Use of endoscopy for gender and ovarian stage determinations in Russian sturgeon (Acipenser gueldenstaedtii) grown in aquaculture

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Abstract

The Russian sturgeon (Acipenser gueldenstaedtii) is native to the Caspian Sea, the Black Sea and the Azov Sea. It used to breed in the main incoming rivers, until dam construction in the mid 20th century blocked upriver spawning migration. Aquaculture of Russian sturgeon has only recently begun, prompted by their declining populations in natural habitats and the rise in meat and caviar prices. However, information on their gonadal development and puberty under culture conditions is incomplete.

Because sturgeons have no external sexual dimorphism and there are no external markers for sexing, internal examination of the gonads must be employed for gender identification as well as to monitor their development. The present study describes endoscopic and histological observations of the gonads of young Russian sturgeons aimed at identifying gender and monitoring ovarian developmental stage in females up to the age of 6 years, when they enter their first puberty cycle, as well as at 7 years of age, when they have completed vitellogenesis, under culture conditions. This information, as related to fish age and size, is of vital importance to commercial farming of Russian sturgeon for caviar production and reproduction.

For gonadal observations in both sexes, we used an endoscopic system consisting of a 4 mm, 14 cm long cystoscope sheath incorporated with fiber-optic light transmission, connected to a halogen cold light source and a miniature videocamera with a control unit attached to a color monitor. This system allowed us viewing of the fish’s abdominal organs, and to save pictures of selected areas of the gonads on a computer as the fish’s personal record. Ovarian biopsies were taken in parallel for histology at typical stages of gonadal development.

Gender could be identified with this system as early as at 3 years of age and the sex ratio under culture conditions of females, males and unidentified gender were 55, 40 and 5%, respectively.

Not only did large differences occur in the developmental stages of female of the same age group, but also ovarian development was highly asynchronous at the early vitellogenic stages. In late vitellogenesis, at the “gray egg” stage (1600–2600 μm diameter), the oocytes were quite regular in size and color, and remained so until the final stages of maturity.

Our study suggests that endoscopy is an efficient method for both gender identification at an early age, and for determination of gonadal developmental stage in sturgeon aquaculture. The ability to see the whole intact gonads of anesthetized fish can reveal important management and research information, with minimal damage or stress to the fish.

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1. Introduction

The Russian sturgeon (Acipenser gueldenstaedtii) is native to the Caspian Sea, the Black Sea and the Azov Sea, and used to breed in their main incoming rivers (Volga, Kura, Don, Kuban and the Dniepr). However, dam construction that started in the mid 20th century blocked upriver spawning migration, preventing the fish from breeding. Therefore, most fish still caught in these water bodies arise from restocking with fingerlings by Russian hatcheries (reviewed by Chebanov and Billard, 2001).

Sexual maturity is reached under natural conditions after 8 to 13 years in males and after 10 to 16 years in females (Hochleithner and Gessner, 2001). However, under aquaculture conditions, sturgeon maturity is usually reached at an earlier age (Doroshov et al., 1997). Aquaculture of Russian sturgeon has only recently begun, prompted by the declining populations in their natural habitats and the rise in meat and caviar prices (Birstein et al., 1997). Nevertheless, information on gonadal development and puberty of these fish in culture is insufficient, although such information on other cultured sturgeon species is available (Amiri et al., 1996; Doroshov et al., 1997; Van Eenennaam and Doroshov, 1998; Linares-Casenave et al., 2003).

In sturgeon aquaculture, where the main purpose is caviar production, a reliable method is needed to separate the fish according to gender. Males are destined to the meat market while females remain in culture for many more years under conditions of optimal growth and development. Sturgeons have no sexual dimorphism, and the absence of external markers for sexing has encouraged the search for a practical technique for internal examination of the gonads for gender identification.

Although sex differentiation in sturgeons is completed at as early as 1 to 2 years of age (Doroshov et al., 1997; Hurvitz et al., 2005), sexing of Russian sturgeon cannot be carried out safely before the age of 3.

In older females approaching puberty, information on ovarian developmental stage is critical for the quality of caviar production. It should be borne in mind that vitellogenesis in cultured sturgeons can last up to 3 or 4 years (Doroshov et al., 1997) with large variations among individual fish of the same age (Amiri et al., 1996). Therefore, estimating the optimal time for caviar harvest requires several examinations of each individual female. In the present study, a comparison was made between endoscopy and histology of the gonads in order to establish a reliable technique for sexing and for monitoring ovarian development in the Russian sturgeon.

2. Materials and methods

2.1. Fish and culture conditions

Russian sturgeons (A. gueldenstaedtii) originated in the Azov Sea were imported in May 1999 as fertilized eggs and reared at “Dan Fish Farms” (Upper Galilee, Israel; 31°30′N, 34°45′E). During the first year, fish were maintained under routine hatchery and nursery conditions, in elongated 5 m³ raceways and running water at 16 °C. After reaching an average weight of 500 g, they were transferred to 250 or 500 m³ rectangular concrete ponds. Under natural photoperiod, water temperature was 13–16 °C in winter (November–February) and 20–24 °C during the rest of the year. They were fed twice a day with 4 mm trout granular feed (Zemach Feed Mills, Zemach, Israel; containing 50% protein and 18% fat), at 0.4 to 1% of their biomass daily, depending on the season and the fish size.

During their first 4 years, 20 randomly selected fish from each age group were sacrificed, biopsies were taken from the gonads for histology and their weight, sex and gonad developmental stage were recorded (Hurvitz et al., 2005). At the age of 5 years, a group of 80 females, randomly selected from previously sexed fish, were stocked in a 40 m³ concrete pond for further monitoring until the age of 7 years, when puberty occurred. Each fish was individually marked by colored and numbered plastic cow ear tags (Hagarin, Israel) attached to one of its pectoral fins. Once a year, in the spring, these fish were examined by endoscopy for gonad development and their weight was recorded. In addition, 10 males were kept for growth rate monitoring and their weight was also recorded annually.

2.2. Endoscopy

For gonadal observation, an endoscope system was constructed. It consisted of a 4 mm cystoscope sheath, 14 cm long and 4 mm in diameter, incorporating an optic light transmission fiber, connected to a halogen cold light source and a miniature videocamera with its control unit connected to a color monitor (Karl Storz, Germany). With this system, the fish’s internal organs could be viewed all along the body cavity and the pictures of selected points could be monitored and saved digitally as an individual record (Fig. 1A, B).

Each fish was anesthetized in a clove oil bath (0.25 mg/l) and placed on a V-shaped base for examination. One 5 to 10 mm incision was made with a sterile scalpel in the abdominal wall through which the cystoscope sheath was inserted (Fig. 1A). The scope was...
equipped with a 5 ml syringe and an internal canal through which the organs could be rinsed with sterile PBS (phosphate-buffered saline) for a clear view. Approximately 1 to 4 ml of PBS was usually needed for the examination and excess fluids were expelled from the fish through the abdominal wall incision soon after the scope was pulled out. After the examination, which lasted for about 2 min, the fish were placed in fresh clean water for recovery. There was no need to suture the incision: it closed by itself without any infection or post-procedural fish mortality. The endoscopic method was also used to monitor gonadal development in both males and females, and gonadal biopsy was taken in parallel for histology, from typical developmental stages. The gonadal biopsy was performed using a metal trocar with a 3×30 mm groove inserted into the ovary through the same incision used for the endoscopy. Approximately 270 mg of tissue was collected by this method from each sampled fish.

2.3. Gender identification (sexing)

The method of endoscopy was employed for gender identification. A total of 10,563, 3.5-year-old Russian sturgeon were examined over a period of 2 months at an average rate of 400 fish per workday. Fish were placed in a 20 m³ concrete pond with fresh 16 °C flowing water from which they were manually taken, anesthetized and examined by endoscopy.

2.4. Histological analysis

Histology was generally performed according to Hurvitz et al. (2005) and Jackson et al. (2006). Briefly, gonadal samples were fixed in Bouin’s fluid for 48 h, and then transferred to 70% ethanol for storage until processing for light microscopy. Paraffin sections of 4 to 7 μm were stained with hematoxylin and eosin. For the description of gonadal stages, the terminology used by Linares-Casenave et al. (2003) was adopted. Mean oocyte diameter was calculated for each fish after measuring five of the largest oocytes present in the fixed tissue before processing. Oocyte diameter was measured using an optical binocular with notches (CETI Belgium), accuracy ±50 μm.

2.5. Statistical analysis

Comparisons if sex ratio departed from the expected 1:1 rate were determined by Chi-square test at 95% significance level, using PRISM 4.02 software (GraphPad, San Diego).

3. Results

3.1. Gender identification (sexing)

During the winter of 2003 (Oct–Feb), fish born in 1999 (4–6 kg BW) were sexed by endoscopy. Fish with gonads composed of large smooth yellowish fatty lobes and a white-pinkish narrow zone containing small but visible semi-transparent spheres (oocytes) were identified as females.

A total of 10,563 fish were examined: 5810 (55%) were found to be females, 4225 (40%) were classified as males and 528 (5%) could not be identified. The male-to-female ratio was not significantly different from the expected 1:1, according to Chi-square test ($p<0.0001$). All fish were 3.5 years of age, averaging 5.3 kg ±1.5 BW with no significant difference between males and
females. The unidentified fish were smaller in size and thinner than the others with an average weight of 2.5 kg ± 0.8. Since the males were later processed for meat production, it was possible to validate the accuracy of the selection by observing the gonads of the processed fish. No females were found among 800 of the processed fish.

### 3.2. Ovarian development

In spring 2004, when the fish were 5 years old, they had an average body weight of 10.2 ± 2.3 kg. They were examined by endoscopy for gonadal development and all females had ovaries at the pre-vitellogenic stage. In spring 2005, when the fish were 6 years old and 11.4 ± 3.2 kg BW, 20% of the females had ovaries with a few white oocytes, representing the initial stage of vitellogenesis (Table 1). However, at the age of 7 years (12.9 ± 3.3 kg BW), the same females were examined again by endoscopy and 52% of them were found to be at different stages of vitellogenesis. Considerable variability in gonadal development was found among individual fish of the same age. Furthermore, a pronounced variability in oocyte diameter, stage and color was found within the same ovary. According to the largest oocytes present in the ovary, by endoscopy and confirmed by histology, five stages of ovarian development were identified: A. pre-vitellogenic; B. white oocytes; C. yellow oocytes; D. gray oocytes; E. black oocytes (Table 2).

In the pre-vitellogenic ovary, the oocytes were relatively uniform in size at 400 ± 50 μm. The first sign of emergence from the pre-vitellogenic stage was the appearance of a few white eggs in the ovary: one or two such oocytes could be seen in a static endoscopic view of 45 ± 5 mm² of the gonadal surface area (Fig. 2A). White oocytes, larger than the surrounding pre-vitellogenic oocytes, had a distinct white color and often protruded above the ovarian surface (Fig. 2B). The prevalence of white oocytes in the ovary varied among individual fish.

At the yellow oocyte stage, yellow oocytes as well as white and pre-vitellogenic oocytes could be seen at the same time (Fig. 2C). At an early gray oocyte stage, yellow, white and pre-vitellogenic oocytes could still be found; however, the shift to the black oocyte stage was seen only after synchronization had been achieved and uniform gonad with gray oocyte had formed (Fig. 2D). This synchronization was accompanied by the complete absorption of the gonadal fat pads. The black oocyte stage was found only at the last endoscopic examination conducted in March 2006, when fish reached the age of 7 years. These could be gray or black in color and 2600 to 3200 μm in diameter. At this stage, the next generation of pre-vitellogenic oocytes could be seen among the large mature follicles (Fig. 2E). The distribution of the gonadal stages of the 80 controlled females during the last examination is shown in Table 2. The average weight of the fish at this time was 12.09 ±

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### Table 1

Summary data on body weight and gonadal stage for age 1–6 years

<table>
<thead>
<tr>
<th>Age (years)*</th>
<th>Body weight (kg)</th>
<th>Gonadal stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>1</td>
<td>0.270±0.084</td>
<td>0.270±0.084</td>
</tr>
<tr>
<td>2</td>
<td>3.335±0.382</td>
<td>3.053±0.666</td>
</tr>
<tr>
<td>3</td>
<td>4.633±0.591</td>
<td>4.217±0.858</td>
</tr>
<tr>
<td>4</td>
<td>6.812±1.53</td>
<td>5.235±1.5</td>
</tr>
<tr>
<td>5</td>
<td>10.215±2.3</td>
<td>7.345±1.8</td>
</tr>
<tr>
<td>6</td>
<td>11.4±3.2</td>
<td>9.876±2.1</td>
</tr>
</tbody>
</table>

*At ages 1–4 years, Females: n=20; Males: n=20. At ages 5–6 years, Females: n=80; Males: n=10.

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### Table 2

Ovarian stages at 7 years of age

<table>
<thead>
<tr>
<th>Stage</th>
<th>Female body weight (kg)±SD</th>
<th>Oocyte diameter (μm)±SD</th>
<th>Egg color</th>
<th>% of females (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-vitellogenic</td>
<td>12.1±2.5</td>
<td>&lt;600</td>
<td>Semi-transparent</td>
<td>45</td>
</tr>
<tr>
<td>White oocyte</td>
<td>12.3±4.5</td>
<td>800±200</td>
<td>White</td>
<td>30</td>
</tr>
<tr>
<td>Yellow oocyte</td>
<td>10.5±2.7</td>
<td>1300±300</td>
<td>White/yellow</td>
<td>13.75</td>
</tr>
<tr>
<td>Gray oocyte</td>
<td>13.4±3.1</td>
<td>2100±500</td>
<td>Yellow/gray</td>
<td>7.5</td>
</tr>
<tr>
<td>Black oocyte</td>
<td>12.6±0.5</td>
<td>3000±400</td>
<td>Gray/black</td>
<td>3.75</td>
</tr>
</tbody>
</table>
3.3 kg. No significant correlation was found between body weight and gonadal stage ($r=0.0225$).

3.3. Histological analysis

The ovaries of 7-year-old females at the pre-vitellogenic stage were characterized by a wide zone of ovigerous lamellae and narrow surrounding fat pads. Oocytes were at the oil droplet and cortical alveolar stages ($200–480 \mu m$ and $480–600 \mu m$, respectively) with surrounding adipose tissue (Fig. 3A). White oocytes (Fig. 3B–C) were at the onset of the yolk-deposition stage. They were larger than $600 \mu m$ in diameter and yolk globules were distributed throughout the cytoplasm. Around the nuclear periphery, many nucleoli were present (Fig. 3B). The outer membranes were composed of the zona radiata, where two layers (interna and externa) could be discerned. A single layer
of granulosa cells as well as the basal lamina and the theca layer surrounded the oocytes (Fig. 3C).

Yellow oocytes were at the mid-vitellogenesis stage and were 1000 to 1600 μm in diameter. Yolk platelets were already distributed throughout (Fig. 3D). Gray oocytes were in the late vitellogenesis stage and were 1600 to 2600 μm in diameter. The cytoplasm contained yolk platelets throughout, and the nucleus was in a central position (Fig. 3E). The black oocyte stage contained fully-grown oocytes in which the nucleus was in a central position or migrating toward the animal pole. The cytoplasm was full of yolk platelets and the vitelline envelope could be seen in the histological cross section with its gelatinous coat (Fig. 3F).

4. Discussion

4.1. Endoscopy

Several methods have been described so far for the examination of sex and gonad developmental stage in sturgeons. The traditional and most common one is to anesthetize the fish and make a 3 to 4 cm incision in the abdomen, through which the gonads can be inspected.
After the examination, the incision is stitched and disinfected. For ripe females, a smaller incision of 1 cm is sufficient to perform a gonadal biopsy with a 4 mm tygon tubing (Conte et al., 1988; Hochleithner and Gessner, 2001). The use of a borescope through the urogenital duct to determine the sex and oocyte maturity stage of sturgeon was described by Kynard and Kieffer (2002). This method, though successful in identifying advanced stages and is harmless to the reproductive organs of the fish, is not accurate for young, immature sturgeons. Hernandez-Divers et al. (2004) described the use of endoscopy for sex determination and gonadal manipulation in the Gulf of Mexico sturgeon (Acipenser oxyrinchus). Recently, an extensive comparison was performed by Wildhaber et al. (2005) in order to analyze the effectiveness of ultrasound versus endoscopy in determining sturgeon gender and stage of maturity. It was concluded, as might be expected, that the success of the methods was dependent on their invasiveness. The least invasive method (i.e. ultrasound) was the least effective while the most invasive (i.e. endoscope through an abdominal incision) was the most effective. Successful use of endoscopy to identify gender has also been reported with one non-sturgeon species, the Arctic charr (Salvelinus alpinus) (Ortenburger et al., 1996).

In the present study, no comparison was made between endoscopy and any of the aforementioned methods. Nevertheless, it was found to be an effective and reliable method in both the field and the laboratory, similar to the results obtained by Wildhaber et al. (2005).

Another commonly used method for staging female gonads is biopsy with the aid of a “trocarr”. The trocar is a sharp metal rod, 3 mm wide and deep with an approximately 50 mm long groove. It is inserted through the abdominal wall into the gonad and gonadal tissue is collected in the groove by rotation. This method, though efficient in the inspection of advanced stages of vitellogenesis or mature males, is problematic at younger stages since only fat tissue is collected and repeated sampling is needed, followed by binocular observation.

Using ultrasound for sexing and staging sturgeons has the advantage of being a non-invasive system. Colombo et al. (2004) have reported 96% accuracy for males, 80% accuracy for females and 86% overall accuracy, while other authors have described 97% accuracy (Moghim et al., 2002). However the use of ultrasound requires a high degree of expertise in order to analyze the images and the younger the fish, the more its accuracy decreases. Unlike ultrasound, endoscopy enables a direct observation of oocyte color, size and distribution and thus, allows a potentially better estimation of their stage of development. Our system also provides approximately 20× magnification of the observed organs, depending on the proximity of the scope to the tissue. This is an important advantage, especially for gender identification in 3-year-old fish when oocytes are only 150 to 180 μm in diameter (Hurvitz et al., 2005) and are not identifiable to the naked eye.

4.2. Gender identification (sexing)

Sturgeons are gonochoristic and evidence for female heterogametic genetic sex determination has been presented for the white sturgeon (Acipenser transmontanus) (Van Eenennaam et al., 1999), bester (Huso huso female × Acipenser ruthenus male) (Omoto et al., 2005) and shortnose sturgeon (Acipenser brevirostrum Lesueur) (Flynn et al., 2006). This suggests the typical avian system for sex determination (WZ female, ZZ male; Devlin and Nagahama, 2002), in which the expected sex ratio of 1:1, operates also in sturgeon. Indeed, Doroshov et al. (1997) found this ratio among hundreds of cultured white sturgeons. However, Flynn et al. (2006) reported that normal, untreated populations of shortnose sturgeon reared by Supreme Sturgeon and Caviar in Canada in culture typically have a sex ratio that is skewed slightly towards females (~56% female). Similarly, our results also show a slight, albeit significant deviation from the expected 1:1 sex ratio in favor of females (55% females, 40% males and 5% unidentified). It is highly unlikely that this deviation implies a different mechanism of sex determination in the Russian sturgeon. Rather, some environmental factors related to the intensive culture system probably favor the female’s survival rate. Though this question was not investigated in this study, the female prevalence over males in the cultured population is a welcome result for the sturgeon culturist.

4.3. Gonadal development

Russian sturgeon males were not the focus of this study except for gender identification. We have shown previously that males have fully differentiated testes as from the age of 2 years and fully mature testes at the age of 4 to 5 (Hurvitz et al., 2005; Jackson et al., 2006). Female gonads are at the pre-vitellogenic stage until the age of 5 years, with ovaries containing oocytes of 180 to 400 μm in diameter (Jackson et al., 2006). In this study, we found that the ovary of a 7-year-old female at the pre-vitellogenic stage (Figs. 2A and 3A) does not differ significantly from that of younger females of the same stage, as described by Hurvitz et al. (2005) and Jackson et al. (2006). The only differences noted were the wider
zone of ovigerous lamellae and the narrower surrounding fat pads. In most of the fish at this stage, oocytes at the oil droplet and cortical alveolar stages (200–480 μm and 480–600 μm, respectively) could be found, compared to the perinucleolus (60–200 μm) and oil droplet (200–400 μm) stage oocytes (Amiri et al., 1996), that were found in 4- to 5-year-old females (Hurvitz et al., 2005; Jackson et al., 2006).

The term “white oocyte” was given to the second developmental stage because of the typical color of these oocytes as seen through the endoscope (Fig. 2B). Histologically, they are at the early yolk stage, corresponding to the onset of vitellogenesis, equivalent to the description of stage 5 of Amiri et al. (1996) for the bester, stage C in Linares-Casenave et al. (2003) for the white sturgeon, or stage C in kaluga (Huso dauricus) and Japanese sturgeon (Acipenser mikadoi) (Omoto et al., 2004). “Yellow oocyte” is the term given to the mid-vitellogenesis stage (Figs. 2C and 3D). “Gray oocyte” corresponds to the late vitellogenesis stage (Figs. 2D and 3E). Both are advanced vitellogenesis stages, similar to those found in other sturgeon species, although some authors have used different names to describe the same developmental stages. Linares-Casenave et al. (2003), for example, used the terms early and mid-vitellogenesis respectively, in white sturgeon. “Black oocytes” are in the migratory nucleus stage (or late vitellogenesis, Linares-Casenave et al., 2003) and in the Russian sturgeon, they are of the minimal diameter of 2.8 mm. At this size they can be harvested for caviar production or be used for reproduction upon completion of maturation.

Since sturgeons are known to have synchronous ovaries (Doroshov et al., 1997), it was surprising to find that during their first puberty cycle, Russian sturgeon females, exhibit asynchronous gonadal development up to the gray oocyte stage. It appears that once a single follicle in the ovary starts its vitellogenic growth, it will continue to develop, irrespective of its neighboring follicles, until it gets to an advanced yolk stage. It will then remain at this stage until other follicles in the ovary reach that point, before continuing to the final stages.

Cultured white sturgeons also exhibit asynchronous gonadal development among females, with vitellogenic growth periods ranging from 16 or 18 months up to 3 or 4 years (Doroshov et al., 1997). Cultured hybrid sturgeon (besters) too shows a large variation among females in the rate of vitellogenic growth (Amiri et al., 1996). However, while the ovarian developmental stage is determined by most investigators according to the size of the most advanced oocytes (Amiri et al., 1996; Doroshov et al., 1997; Van Eenennaam and Doroshov, 1998; Linares-Casenave et al., 2003; Omoto et al., 2005), no detailed description is yet available of the variations in size or other characteristics among oocytes during pubertal development within an individual sturgeon ovary.

4.4. Body size and stage of gonadal maturity

During the first 6 years of culture, male and female Russian sturgeons grow at the same rate except at 4 years of age, when the males are smaller in body weight, probably due to the onset of maturation at that age (Table 1). However, fish body weight is a highly variable parameter, depending, among other things, on culture conditions, so the data found in the present work does not necessarily represent the maximum growth rate of A. gueldenstaedtii in captivity.

At the age of 7 years, only a few of the 80 controlled females were at the black egg stage. However, 55% of the females were already at various stages of vitellogenesis (Table 2). An analysis of ovarian developmental stage versus body weight revealed no significant correlation between these parameters. Similarly, no apparent relationship between body size and oocyte maturity was found in cultured bester (Amiri et al., 1996) or in cultured Siberian sturgeon (A. baeri) (Pelissero et al., 1991), and highly variable pubertal size was found in white sturgeon in both culture and wild populations (Doroshov et al., 1997).

In conclusion, our study suggests that the use of endoscopy in sturgeon aquaculture can be an efficient method for both gender identification at an early age and determination of ovarian developmental stage. The ability to see the whole intact gonads in anesthetized fish can provide an important management tool as well as research information, with minimal damage or stress to the fish. This information, as related to fish age and size, is of vital importance to commercial farming of Russian sturgeon aimed at caviar production and reproduction.

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