Classification of sex and maturity stages of farmed great sturgeon (*Huso huso*) using blood plasma steroid hormone and calcium ion levels

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Abstract

Twenty four farmed great sturgeon, *Huso huso* (including 8 males and 16 females) over 6 years old were used to develop a method for determination of sex and maturity stages. Seasonal gonadal tissue and blood samples were collected from farmed great sturgeon for three years. The sex and stages of maturity were determined by histology and laparoscopy at the beginning and end of experiment. Plasma sex steroid hormone levels [testosterone (T), 17β-estradiol (E_2) and progesterone (P)] were measured by radioimmunoassay, and plasma calcium ion levels were measured by spectrophotometer. Mean concentrations of testosterone, progesterone and estradiol in blood plasma of *H. huso* at maturity stages II, III and IV were 10.86±1.63, 54.14±3.1, 112.41±7.4; 0.84±0.12, 15.66±2.18, 50.75±3.63 ng/ml in males and 9.0±1.39, 6.51± 0.64, 2.95±2.29, 5.45±0.29, 9.47±0.97 and 4.15±0.7 ng/ml in females, respectively. Testosterone and estradiol levels showed significant differences at various stages. Calcium level at stages II, III and IV of sexual maturity in females (8.05 ± 0.09, 10.4 ± 0.34 and 9.6 ± 0.6 mg/dl) was more than males (7.73 ± 0.16, 8.58 ± 0.13 and 8.76 ± 0.11 mg/dl). Results showed that steroid hormone concentration and calcium level of blood plasma in males and females vary between different stages of sexual maturity. Therefore it can be used to determine the stages of sexual maturity in farmed *H. huso*.

Key words: Farmed *Huso huso*, Males and females, Sexual hormones, Calcium, Maturation stage

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Introduction

In recent years, populations of wild sturgeon have decreased sharply in all regions of the northern hemisphere especially in the southern Caspian Sea (Pourkazemi, 2010). Scientists believe that one of the practical ways to prevent the extinction of sturgeon fishes is captive breeding.

Considering the life spawn and late maturity of sturgeon species, especially *Huso huso*, and the lack of morphological indicators for sex determination, it is important to find some techniques to distinguish the males from females at their early stages of maturity in order to separate them for artificial propagation and breeding programs. Great Sturgeon (*H. huso*) is one of the world's most important sturgeon species, because of its rapid growth and adaptation to nutrition regimes and the farming environment. It is one of the main cul tured species of sturgeons in Iran and many other countries for meat and caviar production. Due to the long maturity age and the lack of morphological indicators even at maturity, determination of sex and maturity stage is essential for efficient caviar production. Nowadays, invasive methods such as biopsy (Webb *et al.,* 2002; Chapman and Park, 2005) and semi-invasive methods such as laparoscopy of gonads are used for sex determining and staging of sexual maturation (Matsche *et al.,* 2011). In these methods especially in biopsy, histology of the gonads is used directly for determining sex and sexual maturation stages, which is a time-consuming, costly and invasive method (Kazemi *et al*., 2002 - 2004).

Other studies showed that steroid hormone concentrations in different species of sturgeon vary at the same stages of sexual maturation (Webb and Erickson, 2007). The concentration of these hormones in blood plasma are usually too low to be measurable until the time of sex differentiation when a considerable increase will occur at the start of meiotic division in both males and females (Cuisset *et al*., 1995; Moberg *et al*., 1995; Mojazi Amiri *et al*., 1996; Van Eenennaam *et al*., 1996; Webb *et al*., 1999). 17β-estradiol and calcium ion concentrations in blood plasma of female sturgeon increase during vitellogenesis and decrease at the final stages of sexual maturation (Mojazi Amiri *et al*., 1996 Webb *et al*., 1999; Doroshov *et al*., 1997; Linares-Casenave *et al*., 2003). The concentration of steroid hormones (testosterone and 17β - estradiol) are low in the early stages of maturation, but increase significantly during the vitellogenesis and decrease in the final stages of maturation (Nazari and Ghomi, 2010).

Estradiol hormone is the final yield of the steroidogenesis pathway in the ovarian follicle. Biosynthesis of estradiol is started by exo-endogenous passing of cholesterol into the mitochondrial epithelium of ovarian follicle and activator protein of steroidogenesis (Clark *et al*., 1994; Christenson *et al*., 2001; Stocco, 2002). In other words, gonadotropin hormones activate estradiol secretion and accelerate vitellogenesis (VTG) in liver cells. Therefore, steroid hormones in blood plasma are the most important indicators

for determination of sexual maturity in sturgeon (Webb *et al*., 2000; Ceapa *et al*., 2002; Bahmani *et al*., 2004; Barannikova *et al*., 2004 and Malekzadeh Viaieh, 2006).

Calcium ion levels in blood plasma depend on the amount of VTG plasma steroid hormones, and was therefore used as an index of vitellogenin in sturgeons (Webb *et al*., 2000). The aim of this study was to develop a rapid, simple, economic, and non-invasive method for sex determination, and stages of sexual maturity in farmed *H. huso* based on levels of sexual steroid hormones and calcium ions in blood plasma.

Materials and Methods

This study was carried out at the Rearing and Propagation Department and Physiology and Biochemistry Department of the Dr. Dadman International Sturgeon Research Institute (Iran - Rasht) during 2008 - 2011. A total of 24 over 6 years old farmed beluga fishes (including 16 females and 8 males with mean initial weight 16.42±7.2 and 20.26±5.1 kg and mean initial length 149.78±8.7 and 154.56±14.3 cm, respectively) were used. All fishes were at stage II of their sexual maturation, and transferred to 3 covered circular ponds with 4×4×11.5m dimensions and equipped with aeration system. Eight fish were placed in each pond. Water supply was from Sepidroad River mixed with well water, with dissolved oxygen, temperature and pH averaging 6.14±0.35mg/l, 17.5±1.23^{o}C and 7.65±0.02, respectively. Fish were hand-fed twice daily at a rate of 2-3% of the body weight. The diet consisted of 38-40% protein, 13-15% fat and energy 19.5-20 MJ. Sex determination of fish was done by biopsy and laparoscopy every 6 months at the beginning and the end of the experiment using a Stema Company; model M-CAM1700, 30 degree telescope with 4 mm in size and with length 17.5 cm and with the cold halogen light source, 250 W, Germany).

Blood sampling and biochemical studies were conducted seasonally for 12 seasons. Bleed samples were taken with 5^{cc} syringe from caudal vein at caudal peduncle. . At each time, 3^{cc} blood was collected from each fish and transferred to the Hematology lab for further analysis. Blood plasma were separated using a centrifuge (Labofuge 200, Heraeus Sepatech, Germany) at 3000 rpm for 10 minutes. Samples were stored at -20^{o}C (Pottinger and Carrick, 2001). Steroid hormone levels (including testosterone, progesterone and estradiol) were measured using radio immune assay (RIA) with Immunotech kit and I_{125} (France) and gamma counter LKB (Finland) based on ng/ml. Calcium ion levels were measured using a photometric method and spectrophotometer (UV/VIS – model 6505, Jenway Company, England) and Pars Azmon kit (Iran) with 570 nm wave length at the Hematology lab.

Minimum, maximum, variance and standard error were used to describe biochemical indices. Excel and SPSS17 software were used for data analysis and drawing diagrams. Comparison between 2 groups was done by one-way ANOVA, and Duncan’s test was used for differential studies. Levene’s test was done to test the

equality of variance. An Independent-Sample T-Test was used to detect differences between hormonal and biochemical indices in males and females. Data are presented as Mean ±SE.

Results

Testosterone levels

There were significant difference in testosterone levels between sexual maturity stages II and III; II and IV; III and IV in males and females (p<0.05) (Table1). Testosterone levels showed significant differences between males and females at stages II, III and IV (p<0.05) (Table1).

Progesterone levels

In males, there was significant difference between stages II, III and IV (p<0.05), but there was no significant difference between stages III and IV (p>0.05). In females, significant differences were observed between stages II and III; II and IV, and III and IV (p<0.05) (Table 1). Progesterone levels showed no significant difference between males and females of beluga at stage II, but showed significant differences at stages III and IV (p<0.05) (Table 1).

Estradiol hormone

There was no significant difference between stages II and III (p>0.05), but there were.

Table 1: Testosterone, 17β-estradiol and progesterone and calcium levels changes in males and females of *H.huso* at different stages.

Sex	Sexual stages	Testosterone (ng/ml)	17β-estradiol (ng/ml)	Progesterone (ng/ml)	Calcium (mg/dl)
Males	II	$10.86\pm1.63^{c*}$	$9.0\pm1.39^{a*}$	$^{ns}0.5\pm0.01^{b}$	$^{ns}7.73\pm0.16^{b}$
	III	$54.14\pm3.11^{b*}$	$6.5\pm0.64^{a*}$	$0.5\pm0.07^{a*}$	$8.58\pm0.13^{a*}$
	IV	$112.41\pm7.37^{a*}$	$^{ns}2.96\pm0.29^{b}$	$0.36\pm0.04^{a*}$	$^{ns}8.76\pm0.11^{a}$
Females	II	$0.8\pm0.1^{c*}$	$5.5\pm0.3^{b*}$	$ns0.05\pm0.01^{c}$	$^{ns}8.05\pm0.09^{b}$
	III	$^{*}15.65\pm2.2^{b}$	$9.5\pm0.97^{a*}$	$^{*}0.01\pm0.02^{b}$	$^{*}10.40\pm0.34^{a}$
	IV	$50.75\pm3.6^{a*}$	$^{ns}4.1\pm0.7^{b}$	$0.2\pm0.03^{a*}$	$^{ns}9.6\pm0.6^{a}$

^{abc}: Various letters indicate significant differences at each stages in males and femals

Stars indecate significant differences between each sex*: Calcium ion

significant differences between stage II and IV; III and IV of sexual maturation stages (p<0.05). In females, there was no significant difference between stages II and IV (p>0.05), but there were significant differences between stage II and III; III and IV (p<0.05) (Table.3). Estradiol hormone concentrations showed significant differences between males and females at stages II and III (p<0.05), but not at stage IV.

In males, there was a significant difference between stage III and IV (p<0.05) but not between III and IV of sexual maturity (p>0.05). In females, there was a significant difference between II and III stages (p<0.05), but not between stages II and IV or III and IV (p>0.05). Calcium levels showed no significant difference between males and females at stages II and

IV, whereas there was significant difference at stage III ($P<0.05$) (Table 1).

Discussion

Results of this study indicated that testosterone levels were significantly different between male and female *H.huso* and between stages of sexual maturity ($p<0.05$). At each stage of sexual maturity, testosterone concentrations in blood plasma were greater in males compared to females. The increase in testosterone and decrease in estradiol levels at the final stages of maturity in teleost fish (Nagahama, 1987) and in different species of sturgeon (Frederiek *et al*., 2007) are due to declining the aromatase enzyme activity. Reductions in the levels of testosterone are controlled by gonadotropin hormones, but time-dependent fluctuations of hormones can occur in various species (Bahmani *et al*., 2004).

Differences in mean levels of testosterone in blood plasma between male and female *Acipenser persicus* at stage IV were greater than stage III, and stage III levels were not significantly different from stage II (Bahmani *et al*., 2009). These results differed from those of the present study, because most possibly the testosterone concentration of blood plasma acts as a precursor for estradiol, in female breeders during vitellogenesis at stage III, and rapid division of the second spermatocytes during spermatid production. Testosterone concentration should be significantly higher at stage III than II (Pankhurst, 1997; Nagahama, 2000). In immature fishes, sex steroid hormones levels were low but increased with maturity. These steroid hormones are precursors of estradiol (Dahle *et al*., 2003; Barannikova *et al*., 2004; Sattari, 2010), andthere is a positive relationship among these hormones (Nazari and Ghomi, 2010). In fact, testosterone is the main hormone controlling the reproductive process in sturgeon under natural conditions (Bukovskaya, 1997). There is a constant and positive relationship among steroid hormones of blood plasma (Barannikova *et al*., 2003; 2006).

Bukovskaya and Bayunova (1989) reported that the concentration of testosterone and estradiol of male and female *A. nudiventris* in estuaries and rivers and also in wild individuals of *A. stellatus* and *Huso huso* from the southern area of the Caspian Sea at stage II were not significantly different but they were significantly different at the start of stage III of sexual maturity. An evaluation of the dynamics of sex steroid hormone in *H.huso* at the start of migration to the Volga River showed that testosterone levels in blood plasma in males and females and progesterone levels only higher in males at primary of spring season, but levels of testosterone and progesterone in blood plasma were lower in comparison with spring migratory fishes. Males showed lower levels of testosterone in winter than in spring migratory males (Barannikova *et al.*, 1997). Other authors have shown that testosterone levels in male and female sturgeon increased in spring and reached a maximum at stage IV of sexual maturity, while it decreased in winter in males and females (stage III) (Barannikova, 2003). The reason for this difference was the

earlier stage of sexual maturity in winter migratory breeders than spring migratory brood stocks (Barannikova *et al.,* 1997).

Mean concentration of progesterone and estradiol in farmed *A. nudiventris* breeders were greater than in non spermatic individuals but showed no significant difference. Differences were significant in farmed (mature) female breeders than in immature breeders and were greater in females than males (Bahmani *et al*., 2009).

As in the present study, although progesterone levels decreased in males and females from stage II to stage IV, its mean levels were greater in males tcompared to females (Bahmani *et al*., 2009). It seems that this increase was due to lower requirement and consumption of progesterone hormone in males.

In teleosts testosterone and progesterone concentrations increased during spawning and then decreased sharply (Yan *et al*., 1992; Fontaine *et al*., 1998) and their concentration decreased with development of the ovary and gonad cells especially at vitellogenesis because ovary development is similar to that in sturgeon (Chang *et al*., 1999; Dahle *et al*., 2003; Shafiei Sabet *et al*., 2010). Rapid decrease of estradiol after vitellogenesis and at the final stages of sexual maturity were because aromatase activity ceased resulting from completion of egg development and a sharp reduction of feedback from steroid hormones (Shafiei Sabet *et al*., 2010). However, occasional increases in testosterone in many teleost species occur immediately before spawning and at the final maturity (Chang *et al*. 1999). It seems that cessation of ovary aromatic activates, and no requirement for testosterone and using it for spawning is the major reason to increase testosterone hormone levels at this time. These results were in agreement with the results of the present study.

Progesterone levels showed significant differences in teleost female brood stocks at different stages of sexual maturity (Najafipoor, 2005). Higher levels of progesterone in more mature female breeders compared with the immature breeders was due to the effectiveness of progestin (especially progesterone) in promoting oocytes in some adult teleost, that reaches to its maximum level at spawning time.

Calcium ion

Our results also revealed that calcium levels of blood plasma were significantly different at different stages of sexual maturity in both males and females. Calcium Levels were greater in females than males of *H.huso*. Some studies showed that calcium levels of blood serum in farmed juvenile *H.huso* were similar to those of fresh water teleost and concentrations were the same in males and females (Asadi *et al*., 2006). Others indicated that the concentration of calcium ion varied in different species (Shahsavani *et al*., 2010), genera (Webb *et al*., 2000 and present study) and families (Borges *et al*., 2004). It also varied with age, and environmental conditions (Hoseini *et al*., 2010). The results of present study contrasted with those of Asadi *et al*. (2006). Therefore, in young fish and before vitellogenesis, there were no significant differences in calcium concentration between males and females,

but because of its role in the reproductive cycle and vitellogenine process, calcium concentration is related to age and gonad development cycle (Tsai and Wang, 2000).

Calcium ion concentration is related to the reproductive cycle, and steroid hormones (Najafipoor, 2005; Shafiei Sabet *et al*., 2010), hence it is essential for formation of vitteline granules (Tsai and Wang, 2000). Results of the present study also showed the relationship between calcium ions and reproductive cycle. Calcium ion levels of plasma increased before spawning as a result of an increase in stanius glands in females during ovary development cycle. This increase is due to calcium ion linking to proteins (vitelline granules) of the egg (Ursa and Wandelaar Bonga, 1985). This study also showed that the increase of calcium ion concentration in females was parallel to vitellogenesis, and a significant difference existed between males and females. These results are in accordance with previous studies, demonstrating indicating that the calcium ion concentration was increased with the raise of estradiol hormone in blood plasma at vitellogenesis (Stahl, 2008; Shafiei Sabet *et al*., 2010)

In conclusion, sex determination and staging of sexual maturity in farmed *Huso huso* can be determined by measuring steroidal hormones and some ions and metabolites in blood plasma. The concentration of different indices is different at each stage of sexual development as well as between males and females. It is also interesting that sexual steroid hormones and other indices varied between species of sturgeon and also other fish (Webb *et al.*, 2002; Barannikova, 2003), and even within a species under different (cultural and natural) conditions (Barannikova *et al.*, 1997).

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