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Characteristics of fish yolk proteins and a method for inducing vitellogenin

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**CHARACTERISTICS OF FISH YOLK PROTEINS AND A METHOD FOR
INDUCING VITELLOGENIN**

A Thesis Presented

by

SEAN M. LUCEY

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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DEDICATION

To my lovely wife, Alyson.

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CHAPTER 1
A REVIEW OF THE ROLE OF YOLK PROTEINS AND THEIR PRE-CURSOR
IN VITELLOGENESIS

1.1 Abstract

Teleosts are one of the most diverse groups of vertebrates. They utilize a wide array of reproductive strategies and tactics to overcome the challenges of the many ecological niches they inhabit. The most common reproductive method for teleosts is oviparity. Oviparous animals lay eggs with little or no embryonic development from the mother. The embryos are supplied with nutrition via yolk. Vitellogenesis is the process of the ovary sequestering yolk. It is regulated by exogenous environmental cues that act on the hypothalamus-pituitary-gonad axis. Through a series of hormonal controls, the liver produces the yolk precursor, vitellogenin. Vitellogenin is secreted by the liver and absorbed by the growing oocyte by receptor mediated endocytosis. There it is cleaved into the two main yolk proteins which are subsequently used by the growing embryo. The yolk proteins also play a key role in marine teleosts' ability to osmoregulate their eggs. Presence of vitellogenin in male or juvenile fish is used as a biomarker for estrogenic compounds. There is still much to learn about yolk proteins but methods of induction and isolation are quickly improving concurrently adding to the understanding of fish reproduction.

1.2 Reproductive strategies

There are over 26,000 extant species of fishes, the majority of which are in the infraclass Teleostei (1994). Teleosts display a wide range of morphological, physiological and behavioral traits (Helfman et al. 1997). Because many of the world's fisheries are currently overfished (Boreman et al. 1997; FAO 2002; Worm et al. 2009), there is a growing demand to better understand the dynamics of fish populations and the underlying processes that govern their ability to rebound. Of particular concern for fisheries biologists is the process of reproduction and subsequent recruitment of juveniles to the fishery. Fish reproduction, like fishes themselves, spans a vast array of strategies and tactics (Helfman et al. 1997; Wootton 1998). However, underlying all the various modes of reproduction are common biological processes.

Teleosts are one of the most diverse groups of vertebrates. A major reason for their success has been their ability to utilize various systems of reproduction to exploit a wide range of ecological niches. These reproductive systems are not only biological but also behavioral. Biological systems include various gender differentiation, fertilization methods, and number of spawning cycles. Behavioral systems include mating systems and parental care.

In the northwest Atlantic, principal groundfish (Atlantic cod-*Gadus morhua*, haddock-*Melanogrammus aeglefinus*, pollock-*Pollachius virens*, redfish-*Sebastes* spp., red hake-*Urophycis chuss* and silver hake-*Merluccius bilinearis*; Boreman et al. 1997) and flatfish species are gonochoristic meaning that individuals are born as one sex and

remain that way for the remainder of their lives. Like the majority of teleosts, most are oviparous and lay clutches of numerous small eggs and show little to no parental care. The major exception in the northwest Atlantic principal groundfish is redfish which are ovoviviparous, the eggs develop and hatch within the mother (Collette and Klein-MacPhee 2002). Although there are some instances of semelparous species (species that spawn only once), most exhibit an iteroparous life style. Iteroparity allows a fish the opportunity to repeat spawn. Both strategies allow species to display seasonal timing to maximize conditions that are favorable to their larval stages. These favorable conditions include times when appropriate food, protection from predators, and protection from adverse abiotic conditions are available (Wootton 1998).

Fish also have three main types of ovarian development organization. The three types are synchronous, group-synchronous, and asynchronous. Synchronous spawners develop all of their oocytes at the same time. This is common in semelparous species. Group-synchronous spawners have at least two distinct populations of oocytes, one of larger oocytes and a heterogeneous group of smaller oocytes. This is common in iteroparous species with short spawning seasons and the common organization of the principal groundfish. The final organization, asynchronous, occurs when oocytes of all stages of development are present. This is common for species with protracted spawning seasons and when yolk accumulation relies most heavily on available food resources (Murua and Saborido-Rey 2003).

Similar to the ovarian development organization is spawning pattern. There are two patterns of spawning, total and batch. Total spawners shed all their eggs over a short period of time. Batch spawners release their eggs in multiple batches throughout the spawning season. This strategy allows a species to release eggs over a long time and increase the probability of offspring survival (Murua and Saborido-Rey 2003).

The typical spawning cycle after first maturation of iteroparous fish starts from a resting state and proceeds through a developing state to a ripe or ripe and running state (Figure 1.1). For a more comprehensive study on the maturity stages of the northwest Atlantic, see NEFSC (1993). This cycle of maturity stages is controlled by endogenous factors triggered by exogenous environmental cues. This control ensures the synchronization of gamete production and spawning between the sexes (Mommsen and Korsgaard 2008).

1.3 Vitellogenesis

Environmental cues trigger the hypothalamic-pituitary-gonad axis and in females begin the process of vitellogenesis (Figure 1.2). Vitellogenesis is the process of provisioning eggs with yolk (Wahli et al. 1981; Byrne et al. 1989; Mommsen and Korsgaard 2008). It involves the sequestering of the yolk precursor, vitellogenin (Vg), by the ovary. The process begins with the release of glycoprotein gonadotropins from the pituitary gland (Pankhurst 2008). There are two types of gonadotropins; follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In synchronous spawners, early gonadal growth and development is associated with FSH while LH regulates

maturational events. Both hormones are present in similar levels in asynchronous spawners (Swanson et al. 2003). The two gonadotropins bind to membrane receptors in the ovarian follicle and activate steroid synthesizing enzymes (Pankhurst 2008). The steroid synthesizing enzymes create among other things 17β -estradiol which is the primary hormone responsible for vitellogenesis (Specker and Sullivan 1995). Estradiol is secreted by the ovarian follicles and delivered to the liver where it binds to estrogen receptors in the hepatocyte cytoplasm. This stimulates the synthesis of Vg (Pankhurst 2008).

Vitellogenin, the yolk precursor, is a large glyco-phospho-lipo-protein. It occurs as a dimer with two equal subunits of about 200 kDa (Wahli et al. 1981). Vitellogenin is evolutionarily homologous among a large variety of animals from insects to chickens (Nardelli et al. 1987; Byrne et al. 1989; Hiramatsu et al. 2002a; Patino and Sullivan 2002). Vitellogenin undergoes lipidation, phosphorylation and glycosylation within the liver. Calcium is attached to the phosphorylated portions of Vg to be delivered for skeletal development (Wahli et al. 1981; Hiramatsu et al. 2002c). Vitellogenin is then secreted from the parenchymal liver cells and transported to the ovary where it is taken up by the growing oocytes via receptor-mediated endocytosis. In the ovary, Vg is proteolytically cleaved into the two main yolk proteins, lipovitellin (Lv) and phosvitin (Pv), as well as other small β -components (β') (Wahli et al. 1981; Byrne et al. 1989; Specker and Sullivan 1995; Hiramatsu et al. 2002a). The enzyme responsible for processing Vg is cathepsin D although noteworthy levels of cathepsin B have also been detected (Carnevali et al. 1999; Hiramatsu et al. 2002b).

1.4 Yolk proteins

Lipovitellin, the major nutritional resource for the growing embryo, is a large dimeric lipoprotein, covalently bound to large amounts of lipid. Lipovitellin has two subunits, one ca 115 kDa and a second ca 31 kDa. These subunits are referred to as the heavy chain and light chain respectively (Hiramatsu et al. 2002a). Twenty or more percent of the structure of Lv is composed of lipid. The larger subunit has almost no phosphate while the smaller subunit is highly phosphorylated (Wahli et al. 1981). Only a small proportion of lipid is bound directly to the protein through hydrophobic interaction, with the rest binding through lipid to lipid interactions (Ohlendorf et al. 1978). Besides being the major nutritional source, it appears to have evolutionary and structural relationships to other lipoproteins such as apolipoprotein B, microsomal triglyceride transfer protein, and segments of apolipoprotein B (Thompson and Banaszak 2002).

The other product of Vg processing, phosvitin is a serine rich phosphoprotein (Byrne et al. 1989; Hiramatsu and Hara 1996). Phosvitin composes 3% of the total yolk protein of fishes. Estimates of Pv molecular weight vary greatly because of the cross reactivity of the molecule with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Hiramatsu and Hara 1996). The highly negative charge of Pv allows Vg to ionically bind calcium, thus delivering the minerals the embryo need for skeletal development and other metabolic functions (Patino and Sullivan 2002). Little is understood about β -components (Hiramatsu et al. 2002c).

1.5 Yolk protein's role in fish development

Recent studies have eliminated the idea of one vitellogenin. Several Vg genes are responsible for piscine oogenesis (Matsubara et al. 1999; Reith et al. 2001; Matsubara et al. 2003; Sawaguchi et al. 2006). These different Vg's are believed to result in different forms of the yolk protein lipovitellin and may play divergent roles in fish development including the osmoregulation of pelagic eggs (Reith et al. 2001; Matsubara et al. 2003). Marine teleosts exhibit significant hydration of their oocytes during final maturation, especially those that spawn pelagic eggs (3- to 6- fold increase in volume) (Selman et al. 2001). Hydration allows the eggs to achieve buoyancy by adjusting their specific gravity to that of the surrounding seawater (Craik and Harvey 1987).

Although the physiological mechanisms are not well understood it is believed that hydration occurs by a second proteolysis of yolk proteins. Cathepsin L is the enzyme responsible for the regulation of the second proteolysis, but may play different roles in species that spawn demersal eggs compared to those that spawn pelagic eggs (Matsubara and Koya 1997; Carnevali et al. 1999; Patino and Sullivan 2002). There is also an increase of inorganic ions, specifically Na^+ and K^+ (Matsubara and Koya 1997; Selman et al. 2001). The hydration process is aided by the presence of molecular water channels or aquaporins (Fabra et al. 2005; LaFleur et al. 2005).

This second proteolysis is unique to teleosts and produces a pool of free amino acids (FAA) (Byrne et al. 1989; Thorsen and Fyhn 1996; Selman et al. 2001; Hiramatsu

et al. 2002c; Sawaguchi et al. 2006). The FAA gives the oocyte a mechanism to cope with the hypo-osmotic seawater (marine teleost eggs ~350 mOsm, average seawater ~1000 mOsm) until the osmoregulatory systems develop. Without this unique proteolysis, fish species would be required to be anadromous and return to freshwater to spawn. Anadromy is typical of more ancestral fishes (Finn and Kristoffersen 2007).

Embryogenesis includes the final processing of the yolk proteins. Lipovitellin is further broken down to FAA which serve as a substrate for aerobic energy and protein synthesis (Matsubara and Koya 1997). Multiple forms of Lv could be responsible for heterogeneous eggs where some Lv is degrading for fast development and immediate feeding while other Lv is stored in the gut to allow the larvae to avoid eating until suitable habitat is reached (Hartling and Kunkel 1999). Phosvitin is dephosphorylated for embryonic and larval development, while the β -component does not appear to be processed and is taken up intact to be used by the embryo at a later stage (Hiramatsu et al. 2002c). Lipovitellin also provides a reservoir of water until the drinking mechanisms are developed (Finn and Kristoffersen 2007).

1.6 Yolk proteins as biomarkers

Besides gaining a better understanding of the biological process behind reproduction, yolk proteins are useful for monitoring the environment. In recent years there has been a growing concern about anthropogenic effects on the aquatic environment. Particular concerns exist about the adverse effects of endocrine disrupting chemicals, especially xenoestrogens that mimic natural estrogens. The major sources of

these xenoestrogens are treated and untreated effluents, agricultural and livestock waste, and runoff from sewage treatment plants. Xenoestrogens have been known to bioaccumulate and biomagnify. Male and juvenile fish exposed to large amounts of these chemicals can produce vitellogenin. This makes Vg a useful biomarker of exposure for these estrogenic compounds (Heppell et al. 1995; Hiramatsu et al. 2002c; Fujiwara et al. 2005; Hiramatsu et al. 2006; Johnson et al. 2008; Matozzo et al. 2008).

1.7 Conclusion

In conclusion, the majority of teleosts are oviparous animals that despite using a wide variety of reproductive strategies and tactics have similar underlying biological processes. Vitellogenesis is a process that is similar in a wide range of species yet marine teleosts have modified the procedure to help them deal with the hypo-osmotic environment of the world's oceans. This modification was a key evolutionary adaptation yet there is still much to learn about yolk proteins and their role in reproduction. Although the literature is growing, there is still little understood about lipovitellin (Fujiwara et al. 2005). A major obstacle has been separating Lv from other yolk proteins. Hartling et al. (1997) found a heat stable portion of Lv, which allows heat denaturation of marine eggs to isolate Lv. The next chapter examines the thermodynamic characteristics of Lv in pleuronectids.

The growing concern for environmental impacts and Vg's usefulness as a biomarker for exposure to estrogenic toxins has led to development of many immunoassays. However, Vg is very species specific and tests need to be developed for individual species. The third chapter of this thesis describes the beginning stages of

creating an immunoassay for the New York Bight using a common recreational fish, bluefish (*Pomatomus saltatrix*).

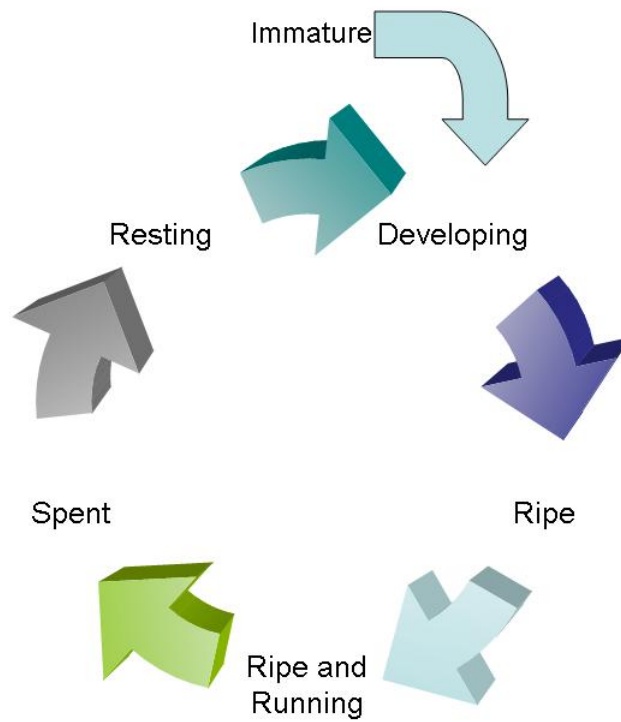


Figure 1.1 – Common reproductive cycle of principal groundfish of the northwest Atlantic.

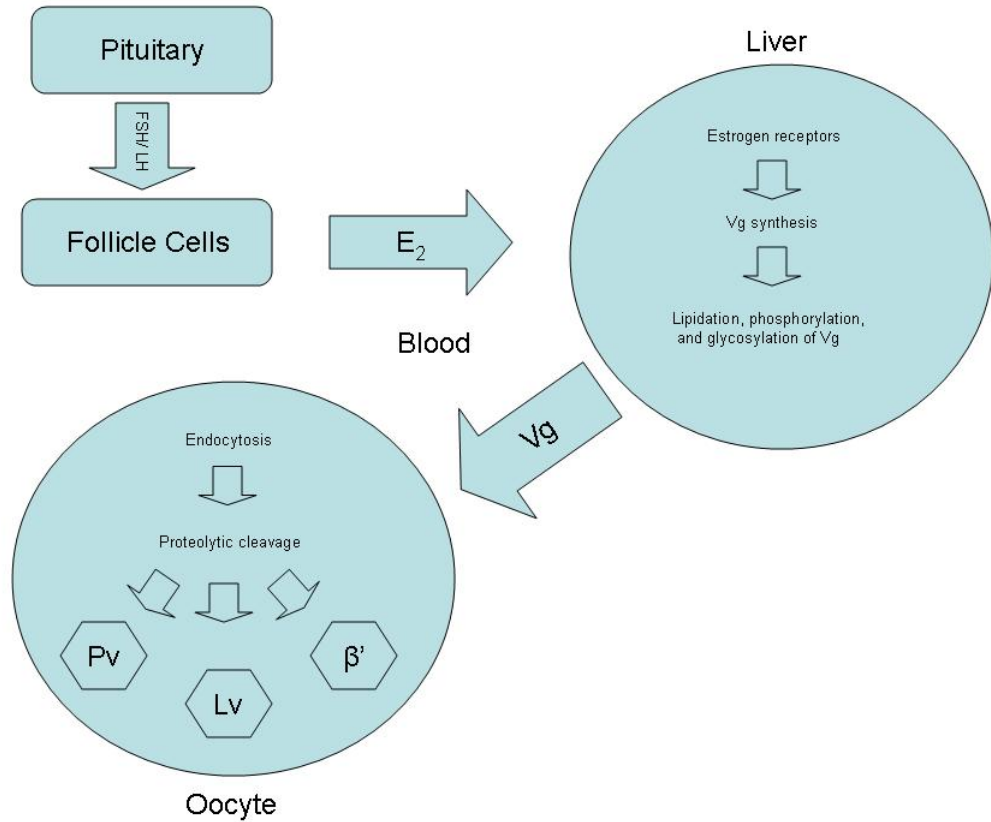


Figure 1.2 – Conceptual diagram of vitellogenesis. Note that many marine teleost have multiple vitellogenin genes that give rise to multiple lipovitellins that are utilized at different times during oocyte maturation. FSH = follicle-stimulating hormone, LH = luteinizing hormone, E₂ = 17β-estradiol, Vg = vitellogenin, Pv = phosvitin, Lv = lipovitellin, and β' = β-components.

CHAPTER 2

**THERMOSTABILITY OF THE YOLK PROTEIN LIPOVITELLIN IN
PLEURONECTIDS USING DIFFERENTIAL SCANNING
CALORIMETRY**

2.1 Abstract

The majority of marine teleosts are oviparous animals. Oviparous animals use yolk proteins as a major source of nutrition for growing embryos. The biggest source of nutrition is the yolk protein lipovitellin. Lipovitellin is a large glyco-phospho-lipo-protein ca. 200 kDa. Large proteins usually denature easily. However, prior evidence shows that fish lipovitellins are thermally stable. Using differential scanning calorimetry, I quantify lipovitellin's thermostability amongst four right-eye flounders (Pleuronectidae: winter flounder, American plaice, witch flounder, and yellowtail flounder). Differential scanning calorimetry allows direct interpretation of all thermodynamic properties; however, Lipovitellin was too large and precipitated before other thermodynamic properties could be determined. Other methods such as circular dichroism will need to be investigated to further study lipovitellin's thermodynamic properties. Pleuronectid lipovitellins all showed high melting points indicative of high thermostability. This shows that despite differing life histories, lipovitellin is conserved.

2.2 Introduction

Fish represent a large portion of the world's biodiversity with well over 26,000 known species (Nelson 1994). Recently, a majority of the world's fish stocks have been recognized as being exploited to or beyond their maximum sustainable levels (Boreman et al. 1997; FAO 2002; Worm et al. 2009). This overexploitation has made it imperative that we improve our knowledge about the biological processes underlying reproduction and recruitment as these are used to forecast population growth. Although fishes display a wide range of strategies and tactics during reproduction, the majority of marine teleosts are oviparous (Helfman et al. 1997; Wootton 1998). Oviparous animals utilize yolk proteins in the development of their embryos, a process known as vitellogenesis (Wahli et al. 1981; Byrne et al. 1989; Specker and Sullivan 1995; Mommsen and Korsgaard 2008).

Vitellogenesis begins with the hepatic secretion of the yolk pre-cursor, vitellogenin (Vg). Vitellogenin is a large dimeric glyco-phospho-lipo-protein which is evolutionarily homologous among a large variety of animals from insects to chickens, not only functionally, but also structurally (Byrne et al. 1989; Hiramatsu et al. 2002a; Patino and Sullivan 2002). Recent studies on Vg have focused on its use as a biomarker for estrogenic toxins because of its presence in males and juveniles exposed to xenoestrogens (Heppell et al. 1995; Hiramatsu et al. 2002c; Fujiwara et al. 2005; Hiramatsu et al. 2006; Matozzo et al. 2008).

Yolk proteins are formed by the proteolytic cleavage of Vg. This cleavage is performed by receptor-mediated endocytosis by the oocyte (Byrne et al. 1989). There are three major groups of yolk proteins, lipovitellins (Lv), phosvitins (Pv), and β -components (β') (Hiramatsu et al. 2002a). Of the yolk proteins, Lv is the major nutritional resource for the growing embryo (Thompson and Banaszak 2002; Walker et al. 2006).

Lipovitellin is a large dimeric lipoprotein, non-covalently bound to large amounts of lipid (Anderson et al. 1998). Lipovitellin has two subunits, one ca 115 kDa and a second of ca 31 kDa. Twenty or more percent of the structure is composed of lipid. The larger subunit has almost no phosphate while the smaller subunit is highly phosphorylated (Wahli et al. 1981). Only a small proportion of lipid is bound directly to the protein through hydrophobic interaction, with the rest binding through lipid to lipid interactions (Ohlendorf et al. 1978). This implies that the lipid molecules exist as a continuous domain that may have their own properties that are able to affect the forces of denaturation outside of the physical structure of the protein. These lipid hydrophobic cores have been shown to play a key role in the folding of the protein and add to its thermostability (Guo et al. 2005).

Despite their evolutionary significance, there have been very few studies conducted on Lv (Fujiwara et al. 2005). Besides being the major nutritional source, it appears to have evolutionary and structural relationships to other lipoproteins such as apolipovitellin, microsomal triglyceride transfer protein, and segments of apolipoprotein B (Thompson and Banaszak 2002; Warriar and Subramoniam 2003). Of even more

interest is Lv's role in the osmotic uptake of water. Lipovitellin breaks down into free amino acids (FAA) which drive the hydration of the eggs, allowing fish to spawn pelagic eggs. In benthic eggs, Lv plays a smaller role as the hydration of the egg is not as severe. Lipovitellin also aids in the osmoregulation of the eggs in the hypo-osmotic environment of the ocean (marine teleost eggs ~350 mOsm, average sea water ~1000 mOsm) until the embryo can develop its own osmoregulatory system (Thorsen and Fyhn 1996; Reith et al. 2001; Selman et al. 2001; Hiramatsu et al. 2002c; Sawaguchi et al. 2006; Finn and Kristoffersen 2007). This same pool of FAA increases the osmolarity of the oocyte and causes an influx of water by aquaporins that provides a reservoir of water until the drinking mechanisms are developed. Both outcomes of this pool of FAA are key evolutionary adaptations that allowed fish to rapidly fill the marine niches and move away from freshwater (Finn and Kristoffersen 2007).

Marine teleosts are known to have multiple Vg genes that give rise to multiple Lv (Reith et al. 2001; Fujiwara et al. 2005; Hiramatsu et al. 2006; Amano et al. 2008a). Multiple forms of Lv are degraded at different stages of development, some to provide immediate feeding and fast development while others are stored in the gut to allow the larvae to develop feeding mechanisms and reach suitable habitat (Hartling et al. 1997). Because of its developmental fate and high lipid content, Lv retains its initial integrity and thus returns to a functional state when stressed (Thompson and Banaszak 2002).

The main reason Lv is under studied is the difficulty in isolating it from the rest of the yolk material. Hartling et al. (1997) developed a method for isolating Lv. Their work

showed that Lv is a mixture of heat stable and heat labile protein in winter flounder (*Pseudopleuronectes americanus*). Heat denaturing eliminates the smaller β -components of yolk protein that can contaminate immunological tests. This high thermostability is a characteristic of Lv has not been reported in any other oviparous species and appears to be unique to teleosts.

This study took the next step and quantified the thermostability of Lv. However, thermostability, which is represented by a high melting point or transition midpoint (T_m), is not the only thermodynamic characteristic that is an important aspect of biological macromolecules. The overall stability of biological macromolecules and their associations is quantified by the standard free energy (ΔG°). There is also an associated change in heat capacity (ΔC_p) as the macromolecule changes thermodynamic states (i.e. unfolds). Integration of C_p versus temperature curve yields the transition enthalpy (ΔH°_m) which gives insights to the secondary structure of the protein (Bruylants et al. 2005; Privalov and Dragan 2007).

The technique known as differential scanning calorimetry (DSC) is the only method for the direct determination of ΔH°_m . DSC has also been used to show the heat stability of Scrombriod myoglobin (Ueki and Ochiai 2004) as well as the instability of selected fast skeletal muscle tropomyosins from across a variety of fish species (Huang and Ochiai 2005). One main limitation of this technique is the requirement of high concentrations of the protein (~1 mg/ml). This could lead to aggregation of the protein or self-association of the native state (Bruylants et al. 2005).

In this study of Lv's thermostability I built off Hartling et al's (1997) work and started with winter flounder Lv. Because of Lv's evolutionary importance it is theorized that it would be greatly conserved, therefore I extended the study to three other members of the family Pleuronectidae. The other three species are American plaice (*Hippoglossoides platessoides*), witch flounder (*Glyptocephalus cynoglossus*), and yellowtail flounder (*Limanda ferruginea*). Although the four species are in the same family they exhibit a variety of life histories. If lipovitellin is conserved the thermodynamic characteristics should be similar amongst the four species. I thus isolated the heat stable Lv via heat denaturation and tested its thermodynamic characteristics using DSC.

2.3 Methods

2.3.1 Study material

Lipovitellin was obtained from late developing to ripe fish ovaries from four pleuronectid species: American plaice, winter flounder, witch flounder, and yellowtail flounder. Ovaries were collected during the Northeast Fisheries Science Center's (NEFSC) bottom trawl surveys (Azarovitz 1981). NEFSC's bottom trawl surveys are conducted in the Northwest Atlantic Ocean and cover the continental shelf from Cape Hatteras, North Carolina to the Gulf of Maine. The seasonal timing of NEFSC's bottom trawl surveys are concurrent with the spawning times of the pleuronectids used in this study.

2.3.2 Heat Denaturation

Ovaries were removed and frozen. Yolk proteins were extracted through homogenization in 0.2N NaCl supplemented with 2 mM phenylmethylsulfonyl fluoride to inhibit proteases. The homogenate was centrifuged for 20 minutes. The supernatant was placed in a 75°C water bath for 3 minutes and centrifuged again. The resulting supernatant was dialyzed in 200 ml of 0.3 N NaCl buffered with 10 mM sodium phosphate and 0.15M KCl for 1 hour and a subsequent 200ml of the same buffer for 2 additional hours to remove any low weight material. The supernatant (3-5 ml) was then added to a Bio Gel A-1.5 column (Bio-Rad, Melville, NY). The sample was eluted at 4°C for approximately 20 hours using a constant flow rate of 8 ml/hour, regulated by a metering pump (Milton Roy Minipump). Fractions were collected at 20 min intervals. The presence of Lv was verified using a uv/vis spectrometer (Perkin Elmer Lambda 2) measuring absorbance at 280 nm and assuming approximately 1 mg/OD unit. The molecular weight was estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) with a Bio-Rad six step high molecular weight standard protein ladder (200, 97, 66, 45, and 31 kDa). The gel was run at a constant voltage (150 V) for 1 hour and was stained with 0.1% Coomassie Blue R-250 (Fisher, Orangeburg, NY) in 40% methanol, 10% acetic acid and de-stained with 10% acetic acid under microwave acceleration. SDS-PAGE was also used to show that smaller weight components of the yolk had been removed, effectively isolating the heat stable Lv.

2.3.3 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was carried out using a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal, Springfield, MA). Sample buffer (0.3N NaCl buffered with 10 mM sodium phosphate and 0.15 M KCl) was compared to the sample by loading 0.5 ml of buffer into the reference well, and 0.5 ml of the protein (1.0-1.5 mg/ml concentration) into the sample well. The cells were then heated from 10 to 110 °C at a rate of 90°/hour. Buffer versus buffer was also run to determine a baseline for the denaturation curve. The resulting denaturation curve was used to calculate the T_m. Samples of winter flounder were run in a similar manner on a TA microcalorimeter (TA Instruments, New Castle, DE) to verify results.

2.4 Results

Lipovitellin was successfully purified from the ovaries via heat denaturation. SDS-PAGE gels showed a significant band present circa 94 kDa in the heat treated serum (Figure 2.1). Secondary bands were also present around 66 kDa. Figure 2.2 shows the step-by-step denaturation process going from multiple bands, the majority of which are low molecular weight (Figure 2.2, column A) to a single band circa 94 kDa (Figure 2.2, column D).

For all four species a single endothermic peak was observed in the range of 86-92°C when using DSC (Figure 2.3). A subsequent crash was observed which is typical of

a protein precipitating out of solution (MicroCal personal communications). To test for reversibility, sequential runs were performed on the samples that resulted in no observable peaks (data not shown). Samples of winter flounder Lv were run on the TA microcalorimeter to avoid the protein coming out of solution but were equally unsuccessful (Figure 2.4). The output from the TA DSC was different from those obtained using the MicroCal DSC; the crash was less noticeable; instead the precipitation was represented by the leveling off the denaturation curve following the T_m . The precipitation was observed on both machines and precludes an accurate estimation of thermodynamic characteristics other than T_m .

2.5 Discussion

The yolk precursor, vitellogenin, breaks down to various yolk proteins to nourish embryos of oviparous animals. Lipovitellin is a large lipoprotein that serves as the major nutritional resource for the embryo. In marine teleosts, Lv serves a secondary function as an integral part of the hydration and osmoregulation of eggs (Thorsen and Fyhn 1996; Reith et al. 2001; Selman et al. 2001; Hiramatsu et al. 2002c; Sawaguchi et al. 2006; Finn and Kristoffersen 2007). It has been theorized that Lv played a key role in the evolution of marine teleosts, allowing them to exploit all the various ecological niches of the world's oceans (Finn and Kristoffersen 2007). Because of this evolutionary significance, one would expect that Lv has been highly conserved despite the divergence of marine teleosts.

The problem with studying Lv has been the presence of other material within the yolk. Hartling et al. (1997) reported a heat stable fraction of lipovitellin in winter flounder. This has allowed for an effective method for isolating the protein. My study isolated not only winter flounder Lv but also three other members of the family Pleuronectidae. The four species display a range of life history traits. Winter flounder are winter/ early spring spawners that have benthic eggs. The other three species produce pelagic eggs that do not contain oil globules (Collette and Klein-MacPhee 2002). Even with these differences SDS-PAGE results showed similar molecular weights within the pleuronectids that was similar to Hartling et al.'s (1997). All species showed a major band circa 94 kDa with a secondary band appearing around 66 kDa. This is a first sign that Lv has been conserved. Slight variations were most likely due to different levels of maturity among individuals as well as the presence of multiple Lvs at different levels of degradation.

Recent studies have reported thermostability of different proteins using DSC (Ueki and Ochiai 2004; Huang and Ochiai 2005). This study shows quantitatively the thermostability of Lv. The four pleuronectids displayed high Tms (86-92°C). This apparent heat stability of Lv has not been reported in any other species and appears to be unique to teleosts. Other denaturation studies found that Vg was much more labile and sensitive to degradation in the presence of urea than its product proteins (Warrier and Subramoniam 2003). In the case of Lv, much of that resistance to unfolding occurs because of strong hydrophobic interactions with the lipid components (Thompson and Banaszak 2002; Guo et al. 2005).

Further insight to the structure of Lv was hoped to be achieved through the use of DSC. Unfortunately the other thermodynamic characteristics (e.g. ΔG° , ΔC_p , ΔH°_m) were unattainable. The high concentration of protein required for DSC caused Lv to precipitate out of solution. During the precipitation the values collected by the calorimeter are no longer indicative of the actual unfolding of the protein. The majority of the scans were conducted on a MicroCal calorimeter. The MicroCal machine uses a coin shaped sample well. It was proposed that a TA microcalorimeter, which uses a coil sample well, might prevent Lv from precipitating. Although the outputs are different from the MicroCal and TA microcalorimeters, the results were effectively the same. This means that other methods such as circular dichroism will have to be explored to determine the structure of Lv and see if the structure has been conserved throughout the evolution of teleosts.

Although the DSC was not able to obtain the full compliment of thermodynamic characteristics, Lv did show conserved properties within the pleuronectids. The T_m values while not exact do demonstrate that Lv is heat stable. SDS-PAGE gels also show that the protein is composed of similar size polypeptides. Further analysis with other methods will have to be explored to show that the structure of Lv has been conserved despite the divergence of marine teleosts. DSC may be the only way to directly calculate the full range of thermodynamic characteristics however it appears not to be the best method when dealing with large macromolecules.

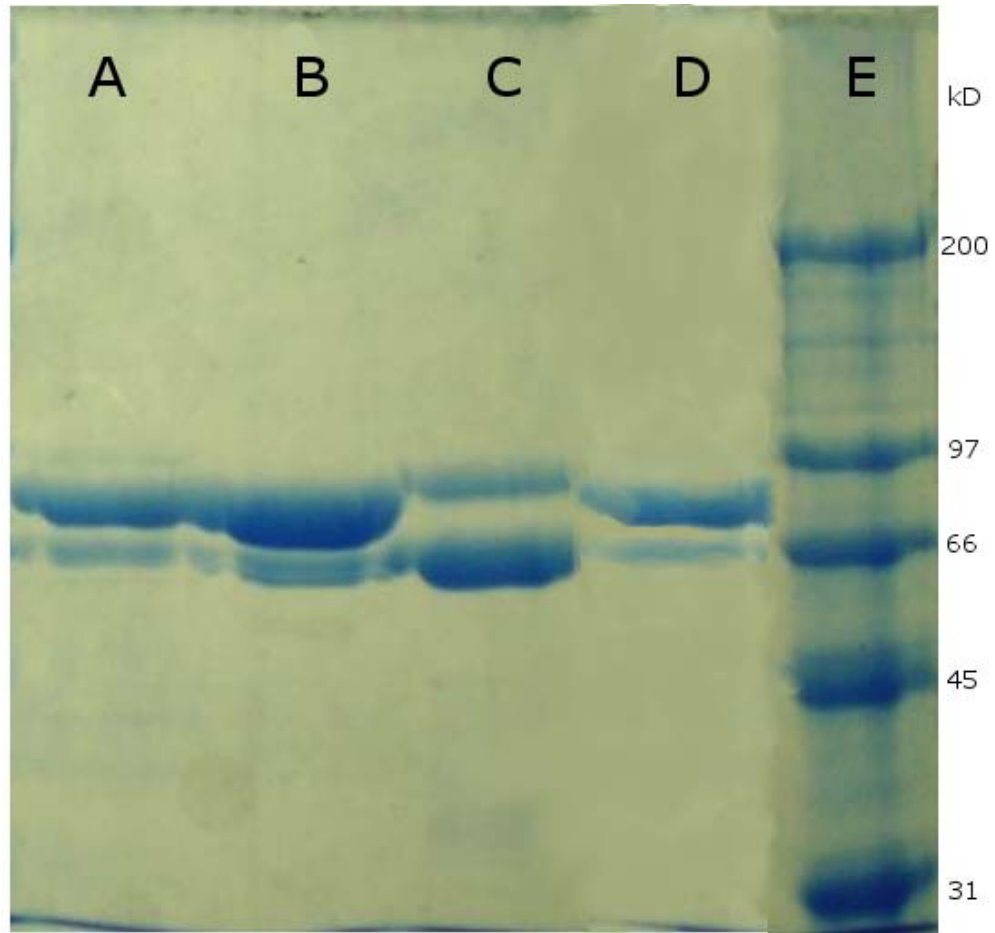


Figure 2.1 – SDS-PAGE gel of the four pleuronectids. Lane A is American plaice, lane B is winter flounder, lane C is witch flounder, and lane D is yellowtail flounder. Lane E contains high molecular weight standards with their corresponding weights to the right.

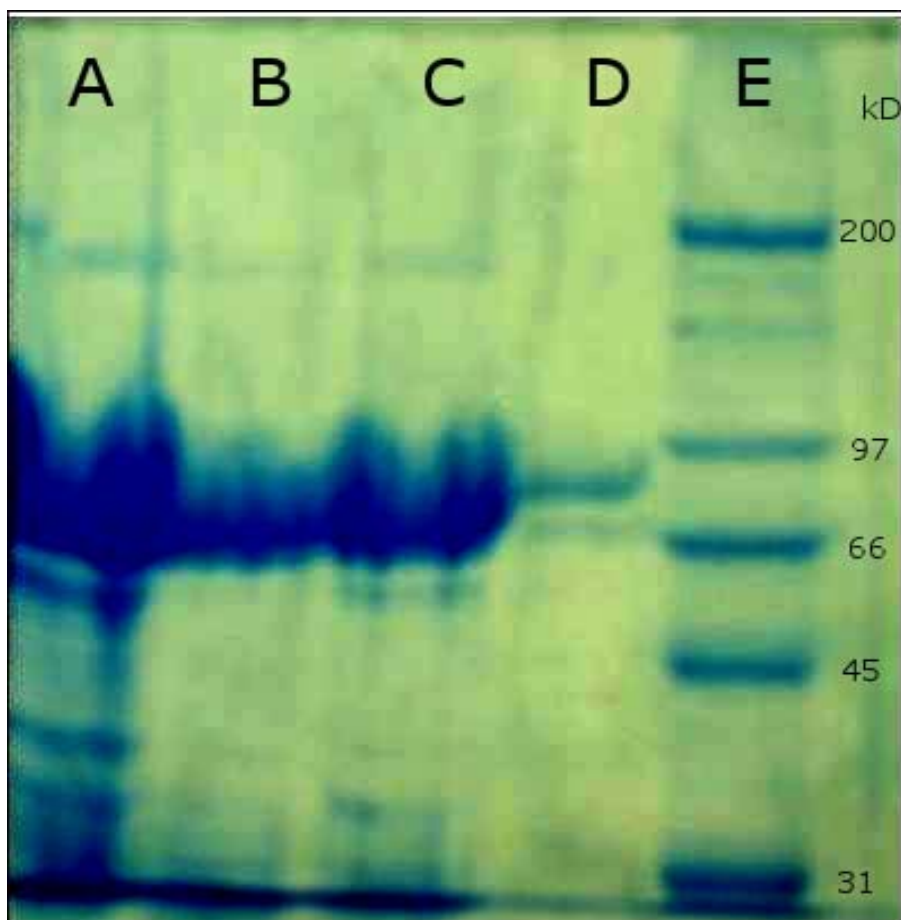


Figure 2.2 – SDS-PAGE gel of step by step heat denaturation of winter flounder Lv. Lane A contains raw ovary sample. Lane B and C are intermediate steps in the process. Lane D is the purified Lv. Lane E contains high molecular weight standards with their corresponding weights to the right.

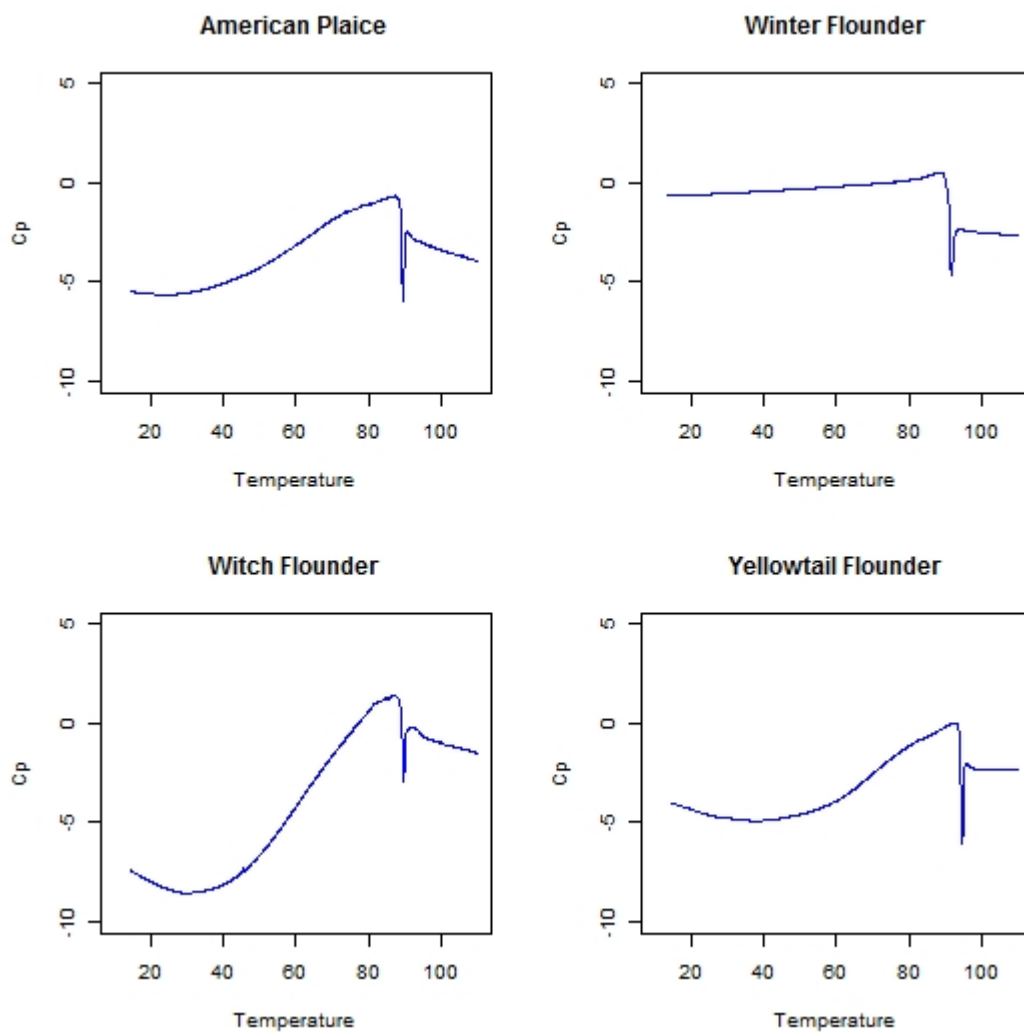


Figure 2.3 – DSC scans of the four pleuronectid species on the MicroCal microcalorimeter.

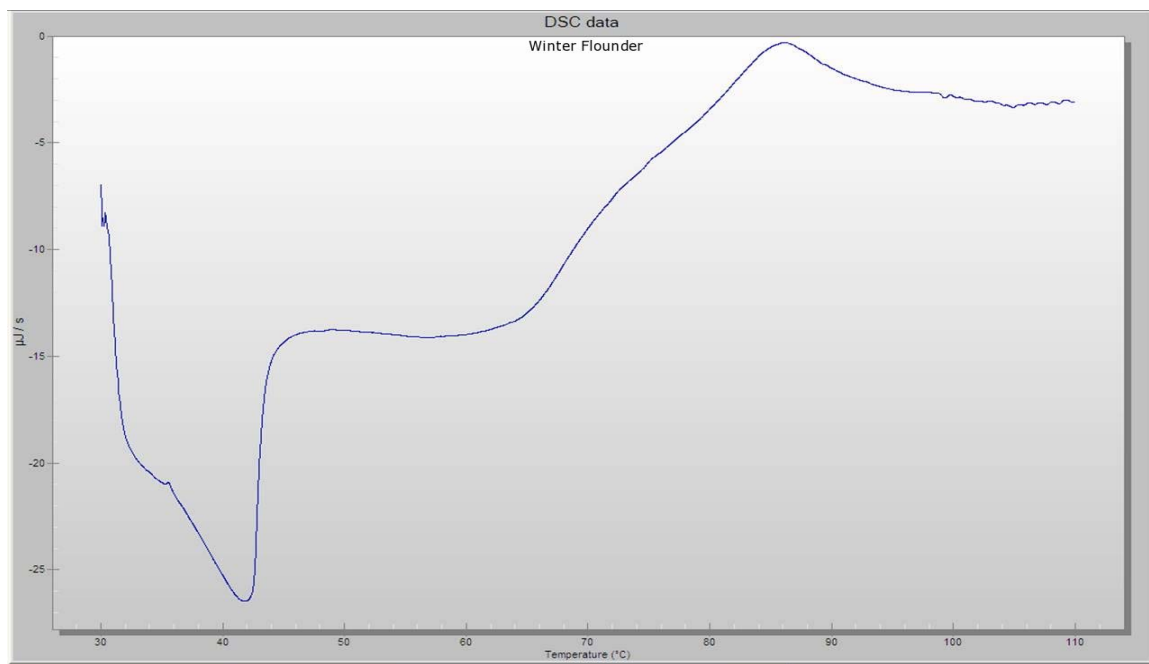


Figure 2.4 – DSC scan of winter flounder on the TA Instrument microcalorimeter.

CHAPTER 3
INDUCTION OF VITELLOGENIN (VG) IN MALE BLUEFISH (*POMATOMUS*
***SALTATRIX*)**

3.1 Abstract

Xenoestrogens are a type of endocrine disrupting chemical that blocks or mimics natural estrogens. They are known to disrupt aquatic life by interfering with natural development and reproduction. A major biological side effect of xenoestrogens is the accumulation of vitellogenin, a female precursor for yolk proteins, in males. This effect has made vitellogenin a useful biomarker for monitoring levels of contamination. Unfortunately, vitellogenin can vary greatly in its immunological and structural characteristics, which means that species-specific assays are necessary. This study took the first step in developing an immunoassay for bluefish (*Pomatomus saltatrix*). Vitellogenin was induced by injecting a group of bluefish with an estrogen, estradiol, and the resulting vitellogenin was isolated from the serum of males. The protein was characterized as vitellogenin by determining its large Stokes radius in gel permeation chromatography combined with its characteristic peptide molecular weight in sodium dodecyl sulfate polyacrylamide gel electrophoresis.

3.2 Introduction

Anthropogenic toxins can have adverse effects on aquatic life. In particular, xenoestrogens, a type of endocrine disrupting chemical that blocks or mimics natural estrogens, can interfere with reproduction and normal development of teleost fish (Heppell et al. 1995; Goksoyr 2006; Scott et al. 2006; Johnson et al. 2008). The major sources of xenoestrogens are treated and untreated effluents, agricultural and livestock waste, and runoff from sewage treatment plants. They have been known to bioaccumulate and biomagnify (Johnson et al. 2008; Matozzo et al. 2008). In response, scientists have developed a number of techniques to monitor xenoestrogen contamination including the use of biomarkers such as the protein vitellogenin (Heppell et al. 1995; Hiramatsu et al. 2002c; Fujiwara et al. 2005; Hiramatsu et al. 2006; Johnson et al. 2008; Matozzo et al. 2008).

Vitellogenin (Vg) is the precursor to yolk proteins in oviparous animals. Vitellogenin is a large glyco-phospho-lipo-protein synthesized in the liver in response to 17β -estradiol (Specker and Sullivan 1995; Hiramatsu et al. 2002c). After undergoing lipidation, phosphorylation and glycosylation, Vg is secreted from the parenchymal liver cells and transported to the ovary via the circulatory system (Wahli et al. 1981; Byrne et al. 1989; Specker and Sullivan 1995; Hiramatsu et al. 2002a). It is found naturally in the blood of maturing female fish, however, one biological response to xenoestrogens is the production of Vg in male and immature fish. This has allowed Vg to serve as a biomarker for xenoestrogens in aquatic environments (Heppell et al. 1995; Hiramatsu et

al. 2002c; Fujiwara et al. 2005; Hiramatsu et al. 2006; Scott et al. 2006; Johnson et al. 2008; Matozzo et al. 2008).

Recently, efforts have been undertaken to monitor the effects of xenoestrogens in the New York City watershed. This watershed covers almost 5200 km² and has over 100 wastewater treatment plants that discharge into it (NYCDEP 2006). This has created multiple sources of possible contamination that could be reaching estuaries and coastal waters. Bluefish (*Pomatomus saltatrix*) are important recreational fish common in the area. They inhabit coastal and inshore waters (Collette and Klein-MacPhee 2002) and have been the focus of other contamination studies (e.g. Deshpande et al. 2002; Burger and Gochfeld 2005). The objective of this study was to collect Vg from male bluefish in order to develop an immunoassay to test for the presence of xenoestrogens.

3.3 Methods

Adult bluefish were captured from the Navesink River in Highlands, New Jersey and held at the James J. Howard Laboratory (National Marine Fisheries Service, Northeast Fisheries Science Center) on Sandy Hook, NJ. Seventeen fish (546-739 mm FL, Table 3.1) were held in four 2.5 m diameter circular tanks supplied with ambient flow-through seawater from Sandy Hook Bay. They were fed daily rations of frozen fish throughout the experiment.

An initial bleed of 5 ml was performed to acquire control serum. Bluefish were anesthetized with Aquí-S (Aquí-S, New Zealand). Zero bleed blood was drawn from the

tail vein of each fish using 5 ml (Becton-Dickinson) disposable syringes coated in heparin (Sigma, Heparin sodium salt, H0777-25KU + 4 ml diH₂O) to prevent clotting. Blood was centrifuged at 1500 rpm in a mini-centrifuge for 20 minutes. Serum was decanted and 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to inhibit proteases. The serum was placed on ice and was frozen within 3 hours. Two fish (1 male and 1 female, Table 3.1) were sacrificed as controls, their gonads macroscopically inspected and tissue samples of gill, liver, and gonad removed and frozen. The tissue samples were not analyzed for this study. Of the remaining fish, 12 were injected with 3 ml 10 mg/ml estradiol. The other three were held as controls. The second bleed occurred 10 days later. Blood was collected in the same manner as before with the exception that 15 ml of blood was drawn. All fish were sacrificed after the second bleed to macroscopically inspect gonads and remove tissue samples.

To determine if Vg had been successfully induced, the serum was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The frozen serum was thawed and centrifuged at 1500 rpm for 20 minutes. Serum was then prepared for SDS-PAGE as described by Laemmli (1970). Samples from male control, female control, and male injected with estradiol were run in adjacent lanes. A six step high molecular weight standard protein ladder (200, 97, 66, 45, and 31 kDa) was also used. The gel was run at a constant voltage (150V) for 1 hour and was stained with 0.1% Coomassie Blue R-250 (Fisher, Orangeburg, NY) in 40% methanol, 10% acetic acid and de-stained with 10% acetic acid under microwave acceleration. Images of the gel were taken with a Nikon D-40 camera.

Once it was determined that a protein had been induced, serum from a male injected with estradiol was subjected to gel chromatography to isolate the protein. This was accomplished by adding the serum to a 0.9x800 cm Bio Gel A-1.5 column (Bio-Rad, Melville, NY) along with 5 mg/ml blue dextran (Pharmacia, MW=2,000,000) as the void volume (V_0) marker and 7 mg/ml potassium hydrogen phthalate (Fisher, MW=204) as the total volume (V_t) marker. The sample was eluted at 4°C for approximately 20 hours using a constant flow rate of 9 ml/hour, regulated by a constant volume metering pump (Milton Roy Minipump). Fractions were collected at 20 min intervals. The presence of the protein was verified using a uv/vis spectrometer (Perkin Elmer Lambda 2).

To further verify the protein as vitellogenin, the Stokes radius was measured. The Stokes radius is the measure of a hard sphere that would diffuse at the same rate as the molecule. The more extended a molecule is the larger the Stokes radius as compared to a compact molecule of the same molecular weight. To do this, the molecular weights of the various peaks were estimated using SDS-PAGE (Laemmli 1970) as above. The peak that resembled the induced protein from the initial gel then had its Stokes radius determined by calculating V_e/V_0 where V_e is the eluted volume of the sample and V_0 the void volume of the column. The value of V_e/V_0 was then plotted on the calibration curve of Duhamel and Kunkel (1983).

3.4 Results

Macroscopic inspection of the gonads revealed that four of the six females showed signs of developing 10 days after the estradiol injection. The two female non-injected controls were also beginning to develop at the time of final sacrifice. The control female sacrificed prior to injection was in a resting state. Developing states were characterized by enlarged ovaries with noticeable vascularization and an orange color. Resting state was characterized by small, flaccid ovaries with little to no vascularization.

The presence of vitellogenic females made it imperative that there were male bluefish. Injected bluefish exhibited a 50/50 sex ratio (6 females, 6 males, Table 3.1). SDS-PAGE showed a significant band present circa 175 kDa in the estrogen injected male, not present in the control male, and lightly present in the control female (Figure 3.1).

Gel permeation chromatography of experimental male bluefish serum on Bio-Gel A-1.5 exhibited three distinct peaks between the void and total volume peaks (Figure 3.2). Further investigation with SDS-PAGE revealed that the second peak contained a protein band circa 175 kDa which is consistent with vitellogenin. By plotting V_e/V_o on the Duhamel and Kunkel (1983) calibration curve it was determined that the Stokes radius of the bluefish vitellogenin is 6.29 nm.

3.5 Discussion

Once thought to be mainly a freshwater problem, there is a growing list of marine teleosts that show signs of oestrogenic effects due to exposure to anthropogenic xenoestrogens (Scott et al. 2006). A side effect exploited by scientists is the increased levels of the female protein vitellogenin in juveniles and males (Heppell et al. 1995; Palumbo et al. 2007; Matozzo et al. 2008). In order to monitor the level of contamination, immunoassays have been developed to vitellogenin as a biomarker. However, the immunological and structural properties of Vg can vary widely among species, even those that are closely related (Heppell et al. 1995). This variation means that species specific assays need to be developed (Watts et al. 2003).

Xenoestrogens are a growing concern in the inshore and coastal waters adjacent to the New York watershed because of the large human population and numerous wastewater facilities that discharge into it. This has demonstrated a need for the development of a biochemical marker for this area. Bluefish have been used as a focal species in this area for many contamination studies (e.g. Deshpande et al. 2002; Burger and Gochfeld 2005). They are an abundant recreational species that are widely available in many areas. Despite being seasonal migrants in the area, juveniles inhabit the estuaries and are exposed to concentrated forms of coastal pollution (Iannuzzi et al. 2004). Using age-0 bluefish as a marker will thus allow for a large coverage area of inshore and coastal waters. However, there are currently no Vg antisera that interact with bluefish.

I encountered a unique problem when obtaining ripe female bluefish as a source of vitellogenin. The Northeast Fisheries Science Center's bi-annual bottom trawl survey did not capture any. The same was true for recreational fishermen that were approached. This meant that I had to develop another method of obtaining bluefish Vg. The problem was solved by inducing Vg in male bluefish.

It is evident when comparing the serums of the control male to the estradiol injected male by SDS-PAGE that an estrogen-induced protein was synthesized. The next step was proving that it is Vg. The protein was found to have a molecular weight around 175 kDa. This is consistent with the findings of Hartling et al. (1997) who found native Vg from winter flounder, *Pseudopleuronectes americanus*, to have a similar molecular weight. By comparing the Stokes radius of the bluefish protein (6.29 nm) to the winter flounder Vg (6.7 nm, Hartling et al. 1997), it once again indicates that the induced protein is Vg. The Stokes radius would indicate that the native form is a dimer since the Stokes radius of an Lv monomer is much smaller. Other studies have also found similar polypeptide weights for Vg using SDS-PAGE (e.g. Palumbo et al. 2007; Amano et al. 2008b).

This study shows that in the absence of developing females, vitellogenin can be successfully induced by injections of the estrogen, estradiol, to a group of fish. This essentially mimics the xenoestrogens and causes the male liver to produce the female protein. Now that there is a supply of bluefish Vg, an immunoassay can be developed for the New York Bight. The next step is to submit the purified protein to a commercial

polyclonal antiserum production protocol that will produce an antiserum. Then an enzyme-linked immunosorbent assay (ELISA) could be developed allowing the monitoring of age-0 bluefish for the presence of vitellogenin which would indicate a high level of xenoestrogens in the water.

Table 3.1 - Adult bluefish (*Pomatomus saltatrix*) captured from the Navesink River in Highlands, New Jersey used in an estradiol injection project to induce vitellogenin (Vg). FL = fork length, TL = total length in mm.

Fish#	FL	TL	Sex	Notes
1	635	704	Female	
2	621	695	Female	
3	546	605	Male	
4	619	690	Male	
5	549	615	Female	Control - Sacrificed prior to injection
6	739	820	Female	
7	635	710	Male	
8	615	690	Male	
9	654	725	Female	
10	615	690	Female	Control - No estradiol injection
11	605	680	Male	Control - No estradiol injection
12	560	625	Female	Control - No estradiol injection
13	615	680	Male	Control - Sacrificed prior to injection
14	632	700	Male	
15	625	690	Male	
16	616	670	Female	
17	630	700	Female	

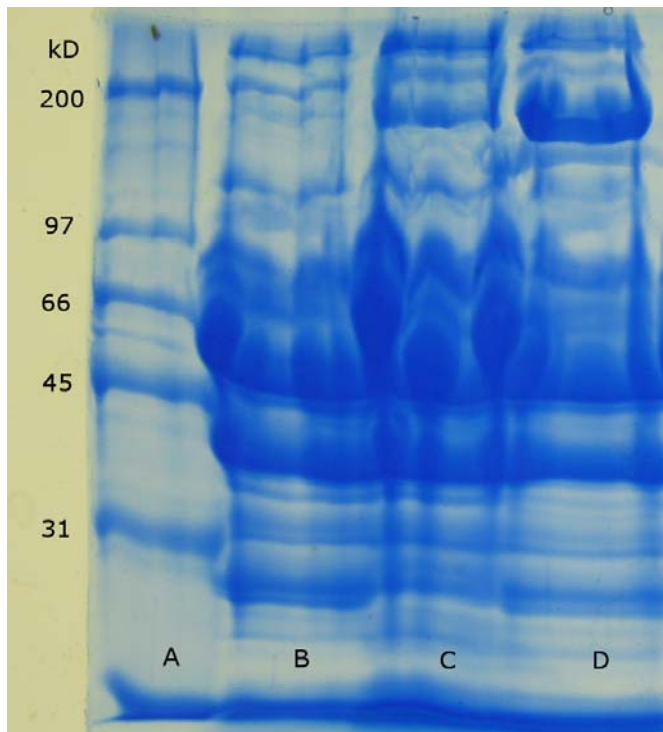


Figure 3.1 - SDS-gel of bluefish serum. Lane **A** is high molecular weight markers with their corresponding weight on the left. Lane **B** is control male (no injection), lane **C** is control female (no injection), and lane **D** is experimental male (estradiol injection). The large band in column **D**, circa 200kDa, is the putative induced Vg.

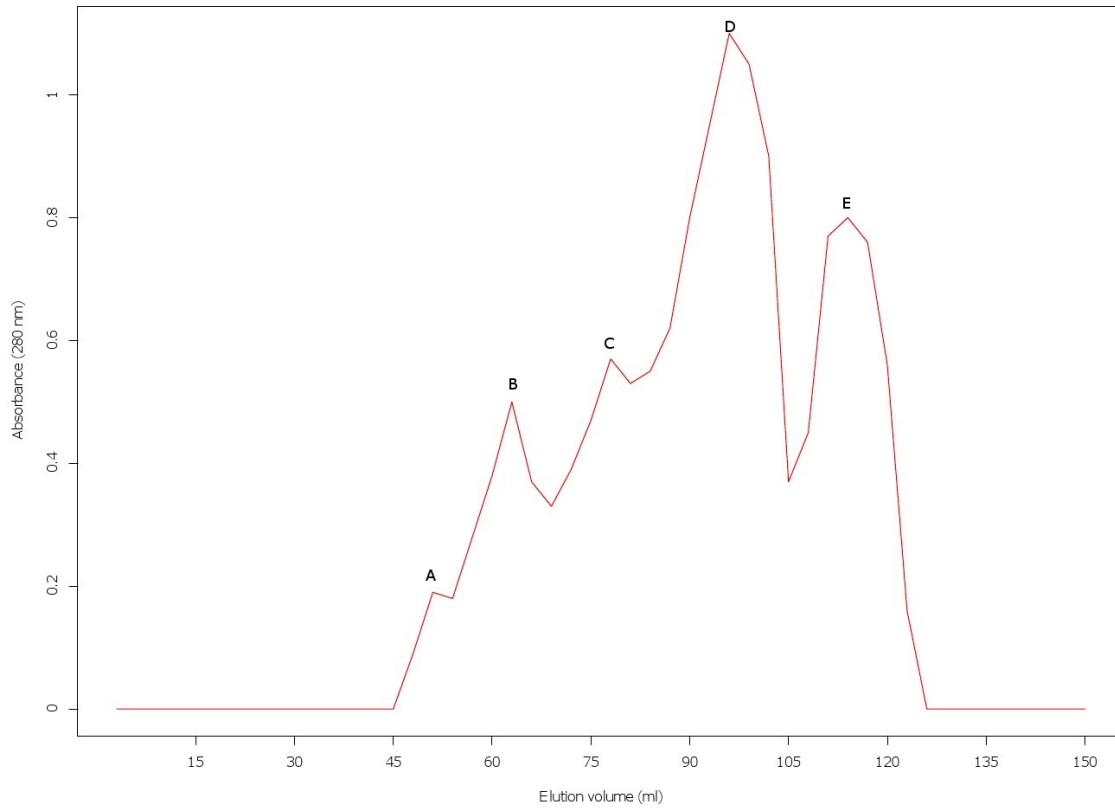


Figure 3.2 - Bio-Gel A-1.5m gel filtration of experimental male bluefish serum. Peak A is the void volume (blue dextran) and peak E is the total volume peak (potassium hydrogen phthalate). The induced V_g is represented by peak C as determined by SDS-Gel (figure 3.3).

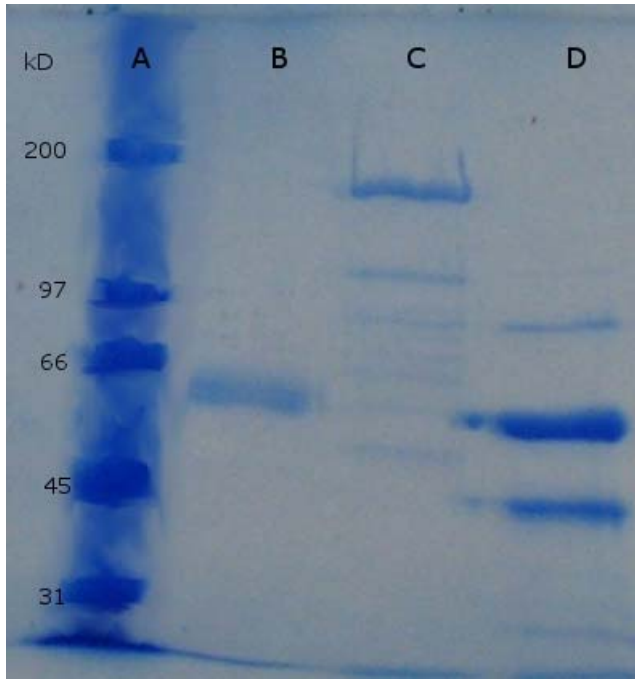


Figure 3.3 – SDS-gel of experimental male bluefish serum after gel filtration. Lanes **B**, **C**, and **D** correspond with volume peaks in Figure 3.2. Lane **A** is high molecular weight markers with their corresponding weight on the left. Lane **C** contains the putative induced Vg.

APPENDIX A

LIST OF ABBREVIATIONS

β'	β -components
ΔC_p	heat capacity
ΔG°	standard free energy
ΔH°_m	transition enthalpy
DSC	differential scanning calorimetry
E_2	17 β estradiol
FAA	free amino acids
FSH	follicle stimulating hormone
kDa	kiloDalton
LH	luteinizing hormone
Lv	lipovitellin
mOsm	milliosmole
NEFSC	Northeast Fisheries Science Center
Pv	phosvitin
SDS-PAGE	sodium dodecyl sulfate polyarylamide gel electrophoresis
T_m	transition midpoint
V_e	eluted volume
V_g	vitellogenin
V_o	void volume
V_t	total volume

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