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Sex identification and sexual maturity stages in farmed great sturgeon, *Huso huso* L. through biopsy

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Summary

Since sturgeons have no external sexual dimorphism and there are no external markers to determine sex, internal examination of the gonads should be used to sex identification. The present study describes the biopsy method and histological observations of the gonads of great sturgeon (*Huso huso*) in both sexes at different age classes. Sex and maturity stages of 226 great sturgeons were identified through gonadal biopsy and histological observations. A 20-25 mm incision was made with a sharp scalpel through the ventral midline between pectoral and pelvic fins, that allowed gonads to be viewed. Determination of sex and maturity stage was successfully performed in all fish. The sex ratio under culture conditions was 1:0.84 (female: male). Most males and females were at mid-spermatogenesis and pre-vitellogenesis stages, respectively. No apparent mortality and infection was observed after surgery and gonad biopsy in fish. Results of this study showed that sex could be identified by this method when fish are 3-year-old or more. Therefore, biopsy technique is a simple and cost-effective tool in sturgeons and has an important role in aquaculture management and conservation benefits.

Key words: Sex identification, Sturgeon, Biopsy

Introduction

Sturgeon species are members of an ancient family of the fish, Acipenseridae. The greatest abundance of *Acipenser* species are found in the Caspian Sea (Birstein, 1993). The great sturgeon or beluga, *Huso huso* (L.) is the largest fish in Eurasian freshwaters and produces the most lucrative caviar and meat in the world. Under natural conditions, it takes a long time for a great sturgeon to be mature while sexual maturity occurs at 10–16-year-old for males and 14–20-year-old for females; the average size and body weight at first spawning is 2 m and 50 kg, respectively (Holčik, 1989). Identifying the sex of sturgeons in early ages is a controversial issue due to their long life, late maturity of first sexual puberty, long time gonadal cycles or non-yearly spawning. Also, sturgeon gametes differ from other fish in the case of ova and sperm by the presence of acrosomes in the spermatozoa and multiple micropyles in the eggs (Keyvanshokooh and Vaziri, 2008; Psenicka *et al*., 2010). Sex identification and the examination of gonadal maturity in sturgeons are a significant operational challenge and an economic liability in artificial rearing. So, males are supplied to market for boneless meat and females are kept for caviar production or as a future broodstock (Doroshov *et al*., 1997).

In sturgeons, sex external secondary characteristics are not distinguishable. Therefore, in pre-spawning phase, sex identification with macroscopic way is an impossible method (females are recognized by enlarged abdomen and male by the spermiation). The most common method is performed routinely at the farm through field activities to determine sex and then the

reproductive conditions are evaluated in sturgeons by a surgical incision and visual examination of the gonads and histological slides prepared from biopsy samples (Conte *et al*., 1988).

Nowadays, there are several techniques to identify sex and characterize the maturation in sturgeons. These options may include blood plasma analyses of steroid levels (Webb *et al*., 2002; Fiest *et al*., 2004), ultrasonography images (Moghim *et al*., 2002), borescope (Kynard and Kieffer, 2002), biopsy (Hallajian *et al*., 2007) and other novel techniques such as endoscopy (Hurvitz *et al*., 2007) and laparoscopy (Falahatkar *et al*., 2009; Falahatkar *et al*., 2011). None of these methods is recommended, as each has its own disadvantages. Blood sampling procedure is time consuming and the analysis of sex hormone requires laboratory equipment, so this procedure does not give immediate results in the field. While ultrasonography is known as a non invasive method, it determines sex accurately and quickly, but the equipment used in this technique is expensive. Also, a skilled person is needed to determine sex and stage of gonadal development (Moghim *et al*., 2002). Although a borescope is a good tool in aquaculture field studies, it has some limitations such as the inability to distinguish a male fish from an immature female and also damage of the funnel-shaped oviduct valve during examination (Kynard and Kieffer, 2002). Endoscopy and laparoscopy are more accurate methods but require expensive tools and skillful persons with good experience and knowledge on fish anatomy to use these methods (Hurvitz *et al*., 2007; Falahatkar *et al*., 2011).

Gonad biopsy is known as a traditional technique to determine sex and its histological examinations have been commonly applied in fish. Non-lethal gonad biopsy technique has been performed widely in sturgeons (Conte *et al*., 1988; Amiri *et al*., 1996; Hallajian *et al*., 2007) and particularly in aquaculture facilities to determine the sex of other fish species (Debas *et al*., 1990; Lokman *et al*., 2001; Matsubara *et al*., 2003; Ashraful Alam and Nakamura, 2008). This procedure is known as an invasive method but gonad biopsies in sturgeon cause minor trauma and remains the most reliable method to determine the sex and the stages of sexual maturity with direct observation of gonads, especially at the early ages (Chapman and Park, 2005). Therefore, the goal of the present study was to determine sex, sexual maturity stages and sex ratio in the great sturgeons by use of gonadal tissue samples through biopsy technique and to identify whether this method is accurate and useful in identification of the sex under culture conditions in this species.

Materials and Methods

Fish and maintenance

The animals examined in the present study were hatched in the Shahid Dr. Beheshti Sturgeon Fish Propagating and Rearing Complex, Rasht, Guilan. One day after hatching, larvae were transferred to the special circular concrete tanks (VNIRO type) and were fed by newly hatched brine shrimp and *Daphnia* after yolk absorption. When the weight of larvae reached to 150 mg, they were transferred to enriched earthen ponds (Yasemi *et al*., 2011). Fish were reared in the earthen ponds and were fed by an artificial diet according to their nutritional requirements during the culture period for a few years. Fish were raised until the age of 3 or more to determine their sex and maturity stages. Gonad biopsy surgery was used to separate sex and evaluate the reproductive conditions in fish.

Gonadal biopsy procedure and other measurements

This study was made in two farms, the Shahid Dr. Beheshti Sturgeon Fish Propagation and Rearing Complex (Rasht, Guilan) and Morvarid Ghorogh Sturgeon Rearing Farm (Talesh, Guilan).

In the first hatchery, 138 fish between the ages of 6 and 14 years from earthen ponds were considered for biopsy. Before that, fish were transferred to a large concrete tank with the volume of 36 m^3. In the second farm, 88 fish between the ages 3 and 6 years were used for biopsy from circular concrete tanks. In both farms fish were deprived from food one day before biopsy. After capture with a special scoop net, fish were removed

from the rearing tank with a canvas stretcher, supporting the entire body to prevent injury and transferred to work table. Before beginning the procedure, some parameters were measured such as total length and body weight. Also, condition factor (CF) was calculated according to the formula below:

$CF = W/L^3 \times 100$

where,
W: Wet weight of fish
L: Total length

A 20-25 mm anterior-posterior incision was made with a sharp scalpel (No. 10 straight blade) through the ventral area of the body wall. The surgical incision site was located as described by Chapman (1989) for white sturgeon *Acipenser transmontanus*. Then, the scalpel was withdrawn to ensure that the incision was not obstructed. For sex identification, visual examination of ovary and testis were used and in some cases a small biopsy sample (5-8 mm) was collected by fine forceps. In this case, forceps were carefully inserted into the body cavity until reaching the gonad. Tissue sample was then cut by sharp scissors and fixed in Bouin's solution for next histological assay. During the operation, gills were kept wet. The abdominal incision was closed with one or two simple sutures, using a needle and a clamp. Fish were injected intramuscularly 1 ml 5% oxytetracycline (Razak, Tehran, Iran) per fish and then place of incision was disinfected with povidone-iodine solution (Kishmedifarm, Kish, Iran) and chloramphenicol spray (Afagh, Tehran, Iran). No deep anesthesia was used during the biopsy process.

Gonadal histology

Samples of gonads were kept in Bouin's solution. Then after 48 h, samples were embedded in paraffin following a schedule to put them into alcohol series for dehydration and clearing. The blocks were sectioned with the thickness of 6 micrometer and stained with haematoxylin and eosin for histological examination (Amiri *et al*., 1996; Van Eenennaam and Doroshov, 1998; Raji and Norouzi, 2010). Sex identification and evaluations of sexual maturity were done by observing the prepared samples under a light microscope according to Amiri *et al*. (1996).

Statistical analysis

Determination of deviation from the expected 1:1 sex ratio for each sturgeon group was done by using the Chi-square test at 95% significance level, with SPSS 13.0 software (Chicago, IL). One-way ANOVA was used to check the difference among the treatments. When differences were observed, Tukey's test was used as post hoc test to find differences among each treatment at the level of 0.05. Independent samples t-test was performed to compare mean weight, length and condition factor of female and male in each age group. Data are presented as mean ± SD.

Results

From 226 examined fish, 123 fish were female and 103 fish were male with overall sex ratio of 1:0.84. Sex of 89.7% of 3-year-old fish, 95.4% of 6-year-old fish and 100% of 14-year-old fish were successfully identified through biopsy procedure. No unknown specimen was found at age 14 (Fig. 1). Sex ratios of fish at different age classes mentioned above were 1:0.45, 1:0.78 and 1:1.23, respectively (Table 1). The female to male ratio was significantly different ($P<0.05$) from the expected 1:1, according to Chi-square test for all age classes. No significant difference was observed between males and females in each age class in terms of weight, total length and CF ($P>0.05$), but significant difference was observed between the weight classes ($P<0.05$, Table 1). Histological assay showed that sex identification in fish with less than 1-year-old (average weight 320 g) is impossible (Fig. 2), but sex can be identified when the age and size of fish increase and ovaries and testes have become differentiated. According to histological assay, 3- and 6-year-old females were in pre-vitellogenic stage (Fig. 3a) and 14-year-old females were in post-vitellogenic stage (Fig. 3b), which is known as migratory nucleus stage. Early spermatogenesis in 3-year-old male (Fig. 4a), mid spermato-genesis in 6-year-old male and late spermatogenesis (Fig. 4b) were present in

Table 1: Characteristics of great sturgeon at different ages with the accuracy of sexing and gonadal stage by biopsy procedure

Sex (n)	Age	Wet weight (kg)	Total length (cm)	Condition factor	Accuracy (%)	Sex ratio (F:M)	Gonadal stage
F (20)	3	7.6 ± 2^{b}	117.7 ± 10.2^{b}	0.46 ± 0.1^{b}	90	1:0.45	Pre-vitellogenesis
M (9)	3	7.9 ± 2.1^{b}	118.8 ± 11.1^{b}	0.46 ± 0.0^{b}	88.9		Early spermatogenesis
F (73)	6	16.3 ± 5.2^{a}	141.7 ± 16.8^{a}	0.56 ± 0.1^{a}	94.5	1:0.78	Pre-vitellogenesis
M (57)	6	16 ± 4.2^{a}	141.4 ± 17^{a}	0.58 ± 0.2^{a}	96.5		Mid-spermatogenesis
F (30)	14	18.2 ± 4.3^{a}	149 ± 9.4^{a}	0.54 ± 0.1^{a}	100	1:1.23	Post vitellogenesis
M (37)	14	18.5 ± 3.2^{a}	148.2 ± 8.4^{a}	0.57 ± 0.1^{a}	100		Spermatogenesis

F: Female, and M: Male

the testes of 14-year-old males.

No infection or mortality was seen during the experiment. All wounds and spots created by biopsy procedures were healed after 3 weeks.

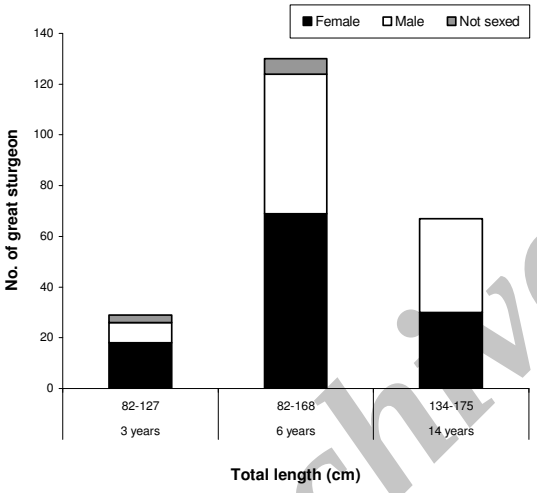

Fig. 1: The number of examined fish from different age and length classes to be sexed through biopsy

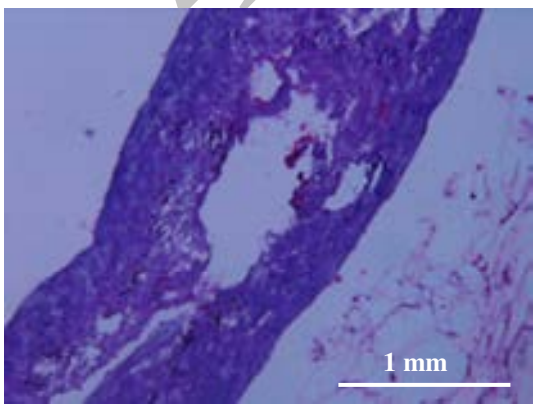

Fig. 2: A histological scheme of gonadal tissue of sub-yearling unsexed great sturgeon (weight= 320 g)

Fig. 3: Light microscopic view maturity stages of great sturgeon female (paraffin sections, haematoxylin and eosin staining). A: Previtellogenic oocyte stage (pvo) and adipose tissue (ad), and B: Magnification of oocyte layer in late previtellogenic (zri: zona radiata interna, zre: zona radiate externa, gr: granulosa cells, tl: thecal layer, and yg: yolk globules)

Discussion

The results of this study showed that a biopsy method and histological examination are useful and efficient techniques to

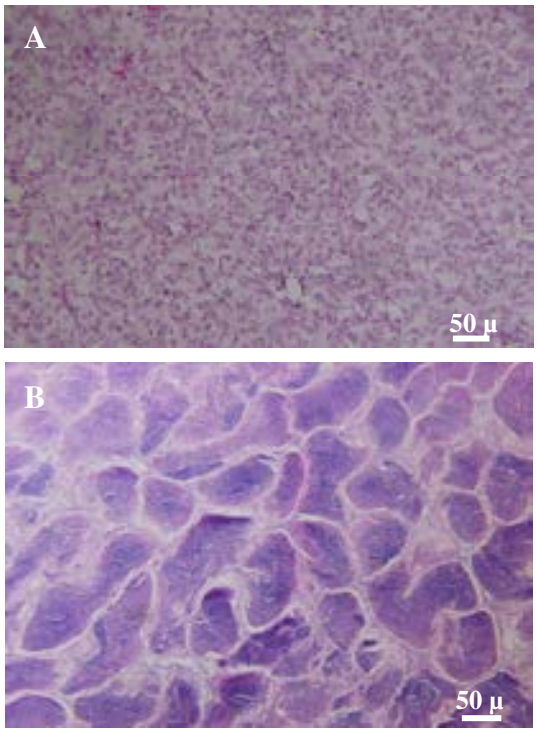

Fig. 4: Light microscopic view maturity stages of great sturgeon male (paraffin sections, haematoxylin and eosin staining). A: Spermatogonia (early spermatogenesis), and B: Spermiation stage

determine sex and maturity stage of 3-year-old great sturgeon. Using biopsy method for great sturgeon, we accurately identified the sex of 96% at different ages. So, this finding demonstrated that the ability of biopsy to distinguish sex was overall greater when compared with other studies based on available techniques like sex hormone criteria (Webb *et al*., 2002; Fiest *et al*., 2004), borescope (Kynard and Kieffer, 2002), endoscopy (Hurvitz *et al*., 2007), laparoscopy (Falahatkar *et al*., 2009; Falahatkar *et al*., 2011) and a combination of methods (Petochi *et al*., 2011) because of direct observation of gonad.

Results show that biopsy is a non-lethal method in sex identification of great sturgeon and all examined fish returned to normal situation without showing any infection, morbidity or even delayed mortality. Growth rate also was not affected (personal observation) following 3 months of biopsy. These findings were proved by previous studies which mentioned gonad biopsy as an effective method to determine the sex in *Anguilla* spp. (Matsubara *et al*., 2003) *Acipencer nudiventris* (Hallajian *et al*., 2007) and *Epinephelus merra* (Ashraful Alam and Nakamura, 2008). The accuracy of biopsy procedure increases by increase in the age of fish and size. Also, taking a proper sample can be a difficult procedure in fish which is not large enough because the germinal portion of gonad or fat tissue is covered by the body wall, so it is the easiest way for the operator to identify sex of advanced gonadal stages or even larger fish.

The result of this study showed a significant deviation from the expected 1:1 sex ratio (1:0.84). Doroshov *et al*. (1997) found 1:1 ratio among cultured white sturgeon. However, Flynn *et al*. (2006) reported that reared shortnose sturgeon *Acipenser brevirostrum* in captivity typically has a sex ratio that is favored relatively towards females (1:0.79). Sometimes sex ratio is changed with culture condition related to intensive culture and environmental conditions. In the present study on great sturgeon, the variations in culture conditions and age classes may affect this sex ratio. In the current study on 226 farmed fish just a slight skew was found in determination of sex ratio with high accuracy rate. Different year classes and additional number of farmed fish need to be sexed carefully to prove a real shift from 1:1. Consequently, increasing the number of inspected fish will probably lead to the estimated sex ratio of 1:1. Further research is needed to find why the sex ratio of sturgeon is changing through the culture period from the expected 1:1.

Histological analysis showed female great sturgeon at the age of 3 and 6 were at the previtellogenic stage and female great sturgeon at the age of 14 were in postvitellogenic stage. Previtellogenic oocytes were recognized by growth of primary oocyte and differentiation of the granulosa cell layer and initiation of secretion of the zona radiate. Also, thickness of zona radiata layer and major growth of oocytes was observed in postvitellogenic stage. Early spermatogenesis, mid spermatogenesis and late spermatogenesis were presented in the testes of 3-, 6- and 14-year-old male great sturgeon, respectively. In most of the fish, at early and late spermatogenesis and late spermatogenesis,

spermatocytes and spermatids were dominant, respectively. Spermatogenic cells are seen surrounding the tubule in early spermatogenesis and progression from early spermatocytes to spermatids could be found through spermatogenesis (Chapman, 1989).

As a result, there are numerous advantages for biopsy that include the following:

1) Direct observation of gonads
2) Quickness of this method
3) Being practical, precise and efficient for field operations and
4) Not expensive

Obviously, when an ovarian follicle or testis sample is needed in order to check the state of maturation, surgery is required. Also, during the aquaculture management of sturgeon, it is necessary to separate the sexes in order to produce meat or caviar. Therefore, one of the most accurate, economical and useful methods is biopsy of fish. Nevertheless, this technique is more stressful and invasive for fish when compared to minimally invasive techniques like endoscopy (Falahatkar *et al*., 2009; Falahatkar and Poursaeid, 2013) or sonography (Moghim *et al*., 2002). So, we recommend comparing various techniques that are used for sex identification to achieve a method with the highest accuracy and the lowest stress.

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