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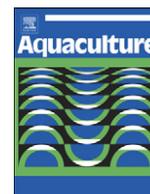


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Investigating the use of plasma testosterone and estradiol-17 β to detect ovarian follicular atresia in farmed white sturgeon, *Acipenser transmontanus*

Mariah J. Talbott^a, Joel P. Van Eenennaam^b, Javier Linares-Casenave^b, Serge I. Doroshov^b, Christopher S. Guy^a, Peter Struffenegger^c, Molly A.H. Webb^{d,*}

^a Montana Cooperative Fishery Research Unit, Montana State University, PO Box 173460, Bozeman, MT 59717, USA

^b Department of Animal Science, One Shields Avenue, University of California, Davis, CA 95616, USA

^c Sterling Caviar, 9149 E. Levee Rd, Elverta CA 95626, USA

^d Bozeman Fish Technology Center, U.S. Fish and Wildlife Service, 4050 Bridger Canyon Rd, Bozeman, MT 59715, USA

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ABSTRACT

To improve quality and yield of caviar in farmed white sturgeon it is essential to correctly assess the stage of ovarian maturity and avoid harvesting females with atretic ovarian follicles. To detect atresia by changes in blood plasma sex steroids, individual females (N = 10) in the late phase of oogenesis were repeatedly bled and their ovaries biopsied before and after onset of ovarian atresia. Follicular atresia was induced by transferring females from cold (10–13 °C) to warm water (20 °C). Ovarian follicle diameter increased and oocyte polarization index decreased in sampled fish over time. Plasma testosterone concentrations in fish with normal follicles were significantly higher, compared to fish with early histological signs of follicular atresia, such as structural changes in the chorion. Plasma estradiol concentrations declined to below detection limit prior to histological signs of atresia in 55% of the fish. Ninety five percent of fish with normal ovarian follicles and 93% of fish with atretic ovarian follicles were correctly classified using a discriminant function analysis based upon plasma testosterone concentrations. Logistic regression models were developed to predict the probability of normal ovaries based on plasma concentrations of sex steroids and can be further refined to improve selection of fish with normal ovaries for caviar harvest.

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1. Introduction

Propagation of sturgeon was first attempted in the mid 19th century in Germany, Russia, and North America to counteract the decline in wild harvest (Williot et al., 2001). Not much progress occurred in sturgeon propagation until the 1950s when advances in sturgeon culture were made in the former Soviet Union to bolster wild sturgeon populations in the Caspian Sea (Binkowski and Doroshov, 1985; Barannikova, 1987; Burtzev, 1999). Sturgeon populations worldwide have continued to decline, leaving many species of sturgeon at risk of extinction (Birstein, 1993; Birstein et al., 1997; Billard and Lecointre, 2001). This has led to strict international regulation with the use of caviar trade quotas by the Convention on International Trade in Endangered Species (CITES). Legal trade in caviar from wild sources has drastically declined over the past 10 years. In response, there has been an increased demand for caviar from several commercially cultured sturgeon species, including white sturgeon. Exports of caviar produced in sturgeon farms worldwide

increased from 1.7 metric tons in 1999 to 14.4 metric tons in 2004 (Raymakers, 2006). Captive produced caviar imports to Europe increased between 1998 and 2006, and 98% of all caviar exported from Europe during this period was from captive bred sturgeon, one-third of which was from white sturgeon (Engler and Knapp, 2008).

To meet the growing demand for caviar from captive raised sturgeon, producers must overcome the challenges of optimizing growth and reducing time to sexual maturity, while simultaneously providing conditions required to complete the normal reproductive cycle. Numerous studies have described the complexity of reproductive cycles in various species of sturgeon (see Doroshov et al., 1997). Farmed white sturgeon exhibits asynchronous onset of first vitellogenesis and biennial ovarian cycles. The ovarian cycle is controlled by environmental factors, such as photoperiod (Moberg et al., 1995), but there is considerable individual seasonal variation among farmed female white sturgeon as to when spawning readiness is reached, varying from February to June (Doroshov et al., 1997; Webb et al., 2001).

Providing the optimal conditions for maturation can be difficult, but if these conditions are not met, it leads to ovarian follicular atresia in some fish, commonly in the late phase of oogenesis. For example, it is advantageous for the caviar industry to increase growth rate and decrease the time to first sexual maturity by raising white sturgeon in water temperatures of 18–20 °C. However, it has been shown that

* Corresponding author. Tel.: +1 406 994 9907.

E-mail address: Molly_Webb@fws.gov (M.A.H. Webb).

holding temperatures higher than 15 °C during the prespawning season invariably induces ovarian atresia in white sturgeon (Webb et al., 2001). This deleterious effect of elevated temperature has been well described in the Caspian and Azov Sea sturgeons (Dettlaff et al., 1993). All major caviar farms in the California and Idaho have modified husbandry practices to include a “vernalization” period by transporting late vitellogenic females to cooler water (10–13 °C) in the fall prior to caviar harvest the following spring. Similar vernalization techniques have been applied to Siberian sturgeon in culture (Williot et al., 1991). This has reduced, but not eliminated the incidence of ovarian atresia at caviar harvest due to individual variation in the chronology of the ovarian cycle and temperature-sensitive ovarian stage. In addition, reduced gamete quality in fish can be caused by other factors, such as management stress (Bayunova et al., 2002) or inadequate nutrition (Bromley et al., 2000).

It would be beneficial for the caviar industry if a quick, reliable, and minimally invasive method to detect the early signs of ovarian follicular atresia was developed. Harvesting, even at the early stage of atresia can cause a reduction in the firmness, flavor, and shelf life of caviar, and sometimes the complete loss of the product, hence reducing profits for the caviar industry. The current method of a quick ovarian biopsy to visually assess follicles for presence or absence of atresia prior to caviar harvest is invasive and cannot detect microscopic changes in the chorion at the onset of atresia. To find an alternative, less invasive method, a group of scientists representing four universities was assembled to gain a better understanding of the biochemical and physiological changes that occur during follicular atresia. Specifically, new methods to identify or predict atresia were tested as an alternative to macroscopic or histological detection. Techniques for detecting atresia explored in this article, and articles that immediately follow, include: short-wavelength near infrared spectroscopy (Servid et al., 2011-this volume), Fourier transform infrared spectroscopy (Lu et al., 2011-this issue), and using plasma sex steroids to discriminate between fish with atretic ovaries and fish with non-atretic ovaries as well as modeling the probability that a fish has non-atretic ovaries (this study). This collaboration provides broad baseline information for potential future commercialization of an alternative, minimally invasive technique to determine the presence or absence of atresia for use by the caviar industry and sturgeon conservation propagation programs.

This paper will focus on the use of blood plasma sex steroids to detect follicular atresia. Both testosterone (T) and estradiol-17 β (E2) play major roles in oocyte development and maturation. The production of T in the thecal cells of the ovarian follicle is stimulated by gonadotropins released into the bloodstream by the pituitary gland. Testosterone is converted into E2 by aromatization in the granulosa cells (Kagawa et al., 1983). Estradiol is subsequently released into the bloodstream and triggers hepatic synthesis of vitellogenin, the yolk precursor protein. Vitellogenin is transported via blood to the ovarian follicles. It is then incorporated into the oocyte via receptor-mediated endocytosis, where it is proteolytically cleaved into yolk proteins (Patino and Sullivan, 2002).

The early stage of atresia cannot be observed macroscopically but can be detected by histology (Linares-Casenave et al., 2002) or by a decrease in plasma concentration of sex steroids (Webb et al., 2001; Linares-Casenave et al., 2002). During late vitellogenesis, T and E2 concentrations in sturgeon blood plasma are elevated until ovulation (e.g. Webb et al., 2001; Barannikova et al., 2002a; Semenkova et al., 2002) or onset of atresia (Webb et al., 2001; Barannikova et al., 2002b; Linares-Casenave et al., 2002). Testosterone and E2 levels were reported to decrease below 1 ng/mL and 0.5 ng/mL, respectively, after visual signs of atresia were evident (Linares-Casenave et al., 2002). In another study, a decrease in plasma sex steroids was reported five weeks prior to visual signs of atresia in cultured white sturgeon (Webb et al. 2001). Therefore, further examination of plasma sex steroid concentrations before and after onset of follicular atresia may

provide useful information for detecting atresia. The objective of this study was to further investigate the relationships between plasma sex steroids and ovarian atresia to develop a tool to detect early signs of ovarian follicular atresia.

2. Materials and methods

2.1. Animals and study sites

Animals were randomly selected for the experiment from the 6-year old stock of white sturgeon females at Sterling Caviar LLC, in Wilton, CA, herein referred to as the warm-water site. Fish were reared at the warm-water site to the late phase of vitellogenesis in 9.1 m diameter, 1.5 m depth, flow-through outdoor tanks, supplied with the well water of constant temperature 20 °C. Dissolved oxygen was maintained above 6 parts per million (ppm). In September 2007, fish were transferred for ‘vernalization’ to a cold-water site, approximately 48 km southeast of the warm-water site, where they were stocked at a density of 5 fish per 3.7 m diameter, 0.9 m deep outdoor covered tanks supplied with clear surface water from Lake Amador (tank temperature 10–13 °C). During the study, fish were fed pelleted sturgeon diet (EWOS®, Surrey, B.C., Canada) at 0.03–0.09% body weight per day at both warm-water and cold-water sites.

2.2. Experimental design

In September 2007, surgical ovarian biopsies were performed on fish at the warm-water site to assess ovarian development. Late-vitellogenic females from the 2001 year class (N = 15, fork length 132–156 cm, weight 25–40 kg) were selected at random, individually tagged with passive integrated transponder (PIT) tags and transported to the cold-water site. Fish were sampled bi-monthly at the cold-water site during November–March, and then transferred to the warm-water site to induce atresia. Sampling at the warm-water site was conducted during April–May using bi-weekly intervals.

2.3. Tissue collection and processing

At each sampling, fish were anesthetized using tricaine methane sulfonate (100 ppm, MS-222) and length, weight, and PIT tag number were recorded. Blood (6–7 mL) was collected from each fish from the caudal vasculature with a 10-mL heparinized vacutainer. Blood samples were placed on ice, centrifuged (3400 rpm for 5 min) and the separated plasma was kept at –80 °C and shipped on dry ice to the Bozeman Fish Technology Center (BFTC) where it was stored at –80 °C until sex steroid analysis. Approximately 50 ovarian follicles per fish were removed by catheter via a 1-cm incision in the abdomen and macroscopically assessed for eggs that were soft and bursting or marbled (black and white) in appearance, indicative of early or mid-atresia, respectively (Linares-Casenave et al., 2002). Follicles were subsequently fixed in 10% phosphate buffered formalin for measurement of follicle diameter, oocyte polarization index (PI), and histological analysis.

The ovarian follicle diameter was measured for 15 follicles per fish (Leica DM 2000, 2.5 \times , RT KE Spot camera). These follicles were then bisected along the animal-vegetal axis for measurement of oocyte PI by image analysis. Oocyte PI, a measure of stage of maturation, is the ratio of the distance of the nucleus from the animal pole to the oocyte diameter.

Follicles designated for histological analysis were processed by dehydration, embedding in paraffin, sectioning at 5 μ m, and staining using periodic acid-Schiff's solution and Hematoxylin and Eosin. Slides were examined for the onset of follicular atresia (Leica DM 2000). The early signs of atresia matched the previous description for white sturgeon (Linares-Casenave et al., 2002) and fish were subjectively classified as two stages: early atresia or mid-atresia.

Early atresia is defined as loss of striation of the chorion (zona radiata) and hypertrophy of the granulosa cells. Mid-atresia is defined as progressive digestion of the chorion and a thickening thecal layer invaded by blood-borne cells (Fig. 1).

2.4. Radioimmunoassays

Plasma steroids were extracted following the methods in Fitzpatrick et al. (1986). For analysis of T, 100 μ l of thawed plasma was extracted twice by adding 2 ml of anhydrous diethyl ether (Mallinckrodt Chemicals, Phillipsburg, NJ), vortexing for 15 s, flash freezing in liquid nitrogen, and decanting the ether mixture into a new test tube. The ether was evaporated under nitrogen. Steroids were resuspended in 1 ml of phosphate-buffered saline with gelatin (PBSG) and thoroughly vortexed. Due to low concentrations of E2 (<1 ng/ml), 200 μ l of plasma was extracted twice using 4 ml of diethyl ether. The remaining extraction steps were the same as described for T. Recovery efficiency was determined to estimate the efficiency of extraction and to provide a correction factor for steroid assay results based upon recovery percentages. Recovery efficiencies for T varied between 74% and 97%, with a mean of 84%. Estradiol recovery efficiencies varied between 70% and 85%, with a mean of 79%.

Concentrations of T and E2 were measured using RIA as described in Fitzpatrick et al. (1986) and modified by Fiest et al. (1990). For E2, 50 μ l of extracted steroid was resuspended in PBSG and analyzed in a competitive binding assay using steroid antibody and tritiated steroid solution. The T antibody had cross-reactivity with androstenedione (0.5%) and dehydroepiandrosterone (0.01%). The E2 antibody had cross-reactivity with estrone (<0.42%), estriol (<0.11%), dihydrotestosterone (<0.055%), T (<0.002%), and ethinylestradiol (<0.008%). Depending upon the concentration of T in the plasma samples, an extract volume of 10, 25, or 50 μ l was used. All samples were analyzed in duplicate and were reanalyzed if the intra-assay and inter-assay coefficients of variation were greater than 5% or 10%, respectively. Steroid levels, determined by RIA, were validated by verifying that serial dilutions were parallel to standard curves. The lower detection limits for T and E2 were 0.5 and 0.1 ng/ml, respectively.

2.5. Statistical analysis

Plasma concentrations of T and E2 were normalized using a \log_{10} -transformation and values for samples that were below the minimum detection limit were substituted with half the minimum detection limit (0.25 ng/ml for T, 0.05 ng/ml for E2). Multivariate analysis of variance (MANOVA) procedures were conducted to compare the concentration of T and E2 among four different groupings in relation to onset of atresia. The four classifications were as follows: late vitellogenic 2 (VTG 2) was two sample dates prior to the histological

detection of atresia; late vitellogenic 1 (VTG 1) was one sample date prior to histological detection of atresia; and early and mid-atresia stages are described in Section 2.3. Comparisons of the treatment means were conducted using the F-test. The accepted significance level was $\alpha = 0.05$.

A stepwise discriminant function analysis (DFA) using T and E2 concentrations was developed to predict the stage of ovarian maturity. To remain in the model, the significance level had to remain below $\alpha = 0.10$. Using the best variable(s) determined in the stepwise procedure, a quadratic DFA was conducted to determine the percent of observations correctly classified into normal (non-atretic) and atretic stages. Cross-validation was used to determine the error rate associated with predicting the stage of atresia using the chosen discriminant functions (see Khattree and Naik, 2000). The MANOVA and DFA analyses were conducted using Statistical Analysis System for Windows, JMP®, version 4 (SAS Institute Inc., Cary, NC).

A logistic regression was used to model the probability of a fish having normal ovaries with plasma T or E2 as the explanatory variables. Atretic observations were assigned to category 0, and normal (non-atretic) observations were assigned to category 1. Phi-hat was calculated to check for overdispersion that may be present due to repeated sampling of individuals. A phi-hat value >1 indicates overdispersion, where $\text{phi-hat} = \Sigma(\text{residuals}^2)/(n - 2)$. Calculated values of phi-hat for both T and E2 were <1, indicating that overdispersion did not occur. Logistic regression analysis, including point estimation of T given specific probabilities and associated 95% confidence intervals (CI), was conducted using R, version 2.8.1. (R Foundation for Statistical Computing, www.r-project.org). Point estimation of E2 was not conducted due to the extremely large size of the 95% CI.

3. Results

3.1. Morphological observations and results

Of the 15 fish originally selected for this study, 11 remained at the end of the experiment. In early November, the aerobic and anaerobic layers of Lake Amador mixed, causing a decrease in dissolved oxygen and an increase in hydrogen sulfide levels at the coldwater site. The combination of decreased water quality and handling stress potentially caused the loss of four fish between November 1st and 16th. Of the 11 fish remaining, one had an over-inflated air bladder that did not return to normal until after transfer to the warm-water site. Data from this fish were not used. During the January sampling, atresia was macroscopically and histologically detected in the first fish; one additional fish initiated atresia between January and March, while still at the cold-water site. After fish were moved to the warm-water site in mid-March, four initiated atresia by April 2nd, one fish by April

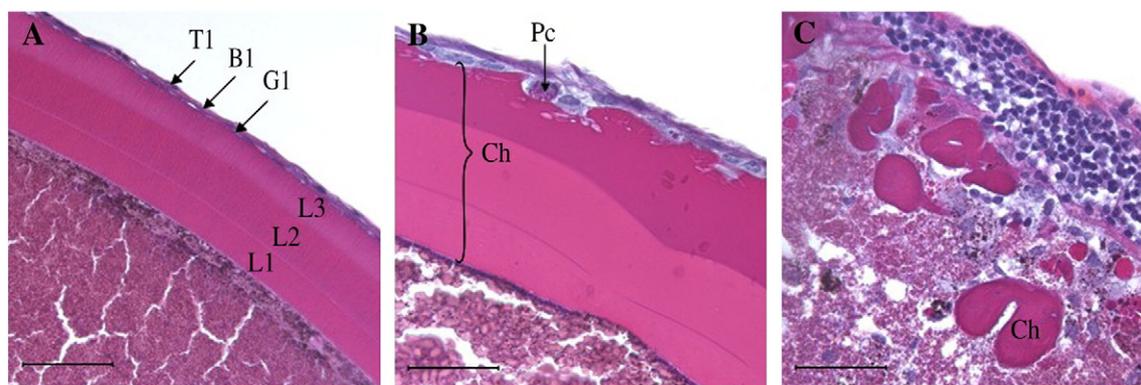


Fig. 1. Histological sections (PAS stain) of white sturgeon ovarian follicles during the progression of atresia. (A) normal follicle; (B) early atresia; and (C) mid-atresia (B1 = basal lamina, Ch = chorion, G1 = granulosa layer, L1, L2, and L3 = three layers of the chorion (Cherr and Clark, 1985), Pc = phagocytic cell, Tl = thecal layer). Scale bars are 50 μ m.

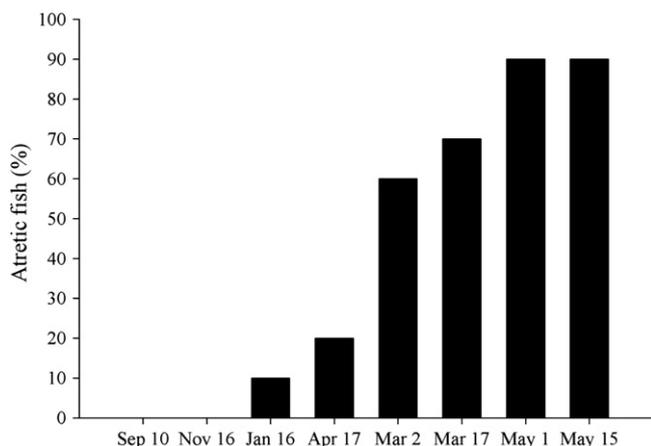


Fig. 2. Sampling dates and cumulative percent of white sturgeon females with atretic ovaries (n = 10). September 2007–May 2008.

17th, and two by May 1st (Fig. 2). At the last sampling on May 15th, one fish had yet to initiate atresia, i.e., atresia was not detectable macroscopically or histologically. Atresia was recognized macroscopically at the time of sampling in three fish that did not show histological signs of early atresia. Conversely, in three separate fish, early atresia was identified histologically before macroscopic signs were present.

In September, the mean follicle diameter (± S.E.M.) and oocyte PI (± S.E.M.) were 2.94 ± 0.03 mm and 0.268 ± 0.02, respectively. Prior to or at early atresia, mean follicle diameter increased to 3.18 ± 0.04 mm and oocyte PI decreased to 0.095 ± 0.012. Follicle diameter and oocyte PI were not measured at mid atresia because follicles became irregular, soft, and the germinal vesicle was no longer distinguishable.

3.2. Steroid hormones

Mean plasma T and E2 concentrations decreased respectively, from 136.2 ng/ml to 43.4 ng/ml, and from 2.2 ng/ml to 1.3 ng/ml, during September to November. The T and E2 concentrations of six fish rebounded and peaked at an average of 95.7 ng/ml and 2.0 ng/ml before decreasing again prior to the histological detection of follicular atresia. Three fish did not rebound from the initial decrease of sex steroids. Two of these were the first to initiate atresia and did so before transfer to the warm-water site. Atresia was detected in the third fish two weeks after being transferred to the warm-water site.

Sex steroids were compared among four different classifications in relation to the onset of atresia. Testosterone concentrations in VTG 2 and VTG 1 groups were significantly different compared to concentrations at early and mid-atresia (Table 1). There was no significant difference in plasma T between late vitellogenic groups VTG 2 and VTG 1 or between early and mid-atresia. Estradiol concentrations in the VTG 2 group were significantly different than in either stage of atresia (Table 1). Estradiol concentrations in the VTG 1 group were not significantly different from VTG 2 or either stage of atresia and

Table 1 Ovarian follicle diameter, oocyte polarization index, and blood plasma testosterone and estradiol concentrations of cultured white sturgeon at: vitellogenic 2 (VTG 2, two sample dates prior to atresia), vitellogenic 1 (VTG 1, one sample date prior to atresia), early atresia (loss of striations of zona radiata and hypertrophy of granulosa cells), and mid-atresia (thick thecal layer, remnants of egg envelope still present). Data are means and S.E.M. (range), n = 9. For testosterone and estradiol different letter superscripts indicate a significant difference between means as tested by MANOVA. Asterisks (*) indicate n = 4; remaining samples (n = 5) were too soft to measure diameter or oocyte polarization index. Dashes indicate follicles were too soft or irregular to measure diameter or oocyte polarization index in mid atresia.

Classification	Follicle diameter (mm)	Oocyte polarization index	Testosterone (ng/ml)	Estradiol (ng/ml)
VTG 2	3.15 ± 0.05 (2.89–3.29)	0.124 ± 0.018 (0.067–0.231)	68.9 ± 11.5 (14.3–106.2) ^a	0.94 ± 0.36 (0.05–2.73) ^a
VTG 1	3.18 ± 0.04 (3.01–3.34)	0.095 ± 0.012 (0.058–0.174)	56.5 ± 12.8 (3.8–112.2) ^a	0.56 ± 0.26 (0.05–1.82) ^{ab}
Early atresia	3.26 ± 0.04 (3.18–3.33)*	0.083 ± 0.006 (0.073–0.100)*	8.2 ± 4.0 (0.3–34.4) ^b	0.06 ± 0.01 (0.05–0.17) ^b
Mid atresia	–	–	1.3 ± 0.2 (0.5–1.8) ^b	0.05 (all observations) ^b

Table 2 Results from stepwise quadratic discriminant function analysis classifying ovarian stage in cultured white sturgeon females using the variable testosterone (T).

Variable	Partial R ²	F-statistics	Wilks' lambda	Average squared canonical correlation
T	0.77	206.0**	0.23**	0.77**

** Denotes p < 0.0001.

there was no significant difference in E2 concentrations between atresia stages.

The stepwise DFA procedure revealed that the model using log₁₀-transformed plasma T concentration data had the greatest discriminative ability to predict ovarian stage (Table 2). Overall, 94% of all observations were classified by ovarian stage using plasma T as the explanatory variable in the DFA, which far exceeds the 50% probability of correctly classifying ovarian stage based on chance alone. The quadratic DFA was able to correctly classify 95% of normal (non-atretic) observations and 93% of atretic observations using T as the explanatory variable (Table 3). Cross validation of the model indicated an error rate of 6%.

Two separate logistic regression models based on either plasma T (Fig. 3) or E2 concentrations (Fig. 4) for modeling the probability of fish having normal ovaries (0 = atretic, 1 = normal, Tables 4) were significant. The explanatory variables T and E2 were log₁₀-transformed to produce the following regression models:

$$p = \exp^{(-4.256+4.660(\log T))} / 1 + \exp^{(-4.256+4.660(\log T))} \quad (1)$$

$$p = \exp^{(5.182+4.629(\log E2))} / 1 + \exp^{(5.182+4.629(\log E2))} \quad (2)$$

where p is the probability of a fish possessing normal ovaries.

The following equation can be used to obtain concentrations of plasma T given a specified probability of a fish having normal ovaries.

$$T = 10^{((\ln(p/(1-p))+4.256)/4.660)} \quad (3)$$

where p is the probability of a fish possessing normal ovaries.

After the logistic regression model for T is validated and modified with a larger number of fish a caviar producer could use an equation similar to Eq. (3) to avoid harvesting fish with atretic ovaries by setting a threshold value of T based on an acceptable probability of harvesting fish with normal ovaries and have an X% certainty that the fish has been properly classified.

4. Discussion

The initial decrease in steroid levels between September and November 2007 in the study fish may be attributed to stress caused by a decrease in dissolved oxygen concentrations due to the turnover of Lake Amador. It has been shown that hypoxic conditions illicit the stress response in Siberian sturgeon, *Acipenser baeri* (Maxime et al., 1995) and white sturgeon (Cech and Crocker, 2002) and that stress

Table 3

Classification summary for determination of ovarian stage from the quadratic discriminant function analysis for cultured white sturgeon females using \log_{10} -transformed plasma testosterone concentrations as the explanatory variable. Bold values are the percentages of observations correctly classified, whereas values not in bold are percentages of misclassified observations; sample sizes (n) are in parentheses.

Actual	Classified		Total
	Normal	Atretic	
Normal	95 (36)	5 (2)	100 (38)
Atretic	7 (2)	93 (25)	100 (27)

causes a decrease in T concentrations in stellate sturgeon, *Acipenser stellatus* (Bayunova et al., 2002; Bukovskaya et al., 1999). It is possible that the initial drop in plasma sex steroids due to stress caused by a decline in dissolved oxygen, initiated atresia in two of three fish whose T and E2 concentrations did not increase after the initial decrease in November. Both of these fish showed histological signs of atresia prior to transfer to the warm-water site. Although plasma cortisol concentrations were not measured, we are presuming that stress may have been a major contributing factor in the initiation of atresia in these two fish. However, this study was designed to determine the best parameters to detect atresia regardless of the cause.

Plasma T concentrations are usually higher in late vitellogenic white sturgeon females compared to E2 concentrations (Webb et al., 2001, 2002). Although the primary role of E2 is to aid in gonadal development, T is involved in other functions, such as positive and negative feedback control of the hypothalmo–pituitary–gonad axis and migratory behavior in sturgeons (Bayunova et al., 2002; Bukovskaya et al., 1999; Ceapa et al., 2002; Safi et al., 1999). Testosterone has also been hypothesized to play an important role in maintaining post-vitellogenic oocytes until acquisition of maturational competence (Kime, 1993). A significant decrease in T during late vitellogenesis, as described in this study, may affect the synthesis of E2 from T. A decrease in E2 production may then compromise ovarian follicle maintenance and may lead to follicular atresia. In each fish within this study, E2 and T decreased concomitantly with follicular atresia. However, T was the more reliable indicator of early atresia because E2 concentrations in more than half the fish fell below the

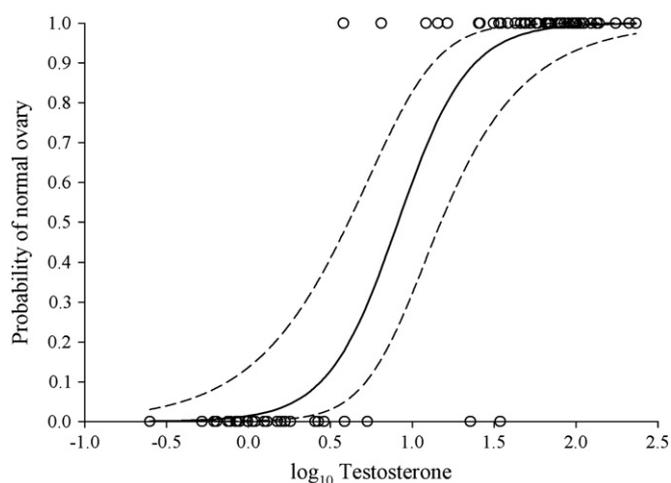


Fig. 3. Model predicting the probability of a white sturgeon female possessing normal ovaries in relation to plasma testosterone concentrations (\log_{10} -transformed). On the y-axis 1 = normal, 0 = atretic. Dashed lines represent 95% confidence intervals. For the model equation, see Eq. (1). $n = 74$.

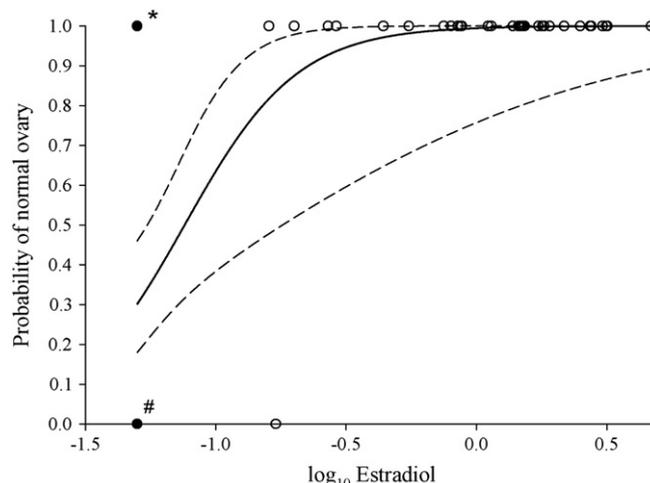


Fig. 4. Model predicting the probability of a white sturgeon female possessing normal ovaries in relation to estradiol concentrations (\log_{10} -transformed). On the y-axis 1 = normal, 0 = atretic. Filled circles indicate overlapping data points. Asterisk indicates 12 data points. Pound symbol indicates 27 data points. Dashed lines represent 95% confidence intervals. For the model equation, see Eq. (2). $n = 74$.

detection limit prior to the onset of atresia. This explains why one sample date prior to detection of atresia (VTG 1) T concentrations, but not E2 concentrations, was significantly different compared to the concentrations detected at early atresia.

Using plasma T is a reliable method to classify fish as having either normal ovaries or atretic ovaries. Being able to distinguish between early and mid-atresia is less important for fish culturists than distinguishing between the presence and absence of atresia. It is less cost prohibitive to err on the side of misclassification of fish with normal ovaries than atretic ovaries. A fish with normal ovaries determined to be atretic could be held for caviar harvest in subsequent years if economically viable, whereas, an atretic fish misclassified as normal would produce substandard caviar or, most likely, no caviar at all. The use of DFA has also been successfully applied to classification of sex and stage of maturity in wild white sturgeon (Webb et al., 2002) and Persian sturgeon, *Acipenser persicus* (Safi et al., 1999; Maleksadeh Viayeh et al., 2006).

A threshold value of T can be determined by using logistic regression modeling to lessen the probability of harvesting white sturgeon with atretic ovaries. For example, if a producer decides that an acceptable probability of harvesting fish with normal ovaries is 0.95 and nothing less, Eq. (3) produces a threshold value of 35.1 ng/ml for T. It should be noted that the 95% CI for the threshold estimate is large corresponding to a 0.95 probability of a fish possessing normal ovaries. A caviar producer may reduce the 95% CI by reducing the probability of harvesting a fish with normal ovaries because the 95% CI for threshold estimates decrease with decreasing probability until 0.5. An equation for determining a threshold for plasma E2 was not given due to the large 95% CI. This was caused by many observations of plasma E2 being below the minimum detection limit for fish with both atretic and normal ovaries. Therefore, it is not advisable to use the logistic regression model for plasma E2 to determine the probability that a fish has normal ovaries.

Before the logistic regression model using T as the explanatory variable is used it needs to be validated with a larger data set that includes different stocks reared under different conditions. Minimally invasive detection of ovarian atresia is important in studies of natural and artificial propagation of the endangered sturgeon species. If used in conservation propagation programs, a separate data set from fish captured in the wild is needed. This type of modeling is advantageous because caviar producers and conservation propagation managers can independently determine a cut-off value of T depending on what

Table 4
Summary of logistic regression models determining probability of normal ovaries using testosterone or estradiol as predictors (β_0 = intercept, β_1 = slope, SE = standard error, Threshold Est = estimate of concentration of steroid (ng/ml) corresponding to 0.95 probability of normal ovaries, SE Threshold Est = standard error of 0.95 threshold estimation).

Model	Predictor	β_0	β_1	SE β_1	p-value	Threshold Est	SE Threshold Est
1	Testosterone	-4.256	4.660	1.092	<0.0001	35.1 (18.5–132.7)*	0.182
2	Estradiol	5.182	4.629	1.633	0.005	0.33 (0.15–17.62)*	0.279

* Data in parentheses are 95% confidence intervals.

they deem as an appropriate probability of selecting fish with normal ovaries.

White sturgeon has a group-synchronous type of ovarian development and a single clutch of vitellogenic follicles (Doroshov et al., 1991). Preovulatory follicular atresia affects all vitellogenic follicles (Linares-Casenave et al., 2002), however it is possible that the rate of atresia across a gonad lobe is not synchronous. This is indicated by the visual observation of early atresia in three fish that could not be detected histologically. Two of these three fish had T concentrations above the 0.95 probability threshold (i.e., normal ovaries) derived from the logistic regression (Eq. (3)). It is possible that only a few follicles were beginning to be resorbed and the majority of follicles were still intact. All observations of early atresia that were identified histologically, but not seen macroscopically, had corresponding concentrations of T below the 0.95 probability threshold. Follicles in the early stages of atresia and follicles of normal structure were observed in the same ovary in seven wild-caught Russian sturgeon *Acipenser gueldenstaedtii*, with mean T concentrations of 71.5 ng/ml, which was greater than mean concentrations of T in six fish with late vitellogenic follicles (61.2 ng/ml) (Barannikova et al., 2002b). The possibility of heterogeneous onset of atresia across ovarian lobes will be further investigated in a future study.

Measuring plasma concentrations of T and E2 is useful for discerning whether or not white sturgeon females have initiated follicular atresia, which allows caviar producers to preselect females for harvest to ensure good quality caviar. This study has not only confirmed previous reports of declining concentrations of T and E2 prior to and after onset of atresia, but it has also attempted classification and probability models that, after validation, may be applicable in both caviar production and conservation propagation programs. At the present time, quantifying T and E2 concentrations by RIA is too time consuming and costly to be beneficial for large caviar producers. This study lays the foundation for further investigation into technology designed for the rapid determination of plasma sex steroid concentrations that would benefit caviar producers and wild sturgeon conservation programs.

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