Today's Date: 11/29/2010. This is the Annual Final Report
Report for FY begin (mm/dd/yyyy): 02/01/2009, end: 10/31/2010

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Project Information
CASG Project No. R/AQ-129
NOAA Grant No. NA04OAR4170038
Actual start date: 02/01/2009
Planned completion date (including extensions); 10/31/2010
Project Title; Soft Egg Syndrome in Farmed White Sturgeon

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Project Hypotheses:
In recent years, aquatic farms raising sturgeon for caviar have experienced an increased ratio of fish with "soft eggs", which have to be sold as an inferior quality product.
Hypotheses: We hypothesize that soft egg syndrome is affected by recent changes in husbandry practices. We will test effects of culture conditions at two rearing sites, two sturgeon diets, and management stress associated with handling and movement of fish between facilities.

The cause of soft eggs is not in the post-harvest processing of caviar, but apparently relates to physiological problems in the oocyte and/or ovary during final maturation, and particularly during egg envelope formation and oocyte maturation.

Project Goals and Objectives: (Figure could not attached to the WEB site)
The long term goal of this research is to increase high quality sturgeon caviar production.
The specific goals of this project are to characterize the molecular structure of the egg envelope from their synthesis and their components' gene expressions, to understand what causes soft eggs, what causes induction of soft egg production during oogenesis, and how to prevent soft egg production in farm reared sturgeon.

Projective Objective (2009-2010) and design:
The project objective are to elucidate the effects of different culture conditions (rearing site, diet, and fish movement) on egg texture, incidence of follicular atresia, and changes in fish weights and plasma sex steroid levels by characterizing and comparing the biochemical composition of the egg envelope and egg lysate between soft and firm eggs; and developing immunoassays for localization of egg envelope proteins.

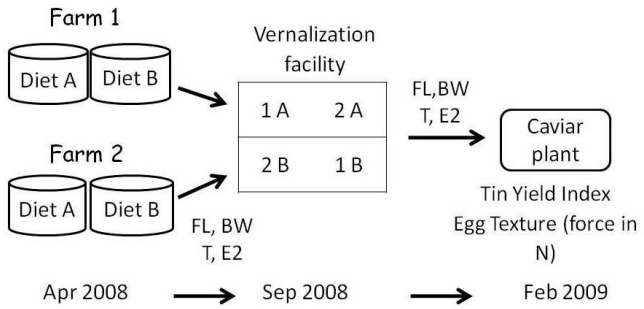


Figure 1. Experimental design

The experiment was designed as a 2 * 2 factorial, with the two farms and two commercial sturgeon diets (modified Atlantic salmon and rainbow trout diets) (Fig.1). From April 2008 to October 2008, two randomly chosen production tanks were fed two different diets on each farm. All the fish were then relocated to a common cold water facility for vernalization, and 23 randomly chosen females from each tank were individually tagged. In the vernalization facility, fish were held in 2 communal tanks and fed using the continuing dietary treatment until March 2009, before caviar harvest. The temperature in each farm and the cold water facility during the experimental period were recorded by temperature probes.
The 23 tagged fish were sampled for blood, body weight and fork length before the transfer to the cold water facility (September 23rd, 25th, October 1st, and 2nd in 2008), and were repeatedly sampled before the caviar harvest (Feb 23^{rd,} 25th, Mar 2nd and 5th in 2009) (Fig.1). At caviar harvest, tin yield and screened yield of all tagged fish were recorded, and egg texture was measured using a Texture Analyzer (Stable Micro System). Ovarian follicles were collected from these tagged females and fixed in a buffered formalin for the Polarization Index (PI: relative distance of germinal vesicle from the animal pole) measurement and histology work.

Briefly describe project methodology:
Egg texture analysis: Firmness (Texture) of the individual fresh eggs were evaluated on-site immediately after separation of the eggs from the ovarian tissue using egg burst force (Newton) measured by a TA.XTPlus Texture Analyzer (Stable Micro System).

Egg size, stage of development, and yield of caviar: Egg was determined by measuring the diameter of 20 eggs and the average weight of an egg in three subsamples. The development stage was evaluated using an egg polarization index (PI) as previously described (Webb et al., 2001). The caviar yield was determined as a total weight of separated eggs divided by fish weight before slaughter.

Fish weight and steroid hormones: Individual weight (+/- 0.5kg) and plasma sex steroids were measured at the warm water sites before transport to the coldwater facility, and at the coldwater facility before transport to the caviar plant. Blood samples were collected with a vacutainer from the caudal vasculature. The plasma testosterone and estradiol concentration were measured by radioimmunoassay in Dr. Webb's laboratory. The measurement used followed the method of Fitzpatrick et al (1986). Intra and interassay variation were less than 5% and 10%, respectively. Detection limit of the assay was 1 ng/ml for testosterone and 0.2 ng/ml for estradiol.

Statistical Analysis: The diameter and PI of eggs were measured using an Image Analyzer in the Aquatic Center at UC Davis. Fifteen eggs were measured from each fish, and all fish were measured in four treatments. Then, the obtained data were analyzed using SAS 9.2 version GLM and MIXED procedures. For Fork length, body weight, condition factor K, and steroid levels (testosterone and estradiol), MIXED procedure with repeated measurement were applied:

$$Y = Diet_i + Farm_j + Diet*Farm_{ij} + Time_k + Fish_{ijkl} + e$$

For egg diameter, PI, egg texture, screen yield, tin yield, screened yield index, and tin yield index, GLM procedure with fixed factors was applied:

$$Y = Diet_i + Farm_j + Diet*Farm_{ij} + error$$

Histological analysis of the egg envelope (Follicular atresia): The incidence of follicular atresia was evaluated by histological observations. Fragments from different sites of both ovaries, prior to separation of fully grown eggs, were fixed in 10% buffered formalin, dehydrated in ethanol series, embedded and sectioned at 4-5 µm in paraffin, stained by hematoxylin & eosin (H &E) and periodic acid Schiff (PAS) stain, and examined under an Olympus compound scope with high resolution objectives. The diagnostic characters and stages of follicular atresia described previously for cultured white sturgeon during late vitellogenesis (Linares et al., 2002) was used. Observations were expressed as proportions of fish with different stages of atresia in each treatment. The one female that had an advanced stage of ovarian atresia at harvest was detectable macroscopically.

SDS-PAGE and Mass Spectrometry of the egg envelope: To clarify the components of the sturgeon egg envelope, SDS-PAGE was performed with the egg envelopes of approximately 30 eggs. Each protein band stained with the Coomassie Brilliant Blue (CBB) R-205 was manually excised using a sterile razor blade, minced well, and collected in a single tube for mass spectrometry analysis and polyclonal antibody production. To assist the cDNA cloning, minced SDS-PAGE gel of the sturgeon egg envelope was digested using sequencing-grade trypsin (Promega) and spotted onto a matrix-assisted laser desorption ionization (MALDI) target. The molecular weights of the tryptic fragments were measured by MALDI peptide fingerprint mass spectra acquired with a time-of-flight (TOF) instrument (MALDI-R, Micromass-Waters). MA Peptide sequencing by MS/MS was accomplished with a nanoflow HPLC interfaced to electrospray ionization (ESI)-ion trap mass spectrometer (LC-MSD Trap, Agilent Technologies). Peptides separated by HPLC underwent fragmentation in the tandem mass spectrometer, and the resulting MS/MS spectrum were used to determine the peptide sequence at the UC Davis Genome Center, Proteomics Core Facility. The MS data from both the tandem mass spectra from the LC-MS/MS experiments and the MALDI-MS peptide fingerprint mass spectra were searched against a subset of protein sequences in either the SWISS-PROT database or the non-redundant NCBI database, using the search program Mascot (Matrix Science, London, UK) or Spectrum Mill (Agilent Technologies). Due to species differences in homologous protein AA sequences, there necessarily are lower identity matches of some peptide sequences.

Describe progress and accomplishments toward meeting goals and objectives:

Please treat the data included in the progress report as confidential, because we are currently writing a paper using those data.

1. The temperature change in two farms and cold water facility:

From April 2008 to October 2008, the temperature of farm2 ranged between 17-27°C, while farm1 temperature ranged from19-22°C.

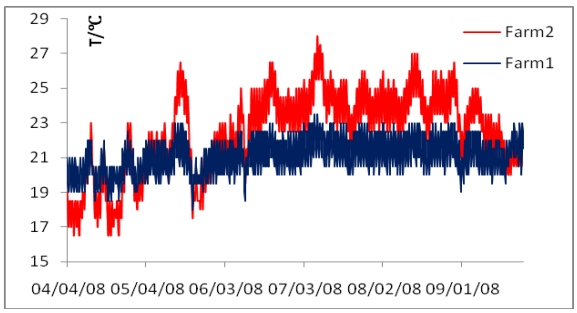


Figure 2. Temperature ranges in the two farms during the experimental period

Farm2 had greater fluctuation of dial temperature than farm1 during this time period. During summer (June to September), the average temperature on farm2 was about 23.6°C higher than on

farm1 (figure 2). In the vernalization facility, temperature was 12 ± 3°C.

2. Body weight: In each experimental group, change of the body weight was measured in the warm facility just before transferring to the cold facility and before harvesting in the cold facility (Fig.3).

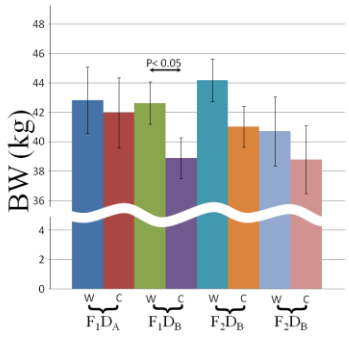
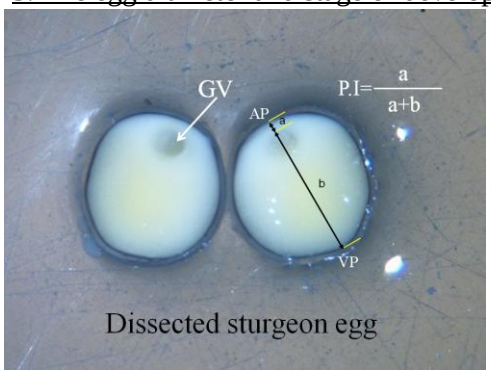


Figure 3. The diet effect on the body weight in the cold facility. F_1: farm1, F_2: farm2, D_A: diet A, D_B: diet B, student T-test was used for statistical analysis.

Interestingly, the fish fed Diet B in farm1 lost significant weight while held in the cold facility. This result suggests that Diet B is a source of less energy than Diet A, or in the cold facility, the accumulated energy source from the Diet B in the warm facility was metabolized faster in the cold facility than that of Diet A. In farm2, the same tendency was observed; however, there was no significantly different effect on the body weight between Diet A and B. The environmental effects, such as temperature, quality of water, or density of the fish held in the tank, may have had an effect on their metabolism, and have hidden the effect of diet.

3. The egg diameter and stage of development, Polarization Index (PI):



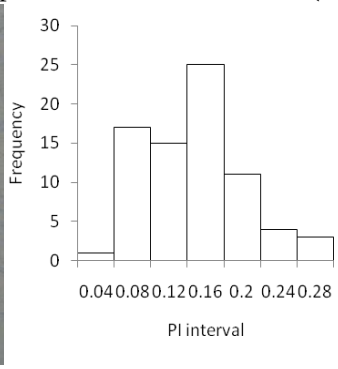


Figure 4. Dissected sturgeon egg for P.I. measurement and frequency distribution of PI (N =76). GV; germinal vesicle, AP; animal pole, VP; vegetal pole

The mean diameter of the egg, PI, and information of other indices obtained from this experiment are summarized in table 1. The frequency distribution of the PI is shown in Figure 3. The polarization index (PI) is a measure of the position of the germinal vesicle (GV), or nucleus, in relation to the animal pole. It is used to determine the degree of oocyte ripeness in sturgeon. Optimally, to optimize yield and quality, females would be sacrificed for caviar when their oocyte PI was 0.10-0.12. As showed in the histograms, PIs in most eggs were between 0.08 and 0.20

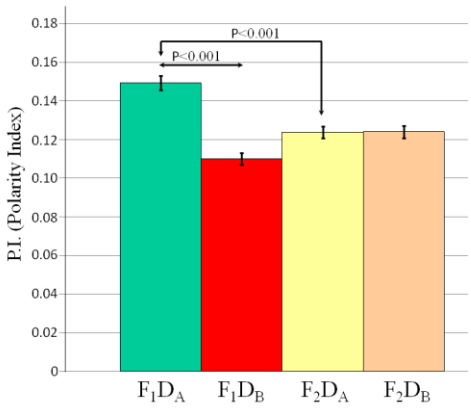


Figure 5. Comparison of the mean value of P.I. obtained the fish treated differently in the warm facility described in Fig. 1. F_1; firm1, F_2, firm2, D_A, diet A, D_B; diet B. A Student T-test was used for statistical analysis.

To identify the effects of diet on the maturation of sturgeon, the mean value of PI obtained from fish treated differently in the warm water facility described in Fig.1 was compared and analyzed using the student T-test (Fig.5). The PIs of the eggs of fish fed Diet A at farm1 was higher than that in fish fed Diet B at farm 1. This result suggests that fish fed Diet B at farm 1, combined with other environmental factors, may have influenced maturation in farm1. Interestingly, this effect was not observed in the fish grown in farm2. It is possible that environment effects present in farm 2 may have hidden the effects of Diet on fish maturation. Of course, the maturation process continues to progress in the cold facility, and the speed of maturation in individual fish may differ in the cold facility. These individual differences make caviar production difficult to control in terms of unifying egg quality. However, the ability to develop tools to determine the timing of ripeness would be beneficial to increase yield and quality of caviar, reduce the number of females undergoing atresia, and to increase the efficiency of the caviar industry.

Application of ultrasound techniques is another good option; however this technology has not progressed far enough to accurately determine egg ripeness,

4. Egg firmness (Texture): Immediately following the extraction of the ovarian mass, the firmness of individual eggs were measured. As the first step, the firmness of the eggs in the different treatments in the warm water facilities were analyzed separately and compared using the student T-Test. The eggs obtained from the fish fed Diet A in both farms showed a significantly higher level of firmness (Fig.6). A comparison of the firmness of eggs from fish fed Diet A, but grown at farm2 did not show any significant difference.

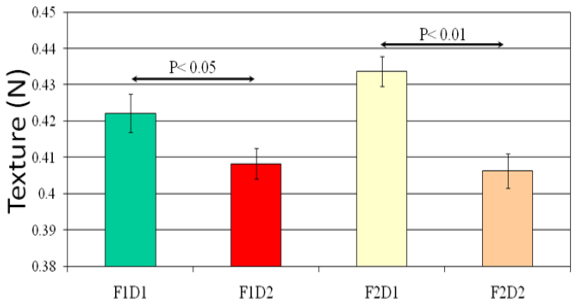


Figure 6. The firmness (texture) of the eggs obtained the fish treated differently in the warm facility described in Fig. 1. F_1: farm1, F_2: farm2, D_A; diet A, D_B: diet B. The number in the left side shows burst force (Newton). A Student T-test was used for statistical analysis.

This result suggest that firmness may depend on the nutrients found in a particular diet fed for 9 month before harvesting, rather than other effects derived from the environment factors found in the two farms. However, our concern is not only with the individual difference among the fish, but also among the individual difference present in each egg of the same fish. To understand and solve soft egg syndrome, it is crucial to identify the molecular mechanisms of egg envelope formation during the oogenesis that is derived from their gene expressions during assemblage of the egg envelope architecture.

5. Steroid Hormones: Changes in the estrogen and testosterone levels measured before vernalization and before harvest of fish treated with different diets and on different farms are described in Fig.1.

Estrogen (E_2): In farm1, the diet produced no significant difference on the estrogen level of the blood plasma.

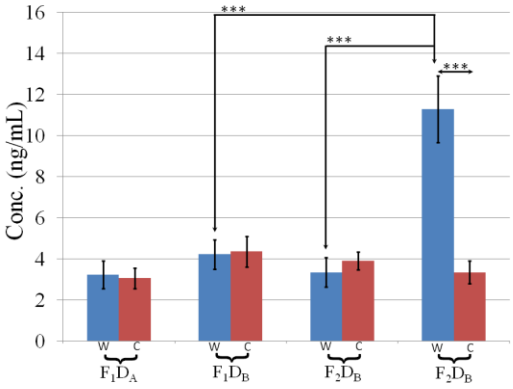


Figure 7. Change of the estrogen level in the blood plasma before and after the vernalization. F_1: farm1, F_2: farm2, D_A; diet A, D_B: diet B. ***: P< 0.001.

The estrogen level of fish grown on farm 1 was not affected during vernalization. Fish grown on farm 2 and fed diet B exhibited estrogen levels that were three times higher in the plasma, but dramatically decreased its level during vernalization. It is difficult to explain why the farm 2, diet B fish showed higher estrogen level in their blood plasma. The high estrogen level only appears in diet B fish; however, we might expect the same observation for farm1. If estrogen is affected only by environmental factors in farm2, the same phenomena should be observed in the fish grown in the same farm2 and fed with diet A. This did not occur. This effect may be caused by not only one factor, but by complex factors such as the influence of diet B and the farms environment effect.

Testosteron (T): Most fish, except the fish grown in the farm2 with Diet A, showed a significant decrease of the T level in the blood plasma during vernalization (Fig.8). This indicates that the lower temperature may directly affect the T level in the blood plasma. Before vernalization, the T level in the fish grown on farm2 was significantly higher than fish in farm1. The drop ratio of the T level, before and after the vernalization, between fish grown on farm1 and those on farm 2 was 30-40% and 10-20% respectively. On both farms, the diets had no effect on the T level in the blood plasma. These results suggested that some environmental factors in farm1 had an influence in maintaining relatively higher levels of T in the blood plasma. Temperature differences are the major environmental difference between these two study farms. Farm 1 exhibited higher temperatures and greater daily and seasonal temperature fluctuations, than those observed on farm 2.

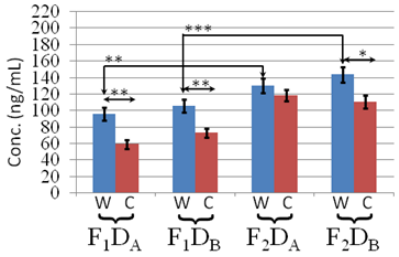


Figure 8. Change of the testosterone level in the blood plasma before and after the vernalization. F_1: farm1, F_2: farm2, D_A; diet A, D_B: diet B. W; warm facility, C; cold facility, *; P< 0.05, **; P< 0.01, ***: P< 0.001.

Further study is necessary to identify the relation between the plasma T level and environmental factors.

In this experiment, the differences in water temperature between the two farms and two different diets showed significant effects on the firmness of the egg, PI and steroid hormones level in the blood plasma. In addition, during vernalization, the fish lost 6% of their weight. One possible reason might be that management stress caused the fish to consume more energy than before, or they possibly stopped, or reduced food consumption during oogenesis.

6. Histological analysis. Histological analysis detected only one case of advanced follicular atresia due to the treatment Diet A × Farm 1. Plasma E_2 concentration in this fish was below the detection limit (< 0.2 ng/ml) and plasma T concentration was also very low (2.64 ng/ml). The normal, non-atretic female shows three striated layers of chorion and evenly spaced cells making up the granulose layer (Fig. 9A). The chorion, basal lamina, and cortical granules in the cytoplasm cortex are stained PAS-positive, but are not stained in the control slide (Fig. 9B). In fish with follicular atresia, the chorion, granulosa layer, cortical alveoli, melanin granules, and yolk platelets are degraded. The dark pigment (product of lipid peroxidation) are aggregated in the ovular space (Fig. 9C, D). The thecal layer is heavily vascularized and exhibits an increase in thickness. The PAS-positive basal lamina is still present at this advanced stage of atresia (Fig. 9D).

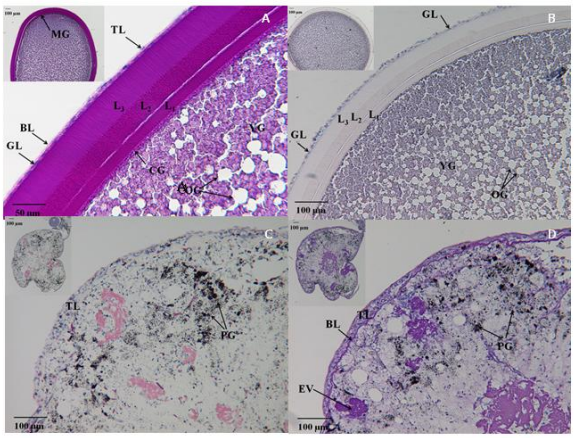


Figure 9. Photomicrographs of histology of ovarian follicles. A: PAS staining of the ovarian follicle. B: PAS control staining. C: H&E staining of late follicular ovarian atresia. D: PAS staining of late follicular ovarian atresia. BL: basal lamina; CG: cortical granules; EV: egg envelope; GL: granulosa cell layer; MG: mlanin granules; OG: oil globules; PG: pigment granules; TL: thecal cell layer; YG: yolk globules.

6. The profiling of egg envelope glycoproteins in each fish: To identify the character of the egg envelope glycoproteins, we used SDS-PAGE on separate samples, using the egg envelope sample obtained from individual fish. Figure 10. represents the common SDS-PAGE pattern of the egg envelope of the fish obtained from each treatment shown in Fig.1. Following our MS/MS analysis, the peptide sequence obtained from the EV3 was matched to that of zona pellucida 3 (ZP3) in zebrafish, gold fish and carp. So EV3 was identified as the sturgeon ZP3. Following the Mass Spectrometric analysis, we are now trying to identify which components of the egg envelope are missing or modified in the soft egg.

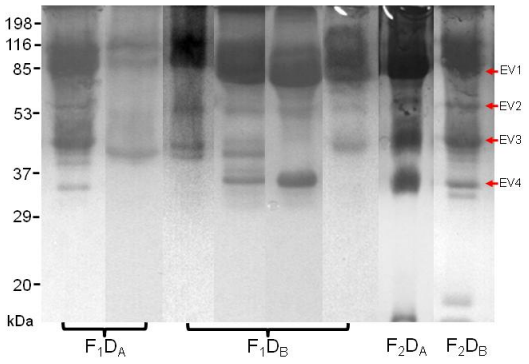


Figure 10. the SDS-PAGE pattern of the egg envelope components of the fish obtained from each treatment shown in Fig.1. The numbers on the left side show the position of the molecular marker. Arrows in right hand side shows the position of the sturgeon egg envelope components. F_1; farm1, F_2: farm 2, D_A; diet A, D_B; diet B shown in Fig.1.

Figure 11 shows that comparison of SDS-PAGE pattern of whole egg lysate in the eggs obtained from fish 307 and 312. The texture of the eggs obtained from fish 312 are softer than those obtained from Fish 307 (Fig. 10a). SDS-PAGE pattern of the whole egg lysate obtained from fish 312 showed the extra band located at 15 kDa. The peptide sequence of this extra band was also

analyzed following MS/MS analysis. The obtained peptide sequence was matched to the sequence of the ZPAX (the component of the egg envelope) in chicken and frog. This data suggested that in the soft eggs, the component of the egg envelope may be genetically abnormally produced or abnormally modified during the construction of egg envelope structure result in the soften the egg texture.

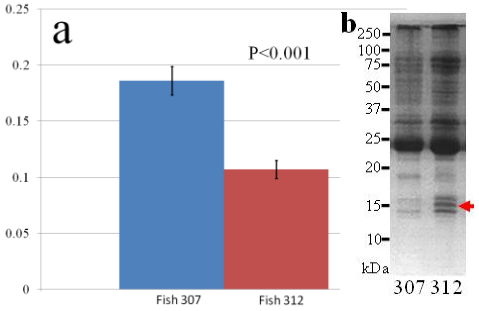


Figure11. The texture of the eggs in Fish 307 and 312 and SDS-PAGE pattern of their whole egg. a: texture, egg burst force (Newton), obtained from fish 307 and 312. bar; standard error, student T-test was used for statistical analysis.

Based on our statistical analysis following the student T-test, diet has an, affect on egg texture. However, our results show that to understand and solve the cause of soft envelope in farmed white sturgeon, it is necessary not only to reconsider diet content, but also to understand the molecular mechanisms of egg envelope formation from their gene expression to egg envelope formation.

Mass Spectrometry of the egg envelope components: As shown in Fig12, the egg envelope consists of 7 components. Following the procedure, 3 peptide sequences from Ev1&2 (mixture), 1 from Ev1, 3 from Ev2, 7 from Ev3, 22 from Ev4, 20 from Ev5, 79 from Ev6, and 4 peptide sequences from Ev7. This information may be useful to obtain the cDNA clones encoding each components of the egg envelope.

Antibody production and immunohistochemistry: Following the procedures described in the original proposal, the rabbit anti sturgeon whole egg envelope suspension antibody, rabbit anti sturgeon egg envelope 1&2 (Ev1&2) antibody, and the mouse anti-sturgeon egg envelope 4 (Ev4) antibody were produced at the UC Davis Comparative Pathology Lab. The immunoreactivities of these antibodies against egg envelope proteins are shown in Figure.12. Anti sturgeon whole egg envelope suspension (anti-WEES) antibody reacted with Ev1~3 intensely and this antibody show very faint reactivities against Ev4~6 and no reactivity against Ev7 (Fig.12B). Anti-Ev1&2 antibody specifically reacted only with Ev1&2 (Fig.12C). Anti-EV4 antibody only reacted with Ev4 in both the ovarian and ovulated eggs (Fig.12D). These results clearly show that anti-Ev1&2 antibody and anti-Ev4 antibody react specifically with the component of the WST egg envelope. To identify the location of Ev1&2 and Ev4 in the ovarian egg envelope and ovulated egg envelope, immunohistochemical analyses were performed using these specific antibodies.

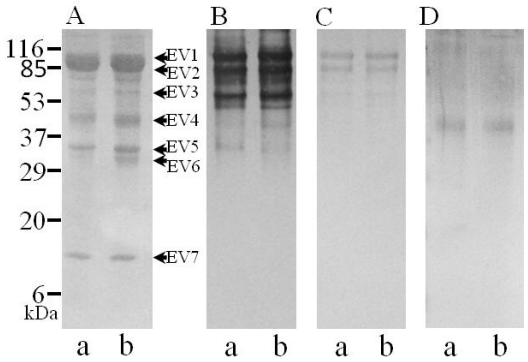


Figure12. SDS-PAGE pattern of the WST egg envelope proteins (A) obtained from ovarian eggs (a) and ovulated eggs (b). and their immunoreactivities against anti-egg envelope suspension antibody (B), anti-Ev1 &2 antibody (C) and anti-Ev4 antibody (C). The numbers in the left side shows the positions of molecular marker (kDa). The arrows in the right side (A) show the position of each egg envelope protein. No immunoreactivities were observed in the negative control (the primary antibody omitted, Data not shown).a; egg envelope extract from ovarian egg, b; egg envelope extract

Project modification:

Modifications were made to obtain the blood, egg, and egg envelope samples for plasma sex steroid analysis. To identify the diet effect on egg envelope structure and texture, the fish were maintained on the same farm, and except for diet, under the same conditions (Temperature and water flow etc). The diet treatment was initiated before vitellogenesis and egg envelope formation. The difference between the original treatment and new treatment was the timing for the treatment. The fish used in the original plan were started on the diet treatment after initiation of vitellogenesis and egg envelope formation. It is possible that the egg envelope formation was

initiated under the same diet conditions. This may be why we could not find a clear difference in the diet effect on egg texture.

Projectoutcome
Please see the section of "**Describe progress and accomplishments toward meeting goals and objectives".**

Impact of project:
We are now confirming our hypothesis that environmental and nutritional changes in the husbandry have affected final caviar quality.
Benefits, commercialization and application of project results:
Sterling Caviar LLC and the Fishery. We have been sharing the results obtained so far, but we are not far enough along to realize the full benefits.

Economic benefits:
We expect that by solving the problem of soft egg syndrome U.S. sturgeon aquaculture will improve and increase the economic benefits provided by US aquaculture in general.
Issue-based forecast capabilities will be used to predict the impacts of a single ecosystem stressor, developed and used for management (i.e., climate change, extreme natural events, pollution, invasive species, and land resource use). Our project will also provide solutions for improvement of the water conditions for fish, particularly white sturgeon, and including endangered species such as green sturgeon, because soft egg syndrome has potential impacts in the eggs of both commercial and natural sturgeon stocks.

Publications:
Conference papers, proceedings, symposia:
Title: Effect of temperature and nutrition on the egg quality in white sturgeon farmed for caviar
Authors: Y. Zhang, S. Doroshov, T. Famula, F. Conte, D. Kueltz, J. Linares-Casenave, J. Van Eenennaam, P. Struffenegger[1], K. Beer[2], and K. Murata [*]
Date:10/29/2009
Conference Title: 6th International Symposium on Sturgeon
Location: Wuhan, China

Peer-reviewed journal articles or book chapters
Title: Husbandry and dietary effects on sturgeon (*Acipenser transmontanus*) farmed for caviar
Authors: Y. Zhang, S. Doroshov, T. Famula, F. Conte, D. Kueltz, J. Linares-Casenave, J. Van Eenennaam, P. Struffenegger[1], K. Beer[2], and K. Murata [*]
Date: submitted
Journal Name: Journal of Applied Ichthyology
Issue/Page Numbers: submitted

Theses, dissertations
Title: Husbandry and dietary effects on sturgeon (Acipenser transmontanus) farmed for caviar
Authors: Yue Zhang
Schools: MASTER OF SCIENCE in Animal Biology in the UNIVERSITY OF CALIFORNIA DAVIS
Date: **06/31/2010**

Workshops and presentations
the 6th International Symposium on Sturgeon in Wuhan, China, October 25-30, 2009, about 500 attendance in the meeting. Effects of temperature and nutrition on the egg quality in white sturgeon farmed for caviar

Dissemination of results
To be accomplished later in the project.

Students
Last Name: Zhang
First Name: Yue
Institution: University of California, Davis
Department: Anima Science
Degree program enrolled in Animal Biology (MS)
Theses/dissertation title: Husbandry and dietary effects on sturgeon (*Acipenser transmontanus*) farmed for caviar
Supported by Sea Grant funds? yes
Start date: 09/01/2008
End date: 06/30/2010

Last Name: McInnis
First Name: Elizabeth
Institution: University of California, Davis
Department: Anima Science
Degree program enrolled in Animal Biology (MS)
Theses/dissertation title
Supported by Sea Grant funds? yes
Start date: 07/01/2010
End date:

Cooperating organizations
Industry: Sterling Caviar LLC, the Fishery

Keywords
white sturgeon, temperature, commercial diet, caviar yield, plasma steroids, egg,