DETERMINING SEXUAL MATURITY IN WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) TO MAXIMIZE YIELD AND QUALITY OF CAVIAR

By

XIAONAN LU

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Chair

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DETERMINING SEXUAL MATURITY IN WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) TO MAXIMIZE YIELD AND QUALITY OF CAVIAR

Abstract

By

Xiaonan Lu, M.S.
Washington State University
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Chair: Barbara A. Rasco

Knowing more about the reproductive status of female sturgeon and being able to predict the proper time to harvest fish roe for caviar is an important factor for producing high yield and consistently uniform caviars. Current techniques for predicting sexual maturity of white sturgeon involves the measurement of oocyte polarization index (PI) that requires an invasive surgical biopsy. Surgical biopsy is stressful to fish and time consuming. New methods would be helpful which are less stressful to fish and also provide additional information about biochemical changes that occur during gonadal maturation and how these changes correlate with roe quality, and to the oocyte PI.

This research examined the feasibility of using Fourier transform infrared (FT-IR) spectroscopy (in the mid-IR range, 4000-400 cm^{-1}) to predict biochemical and potentially morphological changes occurring during vitellogenesis through atresia. Studies were conducted on fish held for a nine month period.

Changes were observed in the FT-IR spectra of sturgeon plasma at selected maturity levels. Differences in plasma biochemical compositions in the steroid region of FT-IR (around 3000 cm^{-1}) were detected along with changes in the concentration of vitellogenin in plasma (around 1080 cm^{-1}). Clear segregation of plasma according to maturity stage was evident using Principal Component Analysis (PCA). It was possible to predict stage of maturity (late vitellogenesis vs. early atresia) about 70% of the time using Soft Independent Modeling of Class Analogy (SIMCA) models. Combination of second derivative transformation, PCA cluster model and SIMCA pass test together precisely predicted maturity stage of sturgeon. A rigorous Partial Least Squares (PLS) model was established to predict PI values between 0.1 and 0.3 (R=0.98, SEP=1.01%) based upon differences observed in spectral features. However, few changes were observed in spectral features of the roe recovered at selected maturity stages indicating that the composition of fish roe is relatively static from vitellogenesis through atresia.

FT-IR spectroscopy combined with various multivariate analyses may provide a useful tool for a more rapid and less invasive assessment of white sturgeon maturity and timing harvest, reducing the need for traditional invasive and stressful surgical biopsy methods for PI determination.

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WENHAI LU & GUIFEN HUANG

CHAPTER ONE
INTRODUCTION

1.1 A review of white sturgeon (*Acipenser transmontanus)* female reproductive physiology and its importance for caviar production

Xiaonan Lu[1], Serge Doroshov[2], Molly Webb[3], Barbara Rasco[1*]

[1]School of Food Science, Washington State University, Pullman, WA 99164-6376, USA

[2]Department of Animal Science, University of California, Davis, CA 95616, USA

[3]Bozeman Fish Technology Center, Bozeman, MT 59715, USA

[*]Corresponding author. Tel: (509) 335-1858; fax: (509) 335-4815. E-mail address: rasco@wsu.edu (B.A. Rasco)

1.1.1 Introduction

Much research is conducted to investigate white sturgeon female reproductive physiology due in part to conservation and restoration activities for threatened and endangered species and also in support of efforts to improve aquaculture practices for these fish. International demand for sturgeon caviar products is focusing the attention of fisheries and food scientists to sturgeon aquaculture, in the United States with white and Siberian sturgeon (i.e. *Acipenser transmontanus* and *Acipenser gueldenstaedtii*) and for these and other sturgeon species around the world. Italy developed large-scale production of caviar using white sturgeon transplanted from California (Caprino *et al.* 2008). France produces significant amounts of caviar by using Siberian sturgeon transplanted from Russia (Williot *et al.* 1998). Israel is developing caviar production by using Russian sturgeon (Hurvitz *et al.* 2005).

Unfortunately, research on sturgeon biology, caviar and fish quality is fragmented and there is limited data and information on aspects of sturgeon biology. Part of this is related to low funding of efforts to support this fishery and its conservation over the years despite the high visibility of sturgeon and loss of the fishery in key areas such as the Caspian Sea. But also, the fish are difficult to study due to the long time required for fish to reach maturity. For example, the farmed white sturgeon females mature in 6-8 years. Various environmental factors and stresses such as water temperature and quality, photoperiod and the availability, type and composition of foods affect the growth and reproductive readiness of sturgeon, but in ways that are yet not well understood (Doroshov *et al.* 1997; LeBreton *et al.* 2004). Furthermore, various

research efforts targeted selected aspects that affect sturgeon female reproduction, but few studies are either well integrated or long term. Since sturgeon is a very complicated fish to study and establishing the model to compare the characteristics of sturgeon with other kinds of fish is intricate and unconvincing, a sustained and continuous research effort is particularly important.

Most of the research on sturgeon female reproduction *in vivo* or *in vitro* is reported by scientists from USA, European countries, Iran or China over the past fifteen years (Doroshov *et al.* 1997; Williot *et al.* 2001; Goncharov 2002; Wei *et al.* 2004; Pourkazemi 2006). Two important review papers about reproduction cycle and mechanisms of oocyte maturation of white sturgeon females are published. One widely cited review paper by Dr. Serge Doroshov published in the *Journal of Environmental Biology of Fisheries* in 1997 discusses observations on the reproductive cycle of cultured white sturgeon (*Acipenser transmontanus*), including sturgeon gonadal cycles, age and body size at puberty and its relationship to sturgeon maturity and more important, hypothetical neuroendocrine reproductive axis of white sturgeon female (Doroshov *et al.*, 1997). His research team at the University of California, Davis published more than fifty research manuscripts about sturgeon reproductive physiology. Russian scientist Boris Goncharov wrote a review paper published in *Journal of Applied Ichthyology* focusing on *in vitro* approaches to study oocyte maturation mechanisms to create better criteria to select sturgeon females for breeding (Goncharov 2002).

This literature review covers sturgeon female reproduction research over the last

fifteen years, especially focusing on the maturity stages from late vitellogenesis to early atresia, a crucial period related to final caviar quality. Relationships of morphological and immunochemical parameters associated with follicular atresia and caviar quality are also analyzed and established.

1.1.2 General discussion of sexual maturity in sturgeon

Sturgeon live in the Northern Hemisphere for quite a long time, first appearing on the fossil record about 200 million years ago. Fifty percent of sturgeon and paddlefish exist in Europe, mostly around the Caspian Sea area, one third exist in Canada and America, and the remainder exist in East Asia and Siberia (Billard *et al.* 2001). Sturgeon reproduce in the freshwater and many migrate to the sea periodically under the neuroendocrine regulation affected by photoperiod, water temperature and some other factors. White sturgeon (*Acipenser transmontanus*) is currently confined to Washington, Oregon, California and Idaho in the United States and to the province of British Columbia in Canada. This species is reared in California farms for meat and caviar production since 1980. The females of farmed white sturgeon mature at an average age of 6-8 years. Observations on the gonadal of reared white sturgeon females manifest a predominately biennial ovarian maturity cycle. Gonadal development in female sturgeon exhibits seasonal forms, with ovarian vitellogenesis starting in the fall and final gonadal maturation achieved during the late spring or early summer in the second year (Doroshov *et al.* 1997; Williot and Brun 1998). However, individual variation of endogenous ovarian cycles of farmed sturgeon

female fluctuated from <1 year to 3-4 years respectively, which may result from different levels of endogenous and exogenous factors and culture stress (Doroshov *et al.* 1997). The sturgeon female reproductive physiology is under the control of neuroendocrine axis, including several important receptor and feedback regulations from the hypothalamus, pituitary, ovary or liver (Fig.1).

1.1.3 Roles of Gonadotropin-releasing hormone, gonadotropin, dopamine and insulin-like growth factor I and regulation of sturgeon maturity

Gonadotropin-releasing hormone (GnRH) and the neurotransmitter dopamine (DA) interwork on the pituitary gonadotropes to regulate the synthesis and release of two kinds of putative gonadotropins (GTH). The gonadotropins control gonadal development, gamete release, and also stimulate sex steroid synthesis in the gonad (Doroshov *et al.* 1997).

Sturgeon brain produces two types of GnRH: a minor form of immonoreactive chicken II GnRH (cGnRH, with an unknown function) and a predominant form of mammalian type of GnRH (mGnRH) controlling reproduction (Sherwood *et al.* 1991; Lescheid *et al.* 1995). The complete amino acid sequence assignment of the sturgeon GnRH is pGlu-His-Typ-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Lescheid *et al.* 1995).

The mechanism of dual gonadotropin control of gonadal development in sturgeon was validated (Moberg *et al.* 1995) and is similar to other species of fish such as salmonids (Swanson *et al.* 1989), rainbow trout (Tyler *et al.* 1991) and other teleosts (Patino *et al.* 2001). Sturgeon gonadotropin-I (GTH-I) appears to conduce and

retain follicular development and vitellogenesis, while sturgeon gonadotropin-II (GTH-II) induces ovarian maturation and final ovulation.

The contents of GTH-I and GTH-II are small in the immature sturgeon pituitary and blood. Along with the beginning of vitellogenesis in sturgeon females, the pituitary concentrations of both gonadotropins increase. GTH-I is the primary gonadotropin in the pituitary at this time. GTH-I concentration in plasma also increases slowly and steadily during vitellogenesis. Prior to final spawn, the pituitary concentration of GTH-II increases dramatically in sturgeon females. It is during this period that GTH-II becomes predominant gonadotropin in the pituitary. The plasma concentration of GTH-II in sturgeon female increases sharply when the fish enters the period of final gonadal maturation and spawning (Fig.2). Furthermore, GTH-II is more potent than GTH-I in inducing germinal vesicle breakdown (GVBD) (Moberg *et al.* 1995).

GTH-I and GTH-II in cultured Siberian and Russian sturgeons were cloned and sequenced recently by French and Israeli scientists (Querat *et al.* 2000; Hurvitz *et al.* 2005). Phylogenetic sequence analysis led to renaming GTH-I and GTH-II as the follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively, as identified in other vertebrates. Based on the experiments with the white sturgeon males, Pavlick and Moberg (1997) suggested that dopamine (DA) acts as an endogenous inhibitor of GnRH-induced pituitary secretion; however, the role of DA in sturgeon female is not clear (Pavlick and Moberg 1997).

German scientists recently reported important role of the insulin-like growth

factor I (IGF-I) system for vitellogenesis in maturing sterlet females (Wuertz *et al.* 2007a; Wuertz *et al.* 2007b). They reported that sterlet females entering vitellogenesis have an increase of IGF-I expression in the ovaries compared than the ones that stayed in pre-vitellogenic period, and another increase at late vitellogenesis prior to spawning, which demonstrated that IGF-I, the substance promoting cell differentiation and growth, is a crucial regulator of gonad maturation, particularly in the ovary.

1.1.4 Sex steroids and vitellogenin during sturgeon maturity

GTH-I circulates in the blood plasma and, upon reaching the ovaries, stimulates the receptor of follicle cells to synthesize estradiol-17β (E_2), the major sex steroid, which is then released into the serum. E_2 diffuses across the membrane of liver cells and binds to the estrogen receptors, resulting in initiation of transcription and translation of vitellogenin (VTG) (Barannikova *et al.* 2002). Female sturgeon vitellogenin is a large molecule (native mw ~440 kDa in sturgeon) composed of a protein backbone phosphoryilated on serine moieties, a large concentration of lipids (~20%), and carbohydrates. It binds calcium and other cations during transport from the liver to the ovary. VTG has a critical function as the precursor of egg-yolk proteins that are major nutritional sources for oviparous species during early development. VTG accumulates in the hepatocytes with advanced modification and is then released back to the serum. Upon reaching the ovaries, VTG passes through follicle cells to reach oocyte surfaces, and is then incorporated into oocytes under the

control of GTH-I (Wheeler *et al.*, 2005). GTH-II stimulates the synthesis and secretion of the maturation-inducing steroids (MIS) by the follicular layer of the oocyte. MIS acts as the messenger to commence meiosis resumption and germinal vesicle breakdown (GVBD) in the oocytes during the final maturity stages (Nagahama *et al.*, 1995).

Sex steroids and VTG in the plasma are good bio-markers for researchers to investigate sturgeon maturity. The general trend for the change in concentrations of each sex steroid and VTG content in sturgeon plasma is elucidated. Estradiol-17β (E_2), the major sex steroid in the plasma of the female fish increases dramatically during vitellogenesis and then decreases when vitellogenesis is completed (Fig.2). VTG concentrations follow the same trend as E_2 due to the fact that E_2 stimulates the liver to produce vitellogenin, which is then incorporated into the developing oocytes (Fig.3). Moberg *et al.* (1991) observed that the hepatic synthesis of VTG in white sturgeon was induced by estrogen. Fujii *et al.* (1991) indicated that vitellogenesis and large E_2 concentrations are correlated in maturing bester (*Huso huso x Acipenser ruthenus*) sturgeon. Moberg *et al.* (1995) reported that both testosterone (T) and E_2 concentration decreased prior to and during final spawning and plasma concentrations of all steroids declined in white sturgeon one month following spawning. Amiri *et al.* (1996) investigated changes in serum concentrations of E_2 and VTG in farmed sturgeon females over one year. The concentrations of these two compounds increased during vitellogenesis, whereas in the post-vitellogenic stage, the concentrations of E_2 declined from 2-4 ng ml^{-1} to 1-2 ng ml^{-1} and VTG from 4-10 mg ml^{-1} to 0.2-0.5 mg

ml^{-1}. Doroshov *et al.* (1997) indicated that average VTG concentration in white sturgeon plasma reaches 7 mg ml^{-1} during late vitellogenesis and decreases significantly before spawning. Linares-Casenave *et al.* (2002) observed that plasma E_2 and T concentrations in white sturgeon decreased to less than 0.5 ng/ml and 1 ng/ml, respectively, after visual signs of atresia were evident. Linares-Casenave *et al.* (2003) also observed that white sturgeon females in the pre-vitellogenic ovarian stage and at the onset of yolk deposition exhibited small VTG concentrations. However, the significant increases in plasma VTG occurred only in sturgeon that developed clutches of the oocytes with detectable crystalline yolk in the cytoplasm (vitellogenic period). Barannikova *et al.* (2004) reported that vitellogenesis and large E_2 concentrations were closely correlated in the maturing sturgeon (*Acipenser gueldenstaedtii*) females.

Changes in the concentrations of 17*α*, 20*β*-dihydroxy-4-pregnen-3-one (17*α*, 20*β*-P) were studied in more detail since it is believed to act as a maturation-inducing steroid (MIS) for final ovulation in fish. Lutes *et al.* (1985, 1987) pointed out that 17 *α*, 20*β*-P performed as a maturation-inducing steroid (MIS) in white sturgeon and also reported that responsive sturgeon females in exogenous induction of ovulation exhibited increased concentrations of 17 *α*, 20*β*-P. Moberg *et al.* (1995) observed that the concentration of 17 *α*, 20*β*-P reached peak concentrations in white sturgeon plasma at final ovulation. Amiri *et al.* (1996) reported that serum concentrations of 17 *α*, 20*β*-P were consistently small (less than 0.2 ng ml^{-1}) throughout the entire reproductive cycle. However, concentrations of this sex steroid increased dramatically

(to 2 ng ml^{-1}) in the ovulated bester sturgeon females compared to non-ovulated individuals when fish were injected with luteinizing hormone releasing hormone analog (LH-RHA). However, the native MIS form in sturgeon is still unknown, because progesterone (P4) and at least two progestagens (17*α*, 20*β*-P and 17,20*β*, 21-trihydroxy-4-pregnen-3-one (17, 20*β*S)) exhibit high potency in stimulating germinal vesicle breakdown (GVBD) *in vitro* and exhibit elevated plasma concentrations at ovulation (Webb *et al.* 2000; Webb *et al.* 2002; Semenkova *et al.* 2002; Semenkova *et al.* 2006; Semenkova *et al.* 2006). Furthermore, androgens such as testosterone (T) and 11-ketotestosterone (11-KT) may also have a role during final oocyte maturation in sturgeon (Semenkova *et al.* 2006). The forms and roles of sturgeon MIS need to be validated in the near future.

In conjunction with sexual development in sturgeon, the characteristics of sturgeon female vitellogenin are also reported by several researchers. Bidwell *et al.* (1991, 1995) observed two VTG polypeptides (190 and 210 kDa, respectively) in plasma and the purified fractions appear to be in accordance with the presence of two VTG mRNA in hepatocytes of white sturgeon. Hiramatsu *et al.* (2002) isolated vitellogenin and its corresponding yolk protein (YP) products, YP1, YP2 and YP3 from serum of estrogen-treated hybrid sturgeon (bester; *Huso huso X Acipenser ruthenus*) and roes from untreated fish respectively. A method using SDS-PAGE followed by western blotting confirmed that YP1, YP2 and YP3 were lipovitellin, β'-component, and phosvitin, individually.

1.1.5 Progression through reproductive cycle from vitellogenesis through atresia

Oocyte maturation in sturgeon includes the orders of germinal vesicle migration, germinal vesicle breakdown (GVBD) and ovulation after the treatment of a dose of 10 μg kg^{-1} of GnRHa followed by intramuscular injection of 4.5 mg kg^{-1} carp pituitary material after 12 hours (Doroshov *et al.* 1997).

As the roe size increases with accumulation of protein and lipid, the nucleus also grows and is then referred to as germinal vesicle (GV). For the first ovarian cycle, the eggs in female sturgeon are arrested in meiosis with gathering adequate yolk proteins and size growth for farmed sturgeon, about 6 to 8 years and for wild sturgeon, about 15 to 32 years until the female reaches puberty. Thereafter, each ovarian cycle may require at least one year or longer in Siberian sturgeon (Williot *et al.* 1998) or two years in white sturgeon (Doroshov *et al.* 1997). Spawning success is primarily influenced by oocyte maturation stages, especially the capability of GVBD (LeBreton *et al.* 2004).

A two-stage concept of oocyte maturation is established for teleost fish (Patino *et al.* 2001). The follicle cells manufacture MIS and respond to MIS during the first stage, along with the onset of GVBD. The second stage comprises additional MIS production from follicle cells and resumption of oocyte meiosis. Ovulated oocytes that can be fertilized are called eggs (Dettlaff *et al.* 1993). With a definitive egg size reaching, the egg enters final maturation and resumes meiosis. At the same time the GV begins to move close to the edge of egg where it comes through GVBD. When the GV is near the edge of the egg plasma membrane, the female is mature adequately

and ready to spawn. The migration of the GV to the periphery of the egg may take a few months, dependent primarily on water temperature and other environmental factors (Webb *et al.* 1999; Webb *et al.* 2001).

With water temperature increase during the spring, germinal vesicle migration progresses in white sturgeon oocytes and oocyte maturation competence (OMC) is achieved, with germinal vesicle GVBD and ovulation occurring after hormonal treatment under culture conditions. However, OMC and GVBD sometimes fail, resulting in ovary regression or atresia. Ovarian atresia is the process by which ovarian follicles are degenerated and subsequently resorbed by the ovary (Linares-Casenave *et al.* 2002).

1.1.6 What Polarization Index (PI) is and how values for this parameter are associated with fish maturity and atresia

Oocyte PI is a ratio of the distance of the germinal vesicle or nucleus from the animal pole to the oocyte animal-vegetal axis diameter and indicates morphogenetic changes in the ovarian follicle occurring during late vitellogenesis leading up to oocyte maturation competence (OMC) (Fig.4). Currently, measuring the PI value is the only means to accurately assess ripeness of white sturgeon females. Dettlaff *et al.* (1993) recommended an oocyte PI of 0.07 or less for selection of female sturgeon broodstock for spawning. Chapman *et al.* (2007) reported that it is better to select females with the PI scores of less than 0.10 for spawning induction, but preferably those females with egg polarization indices of 0.06-0.08.

1.1.7 Factors that result in atresia in sturgeon

Atresia is observed in both wild and farmed sturgeons and associated with stress, unfavorable temperature, photoperiod regimes and non-optimal water quality (Doroshov *et al.* 1994; Doroshov *et al.* 1997). Even though warm water fosters sturgeon to grow and mature rapidly, sturgeon require a cold water environment from post-vitellogenic period to complete maturation and induced ovulation successfully. Webb *et al.* (1999, 2001) provided evidence for the adverse effects of elevated temperature on white sturgeon ovarian development, including low plasma concentrations of sex steroids, arrested germinal vesicle migration, and low spawning success rate. Webb *et al.* (2001) also indicated that unfavorable aquaculture temperatures were validated to reduce the capability of the follicular layers to mediate the maturation response of sturgeon oocytes to gonadotropic hormones, especially GTH-II. Linares-Casenave *et al.* (2002) reported that exposure of white sturgeon females to elevated water temperature during late vitellogenesis stages resulted in follicular atresia and failed reproduction. The onset of atresia, accompanied by quick decline in plasma concentrations of sex steroids and VTG, coincided with hypertrophy of granulosa cells and digestion of the oocyte envelope. The early morphological changes manifested in two weeks after exposure of late vitellogenic sturgeon females to the increased temperature is important to sturgeon caviar production as the early stage of atresia can affect the integrity of the oocyte envelope, rendering sturgeon roe unacceptably soft for processing and preserving (Linares-Casenave *et al.* 2002).

1.1.8 Quality of roe at selected maturity stages from late vitellogenesis to early atresia

Most sturgeon caviar (about 90%) comes from the Caspian Sea. However, each year, thousands of pounds of finished products are often rejected by buyers due to quality defects (Bledsoe *et al.* 2003). There was limited research result about roe quality at different maturity stages. Seagran *et al.* (1954) observed that most amino acids in salmon roe were present in enhancing quantities with increasing maturity. Vuorela *et al.* (1979) reported that protein contents increased with roe maturation up to final spawning and the lipid content reached its maximum at halfway to maturation in the Baltic herring (*Clupea harengus*). Katsiadaki *et al.* (1999) investigated the relationship between cod roe quality and maturity stage. They reported that the roes from vitellogenic ovaries were the A quality, early spawning ovaries were most categorized as B quality, advanced and late spawning ovaries were featured as C quality, and spent ovaries were of the lowest quality grade (D). They also indicated a significant relationship between roe quality and moisture content, the higher quality roe exhibits the smaller moisture content.

In sum, the general information for white sturgeon is that follicular atresia decreases caviar yield and quality. Even the early stage of atresia results in a reduction in the firmness, flavor, and shelf life of caviar, and sometimes completely unacceptable caviar. According to Dettlaff *et al.* (1993), the concentrations of proteins and lipids in sturgeon roes are dependent upon the maturity and overall condition of

the roes in the ovaries. To our knowledge, there are no published research conducted on the plasma sex steroids, protein concentrations and follicle crude compositions related to the firmness, flavor and color of white sturgeon roe collected for caviar production. There were few references to the composition of sturgeon roe (Bledsoe *et al.* 2003; Al-Holy and Rasco 2006) let alone compositional changes in sturgeon roe at selected stages of maturity.

1.1.9 Methods to discriminate sturgeon maturity stages

The current technique for sorting female white sturgeon prior to harvest includes assessing the stage of ovarian maturity by determining oocyte polarization index (PI) value. This technique is accurate but invasive and stressful to fish, time consuming, and not an effective tool when necessary to handle a large number of fish. Therefore, some caviar producers choose not to determine oocyte PI. Instead, ovarian follicles are assessed visually for darkly pigmented follicles, indicative of late vitellogenesis, due to synthesis and deposition of melanin granules in the cortical cytoplasm (Dettlaff *et al.* 1993). Either determination of the oocyte PI value or visual assessment of ovarian follicles was used once in the fall to predict which females to harvest in winter and the following spring. Failing to accurately identify sexually mature fish often results in reduced caviar yield and quality. Thus, researchers are attempting to identify other parameters to determine sturgeon maturity stages more accurately.

Doroshov *et al.* (1994) and Moberg *et al.* (1995) indicated that plasma concentrations of estradiol-17β (E_2) can be used to discriminate vitellogenic stage in

sturgeon. Webb *et al.* (2002) reported that plasma T and E_2 were the best predictors of white sturgeon sex and stage of maturity. Linares-Casenave *et al.* (2003) stated that concentrations of VTG in plasma can differentiate pre-vitellogenic and vitellogenic white sturgeon females. Webb *et al.* (2005) pointed out that by using plasma concentrations of T, 11-KT and E_2, 61% correct classification of pre-vitellogenic females, 86% of vitellogenic females, 92% of post-vitellogenic females, 55% of post-ovulatory females and 29% of atretic females was possible. Malekzadeh Viayeh *et al.* (2006) demonstrated that plasma T and E_2 plus either age, total length, fork length or weight were also good predictors for the determination of stage of sturgeon maturity.

The most viable methods for quantifying sex steroid contents in sturgeon plasma are immunoassay including radioimmunoassay (RIA) (Webb *et al.* 2002) and a more lately developed Enzyme Linked ImmunoSorbent Assay (ELISA) (Nash *et al.* 2000) method. RIA (Tyler *et al.* 1996), western blotting (Hiramatsu *et al.* 2002), ELISA (Linares-Casenave *et al.* 2003) may also determine the concentration of vitellogenin in sturgeon plasma.

1.1.10 Current knowledge about sturgeon caviar and the caviar industry

Sturgeon caviars are the salt-added eggs separated from the supporting connective tissue of sturgeon before curing (Bledsoe *et al.* 2003). The most valuable caviars are harvested from wild sturgeons in Caspian Sea, including Beluga (*Huso huso*), Osetra (*Acipenser gueldenstaedtii*) and Sevruga (*Acipenser stellatus*) caviar,

delicacies famous around the world.

Proximate compositions of wet weight caviar from white sturgeon (*Acipenser transmontanus*) demonstrate that protein constitutes about 25%-30%, lipid about 10%-20%, and ash about 3.5%-6% depending on sturgeon species, living environment, water temperature and other factors (Wirth *et al.* 2000; Caprino *et al.* 2008). Caprino *et al.* (2008) also determined the fatty acid composition and volatile compounds in farmed sturgeon caviars. They indicated that palmitic acid (16:0) (over 20%) and oleic acid (OA, 18:1 n-9) were the most abundant fatty acids followed by docosahexaenoic acid (DHA, 22:6 n-3) and eicopentaenoic (EPA, 20:5 n-3). The large concentration of these fatty acids demonstrated the importance of the saturated fatty acids as the energy stores and PUFA as the temporary reservoir for the further embryonic development. Thirty-three volatile compounds were isolated including 2-alkenals, n-alkanals and 2, 4-alkadienals as the major volatile compounds that affect the flavor and sensory characteristics of caviar.

Over the past few decades, the wild sturgeon stocks were overexploited attributed to an increase demand for caviar, poaching to serve illegal markets, and demand for cash income in the Caspian Sea littoral regions where the fishery is no longer well managed. Exploitation resulted in dramatic decrease of caviar product availability and supply, and loss of reproductively competent individuals from the wild population. This damage to the wild fishery has increased the importance of sturgeon restoration efforts as well as the importance of the production of caviar from aquaculture operations around the world. Because the quality of caviar varies greatly

depending on how and where the fish are raised, researchers are concerned about the differences in caviar qualities, sensory characteristics and chemical components from both wild and farmed sturgeons.

Czesny *et al.* (2000) was able to discriminate roes from specific origins both from wild sturgeon and farmed sturgeon based on egg fatty acid profile, specifically from distinctly different concentrations of stearic and oleic acids. Wirth *et al.* (2002) and Gessner *et al.* (2002) demonstrated that the caviars from farmed sturgeon contained a significant greater concentration of linoleic acid (18:2 n-6) and a smaller concentration of arachidonic acid compared with wild sturgeon caviars.

Cardinal *et al.* (2002) reported that sensory properties of caviar are affected more by the sturgeon species rather than rearing factors and caviars from wild and farmed sturgeons exhibited similar sensory characteristics. A noticeable earthy taste was sometimes observed in both farmed and wild sturgeon caviars attributed to both feed and water quality.

Farming practices and animal husbandry are important factors that can affect sturgeon caviar quality and its sensory characteristics, especially the aspect of feeding. Lipids are still the core compounds of research focus, although other attributes may be important such as the presence of antioxidants and protein quality of the feed. During the vitellogenic period, fatty acids are mobilized from the neutral lipid reserves in sturgeon adipose tissue and absorbed by the eggs, thus influencing triglyceride fatty acid composition of the eggs based on food composition (Wirth *et al.* 2000; Gessner *et al.* 2002; Wirth *et al.* 2002). Lipoprotein biosynthesis in the sturgeon eggs utilizes

saturated and monounsaturated fatty acids to provide metabolic energy, while n-3 PUFA are incorporated into phospholipid fragments of vitellogenin and transferred through the blood into the eggs (Gessner *et al.* 2002; Caprino *et al.* 2008). Unfortunately, fatty acid mobilization in sturgeon eggs is very fragmented. Gessner *et al.* (2002) suggested analysis of lipid contents and lipid categories between farmed sturgeon caviar and wild sturgeon caviar for developing formulated diets. The objective of reducing linoleic (18:2) acid content while increasing arachidonic (20:4) acid level is to improve the quality and nutritional values of farmed sturgeon caviar. Little specific research on lipid mobilization or lipoprotein biosynthesis is available.

Caprino *et al.* (2008) reported that diet mixed with squid oil was responsible for significantly increase egg yield compared to diet mixed with soybean oil due to the different concentrations of n-3 PUFA. However, the overall nutritional and sensory qualities of caviars obtained from farmed sturgeon fed distinctly different compositions of diets were affected very little. Furthermore, feeding time and frequency is another factor affecting fatty acid compositions of sturgeon caviar. Caprino *et al.* (2008) mentioned that significantly greater concentrations of linoleic acid and n-6 PUFA were manifested in the caviars of sturgeon groups fed a diet mixed with soybean oil during the six months prior to harvest compared with the groups fed soybean oil during the last three months prior to harvest. Apparently, more research is required to elucidate the impact of feed on the quality, chemical composition, and sensory characteristics of sturgeon caviar.

The processing techniques and storage conditions also have significant impact on

caviar composition and quality. Gessner *et al.* (2002) indicated that salt content is the most critical factor that determines the gross composition of caviar product, resulting in a large decrease of wet weight of protein and lipid contents. Other processing factors are also considered to determine the final texture, color and quality of caviars, including processing temperature, inappropriate dehydration and excessive lipid removal (Gessner *et al.* 2002; Al-Holy *et al.* 2005). Gussoni *et al.* (2006) studied the effect of storage at 4 ºC on the levels of chemical components in caviars from farmed white sturgeon (*Acipenser transmontanus*) and demonstrated that lipid hydrolysis took place during the caviar storage at 4 ºC for four months (faster in unsalted than in salted eggs). They also excluded that an intensive oxidative process was active in the same storage period.

The economic situation of sturgeon caviar industry was elucidated in detail by Logan *et al.* (1995). Generally, the age of sexual maturity of sturgeon is critically important to economic feasibility of sturgeon caviar production due to the investment risk and return rate resulting from long time sexual maturity of sturgeon. The research group from Aquaculture and Fishery Science Program at the University of California, Davis offered the following suggestions to sturgeon aquaculture firms. If companies only plan to raise sturgeon for meat, then it is more efficient economically to sell the sturgeon when reaching the age of approximate 18.5 mo. When the price of sturgeon roe reaches or exceeds $331 to $441 per kg (1994 prices), a higher rate of return on investment can be obtained by rearing large numbers of fish to a maximum of 10 years and harvesting both meat and roe from the sturgeon. Due to the dramatic

increase in caviar prices over the past five years, a new economic assessment of the industry may be helpful.

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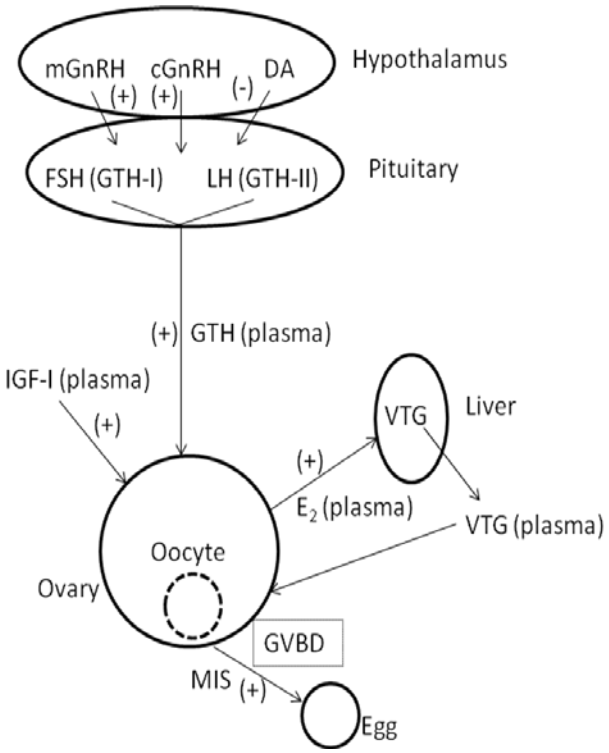

Figure 1. Neuroendocrine reproductive axis of farmed white sturgeon female.

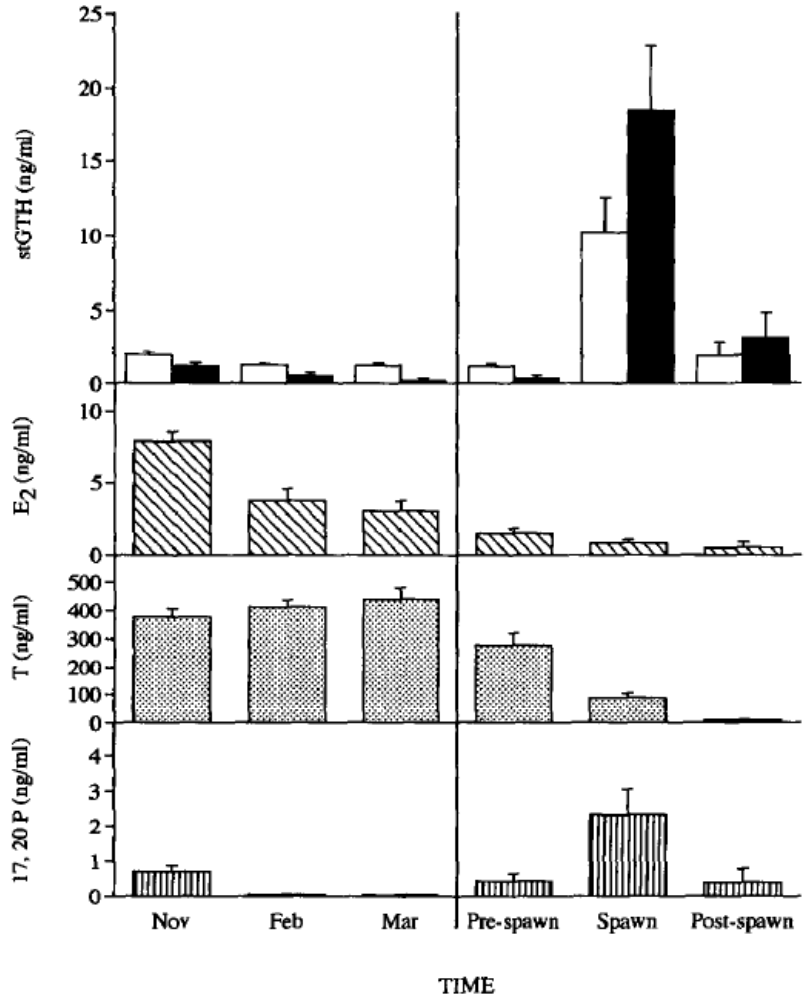

Figure 2. The mean plasma concentration of GTH-I (open bars), GTH-II (solid bars), estradiol-17β (diagonal bars), testosterone (stippled bars) and 17α, 20β-dihydroxy-4-pregnen-3-one (vertical bars) in cultured female sturgeon (n=11) in November, February, March, 36 h prior to spawning (pre-spawn), at spawning and 1 month following spawning (post-spawn) (cited from Moberg *et al.* 1995).

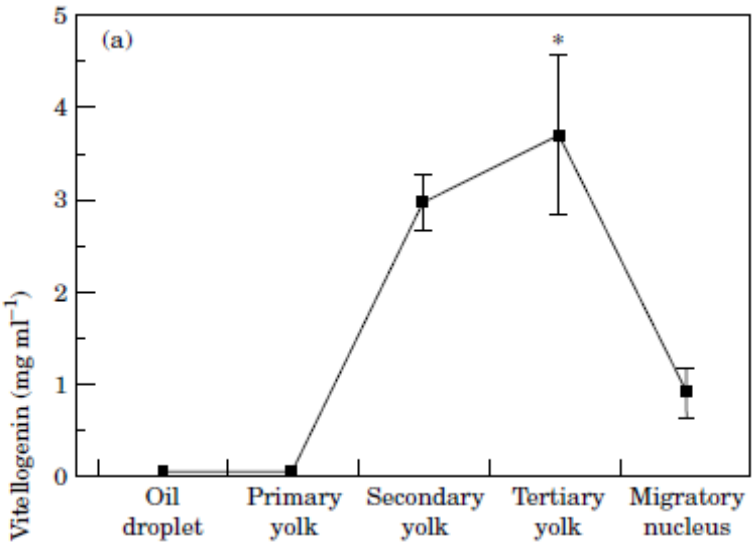

Figure 3. Changes in serum levels of vitellogenin in female bester during the periods of pre-vitellogenesis (oil droplet), vitellogenesis (primary, secondary and tertiary yolk), and late-vitellogenesis (migratory nucleus) (cited from Amiri *et al.* 1996).

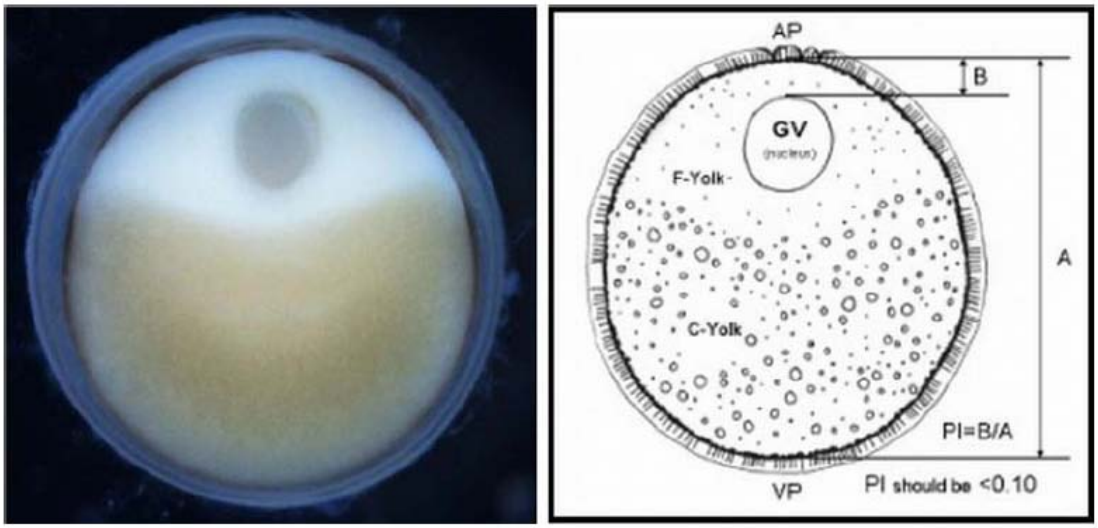

Figure 4. A photograph and drawing of a sturgeon oocyte depicting polarized nucleus (GV), and animal (AP) and vegetal (VP) poles. Segregation of the fine (F-Yolk) and course (C-Yolk) yolk granules helps to orientate the position of the two poles. The follicle PI=B/A; where A is diameter of follicle, excluding the chorion, and B is distance from the top of GV to plasma membrane of follicle (cited from Chapman *et al.* 2007).

1.2 A literature review of Fourier Transform Infrared (FT-IR) Spectroscopy and Multivariate Analysis

Xiaonan Lu and Barbara Rasco

School of Food Science, Washington State University, Pullman, WA 99164-6376, USA

1.2.1 Introduction to FT-IR

Fourier Transform Infrared Spectroscopy (FT-IR) is an advanced technique that can characterize biochemical components in complex matrices and allow for qualitative identification and quantitative estimations based on deformation, bending and ring-vibrations of covalent bonds within the spectral range of 4000-400 cm^{-1}. Particularly important are the CH_2 and CH_3 asymmetric stretch of fatty acids and protein methyl groups, the >C=O stretch of fatty acid esters, the C-N functional groups of proteins, and the asymmetric stretching mode of P=O for phosphate and phosphodiester groups. The utilization of an attenuated total reflectance (ATR) cell with FT-IR spectroscopy provides a technique to assay a wide variety of constituents with little or no sample preparation. FT-IR spectroscopy combined with multivariate analysis is widely utilized in three major research areas in the realm of food science and technology: (1) identification of proteins, lipids and carbohydrate moieties and the changes of their relevant concentrations during food storage and processing (Koca *et al.* 2007), (2) microbial category configuration, especially injured cell (chemical

treatment and heat treatment) detection and bacterial growth phase determination (Burgula *et al.* 2007), and (3) food adulteration (Pappas *et al.* 2008).

1.2.2 FT-IR data pre-processing

Data pre-processing algorithms are useful tools to improve spectra quality and enhance the spectral differences.

The “automatic baseline correction” was applied to raw spectra firstly to adjust any tilted baselines to the horizontal level. Secondly, the “normalization” was performed to compensate for the pathlength effect resulting from the differences among sample thickness. These two steps to process the FT-IR raw spectra are important since during FT-IR ATR spectral collection, sample thickness can change to some extent. For instance, a thick spot of sample can absorb more middle infrared energy than a thin one, resulting in greater peak heights and areas, sometimes peak shifts to a higher or lower wavenumber. Furthermore, different samples may cause the baseline tilted. These two results make spectra comparison and composition quantitative analysis and prediction impossible. The processes of “automatic baseline correction” and “normalizing” to the sample raw spectra make peaks comparable among different samples.

Other data pre-processing methods, such as binning, smoothing followed by second derivative transformation magnifies the manifestation of minor differences among IR spectra. Binning reduces the number of data points in a spectrum by *n* points into one and eliminates the imbalance problem associated with many array

based spectrophotometers. Smoothing eliminates high frequency instrumental noise by adjacent data points. Second derivative transformation separates overlapping absorption bands, eliminates baseline offsets, increases the apparent spectral resolution and provides an estimate of the number of overlapping bands within a region (Lin *et al.* 2004).

1.2.3 Multivariate Analysis

Principal component analysis (PCA) is a vector space transformation technique of reducing a data set to its most predominant features. PCA can reveal the internal of a structure data set and explain the variance in the data. The principle of PCA method determines which major factors affect the differences in the observed spectral features among samples and constructs a two or three dimensional model to segregate them based upon selected variances. The first principal component (PC1) contributes more than second principal component (PC2) to the variation observed between spectra, etc. Loading plots can calculate the contributions of principal components to PCA cluster segregation precisely and clearly. PCA is based upon a second derivative analysis (Lin *et al.* 2005).

Soft Independent Modeling of Class Analogy (SIMCA) is one analytical method of classification based on second derivative and PCA and extensively employed to classify samples according to their analogy to the training samples. The only “fail” or “pass” option is the significant feature of SIMCA, which is also based on the different bio-chemical composition of food matrix and definitely harsher (more difficult to

separate analytical samples from training samples) than PCA cluster classification (Al-Qadiri *et al.* 2006).

Partial Least Squares (PLS) model is a multivariate regression method to establish a relationship between reference values and predicted values, the larger the R value, the better and more vigorous the model (Lin *et al.* 2003).

In sum, all of PCA, SIMCA and PLS require large amounts of data to establish convincing and rigorous models. Empirically, the PCA cluster model requires at least 8-10 spectra from each single sample, the same as PLS, while at least 20 spectra from the same sample is needed to set up the SIMCA model.

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1.3 Justification

The long term goal of this project is to optimize caviar yield and quality from white sturgeon (*Acipenser transmontanus*). It would be helpful for caviar producers to have better analytical methods and tools they could use to produce a consistently uniform caviar. Having a better quantitative indicator of the quality of the sturgeon eggs at harvest would make it possible to standardize processing conditions (*i.e*. predict salt absorption), and maximize caviar yield by harvesting fish eggs at the appropriate size and firmness. Stressing the fish can result in decreasing caviar yield and quality due to follicular atresia (phagocytosis of ovarian eggs). Harvesting a fish too late, in early stage of atresia causes a reduction in the firmness, flavor, and shelf life over caviar, and sometimes the complete loss of the product.

Furthermore, having better measure for predicting sturgeon maturity and egg quality would help with sturgeon broodstock management in culture and conservation propagation programs for endangered sturgeon species.

Currently, the only means to assess ripeness of white sturgeon females and to time harvest is with the measurement of oocyte polarization (PI). This requires surgical biopsy and is stressful to the fish. The PI indicates morphogenetic changes in the ovarian follicle occurring during late vitellogenesis and leading to maturation. .

We need a better understanding of the biochemical and physiological changes that occur during gonadal maturation and how these changes correlate with roe quality, specifically, new methods to predict sturgeon maturity as an alternative to the oocyte PI. An improved method should be non-invasive, minimally stressful to fish, and

quick. Ideally, any new method for predicting maturity should allow the female fish to be sorted in the fall during late vitellogenesis into groups based upon the degree of egg ripeness. Here, Fourier transform infrared spectroscopy (FT-IR) combined with multivariate analysis was examined as a possible replacement for oocyte PI measurement as predictor of ripeness in sturgeon, by taking measurements on both blood plasma and eggs.

CHAPTER TWO

Distinguishing maturity of white sturgeon (*Acipenser transmontanus*) by spectroscopic measurement (Fourier Transform Infrared Spectroscopy) of plasma

XIAONAN LU[1], MOLLY WEBB[2], MARIAH TALBOTT[2], JOEL VAN EENENNAAM[3], AMANDA PALUMBO[3], SERGE DOROSHOV[3], JAVIER LINARES-CASENAVE[3], PETER STRUFFENEGGER[4], BARBARA RASCO[1*]

[1]School of Food Science, Washington State University, Pullman, WA 99163, USA

[2] Bozeman Fish Technology Center, Bozeman, MT 59715, USA

[3] Department of Animal Sciences, University of California, Davis, CA 95616, USA

[4] Marine Harvest, Stolt Sea Farm California LLC, Elverta, CA 95626, USA

* Corresponding author. Tel.: (509) 335-1858; fax: (509) 335-4815. *E-mail address*: rasco@wsu.edu (B.A. Rasco)

Abstract

Fourier Transform Infrared Spectroscopy (FT-IR, 4000-400 cm^{-1}) spectral features of white sturgeon (*Acipenser transmontanus*) plasma (N=40) were determined to predict sexual maturity. Differences in plasma biochemical compositions in the steroid region (around 3000 cm^{-1}) increased markedly as the fish became vitellogenic. Changes in the concentration of vitellogenin were also detected in plasma (around 1080 cm^{-1}). Clear segregation of plasma was possible according to maturity stage (pre-vitellogenic,

vitellogenic, post-vitellogenic and atretic) using Principal Component Analysis (PCA). Oocyte polarization index (PI) values and plasma sexual steroids level could be correlated by PCA. A Partial Least Squares (PLS) model predicted PI values between 0.12 and 0.40 (R=0.95, SEP=2.18%) from spectral features. These results indicate that FT-IR combined with multivariate analysis may provide a tool for rapid assessment of white sturgeon maturity reducing the need for traditional invasive and stressful surgical biopsy methods for PI determination.

Keywords: *FT-IR; white sturgeon; plasma; sex steroids; oocyte polarization index (PI); vitellogenin*

Introduction

Global demand for caviar continues to exceed the supply and aquaculture is the primary source for meeting this demand (Bledsoe *et al.* 2003). Wild sturgeon is overexploited throughout its natural range and is on the endangered list in many parts of the world and in 2005, the U.S. Fish and Wildlife Service restricted the import of beluga sturgeon products from the Caspian Sea region in response to overfishing. Improving methods for cultivating sturgeon will hopefully take some pressure off of the wild fishery and improve the overall health of the wild stocks. However, an important technical consideration in sturgeon aquaculture is how to determine when to harvest the fish. Currently the only way to accurately predict harvest time is with the invasive test for oocyte polarization index (PI).

Oocyte polarization index (PI) and oocyte maturation determination *in vitro* require surgical biopsy. Oocyte PI is a ratio of the distance of the germinal vesicle or nucleus from the animal pole to the oocyte animal-vegetal axis diameter (Doroshov *et al.* 1997) and indicates morphogenetic changes in the ovarian follicle occurring during late vitellogenesis leading up to maturational competence. Optimally, female fish are sacrificed for caviar when their oocyte PI value is around 0.10, a point which has been correlated with optimal yield and quality (Chapman *et al.* 2007). Determination of PI requires 24 hours and is difficult to perform when a large number of fish have to be tested at one time. In current aquaculture practice, farmers take PI determinations once in the fall to predict which female fish are most likely to be sexually mature and ready for harvest in the winter or next spring. Inaccurate predictions result in small

caviar yield and poor caviar quality.

Other assessments for fish maturity involve plasma sex steroid levels. These provide good biomarkers for predicting sturgeon female maturity (Webb *et al.* 2001; Linares-Casenave *et al.* 2002). Several sex steroids are present in the sturgeon plasma matrix, including: 17β-estradiol, testosterone, 11-ketotestosterone, progesterone and 17α, 20α−dihydroxy-4-pregnen-3-one (Burke *et al.* 1984; Nagahama *et al.* 1997; Lokman *et al.* 2002). Plasma 17β-estradiol and testosterone concentrations were reported to decrease to less than 0.5 ng/ml and 1 ng/ml, respectively, after visual signs of atresia were evident (Linares-Casenave *et al.* 2002). A decrease in plasma sex steroids was observed five weeks prior to visual signs of atresia in cultured white sturgeon (Webb *et al.* 2001). Concentrations of 17β-estradiol and testosterone increase during vitellogenesis and in late-vitellogenesis the concentrations of 17β-estradiol declined from 2-4 ng/ml to 1-2 ng/ml while testosterone concentrations remained large (50-60 ng/ml) (Amiri *et al.* 1996). Webb and others (2002) used blood plasma concentrations of testosterone, estradiol and 11-ketotestosterone to classify the stage of gonadal maturity in wild white sturgeon. Currently, the most viable methods for detecting sex steroids in fish plasma are immunoassay including radioimmunoassay (RIA), the standard method for over 20 years (Safi *et al.* 1999; Webb *et al.* 2001; Webb *et al.* 2002) and more recently developed Enzyme Linked ImmunoSorbent Assay (ELISA) methods (Nash *et al.* 2000).

Plasma proteins may serve as biomarkers for maturation of the white sturgeon. For example, either direct assay of vitellogenin, an egg yolk precursor protein

essential for vitellogenesis, or determination of total plasma protein may be useful monitors of sexual maturity. The expression of vitellogenin is dependent upon several sex steroids, with rising steadily in females during sexual maturation (Wheeler *et al.* 2005; Linares-Casenave *et al.* 2002). Blood plasma of vitellogenin can increase over one million-fold (to milligrams per milliliter of plasma) in mature female fish compared with immature females (Tyler *et al.* 1996). Estrogen stimulates the synthesis of vitellogenin, a phospholipoglycoprotein in the liver resulting in an increase in plasma concentrations during vitellogenesis (Doroshov *et al.* 1997). However, vitellogenin decreases when visible signs of atresia (marbled follicles) are present (Amiri *et al.* 1996; Linares-Casenave *et al.* 2002). RIA (Tyler *et al.* 1996), western blotting (Fossi *et al.* 2001), ELISA (Nelsen *et al.* 1998) and assay for alkaline-liable phosphoproteins (ALP) (Kramer *et al.* 1998) are used for vitellogenin quantitation in blood plasma. Vitellogenin is the predominant phosphorous containing protein in fish blood plasma and is relatively easy to detect.

Based on these different biochemical parameters in fish plasma, this study was conducted to determine whether Fourier Transform Infrared Spectroscopy (FT-IR) could be used to monitor changes in one or more of these components and then to correlate these changes with biological predictors of sexual maturity in sturgeon. FT-IR is a technique that can characterize biochemical components in complex matrices for qualitative identification and quantitative estimations. Deformation, bending and ring-vibrations of covalent bonds within the spectral range of 4000-400 cm^{-1} are determined. Utilization of an attenuated total reflectance (ATR) cell with

FT-IR spectroscopy provides a technique to assay a wide variety of constituents with little or no sample preparation. FT-IR spectroscopy combined with multivariate analysis has been widely utilized for identification of proteins, lipids and carbohydrate moieties (Al-Holy *et al.* 2006; Burgula *et al.* 2007). Concentrations of glucose (Petibois *et al.* 1999), protein (Petibois *et al.* 2001), and triglycerides (Petibois *et al.* 2002) in human plasma are determined by FT-IR. In fisheries, FT-IR is used in recent studies on marine ecotoxicology to detect nonylphenol (Cakmak *et al.* 2003) and 17β-estradiol (Cakmak *et al.* 2005) in rainbow trout liver. To our knowledge, no research to date has been conducted using FT-IR to detect sex steroids or vitellogenin in blood plasma, specifically for prediction of physiological or reproductive status. The objectives of this study were to utilize FT-IR spectroscopy combined with multivariate analysis to detect changes in the concentrations of sex steroids, vitellogenin and non-polar lipids in white sturgeon plasma collected at different stages of sexual maturity and to determine whether changes in these spectral features could be correlated with widely used biological parameters such as oocyte polarization index (PI).

Materials and Methods

Study Site Description

The white sturgeon (*Acipenser transmontanus*) females for this study were reared at Sterling Caviar (Wilton, CA) in outdoor tanks (9.1 m diameter, 1.5 m depth). The fish were maintained in good and warm water condition (ca. 20ºC) and exposed to a

natural photoperiod. Dissolved oxygen was continuously monitored and adjusted to stay at or slightly above saturation level. A cold-water site, approximately 48 km southeast of the warm-water site, held late vitellogenic females prior to caviar harvest (3.7 m diameter by 0.9 m deep tanks). The water source was from Lake Amador with tank temperatures varying from 10 to 13ºC. During the period of this study, fish were fed pelleted food manufactured by EWOS® at 3% body weight per day.

Twenty sturgeons (in either late vitellogenic or atretic period) were surgical biopsied in April 2007 for preliminary research study. Another twenty females (fork length 132-156 cm, weight 25-40 kg) were selected at random, individually tagged with passive integrated transponder (PIT) tags in September 2007; the sturgeons were surgical biopsied in September (N=20) and 15 of the 20 fish were in late vitellogenic period (the other five fish were in either pre-vitellogenic or post-vitellogenic period). The 15 late vitellogenic sturgeon were surgical biopsy again in November 2007. Unfortunately, four fish died during this time. Surgical ovarian biopsies were performed on these sturgeons to assess the stage of ovarian development. At each biopsy period, fish were anesthetized using tricane methanesulfonate (MS-222) and length, weight, and PIT tag number recorded.

Plasma and standard preparation

Three and one half milliliters of white sturgeon plasma was collected from the caudal vasculature of 40 fish and shipped frozen by overnight courier to Washington State University (Pullman, WA) for spectral analysis. Twenty sturgeons were biopsied

in April 2007, and an additional 20 fish were biopsied in September and 11 of the 20 fish were biopsied for a second time in November 2007. Oocyte polarization index (PI) values (Doroshov et al., 1997) and the plasma concentrations of sex steroids were determined (Webb et al., 2002) by Mariah Talbott and Dr. Webb in Bozeman Fish Technology Center (Bozeman, MT). Ten microliters of the plasma was applied to a glass slide and the plasma air dried under laminar flow at room temperature (ca. 20 ºC) for 1 h to obtain a uniform dry film. A second 10 µl of the plasma was applied on top of the dried film on the glass slide and air dried by placing under a fume hood for 24 h until the film was visibly dry and homogenous. Triplicate slides were prepared for each plasma sample. Sex steroids powders were purchased from Sigma-Aldrich Co. (St. Louis, MO), or were provided by US Fish and Wildlife Services, Bozeman Fish Technology Center, or the University of California, Davis, CA to identify and standardize FT-IR spectra features. The sex steroid standards included 17β-estradiol, 17α, 20α-dihydroxy-4-pregnen-3-one, 11-ketotestosterone, testosterone and progesterone. Sturgeon vitellogenin was isolated from white sturgeon and then purified (concentration of 26 µg/ml in phosphate buffered saline (PBS)) by Dr. Palumbo in the University of California, Davis (isolation method by Hiramatsu et al., 2002). The plasmas from a pre-vitellogenic sturgeon (one year before breeding) before and after treated with estrogen (vitellogenin was then induced with estrogen) were also collected by Dr. Palumbo, stored under -80 ºC and sent to Washington State University, Pullman for FT-IR spectra analysis to detect sex steroids and sturgeon vitellogenin.

FT-IR spectroscopy

FT-IR spectra were collected using a Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron Inc., San Jose, CA, USA). The glass slide with dried plasma spot was then placed in direct contact with an attenuated total reflection (ATR) zinc selenide (ZnSe) crystal and spectra taken (4000 to 400 cm^{-1}). Thirty spectra were collected at room temperature for each plasma sample, ten for each slide (N=3). FT-IR spectra were also obtained for each of the sex steroid (in powder form, 5 mg powder per analysis) and pure white sturgeon vitellogenin (with phosphate buffered saline). The resolution of FT-IR instrument was set at 4 cm^{-1} with each spectrum composed of a mean of 36 separate scans.

Data analysis

FT-IR data analysis was conducted using OMNIC (Thermo Electron Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, MA, USA) software. The data pre-processing, such as binning, smoothing followed by second derivative transformation (Huang et al., 2001) magnifies and aids in the visualization of small differences among spectra. Binning reduces the number of data points in a spectrum by n points into one. Binning often eliminates the imbalance problem associated with many array-based spectrophotometers (Al-Qadiri et al., 2008). Smoothing eliminates high frequency instrumental noise by adjacent data points (Lin et al., 2004). Second derivative transformation separates overlapping absorption bands, eliminates baseline

offsets, increases the apparent spectral resolution and provides an estimate of the number of overlapping bands within a region (Al-Holy et al., 2006). Data pre-processing algorithms, such as binning (2 cm^{-1}) and smoothing (Gaussian function over 10 cm^{-1}) were employed, followed by a second derivative transformation with a gap value of 12 cm^{-1}. After data pre-processing, principal component analysis (PCA) and partial least squares (PLS) models were developed. Due to the noise resulting from air, PCA and PLS analysis was based on the combination of the wavenumbers of 3600 to 2700 cm^{-1} and 1800 to 900 cm^{-1}.

Principal component analysis (PCA) is one of the most valuable interpretations derived from applied linear algebra and is a common multivariate statistical method for interpreting spectral data variance (Shlens 2005). The principle of PCA method determines which major factors affect the differences in the observed spectral features among samples and constructs a model to segregate plasmas based upon selected sample variances (Lin et al., 2003). The first principal component (PC1) contributes more than second principal component (PC2) to the variation observed between spectra, etc. PCA models can be used to sort plasmas by treatment differences and to confirm which wavenumbers provide the greatest contribution to the plasmas variance. PCA is based upon a second derivative analysis.

Partial Least Squares (PLS) model is a multivariate regression method to establish a relationship between reference value data and predicted value data, the larger the R value, the better and more rigorous the model (Abdi 2003). To establish a PLS model, the first step is to choose the optimal number of latent variables (Lin et al.,

2004). Too many latent variables decrease the precision of the model due to data over-fitting. Too low a number of latent variables will reduce the utility of the model since not all of the relevant data is used for its construction (Huang et al., 2001; Abdi 2003). Being able to establish a robust model and spectral library for predicting a physiological parameter such as sturgeon PI requires sufficient reference data and valid correlations between the reference value (PI) and the predicted value based upon spectral features determined.

Results and discussion

A typical FT-IR spectrum of white sturgeon plasma is presented in Figure 1. This spectrum can be separated into three basic regions: from 3600 cm^{-1} to 2700 cm^{-1}, from 1800 cm^{-1} to 1300 cm^{-1} and from 1300cm^{-1} to 900 cm^{-1}. The first two regions are protein and lipid regions and the third region contains spectral features associated with nucleic acid, polysaccharides and phospholipids. The region between 1800 cm^{-1} and 900 cm^{-1} often contains features for phospholipids, proteins and nucleic acids in complex biological matrices (Lin *et al.* 2005).

A summary of FT-IR absorption band assignments (between 4000 cm^{-1} and 400 cm^{-1}) is presented in Table 1. These band assignments are important for determining how the relative amounts of various components in fish plasma are changing as the fish progresses through vitellogenesis. Plasma is a very complicated biochemical matrix with steroids, polar and non-polar lipids (Webb *et al.* 2002) and proteins including phospholipoprotein such as vitellogenin (Wheeler *et al.* 2005). Fish

vitellogenin is a large molecule (native mw ~440 kDa in sturgeon) composed of the protein backbone phosphoryilated on serine moieties, quite a lot of lipids (~20%), and carbohydrates. It binds calcium and other cations during transportation from liver to the ovary (Bidwell *et al.* 1995). Vitellogenin is internalized by the oocyte via receptor-mediated endocytosis, degrades into two yolk proteins, lipovitellin and phosvitin, which can crystallize to form yolk platelets in the egg cytoplasm (Hiramatsu *et al.* 2002).

Figure 2 provides spectra of various steroids that are critical indicators of fish maturity. In the plasma of female white sturgeon, the concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone fluctuate as the fish becomes sexually mature (Amiri *et al.* 1996; Safi *et al.* 1999; Webb *et al.* 2005). The levels of these have been found to differ significantly in wild and farmed white sturgeon at various stages of sexual maturity (pre-vitellogenic, vitellogenic, post-vitellogenic and atretic) (Amiri *et al.* 1996). Steroid hormones are lipids and with unique spectral features (see Figure 1 and Figure 2) at wavenumbers from 3150 cm^{-1} to 2850 cm^{-1} (Table 1) (Petibois *et al.* 2002).

This is the first report to our knowledge, identifying spectral features of sex hormones in fish blood plasma. There are a number of distinctive spectral features around 3000 cm^{-1} for all of the steroid hormones examined in this study (Figure 2). Three peaks (~ 2960 cm^{-1}, 2929 cm^{-1} and 2850 cm^{-1}) appear to be associated with steroid hormones in plasma. These peaks were also present in sturgeon blood plasma (Figure 1) and were distinct from other spectral features, for example non-polar lipid

(~1700 cm^{-1}), and several proteins (~1650 cm^{-1} and 1540 cm^{-1}) that were important for establishing either PCA or PLS models (Figure 5, 7, and 10) in this study.

Pure vitellogenin (concentration of 26 µg/ml in phosphate buffered saline (PBS)) was isolated from white sturgeon female plasma by Dr. Palumbo in the University of California, Davis with the method from Hiramatsu *et al.* (2002). Figure 3 shows the pure sturgeon vitellogenin spectrum. A clear peak around 1100 cm^{-1} most likely represents vitellogenin, apparent even with spectral noise between 4000 cm^{-1} and 2000 cm^{-1} due to the interfere of the PBS buffer (Fig.3).

Figure 4 shows a comparison of the FT-IR spectra of plasma from the same sturgeon before and after estrogen treatment. Changes in the sex steroids region (~3000 cm^{-1}), lipid region (~1700 cm^{-1}) and around 1080 cm^{-1} (vitellogenin region) were clearly manifested. This result strongly supports our finding about sex steroids and vitellogenin region on the FT-IR spectra.

From Figure 5, significant changes in the spectra were observed in the sex steroids region (~3000 cm^{-1}), lipid region (~1700 cm^{-1}) and around 1080 cm^{-1} for the P=O region. A significant increase in peak amplitude and area was observed around 3000 cm^{-1} corresponding to increases in sex hormone levels a single fish as the fish became more sexually mature from September to November. Similar results were observed for other fish (N=10) from the same experimental treatment. Furthermore, around 1080 cm^{-1} region, which includes nucleic acids, phospholipids and glycogen (Wong *et al.* 1991; Dovbeshko *et al.* 2000), peak height and peak shape changed as the fish matured. Because vitellogenin is a phospholipoglycoprotein and the level will

fluctuate with sturgeon maturity, it can be hypothesized that the region around 1080 cm^{-1} may correlate with changes in vitellogenin levels in sturgeon plasma. Furthermore, an elevated vitellogenin level was expected as the fish oocytes are still depositing yolk and increasing in size from September to November (Amiri *et al.* 1996; Doroshov *et al.* 1997). In the region around 1700 cm^{-1}, which includes spectral features of triglycerides and cholesterol esters (Manoharan *et al.* 1993; Voortman *et al.* 2002) changes were also observed as the fish matured. More research needs to be conducted to determine precisely the biochemical changes that occur as sturgeon mature and how these changes are reflected in the compositional properties of plasma.

As noted in Figure 5, in the region from 1700 to 1200 cm^{-1} (mainly protein region, including protein bending and Amide I and Amide II stretch) there are FT-IR spectral features that may correlate with maturity, although these differences are not as distinctive as those observed in spectral regions associated with sex steroids, lipids and vitellogenin. However, these subtle differences may reveal important information about sexual maturity in white sturgeon. From Figure 6, clearly distinguishable differences in spectra of plasma samples from the same white sturgeon at these two different months were shown, which may not be clearly visible from the FT-IR raw spectra data. There was an increase in peak height around the regions of 1540 cm^{-1} and 1395 cm^{-1}, both of which are amino acids bend and stretch regions. This may be due to an increase in the total plasma protein level that occurs during vitellogenesis (Amiri *et al.* 1996). The peak around 1455 cm^{-1} region shifted slightly to a lower wave number from September to November which may reflect compositional changes

in the lipids and proteins during the vitellogenin formation period (Amiri *et al.* 1996). Further works need to be done to determine what trends are observed throughout the entire maturation cycle of a single sturgeon across an entire year. However, at this point, it appears that FT-IR is at least one technique that can monitor some of the relevant biochemical changes that occur during sturgeon sexual maturation.

Figure 7 shows a clear separation (two-dimensional PCA) between the spectral features of plasma samples taken from a single fish in September and then again in November at a different sexual maturity. Loading plots were used to analyze which spectral region contributed most to this segregation. From Figure 8 (a) and (b), PC1 was around 1700 cm^{-1} and the second, around 3000 cm^{-1}, both of which are the hormone regions. PC1 contributed about 61% to the variation between samples, and PC2, about 13%.

During different maturity periods, the chemical components in white sturgeon plasma will change, to some extent, especially fish hormone level, total lipid level and the level of specific proteins, such as vitellogenin. PCA based model was used to sort plasma samples from different fish at different stages of sexual maturity. A clear separation is shown in Figure 9. The spectra of plasma from atretic fish was clearly separated from those from the three vitellogenic phases indicating that there were differences between vitellogenic phase and atretic phase, and among the three divisions of vitellogenesis that may be useful for determining the level of sexual maturity.

Table 2 shows the oocyte polarization index (PI) values for 31 sturgeon at

different time periods and were within the range of 0.06 and 0.40. Eleven fish were tracked from September to November to monitor changes in the PI values along with fish maturity stages. As sturgeon become more mature, PI values decrease (Table 2 (b)).

Figure 10 is the representative PCA result based on different PI ranges, which reflected the fish maturity levels. There was a clear separation result of the PCA. Then, loading plots were utilized to check which region accounts for PC1. From Figure 11 (a) and (b), the regions around 1700 cm^{-1} and around 3000 cm^{-1} contributed the most to the PCA separation and corresponds to the fish hormone indicating that there is a relationship between hormone level and PI value. These results support the findings of others (Doroshov *et al.* 1998) that as the sex steroids level increases, the PI value decreases as the sturgeon matures.

Partial Least Squares (PLS) models were developed to predict the actual PI value of white sturgeon females (Figure 12). To establish a PLS model, the first step is to choose the optimal number of latent variables (Lin *et al.* 2004). Too many latent variables decrease the precision of the model due to data over-fitting. Too low a number of latent variables will reduce the utility of the model since not all of the relevant data is used for its construction (Huang *et al.* 2001; Abdi 2003).

Validation results for prediction of PI values from spectral features (6 latent variables) between 0.12 to 0.40 from white sturgeon females resulted in a high coefficient of determination (R=0.95) and a relatively low standard error of prediction (SEP=2.18%) based on the regions of 3600 cm^{-1} to 2700 cm^{-1} and 1800 cm^{-1} to 900

cm^{-1} of the FT-IR spectra. This result shows that there is a potential to establish robust PLS model for predicting the PI value of sturgeon from measurements of blood plasma, potentially reducing the need for surgical biopsy.

Conclusions

Biochemical components in white sturgeon plasma including sex steroids and vitellogenin can be detected using FT-IR spectra and used to predict sexual maturity in female fish. FT-IR combined with multivariate analysis (PCA, PLS) may provide a rapid, less invasive and precise assessment method to segregate and sort fish at different maturity stages (pre-vitellogenic, vitellogenic, post-vitellogenic and atretic) and predict oocyte PI values which are currently only available by traditional surgical biopsy. Further works need to be done to determine appropriate parameters throughout the entire maturation cycle of a single sturgeon across an entire year, especially from the late vitellogenic period to the very early atretic period.

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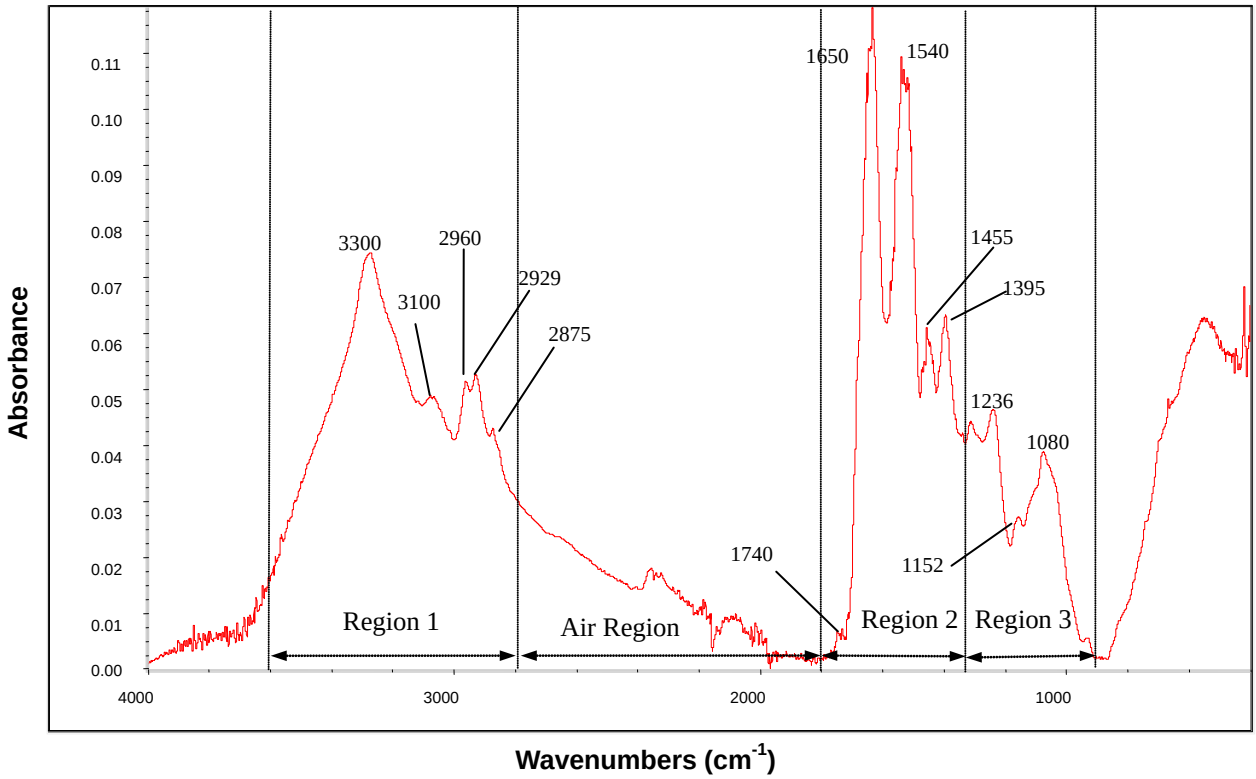
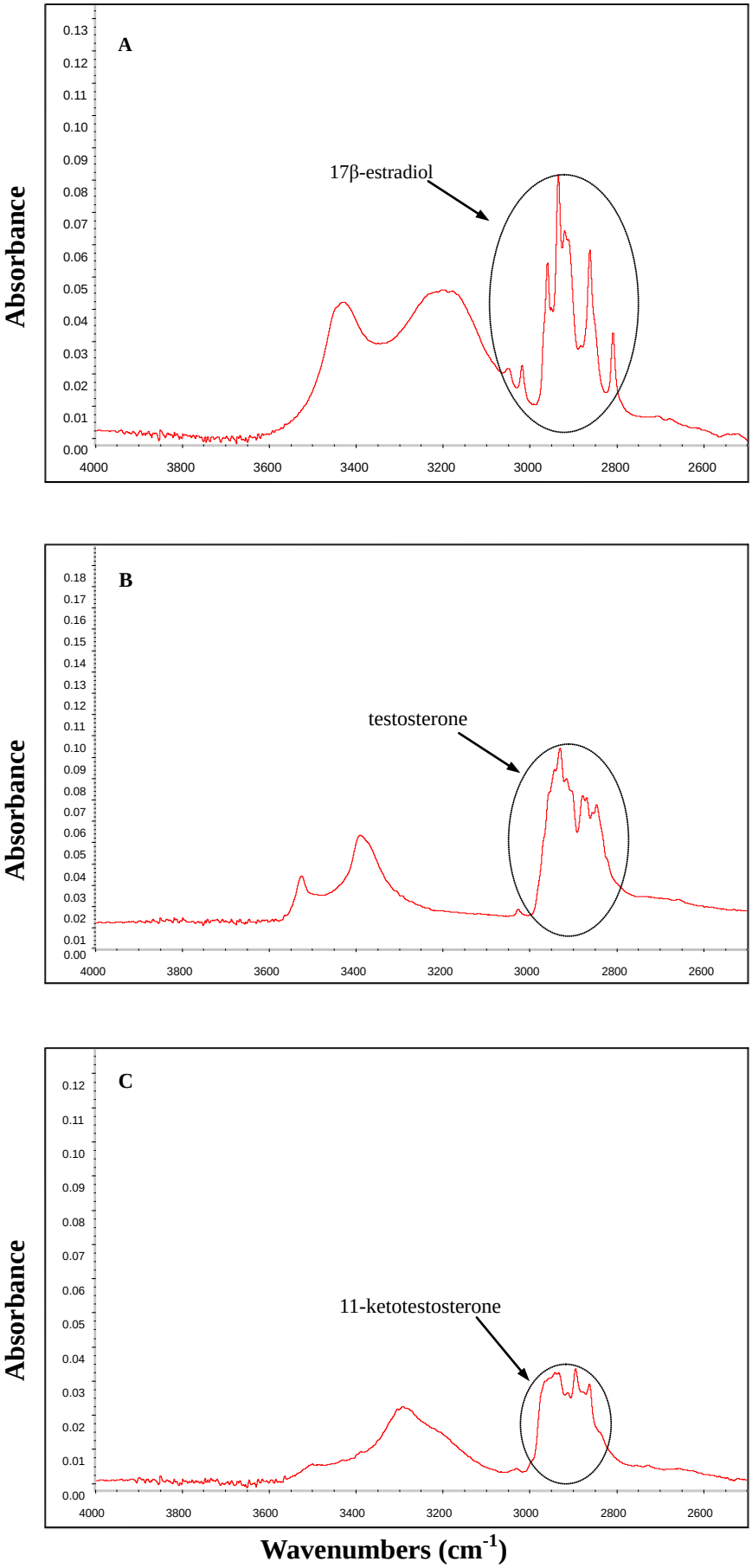


Figure 1 Typical FT-IR absorbance spectrum (before data pre-processing) for white sturgeon (*Acipenser transmontanus*) plasma

Table 1 Absorption band assignments in the FT-IR spectral region*

Wavenumber (cm^{-1})	Spectral Assignment
~3300	N-H stretching of proteins and O-H stretching of polysaccharides
~3100	Olefinic=CH stretching vibration: lipids, cholesterol esters
~2960	CH_3 asymmetric stretch of methyl groups: mainly lipids
~2929	CH_2 asymmetric stretch of methylene groups: mainly lipids
~2875	CH_3 symmetric stretch of methyl groups: mainly lipids
~1740	C=O of ester functional groups: triglycerides, cholesterol esters
~1650	C=O stretch of amides of proteins: Amide I
~1540	N-H bend and C=O stretch of amides of proteins: Amide II
~1455	CH_2 bending: mainly lipids, with little contribution from proteins
~1395	COO^- symmetric stretch: fatty acids and amino acids
~1236	P=O asymmetric stretch: mainly nucleic acids and phospholipids
~1152	CO-O-C asymmetric stretch: glycogen and nucleic acids
~1080	P=O symmetric stretch: mainly nucleic acids and phospholipids

*see Refs. Cakmak *et al.* 2003; Cakmak *et al.* 2006; Dovbeshko *et al.* 2000; Eruhimovitch *et al.* 2006; Haris *et al.* 1999; Lin *et al.* 2003; Manoharan *et al.* 1993; Mariey *et al.* 2001; Nara *et al.* 2002; Petibois *et al.* 1999; Petibois *et al.* 2001; Petibois *et al.* 2002; Petibois *et al.* 2003; Voortman *et al.* 2002; Wong *et al.* 1991.


A
17β-estradiol
B
testosterone
C
11-ketotestosterone
Absorbance
Absorbance
Absorbance
Wavenumbers (cm-1)

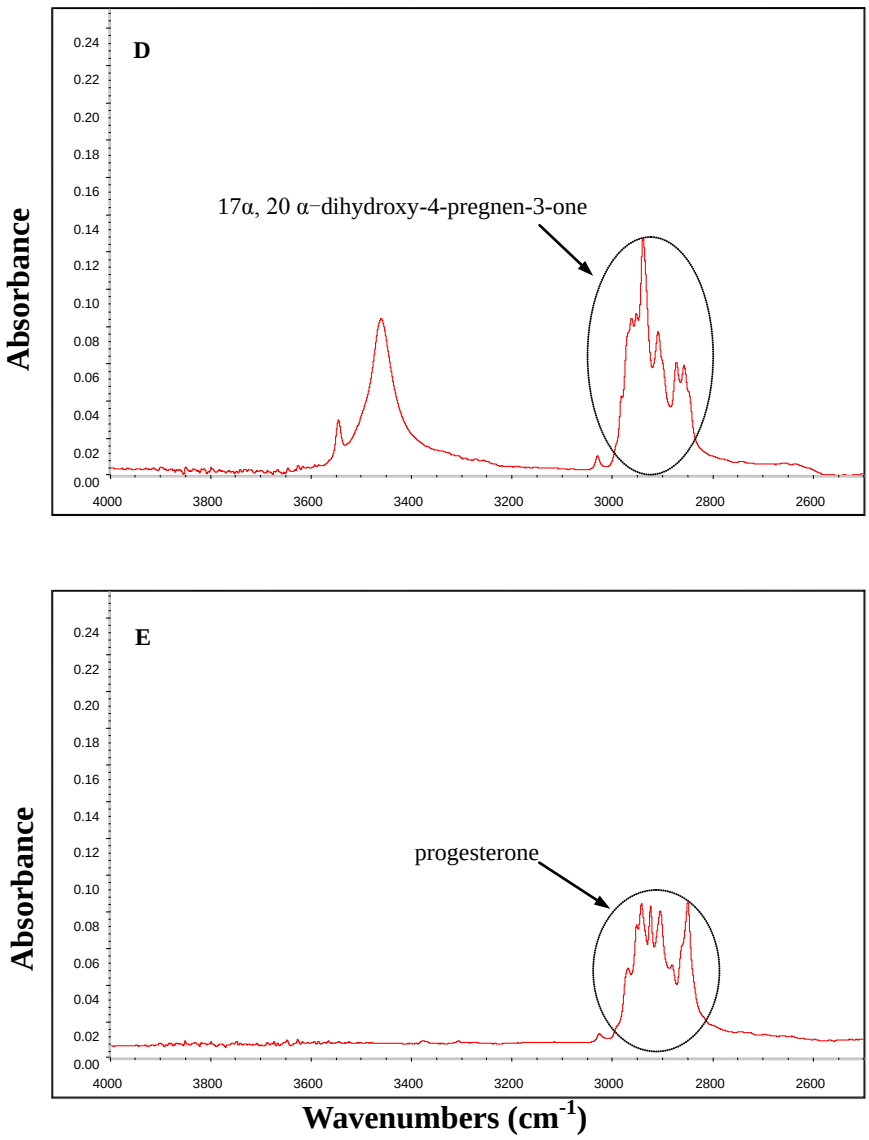


Figure 2 Typical FT-IR spectra for sex steroids: (A) 17β-estradiol (B) testosterone (C) 11-ketotestosterone (D) 17 α, 20α−dihydroxy-4-pregnen-3-one and (E) progesterone.

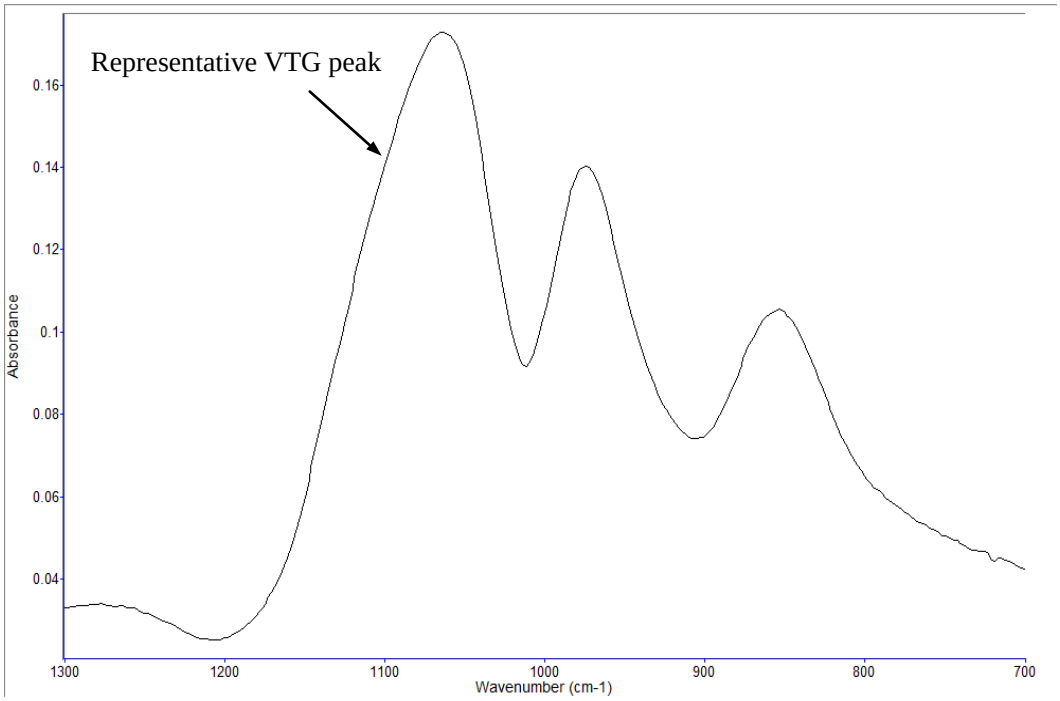


Figure 3 Typical FT-IR spectra for pure white sturgeon female vitellogenin (with phosphate buffered saline).

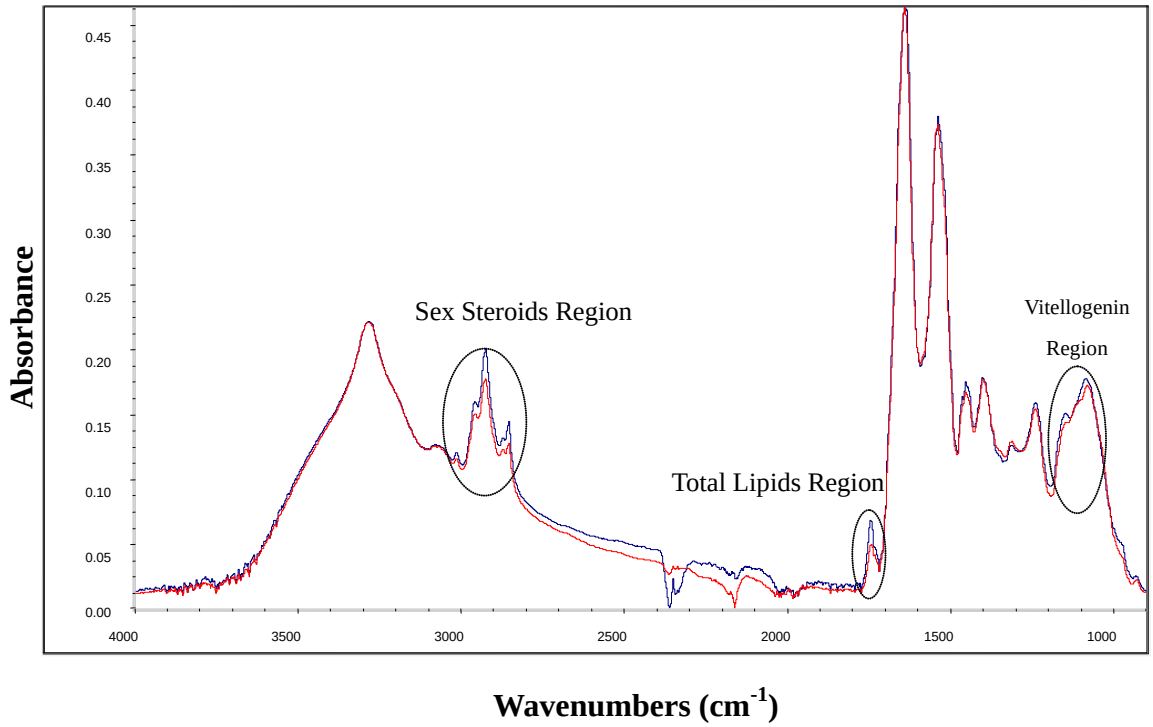


Figure 4 Comparison of FT-IR spectra for plasma from the same white sturgeon female before (Red color) and after (Blue color) estrogen treatment.

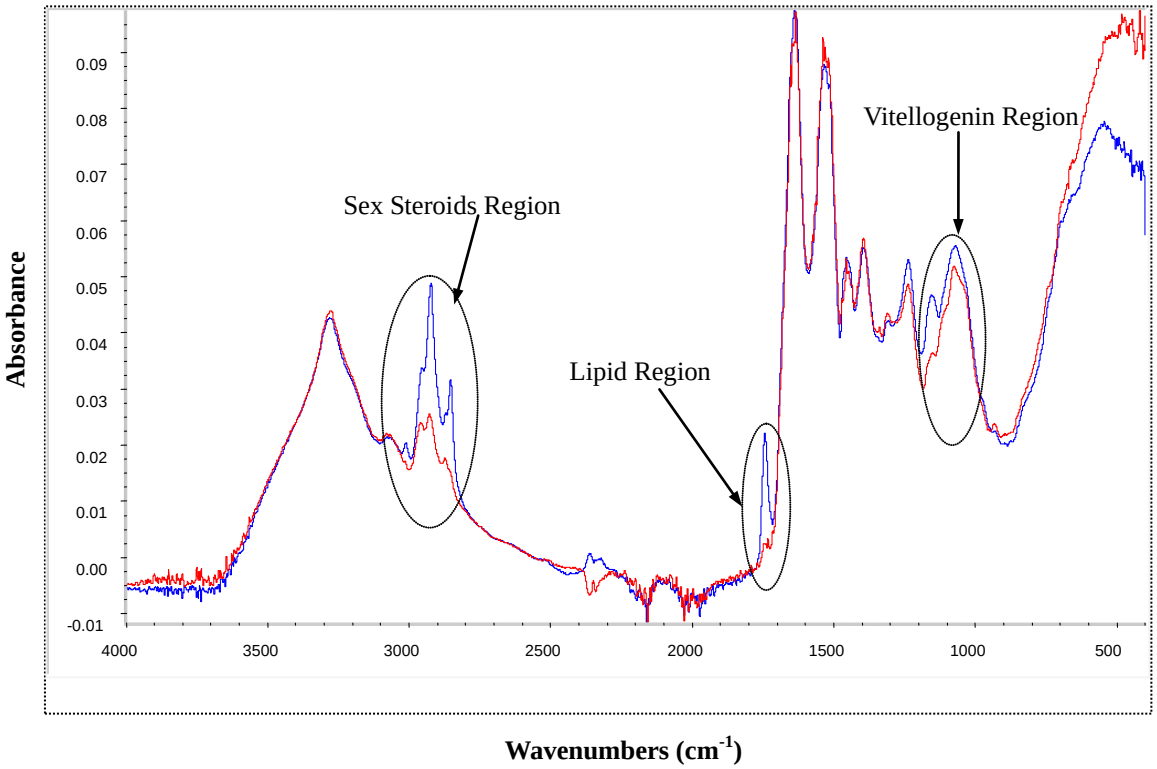


Figure 5 Representative comparison of FT-IR spectra for plasma from the same white sturgeon female at selected months (Red: 2007 September; Blue: 2007 November) as the fish was becoming sexually mature (September PI value: 0.231±0.042, November PI value: 0.129±0.023. Sturgeon was classified as late vitellogenic in both September and November).

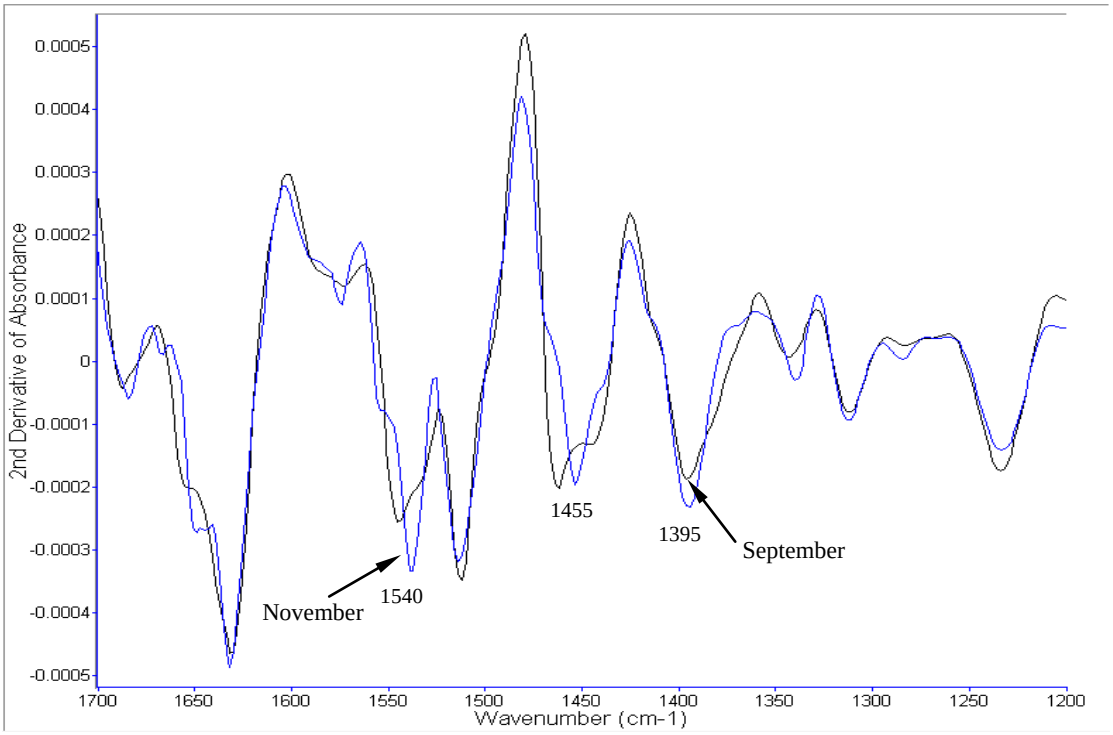


Figure 6 Representative second derivative transformation (12-point gap) of FT-IR spectra of plasma from the same white sturgeon at two selected months (September and November).

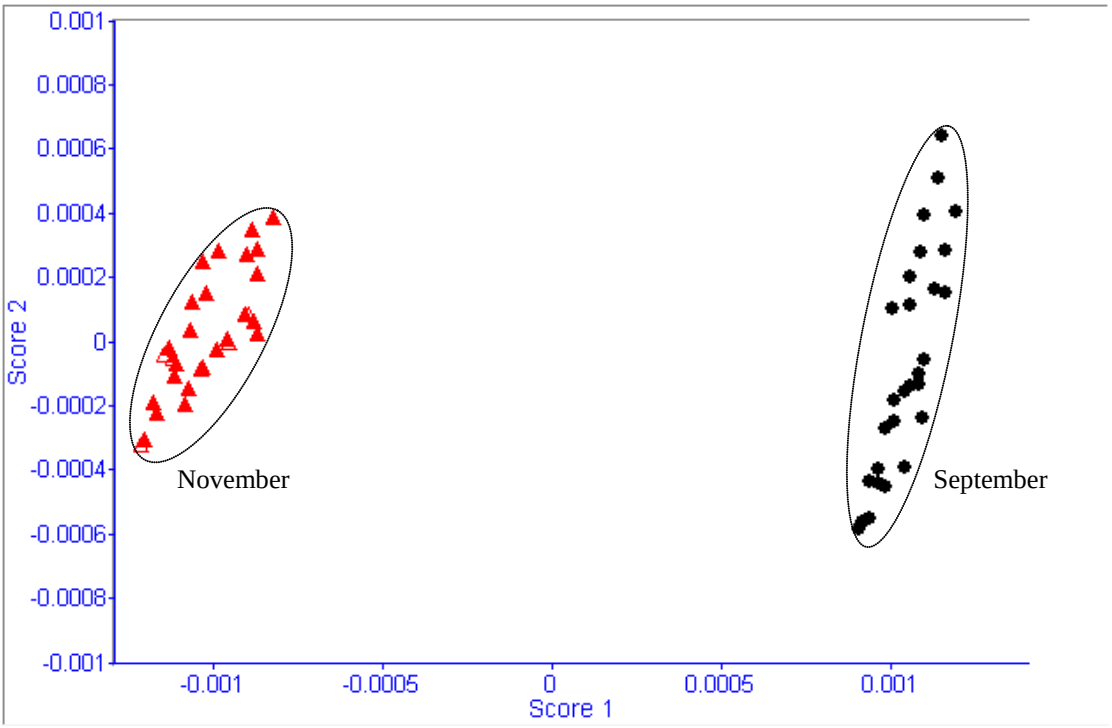


Figure 7 Representative two-dimensional PCA clustering results for plasma from white sturgeon at selected months during sexual maturation.

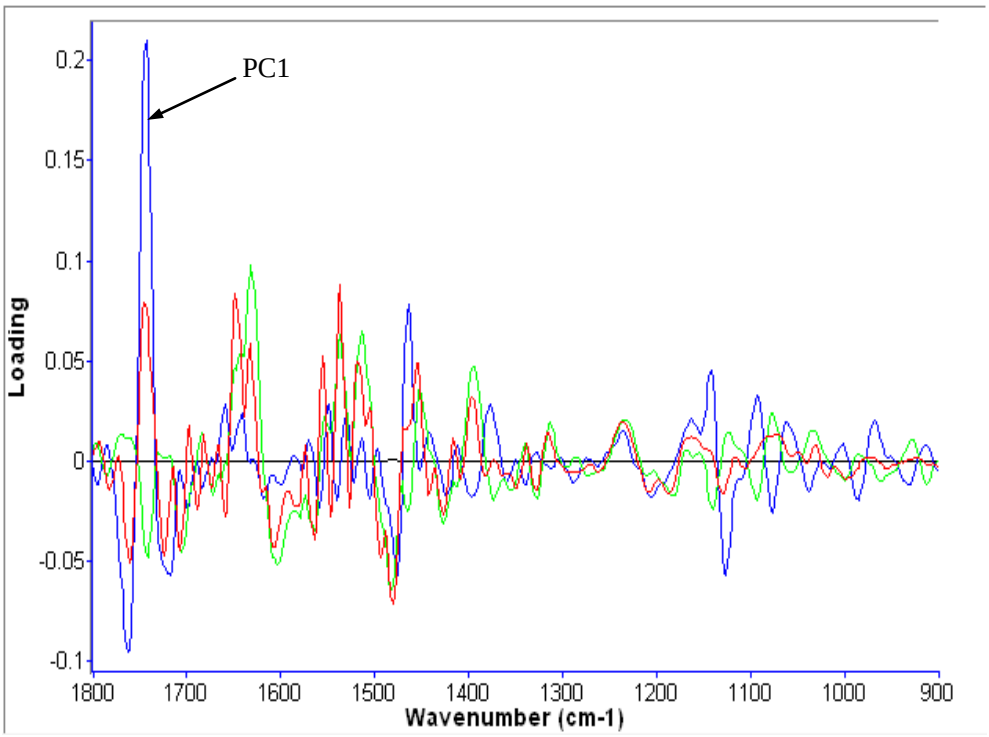


Figure 8 (a) Representative loading plot of first principal component (PC1) obtained from PCA results of FT-IR spectra of plasma from the same white sturgeon female from 1800 cm^{-1} to 900 cm^{-1}.

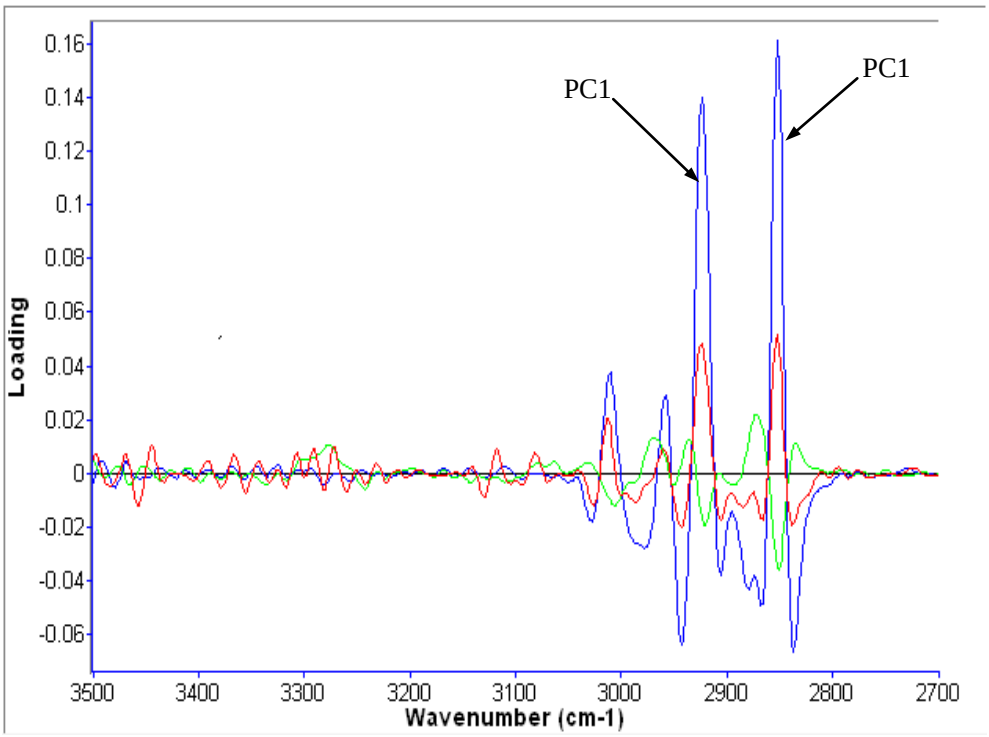


Figure 8 (b) Representative loading plot of first principal component (PC1) obtained from PCA results of FT-IR spectra of plasma from the same white sturgeon female from 3500 cm^{-1} to 2700 cm^{-1}.

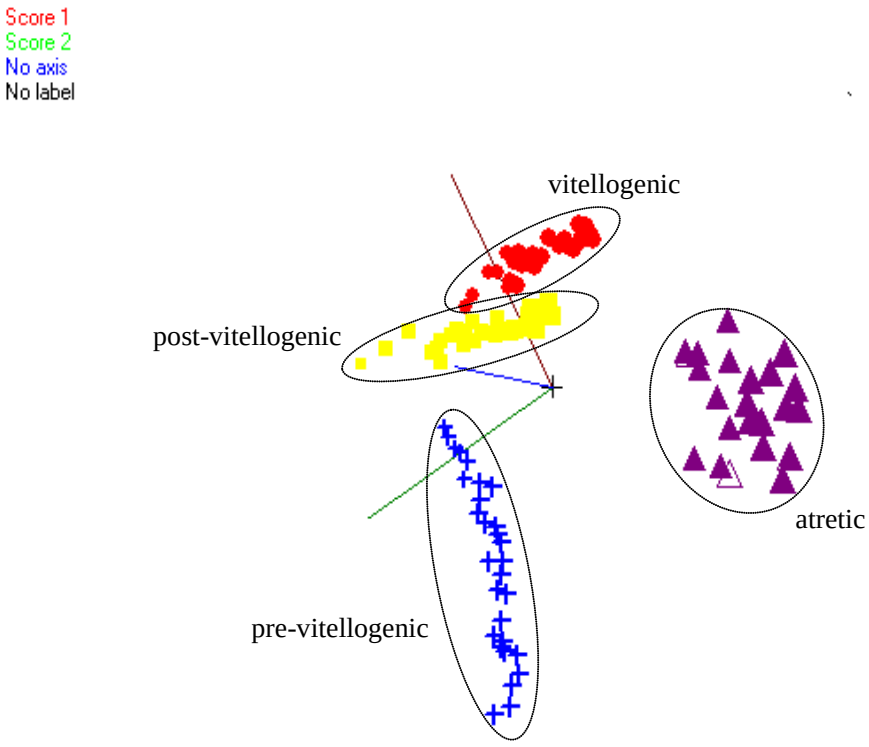


Figure 9 Representative three-dimensional PCA clustering results for stages of maturity of white sturgeon using plasma from selected fish (N=20).

Table 2 (a) Oocyte Polarization Index (PI) values of sturgeons in April 2007

Sample ID	PI (2007-04-09)
1	0.1067
2	0.1736
3	0.0857
4	0.1513
5	0.3109
6	0.3317
7	NA*
8	NA*
9	0.1185
10	0.1439
11	0.1181
12	0.1311
13	0.1277
14	0.1102
15	0.1129
16	0.1768
17	0.0962
18	0.0657
19	0.0840
20	0.2083

* When sturgeons enter the atretic period, the PI value measurement is not available because follicles are too soft.

Table 2 (b) Oocyte Polarization Index (PI) values of sturgeons in September and November 2007

Sample ID*	PI (2007-09-10)	PI (2007-11-16)
182F	0.2310 (0.053)	0.1736 (0.048)
205E	0.2294 (0.027)	0.1360 (0.020)
2E7C	0.2749 (0.081)	0.2411 (0.059)
3D08	0.2814 (0.065)	0.2137 (0.034)
4458	0.3884 (0.054)	0.1919 (0.014)
401A	0.1866 (0.075)	0.2236 (0.050)
483D	0.2892 (0.069)	0.2289 (0.041)
4B34	0.3000 (0.055)	0.1955 (0.020)
6D7E	0.2307 (0.042)	0.1291 (0.027)
795C	0.2371 (0.036)	0.1618 (0.050)
7E07	0.3161 (0.068)	0.1862 (0.030)

*These eleven fish are tracked to monitor the PI value changes from September to November.

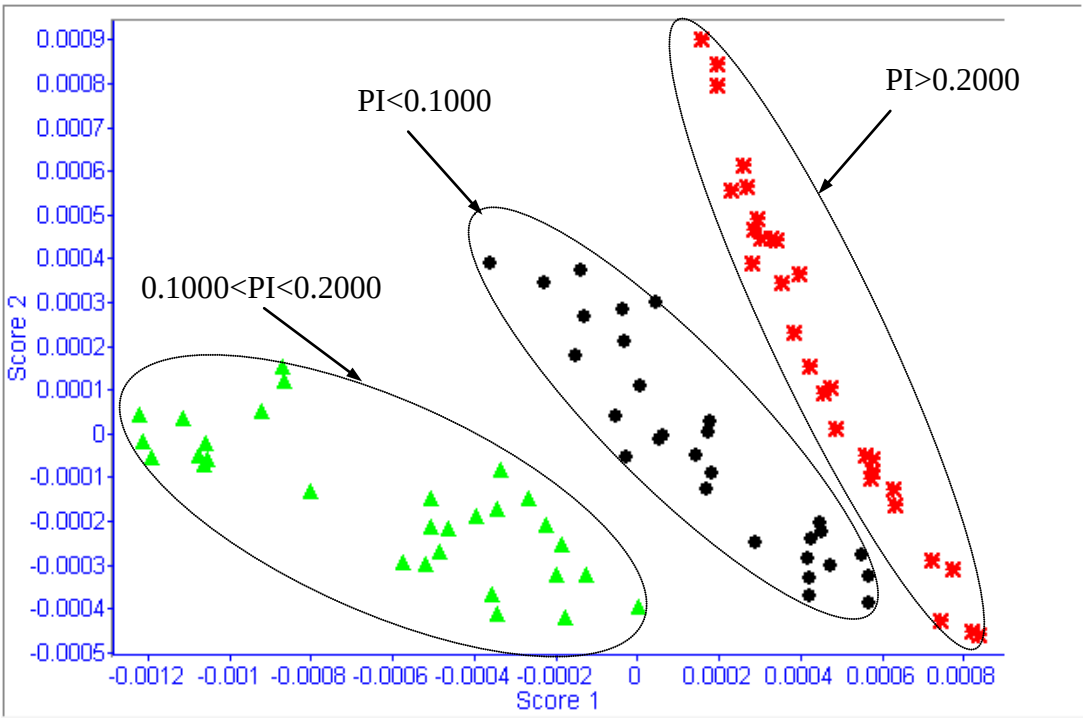


Figure 10 Representative two-dimensional PCA clustering results for fish plasma based on PI values---PI value<0.1000 (spawning in 2 weeks), 0.1000<PI value<0.2000 (spawning in 1 month) and PI value>0.2000 (spawning in 2 months) (N=15).

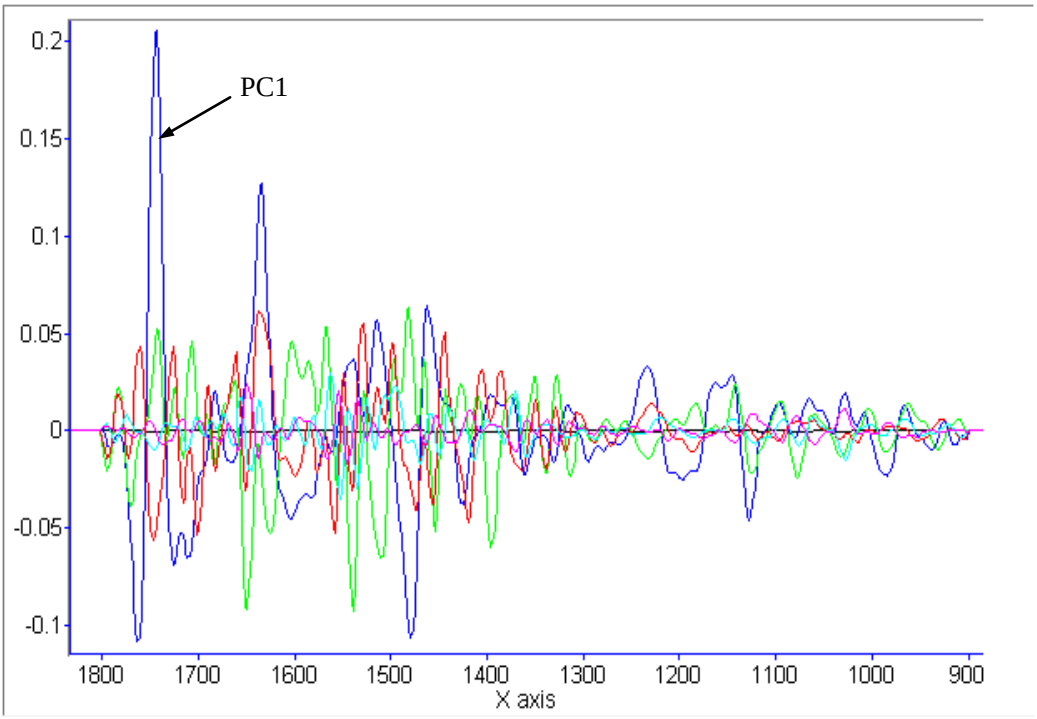


Figure 11 (a) Representative loading plot of first principal component (PC1) obtained from PCA results of FT-IR spectra of plasma based on PI values from 1800 cm^{-1} to 900 cm^{-1}

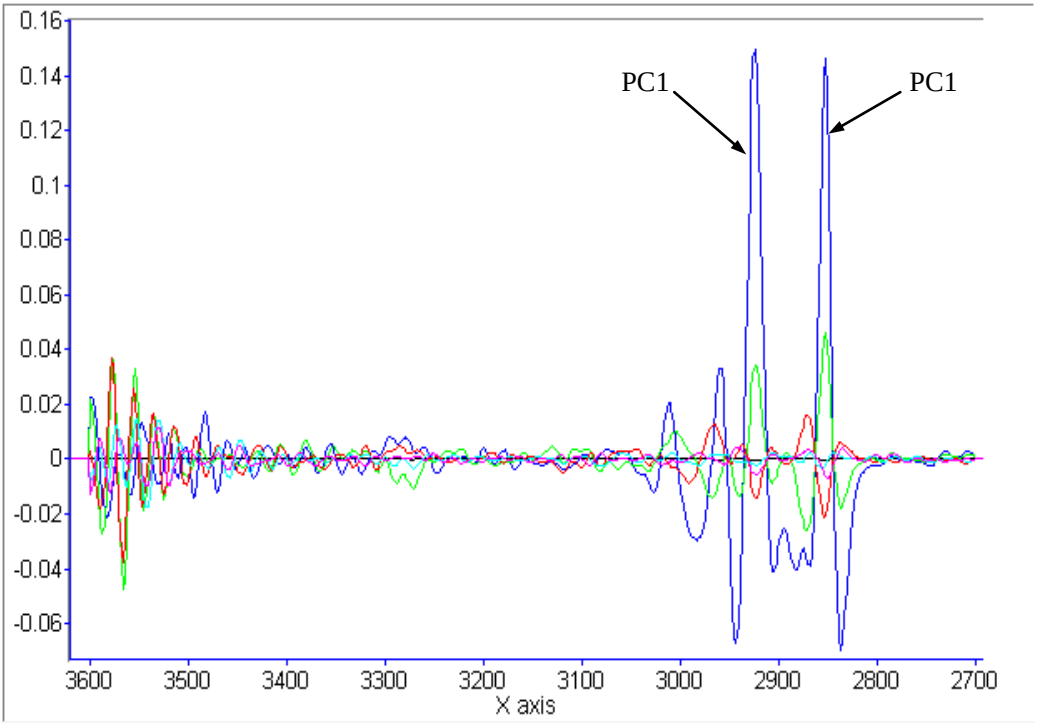


Figure 11 (b) Representative loading plot of first principal component (PC1) obtained from PCA results of FT-IR spectra of plasma based on PI values from 3600 cm^{-1} to 2700 cm^{-1}

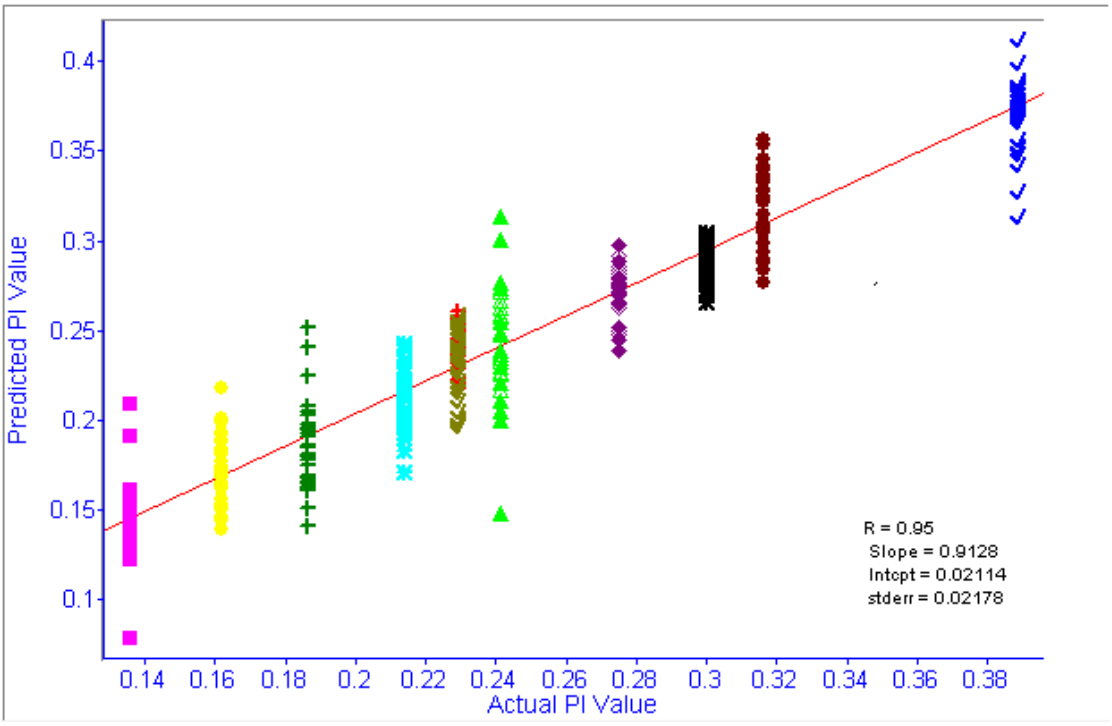


Figure 12 Comparison between the actual and predicted PI values (0.12<PI<0.40) of white sturgeon females (N=11)

CHAPTER THREE

A study of morphological and immunochemical parameters associated with ovarian atresia and quality of caviar in white sturgeon (*Acipenser transmontanus*) females by Fourier Transform Infrared Spectroscopy (FT-IR) and multivariate analysis

XIAONAN LU[1], MARIAH TALBOTT[2], MOLLY WEBB[2], JOEL VAN EENENNAAM[3], JAVIER LINARES-CASENAVE[3], SERGE DOROSHOV[3], BARBARA RASCO[1*]

[1]School of Food Science, Washington State University, Pullman, WA 99163, USA

[2]Bozeman Fish Technology Center, Bozeman, MT 59715, USA

[3]Department of Animal Science, University of California, Davis, CA 95616, USA

[*]Corresponding author. Tel: (509) 335-1858; fax: (509) 335-4815. E-mail address: rasco@wsu.edu (B.A. Rasco)

Abstract

Fourier Transform Infrared Spectroscopy (FT-IR, 4000-400 cm^{-1}) spectral features of white sturgeon (*Acipenser transmontanus*) (N=11) plasma and egg were determined to study the morphological and immunochemical changes from the late vitellogenesis to atresia over a nine month period with the objective of using these changes to predict caviar quality. FT-IR spectra of plasma changed at various maturity levels. Clear segregation of plasma according to maturity stage was evident using Principal Component Analysis (PCA) reflecting changes in the levels of sex steroids and vitellogenin. It was possible to predict stage of maturity (late vitellogenesis vs. early

atresia) about 70% of the time using Soft Independent Modeling of Class Analogy (SIMCA) models. Combination of second derivative transformation, PCA cluster model and SIMCA pass test together can make a precise prediction about the maturity stage of a specific sturgeon female. A rigorous Partial Least Squares (PLS) model was established to predict Polarization Index (PI) values between 0.1 and 0.3 (R=0.98, SEP=1.01%) based upon spectral features. Few changes were observed in spectral features of the roe recovered at selected maturity stages. FT-IR plasma spectra may provide a useful tool for predicting maturity stage and timing of roe harvest.

Keywords:

FT-IR; sturgeon; atresia; plasma; caviar; sex steroids; oocyte polarization index (PI); vitellogenin; PCA; SIMCA; PLS

Introduction

White sturgeon (*Acipenser transmontanus*) were studied for many years by aquaculturists and food scientists as a source of meat and caviar, Investigations of the reproductive physiology of this fish has been ongoing since this is important for determining when fish can be harvested for caviar (Van Eenennaam *et al.* 1996; Billard *et al.* 2001). Ovulation in the sturgeon is under multiple biochemical controls including sturgeon gonadotropin-I (GTH-I), gonadotropin-II (GTH-II) and dopamine (DA). As the sturgeon females progresses from the vitellogenic period to ovulation, the contents of sex steroids and vitellogenin in sturgeon blood and egg follicles fluctuate (Moberg *et al.* 1995; Doroshov *et al.* 1997; Patino *et al.* 2001; Goncharov 2002).

The period from the late vitellogenesis to early atresia is crucial to successful final ovulation and egg yield and quality (Webb *et al.* 2000). Failure to detect final germinal vesicle breakdown (GVBD) will result in the sturgeon enter atresia, which is promoted by raised temperature, photoperiod and stress (Webb *et al.* 1999; Webb *et al.* 2001; Linares-Casenave *et al.* 2002). Ovarian atresia is the process by which ovarian follicles are degenerated and subsequently resorbed by the ovary. Even the very early atretic stage may result in a reduction in the firmness, flavor and shelf life of caviar, and sometimes the complete loss of caviar.

Currently, the only means to assess maturity stage of white sturgeon female is measurement of oocyte polarization index (PI) value requiring invasive surgical biopsy. PI is the ratio of the distance of the germinal vesicle (nucleus) from the animal

pole to the oocyte animal-vegetal axis diameter and indicates morphogenetic changes in the ovarian follicle occurring during late vitellogenesis leading up to oocyte maturation competence (OMC) (Doroshov *et al.* 1997). It is widely accepted that it is better to select females with PI scores of less than 0.1 for spawning induction (Chapman *et al.* 2007). However, this PI measurement method by surgical biopsy is very stressful to sturgeon, which can cause sturgeon females to become reluctant to ovulate and easily go into atresia. Sex steroids and vitellogenin (VTG) are two good bio-markers for investigators to use to monitor sturgeon female maturity conditions. General trend of each sex steroid and VTG content in sturgeon blood during reproduction cycle was elucidated with data compiled from various research papers. Estradiol-17β (E_2), the major sex steroid in female blood plasma increases dramatically during vitellogenesis and reduces when vitellogenesis is completed. VTG has the same trend as E_2 since E_2 stimulates the liver to produce VTG, which is incorporated into the developing oocyte through blood transportation (Moberg *et al.* 1991; Bidwell *et al.*, 1995; Amiri *et al.* 1996; Barannikova *et al.* 2002; Linares-Casenave *et al.* 2003; Barannikova *et al.* 2004). Recently, researchers tried to use sex steroids and VTG as parameters to discriminate and predict sturgeon female maturity stages. Doroshov *et al.* (1994) and Moberg *et al.* (1995) both indicated that plasma concentrations of estradiol can be used to discriminate vitellogenic stages in sturgeon. Webb *et al.* (2002) reported that plasma testosterone (T) and E_2 were the best predictors of sex and stage of maturity. Linares-Casenave *et al.* (2003) reported that concentrations of VTG in plasma can discriminate between pre-vitellogenic and

vitellogenic females. Malekzadeh Viayeh *et al.* (2006) reported that plasma T and E2 plus either age, total length, fork length or weight were the best predictors to distinguish sturgeon maturity stage. However, the data of sex steroids and VTG contents in sturgeon blood during the period of late vitellogenesis to early atresia are limited and fragmented (Webb *et al.* 2005). Radioimmunoassay (RIA) (Webb *et al.* 2002) and Enzyme Liked ImmunoSorbent Assay (ELISA) (Nash *et al.* 2000) are two common methods to determine sex steroids contents in fish blood. Several methods are available to determine vitellogenin content in fish blood, including RIA (Tyler *et al.*, 1996), ELISA (Linares-Casenave *et al.* 2003), western blotting (Hiramatsu *et al.* 2002) and other chemical techniques.

Fourier Transform Infrared Spectroscopy (FT-IR) is an advanced technique that can characterize biochemical functional components in complex matrices and allow for qualitative identification and quantitative estimations within the spectral range of 4000-400 cm^{-1}. Utilization of an attenuated total reflectance (ATR) cell with FT-IR spectroscopy provides a technique to assay a wide variety of constituents with little or no sample preparation (Lin *et al.* 2005). The FT-IR spectral features of sex steroids and vitellogenin in sturgeon female plasma were identified and characterized as explained previously. The objectives of this study were to investigate the morphological (PI value) and immunochemical (sex steroids and vitellogenin content) parameters along the period from late vitellogenesis to atresia and to determine if FT-IR analysis could predict some of these changes. During this study, fifteen sturgeon raised in California were monitored for nine months.

Materials and Methods

Study Site Description

The site description and aquaculture condition are the same as in Chapter 2.

Fifteen late vitellogenic (2001 year class, fork length 132-156 cm, weight 25-40 kg) females were sampled on September 10, 2007 for the first time at the Wilton warm water site of Sterling Caviar, LLC. Sturgeons were individually tagged with passive integrated transponder (PIT) tags and moved the next day to the cold water site and held in two 12' diameter circular tanks. Due to unsatisfied cultured conditions, especially the low dissolved oxygen in cold water during a two weeks period, there were four deaths between November 1st and 16th. White sturgeons were subsequently sampled at two months intervals through March. Two days after the March sampling, the fish were returned to the warm-water site to induce from the late vitellogenesis to ovarian follicular atresia and sampled two more times on April 2, 2008 and April 17, 2008 for the atresia study. At the last sampling ten remaining eleven females were in various stages of ovarian atresia.

Individual sturgeon was captured and held in a water bath of MS-222 (100 ppm) until the decrease of opercular movements to about one beat per 30 seconds. The fish was weighed, measured, and the pit tag number recorded. Blood was collected from the caudal vasculature (2-10 ml heparinized vacutainers) and kept on the ice. Blood will be centrifuged (3400 rpm for 5 min) and the plasma collected. Three and one half milliliters of plasma from each fish during selected sample collection periods were

frozen and sent to Washington State University (Pullman, WA) for FT-IR plasma spectral analysis. Eggs were collected with a catheter (4.5 mm ID rigid teflon tubing) through a small abdominal incision (6-8 mm). Approximately 10 ml of eggs placed into a 2 oz whirl-pak were immediately placed on dry ice and sent by overnight courier to Washington State University (Pullman, WA) for FT-IR egg spectral analysis.

Standard preparation of plasma and caviar

The plasma standard preparation is the same as in Chapter 2.

Three frozen eggs from each sturgeon sample were retrieved randomly from the 2 oz whirl-pak and thawed under room temperature (ca. 20 ºC) for 30 min to receive a soft, flexible and fresh status. Eggs were put in the center of a Petridish. A glass-slide was pressed to the surface of the eggs so as to flatten them. The eggs were covered with the glass slide under the fume hood to be air dried for 24 h to obtain a visibly dried, flat and smooth outer layer.

FT-IR spectroscopy

FT-IR spectra were collected using a Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron Inc., San Jose, CA, USA). The glass slide with plasma dried spot was put in direct contact with an attenuated total reflection (ATR) zinc selenide (ZnSe) crystal. Twenty four spectra were collected at room temperature for each plasma sample, eight for each slide (N=3). The surface of each sturgeon egg

sample was also put in direct contact with the ATR ZnSe crystal. Thirty spectra were collected at room temperature for egg sample, ten for each egg (N=3). The resolution of FT-IR machine was set at 4 cm^{-1} with each spectrum composed of a mean of 36 separate scans.

Data analysis

FT-IR data analysis was conducted using OMNIC (Thermo Electron Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, MA, USA) software. FT-IR spectral features in some regions often look similar because those chemical components in fish plasma have subtle changes during different maturity stages. Therefore, data pre-processing algorithms, such as “automatic baseline correction” and “normalization” were utilized to adjust tilted baselines, compensate for pathlength effect and enhance the spectral differences. Then, other data pre-processing algorithms steps were employed, such as binning (2 cm^{-1}) and smoothing (Gaussian function over 6 cm^{-1}). This was followed by a second derivative transformation with a gap value of 12 cm^{-1}. After data pre-processing, Principal Component Analysis (PCA), Soft Independent Modeling of Class Analogy (SIMCA), and Partial Least Squares (PLS) models were developed. Due to the noise resulting from air, PCA, SIMCA and PLS analysis was based on the combination of the wavenumbers of 3600 to 2700 cm^{-1} and 1800 to 900 cm^{-1}. The characteristics and functions of automatic baseline correction, normalization, binning, smoothing, second derivative, PCA, SIMCA and PLS are indicated in details in Chapter 1 and 2.

Results and discussion

Table 1 provides a list of absorption band assignments for various biochemical functional groups in the FT-IR region between 4000 and 400 cm^{-1}. These bands arise from major chemical components in the plasma and caviar, such as proteins, lipids, polysaccharides and nucleic acids. The different contents of lipids and proteins can contribute to a peak shift to either a higher wavenumber or a lower wavenumber due to affects of particularly biological matrices (Petibois *et al.* 2003). Within the same wavenumber region, there were some minor shifts of peak positions observed in the spectra of plasma and caviar. For example, a peak generally associated with Olefinic=CH stretching vibrations from lipids and cholesterol esters in the plasma matrix was at a slightly higher wavenumber (about 3100 cm^{-1}) than the one in caviar (about 3010 cm^{-1}) (Figure 1).

Representative FT-IR raw spectra of plasma from a single sturgeon during different maturity stages are shown in Figure 1. Peak heights and areas fluctuated in the regions of sex steroids, vitellogenin and total proteins and lipids along with the fish became progressed through the maturity stages. To further discern visual differences in spectral features as the fish matured, a second derivative transformation is often employed (Figure 2).

Second derivative transformation spectra (Figure 2) present clear maturity-dependent differences in the spectral features among plasma samples. For example, fish in early atresia could be clearly differentiated from those in late

vitellogenesis. A peak at 1630 cm^{-1} corresponds to the C=O stretch of amides of proteins (Amide I) (Petibois *et al.* 2001). The peak around 1510 cm^{-1} can be linked to the N-H bend and C=O stretch of amides of proteins (Amide II) (Haris *et al.* 1999). Another peak near 1235 cm^{-1} is associated with the P=O asymmetric stretch mainly from phospholipids and nucleic acids (Wong *et al.* 1991; Dovbeshko *et al.* 2000) (Figure 2). What is more significant is that in spectra of fish in early atresia compared to those in late vitellogenesis, there is a substantial difference in the spectral region associated with vitellogenin (~1080 cm^{-1}). These observed changes in vitellogenin confirm results reported by others about changes in the content of this protein as sturgeon mature (Amiri *et al.* 1996; Webb *et al.* 2001; Linares-Casenave *et al.* 2002). Amiri *et al.* (1996) reported the huge decrease of vitellogenin contents when the fish went through the late vitellogenesis period and into the early atresia period. Linares-Casenave *et al.* (2002) reported the rapid decline in plasma levels of sex steroids and vitellogenin at the onset of atresia, which coincided with hypertrophy of granulose cells and digestion of the oocyte envelop. The unique spectral features of early atresia apparent in the second derivative transformation provide a useful model to monitor and predict progression through these fish maturity stages, especially the onset of atresia. In the future, the maturity stage of a sturgeon may be predicted by spectra of blood plasma using the method described here and examining features in specific regions (*e.g.* Amide I and II, phospholipids and vitellogenin regions).

Figure 3 (a) shows representative FT-IR spectra of the out layer of white sturgeon egg. The peaks around 3010, 2922, 2852, 1743, 1458, 720 cm^{-1} are all

associated with specific stretching, vibration or bending of molecular bonds in lipid molecules (Petibois *et al.* 2002; Cakmak *et al.* 2003), all of which indicate that the surface of the egg has a very high level of lipid. This agrees with the results using other chemical methods to assess the composition of the sturgeon caviar. Bledsoe *et al.* (2003) reported that caviar has a high level of lipid content, especially triglycerides and phospholipids. Wang *et al.* (2008) used High Performance Liquid Chromatography (HPLC) measured a high level of phosphatidylcholine content in sturgeon caviar. Important spectral features for caviar also include a peak around 1147 cm^{-1} which may corresponds to the CO-O-C asymmetric stretch of glycogen and also be associated with nucleic acids. The other two "shoulder" peaks (around 1234 and 1097 cm^{-1}) are likely caused by asymmetric and symmetric stretch of phospholipids (Nara *et al.* 2002; Voortman *et al.* 2002; Cakmak *et al.* 2006). As reported earlier in this thesis, vitellogenin is a phospholipoglycoprotein, composed of the protein backbone phosphoryilated on serine moieties with a large quantity of lipids and carbohydrates. Thus, the region from 1240 to 1080 cm^{-1} could include spectral features for these various chemical constituents of vitellogenin.

Furthermore, there were no apparent protein features for the outer layer of the egg in the Amide I and II region which indicated that spectral features of the protein on the surface of the egg are not that prominent. What is more, the comparison between the plasma IR spectrum and caviar IR spectrum (Figure 3 (b)) reflected that the prominent peak around 3300 cm^{-1} may not be contributed by water. This peak represents the O-H stretch of protein, which would be present in untreated samples as

well as those that had been dehydrated. Some researchers hold the opinion that in a dried sample preparation, that there would be no water features in the spectra and that these features are from protein (Cakmak *et al.* 2003; Nakasato *et al.* 2004; Burgula *et al.* 2007). Others believed that bound water cannot be totally removed and would also contribute to absorbance in this spectral region (Kansiz *et al.* 1999; Lin *et al.* 2005). The IR spectra of the surface of caviar indicate that protein is present at 3300 cm^{-1}, even in sample that were visibly dry. To determine whether this spectral feature was from protein or water, an additional experiment was performed. Sturgeon plasma samples were heated in an oven at 120 ºC, removed and cooled, and the IR spectra collected every two hours. A clear decrease of the peak around 3300 cm^{-1} was observed from the removal of water accompanied by distinct changes in absorbance around 1650 and 1540 cm^{-1}, which are Amide I and Amide II regions (total protein region). These results suggest that the peak around 3300 cm^{-1} most likely represents O-H stretch of protein.

Figure 3(b) shows the comparison between plasma IR spectra and caviar IR spectra. Absorbance in the sex steroids region (around 3000 cm^{-1}), total lipids region (around 1700 cm^{-1}) and vitellogenin region (around 1080 cm^{-1}) are present in both the caviar and plasma. Figure 3(b) illustrates that the vitellogenin content in caviar is likely higher than in plasma following the late vitellogenesis period. This observation agrees with earlier research that shows that vitellogenin is transported from plasma to follicle cells along a capillary network to reach the oocyte surface; this is a critical step to facilitate egg growth in vitellogenesis period (Wheeler *et al.* 2005).

Furthermore, plasma spectra had a more prominent protein features than the outer layer of the sturgeon egg. The FT-IR spectra of the internal contents of the sturgeon egg are very similar to that of plasma, particularly in the Amide I and II regions. Caviar also has a high level of protein (16% to 30%), most of which is located inside the egg rather than on the surface (Bledsoe *et al*. 2003).

A mean centered PCA analysis was conducted based on the second derivative transformation spectra of plasma during different maturity stages in the wavenumber regions of combination of 3600–2700 cm^{-1} and 1800-900 cm^{-1}. Figure 4 shows a representative three-dimensional PCA clustering model for plasma samples from the same fish during different maturity stages. Clear segregations among samples at different maturity stages were observed, demonstrating that PCA could differentiate sturgeon plasma at each maturity stage. It is worth noting that the segregation was clear between late vitellogenic and atresia, even at the very early atretic period (see Fig. 4). Furthermore, the PCA result also reflected that there were no significant changes in IR spectral features from the early atresia to the middle atresia and then finally to the late atresia.

Eleven sturgeons were monitored over a nine month period during which time atresia was induced. Separate chemometric models were developed for each fish. Changes in chemical components that were most important in determining maturity (Table 2) were: sex steroids, vitellogenin and Amide I and II (protein) contributing most to the PCA model, and accounting for more than 70% of the difference observed among spectra. Among the 11 fish, 9 had the same PC distribution (PC1 is sex steroid,

PC2 is vitellogenin and PC3 is Amide I and II). The PC1 for the other two fish was vitellogenin and PC2, sex steroids. Fish (Tag 3D08) was an outlier in this study since she had only entered very early stages of atresia and no further at the point of the last sampling. The other 10 fish progressed through vitellogenesis through late atresia. At this point, it is possible to establish a clear relationship between sex steroid and vitellogenin contents and changes in spectral features of the fish during various maturity stages and we have been able to establish models for this here. When fish proceed from very late vitellogenic period to the atretic period, the sex steroids content and vitellogenin content changed accordingly. This PCA result supports the results of previous studies (Linares-Casenave *et al.* 2002; Webb *et al.* 2005; Webb *et al.* 2007). Linares-Casenave *et al.* (2002) reported that a decrease in plasma estradiol-17β, testosterone and vitellogenin happened as the fish entered the atretic period. Webb *et al.* (2005) reported that testosterone (T), 11-ketotestosterone (11-KT) and E_2 have been found to differ in wild white sturgeon females of various stages of maturity (pre-vitellogenic, vitellogenic, post-vitellogenic, post-ovulatory and atretic). Webb *et al.* (2007) reported that estradiol-17β, testosterone and 11-keto testosterone contents in Oregon green sturgeon (*Acipenser medirostris*) plasma decreased greatly several weeks before ovulation.

Soft independent modeling of class analogy (SIMCA) analysis was performed to classify sturgeon by maturity stages during late vitellogenesis and atresia. Figure 5 shows representative SIMCA pass test results for plasma from the same sturgeon during various maturity stages. A total of 11 fish were analyzed by the SIMCA model

and approximately 70% classification accuracy (between late VTG and atresia) was achieved (Table 6). This SIMCA model was not as rigorous as other SIMCA models established in our lab using simpler biological systems, such as microbial growth and injury (Al-Qadiri *et al.* 2006).

One reason may be that the changes of the chemical components in fish plasma vary progressively during the season and maturity stages, changes in concentration may be very subtle, especially in a narrow time period such as from the late vitellogenesis to early atresia. SIMCA models are most appropriate when for appearance of a new analyte from an enzyme or chemical reaction, such as oxidation of hemoglobin, or for differentiating biological samples based on the presence or absence of a molecule that occurs in one treatment but not in the other. SIMCA models are "harsh" with only 0 (fail) or 1(pass) options, without intermediate states categories, such as is possible with PCA. However, the SIMCA model could be still used to differentiate sturgeon plasma samples between stages where there is either a large relative increase or decrease in a single component. To determine if this model can be effective, more samples will need to be collected.

Fortunately, it appears that a combination of second derivative transformation, PCA cluster model and SIMCA pass test together can make a very precise prediction about the maturity stage of a specific fish. This is a very promising method to be utilized in the fish aquaculture industry in the future to predict sturgeon maturity stage promptly, precisely and non-invasively with only "one drop of plasma" from the sturgeon.

Table 4 summarized the PI values from total 11 sturgeon females during the nine months period study. Except fish 3D08, a general declined trend of PI values were shown along with the sturgeons came through late vitellogenesis to atresia.

Partial least squares (PLS) models were used to establish a calibration model and provide a correlation between reference data (measured values) with FT-IR spectral data (predicted values) (Figure 6). Accordingly, a PLS prediction model was developed to quantify the PI values from different sturgeon during various maturity stages. PI reference values were chosen randomly from eleven fish during different maturity stages, from 0.1 to 0.3, an interval of 0.02 between two data. Validation results of the PLS model with 6 latent variables for predicting PI values yielded a high correlation coefficient (R=0.9874) and a low standard error (0.0101), indicating that an accurate quantification of PI values can be determined from a PLS-based prediction model. Thus, in the future, there will be less of a need to utilize the current surgical biopsy method to determine the PI value. Instead, a small amount of plasma (several drops, about 10 to 20 µl), can be dried on the crystal of an FT-IR detector, spectra collected and a PLS model (library) constructed to predict PI value. The entire process would take only a few minutes, and could hopefully substitute for the current surgical biopsy method.

We did not observe changes in the spectral features of sturgeon eggs from among all 11 fish collected during various maturity stages. Sturgeons in very late vitellogenesis still transport substances from the blood to the follicle, such as vitellogenin, non-polar lipid and sugar (Webb *et al.* 2005). However, measuring the

spectral features of both the shell and internal contents of the sturgeon eggs, and collecting more than two thousand IR spectra, no significant differences were observed in the spectral features of the roe from fish at the different maturity stages, no matter which fish was examined or what the maturity stage was. A rational explanation is that during the late vitellogenesis, when fish is ready to finish dry matter (i.e. yolk protein) accumulation, a massive and rapid uptake of water takes place with the oocyte meiotic maturation and GVBD (Katsiadaki *et al.* 1999). The water absorption is an adaptation that results in a wide spread of the floating eggs. However, the high amount of water content in the eggs can affect the FTIR spectra hugely, which may not reflect the minor change of protein and lipid contents inside of the eggs.

Thus, sex steroids and vitellogenin contents in plasma rather than in the fish egg appear to be better biomarkers for a model to predict and assess sturgeon maturity stages.

Conclusions

The changes of biochemical components in white sturgeon female plasma could be successfully determined by FT-IR following eleven sturgeon females during a nine months period from late vitellogenesis to atresia. Clear segregation of plasma according to maturity stage was evident using Principal Component Analysis (PCA). Soft Independent Modeling of Class Analogy (SIMCA) models can predict stage of maturity (late vitellogenesis vs. early atresia) about 70% of the time. Combination of

second derivative transformation, PCA cluster model and SIMCA pass test together can make a precise prediction about the maturity stage of a specific sturgeon female. A rigorous Partial Least Squares (PLS) model was established to predict Polarization Index (PI) values between 0.1 and 0.3 (R=0.98, SEP=1.01%) based upon spectral features. Unfortunately, few changes were observed in spectral features of the roe recovered at different maturity stages. FT-IR plasma spectra may provide a useful tool for predicting maturity stage and timing of roe harvest.

For further work, fin and urine may be two other materials that could be examined by FT-IR to determine whether correlations could be made between sex hormone or protein levels in these samples and reproductive status of the fish.

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Table 1 Absorption band assignments of FT-IR spectrum

Wavenumber(cm^{-1})	Spectral Assignment
~3300	O-H stretch of water and protein
~3100	Olefinic=CH stretching vibration: lipids, cholesterol esters
~2960	CH_3 asymmetric stretch of methyl groups: mainly lipids
~2929	CH_2 asymmetric stretch of methylene groups: mainly lipids
~2875	CH_3 symmetric stretch of methyl groups: mainly proteins
~2850	CH_2 symmetric stretch of methylene groups: mainly lipids
~1740	C=O of ester functional groups: triglycerides, cholesterol esters
~1650	C=O stretch of amides of proteins: Amide I
~1540	N-H bend and C=O stretch of amides of proteins: Amide II
~1455	CH_2 bending: mainly lipids, with little contribution from proteins
~1395	COO^- symmetric stretch: fatty acids and amino acids
~1236	P=O asymmetric stretch: mainly nucleic acids and phospholipids
~1152	CO-O-C asymmetric stretch: glycogen and nucleic acids
~1080	P=O symmetric stretch: mainly nucleic acids and phospholipids

(a) sturgeon plasma

Wavenumber(cm^{-1})	Spectral Assignment
~3010	Olefinic=CH stretching vibration: lipids, cholesterol esters
~2922	CH_2 asymmetric stretch of methylene groups: mainly lipids
~2852	CH_2 symmetric stretch of methylene groups: mainly lipids
~1743	C=O of ester functional groups: triglycerides, cholesterol esters
~1653	C=O stretch of amides of proteins: Amide I
~1458	CH_2 bending: mainly lipids, with little contribution from proteins
~1377	COO^- symmetric stretch: fatty acids and amino acids
~1234	P=O asymmetric stretch: mainly nucleic acids and phospholipids
~1147	CO-O-C asymmetric stretch: glycogen and nucleic acids
~1097	P=O symmetric stretch: mainly nucleic acids and phospholipids
~967	$C\text{-}N^+\text{-}C$ stretch: nucleic acids

(b) sturgeon eggs

*see Refs. Burgula *et al.* 2007; Cakmak *et al.* 2003; Cakmak *et al.* 2006; Dovbeshko *et al.* 2000; Haris *et al.* 1999; Lin *et al.* 2003; Lu *et al.* 2008; Nara *et al.* 2002; Petibois *et al.* 1999; Petibois *et al.* 2001; Petibois *et al.* 2002; Petibois *et al.* 2003; Voortman *et al.* 2002; Wong *et al.* 1991

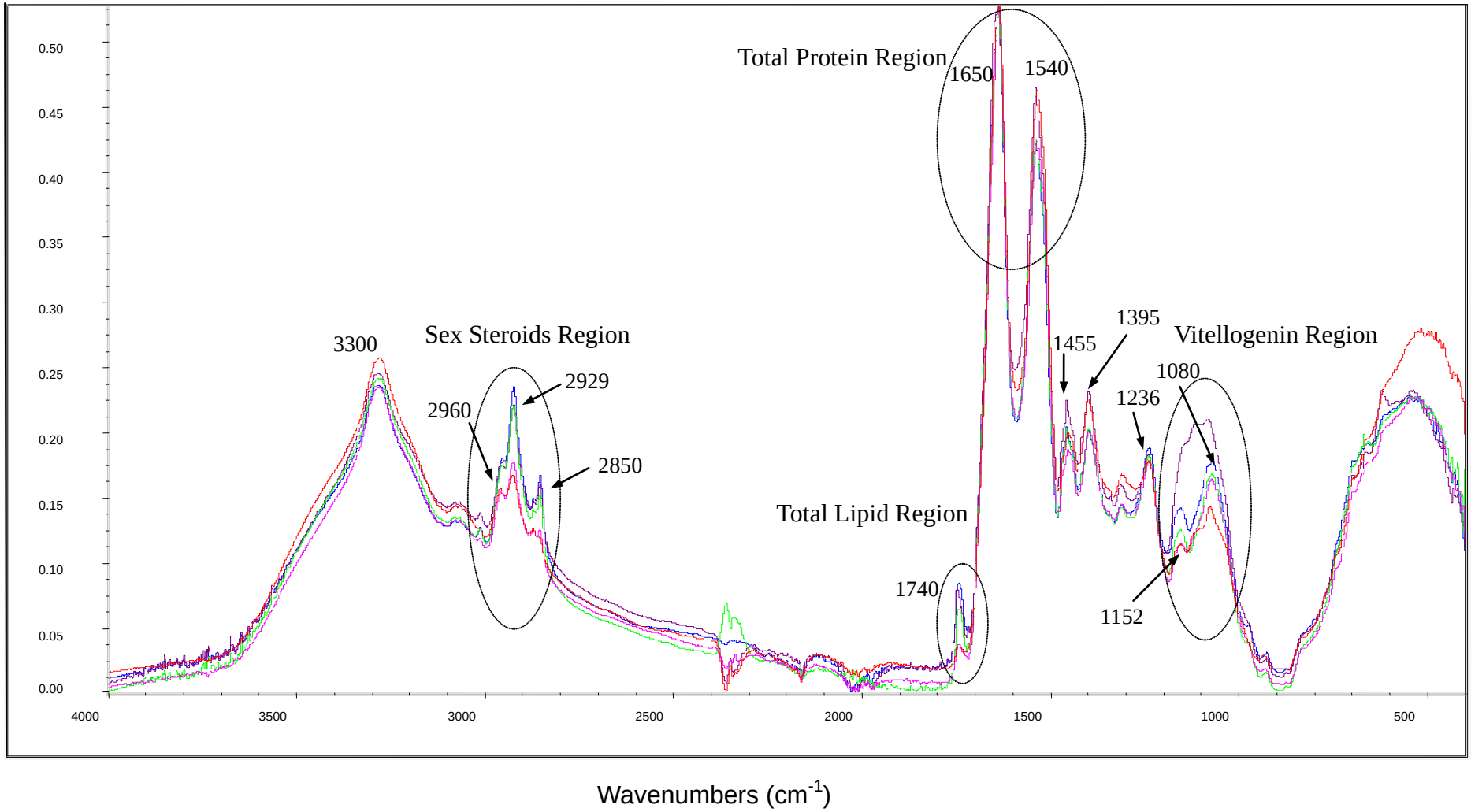

Figure 1 White sturgeon female plasma FT-IR raw spectra analysis and comparison during maturity stages.

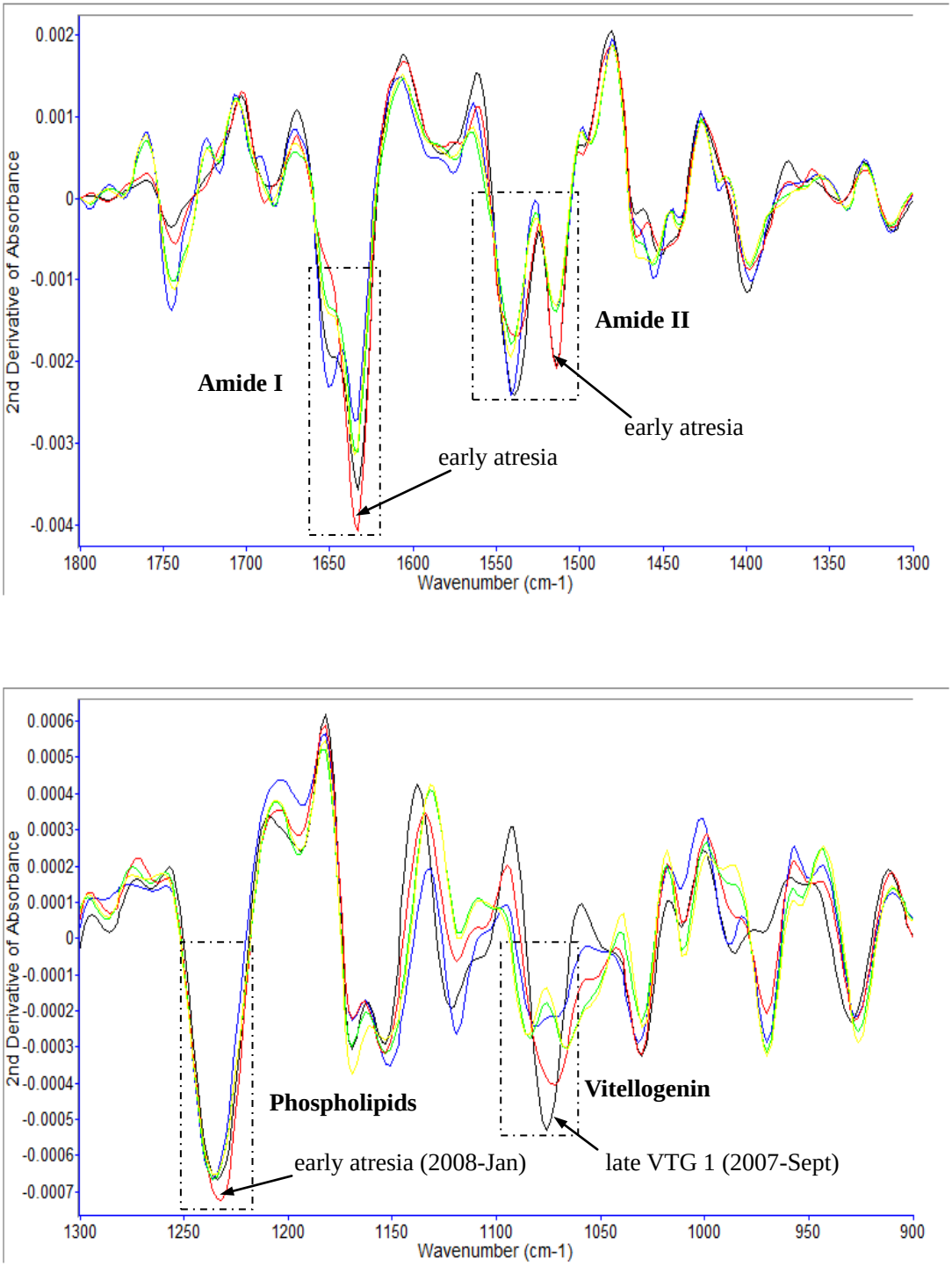

Figure 2 Representative second-derivative transformation of spectra for white sturgeon plasma during maturity stages (from late vitellogenic period to late atretic period, black: late VTG1 (2007-Sept); blue: late VTG-2 (2007-Nov); red: early atresia (2008-Jan); green: middle atresia (2008-Mar); yellow: late atresia (2008-Apr))

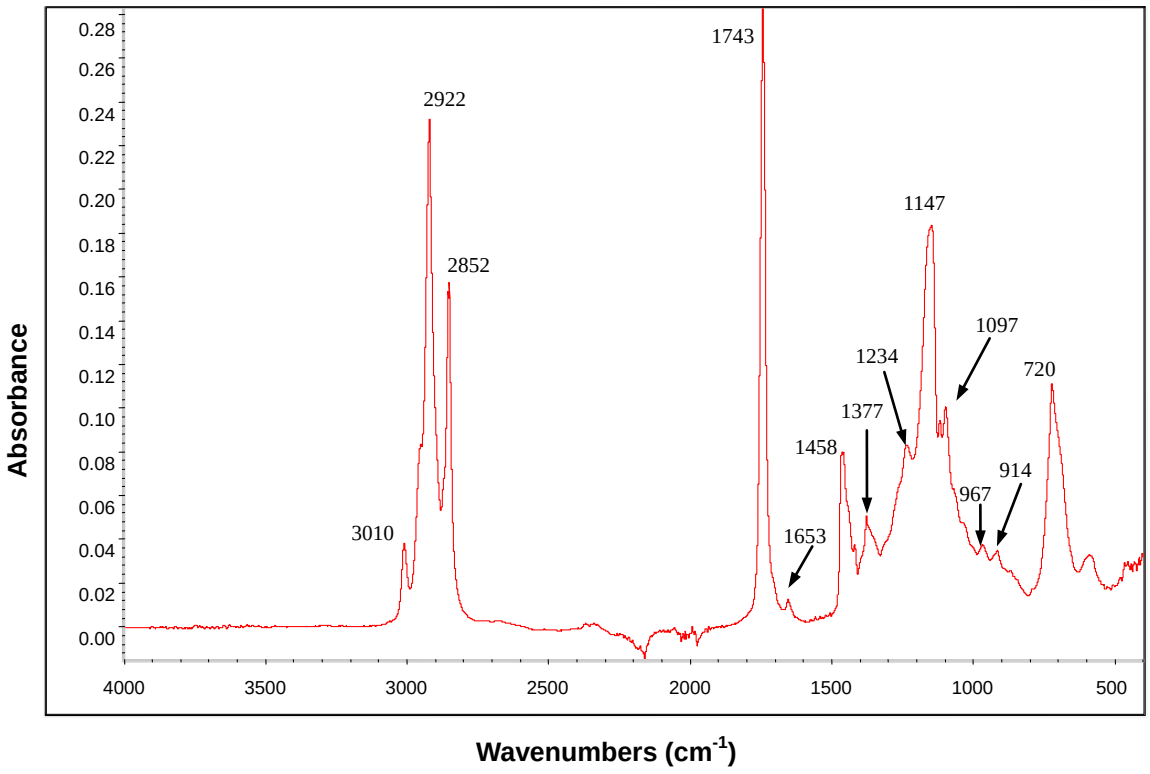

Figure 3 (a) Representative FT-IR raw spectra of white sturgeon egg

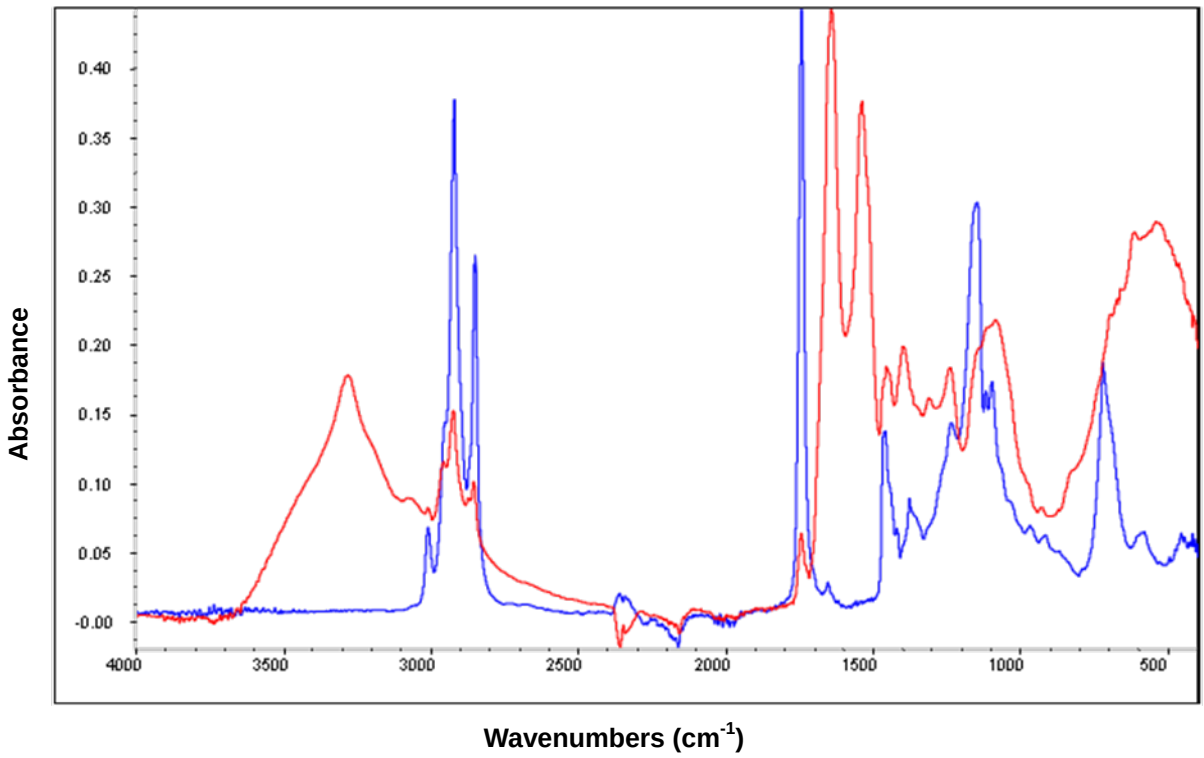

Figure 3 (b) Comparison between white sturgeon plasma and egg FT-IR raw spectra (red: plasma spectrum; blue: egg spectrum)

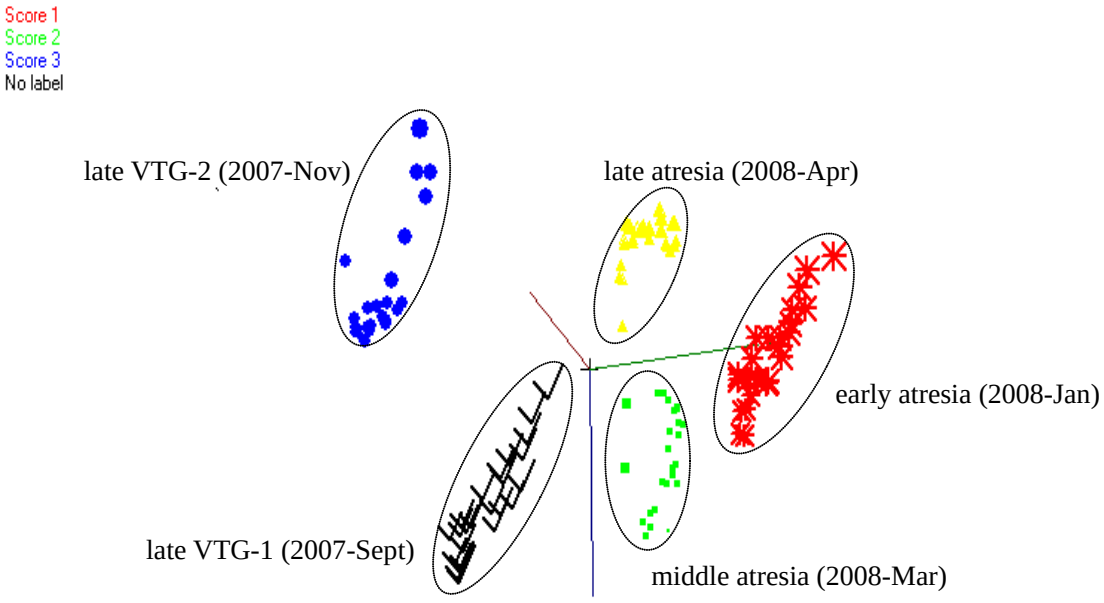

Figure 4 Representative three-dimensional PCA clustering results by maturity stage for white sturgeon plasma

Table 2 Summary of principal component indexes and corresponding loading values of 11 white sturgeon females tracked from late vitellogenesis to late atresia

Fish Tag	PC 1	PC 2	PC 3	Loading 1	Loading 2	Loading 3
182F	sex steroids	vitellogenin	Amide I and II	40.30%	24.63%	11.19%
205E	sex steroids	vitellogenin	Amide I and II	43.15%	16.78%	9.05%
2E7C	sex steroids	vitellogenin	Amide I and II	50.62%	15.32%	8.43%
3D08	sex steroids	vitellogenin	Amide I and II	26.58%	20.32%	12.35%
4458	vitellogenin	sex steroids	Amide I and II	38.05%	19.74%	13.33%
401A	sex steroids	vitellogenin	Amide I and II	47.98%	19.77%	7.32%
483D	sex steroids	vitellogenin	Amide I and II	51.03%	16.88%	6.98%
4B34	vitellogenin	sex steroids	Amide I and II	37.40%	22.52%	14.90%
6D7E	sex steroids	vitellogenin	Amide I and II	44.21%	13.96%	5.42%
795C	sex steroids	vitellogenin	Amide I and II	39.60%	17.31%	10.03%
7E07	sex steroids	vitellogenin	Amide I and II	53.36%	12.94%	6.11%

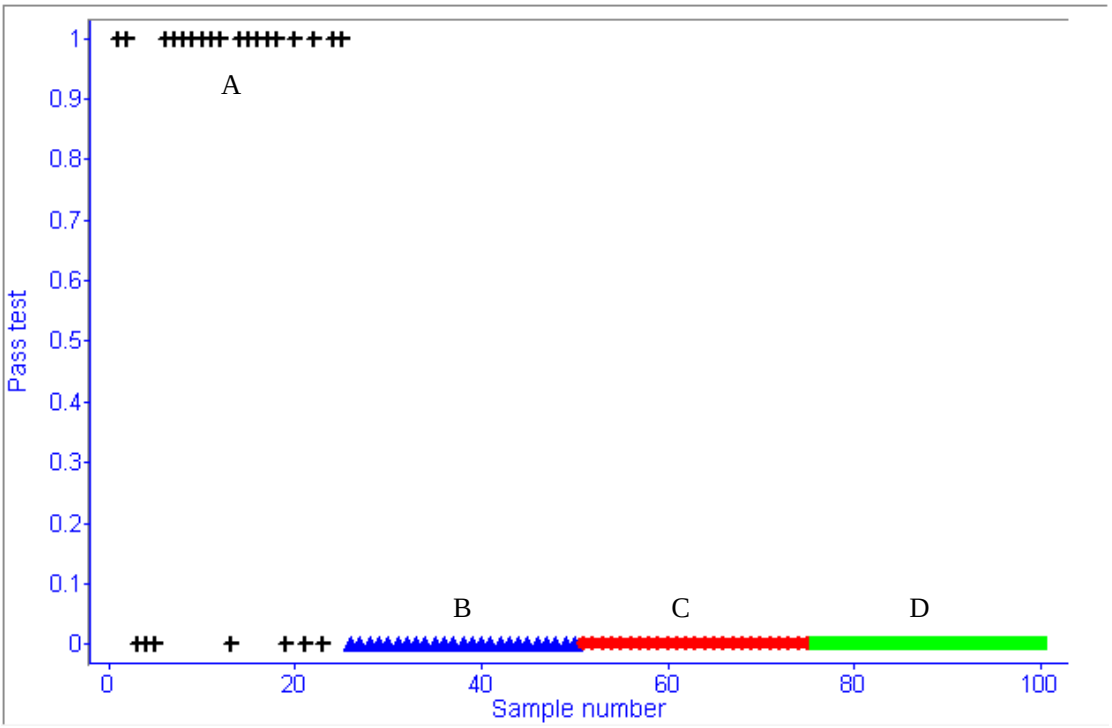

Figure 5 Representative Soft Independent Modeling of Class Analogy (SIMCA) classification of late vitellogenic plasma (A) compared with early atretic (B), middle atretic (C) and late atretic (D) plasma from the same sturgeon

Table 3 SIMCA classification results for late vitellogenesis of each sturgeon female compared to atresia

Sample treatment	No. of correctly classified spectra	% of correctly classified spectra
182F	17	68
205E	18	72
2E7C	18	72
3D08	---	---
4458	16	64
401A	18	72
483D	19	76
4B34	16	64
6D7E	16	64
795C	16	64
7E07	19	76

Table 4 A summary of oocyte polarization index (PI) values of 11 sturgeons during a nine months period

Fish ID	2007/09/10	2007/11/16	2008/1/16	2008/3/17	2008/4/2	2008/4/17	2008/5/1	2008/5/15
182F	0.2310 (0.053)	0.1736 (0.048)	NA	NA	NA	NA	NA	NA
205E	0.2294 (0.027)	0.1360 (0.020)	0.1118 (0.0187)	0.1200 (0.0172)	0.0822 (0.0287)	NA	NA	NA
2E7C	0.2749 (0.081)	0.2411 (0.059)	0.1798 (0.0445)	0.0788 (0.0298)	NA	NA	NA	NA
3D08	0.2814 (0.065)	0.2137 (0.034)	0.1120 (0.0232)	0.0836 (0.0176)	0.0593 (0.0260)	0.0640 (0.0108)	0.0710 (0.0149)	0.0906 (0.0234)
4458	0.3884 (0.054)	0.1919 (0.014)	0.1097 (0.0331)	0.0779 (0.0201)	0.0763 (0.0169)	NA	NA	NA
401A	0.1866 (0.075)	0.2236 (0.050)	0.1305 (0.0292)	0.0959 (0.0284)	0.0676 (0.0179)	NA	NA	NA
483D	0.2892 (0.069)	0.2289 (0.041)	0.0856 (0.0351)	0.0531 (0.0299)	0.0672 (0.0158)	0.0910 (0.0100)	NA	NA
4B34	0.3000 (0.055)	0.1955 (0.020)	0.0998 (0.0299)	NA	NA	NA	NA	NA
6D7E	0.2307 (0.042)	0.1291 (0.027)	0.0800 (0.0120)	0.0726 (0.0154)	0.0731 (0.0162)	NA	NA	NA
795C	0.2371 (0.036)	0.1618 (0.050)	0.1285 (0.0352)	0.0575 (0.0231)	NA	NA	NA	NA
7E07	0.3161 (0.068)	0.1862 (0.030)	0.1134 (0.0280)	0.0962 (0.0115)	0.0913 (0.0165)	0.0998 (0.0154)	NA	NA

(The values with underscore are the data to be used to establish the PLS model. When fish entered atresia, the eggs are too soft to be measured PI value.)

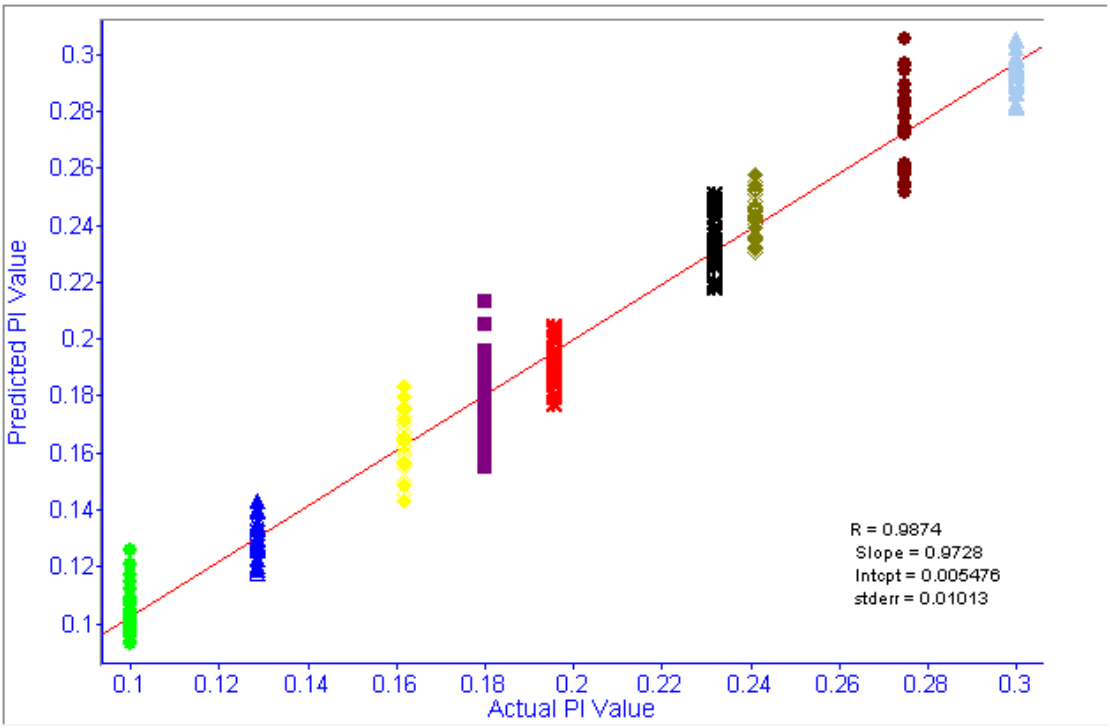

Figure 6 Comparison between the actual and predicted PI values (range: 0.1 and 0.3) for white sturgeon females.

CHAPTER FOUR

CONCLUSIONS AND FUTURE RESEARCH

Biochemical components in white sturgeon plasma including sex steroids and vitellogenin can be detected using FT-IR spectra and used to predict sexual maturity in female sturgeon. FT-IR combined with multivariate analysis (PCA, PLS) may provide a rapid, non-invasive and precise assessment method to segregate and sort sturgeon at selected stages of maturity (pre-vitellogenesis, vitellogenesis, post-vitellogenesis and atresia).

The changes in biochemical components of the blood plasma of white sturgeon females were successfully determined with FT-IR by following eleven sturgeon females during a nine month period from late vitellogenesis to atresia. Clear segregation of plasma according to stage of maturity was evident using Principal Component Analysis (PCA). Soft Independent Modeling of Class Analogy (SIMCA) models accurately predict the stage of maturity (late vitellogenesis vs. early atresia) about 70% of the time. Combination of second derivative transformation, PCA cluster model and SIMCA pass test together provide a precise prediction of the stage of maturity of individual sturgeon females. A rigorous Partial Least Squares (PLS) model was established to predict Polarization Index (PI) values between 0.1 and 0.3 ($R=0.98$, $SEP=1.01\%$) based upon spectral features. Unfortunately, few differences were observed in spectral features of the roe recovered at selected maturity stages.

For future research, the following experiments may be beneficial:

(1) A large number of fish plasma and analysis are necessary to confirm the feasibility of FT-IR combined with multivariate analysis to determine sturgeon female stages of maturity.

(2) Utilize FT-IR to analyze blood or tissue from white sturgeon during the entire reproductive cycle to determine other biochemical changes appropriate for monitoring.

(3) Evaluate tissues other than blood that are less invasive to collect; possible fin or urine analysis with FT-IR may determine whether correlations are identified among sex hormones, protein concentrations and the reproductive status of the sturgeon.

(4) Develop new procedures for FT-IR analysis of sturgeon roe to remove the effect of water on the spectra and make it possible to measure differences in spectral features of roes.

(5) Check the feasibility of using FT-IR to segregate sturgeon sex at an early age by plasma measurement combined with genetic techniques such as Amplified Fragment Length Polymorphism (AFLP).