b}
Very low-density lipoprotein (mg dL^{-1})	19.8 ± 1.4^{b}	27.1 ± 11.6^{b}	52.4 ± 13.7^{a}	19.6 ± 2.1^{b}
Calcium (mg dL^{-1})	7.3 ± 0.9^{b}	10.4 ± 1.7^{a}	12.3 ± 1.7^{a}	7.2 ± 1.1^{b}
Phosphorus (mg dL^{-1})	9.3 ± 1.7^{b}	12.9 ± 1.4^{a}	14.1 ± 2.4^{a}	11.4 ± 1.3^{ab}
Alkaline phosphatase (u L^{-1})	113 ± 13.1^{b}	145.8 ± 10.1^{a}	145.1 ± 26.4^{a}	122.6 ± 4.1^{ab}
Albumin (g dL^{-1})	1 ± 0.1^{b}	1.5 ± 0.4^{ab}	1.9 ± 0.6^{a}	1.1 ± 0.2^{b}
Total protein (g dL^{-1})	1.6 ± 0.4^{b}	2.9 ± 0.3^{a}	2.9 ± 0.4^{a}	2.6 ± 0.3^{a}
Globulin (g dL^{-1})	0.6 ± 0.3	1.3 ± 0.5	0.9 ± 0.5	1.4 ± 0.1
Lipoprotein lipase (pg Ml^{-1})	11 ± 0.7^{a}	11.9 ± 0.7^{ab}	12.4 ± 0.8^{b}	10.8 ± 0.7^{a}
Hepatic lipase (pg Ml^{-1})	11 ± 0.6^{c}	11.9 ± 0.6^{bc}	14.5 ± 0.7^{a}	12.2 ± 0.1^{b}

Values are mean ± SE

Different superscript letters in a row indicate significant differences between the females at different maturity stages ($P < 0.05$)

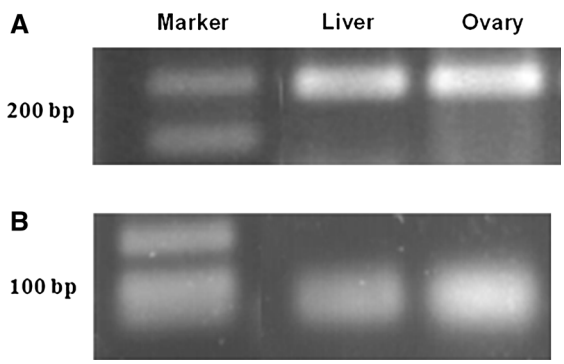

Fig. 2 Electrophoretic result of vitellogenin (**a**) and β-actin (**b**) in ovarian and liver tissues of Sterlet sturgeon *Acipenser ruthenus*

and ovaries of female sterlet during gonadal maturation (Fig. 2). Expression of β-actin, a housekeeping gene, was observed in the liver and ovarian tissues of all female sterlet with an amplification product size of 100 bp (Fig. 2). The relative levels of VTG mRNA in female were detected in both liver and ovarian tissues. The expression level of VTG gene had an increasing trend both in livers and ovaries as ovarian development. The expression level of VTG gene in liver increased from PV stage and reached to maximum at

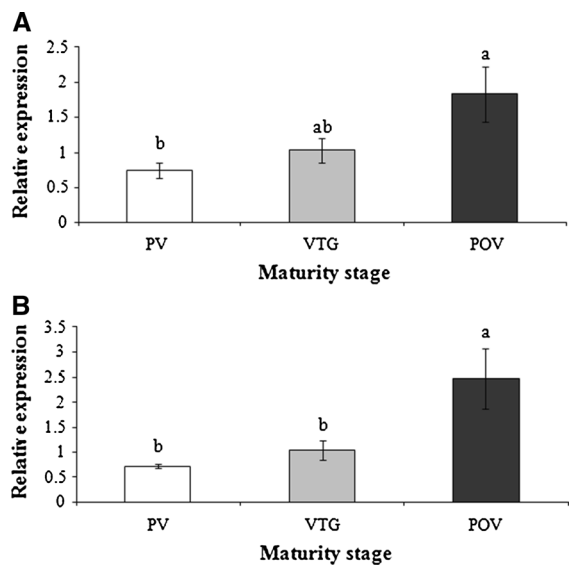

Fig. 3 Real-time quantitative PCR analysis of VTG mRNA levels in liver (**a**) and ovary (**b**) in different developing stages of Sterlet sturgeon *Acipenser ruthenus*. Expression values are normalized by β-actin expressed transcripts. Relative fold difference between stages are presented as mean ± SE ($n = 6$ individuals). Different superscript letters indicate significant differences between the females during developmental stages ($P < 0.05$). *PV* previtellogenic, *VTG* vitellogenic, *POV* post-vitellogenic

POV samples, which was significantly different from POV (Fig. 3a, $F = 4.899$, $df = 2$, $P = 0.04$). The same pattern was found in ovaries VTG mRNA levels, but POV measured samples showed significantly higher level than PV and vitellogenic female (Fig. 3b, $F = 12.248$, $df = 2$, $P = 0.001$).

Discussion

Previtellogenic characterized with small translucent eggs, vitellogenic oocytes with medium-size white eggs, differentiation of the granulosa cell layer, initiation of secretion of the zona radiata and the developing clutches of oocytes, and post-vitellogenic, with large black eggs, thickness of the zona radiata layer and accumulation of large yolk platelets, and oil droplets in the vegetal hemisphere. Beside this, the disintegration of the oocyte nucleus, hypertrophy of granulosa cells, and digestion of the oocyte envelope were observed in light microscopy of atretic follicles as also described in other sturgeon (Linares-Casenave et al. 2002).

The present investigation indicated that plasma metabolites are highly influenced by maturity status. Glucose levels increased during gonadal development and showed significant difference between POV and atretic females. Glucose is an important fuel for metabolism and is oxidized to release energy (Gharaei et al. 2011). This increase may have resulted from the increased metabolic demand during the time of vitellogenesis associated with responding to nutrient mobilization and the energetic over demands of the ovarian growth. This result was consistent with previous study on Persian sturgeon *A. persicus* that glucose increased with maturity in female (Asadi et al. 2006).

Vitellogenesis is crucial for the reproduction of oviparous animals. In female sturgeon sexual development is arrested at previtellogenic stage for many years (Doroshov et al. 1997; Williot and Brun 1998; Akhavan et al. 2015). In the present study, lipid variations during ovarian development confirm a strong link between lipid profile and maturation status. Plasma levels of TAG and total CHOL showed a noticeable increase during gonadal development and peaked at POV. By then, CHOL and TAG levels were significantly higher than those of PV and atretic follicles. It suggests a role of CHOL and TAG to provide the energy needed for coping with the increased metabolic demands during the time of vitellogenesis. The increase in CHOL seen here is attributable to the presence of vitellogenin, the principal yolk protein precursor, which is known to associate with CHOL and also is likely to be associated with HDL component increase in the blood stream. The increased levels of TAG, on the other hand, are more likely associated with increased levels of VLDL, a lipid transporter that has a core that is rich in TAG and that is enclosed by a shell of apolipoproteins (Gibbons and Wiggins 1995; Jonas and Phillips 2008).

Oocyte growth occurs by the uptake of plasma egg yolk precursor proteins, which is derived from the enzymatic cleavage of complex precursors predominantly vitellogenin and during the vitellogenesis phase of oogenesis (Tyler and Sumpter 1996; Kwon et al. 2001; Carnevali et al. 2006). In the current study, the changes that were observed in circulating lipoproteins suggests that not only VTG is the sole source of oocyte lipid, but also lipoproteins may also be involved in transport of lipid to the ovary. Unlike most teleosts, it have been extrapolated that VLDL is the predominant plasma lipoprotein (Iijima et al. 1990, 1995; Wallaert and Babin 1994) and used as the source of neutral lipids for growing oocytes and to a lesser extent by LDL and HDL. It seems that the ovarian lipolysis of circulating lipoproteins is probably a main source of neutral lipids stored in the form of lipid in vitellogenesis (Wallaert and Babin 1994; Wiegand 1996). HDL was significantly higher at POV females in comparison with PV and atretic Sterlet. Taking into account that TAG represents the major component of VLDL (Wallace 1985) and HDL acts to provide CHOL to the gonads (Babin and Vernier 1989), these changes were in agreement with our results considering hyperlipidemic during gonadal development. In this study, LPL and HL activity showed a significant increase in PV Sterlet that indicates these enzymes can play an important role in formation and accumulation of lipid in the oocytes. LPL is the predominant TAG lipase and hydrolyzing TAG in chylomicrons and VLDL. The higher LPL activity is responsible for hydrolysis large amounts of TAG from VLDL into fatty acid which would then be taken up by the oocyte (Mead et al. 2002), whereas HL is both a phospholipase and a TAG lipase and involved in chylomicron remnant and HDL metabolism and plays an important role in the

conversion of VLDL to LDL (Santamarina-Fojo et al. 1998; Mekki et al. 2003; Chatterjee and Sparks 2011). Increased LPL and HL activity during ovarian development significantly higher in PV stage were an effective mechanism to facilitate deposition of lipid and play important role in reproduction and lipid metabolism. This result was in agreement with observations on rainbow trout, *Oncorhynchus mykiss,* European seabass, *Dicentrarchus labrax*, and Siberian sturgeon, *A. baerii*, which demonstrate higher expression of LPL transcript and lipase enzyme activity in the fish ovary during ovarian development (Kwon et al. 2001; Ibanez et al. 2008; Ma et al. 2011). However, in our result, we found that lipase enzymes activity, lipoproteins profile, and lipid profiles decreased dramatically in atretic follicles. These drops could be caused by exceed energy requirements during vitellogenesis, negative energy balance, forcing these fish to rely on stored fats (TAG) to provide their muscles with energy for their growing oocytes. So, this lipid and energy imbalance is probably related to reabsorption and digestion of the yolk in atretic follicles.

Vitellogenin level has very often been assayed indirectly in fish by determination of ALP, CA, and P plasma levels (Pelissero et al. 1989; Moberg et al. 1991; Van Eenennaam et al. 1996; Doroshov et al. 1997; Srivastav and Srivastav 1998; Linares-Casenave et al. 2003; Akhavan et al. 2015). In the present study, variation in ALP, CA, and P was associated with ovarian stage and in agreement with earlier observations (Singh and Srivastav 1990; Linares-Casenave et al. 2003). It is well known that ovaries produce estrogen that stimulates hepatic formation of VTG. In our study, it is clear that increase in VTG levels affects the metabolism of protein-bound fraction of plasma CA and P content.

The protein content in the blood is related to the development of gonad. Among blood proteins, ALB is the major component which takes an active part in blood osmotic regulation, metabolism, and the transport of important compounds including lipids and carbohydrates (Baker 2002). Beside this, ALB binds and transports steroid hormones, including sex hormones (Baker 2002). Babin et al. (2007) mentioned the role of protein in binding to non-esterified in transport of hydrophobic lipids. Therefore, the significant increase found in the blood of Sterlet female in TP and ALB levels in our study was in agreement with previous results in other species (Folmart et al. 1992; Zhang et al. 2014).

Interestingly, Q-RT-PCR results indicated a dual expression of VTG gene. Usually, the liver is considered to be the major site of VTG gene expression in fish species, although in Sterlet females the same VTG gene simultaneously expressed in both liver and ovary during ovarian development. This result was in agreement with some previous literatures (Zmora et al. 2007; Kang et al. 2008; Ding et al. 2010). Based on the VTG expression pattern in livers and ovaries of Sterlet females, VTG exhibits a variable transcript levels during ovarian development. In the current study, a distinct increase in VTG mRNA expression in the ovary was observed during maturation process and peaked at the POV stage. This pattern was also detected in liver, but the rise in the liver sample was lowered and delayed. In this study, VTG mRNA was predominantly expressed in the ovary. This result was in parallel with observations on Korean rose bitterling *Rhodeus uyekii* (Kong et al. 2014) and white cloud mountain minnow *Tanichthys albonubes* that VTG was expressed at a higher level in the ovary than in the liver (Wang et al. 2010). In general, the rise in VTG mRNA expression level in both liver and ovary tissues during vitellogenesis, from previtellogenic to post-vitellogenic stage, occurred which corresponds vitellogenin and egg shell proteins incorporation into the oocyte, and the accumulation of enough nutrients for developing embryos (Lubzens et al. 2010). VTG is apparently the primary transporter of energy to the oocytes, in the form of both yolk protein and lipid (Wallace 1985; Mommsen and Walsh 1988; Roubal et al. 1997). In the current investigation, heterogeneity and asynchrony were evident in Sterlet ovarian samples, especially in VTG stage. So, it is probable in VTG ovarian expression analyses, and variable amounts of oocytes from different developmental stages were presented. This should affect if ovaries are isolated from the fish containing both PV and VTG oocytes.

Overall, in the present study, the increases in biochemical parameters of blood are likely to reflect increased levels of vitellogenin, reinforcing earlier suggestions that vitellogenin-associated variables may be a reliable indirect indicator of vitellogenin level. The variation tendency of plasma lipases activity and lipids concentrations during ovarian development suggest that lipase participates in regulating blood lipid metabolism. It is demonstrated that the VTG gene is expressed in both

ovary and liver tissues in fish. We propose that the livers and ovaries are both the sites of VTG synthesis. Therefore, intense research efforts about the endocrine and lipid mobilization mechanisms, and molecular insights would explain how a given follicle remains at primary growth stage while the adjacent ones progress through vitellogenesis in sturgeon.

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