Regulation of sex steroid production and mRNAs encoding gonadotropin receptors and steroidogenic proteins by gonadotropins, cyclic AMP and insulin-like growth factor-I in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*) at two stages of vitellogenesis

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Abstract

At the completion of vitellogenesis, the steroid biosynthetic pathway in teleost ovarian follicles switches from estradiol-17 β (E2) to maturational progestin production, associated with decreased follicle stimulating hormone (Fsh) and increased luteinizing hormone (Lh) signaling. This study compared effects of gonadotropins, human insulin-like growth factor-I (IGF1), and cAMP/protein kinase A signaling (forskolin) on E2 production and levels of mRNAs encoding steroidogenic proteins and gonadotropin receptors using midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of rainbow trout. Fsh, Lh and forskolin, but not IGF1, increased testosterone and E2 production in MV and L/PV follicles. Fsh increased steroidogenic acute regulatory protein (*star*; MV), 3 β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase (*hsd3b*; MV) and P450 aromatase (*cyp19a1a*; MV) transcript levels. Lh increased *star* mRNA levels (MV, L/PV) but reduced *cyp19a1a* transcripts in L/PV follicles. At both follicle stages, IGF1 reduced levels of *hsd3b* transcripts. In MV follicles, IGF1 decreased P450 side-chain cleavage enzyme (*cyp11a1*) transcripts but increased *cyp19a1a* transcripts. In MV follicles only, forskolin increased *star* and *hsd3b* transcripts. Forskolin reduced MV follicle *cyp11a1* transcripts and reduced *cyp19a1a* transcripts in follicles at both stages. Fsh and Lh reduced *fshr* transcripts in L/PV follicles. Lh also reduced *lhcr* transcripts (L/PV). IGF1 had no effect on gonadotropin receptor transcripts. Forskolin reduced MV follicle *fshr* transcript levels and reduced *lhcr* transcripts in L/PV follicles. These results reveal hormone- and stage-specific transcriptional regulation of steroidogenic protein and gonadotropin receptor genes and suggest that the steroidogenic shift at the completion of vitellogenesis involves loss of stimulatory effects of Fsh and Igfs on *cyp19a1a* expression and inhibition of *cyp19a1a* transcription by Lh.

Keywords: ovarian follicle, gonadotropins, insulin-like growth factor I, rainbow trout, steroidogenesis, gonadotropin receptors

1. Introduction

Development of oocytes of oviparous vertebrates through secondary growth, during which hepatically-derived vitellogenin is taken up by the oocyte and processed (vitellogenesis), and subsequently through maturation/ovulation, is dependent on the somatic cell layers that enclose the oocyte, and together form the ovarian follicle. These somatic cells produce a variety of growth factors, and are also the sites of synthesis of the two major classes of bioactive steroids, estrogens and progestins. In teleost fish, the steroid biosynthetic pathway during vitellogenesis is directed towards production of estradiol-17 β (E2), which stimulates hepatic vitellogenin synthesis, while the completion of vitellogenesis is characterized by major changes in the biosynthetic pathway that lead to the production of maturation-inducing steroids (MIS, 17,20 β -dihydroxy-4-pregnen-3-one [17,20 β -DHP] or 17,20 β ,21-trihydroxy-4-pregnen-3-one). These MIS act to reinitiate meiosis in the oocyte (reviewed by Young et al., 2005; Nagahama and Yamashita, 2008; Lubzens et al., 2010, 2016; Kagawa, 2013). Two pituitary gonadotropins regulate the ovarian follicle's steroidogenic pathway. Follicle-stimulating hormone (Fsh), whose plasma levels peak during vitellogenesis, promotes E2 production, while luteinizing hormone (Lh), whose levels become maximal during the postvitellogenic period, promotes a change in the biosynthetic pathway resulting in MIS production (reviewed by Levavi-Sivan et al, 2010; Lubzens et al., 2010, 2016; Kagawa, 2013).

Associated with the increased ability of the vitellogenic follicle to respond to Fsh and increase E2 production are high levels of follicular Fsh receptors (Fshr) (Kobayashi et al., 2008; Andersson et al., 2009; Guzmán et al., 2014) and high levels of aromatase activity due to increased expression of both the ovarian form of P450 aromatase, encoded by the *cyp19a1a* gene, and of several other genes encoding steroidogenic proteins (Gen et al 2001; Ijiri et al., 2003, 2006; Montserrat et al., 2004; Nakamura et al., 2005; Guzmán et al., 2014 . The switch from E2 to MIS production is characterized by decreased plasma E2 levels and expression of the *cyp19a1a* gene, reduced aromatase activity, increased plasma Lh and follicular Lh receptor (Lhcgr) levels, and an Lh-stimulated increase in 20 β -hydroxysteroid dehydrogenase activity, a process that is transcriptionally regulated (Young et al., 1983;

Nagahama et al., 1985; Nakamura et al., 2005; Senthilkumaran et al., 2002, 2005; Kobayashi et al., 2008; Andersson et al., 2009; Crespo et al., 2012; Guzmán et al., 2014), that leads to conversion of precursor steroids into MIS (reviewed by Nagahama and Yamashita, 2008; Lubzens et al., 2010, 2016; Kagawa, 2013). Knowledge of what initiates the decline in E2-synthesizing capacity at the completion of vitellogenesis is still limited to a few studies that indirectly or directly implicate Lh (e.g., Crespo et al., 2012).

In vitro studies on species such as zebrafish, medaka and goldfish show that heterologous gonadotropin preparations such as human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) can affect activity of particular enzymes and/or transcriptionally regulate one or more steroidogenic protein genes (e.g., Nagahama et al., 1991; Ings and Van Der Kraak, 2006), but the precise roles of Lh and Fsh at different stage of ovarian follicle development in these species remain, for the most part, to be clarified. Studies using highly purified homologous Lh and especially Fsh are less common, and with a few exceptions, are confined to various salmonid species at particular stages of development, particularly previtellogenic (Luckenbach et al., 2011) or preovulatory follicles (Planas et al., 1997, 2000; Crespo et al., 2012). Similarly, changes in *fshr* and *lhcg* transcript levels during ovarian follicle development that are essential to mediate the temporally separate actions of the two gonadotropins have been documented for several species (see Lubzens et al., 2016) but there is limited understanding of how the dynamic expression of these receptors is regulated.

Insulin-like growth factors (Igfs) also appear to participate in the regulation of steroid biosynthesis during vitellogenesis and maturation. In several species, IGF1 and/or IGF2 increase the E2-synthesizing capacity of the follicle (see Reinecke, 2010) and recent work indicates that Igf3, unique to teleosts, regulates transcription of four steroidogenic enzyme genes (Li et al., 2012). In the postvitellogenic follicle, numerous actions of Igfs have been documented, related to increasing responsiveness of the follicle to MIS (“oocyte maturational competence”) through increased expression of the membrane progesterin receptor (see review by Thomas, 2012). All three Igfs have been shown to promote oocyte

maturation alone, but it is unclear if this action is partially mediated through alterations in steroid production.

This study focused on how gonadotropins and IGF1 direct the steroidogenic pathway to E2 production during vitellogenesis and how they may participate in reducing E2 synthesis during postvitellogenic development. We examined the stage- and gonadotropin-specific regulation of expression of four steroidogenic proteins and of two gonadotropin receptors in the ovarian follicles of rainbow trout. We determined the effects of Fsh and Lh on midvitellogenic (MV) follicles and on follicles that were just completing vitellogenesis (late/postvitellogenic, L/PV), when we expected the follicles to lose sensitivity to Fsh and display increased responsiveness to Lh, because of changes in expression of their cognate receptors. We also employed forskolin, which increases adenylyl cyclase activity, to examine the effects of elevated cAMP levels to determine if any identified gonadotropic regulation of expression of target genes involved intracellular cAMP/protein kinase A (cAMP/PKA) signaling, and how that signaling might differ with stage of ovarian follicle development.

2. Materials and methods

2.1. Animals and tissues

Two-year-old virgin female rainbow trout (560-800 g body weight, 33-40 cm fork length) with MV or L/PV ovaries (classification based on follicle diameter and maximum size of follicles achieved in this stock of fish, see Discussion) were sampled from a breeding stock held in flow-through fresh water under ambient conditions. Trout were euthanized in 300 mg/L MS222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO) buffered with sodium bicarbonate. Ovaries were dissected out and kept in trout Ringer's solution (Kagawa et al., 1983) for further in vitro studies. Follicles were also fixed in Bouin's fixative, processed, embedded in paraffin and sectioned using standard histological procedure to determine the morphological characteristics of each stage. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (protocol 4078-01).

2.2. Reagents

Coho salmon Fsh and Lh were purified as described previously (Swanson et al., 1991). Forskolin was obtained from Sigma-Aldrich. Recombinant human IGF1 was purchased from GroPeP Pty Ltd (Adelaide, SA, Australia).

2.3. *In vitro* culture of ovarian follicles

Follicles from each of three females at each stage were isolated from ovarian fragments held in ice-cold trout Ringer's solution. Diameters of 20 intact follicles were measured under a dissecting microscope. From the ovary of each female, MV follicles (10 follicles/well/1 ml Ringer's) or L/PV follicles (10 follicles/well/2 ml Ringer's) were incubated in the presence or absence of Fsh, Lh, human IGF1 (all at 100 ng/ml) or forskolin (10 μ M) in trout Ringer's solution at 12°C for 18 hours with gentle shaking (100 rpm); thus, there were three independent replicates per treatment. Concentrations used were based on those used on previous studies (Maestro et al., 2007; Planas et al., 1997, 2000; Luckenbach et al., 2011) After the incubation, tissue samples were frozen using liquid nitrogen and stored -80°C until RNA extraction. Samples of incubation media were removed and stored at -20°C.

2.4. Expression of genes encoding steroidogenic proteins and gonadotropin receptors

Total RNA was isolated from the ovarian follicles using Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). Total RNA concentrations in extracts were determined using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE). Each RNA sample (200 ng) was incubated with 0.2 units of DNase I Amp Grade (Invitrogen) at room temperature for 15 min to eliminate genomic DNA contamination. Subsequently, DNase I was inactivated by heat denaturation at 65°C for 10 min. Single strand cDNAs were then synthesized using High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR with TaqMan probes (Applied Biosystems) was used to quantify mRNA using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Primer pairs and probes used for the detection of transcript for steroidogenic

acute regulatory protein (*star*), P450 cholesterol side-chain cleavage enzyme (*cyp11a1*), 3 β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase (*hsd3b*) and *cyp19a1a* were identical to those used in previous studies (Kusakabe et al., 2006; Nakamura et al., 2009). Each PCR reaction contained 25 μ l PCR mixture made from 12.5 μ l of ABI Universal PCR Master Mix, 0.9 μ M forward primer, 0.9 μ M reverse primer, 0.2 μ M fluorescent-labeled probe and 3.0 μ l cDNA template. Real-time PCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. To determine the levels of transcripts, cDNAs for a standard curve were generated by reverse-transcription of serial dilutions of each RNA sample. Correlation coefficients of the standard curves ranged from 0.99 to 1.00 and the efficiency of the reaction ranged from 99% to 100% (slope = -3.3). RNA samples not subject to reverse transcription were used to check for genomic DNA contamination during RNA preparation. For the internal control, acidic ribosomal phosphoprotein P0 mRNA, determined previously to be expressed at constant levels in these follicle stages, was used as a reference gene. Primer pairs and probe used for the detection of the reference gene are in Pierce et al. (2004). All quantitative PCR assays for a given target were run on a single plate. Transcript levels were normalized to the reference gene, and the data were calculated as relative abundance per follicle.

2.5 Radioimmunoassay

E2 and testosterone concentrations in media were measured with specific radioimmunoassays, using the same antibodies and methods as described in Kagawa et al. (1981), with tritiated steroids obtained from Perkin-Elmer (Waltham, MA). Sex steroids were extracted from serum using ethyl ether as previously described by Kagawa et al. (1981). All samples were run in triplicate.

2.6. Statistics

Steroid and transcript data comparing effects of treatments were log transformed and analyzed by one-way ANOVA, and control values were statistically compared with treatment values using the Holm-Sidak post hoc test corrected for multiple comparisons. Data on the effects of treatments on transcripts were normalized to the reference gene and

then divided by the mean of the control for ease of viewing relative difference. Data on transcript levels from control incubations of MV follicles and L/PV follicles were log transformed and analyzed using the t-test and are presented normalized to the reference gene to permit comparison between stages. Significance was accepted at $P < 0.05$. All statistical analyses were performed using Prism 6 software (GraphPad Software Inc., San Diego, CA). All the results are expressed as mean $\pm$ SEM, $n=3$.

3. Results

3.1. Oocyte diameter, histology and GSI

MV follicles were 2.51 ± 0.11 mm in diameter, and displayed small yolk globules throughout the ooplasm (data not shown). Donor females had a GSI of 3.22 ± 0.37 . L/PV follicles were 4.42 ± 0.02 mm in diameter, with large yolk globules filling the ooplasm (data not shown). Donor females had a GSI of 12.70 ± 1.67 .

3.2. Follicular testosterone and estradiol-17 β synthesis (Fig. 1)

Treatment of MV follicles with Fsh, Lh, or forskolin resulted in significant 4-5 fold increases in testosterone ($P < 0.01$) and E2 ($P < 0.01$) levels in media, but IGF1 did not significantly alter media levels of either steroid. Testosterone levels after Lh and Fsh treatment of L/PV follicles were significantly higher ($P < 0.01$) than control levels. E2 levels in media after exposure of L/PV follicles to Fsh or Lh were significantly elevated ($P < 0.05$) approximately 3-fold. There was no evidence for an effect of IGF1 on levels of either steroid produced by follicles at either stage. Forskolin stimulated a significant increase in media testosterone (180-fold; $P < 0.001$) and E2 (3-fold; $P < 0.05$) to levels similar to those seen after treatment with gonadotropins.

3.3. Comparison of transcript levels in controls between stages (Fig. 2)

To help inform interpretation of the experimental data, transcript levels from MV and L/PV control follicles after 18 h incubation (data normalized to the reference gene) were compared. Levels of *star* ($P < 0.03$), *cyp11a1* ($P < 0.007$) and *hsd3b* ($P < 0.04$) transcripts in

L/PV follicles were significantly higher than those in MV follicles. Levels of *cyp19a1a* transcripts in MV follicles were significantly higher than those in L/PV ($P<0.005$) follicles. In L/PV follicles, *fshr* transcripts were significantly lower ($P<0.0009$) than those in MV follicles. *Lhcgr* transcripts were significantly higher in L/PV follicles ($P<0.02$).

3.4. *StAR* (Fig. 3)

In MV follicles, Fsh significantly increased ($P<0.05$) *star* transcripts approximately 2-fold but despite the two-fold elevation in mean levels, FSH had no significant effect on L/PV follicles ($P<0.1$). Lh significantly increased (3-fold; $P<0.01$) *star* transcript levels at both stages. IGF1 treatment of follicles at either stage resulted in mean *star* transcript levels that were 50% lower than controls but these differences were only close to being statistically significant ($P<0.01$). In MV but not L/PV follicles, forskolin induced a significant, approximately 2.5-fold increase ($P<0.01$) in *star* mRNA levels.

3.5. *Cyp11a1* (Fig. 3)

Fsh and Lh were without significant effect on *cyp11a1* transcript levels at either stage. Incubation with IGF1 ($P<0.01$) or forskolin ($P<0.05$) resulted in a 50-60% reduction in *cyp11a1* transcript in MV follicles, but not in L/PV follicles.

3.6. *Hsd3b* (Fig. 3)

In MV follicles but not in L/PV follicles, Fsh significantly increased *hsd3b* transcript levels 2-fold ($P<0.05$). In both MV ($P=0.11$) and L/PV follicles, Lh had no significant effect on transcript levels. IGF1 reduced *hsd3b* mRNA levels in both types of follicles by approximately 50-60% ($P<0.05$). Forskolin treatment resulted in a significant ($P<0.01$) 2.5-fold increase in *hsd3b* mRNA levels in MV follicles but had no effect on transcript levels on L/PV follicles.

3.7. *Cyp19a1a* (Fig. 3)

Fsh treatment significantly increased (2-fold, $P<0.05$) *cyp19a1a* transcript levels in MV follicles only. In MV follicles, Lh had no significant effect but reduced ($P<0.01$) levels in

L/PV follicles. In MV (3.5 fold, $P < 0.01$) but not L/PV follicles, IGF1 stimulated a significant increase in *cyp19a1a* mRNA levels. Exposure of both MV ($P < 0.05$) and L/PV ($P < 0.05$) follicles to forskolin resulted in a $>50\%$ reduction in *cyp19a1a* transcript levels.

3.8. *Fshr* (Fig. 4)

Overall, for *fshr* (and *lhcg*), replicates of treated follicles showed much greater variance in response to treatment compared to other gene transcripts measured. Neither Fsh nor Lh had significant effects on *fshr* mRNA levels in MV follicles but transcript levels were significantly lower after incubation of L/PV follicles with Fsh ($P < 0.05$) and Lh ($P < 0.05$). IGF1 had no effect on *fshr* transcripts in MV or L/PV follicles. Forskolin significantly decreased *fshr* mRNA in MV ($P < 0.05$) but not L/PV follicles.

3.9. *Lhcgr* (Fig. 4)

Mean *lhcg* mRNA levels were lower in MV follicles after all treatments but values did not vary significantly from control levels. This trend towards lower *lhcg* transcripts levels after treatment was also apparent in L/PV follicles but only Lh ($P < 0.05$) and forskolin ($P < 0.01$) treatment resulted in a significant reduction in *lhcg* transcript abundance.

4. Discussion

This in vitro study identified a suite of genes in ovarian follicles of rainbow trout whose mRNA levels are regulated by gonadotropins and IGF1. The effects of these hormones on target gene transcript levels are gene-, hormone- and stage-specific. For example, both gonadotropins upregulated *star* mRNA levels in both MV and L/PN follicles, but the control they exerted on *cyp19a1a* differed between upregulation by Fsh in MV follicles and downregulation by Lh in L/PV follicles. IGF1 strongly upregulated *cyp19a1a* mRNA levels in MV follicles but in the same follicles reduced *cyp11a1* and *hsd3b* transcripts. Similarly activation of cAMP/protein kinase A signaling by forskolin resulted in gene- and stage specific effects. This study also found evidence for the potential direct or indirect (via steroids) regulation of *fshr* and *lhcg* expression by their corresponding ligand.

4.1. Follicle stages and steroid production

Classification of follicle stages was based on previous data that used the same breeding stock of rainbow trout as in this study. Mature oocytes of virgin females achieve a maximum diameter of approximately 4.7 mm (Nakamura et al., 2005). Thus, follicles classified as L/PV were either in the terminal stage of vitellogenesis or had completed vitellogenesis. This classification is supported both by the different characteristics of steroid production between MV and L/PV follicles, and in differences in levels of basal transcript levels in controls (discussed further below).

Fsh, Lh and forskolin stimulated steroid production at both stages, with the lower magnitude of response of L/PV follicles indicating that the capacity to respond to a gonadotropic signal with increased E2 production was declining at this stage, in agreement with earlier studies (e.g., Kagawa et al., 1983; Young et al., 1983; Kanamori et al., 1998) and consistent with the relative reduction in *cyp19a1a* transcripts between MV and L/PV follicles of rainbow trout follicles (this study; Nakamura et al., 2005). The stimulatory effects of forskolin agree with the conclusion that acute stimulation of steroid production in vitro is predominantly regulated by the cAMP/PKA signaling pathway in salmonid follicles (Planas et al., 1997, 2000).

As in goldfish vitellogenic follicles (Srivastava and Van Der Kraak, 1994), IGF1 did not affect steroid production at either stage. IGF1 has been reported to increase E2 and MIS production in vitro by preovulatory ovarian follicles of several species, including striped bass (Weber and Sullivan, 2000) and grey mullet (Das et al., 2014). In coho salmon preovulatory follicles, IGF1 inhibited basal and Lh-stimulated testosterone and 17-hydroxyprogesterone production by isolated theca layers but stimulated both E2 and 17,20 β -DHP production by isolated granulosa layers (Maestro et al., 1997). How these divergent effects of IGF1 on coho salmon preovulatory follicles might be mediated is discussed below.

4.2. Steroidogenic protein and gonadotropin receptor mRNA levels between stages

Changes in follicular mRNA levels for six steroidogenic proteins from early vitellogenesis to ovulation in rainbow trout have been reported previously (Nakamura et al., 2005). However, *fshr* and *lhcg* were not measured at that time, and the relative changes between MV and L/PV follicles we report here are informative for interpretation of the experimental results.

The patterns reported on are made with the important caveat that they are based on transcript measurements made after 18 h of incubation. However, the relative differences in transcript levels between the two stages we report on here are very similar to those reported earlier for steroidogenic proteins in rainbow trout ovarian follicles (Nakamura et al., 2005): highest levels of *star*, *cyp11a1* and *hsd3b* transcripts in L/PV follicles; and peak *cyp19a1a* mRNA levels in MV follicles that decline in L/PV follicles. A similar pattern of change has also been reported for coho salmon ovarian follicles (Guzmán et al., 2014).

Fshr transcripts levels in L/PV follicles were 5-6 fold lower than those in MV follicles, and, *lhcg* transcripts were 3-fold higher in L/PV follicles, similar to relative levels in coho salmon ovarian follicles (Guzmán et al., 2014). These difference are in accord with the accepted roles of Fsh in vitellogenesis and Lh in maturation/ovulation (Levavi-Sivan et al., 2010; Lubzens et al, 2010, 2016.) and partially explain the stage-specific differences in response to the treatments in this study, discussed below.

4.3. Effects of in vitro treatment on expression of steroidogenic proteins and gonadotropin receptors

4.3.1. Stage-specific effects of Fsh and Lh

4.3.1.1 Steroidogenic proteins

Star is a protein required for entry of cholesterol into mitochondria, a key rate-limiting step in steroidogenesis. In salmonids, ovarian *star* transcripts progressively increase with follicle development, with the greatest levels occurring during final maturation and ovulation (Kusakabe et al. 2002; Bobe et al., 2004; Nakamura et al., 2005; Guzmán et al., 2014). Fsh

and Lh upregulated *star* transcripts in MV follicles 2-3 fold but only Lh increased transcripts in L/PV follicles. Stimulatory effects of Fsh on *star* transcripts in cortical alveolus stage coho salmon follicles (Luckenbach et al., 2011), and of human chorionic gonadotropin (hCG) on transcript levels in vitellogenic follicles of zebrafish (Ings and Van Der Kraak, 2006) have been reported. Because the Fsh receptor can also bind Lh, but with lower affinity (Levavi-Shivan et al., 2010), the effects of Lh on *star* transcripts in MV follicles could be due to both receptors binding Lh. At a later stage, Lh also induced an approximately 2.5 fold increase in *star* transcripts in preovulatory follicles of brown trout, an action mediated in vitro by tumor necrosis α (Tnfa) (Crespo et al., 2012).

The product of the *cyp11a1* gene encodes P450 cholesterol side-chain cleavage enzyme, required for production of pregnenolone, the first steroid in the biosynthetic pathway. *Cyp11a1* transcript levels increase during vitellogenesis in catfish (Kumar et al., 2000), Arctic char (van Hofsten et al., 2002), rainbow trout (Nakamura et al., 2005), and coho salmon (Guzmán et al., 2014) to peak levels in the postvitellogenic follicle, similar to the pattern seen in control follicles in this study. This study found no evidence that Fsh or Lh acutely regulated *cyp11a1* transcription at either follicle stage. Upregulation of *cyp11a1* transcripts by heterologous gonadotropin preparations in vivo has been reported for Japanese eel ovarian follicles (Kazeto et al., 2006; Ijiri et al., 2006) but hCG had no effect on *cyp11a1* mRNA levels in zebrafish vitellogenic follicles (Ings and Van Der Kraak, 2006). The only significant difference in *cyp11a1* mRNA levels after in vitro treatment of cortical alveolus stage follicles of coho salmon was after 72 h of exposure. (Luckenbach et al., 2011). However, Lh, mediated through Tnfa, increased *cyp11a1* transcripts in preovulatory follicles of brown trout in vitro (Crespo et al., 2012).

The *hsd3b* gene encodes 3 β -hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase. This enzyme catalyzes the transformation of Δ^{5-3} - β -hydroxysteroids to the Δ^{4-3} -keto configuration and is essential for the synthesis of all bioactive steroids. As also seen in this study, progressive increases in *hsd3b* transcripts occur throughout follicle development of Pacific salmonids to a peak at the prematurational stage (Nakamura et al., 2005; Guzmán et al., 2014). Fsh

increased *hsd3b* transcripts in MV follicles, but despite a trend, Lh was without significant effect at this stage. Neither gonadotropin affected *hsd3b* transcripts in L/PV follicles. The lack of response of L/PV follicles may be due to ovarian *hsd3b* expression being maximal at this time in rainbow trout (Nakamura et al. 2005). Both mRNA and protein increased after incubation of early vitellogenic rainbow trout follicles with a partially purified gonadotropin preparation (Young et al., 2000, 2005). Similarly, Fsh increased *hsd3b* transcripts in cortical alveolus stage ovarian follicles of coho salmon in vitro (Luckenbach et al., 2011).

The product of the *cyp19a1a* gene, P450 aromatase, is required for conversion of androgens into estrogens. Highest expression occurs in vitellogenic follicles (e.g., Kumar et al., 2000; Gen et al., 2001; Ijiri et al., 2003; Ings and Van Der Kraak, 2006; Rocha et al., 2009; Garcia-Lopez et al., 2011), with a clear decline in postvitellogenic follicles. A similar pattern occurs in rainbow trout (Nakamura et al., 2005) and coho salmon (Guzmán et al., 2014), with highest expression occurring at a time of peak plasma E2 levels; this was observed in the present study.

The sensitivity of the rainbow trout *cyp19a1a* gene to stimulation by gonadotropins varied with stage and with hormone in the present study. Fsh but not Lh treatment significantly increased *cyp19a1a* transcripts in MV follicles. Similarly, Fsh increased *cyp19a1a* transcripts in early vitellogenic brown trout follicles in vitro (Montserrat et al., 2004). Previous studies on medaka (Nagahama et al., 1991), goldfish (Tan et al., 1986), tilapia (Yoshiura et al., 2003) and zebrafish (Ings and Van Der Kraak, 2006) vitellogenic follicles have reported either stimulation of aromatase activity or increased *cyp19a1a* transcript levels after in vitro stimulation with hCG or PMSG. However, studies with heterologous gonadotropins do not allow firm conclusions to be drawn about which of the endogenous gonadotropins control transcription.

Conversely, in L/PV follicles, Fsh had no effect on *cyp19a1a* transcripts but Lh caused a significant 60% reduction. This observation gives further support to the hypothesis that in

salmonids, the increase in plasma Lh (and *lhcg*r expression) that occurs in the postvitellogenic stages is partially responsible for the reduction in aromatase activity and E2 production that occurs during the switch in the steroidogenic pathway from E2 to 17,20 β -DHP production in salmonids (Maestro et al., 1997; Planas et al., 2000; Young et al., 2005; Lubzens et al. 2010).. *Cyp19a1a* transcripts were reduced in preovulatory follicles of brown trout that underwent maturation in response to Lh (Crespo et al., 2012). *Tnfa* was implicated as a major mediator of Lh's actions on transcript levels for several steroidogenic proteins in the preovulatory follicle. The results of this study extend these findings because they show that Lh can downregulate *cyp19a1a* transcripts in rainbow trout well before oocytes are able to undergo maturation in response to Lh, when *cyp19a1a* transcript levels and aromatase activity are still relatively high compared to those in preovulatory follicles (Nakamura et al., 2005).

4.3.1.2 Gonadotropin receptors

Relative changes in gonadotropin receptor abundance have been documented for several teleost species (Kim et al., 2012; Kobayashi et al., 2008; Andersson et al., 2009; Rocha et al., 2009; Garcia-Lopez et al., 2011; Nyuji et al., 2013; Guzmán et al., 2014). No evidence was found in this study for an upregulation of receptor mRNA levels. Lh and Fsh strongly reduced *fshr* mRNA levels in L/PV follicles and Lh reduced *lhcg*r transcript levels in L/PV follicles. Published studies show a range of effects of gonadotropins on receptor levels. In cortical alveolus stage follicles of coho salmon, Fsh induced a transient reduction in *fshr* transcripts, while 72 h of exposure was required to stimulate an increase in *lhcg*r transcripts (Luckenbach et al. 2011). Lh increased *lhcg*r mRNA levels in explants of immature clownfish ovary (Kim et al., 2012), and also in preovulatory follicles of brown trout (Crespo et al., 2012). Whether some of these effects are direct, or partly or wholly mediated through gonadotropin-induced increased levels of steroids or other factors in incubation media, or through intrafollicular paracrine signaling, is unclear, since E2 upregulated *fshr* and *lhcg*r mRNA levels in black porgy (An et al., 2009) and zebrafish (Liu et al., 2011), and epidermal growth factor abolished E2-induced upregulation of *lhcg*r transcripts in zebrafish follicles (Liu and Ge, 2013).

4.3.2 Effects of IGF1

IGF1 had no effects on steroid production in vitro, but both stimulatory and inhibitory effects of this growth factor on steroidogenic protein transcripts in rainbow trout follicles were identified. The significant reductions in mRNA levels (*cyp11a1*, MV; *hsd3b*, both stages) or trends towards lower levels (*star*, both stages) may partially explain the inhibitory effect of IGF1 on Lh-stimulated steroid production by thecal layers from preovulatory coho salmon (Maestro et al., 1997) and brown trout follicles (Mendez et al., 2005), an action that is mediated in the latter through the mitogen-activated protein kinase (MAPK) signaling pathway. These results diverge from the strong Igf3-induced upregulation of *cyp11a1* transcripts in tilapia ovarian follicles (Li et al., 2012).

Contrary to these inhibitory effects, IGF1 caused a 3.5 fold increase in *cyp19a1a* transcript levels in MV stages, substantially higher than the 2-fold increase seen with Fsh. IGF1 also increased *cyp19a1a* transcripts levels in vitellogenic red seabream follicles (Kagawa et al., 2003), and in tilapia follicles (stage not defined, 180 days after hatching; Li et al., 2012). Since forskolin had no effect at either stage, Fsh's actions on *cyp19a1a* transcript levels could therefore be partly mediated by follicular Igfs, especially as activation of cAMP/PKA signaling suppressed *cyp19a1a* transcript levels. The stimulation by IGF1 of E2 production by granulosa cell layers of preovulatory coho salmon follicles (Maestro et al., 1997) may be linked to increased *cyp19a1a* expression.

4.3.3. Effects of activation of cAMP/protein kinase A signaling

Similar to Fsh and Lh, forskolin increased *star* transcripts in MV follicles by 2-3 fold over controls, but unlike Lh, forskolin had no effect on *star* transcripts in L/PV follicles. As far as we are aware, the result with forskolin is the first direct demonstration of cAMP/PKA signaling regulating transcription of the ovarian follicle *star* gene in teleosts. Based on the differences in relative abundance of *fshr* and *lhcr* transcript levels between stages, the effects of Lh and Fsh on *star* transcripts in MV follicles might be predominantly mediated via the Fshr-cAMP/protein kinase A pathway. However, the lack of effect on *star* transcripts

in L/PV follicles suggests that the stimulatory effects of Lh at this stage were not predominantly mediated through the cAMP/PKA pathway. Other intracellular signaling pathways (e.g., PKC/Ca²⁺, MAPK) pathways or other factors such as Tnfa (Planas et al., 1997; Mendez et al., 2005; Crespo et al., 2012), may participate in the regulation of *star* gene expression at this time.

Forskolin strongly increased *hsd3b* mRNA levels in MV follicles, but was without effect on L/PV follicles. The lack of effect of forskolin on *hsd3b* mRNA levels in late vitellogenic follicles suggests that maintenance of high levels of expression of *hsd3b* in postvitellogenic stages may not depend cAMP/PKA mediation.

Forskolin reduced *cyp19a1a* transcript levels by approximately 50% in follicles at both stages examined. These observations indicate that the increase in *cyp19a1a* transcripts in MV follicles exposed to Fsh is not due to mediation through the cAMP/PKA pathway. Testosterone and E2 levels in media after exposure of follicles to forskolin, Fsh and Lh in this study were very similar, so the potential inhibition of *cyp19a1a* expression by forskolin cannot be due to increased steroid levels. These finding contrast with the increase in aromatase activity that occurred after incubation of vitellogenic follicles of goldfish (Tan et al., 1986) and medaka (Nagahama et al., 1991) with forskolin. In trout, Fsh-induced stimulation of *cyp19a1a* expression may be partially mediated through local Igf (1, 2 or 3) production by the ovarian follicle.

Forskolin significantly reduced *fshr* transcripts in MV follicles and *lhcg* mRNA levels in L/PV follicles, suggesting that gonadotropin-induced reduction in receptor transcript levels discussed above could be mediated through cAMP/PKA signaling. This reduction may not necessarily be solely due to a direct affect on transcription since high concentrations of cAMP analogs (and hCG) reduced mammalian follicle *lhcg* transcripts by reducing *lhcg* mRNA half-life and not through reduced transcription (Menon et al., 2004; Zeleznick et al., 2004). The increased steroid levels in incubation media could mediate some of the effects of gonadotropins and forskolin on *fshr* and *lhcg* transcripts in rainbow trout. However, the

reduction of *fshr* mRNA levels after exposure of L/PV follicles to IGF1 cannot be attributed to steroids, since IGF1 had no effect on steroid production at any stage.

4.4 Summary and Conclusions

This study (summarized qualitatively in Table 1) demonstrates that steroidogenesis during vitellogenesis and the stage-specific changes in the steroidogenic pathway of salmonids at the termination of vitellogenesis depends on specific, stage-dependent stimulatory and inhibitory actions of Fsh and Lh on *star*, *hsd3b* and *cyp19a1a* mRNA levels. The study also further implicates Igfs in regulating follicle steroidogenesis. The strong upregulation of MV follicle *cyp19a1a* transcripts by IGF1 provides further evidence for multiple roles of Igfs in follicle development and maturation but also raises the possibility that Fsh's actions on *cyp19a1a* transcript levels could be partly mediated by follicular Igfs. A key finding was the Lh-induced reduction of *cyp19a1a* transcript levels in L/PV follicles, which further supports the idea that increased LH signaling in the postvitellogenic follicle reduces the ability of the follicle to produce E2 (Crespo et al., 2012). The main finding on regulation of gonadotropin receptor transcripts was the reduction of transcripts by gonadotropins, which may be mediated through cAMP/protein kinase A. The stage-dependent reduction of transcript levels of some genes in response to forskolin and IGF1 could be due both to direct or indirect actions (via steroids) leading to transcriptional downregulation, and/or effects on mRNA half-life or other processes.

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Table 1

Summary of effects of treatments on mRNA levels in midvitellogenic (*MV*) and late/postvitellogenic follicles (*L/PV*) of rainbow trout

Gene	Treatment							
	Fsh		Lh		IGF1		Forskolin	
	Stage							
	MV	L/PV	MV	L/PV	MV	L/PV	MV	L/PV
<i>fshr</i>	0	-	0	-	0	0	-	0
<i>lhcgr</i>	0	0	0	-	0	0	0	-
<i>star</i>	+	0	+	+	0	0	+	0
<i>cyp11a1</i>	0	0	0	0	-	0	-	0
<i>hsd3b</i>	+	0	0	0	-	-	+	0
<i>cyp19a1a</i>	+	0	0	-	+	0	-	-

0, no effect

+, significantly higher than control value ($P < 0.05$)

-, significantly lower than control value ($P < 0.05$)

Figure Legends

Figure 1 Estradiol-17 β (E2) and testosterone (T) levels in incubation media after in vitro treatment of midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of rainbow trout. Follicles were incubated in the presence or absence (C) of Fsh, Lh, IGF1 (all at 100 ng/ml) or forskolin (Forsk; 10 μ M) in trout Ringer's solution at 12°C for 18 h. Levels of steroids were measured by radioimmunoassay. Each bar represents mean $\pm$ SEM of three independent replicates. Asterisks above bars indicate statistically significant differences from control values: *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 2 Comparison of transcript levels encoding gonadotropin receptors and steroidogenic proteins in midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of rainbow trout. Follicles were incubated for 18 h in trout Ringer's solution at 12°C for 18 h. RNA was extracted and subject to quantitative PCR. Data are normalized to levels of the reference gene (acidic ribosomal phosphoprotein P0). Each bar represents mean $\pm$ SEM of three independent replicates. Asterisks above L/PV bar indicates significant difference from MV value: *, P<0.05; **, P<0.01; ***, P<0.001

Figure 3 Effect of Fsh, Lh, IGF1 or forskolin on levels of steroidogenic protein transcripts in midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of rainbow trout. Follicles were incubated in the presence or absence (C) of Fsh, Lh, IGF1 (all at 100 ng/ml) or forskolin (Forsk; 10 μ M) in trout Ringer's solution at 12°C for 18 h in trout Ringer's solution. RNA was extracted and subject to quantitative PCR. Data were normalized to levels of the reference gene (acidic ribosomal phosphoprotein P0) and are presented as fold-change compared to control levels (control =1). Each bar represents mean $\pm$ SEM of three independent replicates. Asterisks above bars indicate statistically significant differences from control values: *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 4 Effect of Fsh, Lh, IGF1 or forskolin on levels of gonadotropin receptor transcripts in midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian follicles of

767 rainbow trout. Follicles were incubated in the presence or absence (C) of Fsh, Lh, IGF1 (all
768 at 100 ng/ml) or forskolin (Forsk; 10 μ M) in trout Ringer's solution at 12°C for 18 h in
769 trout Ringer's solution. RNA was extracted and subject to quantitative PCR. Data were
770 normalized to levels of the reference gene (acidic ribosomal phosphoprotein P0) and are
771 presented as fold-change compared to control levels (control =1). Each bar represents mean
772 $\pm$ SEM of three independent replicates. Asterisks above bars indicate statistically significant
773 differences from control values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4