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**INVESTIGATING FACTORS CONTRIBUTING TO REDUCED EMBRYO SURVIVAL IN
FARM RAISED ATLANTIC SALMON, *Salmo salar* L.**

by

LeeAnne Rose Thayer

B.S. University of New Hampshire, 2008

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Marine Biology)

The Graduate School

The University of Maine

August 2017

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Dissertation Advisor: Dr. Heather Hamlin

An Abstract of the Dissertation Presented
in Partial Fulfillment of the Requirements for the
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(in Marine Biology)

August 2017

Embryo mortality of Atlantic salmon, *Salmo salar* Linnaeus 1758, has been increasing for more than a decade in the State of Maine, a leading producer of this species in the United States. Increasing embryo mortality not only creates a financial bottleneck for farms but also prevents the sale of surplus eggs as an additional source of revenue. Blood and egg samples were collected at three Maine Atlantic salmon farms from female broodstock at the time of spawning over a 2-year period. Correlative factors for reduced embryo survival were investigated by measuring egg and maternal plasma concentrations of 17 β -estradiol (E2), 11-ketotestosterone (11-KT), testosterone and calcium, as well as maternal hepatic ethoxyresorufin-O-deethylase (EROD) activity and standard length. Significant positive correlations were found between maternal plasma concentrations of E2 and 11-KT and embryo survival. Interestingly, there was no correlation with egg concentrations of sex steroids and embryo survival, suggesting that embryo mortality does not likely rest with the maternal deposition of sex steroids into the egg, but with another hormone regulated process related to egg assembly, ovulation, or post-ovulatory ageing.

E2 and 11-KT in the plasma, ovarian fluid (OF) and eggs of female broodstock Atlantic salmon were measured to determine possible sources of sex steroids to the eggs. There was a positive correlation between OF hormone concentration and egg hormone concentration but no correlation between maternal plasma hormone concentration and egg hormone concentration, suggesting OF plays an important role in oocytes at the time of spawning.

Although the correlations in this study help us to understand the reproductive biology of the Atlantic salmon, this information applies only to the time of spawning. Therefore, annual hormone profiles of the northern strain of Atlantic salmon were created by plotting monthly average hormone concentrations of plasma E2 and 11-KT in female broodstock Atlantic salmon over the course of a year to better understand the reproductive biology of this commercially important species.

To My Grandfather, John C. Woodcock

Captain of the Janice R.

ACKNOWLEDGMENTS

The path that has led me down the road of earning a Ph.D. has been full of personal growth, making meaningful relationships, and plenty of adventure. I first need to thank David Berlinsky for having a passion for aquaculture that is contagious and thankfully rubbed off on me; I love this field and feel right at home. If it wasn't for his mentorship while I was an undergraduate at the University of New Hampshire then I wouldn't be here today. Secondly, I need to thank my advisor Heather Hamlin for taking a chance on me and accepting me as her first graduate student. I feel so grateful to have worked on this project with her. Heather allowed me to be involved with the decisions pertaining to the experimental design and methodology of this research and I can't thank her enough for building my confidence and leadership skills. I was able to learn new lab techniques from Heather and the experience I gained was nothing short of fun. Among RIA's and EIA's, Heather taught me the wonderful process of histology, a technique I became obsessed with and I thank you so much for that!

I would also like to thank the rest of my committee for supporting me through this entire process. Adria Elskus gave the best advice at our weekly lab meeting; whether it was practicing a presentation, going through a poster draft, or brainstorming research ideas, she always took the time to give thoughtful comments. Murray Hall has never been the same without you. Mary Tyler, where does one even begin to say 'thank you' to her? Mary is such a fearless woman and extremely innovative in her classroom techniques, she is not afraid to step outside the box and I am so grateful to have crossed paths with her. Ian Bricknell, he was one of the first people to boost my confidence and encourage me to enter graduate school. He saw my potential and because of that I have been able to reach for my wildest dreams, thank you so much!

My research would not be possible without the help of many people. I would like to give a huge thank you to the salmon farmers that I collaborated with and for letting me collect my samples during the most stressful time of year, spawning season! Thank you to the National Cold Water Marine Aquaculture Center's William Wolters, Brian Peterson, Melissa Alberts, Ryan Hastey, Gary Burr, and Davin O'Connell as well as Cooke Aquaculture's Greg Lambert and Brian Wheeler. It was such a pleasure working with everyone and seeing them each year during the coldest winter months. I will definitely miss all of the sampling days. I also need to thank everyone who was willing to wake up in the early winter mornings to help collect my samples with me: Alyssa Freitag, Deborah Bouchard, Sarah Turner, Scarlett Tudor, Sarah Conlin, Meghan Capps, and Jonathan Thayer.

I also need to thank the Aquaculture Research Center's Neil Greenburg and Robert Harrington for all of their help with my year long salmon study that took place. They were instrumental in making my sampling days go smoothly and making sure all of the equipment was up and running in the recirculating aquaculture system.

Lastly, I want to thank my ENTIRE family who continue to always believe in me and have never stopped rooting for me, thank you! Biggest thanks to my Mom and Dad who raised me on a farm and taught me early about proper work ethic and the "can-do" way of life. I am so blessed to have them as my parents and role models. Thank you to my big sister, Chrissy Burr, who I always looked up to and wanted to be exactly like growing up. Thank you for always putting up with me (even today) and never making me feel anything less than loved and accepted. Thank you to my loving husband who has supported all of my life and career decisions and has even saved me on occasion when I needed help in the lab or field...like that one time at Stinson's when he got covered head to toe in herring "juice", what a ride it has been!! Jonny, you

have given me more than I could ever dream of and so much more, thank you. I can not wait to see what the future has in store for us!

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LIST OF ABBREVIATIONS

3 β HSD	3 β -hydroxysteroid Dehydrogenase
11-KT	11-ketotestosterone
ANOVA	Analysis of Variance
DHP	17 α ,20 β -dihydroxy-4-pregnen-3-one
DO	Dissolved Oxygen
DPO	Days Post Ovulation
DPS	Distinct Population Segment
DTT	Dithiothreitol
E2	17 β -Estradiol
EDCs	Endocrine Disrupting Contaminants
EIA	Enzyme Immunoassay
EROD	Ethoxyresorufin-O-deethylase
ESA	Endangered Species Act
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormones
HPG	Hypothalamic-pituitary-gonad
HPO	Horseradish Peroxidase
ISA	Infectious Salmon Anemia
LH	Leutenizing Hormone
MeOH	Methanol
NCWMAC	National Cold Water Marine Aquaculture Center
NMFS	National Marine Fisheries Service

OF	Ovarian Fluid
P	Pregnenalone
P4	Progesterone
P450 _{scc}	P450 side-chain cleavage
PBS	Phosphate Buffered Saline
PAH	Polycyclic Aromatic Hydrocarbons
PHH	Planar Halogenated Hydrocarbons
RAS	Recirculating Aquaculture System
RIA	Radio-Immunoassay
SEM	Standard Error of the Mean
SE	Standard Error
StAR	Steroidogenic Acute Regulatory Protein
T	Testosterone
T ₃	Triiodo-L-thyronine
USDA	United States Department of Agriculture
USFWS	United States Fish and Wildlife Service
TH	Thyroid Hormone
Vtg	Vitellogenin

CHAPTER 1

INTRODUCTION

1.1. Background

1.1.1. Overview of Reproductive Endocrinology in Fishes

Environmental cues are key factors in reproductive success for arguably all species. Changing of the tides, temperature fluctuations, light cycles, salinity changes, and food availability are just a few examples of signals from the environment that trigger the hypothalamic-pituitary-gonad (HPG) axis in teleosts (Lubzens *et al.* 2010). Environmental signals trigger the hypothalamus to produce gonadotropin releasing hormones (GnRH) which stimulate the anterior pituitary to produce follicle stimulating hormone (FSH) and leutenizing hormone (LH). FSH and LH circulate throughout the body and target organs by binding to their respective receptors, FSH-R and LH-R; this will trigger processes such as gonadal steroidogenesis. The gonad will produce sex steroids such as the estrogen 17 β -estradiol (E2) and the androgen 11-ketotestosterone (11-KT). In females, E2 is responsible for triggering growth of the gonad, vitellogenesis, oogenesis, oogonial proliferation and sexual maturation (Grier 2000). The hormone 11-KT is responsible for stimulating sexual behavior and secondary sexual characteristics (Gilbert 2010). Although 11-KT is considered the primary androgen in fish, concentrations of testosterone (T) in females can meet or even exceed those found in males (Macgregor, Dindo & Finucane 1981; Scott, Bye & Baynes 1980) suggesting it plays an important role in female maturation. Although it is known that T serves as the precursor of E2 by aromatization (Gilbert 2010) and helps to stimulate sexual behavior (Scott *et al.* 1980), its role in fish is still not fully understood. When compared to T, 11-KT is considered the more potent androgen in teleosts and is particularly high in females in some species of salmonids.

All sex steroids, such as E2, 11-KT, and T are derived from a cholesterol precursor. Inside the cell, cholesterol gets mobilized to the outer mitochondrial membrane where steroidogenic acute regulatory (StAR) protein transports it from the outer to the inner mitochondrial membrane. This transportation step is thought to be the rate limiting step in steroidogenesis (Stocco 1999). Cholesterol is then cleaved by the enzyme P450 side-chain cleavage (P450_{scc}) to form pregnenolone (P), the first steroid hormone. Pregnenolone is converted to progesterone (P4) by 3 β -hydroxysteroid dehydrogenase (3 β HSD), and P4 can enter one of several pathways in order to produce other steroid hormones, including E2, T, and 11-KT.

1.1.2. History of Atlantic Salmon Farming in Maine

Historically, the Atlantic salmon has been an important fish for food and sport. Records of Native Americans and European settlers eating salmon date back to the 1700's (Tiro 2016). Due to overfishing, dams, and environmental factors, Atlantic salmon populations have dramatically declined. In 1870, the first attempt at restoring Maine's salmon population was made by opening a salmon hatchery: The US Fish and Wildlife Service's Craig Brook Hatchery in Bucksport. The purpose of this hatchery is to catch Atlantic salmon broodstock in local rivers for gamete collection and egg fertilization. Once the eggs have hatched, fry are kept at the hatchery for several weeks of conditioning before they are released into the wild. Despite the restocking efforts made towards reviving Atlantic salmon in Maine, their numbers have never recovered and in 2000, the Endangered Species Act (ESA) listed Atlantic salmon as endangered (NRC 2004).

Salmon farming in Maine started in the 1970's, with most of the production coming from Washington and Hancock counties, which comprise Down East Maine. In 2000, the salmon industry of Maine was valued at approximately \$60 million (NRC 2004). Unfortunately, infectious salmon anemia (ISA) spread from New Brunswick, Canada down to Maine in 2001 (Veneman 2001), and by 2002 all of the salmon farms in Cobscook Bay were infected. In an attempt to eradicate the disease, the farms were forced to destroy their fish and sanitize all of their equipment. This resulted in a significant layoff of jobs in the Down East area of Maine. Although the federal government committed \$16.4 million over two years to help with ISA prevention and costs accrued to the farms, the salmon industry of Maine has only recently recovered from the ISA outbreak. Today, Atlantic salmon net pen operations in Maine produce three to four million fish a year through on demand purchasing where fish are delivered to the market within 24-36 hours (NRC 2004).

1.1.3. Research Objectives

The purpose of these studies is to investigate correlations between embryo survival and critical factors that are involved in the reproductive success of Atlantic salmon, which could highlight potential causes of declining embryo survival. Unlike many teleost eggs that are clear, salmon eggs are an opaque/orangey color, which makes it impossible to see cell division and determine if eggs are developing properly once fertilized. Since it is difficult to determine proper embryo development after fertilization, embryos are raised until the eyes are visible inside the chorion, a stage in salmonid embryo development commonly called “eye up” or the “eyed stage”. This is unlike other fish species that have clear eggs which can be discarded right after fertilization if there is problem since it can be seen visually. Once the salmon embryos have

made it to the eyed stage, a process that takes approximately two months, unfertilized eggs or non-viable embryos can be discarded.

With this research, it is hoped that a knowledge base will be developed that will provide a tool for salmon farms to help improve embryo survival. This will save time, money, space, and peace of mind during the most stressful time of year for hatcheries.

CHAPTER 2

INVESTIGATING FACTORS CONTRIBUTING TO REDUCED EMBRYO SURVIVAL IN FARM RAISED ATLANTIC SALMON, *Salmo salar* L.

2.1. Introduction

The United States (U.S.) currently imports more than 90% of its seafood, resulting in a seafood trade deficit of more than 10 billion dollars; this is the greatest agricultural trade deficit in the U.S. (FAO, 2011). With capture fisheries at or exceeding their maximum sustainable yield for nearly all commercially important species, the growing trade deficit has encouraged the growth of domestic aquaculture. Aquaculture currently accounts for approximately 50% of U.S. seafood imports, with Atlantic salmon being the second leading import (FAO, 2011). Imports of Atlantic salmon were valued at more than \$1 billion dollars in 2013, highlighting a tremendous opportunity for domestic growth. In the U.S., Atlantic salmon are listed under the Endangered Species Act, and consequently all of its production is from aquaculture sources (Greenhalgh & Sutterby 2005). Two decades ago, hatchery managers in Maine could rely on greater than 80% embryo survival, but today, survival rates have dropped by nearly half (Figure A.1.). Embryo and fry production is labor intensive and costly, and reductions in embryo survival have caused a considerable financial bottleneck in the hatchery process in New England. In addition, hatchery operations can no longer rely on the sale of surplus eggs as an additional source of revenue.

In New England, a leading producer of Atlantic salmon in the U.S., commercial aquaculture production has dropped more than 35% since 2000 (Kocik & Sheehan 2006), due in large part to reduced embryo survival. A principal goal of commercial hatchery operations is to maximize the production of high quality eggs from each broodfish; the full potential of a hatchery can only be realized if it can provide a predictable and reliable supply of high quality

eggs and fry at set times of the year. High quality eggs display low levels of mortality from fertilization to hatching, and develop healthy and fast growing fry and juveniles, however, there is no general agreement on the percentage of surviving larvae that defines a high quality batch of eggs. Studies have shown nutrition (Yamamoto *et al.* 2011), stress (Mingist *et al.* 2007), temperature (Pankhurst *et al.* 1996), photoperiod (Taranger *et al.* 1998), diet (Watanabe *et al.* 1974), and endocrine status of the female (Taranger *et al.* 1998) to significantly affect egg quality and production. Few studies, however, assess variations in egg quality that are commonly seen between same strain females cultured in the same tank and treated under the same conditions (Bromage *et al.* 1992). In Atlantic salmon, stages of embryonic development prior to the eyed-stage (stage in which the eyes are visible) are generally thought to be more sensitive and vulnerable to mortality, therefore the eyed-stage is a critical benchmark in embryonic growth.

Successful egg fertilization, incubation and embryo development requires the coordination of numerous physiological processes, many of which are controlled by hormones (Lubzens *et al.* 2010; Weltzien *et al.* 2014). Unlike placental mammals that can adjust hormone signaling throughout fetal growth, oviparous, or egg laying species, must rely on maternal hormone investments in the oocyte prior to egg release. Although the chorioallantoic membrane in the eggs of some oviparous terrestrial species, such as the chicken (*Gallus gallus* L.), has been shown to possess mild steroidogenic activity (Albergotti *et al.* 2012), this has not been shown in fishes and maternal steroids deposited to the yolk are believed to be the primary hormone reservoir directing embryonic development prior to gonadogenesis (Albergotti *et al.* 2009; Pikulkaew *et al.* 2010). Therefore, altered maternal hormone concentrations during oogenesis

that modify hormone deposition into the egg could have serious consequences for embryo survival.

17 β -estradiol, T and 11-KT have been reported to show seasonality in female Atlantic salmon as well as other salmonids; (Fitzpatrick, Vanderkraak & Schreck 1986; Kime & Manning 1982; Taranger *et al.* 1998), and are arguably the most well-studied hormones in fishes.

Although T is typically viewed as the primary androgen in many vertebrates, 11-KT is a principal androgen in fish and is particularly high in female salmonids (Borg 1994). The primary estrogen, E2, is responsible for regulating gonadal growth and sexual maturation, as well as the production of vitellogenin and the sequestration of yolk proteins from the liver into the egg.

Therefore, concentrations of both E2 and vitellogenin tend to fluctuate together, showing peak concentrations around the onset of ovulation (Leet, Gall & Sepulveda 2011). Egg quality is directly correlated with the ability of mature females to undergo seasonal fluctuations in sex steroid hormones (Deane & Woo 2003; Mingist *et al.* 2007; Raine & Leatherland 2003).

Throughout the year, the hormonal status of the female broodfish will affect gonad and oocyte development as well as oocyte maturation and ovulation (Brooks, Tyler & Sumpter 1997).

Nutrient and hormone deposition into an egg is maternally regulated throughout embryogenesis and ends upon ovulation, afterwhich only water, and certain contaminants in the water, are known to penetrate the ovulated egg (Holliday & Jones 1967). Therefore, incorporation of all elements of an egg that will determine its quality must be present before ovulation. Certain nutrients, such as calcium, have been shown to influence egg quality, and calcium additions to the aquatic environment have been shown to improve embryo survival (Danner 2008).

Numerous contaminants have been shown to alter egg quality and embryo development. Given the ubiquitous nature of many endocrine disrupting contaminants (EDCs), some level of contaminant exposure to broodfish is likely. A biomarker for EDC exposure, through induction of the CYP1A subfamily, was first proposed in 1975; today it has become one of the most utilized assessments for *in-vivo* contaminant exposure and has been demonstrated in over 150 fish species. The CYP1A subfamily is of toxicological significance because of its involvement with toxin clearance (Guengerich & Liebler 1985), and its induction is measured through ethoxyresorufin-O-deethylase (EROD) activity in the liver. One of the most useful aspects of EROD activity is that the enzyme's concentration increases with increasing exposure to toxins (Whyte *et al.* 2000). An extensive list of contaminants have been examined for EROD activity potential, but the most common EDCs that are well established for EROD activity include certain planar halogenated hydrocarbons (PHHs) and polycyclic aromatic hydrocarbons (PAHs) along with other structurally similar compounds (Whyte *et al.* 2000), which include commonly used lipophilic chemicals (Qian *et al.* 2004). The induction of EROD activity has been correlated with affecting the reproductive system of fish by reducing serum sex steroid concentrations (Larsson *et al.* 1988).

The purpose of this study is to investigate correlations between embryo survival and critical factors that are involved in the reproductive success of hatchery raised Atlantic salmon, to highlight potential causes of declining embryo survival. As this study seeks to understand the decline in embryo survival that is experienced throughout Maine hatcheries and not just an individual hatchery, we must pool the data for all hatcheries. Pooling data from all hatcheries, and years, will allow the likely cause(s) of embryo decline to rise above all of the variation. Since hormones regulate almost every aspect of oogenesis and embryogenesis, it is hypothesized

that sex steroid hormone concentrations in the mother and/or the eggs will be different in egg batches that have high survival to the eyed stage (80% and above) versus egg batches with low survival (79% and below) to the eyed stage.

2.2. Methods

2.2.1. Sampling Sites

National Cold Water Marine Aquaculture Center: The Agricultural Research Service (ARS) is a scientific organization of the United States Department of Agriculture (USDA) which runs the National Cold Water Marine Aquaculture Center (NCWMAC), an Atlantic salmon research facility that focuses on developing a selective breeding program in order to provide more efficient and sustainable farming of Atlantic salmon. The NCWMAC facility raises their broodstock in a recirculating aquaculture system (RAS) that uses water from either Hog Bay (for seawater) or well water (for freshwater) depending on the strength of salinity the facility demands. Broodstock are photo-thermally manipulated throughout the year and are grown in fiberglass tanks.

Cooke Aquaculture's Bingham Site: Cooke Aquaculture's Bingham hatchery is a RAS that runs only on fresh well-water. The broodstock are raised in covered outdoor cement tanks with photo-manipulation.

Cooke Aquaculture's Eastport Site: Cooke Aquaculture's Eastport site, known as Deep Brook Cove, is a sea-cage site in which broodstock are exposed to full-strength seawater as well as ambient photoperiod and temperature.

2.2.2. Sample Collection

At the time of egg stripping, female broodfish were euthanized with tricaine methanesulfonate (MS-222, Western Chemical, Ferndale, WA.), standard length measured and blood was collected from the caudal vein using 10mL lithium heparin coated BD VacutainersTM (Fisher Scientific, Pittsburg, PA.). Vacutainers were placed on ice (4-6 h) until centrifugation for ten minutes at 4°C between 700 and 1000 g; plasma was stored at -80°C until analyzed. Liver and egg tissue from each female was frozen in liquid nitrogen until analyzed.

2.2.3. Assay Validations

Plasma and egg samples were thawed on ice, extracted twice with diethyl ether to enhance extraction efficiency, dried under filtered air, and reconstituted with appropriate buffer. All samples were validated from a pool of five animals by ensuring that serially diluted samples ran parallel to serially diluted standards. Intra- and inter-assay variances were made from a pool of five female plasma samples. Only samples that fell within 80% of the standard curve were used for analysis. No samples underwent multiple freeze-thaw cycles.

2.2.4. 11-KT Enzyme Immunoassay

Plasma samples were thawed on ice and 11-KT concentrations were analyzed according to instructions provided by a commercial kit (Cayman Chemical Co., Ann Arbor, MI). The plate provided in the kit is coated with mouse anti-rabbit antibodies and 50 µl of sample, standard, EIA buffer or DI water was added to the appropriate wells. Tracer and antiserum was then added at 50 µl each and the plate was incubated at 20°C for 18 hrs. Plates were allowed to reach room temperature for 30 min before washing 5 times with wash buffer, 200 µL of Ellman's reagent was added to each well and incubated in the dark at room temperature on a plate shaker. Plates were read at 412 nm using a microplate reader (Molecular Devices, Sunnyvale, CA.). Standards,

blanks, total activity, nonspecific binding, intra- and inter-assay variance and samples were all run in duplicate. Intra- and inter-assay variance was 5.5% and 6.9%, respectively.

2.2.5. E2 and T Radio-immunoassays

A solid-phase radio-immunoassay was conducted to analyze E2 and T hormones using 96-well plates (protein A Flash Plate Plus; Perkin Elmer, Boston, MA). Plasma samples were thawed on ice, the steroid fraction was extracted twice with diethyl ether, dried with filtered air and reconstituted in phosphate buffered saline (PBS, pH 8.0). Antibodies for each hormone were used from the same lot number throughout the study (Fitzgerald Industries, Concord MA). Antibodies were diluted in PBS and this was coated onto the plate at 100 µl per well and incubated for two hours at room temperature. After, plates were rinsed twice with PBS, 100 µl of sample, standard or DI water was added to the wells and then radiolabelled T or E2 was added at 12000 cpm per 100 µl. Plates were then incubated at room temperature for three hours in the dark on a plate shaker. Intra- and inter-assay variance for E2 and T were 10.1% and 10.7%, and 12.7% and 8.2%, respectively. Plasma samples, standards, blanks, non-specific binding, were added to the plate in duplicate. Plates were read using a Microbeta 1450 Trilux counter (Perkin Elmer, Waltham, MA.).

2.2.6. Calcium Assay

Plasma samples were thawed on ice and calcium concentrations were measured using instructions provided by a commercial kit (Cayman Chemical Co, Ann Arbor, MI). Plasma samples were diluted in assay buffer and 20 µl of the sample, standard, and blanks were added to the plate in duplicate. 100 µl of Working Detector Reagent was added to each well, the plate was shaken for 25 sec and incubated for 5 minutes at room temperature. Plates were read at 575 nm

using a microplate reader (Molecular Devices, Sunnyvale, CA.). Intra- and inter-assay variance was 7.4% and 8.7%.

2.2.7. EROD Assay

All chemicals required for the following procedures were purchased through Sigma-Aldrich Chemical Company (St. Louis, MO.) except for dithiothreitol (DTT) and high purity, gas-chromatography grade methanol (MeOH) which was purchased from ACROS Organics (a division of Fisher Scientific, Pittsburg, PA.). Control and “hot” microsomes from rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) were generously provided by Adria Elskus. Hot microsomes came from trout that had been previously treated with β -naphthoflavin, a contaminant known to induce EROD activity and control microsomes were from trout not treated with the contaminant.

Livers were removed from liquid nitrogen, immediately weighed and homogenized in an ice bath using 50 mM TRIS buffer (pH 7.4) at a ratio of 10 mL buffer per gram of liver. Livers were homogenized using a Wheaton Potter-Elvehjem tissue grinder and PTFE pestle (Fisher Scientific, Pittsburg, PA.) after 5-8 passes of the pestle and microsomal fractions of the homogenate were obtained by methods similar to (Stegeman, Binder & Orren 1979), however, the three centrifuge speeds and times differed as follows: 1) 1,500 xg for 10min, 2) 10,000 xg for 10 min and 3) 100,000 xg for 60min. After the third centrifugation, the microsomal pellet was resuspended in TEDG buffer at a ratio of 1 mL buffer per gram of liver weight. For each female, two aliquots of liver microsomes were placed in cryovials and stored in liquid nitrogen until future use in determining the protein concentration and EROD activity.

Microsomal protein was measured using the PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, IL.) following the instructions for the Microplate Procedure. All standards, blanks and samples were run in duplicate and read on a microplate reader at 562 nm (Molecular Devices, Sunnyvale, CA.).

Microsomal EROD activity was measured and determined through fluometric methods previously described by Hahn *et al.* (1993). The reaction mixture used for this study contained 150 μ L of 7-ethoxyresorufin buffer (7-ethoxyresorufin diluted to 2.67 μ M in methanol), 10 μ L of microsomes and 40 μ L of 6.68 mM NADPH. All standards and samples were run in triplicate on a 48-well CoStar plate using a microplate flurometer (BioTek, Synergy 4, Wilmington, DE.).

2.2.8. Statistical Analyses

The relationship between eyed-egg percentage and all correlating factors were examined using linear regression analyses except for EROD activity; this was the only assay which had a control, therefore an analysis of variance (ANOVA) was used to determine statistical significance from the control. Shapiro-Wilk and Levene's test were used to determine homogeneity between distribution and variance, respectively.

The aim of this study was to investigate trends that span across all years and all hatchery conditions, therefore, data for both years (2011 and 2012) and amongst all three hatcheries were pooled for each regression.

2.3. Results

Regressions for each endpoint are shown in the Appendix (page 58). Significant correlations were difficult to determine visually in the regressions, therefore the results are presented as a mean for females which have been grouped by increments of 10% eyed-egg survival (Figs 2.1 to 2.6). Error bars are $\pm$ standard error of the mean (SEM).

2.3.1. Female Plasma and Egg Sex Steroids

There were no significant correlations between female plasma T (Fig.2.1.A) and embryo survival to the eyed-stage. Significant positive correlations ($P \leq 0.001$) were observed between female plasma 11-KT (Fig. 2.1. B) and E2 (Fig. 2.2. A) with embryo survival to the eyed stage. There was no significant correlation between egg hormones (Fig. 2.3. A, B and Fig. 2.4. A) with survival of embryos to the eyed-stage.

2.3.2. Female Plasma and Egg Calcium

No significant correlations were observed between female plasma calcium and survival to the eyed stage (Fig. 2.2. B) or between egg calcium concentration and survival of embryos to the eyed stage (Fig. 2.4. B).

Figure 2.1. Plasma T and 11-KT. Variation in mean ($\pm$ SE) plasma (A) testosterone (pg/mL) and (B) 11-ketotestosterone (pg/mL) concentrations in adult female Atlantic salmon versus % embryos reaching the eyed stage. *n*, number of females analyzed in each percentile. There is a significant correlation between the hormone 11-ketotestosterone (B) with % of embryos reaching the eyed stage.

Figure 2.2. Plasma E2 and Calcium. Variation in mean ($\pm$ SE) plasma (A) estradiol (pg/mL) and (B) calcium (mg/dl) concentrations in adult female Atlantic salmon versus % embryos reaching the eyed stage. *n*, number of females analyzed in each percentile. There is a significant correlation between the hormone estradiol (A) with % of embryos reaching the eyed stage.

Figure 2.3. Egg T and 11-KT. Variation in mean ($\pm$ SE) egg (A) testosterone (pg/mL) and (B) 11-ketotestosterone (pg/mL) concentrations in adult female Atlantic salmon versus % embryos reaching the eyed stage. *n*, number of females analyzed in each percentile. There are no significant correlations between the hormones testosterone (A) and 11-ketotestosterone (B) with % of embryos reaching the eyed stage.

Figure 2.4. Egg E2 and Calcium. Variation in mean ($\pm$ SE) egg (A) 17 β -estradiol (pg/mL) and (B) calcium (mg/dl) concentrations in adult female Atlantic salmon versus % embryos reaching the eyed stage. *n*, number of females analyzed in each percentile. There are no significant correlations between the hormones estradiol (A) and calcium (B) with % of embryos reaching the eyed stage.

Figure 2.5. Liver EROD Activity. Variation in mean ($\pm$ SE) hepatic ethoxyresorufin-O-deethylase (EROD) activity in adult female Atlantic salmon compared to control and hot samples. N, number of females analyzed in each percentile. There is no statistical significance between female hepatic EROD activity when compared to the control.

Figure 2.6. Female Standard Length. Variation in mean ($\pm$ SE) standard length of adult female Atlantic salmon versus % of embryos reaching the eyed stage. n, number of females analyzed in each percentile. There is no significant correlation between female standard length and % of embryos reaching the eyed stage.

2.3.3. Female Hepatic EROD Activity

Female hepatic EROD activity was not statistically different ($P \leq 0.05$) between females compared to control (Fig. 2.5).

2.3.4. Female Length

No significant correlations were found between female standard-length and survival of embryos to the eyed stage (Fig. 2.6).

2.4. Discussion

This is the first study to show that concentrations of maternal plasma E2 and 11-KT positively correlate with embryo survival in farm-raised Atlantic salmon. Interestingly, egg concentrations of sex steroids did not correlate with embryo survival. This indicates that embryo mortality does not likely rest with maternal deposition of sex steroids into the egg, but with another hormone regulated process related to egg assembly, ovulation and/or post-ovulatory aging.

Maternal hormones play key roles in directing the assembly of the egg (as reviewed by Lubzens, et al 2010). Notably, maternal E2 regulates the synthesis of vitellogenin from the liver, which is sequestered by the developing oocyte and serves as a primary constituent of egg yolk (Plack et al 1971). A number of egg components, including vitamins, metals, hormones and binding proteins can enter the oocyte during this receptor-mediated uptake of vitellogenin, either bound to vitellogenin or adventitiously (Brooks *et al.* 1997). In addition, maternal RNAs are deposited into the yolk that then encode for growth factors, receptors and hormones, which fulfills the regulatory needs of the embryo for a variety of physiological functions including growth, osmoregulation and organogenesis, until their own systems become competent (Lam et al., 1994).

Previous studies in Atlantic salmon have shown E2, 11-KT and T to peak around the time of spawning and show precipitous post-ovulatory declines (Stuartregor, Sumpter & Dodd 1981; Taranger *et al.* 1998). The current study showed that those salmon that produced eggs with low embryo survival had lower plasma concentrations of E2 and 11-KT, which could indicate a longer period of post-ovulatory aging. Post-ovulatory aging is a considerable problem in commercial aquaculture, and contributes significantly to embryo mortality in a number of species, including salmonids (Bobe & Labbe 2010; de Gaudemar & Beall 1998; Fauvel *et al.* 1993; Flett *et al.* 1996). The time period from ovulation to non-viability of the egg varies greatly among species and culture temperatures. In rapidly developing species such as tilapia (*Oreochromis moassmbicus* Peters, 1852) and goldfish (*Carassius auratus* L.), ovulated eggs retained in the ovarian cavity can begin to overripen within 1.5h (Harvey & Kelley 1984) and 12h, respectively (Formacion *et al.* 1995). In other fish species, such as salmonids, this period can last several days, to weeks, depending on conditions and temperatures (de Gaudemar & Beall 1998; Springate *et al.* 1984). De Gaudemar and Beall (1998) held female Atlantic salmon for varying lengths of time after ovulation before allowing them to volitionally spawn with males. The females were held from 1 to 12 days post-ovulation before being released into individual spawning channels. In females that had just ovulated, 78.5% of eggs the researchers recovered were viable, whereas in females that had been held for at least a week, viability decreased to 59.5%. Studies of salmonids reared in captivity report similar findings, with reduced embryo survival when artificial fertilization occurred several days or weeks following ovulation (Bry 1981).

Previous studies in coho salmon (*Oncorhynchus kisutch* Walbaum, 1792) examining a steady increase in embryo mortality over a nearly 20 year span, found that temperature-induced overripening was the most likely cause (Flett *et al.* 1996). Similar to our study, the female coho salmon showed lower concentrations of sex steroids than fish of the same genetic stock released in another region that were not experiencing increasing embryo mortality. Cross-fertilization studies indicated the problem originated from eggs, not milt, and embryo mortality in coho salmon did not appear to be related to egg contaminants (heavy metals or organochlorines), egg size, total lipid, protein, or ion concentration (Morrison, Leatherland & Sonstegard 1985a; Morrison, Leatherland & Sonstegard 1985b). Researchers concluded that the lower concentrations of plasma sex steroids, the presence of overripe eggs in the body cavity, along with the unilateral nature of egg release in some individuals, were strong support for an impairment in timing of ovulation. These fish experienced elevated temperatures during critical periods during vitellogenesis, and it was speculated that this caused an asynchronous maturation of the vent and the gonad. Although the eggs had ovulated, they could not be released from the vent via abdominal massage. It is common practice to assess the readiness of female salmon to spawn by examining their abdominal morphology and their ability to release eggs following abdominal pressure. These studies indicate that this method will not always be reliable if female vent maturation is impaired and eggs are incapable of exiting the vent. It is also possible that the elevated temperatures led to asynchronous ovulation, which would lead to portions of eggs being overripe, while others are not yet viable. Taken together, these studies support the possibility that the increased embryo mortality experienced by Atlantic salmon in Maine could be due to post-ovulatory aging and fish should be evaluated for vent maturation and asynchronous ovulation.

Given the global trend of increasing temperatures, it is possible this could be a contributing factor, though other environmental factors could presumably have an influence on timing as well. Taranger and Hansen (1993) found significant decreases in egg survivability to the eyed stage (94.1% to 76.6%) from Atlantic salmon females raised at either cold temperatures (5-7°C) or warm temperatures (13-14°C), respectively, during the spawning season. At the end of the study, mean ovulation of females raised at 13-14°C was delayed by 5 weeks, with a total of 43% of the females never having ovulated, showing that high temperatures can not only delay, but even inhibit ovulation. Studies conducted on brook trout (*Salvelinus fontinalis* Mitchill, 1814) and rainbow trout (*O. mykiss*) also linked high temperatures to increased post-ovulatory aging (Aegerter & Jalabert 2004; Hokanson *et al.* 1973). Given trends in climate change and global warming, it is possible increasing degree days could also be a driver for elevated embryo mortality in Atlantic salmon, although research is needed to examine this relationship.

While improper egg assembly or contaminant exposure cannot be ruled out, our results showed that EROD activity in the liver, a key indicator of contaminant exposure, was similar across females producing high or low embryo survival. Calcium content of the eggs, a component that has been associated with embryo survival in trout, was also similar in eggs of females producing high or low embryo survival. Although hormone and calcium contents of the eggs are just two of many factors necessary for proper egg assembly, these results nonetheless lend support to post-ovulatory aging, versus altered egg composition, as a major factor in decreasing embryo survival in farm-raised Atlantic salmon. Female size also did not correlate with embryo survival, as has been shown in other species (Bromage *et al.* 1990).

This study is the first to examine correlations of sex steroid concentrations in maternal plasma, as well as the eggs, with embryo mortality in Atlantic salmon. This study showed that egg concentrations of E2, T and 11-KT are not a good predictor of embryo survival, while maternal plasma concentrations of E2 and 11-KT at the time of spawning are positively associated with increased embryo survival. More work is needed to understand the mechanisms involved, including the role of post-ovulatory aging on egg competence in the commercial culture of Atlantic salmon.

CHAPTER 3

CORRELATIONS OF PLASMA, OVARIAN FLUID, AND EGG SEX STEROIDS, 17 β -ESTRADIOL AND 11-KETOTESTOSTERONE, AT THE TIME OF SPAWNING IN ATLANTIC SALMON, *Salmo salar* L.

3.1. Introduction

The incorporation of compounds, such as hormones, trace metals, vitamins and minerals, into an oocyte during oogenesis is an important process for successful egg development and survival. Although the roles of these molecules in the oocyte are fairly understood, the mechanism for deposition is not. Sex steroids are produced by the follicular layers of the oocyte (together called the follicle), the theca cells produce androgens while the granulosa cells produce estrogens. Sex steroids produced by the follicle can either be transported inside the oocyte or outside to the surrounding ovarian fluid (OF). Prior to follicle formation, sex steroids found in the oocyte can theoretically only come from maternal sources. Therefore, potential sources of sex steroids to be sequestered by the developing oocyte could come from either maternal plasma and/or the follicle, but the relative contribution of each of these sources to oocyte composition is unclear.

Major stages of egg development include the formation of primary germ cells and their transitions into oogonia, then primary oocytes, then secondary oocytes, until finally maturation and ovulation of a fertilizable egg (an oocyte free from its' follicular layers). Secondary oocyte growth can be distinguished by the considerable growth of the oocyte during vitellogenesis, a period when the oocyte accumulates nutritional reserves later needed for survival and successful development. During vitellogenesis, vitellogenin (Vtg) is released from the liver by a positive feedback loop with 17 β -estradiol (E2) and is sequestered into the oocyte. At this time, the oocyte

also accumulates maternal RNAs. Since secondary oocyte growth requires the oocyte to allow many maternally derived chemicals to be sequestered, the majority being Vtg, it would seem fit for maternal sex steroid deposition to occur at this time as well.

Studies that have examined this question in fish have focused primarily on the mechanism of thyroid hormone (TH) sequestration. It was originally proposed that since hormones travel by binding to transport proteins, that vitellogenin (Vtg) could be a potential transport protein for TH. Although MacKenzie et al. (1987) showed some evidence for TH transport by Vtg, there is strong support that Vtg is not the mechanism for TH deposition into the oocyte. Flett and Leatherland (1989) were unable to show significant binding of ^{125}I -labeled THs to Vtg; the TH was found to be associated with smaller molecular weight proteins. Brown et al. (1989) also show evidence that Vtg is not the transport protein, nor is vitellogenesis required for TH transport, since they found triiodo-L-thyronine (T_3) enters the oocyte throughout maturation, a period during oogenesis where the incorporation of Vtg has ceased. Another chemical that has been studied to better understand Vtg as a transport protein is through horseradish peroxidase (HPO), an electron-dense tracer molecule. Sellman and Wallace (1982) injected sheepshead minnows (*Cyprinodon variegates variegates* Lacépède, 1803) intraperitoneally with HPO during vitellogenesis to see if HPO would be transported by Vtg or by some other mechanism (Selman & Wallace 1982). HPO was transported to the yolk of the egg by pore canals on the vitelline envelope, providing evidence that Vtg may not be required for the transport of certain chemicals.

The above studies provide information on the sources and mechanisms for egg sequestration of two chemicals, TH and HPO. TH enters the oocyte by attaching to small molecular weight proteins found in the blood of the female while HPO enters the oocyte through pore canals; these sources and mechanisms serve as models for other chemicals such as sex

steroids. This study aims to better understand the sources of E2 and 11-KT that are deposited into the egg of the Atlantic salmon. Understanding the sources of sex steroid deposition into the oocyte could help to identify how this system can be disrupted and aid in identifying causes of altered deposition. It must be remembered that this study focuses on measuring sex steroids in the egg, OF and plasma at a single time point, the time of spawning, therefore any correlations that may be found will give insight into the source of egg sex steroids, not necessarily mechanisms of steroid deposition.

3.2. Methods

3.2.1. Sample Collection

Plasma, ovarian fluid and eggs were collected from 4 year old female Atlantic salmon broodstock reared at Cooke Aquaculture's Bingham, Maine hatchery. All samples were taken at time of spawning. Fish were anesthetized with tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA, USA), euthanized with blunt force concussion, and then blood samples were collected by puncturing the caudal vein with a 22 gauge needle into a lithium heparin coated BD Vacutainer™ (Fisher Scientific, Pittsburg, PA, USA). Females were then stripped of eggs by abdominal incision and eggs were collected in a plastic container. From each female, approximately 8-10 eggs and 1.75 mL of ovarian fluid was collected using a plastic transfer pipette. Sample tubes were all placed on ice for approximately 3 hours prior to processing. Blood was then centrifuged for 10 minutes at 4°C at 1000xG and the plasma portion was stored at -80°C, along with the ovarian fluid and egg samples, until analyzed.

3.2.2. Assay Validations

Plasma, ovarian fluid, and egg samples were thawed on ice, extracted twice with diethyl ether to enhance extraction efficiency, dried under filtered air, and reconstituted with buffer. All samples were validated from a pool of four to five animals by ensuring that serially diluted samples ran parallel to serially diluted standards. Only samples that fell within 80% of the standard curve were used for analysis. No samples underwent multiple freeze-thaw cycles.

3.2.3. Radio-immunoassay

Solid-phase radio-immunoassays were conducted to measure E2 in plasma samples according to methods previously described in Chapter 2. Antibodies used for all plates were from the same lot number (Fitzgerald, Concord, MA, USA) and samples were reconstituted in PBS, pH 8.0. Standards, samples, blanks, non-specific binding, total activity, intra- and interassay variances were all run in duplicate. Plates were read using a Microbeta 1450 Trilux counter (Perkin Elmer, Waltham, MA, USA) and intra- and inter-assay variance for E2 plasma samples were 10.1% and 10.7%, respectively.

3.2.4. Enzyme Immunoassays

Plasma 11-KT, ovarian fluid and egg E2 and 11-KT were measured using enzyme immunoassays following protocols provided in Chapter 2. Standards of 11-KT and E2, samples, blanks, non-specific binding, total activity, intra- and inter-assay variances were all run in duplicate. Intra- and inter-assay variances, respectively, were as follows: Plasma 11-KT, 5.5% and 6.9%; ovarian fluid E2, 9.8% and 8.2%; ovarian fluid 11-KT, 5.9% and 6.7%; egg E2, 11.3% and 9.5%; and egg 11-KT, 10.4% and 11.7%. All plates were read at 412 nm using a Synergy 4 microplate reader (BioTek, Wilminton, DE, USA).

3.2.5. Statistical Analyses

The relationships between both plasma and ovarian fluid sex steroid hormones with egg hormones were examined using linear regressions performed by the statistical package JMP® version 13.0.0 (Cary, NC, USA).

3.3. Results

3.3.1. Female Plasma and Egg Sex Steroids

There were no significant correlations between either female plasma E2 ($p=0.43$) or 11-KT ($p=0.90$) with egg hormones (Fig. 3.1 A and B).

3.3.2. Female Ovarian Fluid and Egg Sex Steroids

There were significant correlations between egg hormones and both female ovarian fluid E2 and 11-KT with egg hormones ($p\leq 0.05$) (Fig. 3.2 A and B).

Figure 3.1. Plasma Versus Egg E2 and 11-KT. Linear regression of female Atlantic salmon plasma estradiol concentrations (pg/mL) versus egg estradiol concentrations (pg/mL) (A) and linear regression of female Atlantic salmon plasma 11-ketotestosterone concentrations (pg/mL) versus egg 11-ketotestosterone concentrations (pg/mL) (B).

Figure 3.2. Ovarian Fluid Versus Egg E2 and 11-KT. Linear regression of female Atlantic salmon ovarian fluid estradiol concentrations (pg/mL) versus egg estradiol concentrations (pg/mL) (A) and linear regression of female Atlantic salmon ovarian fluid 11-ketotestosterone concentrations (pg/mL) versus egg 11-ketotestosterone concentrations (pg/mL) (B).

3.4. Discussion

A variety of compounds such as hormones, minerals, trace metals, vitamins, and amino acids are present in the egg, however, very little is known about their sources and mechanisms of sequestration by the developing oocyte. This study shows a positive correlation between sex steroids (E2 and 11-KT) in the eggs of Atlantic salmon with the surrounding OF at the time of spawning. In addition, the study shows no correlation between sex steroids (E2 and 11-KT) in the eggs and with maternal plasma. These findings suggest that the follicle layers could be key sources of egg sex steroid concentrations, and that sex steroid concentrations in maternal plasma may not play a significant role at the time of spawning.

Raine and Leatherland (2003) saw similar results in rainbow trout using T_3 . They found that ovulated eggs immersed in a low and high dose bath of T_3 were able to take up the hormone in appropriate proportions. Also, when T_3 -supplemented oocytes were transferred to clean OF, the T_3 in the OF increased, showing transfer of the hormone from the oocyte to the OF. However, in these trials T_3 was not measured in the eggs and the OF at the same time, therefore, there is no way to infer if there was an equilibrium of T_3 concentration between the OF and the eggs, only that there was a transfer of the hormone between the OF and eggs and vice versa. Authors suggest there is a passive fluxing of T_3 along a concentration gradient between the oocyte and the OF. This suggests that the oocyte and steroids in the OF are in a state of constant communication. If ovulated eggs are able to uptake chemicals passively in the OF, this could lead to studies that immerse oocytes in mediums containing chemicals thought to improve embryo survival to see if the chemical is sequestered and if it affects embryo survival.

The OF in fish is classically known to aid in sperm motility and fertilization, however, studies have also revealed its importance in preventing egg overripening as well as creating a jelly coat needed for some eggs. Species of fish that attach their eggs to substrates, such as salmonids, need to generate an adhesive jelly coating around the oocyte to allow their eggs to adhere. Mansour and Patzner (2009) discovered certain proteins in the OF are responsible for reacting with the chorion to create the jelly coat in the Eurasian perch (*Perca fluviatilis* L.) (Mansour, Lahnsteiner & Patzner 2009). Formacion et al. (1995) measured serum and OF steroids at 0, 3, 6, 12, 18, and 24 hrs post ovulation in goldfish (*C. auratus*). The steroids P4, E2, and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) were all present at higher concentrations in the OF than in the serum; P4 concentration in the OF was 6-10 times higher than in the serum, E2 was 4 times higher, and DHP was 2-5 times higher. This study revealed that ovulated eggs are bathed in OF that contains higher levels of steroids than maternal serum, suggesting that steroids in the OF are likely to have a direct effect on the oocytes. To determine this, Formacion et al. (1995) immersed ovulated eggs in a bath of either OF containing an anti-serum against P4 (anti-P4 group) or a heat denatured anti-P4 (control group). Results showed oocytes bathed in anti-P4 had accelerated overripening, suggesting P4 plays an important role in delaying overripening.

Although this study shows a correlation between egg and OF hormone concentrations at the time of spawning, it does not indicate when hormone deposition to the egg occurred and what source(s) it was from. If hormones are deposited throughout oocyte development, there is still potential for maternal plasma to be a source. Future studies should aim to collect maternal plasma, OF, and egg samples at various time points during development and after ovulation to determine how all three factors influence one another's hormone concentration.

In conclusion, E2 and 11-KT concentrations in Atlantic salmon oocytes appear to be related to the concentration of steroids in the surrounding OF, not the maternal plasma. This information can be used as an aid in developing future studies that aim to understand the sources and mechanisms of sex steroid deposition into the oocyte.

CHAPTER 4
ANNUAL HORMONE PROFILES OF 17 β -ESTRADIOL AND
11-KETOTESTOSTERONE IN THE NORTH AMERICAN
STRAIN OF FEMALE ATLANTIC SALMON,
***Salmo salar* L., BROODSTOCK**

4.1. Introduction

Since the early 19th century, wild Atlantic salmon populations in New England have been severely depleted and today, Maine is the last state to observe runs of Atlantic salmon. The National Marine Fisheries Service (NMFS) and United States Fish and Wildlife Service (USFWS) have labeled anadromous Atlantic salmon that occur in Maine rivers from the Androscoggin to the Dennys as a Distinct Population Segment (DPS) (NRC 2004). The DPS was listed as endangered under the Endangered Species Act in 2000 and despite conservation efforts, returns of adult Atlantic salmon to the Maine rivers are extremely low. Commercial fishing for anadromous Atlantic salmon as well as a long beloved past time of recreational fishing are no longer an opportunity for New Englanders.

Although salmon farming in Maine has become the most profitable aquaculture species in the state, since 2000 production has dropped by about 35% (Kocik & Sheehan 2006). Although a major contributor to these losses is low survival of embryos it highlights a need for increased knowledge of Atlantic salmon reproductive endocrinology. Compared to other salmonids that are well studied (Crim & Idler 1978; Dye *et al.* 1986; Fitzpatrick *et al.* 1986; Mayer *et al.* 1992; Scott *et al.* 1980; Slater, Schreck & Swanson 1994; Taranger *et al.* 1998; Vikingstad *et al.* 2016), little is known about the sex steroid profiles during an annual or reproductive season in North American strains of Atlantic salmon. An understanding of seasonal hormone profiles for any

commercially important species is a hallmark of baseline endocrinological knowledge.

Determining these profiles may increase our understanding of reproductive function and could ultimately contribute to increased production for both hatcheries and recovery efforts.

Certain estrogens and androgens have been correlated with reproductive status and behavior in many fish species with arguably the most well-studied being E2 and 11-KT. In many fish species that have a reproductive cycle that occurs once a year, plasma E2 concentrations in females generally peak during the growth stage of oocytes, known as vitellogenesis, and then concentrations drop dramatically as gonadal development progresses (Alvarado *et al.* 2015). Circulating E2 stimulates the liver to release vitellogenin, an egg yolk precursor, which then travels to the ovary and is taken up by the eggs (Campbell & Idler 1976). Vitellogenesis is the main contributor to the enormous growth and nutritional input experienced by salmonid eggs (Grier 2000). Concentrations of plasma 11-KT as well as T, peak about a month after E2 peaks, and then concentrations drop dramatically right before ovulation (Mayer *et al.* 1992; Taranger *et al.* 1998; Wingfield & Grimm 1977). Androgens are classically known for regulating male sexual behavior and characteristics (Borg 1994), but they have also been suggested to be responsible for female sexual behavior, as well as expressing secondary sexual characteristics (Kazeto *et al.* 2011) which explains why concentrations peak right before ovulation. Concentrations of 11-KT have been shown to be particularly high in female salmonids compared to T (Borg 1994), and peak androgen concentrations in females are typically higher than in males suggesting that androgens play a vital role in female reproduction and sexual maturation (Campbell *et al.* 1980; Mayer *et al.* 1992).

The primary goal of this study was to determine the annual hormone profile of E2 and 11-KT in plasma of North American strains of female Atlantic salmon. We also aimed to determine if steroid hormone profiles were altered by the frequency of sampling. The findings of this project will not only increase our baseline understanding of the reproductive endocrinology of North American strains of Atlantic salmon, but will provide refined blood sampling protocols for salmonid endocrine research. Findings from this study will be commercially relevant, since fish will be raised in a RAS while being photo-thermally manipulated.

4.2. Methods

4.2.1. Experimental Animals

Four year old Atlantic salmon broodstock were donated by the United States Department of Agriculture Agricultural Research Service (USDA ARS) facility in Franklin, Maine and transported to the University of Maine's Aquaculture Research Center (ARC) on September 18, 2015.

4.2.2. Experimental Design

The system consisted of six fiberglass tanks (2,000 l each), a header tank (600 l) which was aerated and filled with polyethylene biofilter media, a sump (230 l), a drum filter (Faiver, Model 40 Series, Baum-Les-Dames, France) to remove suspended solids and a heat exchanger to chill the water. System water was sourced from a freshwater well and was partially recirculated so that water in the entire system was replaced with water every 25-26 hrs. Water chemistry testing for ammonia, nitrite and nitrate was measured with a SMART3 colorimeter (LaMotte Company, Chestertown, MD, USA) and pH was measured with a pHTestr® 20 (Oakton Instruments, Vernon Hills, IL, USA) once a week. Water dissolved oxygen (D.O.) and temperature per tank were recorded at least once a day, using the OxyGuard Handy Polaris 2

Portable DO Meter (OxyGuard International A/S Farum, Denmark). The D.O. was maintained at approximately 8.0-11.0 ppm, depending on the month, and temperature followed a regime to allow for reproductive development throughout the year (Fig. 4.1). Photoperiod simulated the natural daylight pattern for Old Town, Maine to also allow for reproductive development throughout the year (Fig. 4.2).

Figure 4.1. Temperature Profile. Temperature experienced by Atlantic salmon broodstock from start of the experiment in September 2015 to the end of the experiment in August 2016.

Figure 4.2. Photoperiod. Light cycle experienced by Atlantic salmon broodstock from the start of the experiment in September 2015 to the end of the experiment in August 2016. Photoperiod simulated the ambient light cycle in Old Town, Maine.

There were three treatments with two tanks per treatment for a total of six tanks. The treatments were based on bleeding regimes to determine if any variation in plasma hormones was due to bleeding frequency. Fish in treatment 1 were bled once a month, fish in treatment 2 are bled every other month, and fish in treatment 3 were only bled on the first day of sampling, in November during the spawning season and then again on the last day of the experiment. This treatment is referred to as F,S,L for “first, spawning, last” bleeding months. Tanks were randomly assigned to treatments and fish were also randomly assigned to a treatment and tank. Two to three males were placed in each tank with eight to nine females. Fish were not fed during the reproductive season (October, November and December). In January, after reproduction, fish were fed 0.75% of their body weight once a day, six times a week with a commercial pellet

(9.0mm Bio-Oregon). Tanks were generally self-cleaning however any uneaten food that was not removed was siphoned at least once a week.

4.2.3. Blood Collection

Blood samples were collected from fish in designated tanks according to Table 1. Fish were anesthetized with buffered MS-222 (Western Chemical, Inc., Ferndale, WA, USA) at a concentration of 60 mg/L and blood was collected by puncture of the caudal vein with a 10mL syringe and a 21 gauge needle. Approximately 4 mL of blood was collected at each sampling period in lithium heparin Vacutainer™ tubes (BD, Franklin Lakes, NJ, USA) and stored on ice for approximately 45 minutes before centrifugation (1000xG, 4°C, 10 minutes). Plasma was stored at -80°C until analyzed.

Table 4.1. Bleeding Schedule. Bleeding schedule for each tank based on their blood sampling treatment (F,S,L = **F**irst month, **S**pawn month of November, **L**ast Month).

Month	Bleeding Schedule		
	Monthly	Bi-Monthly	F,S,L
September	X	X	X
October	X		
November	X	X	X
December	X		
January	X	X	
February	X		
March	X	X	
April	X		
May	X	X	
June	X		
July	X	X	
August	X	X	X

4.2.4. Spawning

During the months of November and December, females were inspected for ovulation by massaging the abdomen to determine if eggs flowed freely from the oviduct. Ovulated eggs were collected in individual buckets and then placed in a strainer to remove ovarian fluid. Eggs were then rinsed with a prepared diluent (simulated ovarian fluid) and placed back in the bucket with enough diluent to cover the eggs. Approximately 1mL of sperm from two males were mixed with each egg batch and allowed to sit for five minutes to allow fertilization. Eggs were then rinsed with system water and allowed to water harden for one hour. Egg batches were incubated individually in heath trays (MariSource, Fife, WA, USA) with freshwater (water temperature ranging from 6-7°C) and survival of embryos were determined by counting individual embryos that reached the eyed stage. Embryos were treated three times a week with formalin according to the *Guide to Using Drugs, Biologics, and other Chemicals in Aquaculture* Companion Treatment Calculator provided online by the American Fisheries Society (Bethesda, MD, USA) to prevent fungal infection. Dead embryos were removed in the dark using a red lamp and forceps once a week.

4.2.5. Assay Validations

Plasma hormones were validated from a pool of five animals to verify that curves from serially diluted samples ran parallel to serially diluted standards. No plasma sample underwent multiple freeze-thaw cycles and all standards, samples, intra- and interassay variances were run in duplicate.

4.2.6. E2 and 11-KT Enzyme Linked Immunoassays

Plasma samples were thawed, extracted twice with diethyl ether, dried with filtered air and reconstituted in ELISA buffer. Concentrations of E2 and 11-KT were analyzed according to instructions provided by the commercial ELISA kits (Cayman Chemical Co., Ann Arbor, MI, USA) specific to each hormone. E2 and 11-KT samples were plated in the same manner as in the methods for the 11-KT Enzyme Immunoassay in Chapter 2. Intra- and interassay variance for E2 and 11-KT were 14.8% and 8.3%, and 10.9% and 7.7%, respectively. Plates for E2 and 11-KT were read at 412nm using a microplate reader (Synergy 4; Biotek, Wilmington, DE, USA).

4.2.7. Statistical Analyses

Statistical analyses were performed using the statistical package JMP version 13.0.0 (Cary, NC, USA). Shapiro-Wilk and Levene's tests were used to compare homogeneity between distributions and variances, respectively. Analysis of variance (ANOVA) for hormones was used to compare differences among treatment groups (months and bleeding schedule). If significance was determined ($p \geq 0.05$) a Tukey's post hoc test was used to determine differences among treatment means. A two-sample t-test was used to determine any difference between spawning date and survival to eyed stage.

4.3. Results

4.3.1. Sex Steroids

Plasma concentrations of E2 started to increase in July and August with peak concentrations occurring in September, thereafter declined to low levels through June (Fig 4.3 A). Plasma 11-KT concentrations remained relatively low throughout the year except for a dramatic peak in concentrations in October (Fig. 4.3 B).

Figure 4.3. Annual Hormone Profile of E2 and 11-KT. Monthly plasma estradiol (A) and 11-ketotestosterone (B) concentrations (mean $\pm$ SE) of Atlantic salmon female broodstock from September 2015 through August 2016.

4.3.2. Bleeding Frequency

September 2015 was the start of the current study and all females had starting concentrations of E2 and 11-KT that were statistically similar between bleeding frequency treatments (Fig. 4.4). By the end of the study, August 2016, females that were bled monthly or every other month still had statistically similar concentrations of E2 and 11-KT to fish bled only at the beginning, at spawning and at the end of the trial (Fig. 4.5).

Figure 4.4. Plasma E2 and 11-KT in September. Plasma estradiol (A) and 11-ketotestosterone (B) concentrations (mean $\pm$ SE) of Atlantic salmon female broodstock per bleeding schedule treatment in the month of September 2015. Means with no superscript are not significantly different ($p \geq 0.05$). (F,S,L = First, Spawning, and Last).

Figure 4.5. Plasma E2 and 11-KT in August. Plasma estradiol (A) and 11-ketotestosterone (B) concentrations (mean $\pm$ SE) per bleeding schedule treatment in the month of August 2016. Means with no superscript are not significantly different ($p \geq 0.05$). (F,S,L = First, Spawning, and Last).

4.3.3. Spawning Date

Spawning that occurred in November had statistically significant higher embryos that survived to the eyed stage than the December spawn ($p \geq 0.05$) (Fig. 4.6).

Figure 4.6. Eye Up. Percent eye up in the months of November and December (mean $\pm$ SE) in Atlantic salmon ($n = 18$ and $n = 15$ respectively). Means with superscript are significantly different ($p \geq 0.05$).

4.4. Discussion

This is the first study to describe the annual sex steroid profiles of E2 and 11-KT in North American strains of Atlantic salmon female broodstock raised in a RAS. In the present study, plasma concentrations of E2 peaked about two months before ovulation, coincident with vitellogenesis and oocyte growth. This agrees with what has been found in other salmonids such as Atlantic salmon grown in Tasmania (Taranger *et al.* 1998), pink salmon (*Oncorhynchus gorbuscha* Walbaum, 1792) (Dye *et al.* 1986), chinook salmon (*Oncorhynchus tshawytscha* Walbaum, 1792) (Slater *et al.* 1994), Arctic charr (*Salvelinus alpinus* L., 1758) (Mayer *et al.* 1992), and rainbow trout (Scott, Sumpter & Hardiman 1983). In the present study, plasma concentrations of 11-KT peak after E2 levels declined, just prior to ovulation, suggesting that 11-KT plays an important role in sexual maturation and possibly behavior, which has also been shown in other female teleosts (Alvarado *et al.* 2015; Pham *et al.* 2012). Alvarado *et al.* (2015)

measured sex steroids during sexual development in wild southern Hake (*merluccius australis* Hutton, 1872); E2 peaked during vitellogenesis and declined prior to ovulation. Pham et al. (2012) measured sex steroids in waigieu seaperch (*Psammoperca waigiensis* Cuvier, 1828) and found high levels of 11-KT in the plasma of females during the months of sexual maturation.

Ovulation was defined as the period when eggs flowed freely out of the oviduct, which occurred in the months of November and December. During both months, females were checked for ovulation. Five females had free flowing eggs during both November and December, while most females (n = 28) ovulated in either November or December; these egg batches appeared visually similar and there were no indications of “bad” or overripe eggs. Interestingly, egg survival to the eyed stage ranged from 55-93% from eggs taken in November for those five females, but when the eggs were taken in December from the same females, all five batches had no eggs that reached the eyed stage. This suggests that the window of egg viability after ovulation is less than a month. Several papers that have studied the optimal time to strip eggs after ovulation in rainbow trout found that approximately 4-7 days post ovulation (DPO) will achieve maximum egg survival (Bry 1981; Sakai *et al.* 1975; Springate *et al.* 1984). When eggs are retained in the body cavity 9-12 DPO at approximately 13°C there is a significant decline in egg survival, and this window is even shorter with increasing temperature (Bry 1981).

Photoperiod and temperature are important (possibly the two most important) variables in achieving successful teleost reproduction. Many aquacultured species have been induced to spawn out of their natural reproductive season solely through light and temperature cycle manipulations (Jonsson & Jonsson 2009; Norberg *et al.* 2004; Taranger *et al.* 1998; Vikingstad *et al.* 2016; Pankhurst *et al.* 1996). Since the animals for this study were spawned in an aquaculture

facility, we needed to provide a photoperiod and temperature regime that reflected ambient sunlight and temperatures throughout the year in Old Town, Maine.

Stress can result in physiological changes that can interfere with sampling endpoint measurements and ultimately alter the results. Examples of stress that can impact fish physiology include malnourishment (Yamamoto *et al.* 2011), confinement stress (Campbell, Pottinger & Sumpter 1994) and handling associated with multiple bleedings (Hamlin *et al.* 2008). This study showed that animals that were bled monthly did not differ in plasma hormone concentrations relative to animals bled every other month, or in fish sampled only at the beginning of trial, during spawning and at the end of the trial (Figs. 4-4 and 4-5). This study validates the use of monthly capture, bleeding and confinement stress for routine hormone sampling, as it does not appear to alter plasma concentrations of sex steroids. Further, fish sampled monthly showed a similar oocyte development as those sampled less frequently.

Although monthly sampling had no significant effect on the sex steroid concentrations of the female salmon, it is worth mentioning that handling did not come without risks. During each sampling point, considerable care was taken to ensure each fish was handled carefully, however, some females were unintentionally dropped when transferring them from their tank to the sampling station, and we decided to remove these females from the analyses. On one occasion, a few females were overdosed with MS-222 and these fish were also removed from the experiment. Since these females were part of the monthly and bi-monthly bleeding treatments, mortality was inevitably higher in those treatments compared to the F,S,L treatment.

In summary, this study is the first to describe the annual plasma hormone profiles of E2 and 11-KT in North American strains of female Atlantic salmon raised in a RAS. This information will help to increase our understanding of the physiology and reproductive endocrinology of Atlantic salmon. This study also suggests that monthly blood sampling in Atlantic salmon will not impact sex steroid profiles and likely has little negative impact on reproduction.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1. Summary

Causes for decreased embryo survival in farm raised Atlantic salmon are first addressed in Chapter 1. Since hormones regulate almost every aspect of reproduction, it is important to understand if there is a correlation between embryo survival and certain hormones that are important in salmon reproduction. After visiting three farms and sampling over 300 female Atlantic salmon, a significant positive correlation was found between embryo survival and levels of plasma E2 and 11-KT in female Atlantic salmon. Although these correlations were found, it is important to note that all samples were taken at the time of spawning, giving only a snapshot in time of hormone concentration in each female. Therefore, where each female is in their annual hormone cycle cannot be determined. For example, peak concentrations of plasma E2 and 11-KT correlate with high embryo survival, but for females with lower concentrations of these hormones, there is no way to determine if the females: a) are about to reach the peak concentration, b) have already come down from the peak, or c) will never reach the peak because they did not go through a classic hormone cycle.

Interestingly, hormones in the egg at the time of spawning showed no significant correlations with embryo survival, raising questions about steroids in the egg and their potential source. Chapter 3 investigates these questions and results showed a significant correlation between hormone concentrations in the egg with OF hormone concentrations, not with plasma hormone concentrations. Results suggest that the steroidogenic follicle layers could be key sources of egg sex steroid concentrations.

The snapshot of hormone concentrations in each female Atlantic salmon at the time of spawning, seen in Figures 2.1 and 2.2, led to the question: what is their annual hormone cycle? Chapter 4 describes the first study to document the annual cycle of the hormones E2 and 11-KT in female broodstock of the northern strain of Atlantic salmon. Peak concentrations of E2 occur two months prior to ovulation, signifying induction of vitellogenesis; peak concentrations of 11-KT occurred one month prior to ovulation, signifying induction of sexual behavior and secondary sexual characteristics.

5.2. Future Directions

Although the current study provides a small piece to the embryo survival puzzle, future studies should examine the following to build upon these data and enhance the knowledge of Atlantic salmon reproduction:

a) Understand why farmed female Atlantic salmon are not synchronously cycling through their hormone cycles. Differences in hormone concentrations among females during peak spawning months suggest they will not synchronously spawn and therefore timing of egg collection is a key to successful embryo survival. Once a female has ovulated her eggs, they must be collected within a certain window of time before going atretic. This window of egg viability has been studied in salmonids and varies considerably from study to study (Aegerter & Jalabert 2004).

b) Understand why farmed salmon do not spawn volitionally. If females were able to release their ovulated eggs into their tank at the precise time for optimal fertilization it would eliminate the speculation and subjectivity as to when the females are ready to spawn. This would reduce labor required for sorting fish for spawning readiness, a process that likely causes increased stress to the fish.

Environmental and social cues trigger wild, sexually mature female Atlantic salmon to dig a hole in the gravelly bottom of their natal river by thrusting their tail back and forth. Once a small hole (called a redd) about 10-30 cm deep, has been carved out of the stream bottom, eggs and milt can be released into it and the female can cover the fertilized eggs with the gravel she had previously removed; eggs can be buried up to a foot in gravel. Farmed salmon do not have the cues to stimulate a natural spawning response, therefore future studies should try to imitate the natural stream environment and conditions that wild Atlantic salmon traditionally spawn in to help induce volitional spawning in farmed Atlantic salmon.

c) In this study there was no significant correlation between embryo survival and female EROD activity. Although this suggests that contaminants are not responsible for reduced embryo survival, we must keep in mind that EROD activity is only activated by chemicals structurally similar to PHHs and PAHs. There are many other types of chemicals found in the aquatic environment that have the potential to reduce embryo survival, such as personal care products, pharmaceuticals, and pesticides/herbicides/insecticides, therefore future studies should determine if other contaminants are contributing to the decrease in embryo survival.

5.3. Conclusions

The description of sex steroids and egg hormone regulation will help to improve our understanding of Atlantic salmon reproduction. Expanding our knowledge in Atlantic salmon reproduction will greatly benefit Maine's salmon aquaculture industry since a better understanding of the mechanisms that lead to high embryo survival could allow a reduction in the number of broodstock to spawn, number of eggs to stock, hatch trays and tanks needed to house the eggs, and reduce the amount of time and effort required for husbandry.

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APPENDIX: SUPPLEMENTAL FIGURES

Figure A.1. Oquossoc Eye Up. Yearly average % of embryos reaching the eyed stage for all females spawned for Cooke Aquaculture's Oquossoc, Maine Atlantic salmon hatchery.

Figure A.2. Linear Regression of Plasma T and 11-KT. Simple linear regressions of plasma (A) testosterone (pg/mL) and (B) 11-ketotestosterone (pg/mL) concentrations in female Atlantic salmon plasma versus % of embryos reaching the eyed stage. There is a significant positive correlation between the hormone 11-ketotestosterone (B) with % of embryos reaching the eyed stage. There is no significant correlation between testosterone (A) in the female plasma with % of the embryos reaching the eyed stage.

Figure A.3. Linear Regression of Plasma E2 and Calcium. Simple linear regressions of plasma (A) 17 β -estradiol (pg/mL) and (B) calcium (mg/dl) concentrations in female Atlantic salmon plasma versus % of embryos reaching the eyed stage. There is a significant positive correlation between the hormone 17 β -estradiol (A) with % of embryos reaching the eyed stage. There is no significant correlation between calcium (B) in the female plasma with % of the embryos reaching the eyed stage.

Figure A.4. Linear Regression of Egg T and 11-KT. Simple linear regressions of (A) testosterone (pg/mL) and (B) 11-ketotestosterone (pg/mL) concentrations in Atlantic salmon eggs versus % of embryos reaching the eyed stage. There are no significant correlations between testosterone (A) and 11-ketotestosterone (B) in the eggs and with % of the embryos reaching the eyed stage.

Figure A.5. Linear Regression of Egg E2 and Calcium. Simple linear regressions of (A) 17β-estradiol (pg/mL) and (B) calcium (mg/dl) concentrations in Atlantic salmon eggs versus % of embryos reaching the eyed stage. There are no significant correlations between 17β-estradiol (A) and 11-ketotestosterone (B) in the eggs and with % of the embryos reaching the eyed stage

Figure A.6. Linear Regression of Female Length. Simple linear regression of female standard length versus % of embryos reaching the eyed stage. There is no significant correlation between female standard length and % of embryos reaching the eyed stage.

BIOGRAPHY OF THE AUTHOR

LeeAnne Rose Thayer was born in Cumberland, Maine to her parents Jonathan and Roberta Burr. Growing up on a farm, she entered 4-H at the age of 9, joining the Pig Club and raising a racing pig. Quickly after, she started raising pigs for market and she joined two other 4-H clubs: the Sheep Club and the Steer Club, raising sheep and steer for market. While raising her animals, LeeAnne also started working with her Grandfather, John C. Woodcock, at the age of 9, filling the lobsterman's baitbags while docked on shore. At the age of 12, LeeAnne was able to haul traps with her Grandfather and became his Stern-man. Finally, at the age of 18, she legally became his apprentice and received a license to haul thirty of her own traps. Growing up, it was easy to tell that LeeAnne would want to have a career in agriculture.

In 2004, LeeAnne graduated from Greely High School and started her undergraduate career at the University of New Hampshire (UNH) where she quickly became engulfed in the aquaculture field as well as SCUBA diving. In 2008, she graduated from UNH with a bachelor's degree in marine and freshwater biology and with a Master SCUBA diver certification. After graduating from UNH, she started working for the University of Maine's Center for Cooperative Aquaculture Research Center (CCAR) as a research technician. There, her love for aquaculture grew even deeper. In 2011, LeeAnne was given the opportunity to work with Dr. Heather Hamlin on a project that involved aquaculture in Maine, which lead her to the Graduate School at the University of Maine in Orono working with Atlantic salmon.

Scholarly Publications:

Freitag, A.R., Thayer, L.R., Hamlin, H.J. (2016) Effects of elevated nitrate on early thyroid morphology in Atlantic salmon, *Salmo salar*. *Journal of Applied Ichthyology*, 32, 296-301.

McCoy, J.A., Hamlin, H.J., Thayer, L.R., Guillette, L.J.Jr., Parrot, B.B. (2016) The influence of thermal signals during embryonic development on intrasexual and sexually dimorphic gene expression and circulating steroid hormones in American alligator hatchlings (*Alligator mississippiensis*). *General and Comparative Endocrinology*, 238, 47-54.

Thayer, L.R. & Hamlin, H.J. (2015) Investigating factors contributing to reduced embryo survival in farm-raised Atlantic salmon, *Salmo salar*. *Aquaculture Research*, 47, 3980-3989

Freitag, A.R., Thayer, L.R., Leonetti, C., Stapleton, H.M. & Hamlin, H.J. (2015) Effects of elevated nitrate on endocrine function in Atlantic salmon, *Salmo salar*. *Aquaculture* 436, 8-12.

Professional Memberships:

World Aquaculture Society

LeeAnne is a candidate for the Doctor of Philosophy degree in Marine Biology from the University of Maine in August 2017.