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Full Length Research Paper

Early sex identification in cultured beluga (*Huso huso*) using plasma steroid hormones

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This study is aimed at evaluating when sex could be determined in beluga by examining levels of plasma steroids. Blood was taken from beluga from two stations (Shahid Rajaee: SR, Sari farm: SI) which were kept at 19°C at the age of 24 and 36 months and plasma testosterone (T), 11-ketotestosterone (KT) and estradiol (E2) concentrations and plasma calcium were determined. The results revealed that, plasma T and KT levels were significantly higher in males than females in both stations at the age of 24 and 36 months ($P<0.05$). There were no differences in E2 and calcium concentrations between males and females at both ages. In addition, the results showed that, plasma T increased significantly in both locations, with increasing age from 24 to 36 months. Histological analysis of gonads revealed that all but six of the SI farm fish at the age of 24 months had well differentiated testes with spermatogonia undergoing mitosis in cysts (Stage 2). The six of the population in SI farm, which had no differentiated gonad, had also the lowest weight in comparison to differentiated fish. It also appears that proliferation of spermatogonia is necessary for production of androgens and growth rate is influenced by water temperature. The results of this study revealed that, plasma T and KT can be used as good markers to determine the sex in the early life stage of beluga.

Key words: Sturgeon, cultured beluga, sex determination, sex steroids.

INTRODUCTION

Sturgeon populations worldwide are decreasing due to over-fishing, pollution and habitat distraction (Birstein, 1993; Williot and Brun, 1998; Billard and Lecointre, 2001; Feist et al., 2004; Ghomi et al., 2010). Consequently, in recent years, the intensive culture of certain sturgeon has been developed as an alternative to other more traditional fish species such as salmonids and cyprinids. The beluga sturgeon (*Huso huso*) is an increasingly important aquaculture species in Russia, Eastern Europe, Turkey, Japan and Iran because of the dwindling natural sources for its caviar and meat. This fish is a commercially important fish and is commonly processed into frozen fillets. Therefore, the use of captive broodstock and aquaculture of sturgeon may be advisable for both meat and caviar production (Williot and Brun, 1998; Feist et al., 2004). In an economic point of view, in sturgeon aquaculture, it is important that fish sex be determined early so that the male fish can be cultured for meat production and females used for caviar. Due to the late age of the first maturity, long gonadal cycles and non-yearly spawning of sturgeon, the sex of sturgeon cannot be externally determined until pre-spawning (Williot and Brun, 1998; Feist et al., 2004).

Normally, sexual maturity is reached at 18 years for beluga females and 14 for males in Caspian Sea (17 ppt in salinity, and temperature of 9°C). However, in Iran (Shahid Rajaee Sturgeon Fish Farm, Sari, Iran) where

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Abbreviations: T, Testosterone; **KT,** 11-ketotestosterone; **E2,** estradiol; **SR,** Shahid Rajaee; **SI,** Sari; **H and E,** Hematoxilin-Eosin.

higher temperature freshwater are being used, maturity occurs at the age of nine, while sex determination using biopsy could be performed at the age of four (Nazari et al., 2009). It is well known that water temperature is the main contributor to the process of testicular development (Feist et al., 2004).

There are some methods which have being used for sex determination. Surgical examination or biopsy of the gonads leads to identification of sex, based on either obvious female or male or as unknown sex. Although survival rate is nearly 100%, this method is invasive. A less invasive procedure for determining the sex of fish, such as blood drawing, may lead to an earlier characterization of sex (Feist et al., 2004). It has been documented that the sex steroid levels in sturgeon remain low until the onset of gonadal growth (Doroshov et al., 1997). Both testosterone (T) and 11-ketotestosterone (KT) are found in males, the latter being the major androgen responsible for testicular development (Jiang et al., 1996; Miura et al., 1996; Nagahama, 1999; Devlin and Nagahama, 2002). In addition, maturation steroids such as 17α, 20ß-dihydroxy-4-pregnen-3-one (17α, 20ß-DP), as well as other direct precursors of these hormones (from pregnenolone) have been studied in fish. A variety of non-classical sex steroids are also produced in fish gonads (Kime, 1993). 17ß-Estradiol (E2) is found at much higher levels in females than males, and is believed to be the major sex steroid responsible for inducing and maintaining ovarian development (Devlin and Nagahama, 2002). Further more, E2 increases during the vitellogenesis (Doroshov et al., 1997; Web et al., 1999; 2001; 2002).

The study aims to determine early sex in cultured beluga (*H. huso*) in high temperature water by examining the plasma concentration of T, KT and E2 and the blood calcium in 24 and 36 months fish in the two different farms.

MATERIALS AND METHODS

Fish culture and sampling

In this study, two different populations of beluga at the age of 24 and 36 months, from two sturgeon farms were studied. All fish in Shahid Rajaee (SR) farm (24 months: n =17; 36 months: n = 17) and Sari (SI) farm (24 months: n = 32; 36 months: n = 30) were reared in concrete tanks (8 m diameter, 1.5 m depth) with water constantly overflowing with the temperature of 17 - 21°C (Oxygen saturation: 71 - 83%, pH: 6.9 - 7.4). Fish in two farms were fed with minced crucian carp (*Carassius auratus*) at the early stage of life. After three months of rearing, fish in SR farm were fed by mixed Kutum (*Rutilus frisii kutum*) feed (37.5% protein, 10.7 fat, 8.5% moisture) and minced crucian meat, in a ratio of 3:1 (w/w) as the semi-moist pellet feed (23% protein, 5.6% fat, 49.46% moisture), while dried-pellet was used for SI farm (44% protein, 10% fat). Feeding rates were same in both farms; for fish less than 1 kg, 5 - 8% of body weight was used, while for fish more than 1 kg, 3% of body weight was used (Nazari et al., 2009).

For analysis of sex steroid hormones, blood samples (5 mL) of fish were taken from the caudal vein and the blood serum was extracted by centrifuge (3000 g, 5 min) and then stored at -20°C until analysis according to Kopp et al. (2009) with slight modification. At each sampling, fish were weighted and total length was measured.

Radioimmunoassay

Steroids T, KT, E2 levels and blood calcium concentration were determined in SR farm, while only T and KT were determined in SI farm. The steroids T, KT and E2 were extracted from plasma according to the method of Fitzpatrick et al. (1986) which has been modified by Fiest et al. (2004). Briefly, 100 µl of plasma was extracted twice using 2 ml of diethyl ether. Tubes were vortexed after the addition of ether, and the aqueous phase was removed by snap-freezing in liquid nitrogen. Combined extracts responded in 1 ml of sodium azide buffer solution. Estradiol and testosterone were measured by RIA antisera (125I-labled estradiol, 125I-labled testosterone) using Immunotech kit (Beckman Coulter Co.). All samples were analyzed in duplicate. The intra- and inter-assay coefficients of variation for all assays were less than 12.1 and 11.2%, respectively for E2, and less than 14.8 and 15%, for T. Steroid levels determined by RIA were validated by verifying that serial dilutions were parallel to standard curves. The average extraction efficiencies for E2 and T were 83 and 91%, respectively.

KT was measured using enzyme linked immunosorbent assay (ELISA) method. This assay is based on the competition between 11-KT and 11-KT-acerylcholinesterase (AChE) conjugate (11-KT tracer) for a limited number of 11-KT-specific rabbit antiserum binding sites. Sturgeon plasma KT levels were determined using kit (Cayman Co., USA) with ELISA instrument (SLT Co., Austria). Plasma calcium concentrations were measured by colorimetric method using autoanalyzer (Technicon Co., USA), Man kit (Kopp et al., 2009).

Histology

All fish were biopsied and gonad tissues were subjected to standard histological procedures (Ostaszewska et al., 2005). Briefly, the samples were collected and stored at 10% phosphate buffered formalin, dehydrated in graded ethanol and embedded in paraffin. Serial sagittal sections, 3 - 5 µm thick, were cut from each block, mounted on glass slides, dried using water bath at 48 - 50°C, and stained with Hematoxilin-Eosin (H and E), then examined by light microscopy.

Statistical analysis

Two group's data were analyzed by unpaired t-test using the Statistical Package for the Social Sciences (SPSS) software, release 16.0 (SPSS Inc., Chicago, IL, USA). Probability of 5% was used for significant determination.

RESULTS

The photographs of the gonadal histology are shown in Figure 1 A and B. The results of histological analysis of the fish gonads in both SR and SI farm showed that all 36 months fish in both farms had differentiated gonads and were in stage 2. But, 6 of 32 fish at the age of 24 months in SI farm did not have differentiated gonad, but the rests were sex determined.

The data from histology showed that all males from SR and SI farm at the age of 24 and 36 months had well

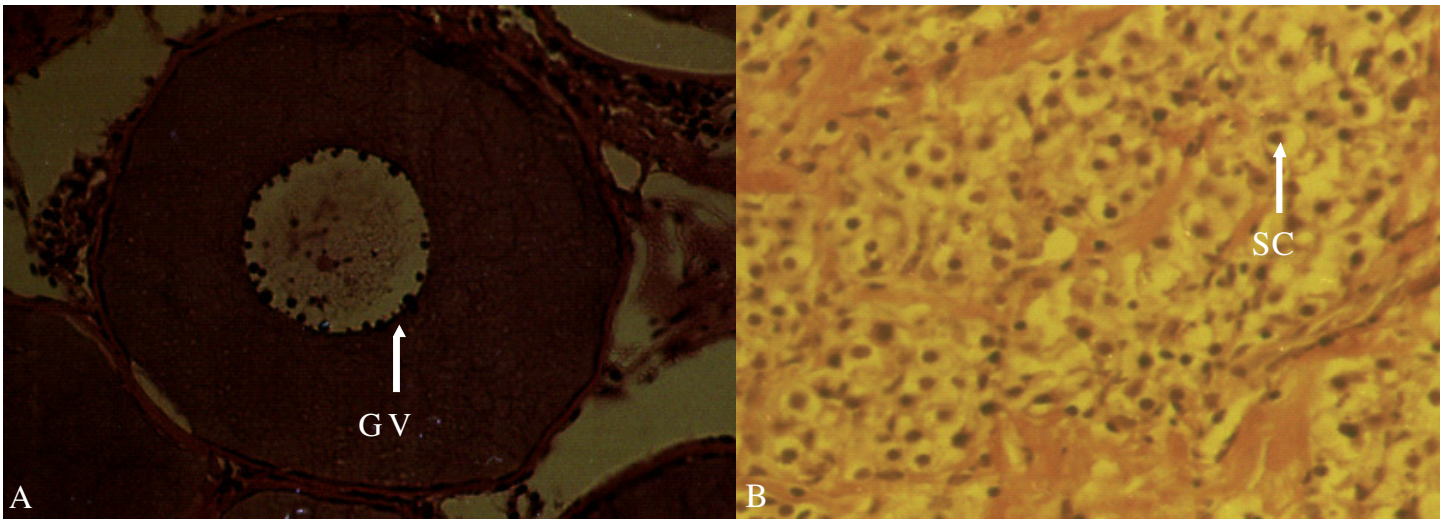

Figure 1. Light micrographs showing 36 months beluga gonads. A: Stage II ovary, B: Stage II testis. GV: Germinal vesicle, SC: spermatocyte.

Table 1. Body weight of beluga in SR and SI farms at two ages[1].

Location	Sex	Age (month)	
		24	36
SR	M	3880 ± 490	4463 ± 672
	F	3872 ± 390	4330 ± 318
SI	M	3530 ± 347	3853 ± 230
	F	3467 ± 423	3768 ± 385

[1]Mean ± SD

differentiated testes with cysts containing spermatocytes (SC) (Figure 1B). Body weight of the fish in this study showed that SR fish had more weight than SI at both ages (Table 1).

The plasma steroids (T and E2) levels and calcium concentration for SR farm are shown in Table 2A. Testosterone concentrations in plasma for SI farm are presented in Table 2B. The results of this study revealed that significant difference could be observed between males and females sturgeon with respect to plasma T ($P<0.05$). Hence, plasma T in males' sturgeon was higher than females in 24 and 36 months fish in two stations. T concentration in 24 and 36 months fish in SR farm were significantly higher than fish in SI farm ($P<0.05$). Concentration of E2 and calcium were relatively high in both males and females (>5.7 ng/ml for E2, >5.66 mg/ml for Calcium) and no significant differences between either sex or age were found ($P>0.05$).

Scattergrams of T in SR and SI farms are shown in Figure 2 and 3. It is obvious that, in both stations, levels of T in males had begun to increase at the age of 24 months. At this age, no female had values of T more than 0.2 ng/ml, while eight of the 13 males in SI farm and two of the 10 males in SR farm showed values more than 0.2 ng/ml. At the age of 36 months T levels were increased in males in both farms, and in all females in both farms remained low. In SI farm, 24 months fish with low weight (2270 g) had extremely low level of T in comparison to larger group (Mean weight: 3880 g).

The results of KT are shown in Figure 4. The results of this study revealed that there is a significant difference between males and females with respect to KT level, for both ages, which were higher in males than in females. At both ages, no female had levels of KT more than 1 ng/ml, whereas all males had levels more than 0.5 ng/ml.

DISCUSSION

Findings of the current study from two farms showed that a technique with less invasive effects on brooder could be employed at the age of 24 and 36 months at culture temperature of 19°C for beluga fish sex determination. Previous researchers have examined gametogenesis, plasma gonadotropins and sex steroid profiles in adult white sturgeon (Moberg et al., 1995; Doroshov et al., 1997; Webb et al., 1999, 2001, 2002), and adult bester (Amiri et al., 1996a; Amiri et al., 1996b). However, only study about plasma sex steroids in the early stage of life of sturgeon is related to Feist et al. (2004) experiment which is related to white sturgeon (*Acipenser transmontanus*). Current study provides the first information regarding plasma sex steroids during the period of early gonadal development in beluga. Amiri et al. (1996a) have mentioned that T value in immature group was low (5-15 ng/ml), while it was higher in our results. They have also reported that androgen levels had increased during sper-matogenesis stage (2 - 4). Frantzen et al. (1997) showed that plasma T and E2 concentrations were increased dramatically in pre-adult Arctic charr (*Salvelinus alpines*), while, they have

Table 2A. Plasma steroids (T, E2) and calcium concentrations in 24 and 36 months beluga in SR farm.

Plasma steroid	Sex	n	Stage	Mean ± SD	P-value
24 months					
T (ng/ml)	M	10	2	0.485 ± 0.41	0.031
	F	7	2	0.087 ± 0.042	
E2 (ng/ml)	M	10	2	11.02 ± 4.8	0.407
	F	7	2	12.77 ± 2.5	
Calcium (mg/ml)	M	10	2	7.52 ± 0.32	0.178
	F	7	2	7.2 ± 0.57	
36 months					
T (ng/ml)	M	10	2	1.40 ± 1.14	0.007
	F	7	2	0.028 ± 0.019	
E2 (ng/ml)	M	10	2	11.34 ± 2.67	0.516
	F	7	2	10.32 ± 3.6	
Calcium (mg/ml)	M	10	2	7.39 ± 0.69	0.804
	F	7	2	7.46 ± 0.37	

Table 2B. Plasma steroid (T) concentrations in 24 and 36 months beluga in SI farm.

Plasma steroid	Sex	n	Stage	Mean ± SD	P-value
24 months					
T (ng/ml)	M	13	2	0.163±0.14	0.019
	F	13	2	0.058±0.04	
36 months					
	M	13	2	0.251±0.2	0.004
T (ng/ml)	F	13	2	0.081± 0.046	

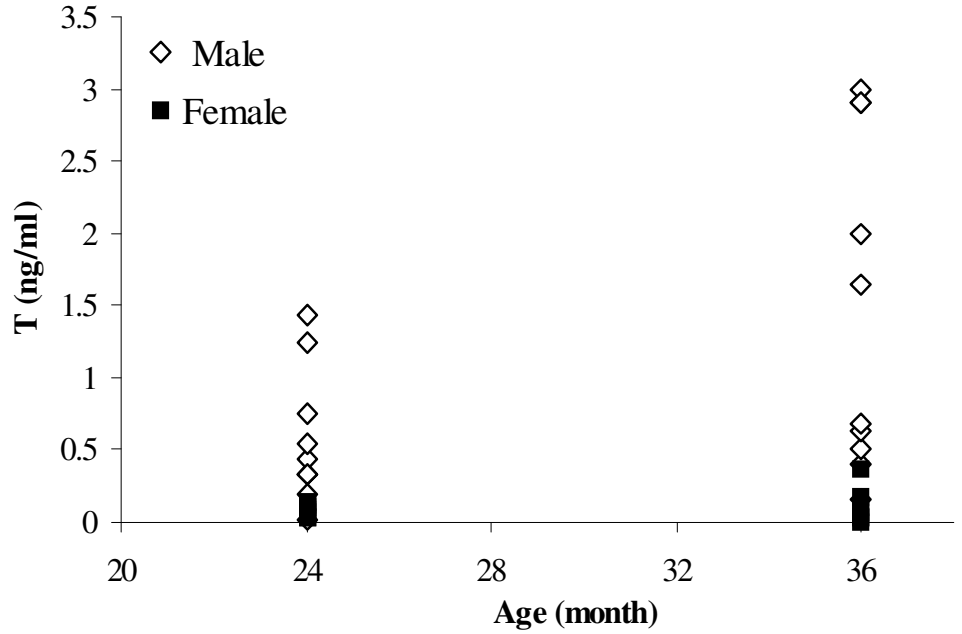

Figure 2. Scattergram of plasma testosterone (T) concentration in males and females beluga raised in Shahid Rajaee (SR) farm.

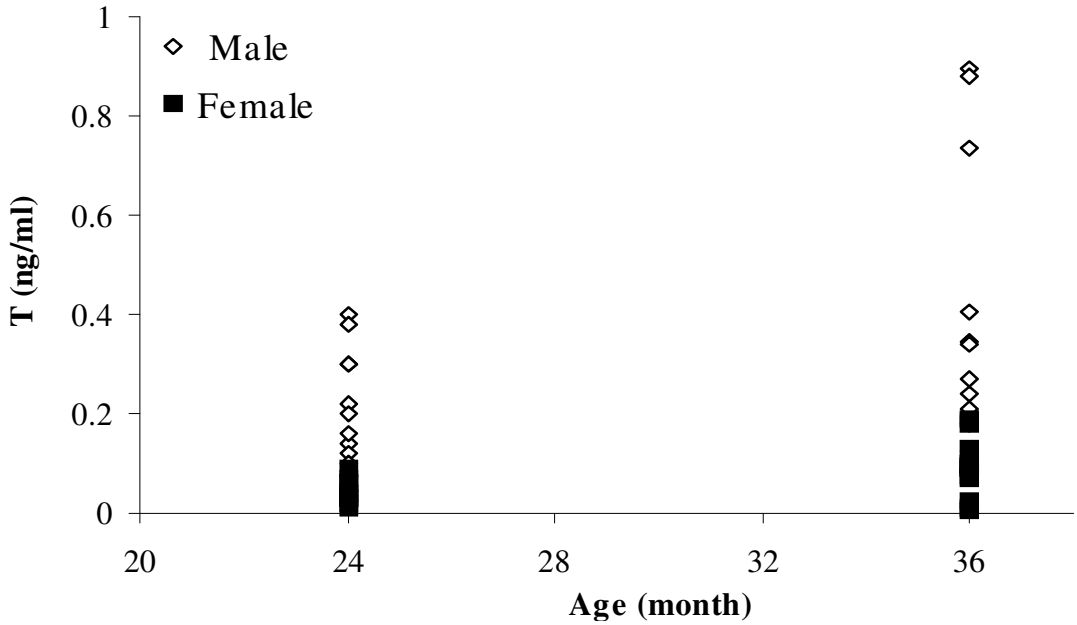

Figure 3. Scattergram of plasma testosterone (T) concentration in males and females beluga raised in Shahid Rajaee (SR) farm.

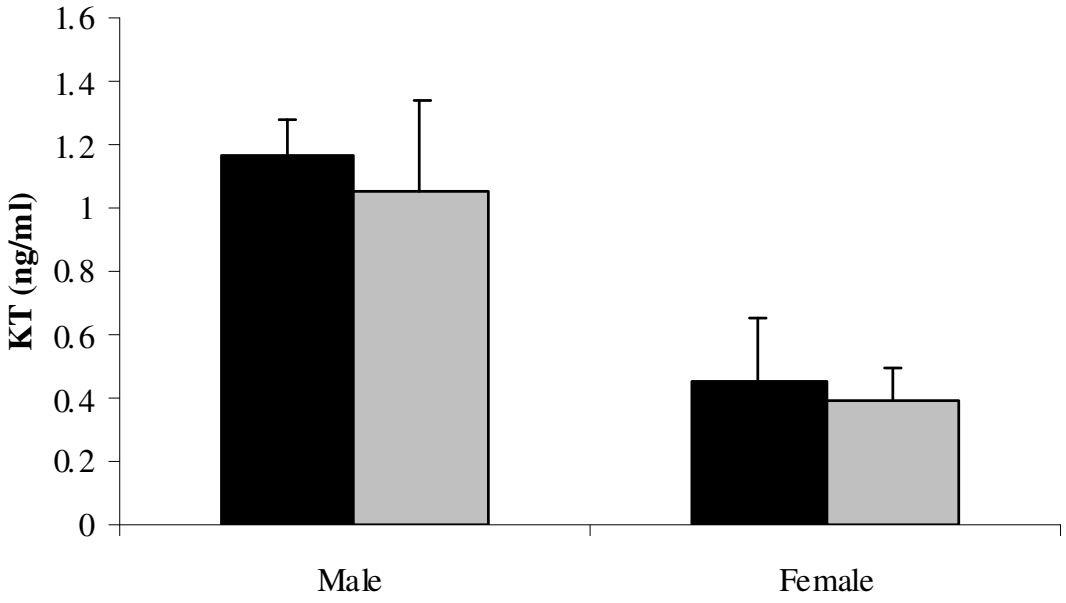

Figure 4. Beluga plasma KT (ng/ml) in males and females at 24 months (■) and 36 (■) months fish (Error bars show SD).

indicated that plasma T and E2 concentrations were rapidly increased during ovulation.

In the current study, plasma T levels in males in both stations were significantly higher than females ($P<0.05$). Hence, all fish at the age of 24 and 36 months with T concentration more than 0.2 ng/ml were male. The same results were observed by Feist et al. (2004). Their findings showed that, all white sturgeon (*A. transmontanus*) fish with androgens levels greater than 2 ng/ml at the age of 21 and 27 months, were male.

Our findings revealed that 6 of 32 fish from SI farm at the age of 24 months were not sex determined based on the plasma androgen hormone levels. It could be explained by fish growth rate in comparison to other fish.

Fish with unidentified sex had a low level of T and lowes weight (2270 g) in comparison to other fish (3880 g) with identified sex. Feist et al. (2004) demonstrated that, white sturgeon males from Oregon and Idaho locations could not be differentiated from females based on plasma androgens. None of these males had entered stage 2 suggesting that this stage is associated with elevating plasma androgens. They assumed that, Oregon and Idaho fish grew slower than those raised in warmer water in California, where males were capable of producing androgens. This suggests that growth rate, rather than body size is the main contributor to the process of testicular development. This stunted male would most likely be discarded early in an aquaculture setting.

In current study, the plasma KT results revealed that there is a significant difference between males and females. KT level in males was more than 1 ng/ml, whereas no females had more than 0.5 ng/ml. The same results have been reported by Fiest et al. (2004) for sturgeons. Synthesis of KT has been identified in the testis from teleosts and its levels are correlated with male gonadosomatic index with an increase during spermiation (Fostier et al. 1983).

Analysis of KT in plasma revealed that this androgen is greater in males than females and successfully used for sex identification in salmonids (Sangalang and Freeman, 1988), which is in agreement with our findings. The possible use of this androgen for sex identi-fication in Siberian sturgeon, *Acipenser baerii* was suggested by Cuisset et al. (1994). In immature Siberian sturgeon, plasma levels of KT were significantly different between males and females in a way that in females, it never exceeded 2.5 ng/ml while in some males its levels reached as high as 170 ng/ml. This observation is in good agreement that KT is a male-specific hormone (Fostier et al., 1983).

In this study, plasma E2 and calcium were found relatively same in both males and females. Feist et al. (2004) reported that in 18 to 30 months white sturgeon fish, plasma E2 levels did not have any significant difference with either sex or location. Webb et al. (1999) found high levels of plasma calcium concentrations in both male and female white sturgeon, which is in agreement with our results in the current study. In an economical point of view, it is important to have the ability to determine the sex of sturgeon at younger ages. An analysis of the joint meat and caviar production systems for sturgeon showed that maximum profitability for separation of males and females occurred at the age of 18.5 months (Logan et al., 1995) and 21 months for white sturgeon (Feist et al., 2004).

In this study, we have shown that there is an ability to separate males and females of beluga sturgeon at the age of 24 months based on plasma androgen levels with an un-invasive method. In Iran, beluga sturgeon is being cultured in concrete tanks using warm fresh water, both for meat and caviar production. In our previous study, we found that using warm water could reduce maturity age by nine years in beluga (Nazari et al., 2009). Hence, early sex determination may gain important advantage in econ-omic term.

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