ROLE OF TRANSFORMING GROWTH FACTOR BETA SUPERFAMILY (TGFβ) ON STEROIDOGENIC ENZYME EXPRESSION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) OOCYTE SOMATIC LAYER ISOLATES

By

Hannah R. Parrott

An Abstract
of a thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
in the Department of Biology and Agriculture
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May, 2014
ABSTRACT

By

Hannah R. Parrott

Increasing the knowledge on the regulation of steroid synthesis in teleost fish is key to optimizing the production of competent follicles (fecundity), which will assist the aquaculture industry in meeting the ever-increasing demand for consumable fish. The objectives of this project are to compare the steroidogenic enzyme activity of steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase (cyp19a1) enzymes expressed by the somatic follicular layers of both immature and mature oocytes when treated with BMP4/7 and Activin A. We also investigated the role of a new receptor blocker, DMH-1, to see if it is an effective regulator in steroidogenesis and final oocyte maturation. The enzyme StAR and 3β-HSD are required for both estradiol (E2) and maturation inducing hormones (MIH) production, whereas cyp19a1 is only required for E2. As all steroids are synthesized from a common precursor, it is the alteration of these enzyme’s expression profiles that dictates the steroid being synthesized.

Quantitative Real-Time PCR (qPCR) was performed to measure steroidogenic enzyme mRNA levels. Quantification of the gene expression were determined by the [delta][delta]-CT method normalized to the standard housekeeping gene EF1 alpha. A 2-way Analysis of Variance (ANOVA) demonstrated a significant difference in steroidogenic enzyme expression based on maturity level. In contrast, independent treatments with ActA, BMP4/7, and DMH-1 showed no significant effect in regulating steroidogenesis.
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May, 2014
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LIST OF ABBREVIATIONS

17α-HPO - 17α-hydroxyprogesterone

20β-HSD - 20β-hydroxysteroid dehydrogenase

3β-HSD - 3β-hydroxysteroid dehydrogenase

ActA - Activin βA,βA

ANOVA - Analysis of Variance

BAMBI - BMP and activin membrane-bound inhibitor

BCP - 1-bromo-3chloropropane

BMP - Bone morphogenetic protein

cAMP - Cyclic adenosine monophosphate

cDNA - complementary DNA

CERC - Columbia Environmental Research Center

Ct - cycle threshold

Cyp11a1 - Cholesterol side-chain-cleavage cytochrome P450

Cyp19a1 - P450 aromatase

DMH-1 - dorsomorphin homologue 1

DNA - Deoxyribonucleic acid

E 2 - 17β-estradiol

EF1a - Elongation factor-1-alpha

EST - Expressed sequence tag

EtOH - Ethanol

FAO - Food and Agriculture Organization of the United Nations

FSH - Follicle-stimulating hormone

FSHr - FSH receptor
GFP - Green fluorescence protein
GnRH - Gonadotropin-releasing hormone
GPCR - Gs-protein-coupled receptor
GtH - Gonadotropin
GV - Germinal vesicle
GVBD - Germinal vesicle breakdown
HPG-axis - Hypothalamic-pituitary-gonadal axis
IPA - Isopropanol
LH - Luteinizing hormone
LHr - LH receptor
MDC - Missouri Department of Conservation
MIH - Maturation inducing hormone
MPF - Maturation promoting factor
MRC - Molecular Research Center
mRNA - messenger RNA
NF - Nuclease free
P4 - Progesterone
P450c17 - Cytochrome P450c17
PGC - Primordial germ cell
PKA - Protein kinase A
qPCR - Quantitative polymerase chain reaction
RFU - Relative fluorescence units
RISC - RNA-induced silencing complex
RNA - Ribonucleic acid
RNAi - RNA interference
rRNA - ribosomal RNA
SEM - Standard error of measurement
siRNA - Small interfering RNA
StAR - Steroidogenic acute regulatory
STF - Standard temperature and force (defined as 4°C and 12,000g)
T4 - testosterone
TGFβ - Transforming Growth Factor β
tMM - Trout mineral medium
USDA - United States Department of Agriculture
Vg - Vitellogenin
ΔΔCt - delta delta Ct method
CHAPTER 1: NATURE AND SCOPE OF THE STUDY

General Introduction:

In recent years, there is a pressing need for higher efficiency egg production and enhanced viability of progeny to alleviate pressure on our natural resources due to overfishing and an increasing demand for marketable products. In the United States, this form of agriculture is one of the fastest growing global food production systems, and now contributes around 13% of world animal-protein supply (FAO, 2013). One of the main goals of the aquaculture industry is to maximize the production of viable eggs that result in high offspring survival rates. Until recently, most of the research efforts were focused on endocrine regulation of spawning and optimizing rearing protocols. The molecular, biochemical and physiological mechanisms that lead to the production of so-called “high quality eggs,” has largely been ignored in the available literature. Studies on teleost fish reproduction and work on the optimization of the number of competent follicles in female fish will assist the aquaculture industry in meeting the ever increasing demand for fish. The following review discusses the process of fish oocyte maturation and describes important steroids influencing oocyte maturation.

The objectives of this project are to compare the steroidogenic enzyme activity of StAR, 3B-HSD, and cyp19a1 enzymes expressed by the somatic follicular layers of both immature and mature oocytes when treated with BMP4/7 and Activin A. We also investigated the role of a new receptor blocker, DMH-1, to see if it is an effective regulator in steroidogenesis and final oocyte maturation. Knowing that estrogens produced by the follicle regulate the production of vitellogenin, the yolk precursor protein, which results in enlargement of the follicle during the early growth phase and that progesterone is needed for final follicular maturation and ovulation
(Nagahama & Yamashita, 2008), understanding the expressions of these steroidogenic enzymes will provide information that could better predict the timing of spawning for higher production efficiency.

**Regulation of Oogenesis in Rainbow Trout:**

Ovaries within the female rainbow trout are located bilaterally along the sides of the abdominal cavity. Unlike mammals who are born with a predetermined number of primary (1º) oocytes and lack oogonia, which arise from a primordial germ cell and later differentiate into an oocyte in the ovary, teleosts produce an indeterminate amount of oocytes and can form a sustainable population of oogonia throughout their lifespan. During the process of oogenesis, these oogonia form into 1º oocytes via mitosis. Each ovarian follicle within the ovary contains the oocyte, surrounded by layers of steroid hormone secreting cells, the theca and granulosa layers (Figure 1). Between teleost species, large differences occur in the number and nomenclature of the stages in ovarian development. Teleost ovarian development can be broken down into six distinct stages: oogenesis, primary growth, cortical alveolus, vitellogenesis, maturation and ovulation (Nagahama, 1983). Discussion of the stages will be limited to the primary growth stage, vitellogenesis, and maturation. These phases are characterized by different metabolic and physiological events taking place inside the ovarian follicles, changes in the rate of production of hormones by the follicles, and the nature of the main hormones produced, particularly the ovarian steroids (Leatherland et al., 2003; Nakamura et al, 2005; Thomas et al., 2004).

Oocyte maturation, otherwise known as oogenesis, is a multi-step process involving an immature ovum (Figure 2). The oocyte originates in the female ovary during gametogenesis where primordial germ cells divide to produce an oogonium. During oogenesis the oogonium
becomes a primary oocyte (Wu et al., 2000). In teleosts, as in other non-mammalian vertebrates, the principal events responsible for the growth of oocytes are due essentially to the accumulation of yolk proteins within their cytoplasm (Devlin & Nagahama, 2002). This phase known as the primary growth phase is broken into two sub-stages, the chromatin nucleolar and perinucleolar. The chromatin nucleolar stage leads to an approximate 10-fold increase in the size of the oocyte and decreases the nuclear-ooplasm ratio (Selman & Wallace, 1989). The enlargement of the nucleus into the germinal vesicle (GV) and the elaboration of the organelles results from the perinucleolar phase. During this time the oocyte remains in meiotic arrest, at the end of prophase I and in the diplotene stage. By the end of the primary growth stage the oocyte becomes enveloped by thecal and granulosa cells, creating an ovarian follicle (Wallace & Selman, 1981). The primary growth phase at this time is considered previtellogenic growth. After the oocyte completes its growth, the follicle-enclosed oocyte undergoes meiotic resumption (completion of the first meiotic division followed by progression to metaphase II), where it enters into meiotic suspension again. This is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte.

Progression of the oocyte into the vitellogenic stage requires action by the pituitary (Khoo, 1979) (Figure 3). The anterior pituitary gonadotroph cells release the gonadotropin (GtH) follicle-stimulating hormone (FSH). This production of FSH stimulates the production of ovarian estradiol (E2) (Wallace & Selman, 1985). The E2 then induces the production and release of vitellogenin (Vg) (Nagahama, 1984). Vitellogenin is an egg yolk precursor protein that produces lipoproteins and phosphoproteins that make up most of the protein content of yolk. It is the source of nutrients during early development (Patino & Sullivan, 2002). Vitellogenin is produced in the liver and then is transported to the oocyte, resulting in oocyte enlargement. This
enlargement of the oocyte via Vg uptake is a manifestation of vitellogenesis (Selman & Wallace, 1982). According to Tyler and coworkers, vitellogenesis can be divided up into multiple stages based on the diameter of the oocytes; Pre-vitellogenesis (<0.5 mm), late pre-vitellogenesis (0.51-0.65 mm), early vitellogenic (0.66-1.1 mm), mid-vitellogenic (1.11-2.1 mm), late vitellogenic (2.11-4 mm), and competent (indicated by germinal vesicle breakdown (GVBD)) (Tyler et al., 1994). All these stages were based on the description summarized by Nagahama (1983).

At the competent stage the growth of the oocyte reaches its maximum, E2 production peaks, and the oocyte reaches maturation or meiotic maturation (Nagahama, 1994). This is a critical step in the oogenetic process and must occur prior to ovulation. During the course of fish maturation, oocytes undergo drastic morphological changes associated with progression of the meiotic cell cycle (Tokumoto, 2011). These changes are a prerequisite for successful fertilization and consist of germinal vesicle breakdown (GVBD), which marks the onset of meiosis I (Barkataki et al., 2012), chromosome condensation, assembly of meiotic spindles, formation of the first polar body, and hydration, which causes the oocyte to become transparent (Nagahama & Yamashita, 2008).
Figure 1: Follicular oocyte with Thecal and Granulosa layers
Follicular maturation begins with the oocytes ability to respond to a maturation-inducing hormone (MIH). In fish, oocyte maturation occurs within the ovarian follicle and is triggered by a progestogen. The MIH is produced by follicular layers in response to luteinizing hormone (LH) stimulation. In rainbow trout, \textit{17α,20b-dihydroxy-4-pregnen-3-one (17α,20β-DP)} is the MIH (Fostier et al., 1973; Nagahama & Adachi, 1985). During the initial MIH-independent phase, LH induces the oocytes to become maturationally competent by upregulating MIH receptor concentrations and the steroidogenic enzymes required for MIH synthesis (Patino & Thomas, 1990; Thomas et al., 2001). In the second MIH-dependent phase, the MIH triggers the resumption of meiosis, migration of the nucleus to the animal pole, and its dissolution (i.e., GVBD) in preparation for fertilization by sperm (Patino & Thomas, 1990). The MIH binds to specific receptors on the oocyte cell surface to activate intracellular transduction pathways that induce maturation by a non-genomic mechanism (Thomas et al., 2002).

Several bodies of evidence, in multiple fish species, further indicate that somatic compartments of the ovarian follicle play an important role to allow acquisition of oocyte competence (Stacey, 1984). Studies have subsequently demonstrated that many genes are actively expressed in somatic follicular layers during final oocyte maturation, including genes coding for steroidogenic enzymes, paracrine factors, gonadotropin receptors, and cell-to-cell communication genes, such as connexins (see Lubzens et al., 2010; Patino & Sullivan, 2002 for review).
Figure 2. Oocyte growth and maturation

<table>
<thead>
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<th>G2</th>
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<td>Ana/Telophase I</td>
<td>Metaphase II</td>
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<td>M-phase</td>
<td>M-phase</td>
<td>M-phase</td>
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<tr>
<td>Oocyte Maturation</td>
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Oogonium → 1° Oocyte → 2° Oocyte

Pituitary Independent

FSH & E₂ Dependent

LH & MIH Dependent

Figure 3. Disbursement of growth and maturation steroids 17β-estradiol (E₂) and maturation inducing hormone (MIH).
The Hypothalamic-Pituitary-Gonadal Axis

In teleosts, like in other vertebrates, the steroid production functions that are part of puberty and reproductive growth are controlled by the hypothalamic–pituitary–gonadal axis (HPG) (Bobe et al., 2004; Luckenback et al., 2008) (Figure 4). The hypothalamus is responsible for the production of gonadotropin-releasing hormones (GnRHs), which stimulates the pituitary to release gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Nakamura et al., 2005 & 2009). They act on somatic cells in the gonads, and thereby regulate gonadal biosynthesis of steroid hormones. These studies generally support the hypothesis that ovarian steroidogenesis in fish is regulated by pituitary gonadotropins (GtH), acting through their G-protein linked receptors (GtH-R) on the plasma membrane of the thecal and granulosal cells (Young et al., 2005; Campbell et al., 2006). Binding of the GtHs to their respective GtH-Rs initiates the activation of adenylate cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP) levels (Paul et al., 2010). Increases in intracellular cAMP levels rapidly activate intracellular reserves of steroidogenic acute regulatory (StAR) protein, and initiate intracellular signaling cascades that increase the expression of several genes, including StAR and genes that encode for steroidogenic enzymes (Ings & Van Der Kraak, 2006; Nagahama & Yamashita, 2008)

FSH and LH have similar steroidogenic potency during early phases of gametogenesis, while LH is generally more potent than FSH during late stages of gametogenesis (Suzuki et al., 1988; Planas & Swanson, 1995, Planas et al., 1997). Although the precise role of FSH and LH in regulating follicular steroidogenesis over the course of ovarian development has not been described, the variation in the circulating levels of FSH and LH during sexual maturation (Swanson et al., 1989, Prat et al., 1996) and in the type and localization of FSH and LH receptors
in the ovary would suggest that maturation-associated changes in the steroidogenic effects of FSH and LH may indeed occur in the salmon ovary. It is known that during secondary oocyte growth, plasma levels of FSH are elevated while the circulating levels of LH are extremely low or nondetectable. At this stage, the type I GtH receptor has been shown to be present in both theca and granulosa cells. However, during final oocyte maturation (FOM), plasma levels of LH increase as FSH levels decline in parallel with the appearance of the type II GtH receptor in granulosa cells (Yan et al., 1992, Miwa et al., 1994) In addition, during the transition from vitellogenesis to FOM, the changes in circulating levels of FSH and LH occur concomitantly with changes in the production of two major ovarian steroids: estradiol-17b (E2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), respectively. These changes in plasma steroid levels occur as a result of a steroidogenic shift in the salmonid ovary, from the production of E2 to the production of 17α,20β-DP (Goetz et al., 1987, Nagahama et al., 1995).

Due to the significant positive correlation between the plasma levels of FSH and E2, as well as between the plasma levels of LH and 17α,20β-DP (Swanson, 1991; Oppen-Berntsen et al., 1994; Slater et al., 1994), it is possible that FSH and LH play important roles in regulating the steroidogenic shift in the ovary, which determines the transition from the vitellogenic state to the maturational state.
Figure 4. Hypothalamic-Pituitary Gonadal Axis
Steroidogenesis

Cells in specific tissues, such as gonadal cells, synthesize steroid hormones in a biological process known as steroidogenesis. This pathway is orchestrated by the conversion of cholesterol into steroid hormones (Figure 5). In follicular tissues, cAMP-activated (phosphorylated) StAR protein acts as the rate-limiting step, transporting cholesterol into the inner mitochondrial membrane (Stocco and Clark, 1997). In the inner mitochondrial chamber, the enzyme Cyp11a1 (also known as P450scc) side chain cleavage catalyses the first step of steroid synthesis. Cholesterol is converted to pregnenolone (P5) and associated with the inner membrane (Leatherland et al., 2010). P5 is then further converted to 17α hydroxypregnenolone by Cyp17a1.

In the thecal cells, 17α hydroxypregnenolone is converted into androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD), where it is transported into the granulosa cells and transformed by the granulosa cell’s smooth endoplasmic reticulum enzymes to form androgens. Androstenedione is converted to estrone (E1) by Cyp19a1 and then 17β-HSD forms estradiol (E2). The enzymatic activity of Cyp19a1 is thought to be the rate-limiting step in estrogen biosynthesis (Simpson et al., 1994) and changes in Cyp19a1 enzyme activity or expression of Cyp19a1 genes have been shown to be major regulators of the gonadal production of E2 during reproduction and development (Chang et al., 1997). Within the granulosa cells pregnenolone is converted directly into progesterone (P4) by 3β-HSD (Havelock et al., 2004). The in vitro studies of steroid hormone synthesis by isolated whole follicles or separated theca and granulosa cell preparations have proved to be valuable methods to evaluate the direct actions of various hormones on theca and granulosa cell steroidogenesis.
During final oocyte maturation, a shift in steroidogenesis occurs in ovarian follicles, mediated by changes in gene expression of steroidogenic enzymes like ovarian Cyp19a1 and 20β-hydroxysteroid dehydrogenase (20β-HSD) (Senthilkumaran et al., 2004). This is a switch from predominantly FSH toward higher levels of LH, which leads to the production of MIH instead of estradiol (E2).
Figure 5. Steroidogenesis pathway with key enzymes
**Transforming Growth Factor β-Superfamily**

TGFβ Superfamily members are broken up into three subfamilies that include TGFβ’s, activin, inhibin, and bone morphogenetic proteins (BMPs). The main role of the TGFβ superfamily is to activate G protein-coupled receptor involved in up-regulating growth and development pathways. Then through a cascade of events, intracellular gene regulatory proteins called SMADS, transduce TGF signals to the nucleus. These molecules ultimately interact with other transcription factors in the nucleus to stimulate TGFβ-specific genes, and therefore directly influencing gene expression (Kohli et al., 2003).

**Bone morphogenetic proteins**

Bone morphogenetic protein (BMP) was named in 1965 for its activity of inducing bone formation (Urist, 1965). Bone morphogenetic proteins are morphogens belonging to the transforming growth factor-β (TGF-β) superfamily, and include more than 35 members (Shimasaki et al., 2004). BMPs are multi-functional cytokines that participate in cell proliferation, differentiation, and apoptosis in various tissues (Tsuchida et al., 1996). There are two major types of membrane-bound receptors in the TGF-β superfamily; type I and type II receptors. The difference between type I and type II receptors are that type I receptors contain a GS domain, which aid in signal transduction (Heldin et al., 1997). A real-time PCR-based gene expression survey was performed in the rainbow trout to investigate the expression profiles of some target genes, including BMPs, during maturation competence acquisition or oocyte maturation. In maturing females and those females that acquired high competence to mature, BMP7 increased in expression, whereas BMP4 expression increased later at the time of oocyte maturation, suggesting a potential role for BMPs in the control of oocyte maturation in the rainbow trout (Bobe et al., 2004).
A similar study was performed later in the rainbow trout, focusing on selected target genes during gonadal differentiation and early gametogenesis. The gene BMP7 was found to be among the group of genes with the highest relative expression in the ovary during gonadal differentiation and at the beginning of gametogenesis, whereas BMP4 showed a similar expression profile in testis and ovary (Baron et al., 2005). Recently, a study on the spatiotemporal expression of BMP4, BMP7, and GDF9 in the rainbow trout ovary showed that these genes reached their highest expression at previtellogenic stage during folliculogenesis, and their expression was concentrated in the oocyte (Lankford & Weber, 2010). In terms of developmental roles of BMPs, BMP4 helps in the establishment of left-right asymmetry while bmp7 participates in dorsal-ventral patterning (Miyazono, 2000). Changes in the expression of this family have been characterized in the ovary of rainbow trout during gonadal differentiation and the onset of gametogenesis (Baron et al. 2005), as well as in the somatic cells during maturation (Bobe et al., 2003, 2004). BMP4 and BMP7 are both expressed in the oocyte and in the follicular layers suggesting that BMPs are directly involved in the regulation of steroidogenesis (Lankford and Weber, 2010). However, the mechanisms and patterns of the expression of both Activin A and BMP groups have not been well studied during oocyte maturation.

**Activin A**

Activin A is an important member of the transforming growth factor β (TGFβ) superfamily and its role in gonadal functions has been extensively studied in mammals but not in lower vertebrates (Mather et al., 1997). Activin A is a dimeric polypeptide consisting of two β-monomers βA βA (Act-A), βA βB (Act-AB), and βB βB (Act-B) (Hillier, 2001). Previous studies have demonstrated that Activin A stimulated oocyte maturation and increased GVBD in a
dose-dependent manner in different species of fish (Selman & Wallace, 1989). However, further investigations into the roles of activin during oocyte maturation and steroidogenesis are needed.

**DMH1 inhibitor:**

Endogenous extracellular ligand trap proteins (antagonists) such as Noggin, Follistatin, and Chordin oppose the actions of BMP and TGF-β1 ligands in vivo (Shi & Massague, 2003). An excellent example of this concept is Dorsomorphin (DM) and its family of analogs (Nagahama et al., 1995). These structurally-related compounds differentially target BMP signaling. DMH-1 is a recently developed highly selective small molecule BMP inhibitor that functions similarly to those listed above (Hao et al., 2014). However, according to Neely et al. (2012), unlike the more robust recombinant proteins, he states that using smaller molecules like DMH-1 will have several advantages. These smaller molecules, like DMH, can be produced in large quantities and of high purity, relatively cheaply. They are more stable and can diffuse more readily into tissues. Also, compared to different recombinant proteins, the effectiveness of smaller chemical molecules are more consistent.
CHAPTER 2: EFFECTS ACTA AND BMP4/7 CHIMERA ON STEROIDOGENIC ENZYME MRNA EXPRESSION

Introduction:

In an oocyte there are various growth factors that could be involved in follicle growth. It has been established (Shimaski et al., 2004) that intraovarian factors belonging to the transforming growth factor β superfamily; including Activins and BMPs are synthesized by granulosa and thecal cells and that they have roles as autocrine/paracrine regulation of follicular growth and development. GDF9 and BMP15 are widely studied and their importance in reproductive development was demonstrated using knockout mice. A number of studies have suggested the importance of BMPs in mammalian reproduction but few studies have looked into teleost reproduction. BMPs have different functions in various species. In the mouse, BMP4 and BMP7 have different expression patterns in limb patterning and development, while they are also involved in kidney development (Lee et al., 2001). It is believed that BMPs form both homodimers and heterodimers, with heterodimers are more active in in vivo and in vitro (Kohli et al., 2005). These dimers are involved in the binding to the type II receptors leading to steroid production within the lutenization process. Lutenization leads to an oestrogenic to progestogenic shift in follicular steriodogenesis, through the up-regulation of components in the steroidogenic pathway in both thecal and granulosa cells. These include StAR, 3β-HSD. Cyp19a1 (aromatase) requires a conversion of androgens to estrogens by the granulosa cells, leading to a decrease after preovulatory LH surges (Nagahama, 1997). Therefore, the objective of this study is to compare the effects of BMP4/7 and Activin A on steroidogenic enzyme activity of StAR, 3B-HSD, and cyp19a1 enzymes expressed by the somatic follicular layers in both immature and mature oocytes. We also investigated the role of a new receptor blocker, DMH-1, to see if it is an effective regulator in steroidogenesis and final oocyte maturation.
Materials and methods:

Fish care and sampling:

The rainbow trout used in the temporal analysis of mRNA and the steroidogenic portion of this study were obtained from The Shepherd of the Hills Hatchery in Branson, Missouri, USA. Trout were maintained in 48x42x36 tanks equipped with chillers biological filtration and air circulation. Temperatures were kept between 16-19°C and fish were fed 1% of their total body weight. Water quality was monitored weekly and water changes were done if optimal water quality was not met. (IACUC Protocol #: 13-3227) Female trout were anesthetized by immersing in MS-222 (150 mg/L – Finguel; Argent, Redmond, WA), weighed and decapitated. Fish were categorized into mature and immature stages based on the size of the follicle. Follicle sizes < 1mm were considered immature while follicles with a visible GV are considered mature. Collecting 4 animals in the exact stages were difficult to obtain. Upon sampling, ovaries were excised and weighed from 4 mature and 4 immature female rainbow trout, then placed in ice cold sterile trout mineral medium pH: 8.0 (TMM; Bobe et al., 2003). (Table 1).

Table 1. Trout Mineral Medium (1L)

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</tr>
<tr>
<td>Glucose</td>
<td>5.6</td>
<td>180.2</td>
<td>1009.1</td>
<td>1.0091</td>
</tr>
<tr>
<td>Heps</td>
<td>20</td>
<td>260.3</td>
<td>5206.0</td>
<td>5.2060</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Penicillin</th>
<th>10,000 U/mL</th>
<th>100,000 U/L</th>
<th>Combined with Strep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strep</td>
<td>10mg/mL</td>
<td>100mg/L</td>
<td>10mL total</td>
</tr>
<tr>
<td>BSA</td>
<td>0.10%</td>
<td>0.10%</td>
<td>1 g</td>
</tr>
</tbody>
</table>

*MgCl2-6H2 substituted for MgCl2, keep Rnase free. Bobe, 2003
Somatic cell separation:

A total of 25 sets of somatic follicular layers (i.e., theca and granulose layers) were collected in triplicate from 4 individual fish in the first study and 5 fish in the second study. The follicular layers were isolated by mechanically rupturing the oocyte with fine forceps and discarding the ooplasm. Tissues were then placed into 24-well falcon plates with 1mL TMM. The medium was then removed and 1.5mL of fresh TMM was added along with 1.5 µl to bring final concentration to 100ng/mL of each treatment peptide (Figure 6). Peptide treatments were performed in triplicate. Plates were incubated overnight at 14°C under constant orbital oscillation. After incubation, the tissues were then removed from the wells and placed in 1.5mL snap-cap vials, flash frozen in liquid nitrogen and stored at -80°C until used for RNA isolation. RNA was isolated as described below (Lankford & Weber 2010).

Figure 6. Peptide Treatment Plate Set-up
RNA isolations:

To isolate the RNA 1 mL of RNAzol RT™ was added to each 1.5 mL round-bottom snap-cap vial containing the previously frozen tissue layers along with a sterile 3mm stainless steel bead. Samples were homogenized using 2 x 1 min cycles at 30 cycles/sec in a Qiagen TissueLyser (Retsch, Newtown, PA), and allowed to stand for 5 min before centrifuging at 12,000 x g for 5 min at 4°C. Supernatant from the samples was removed and added to 400 μL of nuclease free sterile water (NF water), then incubated at room temperature for 15 min. After the incubation period, samples were centrifuged at 12,000 x g for 15 min at 4°C and the upper clear aqueous layer was then removed. After the extraction, 400 μL of 75% EtOH per mL of RNAzol™ was added to 1 mL of the clear aqueous layer to precipitate out the RNA before incubating for 20 min at room temperature. Samples were then centrifuged at max speed (20,817 x g) for 8 min at 4°C. Precipitation resulted on a small translucent pellet of RNA. The supernatant was removed and the pellets were washed once with 1.5mL of 75% EtOH and twice with 500μL of 75% EtOH. Ethanol was aspirated off after each wash.

A DNase treatment and RNA re-extraction was added to assure the removal of any genomic DNA contamination. The pellet was then dissolved in 12-40μL of NF-water and either flash frozen in a -80°C freezer or incubated at 55-60°C for 10-15 min to allow complete reconstitution. Lastly, 2μL of each RNA sample was used to determine the quantity of RNA using a NanoDrop ND-1000 (Wilmington, DE USA) and the integrity was verified through analysis of the 260/280 ratio and 1.5% agarose electrophoresis.
Complementary DNA:

The cDNA was synthesized by allowing 1µg (2µL) random primers (Promega, Madison, WI) to anneal to 2 µg of RNA for 5 min at 70° C in a final volume of 10 µL. Then 15 µL of reverse transcription Master mix (Table 2) was added and incubated at 37° C for 1 hr. The cDNA was then diluted 1:5 prior to quantitative real-time polymerase chain reaction (qPCR).

Table 2. RT Random Hex Master Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>conc</th>
<th>Vol/tube (µL)</th>
<th>Vol for mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMLV buffer</td>
<td>5X</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>10mM each</td>
<td>1.25</td>
<td>62.5</td>
</tr>
<tr>
<td>rRNasin</td>
<td>40 U/µL</td>
<td>0.625</td>
<td>31.25</td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>200 U/µL</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>NF water</td>
<td></td>
<td>7.125</td>
<td>356.25</td>
</tr>
<tr>
<td>total vol (µL)</td>
<td></td>
<td>15</td>
<td>750</td>
</tr>
<tr>
<td># rxns</td>
<td></td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Quantitative Real-Time Polymerase Chain Reaction:

Amplification and expression assays were conducted using a Bio-Rad iQ™5 optical module mounted on a Bio-Rad iCycler™ (Bio-Rad, Hercules, CA). Quantitative RT-PCR reactions were carried out in triplicate using 2.5µL of the diluted cDNA mix with 12.5µL 1x SYBR Green PCR Master Mix (SABiosciences, Valencia, CA), and 10µL of previously optimized primer mix in a 25 µL total reaction (Table 3).

The thermal cycling profile was 95.0°C for 30 s, 2 cycles of 95.0°C for 1 min, 95.0°C for 10 min, 50 cycles of 95.0°C for 20 s then 60.0°C for 1min, 95.0°C for 1 min, then 55.0°C for 1 min and 81 cycles of 55.0°C for 10 sec increasing temperature 0.5°C each cycle. Cycle threshold (Ct) was set to 10% of the fluorescence units (RFU). Quantification of the gene expression were determined by the [delta][delta]-CT (ΔΔ-CT) method normalized to the standard housekeeping gene EF1α.
Statistical Analysis:

With the level of significant difference set at $p \leq 0.05$, all data was analyzed by SigmaPlot 12.3. The relative expression of $3\beta$-HSD, Cyp19a1 and StAR from four mature fish vs. four immature fish were compared using a 2-way Analysis of Variance (ANOVA) model followed by a Tukey pairwise multiple comparison test. Kruskal-Wallis one way Analysis of Variance on Ranks was used to determine the differences in enzyme expression levels from the different peptide treatments: Activin A, BMP4/7 and DMH-1. Data was reported as means ± standard error of measurement (SEM).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1α</td>
<td>800</td>
<td>600</td>
</tr>
<tr>
<td>StAR</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>$3\beta$-HSD</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>Cyp19a1</td>
<td>800</td>
<td>800</td>
</tr>
</tbody>
</table>
Results:

*StAR* - The mean expression of StAR in immature fish was not significantly different when treated with Activin A, BMP4/7 or DMH-1. However, there appears to be a non-statistical increase in StAR expression levels when treated with ActA and DMH-1, while treatment with BMP4/7 appears to have had a slight inhibitory effect on the production of StAR. In mature fish, there was no significant difference between the treatments, but similar to above, there appears to be an increase in StAR expression in the presence of ActA. Both BMP4/7 and DMH-1 appeared to have little or no effect on StAR mRNA expression levels in mature fish. It is notable that there appears to be an increase in StAR expression in immature fish when treated with DMH-1, while in the mature fish there was little change in expression levels. When comparing mean StAR mRNA expression levels based on the level of maturity, there was a significant difference in expression levels (2-way ANOVA on ranks, P = 0.043).
Figure 7. Mean ± SEM of StAR mRNA relative enzyme expression from 4 mature vs. 4 immature fish. All treatments were normalized around the control. There was no significant difference among treatment groups. There was a significant difference among the maturity level (2-Way ANOVA P ≤ 0.05)
**3β-HSD** – Mean mRNA expression of 3β-HSD was not significantly different among treatments in immature fish (Figure 8). However, treatment with DMH-1 appeared to increase 3β-HSD expression, while treatments with ActA and BMP4/7 reduced the expression level. There were also no significant differences in expression in the mature fish. While no treatments affected 3β-HSD expression levels in the specific maturity groups, similar to StAR expression, immature mRNA expression was lower than mature. Again, it is notable that there appears to be an increase in 3β-HSD expression in immature fish when treated with DMH-1, while in the mature fish there was little change in expression levels. Furthermore, within the treatments, there was a significant difference in 3β-HSD expression in immature fish vs. mature fish when treated with either ActA or BMP4/7 (Figure 7).

**Cyp19a1** - There was a significant difference in expression levels from immature fish to that of mature fish (2-way ANOVA, P = 0.038). Overall, treatments with ActA, BMP4/7 and DMH-1 do not appear to impact Cyp19a1 expression levels in either immature or mature fish.
Figure 8. Mean ± SEM of 3β-HSD mRNA relative enzyme expression from 4 mature vs. 4 immature fish. All treatments were normalized around the control. There was a significant difference among the maturity levels (2-Way ANOVA P ≤ 0.05).
Figure 9. Mean ± SEM 3β-HSD mRNA enzyme expression treated with 100ng/mL ActA and BMP4/7. There was a significant difference in 3β-HSD expression among the ActA and BMP4/7 treatments (Mann-Whitney Rank Sum Test, $P = 0.029$).
Figure 10. Mean ± SEM of Cyp19a1 mRNA relative enzyme expression from 4 mature vs. 4 immature fish. All treatments were normalized around the control. There was a significant difference among the maturity level (2-Way ANOVA P ≤ 0.05).
Figure 11. Mean ± SEM of StAR and 3β-HSD mRNA relative enzyme expression from mature vs. immature fish treated with 100ng/mL DMH-1. All treatments were normalized around the control. All data showed no significant difference (Independent T-Test $P \geq 0.05$)
Table 4. Non-normalized control mean mRNA expression

<table>
<thead>
<tr>
<th>Steroidogenic Enzyme</th>
<th>Mature</th>
<th>SEM</th>
<th>Immature</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>StAR</td>
<td>0.5667</td>
<td>0.1868</td>
<td>0.0354</td>
<td>0.0150</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>1.2095</td>
<td>0.3866</td>
<td>0.2116</td>
<td>0.0600</td>
</tr>
<tr>
<td>Cyp19a1</td>
<td>0.3087</td>
<td>0.0980</td>
<td>0.2914</td>
<td>0.2413</td>
</tr>
</tbody>
</table>
Discussion:

This investigation was designed to gain information on ovarian molecular regulators with a focus on the paracrine impact of the TGFβ superfamily on the process of steroidogenesis. Specifically, the functional role of ActA, BMP4/7 dimer, and a receptor antagonist, DMH-1, to alter the expression of key steroidogenic enzymes was investigated in theca/ granulosa cell layer isolates. The isolate method was utilized in this study as previous work in our lab had investigated a similar set of questions, but did so in whole follicles. The responses were slight, if any in that study, so it was postulated that the peptide treatment might have had limited access to the somatic cells of the follicle due to the chorion layer of the follicle. Overall, I found very little information to suggest significant differences exist between any of the treatments and the steroidogenic enzyme mRNAs investigated in this study (Figures 5, 6, & 8) but did find statistical differences that indicate a difference in the expression levels on the enzymes between maturational stages (Figure 7). Furthermore, there appears to be more impact with DMH-1 on immature layers than it has on mature layers (Figure 9). This could suggest that members of the TGFβ superfamily has greater impacts on the early development of the follicle that in the maturation stages. Specific response of each treatment will be discussed and incorporated into the literature individually.

Activin A – Activin A is a dimeric protein that structurally belongs to the TGFβ superfamily. In addition to stimulating FSH production from the pituitary, activin has been shown to play roles in cell proliferation, differentiation, apoptosis, (Chen et al., 2006) metabolism, homeostasis, immune response, wound repair, (Sulyok et al., 2004) and endocrine function. Expression of ActA StARts low in early stages and then increases during Vg stages (Ge et al., 1993; Lankford & Weber, 2010). At the primary growth phase, the follicle is pituitary
independent, but the follicle continues to progress into the 2° growth phase suggesting some level of local regulation at the level of the oocyte itself. It is well known that the pituitary stimulates follicles with FSH to increase estradiol production during early 2° growth leading to vitellogenesis (Nagahama, 1994). Within this study, ActA appears to increase StAR in both mature and immature fish. StAR is the general transporter of cholesterol into the mitochondrial membrane that acts as the rate-limiting step in steroidogenesis, so within the fish, a treatment with activin A seems to allow more StAR to be introduced into the system, resulting in more steroids to be produced. However, within the immature fish, there appears less 3β-HSD being produced, while in mature fish there was little effect on expression. The enzyme 3β-HSD is responsible making the cholesterol backbone viable for sex steroid production (Havelock et al., 2004), suggesting that exposure to ActA prematurely might block 3β-HSD production in immature fish and potentially preventing early maturation before the follicle is complete enough to enter the growth phases.

The conversion of sex steroids into estradiol is mediated by the action of Cyp19a1 (aromatase). Within immature and mature fish, ActA produced no significant changes in Cyp19a1 expression, suggesting it is not necessary for up-regulation of sex steroids to E2. Overall, analyses of these results are difficult to interpret due to the fact there was high variation in enzyme expression between fish possibly caused by small differences within the maturity stages of the fish. Increasing sample size of both immature and mature fish will possibly compensate variance found within the maturity stages, but it is likely that having the ability to sample many fish in search of animals that are all at a very similar stage is required either way.

*BMP4/7* - BMPs have been demonstrated to have a pivotal role in the regulation of reproduction in the gonads (Shimasaki et al., 1999). In mice it was shown that BMP4 had a role
in primordial germ cell (PGC) formation by demonstrating that BMP4 knockout mice failed to produce PGC, concluding that BMP4 was required for PGC formation (Huang et al., 2001). By directly acting on the pituitary cells, BMP7 increases FSH synthesis and secretion (Otsuka & Shimasaki, 2002). BMP7 is also known to inhibit apoptosis in several tissues. (Dudley et al., 1995; Luo et al., 1995) and therefore could be hypothesized that it is part of the mechanism that promotes follicle survival by enhancing granulosa proliferation and suppressing apoptosis. In the rat granulosa cells, BMP7 was shown to augment FSH induced expression of Cyp19a1, while inhibiting expression of StAR. Additionally, BMP7 was shown to inhibit ovulation and progesterone production (Lee et al. 2001). Given that progesterone is a key player for ovulation, inhibition of progesterone would suggest BMP7 could be connected to the mechanism behind ovulation inhibition. In this experiment we used BMP4/7 chimera because recent evidence has shown that BMP4 and BMP7 work synergistically during ovarian regulation, thus providing a more robust response (Logeart-Avramoglou et al., 2006).

Treatment with BMP4/7 appears to decrease 3β-HSD expression, while blocking with DMH-1 appears to increase. If true, these data would suggest BMP4/7 attenuates sex steroid synthesis in immature fish. This is opposite to its suggested role as a peptide that increases the capacity to produce E2 in mammals, however, that effect is likely to be highly stage specific and previous studies in fish have never looked into this response. Similarly to above, expression of 3β-HSD in immature animals was significantly lower than that of mature fish when treated with BMP4/7, suggesting that exposure to BMP4/7 might block 3β-HSD production in immature fish and potentially preventing early maturation.

DMH-1 – In this study we searched for an appropriate blocker of the TGFβ superfamily that we could use to investigate a loss-of-function in contrast to the peptides used above. There
are several candidates that are natural regulators of the BMP4, 7 and 4/7 that could be used to modify the system. Some of these include follistatin, noggin, chordin, and dorsomorphin (Nagahama et al., 1995). Follistatin is an inhibitor of pituitary FSH secretion and therefore directly opposed activin’s actions. Male and female mice that overexpress follistatin have shown to exhibit numerous defects in the testis, ovary, and hair. (Guo et al., 1998). A recent study showed that follistatin can act to augment certain BMP7 actions, but inhibits others (Amthor et al., 2002) Noggin prevents BMPs from binding their receptors (Zimmerman et al., 1996). Chordin exhibits similar functions as noggin, but has a higher affinity to bind to BMP 2 and 4. Dorsomorphin blocks SMAD 1/5/8 phosphorylation by inhibiting binding of the BMP type I receptors ALK2, ALK3 and ALK6 (Yu et al., 2008). All of the above mention molecules have their advantages, but most have the disadvantage of being either too large molecularly or are not available through commercial sources.

Within this study we looked at an analog of dorsomorphin (DMH-1). This commercially available molecule is also considered a small organic molecule (SOM), which when well characterized have highly specific function on target proteins and they may only act on one of many functions a molecule may perform (Stockwell, 2004). To my knowledge this compound had never been used on fish ovarian tissues. In our hands, it appears to have disparate effects based on maturity (Figure 9). Within the immature fish, treatments with DMH-1 appears to increase steroidogenic enzyme expression, while in mature fish there is little change in expression. Further studies into the effects of DMH-1 need to be established to understand the mechanisms behind this new inhibitor and the molecules it inhibits, but our results taken together, indicate that the class of molecules DMH-1 inhibits must have more function in the immature stages than in mature stages, if there is an effect at all.
Mature vs. Immature – This study revealed a significant difference in steroidogenic enzyme expression when comparing mature fish and immature fish. Non-normalized immature mRNA expression was lower than mature, which is consistent with the reduced general steroid levels in immature fish (Table 4). As the fish reaches competency, treatments with ActA and BMP4/7 appear to have little or no effect on mRNA expression (Figures 6 & 8). Our results suggest that treatments with ActA, BMP4/7 and DHM-1 may be dependent on maturity.

Lankford and Weber (2010) found that mRNA expression of StAR and 3β-HSD were stage dependent and expression levels increased between late vitellogenesis and competency, while cyp19a1 appeared to decrease slightly. StAR mRNA expression showed a significant difference in the level of maturity but there was no significant difference between treatments. There appears to be up-regulation in the general transporter, StAR, in both mature and immature animals when treated with ActA, but BMP4/7 and DMH-1 had little effect on StAR expression in the mature fish. This would suggest that ActA might be simply upregulating capacity but not the specific steroid synthesized.

3β-HSD mRNA expressions in immature fish were significantly lower than mature, but there was no significant difference among the treatments. Previous data presented by Lankford and Weber (2010) suggest that TGFβ members are rare in mature stages, so no effect in expression was expected. Within the immature fish BMP4/7 appears to be impacting steroidogenesis negatively based on the lower enzyme mRNA expressions, while in mature fish there is little change in expression. However, one needs to be careful about interpreting mRNA data, as a reducing could either mean the protein is being made at a higher rate that the transcript or that there is less need for the protein so less transcript is being made. In addition, for StAR and 3β-HSD expression, immature mRNA expression was lower than mature, which is
consistent with the reduced estrogen levels in immature fish. Throughout this study slight maturity stage differences among the fish could have attributed to the high variation observed between treatment groups. With the limited access to fish that we had in this work we could not establish even four fish that were at the exact immature or mature sub-stage. This mixture of sub-stages within the stages is problematic, as these fish are aggressively developing follicles. This means that a week’s difference in stage would likely result in a very different response on the molecular side.

**Future Investigations**

This investigation into the comparison of steroidogenic enzyme activity of StAR, 3B-HSD, and cyp19a1 enzymes expressed by the somatic follicular layers showed to be largely dependent on maturity of the animal. To further elucidate the effects of the TGFβ members of steroidogenesis, this study needs to have a larger sample size and fish of the same maturational competence level to decrease variation among the treatment groups. This investigation provided some insight on more promising peptides to use as treatments and which steroidogenic enzymes to focus on. To investigate the effects ActA, BMP4/7 have on final maturation, additional follicles should be sampled and treated with these peptides, then scored for GVBD. This would provide necessary evidence to determine regulation of follicular maturation. Further investigations into the functions of DMH-1 need to be examined as well to see if it is an effective regulator of steroidogenesis.
LITERATURE CITED


Prat F, Sumpter JP, Tyler CR. 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (Oncorhynchus mykiss). *Biology of Reproduction* 54: 1375–1382.


