DETERMINING MORHPOLOGICAL AND BIOCHEMICAL PARAMETERS ASSOCIATED WITH OVARIAN FOLLICULAR ATRESIA AND CAVIAR QUALITY AND YIELD IN CULTURED WHITE STURGEON (*Acipenser transmontanus)*

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ABSTRACT

Harvesting sturgeon Acipenseridae when the ovarian follicles have the appropriate firmness and size is important for the caviar industry in order to produce a consistently uniform product and maximize caviar yield. Therefore, it is beneficial for the caviar industry to detect fish with atretic ovarian follicles prior to harvest, which can cause a decrease in caviar grade or complete loss of the product and to harvest fish at the correct stage of ovarian maturity to produce the highest quality and yield of caviar possible. The objectives of this study were to find a parameter that can detect early signs of ovarian follicular atresia by measuring blood plasma concentrations of sex steroids and determine if correlations exist among plasma sex steroid concentrations, morphological characteristics, and caviar yield and quality. To achieve the first objective, blood and ovarian follicles were collected repeatedly from 15 fish prior to and after inducing follicular atresia. Plasma testosterone (T) was the best indicator of the onset of atresia. Logistic regression models were used to illustrate how a threshold value of T can be determined to decrease the probability of harvesting white sturgeon with atretic ovaries. To achieve the second objective, biological samples (blood, ovarian follicles) and morphological measurements were collected from white sturgeon at caviar harvest (n = 20 per month) for five months. Analyses of parameters associated with caviar quality were limited because 92% of fish produced the same quality of caviar. Plasma E2 was correlated with caviar yield, caviar yield as a percent of body weight, and gonadosomatic index (GSI), whereas T was not correlated with either caviar yield or caviar yield as a percent of body weight. Ovarian fat varied greatly among individuals. Consequently, the ovarian stage associated with caviar yield could not be determined by measuring morphological parameters indicative of ovarian maturity. Post-hoc analyses were conducted to determine if parameters differed among ovarian fat categories. In the future, this study may benefit sturgeon conservation propagation programs by improving techniques for detection of ovarian atresia and offering a less-invasive method for estimating fecundity by utilizing the correlation between plasma E2 and GSI.

CHAPTER 1

PLASMA SEX STEROID CONCENTRATIONS DURING ONSET OF OVARIAN ATRESIA IN CULTURED WHITE STUREGON

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; Burtsev 1999). Sturgeon populations worldwide have continued to decline, leaving many species of sturgeon at risk of extinction (Billard and Lecointre 2001). This has lead 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 imported 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). Cultured white sturgeon exhibit 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 cultured 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 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 caviar farms in 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. This has reduced, but not eliminated, the incidence of ovarian atresia at caviar harvest. Individual variation in the chronology of the ovarian cycle and the temperature-sensitive ovarian stage continue

to result in follicular atresia. In addition, ovarian follicular atresia can be induced by many other factors, such as management stress or inadequate water quality and nutrition.

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. New techniques for detecting atresia include: short-wavelength near infrared spectroscopy (Servid et al. in review), Fourier transform infrared spectroscopy (Lu et al. in review), and steroid analysis by radioimmunoassay (RIA) (this chapter). This collaboration provides broad baseline information for the potential future commercialization of an alternative, minimally invasive technique to determine presence or absence of atresia for use by the caviar industry and sturgeon conservation propagation programs.

This chapter will focus on the use of blood plasma sex steroid parameters to detect follicular atresia. 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 the

plasma concentration of sex steroids (Webb et al. 2001; Linares-Casenave et al. 2002). Circulating estradiol-17β (E2) and its precursor testosterone (T) are essential for development, growth, and maintenance of ovarian follicles. During late vitellogenesis, T and E2 concentrations in sturgeon blood plasma are elevated until ovulation (e.g. Webb et al. 2001; Barannikova et al. 2002b; Semenkova et al. 2002) or onset of atresia (Webb et al. 2001; Barannikova et al. 2002a; 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, 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.

Methods

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 at or slightly above air saturation using tank oxygenation. 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 held in 3.7-m diameter, 0.9-m deep outdoor tanks supplied with the 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.

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.

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 heprainized vacutainer. Blood samples were placed on ice, centrifuged (3400 rpm for 5 minutes) 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. 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, 2.5x, 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 μm, 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 a 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 (Figure 1.1).

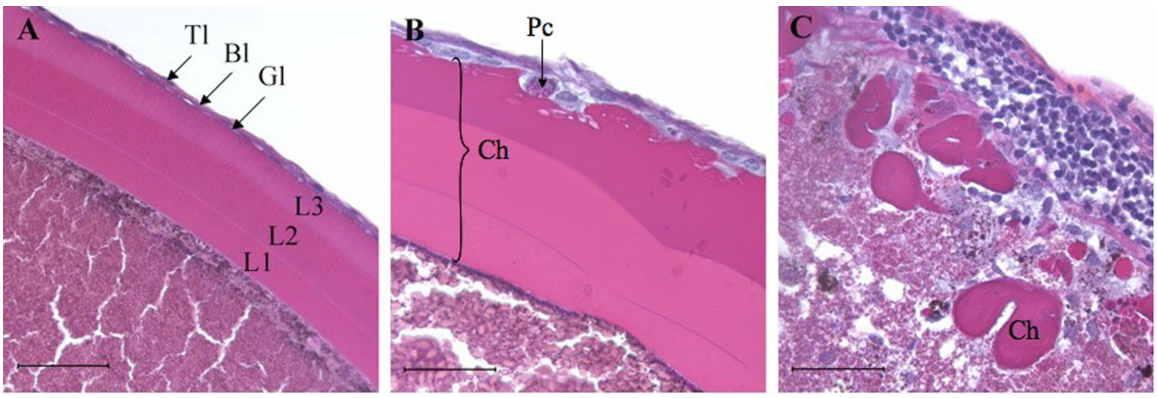

Figure 1.1. Histological sections (PAS stain) of white sturgeon ovarian follicles during the progression of atresia. (A) normal follicle; (B) early atresia; (C) mid-atresia (Bl = basal lamina, Ch = chorion, Gl = granulosa layer, L1, L2, L3 = three layers of the chorion, Pc = phagocytic cell, Tl = thecal layer). Scale bars are 50 µm.

Radioimmunoassays

Plasma steroids were extracted following the methods in Fitzpatrick et al. (1986). For analysis of T, 100 µl of thawed plasma was extracted twice by adding 2 ml of anhydrous diethyl ether (Mallinckrodt Chemicals, Phillipsburg, NJ), vortexing for 15 seconds, 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 µl 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 Feist et al. (1990). For E2, 50 μl of extracted steroid was resuspended in PBSG and analyzed in a competitive binding assay using steroid antibody and tritiated steroid solution. Depending upon the concentration of T in the plasma samples, an extract volume of 10, 25, or 50 μl was used. All samples were analyzed in duplicate and were reanalyzed if the intra-assay and inter-assay coefficient of variation was greater than 5% or 10%, respectively. Steroid levels, determined by RIA, were validated by verifying that serial dilutions were parallel to standard curves. The minimum quantifiable concentrations (MQC) for T and E2 were 0.5 and 0.1 ng/ml, respectively.

Statistical Analysis

Plasma concentrations of T and E2 were normalized using a $\log_{10}$-transformation and values for samples that were below the MQC were substituted with half the MQC (0.25 ng/ml for T, 0.05 ng/ml for E2) (Glass and Gray 2001). Multivariate analysis of variance (MANOVA) procedures were conducted to compare concentration of T and E2 among four different classification groups 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; early and mid-atresia (see previous section Tissue Collection and Processing). 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 variable. 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 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 (Faraway 2006). 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.

Results

Morphological Observations

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 contributed to 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 17th, and two by May 1st (Figure 1.2). 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.) was 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.

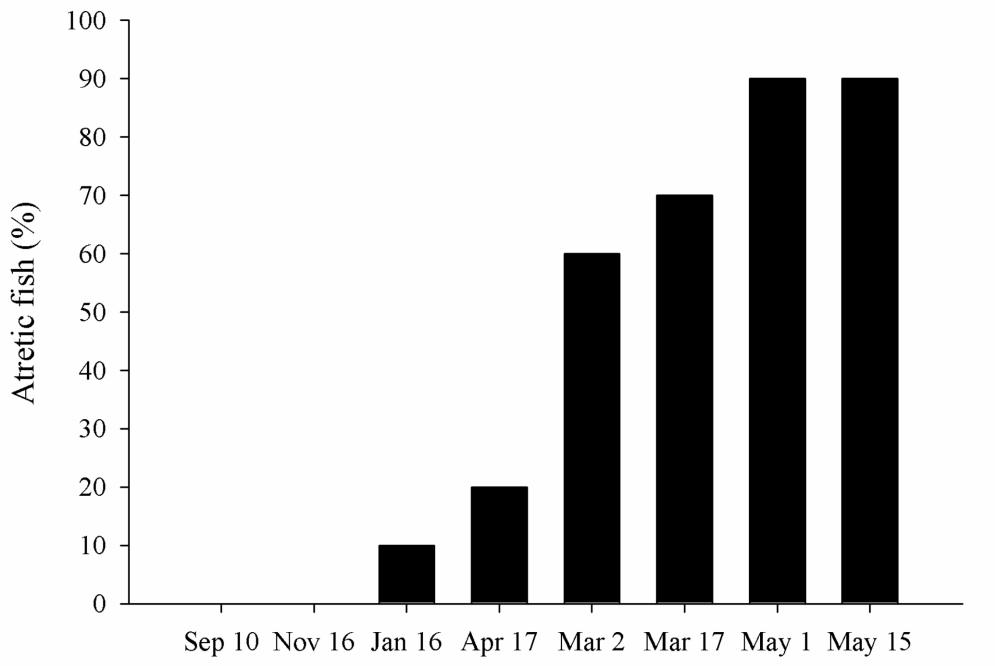

Figure 1.2. Sampling dates and cumulative percent of white sturgeon females with atretic ovaries (N = 10). September 2007 – May 2008.

Steroid Hormones

Mean plasma T and E2 concentrations decreased from 136.2 ng/ml to 43.4 ng/ml, and from 2.2 ng/ml to 1.3 ng/ml, respectively, 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, respectively, 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 fish 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 1.1). 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 1.1). Estradiol concentrations in the VTG 1 group were not significantly different from VTG 2 or either stage of atresia, and there was no significant difference in E2 concentrations between atresia stages.

The stepwise DFA procedure revealed that the model using $\log_{10}$-transformed plasma T concentration data had the greatest discriminative ability to predict ovarian stage (Table 1.2). Overall, 94% of all observations were correctly 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 1.3). Cross validation of the model indicated an error rate of 6%.

Table 1.1. 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 ± SEM (range), N = 9. Different letter superscripts indicate a significant difference between means. 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 1.2. Results from stepwise quadratic discriminant function analysis classifying ovarian stage in cultured white sturgeon females using plasma testosterone (T) or estradiol (E2). Dashes indicate no value was calculated for E2 because it was not a significant parameter.

Parameter	Partial R^2	F-statistic	Wilks' lambda	Average squared canonical correlation
$\log_{10}$ T	0.77	206.0**	0.23**	0.77**
$\log_{10}$ E2	0.03	2.2*	–	–

* denotes $p > 0.10$
** denotes $p < 0.0001$

Table 1.3. 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.

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

Two separate logistic regression models based on either plasma T (Figure 1.3) or E2 concentrations (Figure 1.4) for modeling the probability of a fish having normal ovaries (0 = atretic, 1 = normal, Table 1.4) were significant. The explanatory variables T and E2 were log_{10}-transformed to produce the following regression models:

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

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

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

Table 1.4. 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

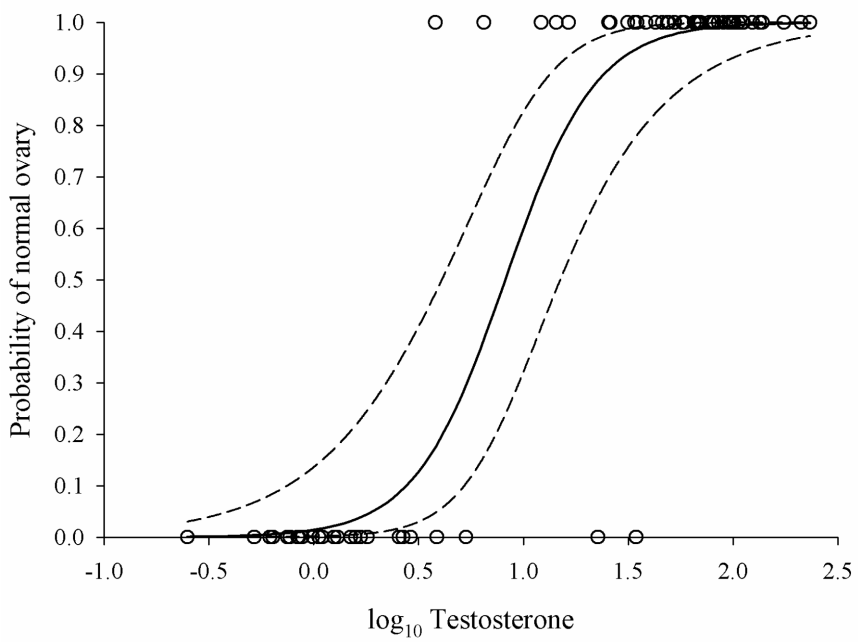

Figure 1.3. Model predicting the probability of a white sturgeon female possessing normal ovaries in relation to plasma testosterone concentrations (log_{10}-transformed). On the y-axis 1 = normal, 0 = atretic. Dashed lines represent 95% confidence intervals. For the model equation, see Eq. 1. N = 74

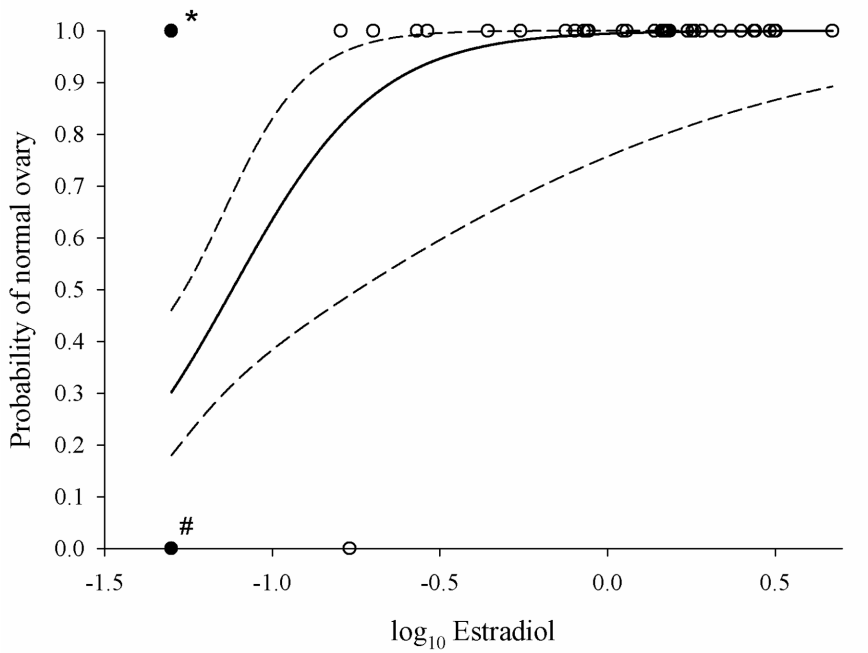

Figure 1.4. Model predicting the probability of a white sturgeon female possessing normal ovaries in relation to $\log_{10}$-transformed estradiol concentrations. On the y-axis 1 = normal, 0 = atretic. Filled circles indicate overlapping data points. Asterisk indicates 12 data points. Pound symbol indicates 27 data points. Dashed lines represent 95% confidence intervals. For the model equation, see Eq. 2. N = 74.

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

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

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

A caviar producer could use Equation 3 to avoid harvesting fish with atretic ovaries by setting a threshold value of T based upon an acceptable probability of harvesting fish with normal ovaries and have an X% certainty that the fish has been properly classified.

Discussion

Both T and E2 are essential for 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 (Patiño and Sullivan 2002). A significant decrease in plasma 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.

Plasma T concentrations are usually higher in late vitellogenic white sturgeon females compared to E2 concentrations (Webb et al. 2001; Webb et al. 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-pitutary-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). 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 minimum

quantifiable concentration 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, were 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 or 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; Malekzadeh 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 an acceptable probability of harvesting fish with normal ovaries is 0.95 and nothing less, equation 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 MQC 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 they deem as an appropriate probability of selecting fish with normal ovaries.

White sturgeon have 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). 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. The possibility of

heterogeneous onset of atresia across ovarian lobes will be further investigated in a future study.

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 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, I am 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.

Measuring plasma concentrations of T and E2 was useful for discerning whether or not white sturgeon females have initiated follicular atresia, which will 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. Using technology that can rapidly determine plasma E2 concentrations may serve as a surrogate for gonadal biopsies currently used to select post-vitellogenic females from the wild for broodstock purposes. The less invasive nature of blood collection may decrease stress due to prolonged handling and eliminate or reduce the risk of infection from biopsies.

CHAPTER 2

INVESTIGATING MORPHOLOGICAL PARAMETERS AND BLOOD PLASMA SEX STEROIDS AT THE TIME OF CAVIAR HARVEST IN CULTURED WHITE STURGEON

Introduction

Humans have utilized sturgeons for thousands of years. Aristotle mentioned the use of sturgeon swim bladders to produce a glue-like substance in 350 B.C. The first documented use of the word caviar has been traced back to Batu Khan, grandson of Ghengis Khan, in a letter from 1240 A.D. describing a meal he was served in a town north of Moscow (Sternin and Doré 1993). Non-pressed, fresh caviar was enjoyed in local Russian markets but could not be distributed widely until railroads were built throughout Europe and refrigeration technology was invented in the early 19th century. In the mid-1800s, traveling Russian aristocrats introduced the European elite to the delicacy of caviar in its freshest form (Carey 2005). Since this time, caviar has been a sought after delicacy throughout the world. The high demand for caviar has lead to the exploitation of many species of sturgeon in the Northern Hemisphere. Overfishing, habitat degradation, pollution, and altered temperature and flow regimes caused by the construction of dams has lead to 17 species of sturgeon being listed as endangered by the International Union for Conservation of Natural Resources (IUCN) (IUCN 2001).

The most prized caviar comes from species of sturgeon found in the Caspian Sea including beluga *Huso huso*, sevruga or stellate *Acipenser stellatus*, and sterlet *Acipenser*

ruthenus. The commercial harvest of these species from the Caspian Sea has declined since its peak in the early 20th century at 39,400 tons to 2,900 tons in 1995 (Ivanov et al. 1999). Spawning populations of beluga sturgeon have declined from 2,600 tons in 1970 to 300 tons in 1995 (Ivanov et al. 1999). This decline prompted the U.S. to list the beluga sturgeon as endangered in 2005 and subsequently impose a ban on the import of caviar from beluga sturgeon in 2006.

In response to the decline in harvest of wild sturgeons and bans placed on imports of wild caviar, there has been greater demand for captive breeding and rearing of sturgeon for caviar production. White sturgeon have been a top choice for farm-raised caviar because of their relatively fast growth rate, large eggs, and high meat yield. In the late 1970s, research at the University of California-Davis was initiated to enhance the effectiveness of propagating white sturgeon and commercial production efforts began in the mid 1980s in California. Since then, white sturgeon have been utilized by the caviar industry worldwide including production in Germany, France, Greece, Italy, Israel, South America, and Canada.

Addressing the variability in ovarian maturation among individual fish may help the caviar industry increase caviar yield and quality and produce a more consistent product. While the ovarian cycle is controlled by environmental factors, such as photoperiod (Moberg et al. 1995), there is significant individual variation in the biological clock of cultured female white sturgeon, *i.e.,* spawning readiness is reached in February to June (Doroshov et al. 1997; Webb et al. 2001). Measurement of the oocyte polarization index (PI) is a commonly used technique to determine follicle maturity and

spawning readiness in sturgeon. Nevertheless, this technique is time consuming and often not a feasible option for large-scale caviar producers.

In California, white sturgeon are reared in warm-water (18-20°C) until the age of 6. In the fall of their 6th year, females are checked for darkly pigmented follicles by ovarian biopsy, which indicates the presence of late vitellogenic ovaries. These vitellogenic females are then transported to a cold-water site (10–13°C) to mimic the natural thermal regime of temperate rivers in which white sturgeon have adapted. In late winter and spring, fish are chosen at random from the cold-water site for caviar harvest. Ovaries are visually assessed for the presence of atresia by ovarian biopsy immediately prior to caviar harvest; follicle maturity, *i.e.,* oocyte PI, is not assessed prior to caviar harvest. This often results in decreasing caviar yield and quality because the stage of maturity is unknown, and it therefore may not be optimal at the time of harvest.

The ideal technique for determining follicle maturity immediately prior to caviar harvest would be quick, accurate, and less invasive than an ovarian biopsy. Measuring blood plasma concentrations of testosterone (T) and 17-β estradiol (E2) is a less-invasive technique that has been used to classify wild white sturgeon females into various stages of maturity (pre-vitellogenic, vitellogenic, post-vitellogenic, post-ovulatory, and atretic) (Webb et al. 2002; Chapter 1). During late vitellogenesis, T and E2 concentrations in sturgeon blood plasma are elevated until ovulation (e.g. Webb et al. 2001; Barannikova et al. 2002b; Semenkova et al. 2002) or onset of atresia (Webb et al. 2001; Barannikova et al. 2002a; Linares-Casenave et al. 2002; Chapter 1).

Measuring blood plasma sex steroid concentrations may be a minimally invasive technique used to determine the optimal time to harvest caviar and therefore enhance the consistency of quality and yield for the caviar industry. Before a rapid blood plasma sex steroid assay technique can be developed, relationships among blood plasma sex steroids, morphological parameters, and caviar quality and yield must be investigated.

Previous research conducted at the University of California-Davis found a negative correlation between caviar yield and oocyte PI (Doroshov and Van Eenennaam 1998). This study was conducted to validate the correlation between caviar yield and oocyte PI, investigate if blood plasma sex steroids were correlated with oocyte PI, and determine if other correlations existed among plasma sex steroid concentrations, morphological characteristics, and caviar yield and quality (firmness, flavor, color).

Contradictory to previous research, there was no correlation observed between oocyte PI and caviar yield in the year class of fish used for this study. Ovarian adipose tissue was found to vary greatly among individual fish and significantly influenced caviar yield. Therefore, fish were classified post-hoc into three groups according to the amount of ovarian adipose tissue present to further investigate differences among these groups and investigate how ovarian adiposity may affect ovarian physiology.

Methods

Animals and Study Sites

Animals were randomly selected from the 6-year old year class of late-vitellogenic white sturgeon females being held at Sterling Caviar's cold-water site,

southeast of Sacramento, CA. Fish were held in 3.7-m diameter, 0.9-m depth outdoor tanks supplied with the surface water from Lake Amador (tank temperature 10–13°C). The day before caviar harvest, fish were transported to the caviar-processing site, located 24 km north of Sacramento, CA, and held overnight in two 9.1-m outdoor concrete tanks at 20–22°C. Prior to this study, fish were fed a pelleted sturgeon diet (EWOS®, Surrey, B.C., Canada) at 0.03–0.09% body weight per day.

Experimental Design

From February through June 2008, 100 white sturgeon females (20 per month) were randomly selected for caviar harvest. Each fish was tagged for individual recognition with a combination of pectoral fin punches and barbel clips.

Tissue Collection and Processing

One day prior to caviar harvest, 6 mL of blood was sampled from the caudal vasculature with a heparinized vacutainer for measurement of plasma T and E2 by radioimmunoassay (see Chapter 1 Methods for radioimmunoassay detail). Fish were transported to the caviar-processing site and held overnight. Fish were euthanized, weighed, total and fork length (FL) measured, condition factor (K) calculated (body weight / FL^3 x 100000), and the ovaries removed and weighed for calculation of gonadal somatic index (GSI) ((gonadal weight/body weight) x 100). Ovaries were screened to remove previtellogenic oocytes, adipose tissue, and somatic tissue. Remaining eggs were salted to create the final caviar product. A final caviar weight was

taken for calculation of 1) caviar yield (kg/fish); 2) caviar yield as a percent ovarian weight (CY%OW), calculated as ((CY/OW) x 100)); and 3) caviar yield as a percent of body weight (CY%BW), calculated as ((CY/BW) x 100).

Prior to screening of ovaries, but after ovarian weight was measured, a sub sample of approximately 30 ovarian follicles was removed from the ovary and fixed in 10% buffered formalin for measurement of oocyte PI and follicle diameter (see Chapter 1 for oocyte PI and follicle diameter measurement description).

A 28-g sample of the final caviar product (salt added) was used to assess egg quality (color, firmness, and flavor) by Sterling Caviar, LLC. All caviar processing occurred in refrigerated rooms (7 to 8°C). Caviar grade was determined based on the following system:

Grade A: firm, large, good tasting eggs

Grade B: firm, small, good tasting eggs

Grade C: not firm, large, good tasting eggs

Grade R: Grades A, B, or C downgraded if chorions were broken or eggs had two colors

Statistical Analysis

The Shapiro-Wilk test for normality was used to check for non-normal distributions in the data of each parameter. Estradiol data was $\log_{10}$-transformed to achieve normality. No other parameters were transformed. Pearson's correlation coefficients were calculated to detect correlations between parameters measured at time

of caviar harvest (body weight, fork length, oocyte PI, follicle diameter, E2, T, ovarian weight, the ratio of plasma E2 to ovarian weight (E2:OW, ng/ml·kg^{-1}), caviar yield, GSI, CY%BW, CY%OW). The accepted significance level was $\alpha = 0.001$. All correlations investigated were analyzed for possible nonlinear relationships by creating quantile plots (Q-Q plots) and plotting fitted values versus residuals to look for patterns indicative of nonlinear relationships. One-way analysis of variance was used to compare each parameter among sample dates.

To find the best multiple linear regression model to explain caviar yield Akaike's Information Criterion (AIC; Akaike 1973) with a small sample size adjustment (AIC_c; Hurvich and Tsai 1989) was calculated for models containing all possible combinations of the independent variables FL, oocyte PI, T, E2 (log_{10}-transformed), follicle diameter, body weight, and ovarian weight. Models were ranked depending on corresponding AIC_c values (rank 1 = lowest AIC_c) and models with Akaike differences (Δ_i; where i = the model rank) greater than 7 were considered to be poorly supported and were not analyzed further (Burnham and Anderson 2002). All models with $\Delta_i < 7$ were evaluated for significance of estimated parameters. All estimated parameters needed to be significant ($p < 0.05$) and multicolinearity (assessed by calculating the variance inflation factor) could not be present for a model to be included in the final list of best possible models. For each model in the final list, Δ_i and evidence ratios (w_1/w_j; where w_1 is the Akaike weight for the highest ranked model and w_j is the Akaike weight for the j^{th} highest ranked model) were calculated. Evidence ratios were interpreted as the likelihood that the highest ranked

model was the best model relative to the j^{th} highest ranked model given that one of the models in the set must be the Kullback-Leibler best model (Kullback and Leibler 1951; Burnham and Anderson 2002).

Fish were separated post-hoc into three different categories based on ovarian fat content to explore if parameters measured differed among ovarian fat categories. Fish were assigned to each category based on the following conditions: high fat (HF) = less than 50% of ovary was used as caviar, medium fat (MF) = 50–65% of ovary was used as caviar, low fat (LF) = greater than 65% of ovary was used as caviar. One-way analysis of variance was used to compare each parameter among ovarian fat categories. Three different analysis of covariance (ANCOVA) models were employed to explore if ovarian adiposity influenced the relationship between E2 and CY%BW, E2 and oocyte PI, or T and oocyte PI. An ANCOVA for T and CY%BW was not explored because there was no significant correlation between T and CY%BW when analyzed by Pearson's correlation coefficient. The interaction term E2 ∗ ovarian fat category was removed from an ANCOVA model if it was not significant at $p > 0.05$.

To detect differences in caviar yield, oocyte PI, follicle diameter, E2, and T between caviar qualities, three stratified (by ovarian fat category), random sub-samples of five fish with grade C caviar were compared by student's t-test to the five fish with grade A+B (lumped) caviar. All statistical analyses were performed using R, version 2.8.1. (R Foundation for Statistical Computing, www.r-project.org).

Results

Ninety-two of the 100 fish sampled in 2008 produced grade C, 4 fish produced grade B, one produced grade A, one produced grade R caviar, and 2 fish had atretic ovaries (eggs not suitable for caviar). No differences in caviar yield, oocyte PI, follicle diameter, E2, or T were found between grade C and grade A+B (lumped) caviar. Data analyses focused on correlations with caviar yield and between morphological and biochemical parameters.

Body weight did not differ among sample dates (Table 2.1). However, follicle diameter increased and oocyte PI (Table 2.1) decreased from February to June, indicating that more fish in April, May, and June had reached full maturity (oocyte PI < 0.10) than in February or March. There was no significant difference in mean caviar yield among sample dates (Table 2.1). Furthermore, there was no correlation between caviar yield and oocyte PI or follicle diameter (Table 2.2). Follicle diameter was negatively correlated with oocyte PI (Figure 2.1).

Table 2.1. Comparison of parameters at each sample date during 2008 white sturgeon caviar harvest. Data are means ± SEM. Different letters indicate significant differences between means within parameters among sample months (BW = body weight, FD = follicle diameter, PI = oocyte polarization index, CY = caviar yield, T = plasma testosterone, E2 = plasma estradiol).

Parameter	Feb	Mar	Apr	May	June
BW (kg)	34.5 ± 1.5 ***a***	34.2 ± 1.5 ***a***	33.6 ± 1.2 ***a***	34.7 ± 1.6 ***a***	33.7 ± 1.63 ***a***
FD (mm)	3.05 ± 0.02 ***ab***	3.02 ± 0.02 ***a***	3.11 ± 0.02 ***bc***	3.17 ± 0.02 ***c***	3.17 ± 0.02 ***c***
PI	0.143 ± 0.012 ***a***	0.120 ± 0.009 ***ab***	0.097 ± 0.008 ***ac***	0.078 ± 0.007 ***c***	0.073 ± 0.006 ***c***
CY (kg)	2.38 ± 0.13 ***a***	2.63 ± 0.20 ***a***	2.62 ± 0.20 ***a***	3.00 ± 0.25 ***a***	2.52 ± 0.16 ***a***
T (ng/ml)	139.3 ± 12.9 ***a***	159.7 ± 9.5 ***ab***	172.2 ± 12.4 ***ab***	187.9 ± 14.1 ***b***	186.0 ± 11.0 ***ab***
E2 (ng/ml)	3.95 ± 0.70 ***a***	5.72 ± 1.40 ***a***	4.62 ± 1.31 ***a***	4.34 ± 1.42 ***a***	2.01 ± 0.26 ***a***

Table 2.2. Pearson's correlation coefficients between parameters measured at caviar harvest from white sturgeon. Bold values indicate significance of $p < 0.001$ (PI = oocyte polarization index, FD = follicle diameter, E2 = plasma estradiol, T = plasma testosterone, BW = body weight, FL = fork length, OW = ovarian weight, CY = caviar yield, GSI = gonadosomatic index, CY%BW = caviar yield as percent body weight, CY%OW = caviar yield as percent ovarian weight).

Parameter	PI	FD	E2	T	BW	FL	OW	CY	GSI	CY%BW	CY%OW
PI	–	**-0.390**	0.173	**-0.369**	-0.063	-0.137	-0.004	0.0008	0.061	-0.0007	-0.082
FD		–	-0.149	0.246	-0.037	0.003	-0.019	0.136	-0.004	-0.168	0.273
E2			–	0.049	-0.004	-0.148	**0.328**	**0.420**	**0.516**	**0.474**	0.147
T				–	0.024	-0.030	0.016	-0.027	-0.017	-0.057	-0.074
BW					–	**0.849**	**0.787**	**0.531**	0.229	-0.077	**-0.377**
FL						–	**0.578**	**0.378**	0.048	-0.144	-0.277
OW							–	**0.830**	**0.770**	**0.423**	-0.195
CY								–	**0.755**	**0.789**	**0.354**
GSI									–	**0.751**	0.039
CY%BW										–	**0.680**
CY%OW											–

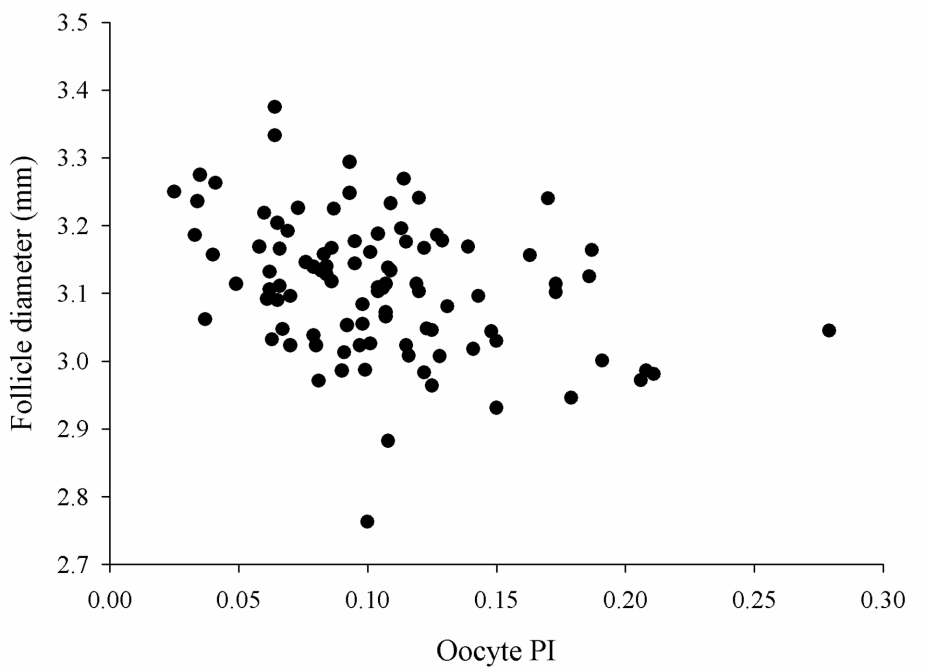

Figure 2.1. Scatter plot of follicle diameter and oocyte polarization index (PI) of white sturgeon at time of caviar harvest, 2008 (r = -0.390, N = 98, $p < 0.001$).

Plasma T increased through time with significant differences between February and May (Table 2.1). Regression analysis revealed a significant, but weak correlation between plasma T and oocyte PI (Table 2.2, Figure 2.2). There was no correlation between plasma T and caviar yield. Plasma E2 did not differ among sample dates and was not correlated with oocyte PI (Table 2.1 and 2.2). Estradiol was positively correlated with caviar yield (Table 2.2, Figure 2.3), CY%BW (Table 2.2, Figure 2.4), and with GSI (Table 2.2).

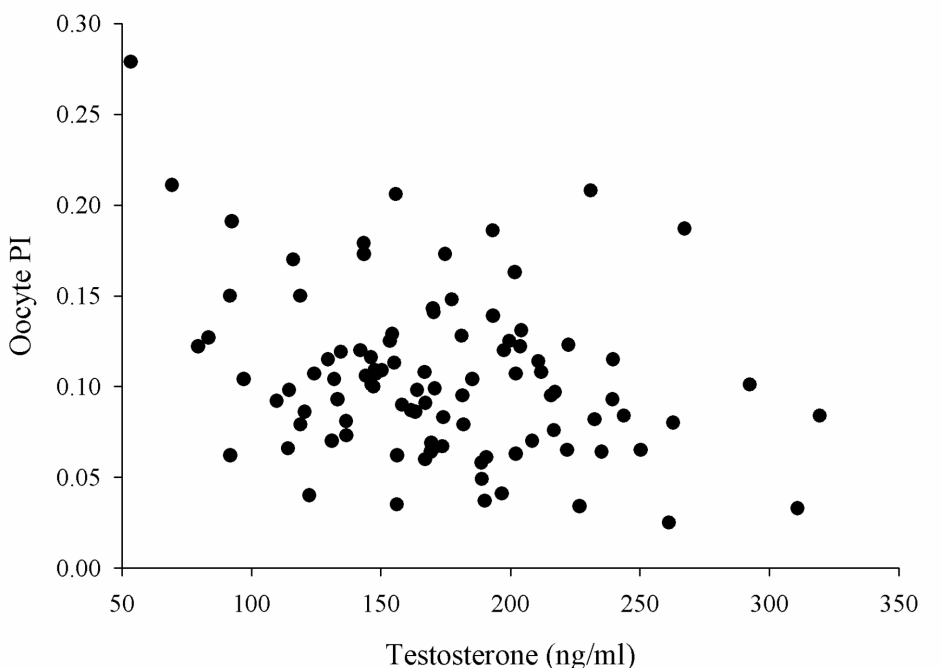

Figure 2.2. Scatter plot of oocyte polarization index (PI) and plasma testosterone of white sturgeon at caviar harvest, 2008 (r = -0.369, N = 98, $p <$ 0.001).

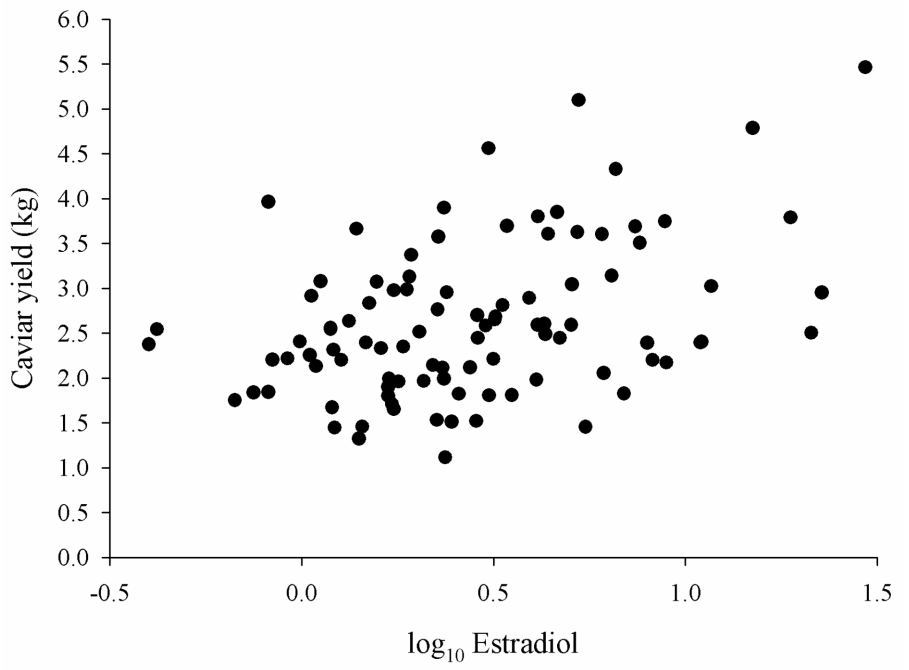

Figure 2.3. Scatter plot of caviar yield and plasma estradiol (log_{10}-transformed) of white sturgeon at caviar harvest, 2008 (r = 0.420, N = 98, $p <$ 0.001).

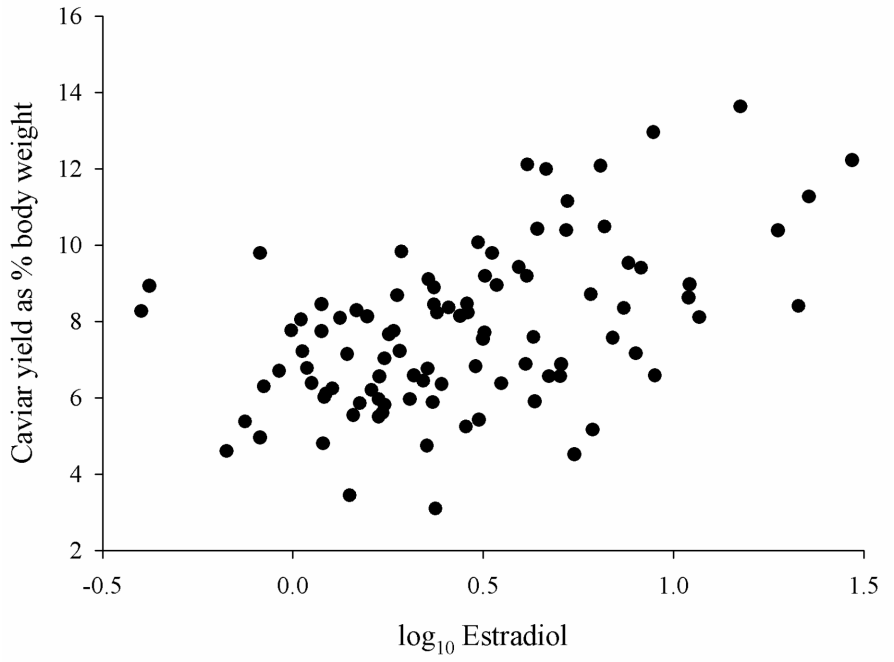

Figure 2.4. Scatter plot of caviar yield as percent body weight and plasma estradiol (log_{10}-transformed) of white sturgeon at caviar harvest, 2008 (r = 0.474, N = 98, $p < 0.001$).

The parameter most correlated with caviar yield was ovarian weight (Table 2.2, Figure 2.5). The model chosen to be the best (lowest AIC_c among models with all parameters significant, no multicolinearity among independent variables) to explain caviar yield included ovarian weight, follicle diameter, body weight, and plasma E2 (log_{10}-transformed) (Table 2.3).

The CY%BW was not correlated with body weight. Furthermore, there was a negative correlation between CY%OW and body weight (Table 2.2, Figure 2.6). The parameter with the greatest correlation to CY%OW was CY%BW (Table 2.2, Figure 2.7). There was a large variation in CY%OW (range, 24–78%) among fish sampled, indicating varying degrees of ovarian adiposity within the same year class.

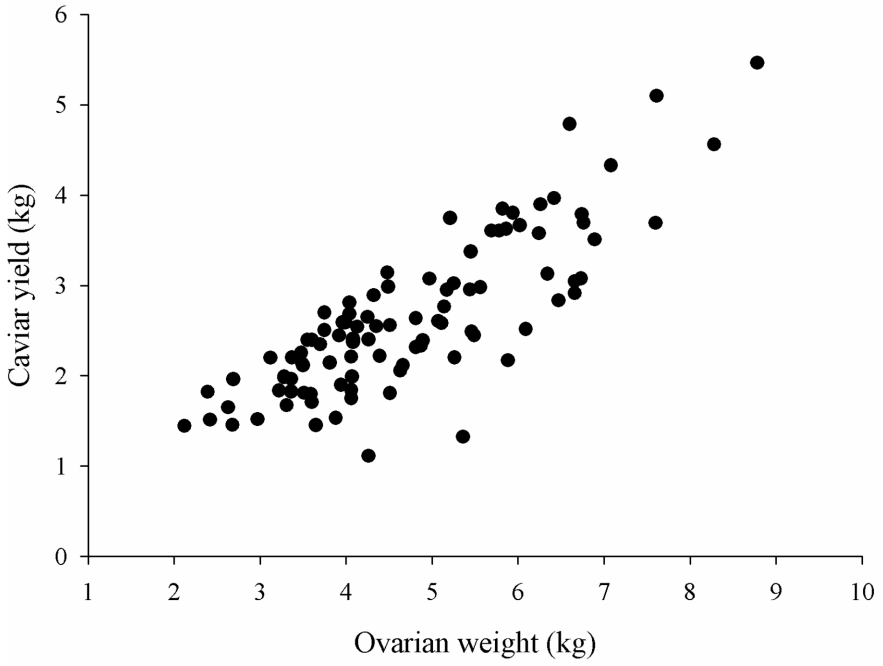

Figure 2.5. Scatter plot of caviar yield and ovarian weight of white sturgeon at time of harvest, 2008 (r = 0.830, N = 98, $p < 0.001$).

Table 2.3. Ranking of multiple linear regression models according to Akaike's information criterion adjusted for small sample size (AIC_c), AIC_c differences (Δ_i), and evidence ratios (w_1/w_j) for three models explaining caviar yield in cultured white sturgeon (BW = body weight, E2 = plasma estradiol (log_{10}-transformed), FD = follicle diameter, OW = ovarian weight), N = 98. Number of estimated parameters (k) also given.

Rank	Independent variables included	k	AIC_c	Δ_i	w_1/w_j
1	E2, FD, BW, OW	5	120.38	0.00	
2	FD, BW, OW	4	121.97	1.59	2.21
3	BW, OW	3	126.46	6.08	20.91

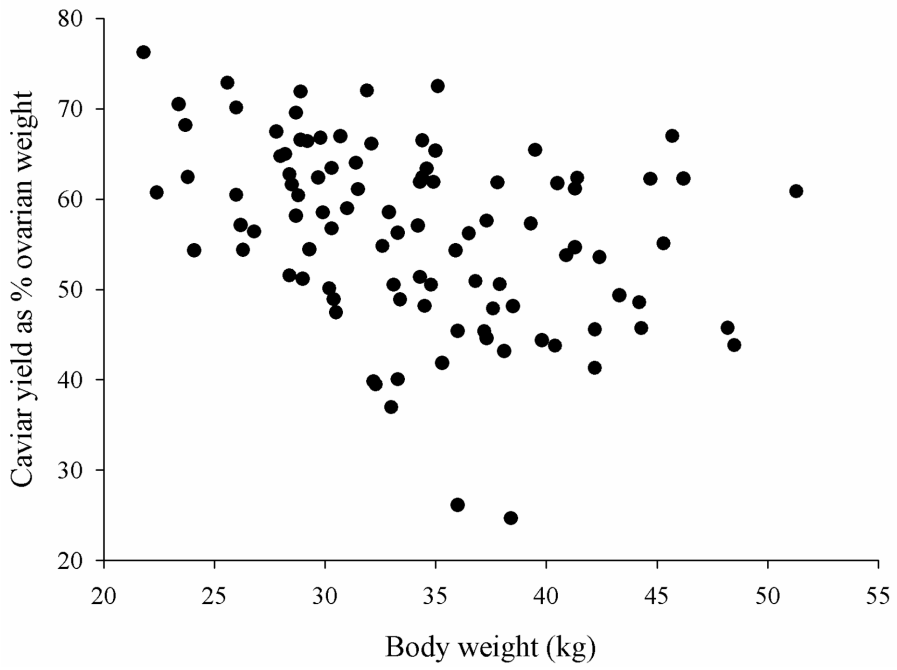

Figure 2.6. Scatter plot of caviar yield as percent ovarian weight and body weight of white sturgeon at time of harvest, 2008 (r = -0.377, N = 98, $p <$ 0.001).

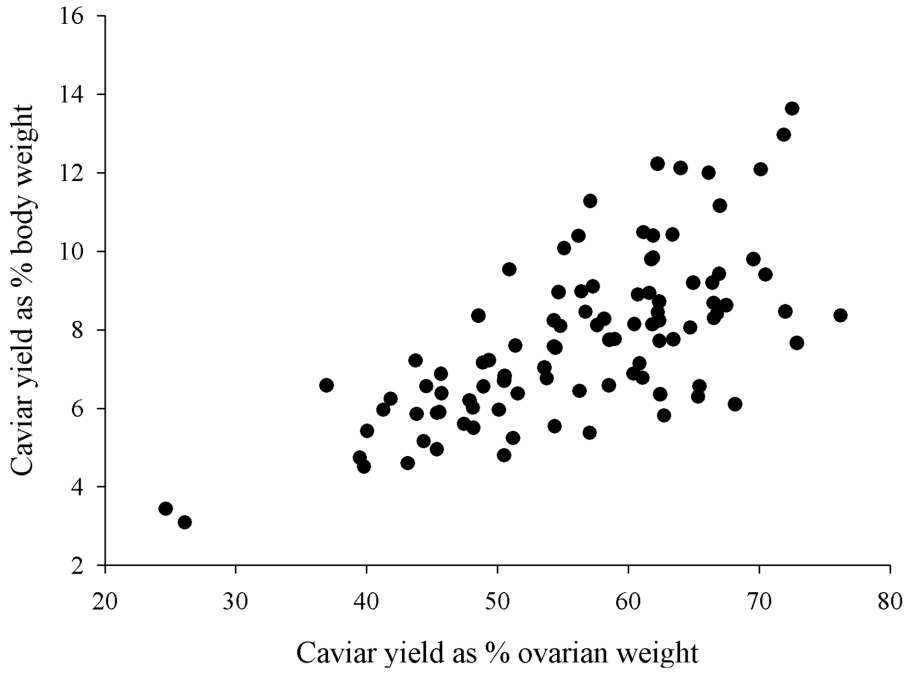

Figure 2.7. Scatter plot of caviar yield as percent body weight and caviar yield as percent ovarian weight of white sturgeon at time of harvest, 2008 (r = 0.680, N = 98, $p < 0.001$).

Ovarian Fat Categories

The MF group had the greatest number of fish (N = 51), followed by the HF group (N = 28) and the LF group (N = 19). Oocyte PI, ovarian weight, and GSI did not differ among ovarian fat categories (Table 2.4). The HF group had the highest mean body weight, smallest follicle diameter, and lowest CY%BW and CY%OW (Table 2.4). The LF group had the lowest mean body weight and K, but produced the highest CY%BW and CY%OW and had larger eggs than the HF group (Table 2.4). The amount of plasma E2:OW was higher in the LF group than the HF group, but E2:OW in the MF group was not significantly different than either the LF or HF groups (Table 2.4).

Table 2.4. Comparison of parameters measured in white sturgeon among ovarian fat categories during 2008 caviar harvest. Data are means ± SEM. Different letters indicate significant differences between means ($p < 0.05$). High Fat, N = 28; Medium Fat, N = 51; Low Fat, N = 19 (BW = body weight, K = condition factor, PI = oocyte polarization index, FD = follicle diameter, T = testosterone, E2 = estradiol, OW = ovarian weight, E2:OW = ratio of estradiol to ovarian weight, GSI = gonadosomatic index, CY = caviar yield, CY %BW = caviar yield as a percentage of body weight, CY%OW = caviar yield as a percentage of ovarian weight).

Parameter	High Fat	Medium Fat	Low Fat
BW, kg	37.6 ± 1.0 ***a***	33.7 ± 0.9 ***b***	30.4 ± 1.3 ***b***
K	1.07 ± 0.017 ***a***	1.07 ± 0.015 ***a***	1.00 ± 0.023 ***b***
PI	0.103 ± 0.008 ***a***	0.103 ± 0.014 ***a***	0.103 ± 0.011 ***a***
FD, mm	3.07 ± 0.018 ***a***	3.12 ± 0.014 ***b***	3.13 ± 0.022 ***b***
T, ng/ml	162.2 ± 9.0 ***a***	184.3 ± 7.7 ***a***	155.4 ± 9.3 ***a***
E2, ng/ml	3.11 ± 0.45 ***a***	4.19 ± 0.78 ***a***	5.73 ± 1.21 ***a***
OW, kg	5.1 ± 0.2 ***a***	4.8 ± 0.2 ***a***	4.2 ± 0.3 ***a***
E2:OW, ng/ml kg^{-1}	0.62 ± 0.09 ***a***	0.83 ± 0.12 ***ab***	1.38 ± 0.30 ***b***
GSI, %	13.5 ± 0.4 ***a***	14.0 ± 0.4 ***a***	13.5 ± 0.7 ***a***
CY, kg	2.2 ± 0.1 ***a***	2.8 ± 0.1 ***b***	2.9 ± 0.2 ***b***
CY%BW	5.8 ± 0.2 ***a***	8.1 ± 0.2 ***b***	9.3 ± 0.5 ***c***
CY%OW	43.1 ± 1.2 ***a***	57.9 ± 0.6 ***b***	69.9 ± 0.7 ***c***

The correlation between CY%BW and plasma E2 did not differ among ovarian fat categories as indicated by the interaction term (E2 ∗ Fat) not being significant in the ANCOVA model A (Table 2.5, Figure 2.8). The data for each fat category was highly scattered causing overlap in the data among all ovarian fat categories (Figure 2.8). The y-intercepts did differ among ovarian fat categories, with the HF group displaying the lowest y-intercept (Table 2.4, Figure 2.8).

Although the relationship between plasma E2 and oocyte PI did differ among fat categories as indicated by the significant interaction term in the ANOVA model B, y-intercepts did not differ among fat categories (Table 2.5, Figure 2.9). The slope of the MF group was not significantly different than 0, which was the cause of the significant interaction term (E2 ∗ Fat). Ovarian fat category did not influence the relationship between oocyte PI and T and slopes were also not different (Table 2.5). Therefore, regression lines were not plotted in Figure 2.10.

Table 2.5. ANCOVA results for A) differences in caviar yield as percent body weight (CY%BW) assessed at different ovarian fat categories (Fat) with plasma estradiol (E2) as the covariate, B) oocyte polarization index (PI) assessed at different ovarian fat categories with plasma E2 as the covariate, and C) oocyte PI assessed at different ovarian fat categories with plasma testosterone (T) as the covariate. N = 98 (sig. = significant, n.s. = not significant). All E2 data is $\log_{10}$-transformed.

Dependent Variable	Source	df	MSE	F Value	p value	Interpretation
A) CY%BW						
	E2	1	98.83	43.86	< 0.001	sig. slope different than 0
	Fat	2	52.80	23.43	< 0.001	sig. intercepts different
	E2 ∗ Fat	2	2.75	1.22	0.300	n.s. slopes not different
B) Oocyte PI						
	E2	1	0.0149	8.30	0.006	sig. slope different than 0
	Fat	2	0.0008	0.44	0.643	n.s. intercepts not different
	E2 ∗ Fat	2	0.0082	4.60	0.013	sig. slopes different
C) Oocyte PI						
	T	1	0.0195	10.23	0.002	sig. slope different than 0
	Fat	2	0.0006	0.35	0.708	n.s. intercepts not different
	T ∗ Fat	2	0.0002	0.10	0.901	n.s. slopes not different

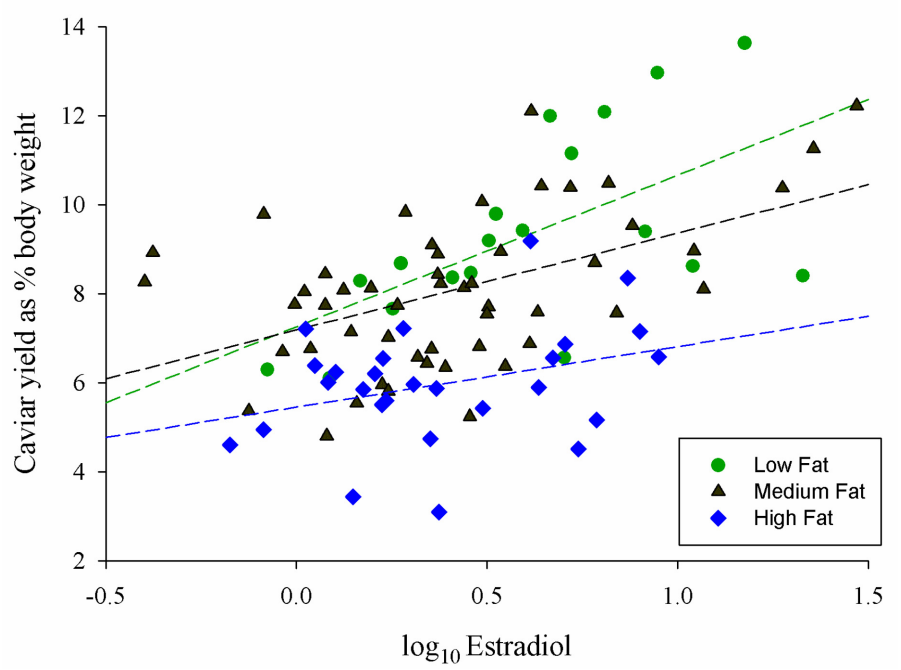

Figure 2.8. Caviar yield as percent body weight plotted relative to plasma estradiol (log_{10}-transformed) among cultured white sturgeon at time of caviar harvest, 2008. Ovarian fat category and linear regressions are indicated for clarity (Low Fat, N = 19; Medium Fat, N = 51; High Fat, N = 28).

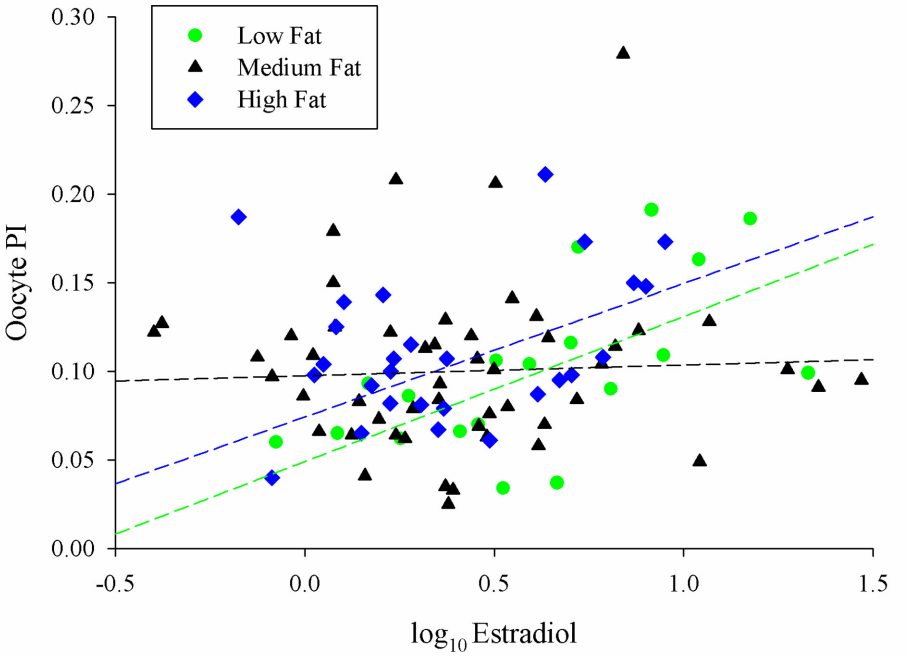

Figure 2.9. Oocyte polarization index (PI) plotted relative to plasma estradiol (log_{10}-transformed) among cultured white sturgeon at time of caviar harvest, 2008. Ovarian fat category and linear regressions are indicated for clarity (Low Fat, N = 19; Medium Fat, N = 51; High Fat, N = 28).

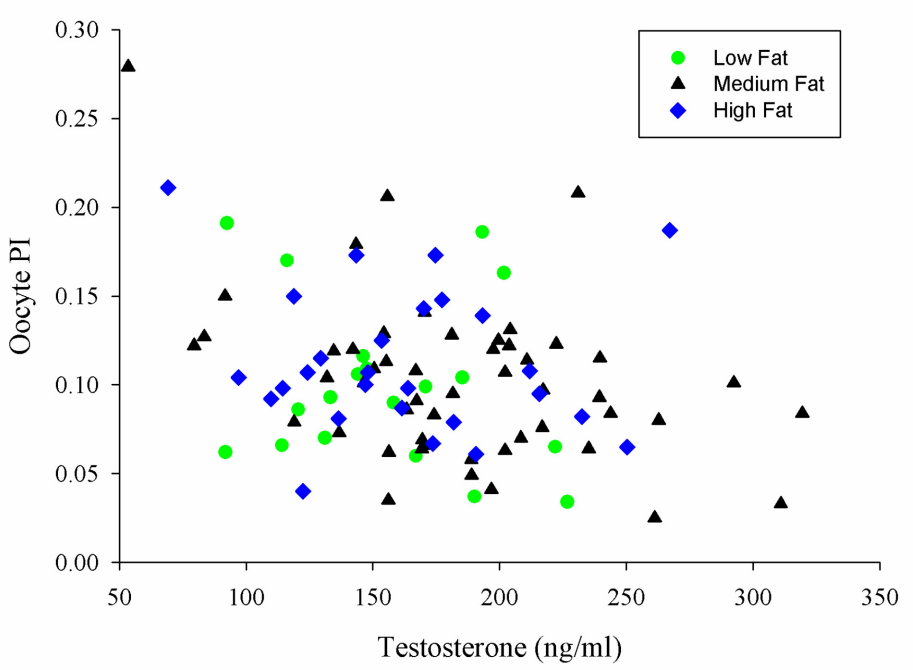

Figure 2.10. Oocyte polarization index (PI) plotted relative to plasma testosterone among cultured white sturgeon at time of caviar harvest, 2008. Ovarian fat category is indicated for clarity (Low Fat, N = 19; Medium Fat, N = 51; High Fat, N = 28).

Discussion

No differences were found between caviar quality grades. However, this analysis was limited by a low samples size of grades other than C because 92% of fish produced grade C, soft caviar. Reduced caviar quality has affected the caviar from the California producers for several years. Scientists at UC Davis are currently investigating possible causes that are leading to reduced caviar quality.

Caviar yield was not correlated with morphological parameters indicative of ovarian maturity. The assumption that oocyte PI is negatively correlated with caviar yield was based on previous broodstock management reports (Doroshov and Van Eenennaam 1998) that showed an increase in caviar yield as oocyte PI decreased. This study tested

that assumption and the idea that if plasma T or E2 were shown to be strongly correlated with oocyte PI, it would provide a tool to identify when to harvest a female fish for the highest yield. Even though oocyte PI decreased and follicle diameter increased in May and June, indicating fish had reached full maturity, there was no difference in caviar yield among harvest months. Furthermore, oocyte PI was not correlated with caviar yield and the correlation between oocyte PI and plasma E2 was influenced by ovarian adiposity in the year class of fish used for this study. This signifies that other factors influenced the yield of caviar to a greater extent than stage of maturity.

Faster growing fish within the same year class did not increase production rate as indicated by a lack of correlation between CY%BW and body weight. In addition, there was a negative correlation between CY%OW and body weight, meaning, in general, that larger fish have fattier gonads. This is further supported by the finding that fish in the HF group had a greater mean BW than fish in the MF or LF group. The positive correlation between CY%OW and CY%BW indicates that fish with less ovarian adipose tissue produce more caviar per kg of body weight than fish with greater ovarian adipose tissue.

The large variation in CY%OW, caused by varying degrees of ovarian adiposity, is the most probable confounding factor leading to a the lack of an observed correlation between ovarian maturity measurements (oocyte PI and follicle diameter) and caviar yield in this study. Many studies have described the presence and deposition of adipose tissue in sturgeon gonads as the fish progress through maturity, but none have documented high adiposity in ripe or spawning females. Flynn and Benfey (2007) described the shortnose sturgeon *Acipenser brevirostrum* gonad as being comprised of a

gametogenic portion and an adipose portion. Adipocytes were present 72 days post hatch in shortnose sturgeon and were located medially along the gonad as opposed to the lateral position of germinal epithelium (Flynn and Benfey 2007). In Adriatic sturgeon *Acipenser naccarii*, Grandi and Chicca (2008) observed a similar medial formation of adipose tissue and lateral formation of germinal tissue in the ovary. The gonads of wild immature female shovelnose sturgeon *Scaphirhynchus platorynchus* possessed large amounts of adipose tissue, which gradually decreased with increasing ovarian maturity, with little or no adipose tissue present in spawning females (Colombo et al. 2007; Wildhaber et al. 2007). Hurvitz et al. (2007) also documented absorption of adipose tissue when ovarian maturity transitioned from vitellogenesis to post-vitellogenesis in Russian sturgeon *Acipenser gueldenstaedtii*. A study conducted on European sea bass *Dicentrarchus labrax* concluded that the there was high activity of lipoprotein lipase in the ovary during vitellogenesis and a concomitant gross reduction of the adipose tissue mass during the progression from early vitellogenesis until spawning (Ibáñez et al. 2008). These studies have hypothesized that ovarian fat is an important energy source for gonadal development.

The role of ovarian fat has also been studied in paddlefish *Polyodon spathula*. Scarnecchia et al. (2007) observed large gonadal fat bodies (GFBs) in ripe paddlefish ovaries in young spawning females (ages 15–18 years). These GFBs are absorbed over time as the fish age (ages >24 years). Gonadal fat body stores have been hypothesized to be important, steady energy stores for paddlefish that live in severe climates with long

winter seasons that provide little or no food. Variation in GFB size among same aged paddlefish may be due to widely varying feeding conditions (Scarnecchia et al. 2007).

It is unlikely that the large variation in ovarian fat observed in cultured white sturgeon is due to varying amounts of food availability. However, it is possible that energy rich commercially produced diets can cause an energy imbalance, contributing to the high adiposity observed in cultured sturgeon. Differing genotypes have been attributed to varying degrees of obesity in many mammalian species (Taylor and Poston 2006) as well as rainbow trout *Onchorhynchus mykiss* (Quillet et al. 2005, 2007) and may be a factor in ovarian fat deposition in sturgeon.

Although the weight of the adipose tissue associated with the ovaries was not directly measured in this study, the relative amount of fatty tissue was estimated by calculating the percent of ovarian weight that was not used as caviar. The fish in the LF group were the most efficient producers of caviar, with the highest CY%OW and CY%BW, as well as the largest follicle diameter. Fish in the LF and MF groups weighed less than the fish in the HF group. It is possible that bigger fish were more metabolically efficient than smaller fish. They not only grew faster but also stored extra energy as ovarian adipose tissue. It is also possible that fish with less ovarian adipose tissue have larger ovarian follicles than fish with greater ovarian adipose tissue because more energy is focused on yolk production rather than adipose tissue production.

Fish size has been shown to be partially genetically determined in stellate sturgeon (Riabova et al. 2006) and pink salmon *Oncorhynchus gorbuscha* (Beacham and Murray 1988; Smoker et al. 1994). Ovarian adiposity may also be partially genetically

determined because it is correlated with fish size. Further research investigating the cause of high adiposity in post vitellogenic white sturgeon ovaries is needed because ovarian fat content has a significant effect on caviar yield.

Ovarian adiposity may also effect how T and E2 regulate 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 aromatase 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 developing ovarian follicles. It is then incorporated into the oocyte via receptor-mediated endocytosis, where it is proteolytically cleaved into yolk proteins (Patiño and Sullivan 2002). An endocrine shift occurs as vitellogenesis is terminated and oocyte maturation begins. This is associated with a switch in the ovarian follicle steroidogenic pathway from the production of E2 to the production of the maturation-inducing steroid (MIS) needed for the resumption of meiosis (Nagahama and Yamashita 2008). In this study, fish in the LF and HF groups with low oocyte PIs (> 0.10), signaling the completion of vitellogenesis and potential for maturational competence (MC), had lower plasma E2 concentrations than fish in the MF group. It is plausible that these fish had reduced plasma E2 concentrations because they were closer to the steroidogenic transition from estrogens to progestins than fish with greater oocyte PI measurements. The reduction in plasma E2 prior to MC has previously been observed in sturgeons (Barannikova et al. 2004; Mojazi Amiri et al. 1996).

The relationship between oocyte PI and plasma E2 did differ among ovarian fat categories. However, the reason for this is unclear. The LF group and HF group showed a similar correlation (slope) between oocyte PI and plasma E2, whereas the MF group was different from either the LF or the HF group. This seems counterintuitive, as it would be expected that the LF group would be more similar to the MF group than the HF group. Furthermore, ovarian fat did not influence the relationship between oocyte PI and plasma T or between CY%BW and plasma E2. One possible explanation for this is that the designation of ovarian fat categories was subjective and was not biologically relevant. In the future it, may be more prudent to designate only two ovarian fat categories, fish with high ovarian fat and fish with low ovarian fat that resemble the ovarian fat content of wild sturgeon. A second explanation may be that the scatter in the plasma sex steroid, oocyte PI, and CY%BW data is too great to make meaningful conclusions. It is difficult to explain the large variation in plasma T and E2 concentrations in fish with similar oocyte PIs, but one factor may be genetic differences.

Estradiol has been shown in numerous studies (Crim and Idler 1978; Fujita et al. 2005; Haukenes et al. 2008; Shultz 1984) to increase with increasing GSI. However, these studies include fish in the pre-vitellogenic stage as well as the vitellogenic and post-vitellogenic stages. To the best of my knowledge, no other studies have shown a positive relationship between E2 and GSI or between E2 and CY%BW within post-vitellogenic females. A study conducted on plainfin midshipman (*Porichthys notatus*) found that, in general, females with the largest body mass also had the largest ovaries and the highest

levels of plasma E2 and T during the pre-nesting period (Sisneros et al. 2004). Nevertheless, E2 and GSI were not correlated among ripe female plainfin midshipman.

A possible explanation for the relationship between plasma E2 and CY%BW may be as simple as proportionality; more E2 is needed to maintain larger ovaries that contain greater number of follicles in fish of the same size. It is possible that fat surrounding the follicles may decrease the number of potential follicles an ovary can produce simply by displacement. A reduction in the number of follicles in turn reduces the amount of E2 produced, causing the fish in the HF group to produce less E2 per kg of ovary than the LF group. These findings suggest that ovarian fat interferes with a consistent relationship between E2 production and number of follicles per kg of body weight, as well as reducing the amount of E2 produced per kg of ovary.

Two regions of fatty tissue were observed in the ovaries of sampled fish: the clusters of fat surrounding follicles in the germinal part of the ovary and the more significant, discrete portions of fat attached along the medial sides of the ovaries. Unfortunately, the location of the majority of ovarian fat in each fish was not recorded. It would be worth investigating if differences occur between the two types of ovarian fat deposition (germinal clusters or medial) in regards to morphological and plasma sex steroid parameters in a future study.

It may be possible for sturgeon conservation managers to use the relationship between plasma E2 and CY%BW to estimate fecundity in ripe female wild white sturgeon following validation of this observation. Caviar yield would need to be converted to number of eggs per kg, an estimate of fecundity. The correlation between

plasma E2 and CY%BW may potentially be stronger among wild fish because high ovarian adiposity in late-vitellogenic ovaries is not as commonly observed as is in cultured fish. Estimates of fecundity are an important component of fish population estimates and monitoring fish population structure over time. Using concentrations of plasma E2 may be a non-lethal, minimally invasive alternative for estimating fecundity in endangered sturgeon species where estimation of fecundity by lethal methods is not possible.

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