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-17β (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), 3β-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 *lhcgr* transcripts (L/PV). IGF1 had no effect on gonadotropin receptor transcripts. Forskolin reduced MV follicle *fshr* transcript levels and reduced *lhcgr* 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-17β (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, 17,20β-dihydroxy-4-pregnen-3-one [17,20β-DHP] or 17,20β,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 20β-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 *lhcgr* 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 progestin 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).

144
145 *2.2. Reagents*
146 Coho salmon Fsh and Lh were purified as described previously (Swanson et al., 1991).
147 Forskolin was obtained from Sigma-Aldrich. Recombinant human IGF1 was purchased
148 from GroPeP Pty Ltd (Adelaide, SA, Australia).
149
150 *2.3. In vitro culture of ovarian follicles*
151 Follicles from each of three females at each stage were isolated from ovarian fragments
152 held in ice-cold trout Ringer's solution. Diameters of 20 intact follicles were measured
153 under a dissecting microscope. From the ovary of each female, MV follicles (10
154 follicles/well/1 ml Ringer's) or L/PV follicles (10 follicles/well/2 ml Ringer's) were
155 incubated in the presence or absence of Fsh, Lh, human IGF1 (all at 100 ng/ml) or forskolin
156 (10 μM) in trout Ringer's solution at 12°C for 18 hours with gentle shaking (100 rpm); thus,
157 there were three independent replicates per treatment. Concentrations used were based on
158 those used on previous studies (Maestro et al., 2007; Planas et al., 1997, 2000; Luckenbach
159 et al., 2011) After the incubation, tissue samples were frozen using liquid nitrogen and
160 stored -80°C until RNA extraction. Samples of incubation media were removed and stored
161 at -20°C.
162
163 *2.4. Expression of genes encoding steroidogenic proteins and gonadotropin receptors*
164 Total RNA was isolated from the ovarian follicles using Trizol reagent as described by the
165 manufacturer (Invitrogen, Carlsbad, CA). Total RNA concentrations in extracts were
166 determined using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE). Each
167 RNA sample (200 ng) was incubated with 0.2 units of DNase I Amp Grade (Invitrogen) at
168 room temperature for 15 min to eliminate genomic DNA contamination. Subsequently,
169 DNase I was inactivated by heat denaturation at 65˚C for 10 min. Single strand cDNAs
170 were then synthesized using High-Capacity cDNA archive kit (Applied Biosystems, Foster
171 City, CA). Real-time quantitative PCR with TaqMan probes (Applied Biosystems) was
172 used to quantify mRNA using the ABI PRISM 7300 sequence detection system (Applied
173 Biosystems). Primer pairs and probes used for the detection of transcript for steroidogenic

acute regulatory protein (*star*), P450 cholesterol side-chain cleavage enzyme (*cyp11a1*), 3β-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 µl PCR mixture made from 12.5 µl of ABI Universal PCR Master Mix, 0.9 µM forward primer, 0.9 µM reverse primer, 0.2 µM fluorescent-labeled probe and 3.0 µl 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 ± 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-17β 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 *lhcgr*), 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 *lhcgr* mRNA levels were lower in MV follicles after all treatments but values did not vary significantly from control levels. This trend towards lower *lhcgr* 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 *lhcgr* transcript abundance.

4. Discussion

This in vitro study identified a suite of genes in ovarian follicles of rainbow tout 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 *lhcgr* expression by their corresponding ligand.

294

295 *4.1. Follicle stages and steroid production*

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

303

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

313

314 As in goldfish vitellogenic follicles (Srivastava and Van Der Kraak, 1994), IGF1 did not
315 affect steroid production at either stage. IGF1 has been reported to increase E2 and MIS
316 production in vitro by preovulatory ovarian follicles of several species, including striped
317 bass (Weber and Sullivan, 2000) and grey mullet (Das et al., 2014). In coho salmon
318 preovulatory follicles, IGF1 inhibited basal and Lh-stimulated testosterone and
319 17-hydroxyprogesterone production by isolated theca layers but stimulated both E2 and
320 17,20β-DHP production by isolated granulosa layers (Maestro et al., 1997). How these
321 divergent effects of Igf1 on coho salmon preovulatory follicles might be mediated is
322 discussed below.

323

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 *lhcgr* 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, *lhcgr* 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 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 of *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 3β-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

384 increased *hsd3b* transcripts in MV follicles, but despite a trend, Lh was without significant
385 effect at this stage. Neither gonadotropin affected *hsd3b* transcripts in L/PV follicles. The
386 lack of response of L/PV follicles may be due to ovarian *hsd3b* expression being maximal
387 at this time in rainbow trout (Nakamura et al. 2005). Both mRNA and protein increased
388 after incubation of early vitellogenic rainbow trout follicles with a partially purified
389 gonadotropin preparation (Young et al., 2000, 2005). Similarly, Fsh increased *hsd3b*
390 transcripts in cortical alveolus stage ovarian follicles of coho salmon in vitro (Luckenbach
391 et al., 2011).
392
393 The product of the *cyp19a1a* gene, P450 aromatase, is required for conversion of androgens
394 into estrogens. Highest expression occurs in vitellogenic follicles (e.g., Kumar et al., 2000;
395 Gen et al., 2001; Ijiri et al., 2003; Ings and Van Der Kraak, 2006; Rocha et al., 2009;
396 Garcia-Lopez et al., 2011), with a clear decline in postvitellogenic follicles. A similar
397 pattern occurs in rainbow trout (Nakamura et al., 2005) and coho salmon (Guzmán et al.,
398 2014), with highest expression occurring at a time of peak plasma E2 levels; this was
399 observed in the present study.
400
401 The sensitivity of the rainbow trout *cyp19a1a* gene to stimulation by gonadotropins varied
402 with stage and with hormone in the present study. Fsh but not Lh treatment significantly
403 increased *cyp19a1a* transcripts in MV follicles. Similarly, Fsh increased *cyp19a1a*
404 transcripts in early vitellogenic brown trout follicles in vitro (Montserrat et al., 2004).
405 Previous studies on medaka (Nagahama et al., 1991), goldfish (Tan et al., 1986), tilapia
406 (Yoshiura et al., 2003) and zebrafish (Ings and Van Der Kraak, 2006) vitellogenic follicles
407 have reported either stimulation of aromatase activity or increased *cyp19a1a* transcript
408 levels after in vitro stimulation with hCG or PMSG. However, studies with heterologous
409 gonadotropins do not allow firm conclusions to be drawn about which of the endogenous
410 gonadotropins control transcription.
411
412 Conversely, in L/PV follicles, Fsh had no effect on *cyp19a1a* transcripts but Lh caused a
413 significant 60% reduction. This observation gives further support to the hypothesis that in

414 salmonids, the increase in plasma Lh (and *lhcgr* expression) that occurs in the
415 postvitellogenic stages is partially responsible for the reduction in aromatase activity and
416 E2 production that occurs during the switch in the steroidogenic pathway from E2 to
417 17,20β-DHP production in salmonids (Maestro et al., 1997; Planas et al., 2000; Young et al.,
418 2005; Lubzens et al. 2010).. *Cyp19a1a* transcripts were reduced in preovulatory follicles of
419 brown trout that underwent maturation in response to Lh (Crespo et al., 2012). Tnfa was
420 implicated as a major mediator of Lh's actions on transcript levels for several steroidogenic
421 proteins in the preovulatory follicle. The results of this study extend these findings because
422 they show that Lh can downregulate *cyp19a1a* transcripts in rainbow trout well before
423 oocytes are able to undergo maturation in response to Lh, when *cyp19a1a* transcript levels
424 and aromatase activity are still relatively high compared to those in preovulatory follicles
425 (Nakamura et al., 2005).
426
427 *4.3.1.2 Gonadotropin receptors*
428 Relative changes in gonadotropin receptor abundance have been documented for several
429 teleost species (Kim et al., 2012; Kobayashi et al., 2008; Andersson et al., 2009; Rocha et
430 al., 2009; Garcia-Lopez et al., 2011; Nyuji et al., 2013; Guzmán et al., 2014). No evidence
431 was found in this study for an upregulation of receptor mRNA levels. Lh and Fsh strongly
432 reduced *fshr* mRNA levels in L/PV follicles and Lh reduced *lhcgr* transcript levels in L/PV
433 follicles. Published studies show a range of effects of gonadotropins on receptor levels. In
434 cortical alveolus stage follicles of coho salmon, Fsh induced a transient reduction in *fshr*
435 transcripts, while 72 h of exposure was required to stimulate an increase in *lhcgr* transcripts
436 (Luckenbach et al. 2011). Lh increased *lhcgr* mRNA levels in explants of immature
437 clownfish ovary (Kim et al., 2012), and also in preovulatory follicles of brown trout
438 (Crespo et al., 2012). Whether some of these effects are direct, or partly or wholly mediated
439 through gonadotropin-induced increased levels of steroids or other factors in incubation
440 media, or through intrafollicular paracrine signaling, is unclear, since E2 upregulated *fshr*
441 and *lhcgr* mRNA levels in black porgy (An et al., 2009) and zebrafish (Liu et al., 2011), and
442 epidermal growth factor abolished E2-induced upregulation of *lhcgr* transcripts in zebrafish
443 follicles (Liu and Ge, 2013).

444

445 *4.3.2 Effects of IGF1*

446 IGF1 had no effects on steroid production in vitro, but both stimulatory and inhibitory
447 effects of this growth factor on steroidogenic protein transcripts in rainbow trout follicles
448 were identified. The significant reductions in mRNA levels (*cyp11a1*, MV; *hsd3b*, both
449 stages) or trends towards lower levels (*star*, both stages) may partially explain the
450 inhibitory effect of IGF1 on Lh-stimulated steroid production by thecal layers from
451 preovulatory coho salmon (Maestro et al., 1997) and brown trout follicles (Mendez et al.,
452 2005), an action that is mediated in the latter through the mitogen-activated protein kinase
453 (MAPK) signaling pathway. These results diverge from the strong Igf3-induced
454 upregulation of *cyp11a1* transcripts in tilapia ovarian follicles (Li et al., 2012).

455

456 Contrary to these inhibitory effects, IGF1 caused a 3.5 fold increase in *cyp19a1a* transcript
457 levels in MV stages, substantially higher than the 2-fold increase seen with Fsh. IGF1 also
458 increased *cyp19a1a* transcripts levels in vitellogenic red seabream follicles (Kagawa et al.,
459 2003), and in tilapia follicles (stage not defined, 180 days after hatching; Li et al., 2012).
460 Since forskolin had no effect at either stage, Fsh's actions on *cyp19a1a* transcript levels
461 could therefore be partly mediated by follicular Igfs, especially as activation of cAMP/PKA
462 signaling suppressed *cyp19a1a* transcript levels. The stimulation by IGF1 of E2 production
463 by granulosa cell layers of preovulatory coho salmon follicles (Maestro et al., 1997) may be
464 linked to increased *cyp19a1a* expression.

465

466 *4.3.3. Effects of activation of cAMP/protein kinase A signaling*

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

474 in L/PV follicles suggests that the stimulatory effects of Lh at this stage were not
475 predominantly mediated through the cAMP/PKA pathway. Other intracellular signaling
476 pathways (e.g., PKC/Ca^{2+}, MAPK) pathways or other factors such as Tnfa (Planas et al.,
477 1997; Mendez et al., 2005; Crespo et al., 2012), may participate in the regulation of *star*
478 gene expression at this time.
479
480 Forskolin strongly increased *hsd3b* mRNA levels in MV follicles, but was without effect on
481 L/PV follicles. The lack of effect of forskolin on *hsd3b* mRNA levels in late vitellogenic
482 follicles suggests that maintenance of high levels of expression of *hsd3b* in postvitellogenic
483 stages may not depend cAMP/PKA mediation.
484
485 Forskolin reduced *cyp19a1a* transcript levels by approximately 50% in follicles at both
486 stages examined. These observations indicate that the increase in *cyp19a1a* transcripts in
487 MV follicles exposed to Fsh is not due to mediation through the cAMP/PKA pathway.
488 Testosterone and E2 levels in media after exposure of follicles to forskolin, Fsh and Lh in
489 this study were very similar, so the potential inhibition of *cyp19a1a* expression by forskolin
490 cannot be due to increased steroid levels. These finding contrast with the increase in
491 aromatase activity that occurred after incubation of vitellogenic follicles of goldfish (Tan et
492 al., 1986) and medaka (Nagahama et al., 1991) with forskolin. In trout, Fsh-induced
493 stimulation of *cyp19a1a* expression may be partially mediated through local Igf (1, 2 or 3)
494 production by the ovarian follicle.
495
496 Forskolin significantly reduced *fshr* transcripts in MV follicles and *lhcgr* mRNA levels in
497 L/PV follicles, suggesting that gonadotropin-induced reduction in receptor transcript levels
498 discussed above could be mediated through cAMP/PKA signaling. This reduction may not
499 necessarily be solely due to a direct affect on transcription since high concentrations of
500 cAMP analogs (and hCG) reduced mammalian follicle *lhcgr* transcripts by reducing *lhcgr*
501 mRNA half-life and not through reduced transcription (Menon et al., 2004; Zeleznick et al.,
502 2004). The increased steroid levels in incubation media could mediate some of the effects
503 of gonadotropins and forskolin on *fshr* and *lhcgr* 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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References

An, K.W., Lee, K.Y., Yun, S.G., Choi, C.Y., 2009. Molecular characterization of gonadotropin subunits and gonadotropin receptors in black porgy, *Acanthopagrus schlegeli*: effects of estradiol-17β on mRNA expression profiles. Comp. Biochem. Physiol. B 15, 177–188.

Andersson, E., Nijenhuis, W., Male, R., Swanson, P., Bogerd, J., Taranger, G.L., Schulz, R.W., 2009. Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. Gen. Comp. Endocrinol. 163, 329-39.

Bobe, J., Nguyen, T., Jalabert, B., 2004. Targeted gene expression profiling in the rainbow trout (*Oncorhynchus mykiss*) ovary during maturational competence acquisition and oocyte maturation. Biol. Reprod. 71, 73-82.

Crespo, D., Evaristo L. Mañanós, E.L., Roher, N., MacKenzie, S.A., Planas, J.V., 2012. Tumor necrosis factor alpha may act as an intraovarian mediator of luteinizing hormone-induced oocyte maturation in trout. Biol. Reprod. 86, 1-12.

Das, P., Pramanick, K., Mukherjee, D., Maiti, B.R., 2014. In vitro induction of oocyte maturation and steroidogenesis by gonadotropins, insulin, calcitonin and growth factor in man estuarine flat head grey mullet, *Mugil cephalus* L. Fish Physiol. Biochem. 40, 105-116.

Erickson, G.F., Garzo, V.G., Magoffin, D.A., 1989. Insulin-like growth factor-I regulates aromatase activity in human granulosa and granulosa luteal cells. J. Clin. Endocrinol. Metab. 69, 716-24.

García-López, A., Sánchez-Amaya, M.I., Prat, F., 2011. Targeted gene expression profiling in European sea bass (*Dicentrarchus labrax* L.) follicles from primary growth to late vitellogenesis. Comp. Biochem. Physiol A160, 374-380.

Gen, K., Okuzawa, K., Kumakura, N., Yamaguchi, S., Kagawa, H., 2001. Correlation between messenger RNA expression of cytochrome P450 aromatase and its enzyme activity during oocyte development in the red seabream (*Pagrus major*). Biol. Reprod. 65, 1186-1194.

Guzmán, J.M., Luckenbach, J.A., Yamamoto, Y., Swanson, P. 2014. Expression profiles of

560 Fsh- regulated ovarian genes during oogenesis in coho salmon. PLoS ONE 9, e114176.
561 Ijiri, S., Kazeto, Y., Lokman, P.M., Adachi, S., Yamauchi, K., 2003. Characterization of a
562 cDNA Encoding P-450 aromatase (CYP19) from Japanese eel ovary and its expression
563 in ovarian follicles during induced ovarian development. Gen. Comp. Endocrinol. 130,
564 193-203.
565 Ijiri, S., Takei, N., Kazeto, Y., Todo, T., Adachi, S., Yamauchi, K., 2006. Changes in
566 localization of cytochrome P450 cholesterol side-chain cleavage (P450scc) in Japanese
567 eel testis and ovary during gonadal development. Gen. Comp. Endocrinol. 145, 75-83.
568 Ings, J.S., Van Der Kraak, G.J., 2006. Characterization of the mRNA expression of StAR
569 and steroidogenic enzymes in zebrafish ovarian follicles. Mol. Reprod. Devel. 73,
570 943-54.
571 Kagawa, H., Takano, K., Nagahama, Y., 1981. Correlation of plasma estradiol-17β and
572 progesterone levels with ultrastructure and histochemistry of ovarian follicles in the
573 white-spotted char, *Salvelinus leucomaenis*. Cell Tiss. Res. 218, 315-329.
574 Kagawa, H., Young, G., Nagahama, Y., 1983. Relationship between seasonal plasma
575 estradiol-17β and testosterone levels and in vitro production by ovarian follicles of
576 amago salmon (*Oncorhynchus rhodurus*). Biol. Reprod. 29, 301-309.
577 Kagawa, H., Gen, K., Okuzawa, K., Tanaka, H., 2003. Effects of luteinizing hormone and
578 follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and
579 P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*.
580 Biol. Reprod. 68, 1562-158.
581 Kagawa, H., 2013. Oogenesis in teleost fish. Aqua-BioScience Monographs, Vol. 6, No. 4,
582 pp. 99–127.
583 Kanamori, A., Adachi, S., Nagahama, Y., 1988. Developmental changes in steroidogenic
584 responses of ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) to chum
585 salmon gonadotropin during oogenesis. Gen. Comp. Endocrinol. 72, 13-24.
586 Kazeto, Y., Adachi, S., Yamauchi, K., 2006. Cloning and characterization of a cDNA
587 encoding cholesterol side-chain cleavage cytochrome P450 (CYP11A1):
588 tissue-distribution and changes in the transcript abundance in ovarian tissue of Japanese
589 eel, *Anguilla japonica*, during artificially induced sexual development. J. Steroid

590 Biochem. Mol. Biol. 99, 121-128.
591 Kim, N.N., Habibi, H.R., Lee, J., Choi, C.Y., 2012. Effects of recombinant gonadotropin
592 hormones on the expression of vitellogenin, gonadotropin subunits and gonadotropin
593 receptors in cinnamon clownfish, *Amphiprion melanopus.* Comp. Biochem. Physiol. B
594 162, 73-80.
595 Kobayashi, T., Pakarinen, P., Torgersen, J., Huhtaniemi, I, Andersen, O., 2008. The
596 gonadotropin receptors FSH-R and LH-R of Atlantic halibut (*Hippoglossus*
597 *hippoglossus*)-2. Differential follicle expression and asynchronous oogenesis. Gen.
598 Comp. Endocrinol. 156, 595-602.
599 Kumar, R.S., Ijiri, S., Trant, J.M., 2000. Changes in the expression of genes encoding
600 steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a
601 reproductive cycle. Biol. Reprod. 63, 1676-1682.
602 Kusakabe, M., Todo, T., McQuillan, H.J., Goetz, F.W., Young, G., 2002. Characterization
603 and expression of steroidogenic acute regulatory protein (StAR) and MLN64 cDNAs in
604 trout. Endocrinol. 143, 2062-2070.
605 Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young G., 2006. Changes in mRNAs
606 encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors
607 for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189,
608 541-554.
609 Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives
610 on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412-437.
611 Li, M., Wu, F., Gu, Y., Wang, T., Wang, H., Yang, S., Sun, Y., Zhou, L., Huang, X., Jiao, B.,
612 Cheng, C.H., Wang, D. 2012. Insulin-like growth factor 3 regulates expression of genes
613 encoding steroidogenic enzymes and key transcription factors in the Nile tilapia gonad.
614 Biol. Reprod. 86, 1-10.
615 Liu, K.C., Lin, S.W., Ge, W., 2011. Differential regulation of gonadotropin receptors (fshr
616 and lhcgr) by estradiol in the zebrafish ovary involves nuclear estrogen receptors that are
617 likely located on the plasma membrane. Endocrinol. 152, 4418-4430.
618 Liu, K.C., Ge, W., 2013. Differential regulation of gonadotropin receptors (fshr and lhcgr)
619 by epidermal growth factor (EGF) in the zebrafish ovary. Gen. Comp. Endocrinol. 18,

288–294.
Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165, 367-389.
Lubzens, E., Bobe, J., Young, G., Sullivan, C.V., 2016. Maternal investment in oocytes and eggs: the molecular cargo and its contributions to fertility and early development. Aquaculture (in press).
Luckenbach, J.A., Dickey, J.T., Swanson, P., 2011. Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors in vitro during early secondary oocyte growth in coho salmon. Gen. Comp. Endocrinol. 171, 52-63.
Maestro, M.A., Planas, J.V., Moriyama, S., Gutiérrez, J., Planas, J., Swanson, P., 1997. Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. Gen Comp Endocrinol. 106, 189-201.
Méndez, E., Montserrat. N., Planas, J.V., 2005. Modulation of the steroidogenic activity of luteinizing hormone by insulin and insulin-like growth factor-I through interaction with the cAMP-dependent protein kinase signaling pathway in the trout ovary. Mol. Cell. Endocrinol. 229, 49-56.
Menon, K.M., Munshi, U.M., Clouser, C.L., Nair, A.K., 2004. Regulation of luteinizing hormone/human chorionic gonadotropin receptor expression: a perspective. Biol. Reprod. 70, 861-866.
Montserrat, N., González, A., Méndez, E., Piferrer, F., Planas, J.V., 2004. Effects of follicle stimulating hormone on estradiol-17β production and P-450 aromatase (CYP19) activity and mRNA expression in brown trout vitellogenic ovarian follicles *in vitro*. Gen. Comp. Endocrinol. 137, 123–131.
Nagahama, Y., Young, G., Adachi, S., 1985. Effect of actinomycin D and cycloheximide on gonadotropin-induced 17α,20β-dihydroxy-4-pregnen-3-one production by intact follicles and granulosa cells of the amago salmon, *Oncorhynchus rhodurus*. Dev. Growth Differ. 27, 213–221.
Nagahama, Y., Matsuhisa, A., Iwamatsu, T., Saki, N., Fukada, S. 1991. A mechanism for the

650 action of pregnant mare serum gonadotropin on aromatase activity in the ovarian
651 follicles of the medaka, *Oryzias latipes*. J. Exp. Zool. 259, 53-58.
652 Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Dev. Growth
653 Diff. 50, S195-S219.
654 Nakamura, I., Evans, J.C., Kusakabe, M., Nagahama, Y., Young, G., 2005. Changes in
655 steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in
656 ovarian follicles during ovarian development of rainbow trout (*Oncorhynchus mykiss*).
657 Gen. Comp. Endocrinol. 144, 224-231.
658 Nakamura, I., Kusakabe, M., Young, G., 2009. Differential suppressive effects of low
659 physiological doses of estradiol-17β in vivo on levels of mRNAs encoding steroidogenic
660 acute regulatory protein and three steroidogenic enzymes in previtellogenic ovarian
661 follicles of rainbow trout. Gen. Comp. Endocrinol. 163, 318-323.
662 Nyuji, M, Kitano, H., Shimizu, A., Lee, J.M., Kusakabe, T., Yamaguchi, A., Matsuyama, M.,
663 2013. Characterization, localization, and stage-dependent gene expression of
664 gonadotropin receptors in chub mackerel (*Scomber japonicus*) ovarian follicles. Biol.
665 Reprod. 88, 1-14
666 Pierce, A.L., Dickey, J.T., Larsen, D.A., Fukada, H., Swanson, P., Dickhoff, W.W., 2004. A
667 quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the
668 study of GH regulation of IGF-I gene expression in primary culture of salmon
669 hepatocytes. Gen. Comp. Endocrinol. 135, 401-411.
670 Planas, J.V., Goetz, F.W., Swanson, P., 1997. Stimulation of brook trout ovarian
671 steroidogenesis by gonadotropins I and II is mediated by the cyclic adenosine
672 3',5'-monophosphate/protein kinase A pathway. Biol. Reprod. 57, 647–654.
673 Planas, J.V., Athos, J., Goetz, F.W., Swanson, P., 2000. Regulation of ovarian
674 steroidogenesis in vitro by follicle-stimulating hormone and luteinizing hormone during
675 sexual maturation in salmonid fish. Biol. Reprod. 62, 1262-1269.
676 Reinecke, M. 2010. Insulin-like growth factors and fish reproduction. Biol. Reprod. 82,
677 656–661.
678 Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal
679 expression of gonadotropin receptors, steroidogenic acute regulatory protein and

680 steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol. 162, 265–275.
681 Senthilkumaran, B., Sudhakumari, C. C., Chang, X. T., Kobayashi, T., Oba, Y., Guan, G.,
682 Yoshiura, Y., Yoshikuni, M., Nagahama, Y., 2002. Ovarian carbonyl reductase like
683 20β-hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression
684 during natural and gonadotropin-induced meiotic maturation in Nile tilapia. Biol. Reprod.
685 67, 1080– 1086.
686 Senthilkumaran, B., Yoshikuni, M., Nagahama, Y. 2004. A shift in steroidogenesis
687 occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocrinol. 215,
688 11–18.
689 Srivastava, R.K., Van Der Kraak, G.,1994. Insulin as an amplifier of gonadotropin action
690 on steroid production: mechanisms and sites of action in goldfish prematurational
691 full-grown ovarian follicles. Gen. Comp. Endocrinol. 95, 6-70.
692 Swanson, P., Suzuki, K., Kawauchi, H., Dickhoff, W.W., 1991. Isolation and
693 characterization of two coho salmon gonadotropins, GtH I and GtH II. Biol. Reprod. 44,
694 29–38.
695 Tan, J.D., Adachi, S., Nagahama, Y. 1986. The in vitro effects of cyclic nucleotides,
696 cyanoketone, and cycloheximide on the production of estradiol-17β by vitellogenic
697 ovarian follicles of goldfish (*Carassius auratus*). Gen. Comp. Endocrinol. 63, 110-116.
698 Thomas, P., 2012. Rapid steroid hormone actions initiated at the cell surface and the
699 receptors that mediate them with an emphasis on recent progress in fish models. Gen.
700 Comp. Endocrinol. 175, 367-383.
701 von Hofsten, J., Karlsson, J., Jones, I, Olsson, P.E., 2002. Expression and regulation of
702 fushi tarazu factor-1 and steroidogenic genes during reproduction in Arctic char
703 (*Salvelinus alpinus*). Biol. Reprod. 67, 1297-1304.
704 Weber, G.M., Sullivan, C.V., 2000. Effects of insulin-like growth factor-I on in vitro final
705 oocyte maturation and ovarian steroidogenesis in striped bass, *Morone saxatilis*. Biol.
706 Reprod. 63, 1049–1057
707 Yoshiura, Y., Senthilkumaran, B., Watanabe, M., Oba, Y., Kobayashi, T., Nagahama, Y.,
708 2003. Synergistic expression of AD4BP/ SF-1 and cytochrome P-450 aromatase (ovarian
709 type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests

transcriptional interaction. Biol. Reprod. 68, 1545–1553.Young, G., Kagawa, H., Nagahama, Y., 1983. Evidence for a decrease in aromatase activity in the ovarian granulosa cells of amago salmon (*Oncorhynchus rhodurus*) associated with final oocyte maturation. Biol. Reprod. 29, 310-315. Young, G., Todo, T., Kusakabe, M., Kobayashi, T., Nagahama, Y., 2000. 3β-hydroxysteroid dehydrogenase gene: sites of expression in trout gonads and cAMP-dependent regulation. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson and S.O. Stefansson, Editors, Reproductive Physiology of Fish, Institute of Marine Research, Bergen, p. 202.

Young, G., Lokman, P.M., Kusakabe, M., Nakamura, I., Goetz F.W. 2005. Gonadal steroidogenesis in teleost fish. In: Molecular Aspects of Fish and Marine Biology (C. Hew series ed.), volume 2: Hormones and their receptors in fish reproduction (N. Sherwood and P. Melamed eds), pp. 155-223. World Scientific Press, Singapore.

Zeleznik, A.J., 2004. The physiology of follicle selection. Reprod. Biol. Endocrinol. 2:31.

Table 1

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

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

0, no effect

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

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

737 **Figure Legends**

738

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

746

747 **Figure 2** Comparison of transcript levels encoding gonadotropin receptors and
748 steroidogenic proteins in midvitellogenic (MV) and late/postvitellogenic (L/PV) ovarian
749 follicles of rainbow trout. Follicles were incubated for 18 h in trout Ringer's solution at
750 12°C for 18 h. RNA was extracted and subject to quantitative PCR. Data are normalized to
751 levels of the reference gene (acidic ribosomal phosphoprotein P0). Each bar represents
752 mean ± SEM of three independent replicates. Asterisks above L/PV bar indicates significant
753 difference from MV value: *, P<0.05; **, P<0.01; ***, P<0.001

754

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

764

765 **Figure 4** Effect of Fsh, Lh, IGF1 or forksolin on levels of gonadotropin receptor
766 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 μM) 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 ± SEM of three independent replicates. Asterisks above bars indicate statistically significant
773 differences from control values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

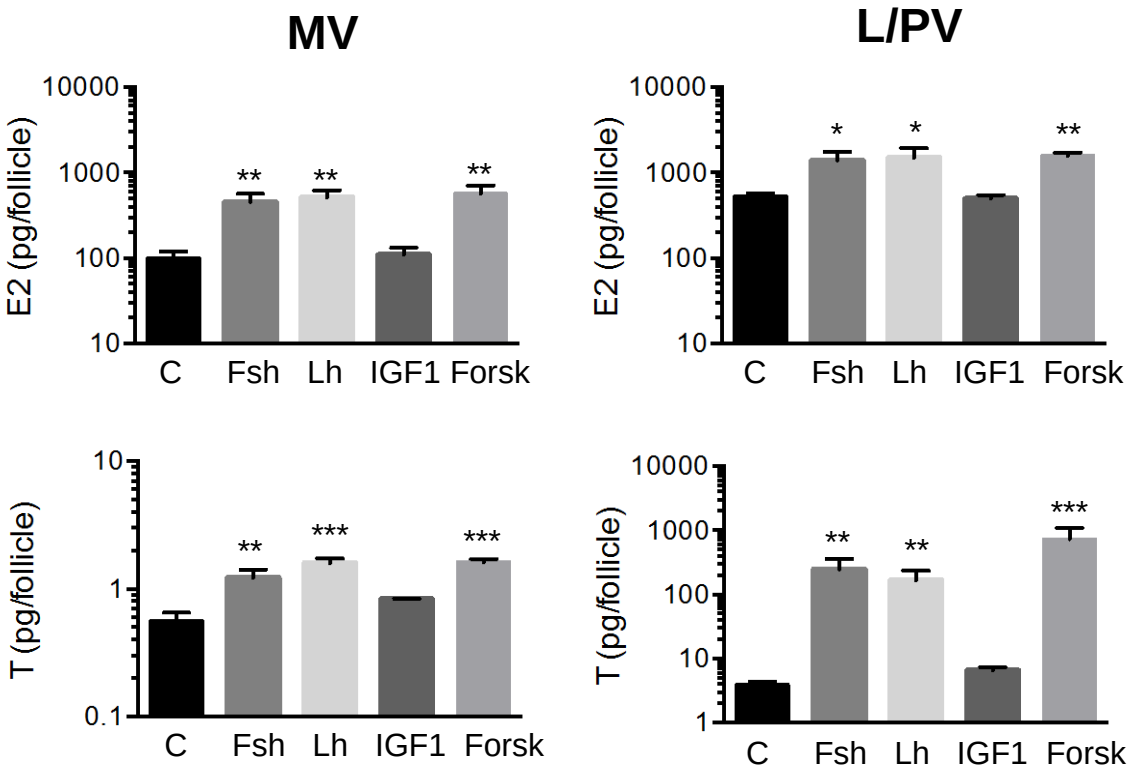
MV
L/PV
E2 (pg/follicle)
T (pg/follicle)
10000
1000
100
10
1
0.1
C
Fsh
Lh
IGF1
Forsk
*
**
***

FIGURE 1

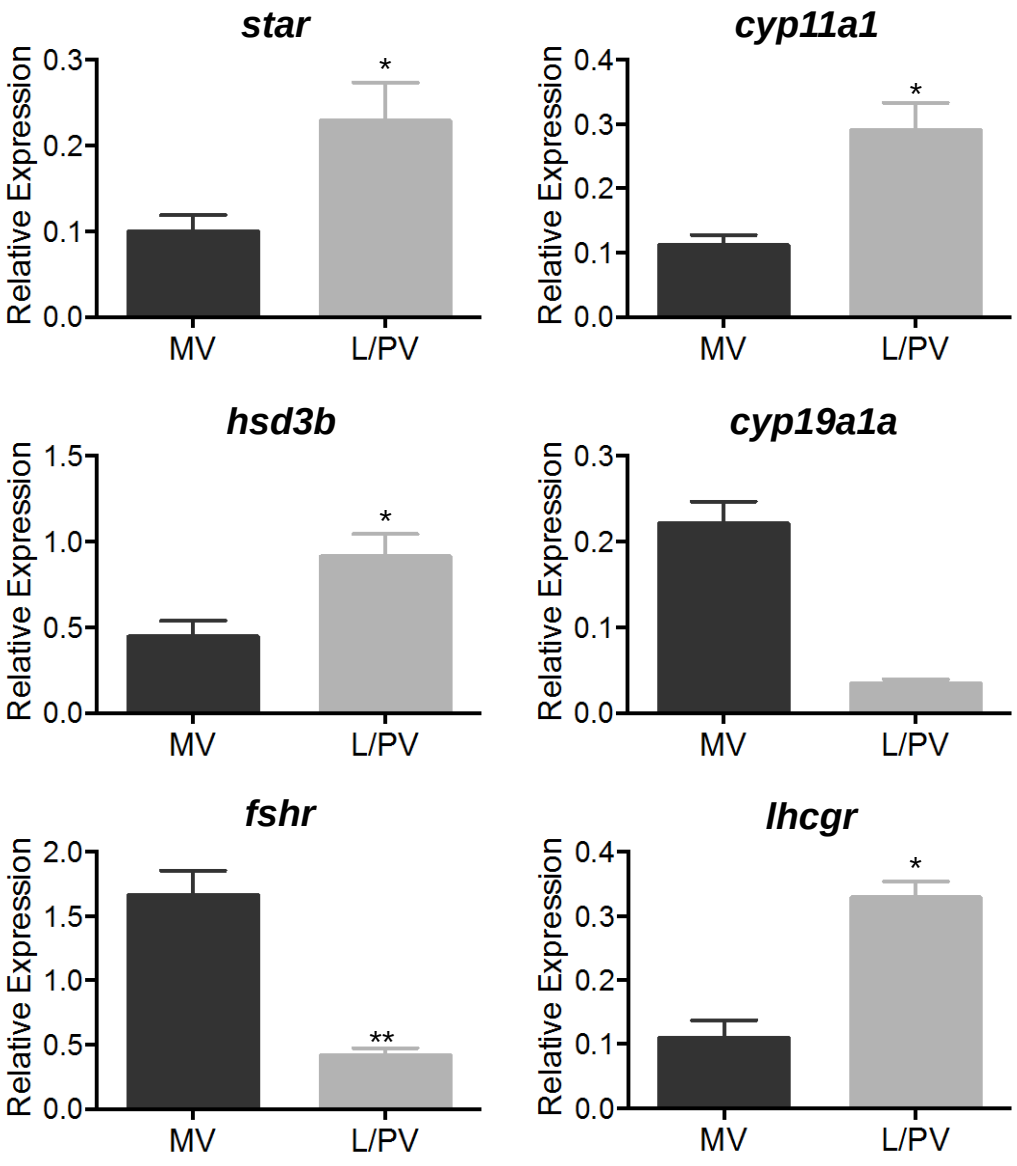
star
Relative Expression
0.3
0.2
0.1
0.0
*
MV
L/PV
cyp11a1
Relative Expression
0.4
0.3
0.2
0.1
0.0
*
MV
L/PV
hsd3b
Relative Expression
1.5
1.0
0.5
0.0
*
MV
L/PV
cyp19a1a
Relative Expression
0.3
0.2
0.1
0.0
MV
L/PV
fshr
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
MV
L/PV
lhcgr
Relative Expression
0.4
0.3
0.2
0.1
0.0
*
MV
L/PV

FIGURE 2

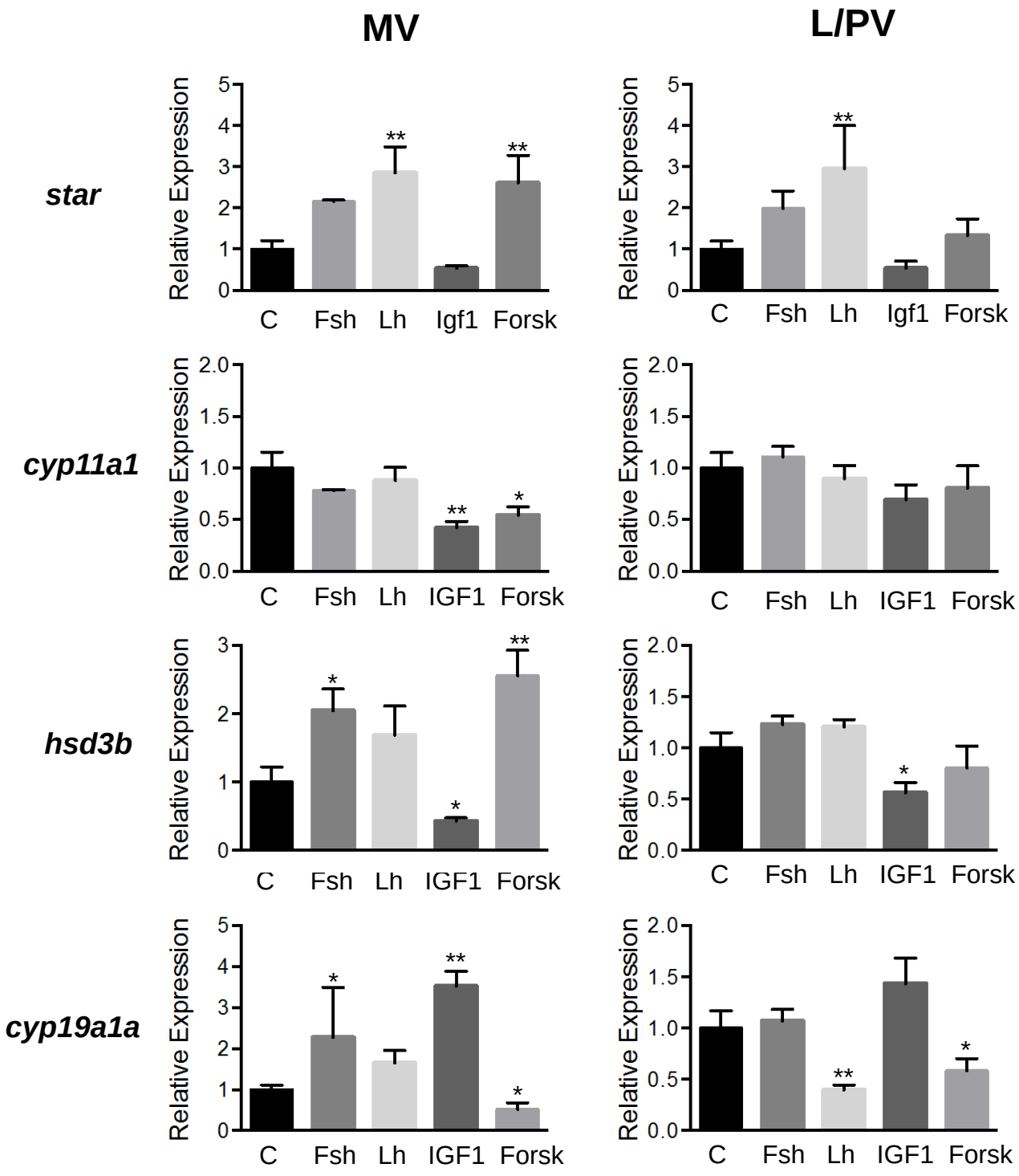
MV
L/PV
star
cyp11a1
hsd3b
cyp19a1a
Relative Expression
C
Fsh
Lh
Igf1
IGF1
Forsk

FIGURE 3

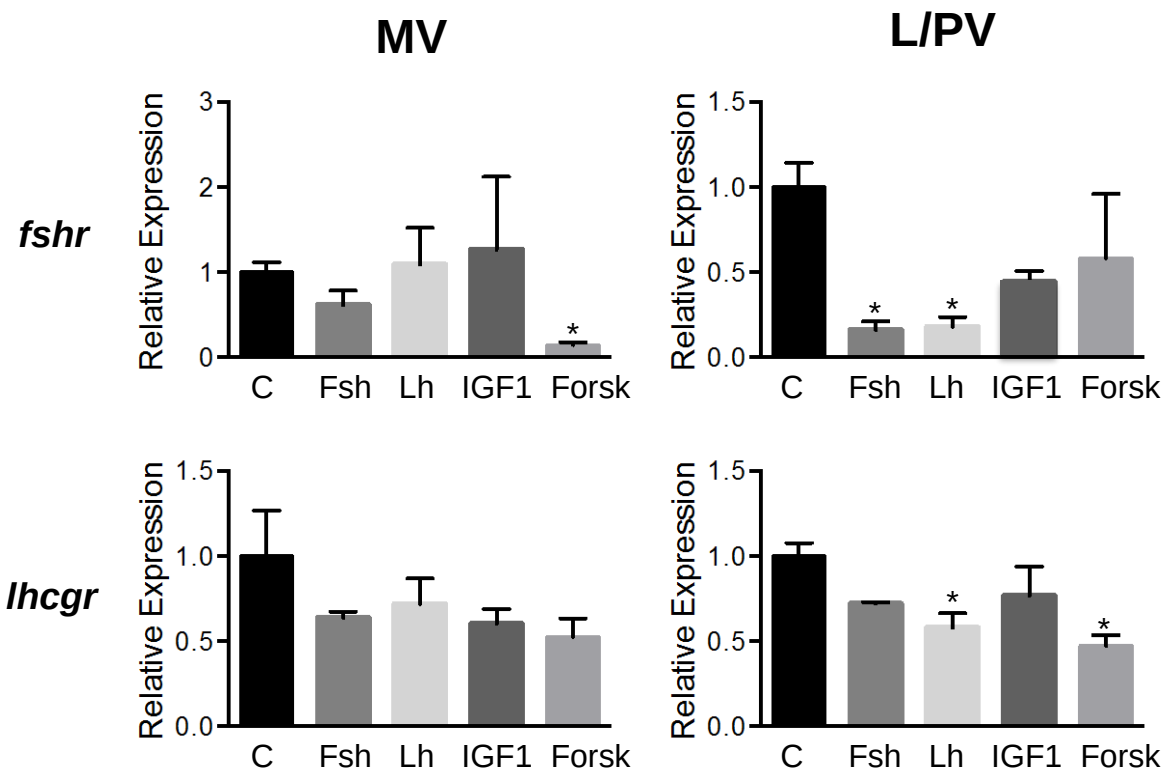
MV
L/PV
fshr
lhcgr
Relative Expression
3
2
1
0
1.5
1.0
0.5
0.0
C
Fsh
Lh
IGF1
Forsk
*

FIGURE 4