

Article

Transcriptional Responses of HPG-Axis Genes and Hepatic *vtg* to 11-Ketotestosterone in Previtellogenic Sterlet (*Acipenser ruthenus*)

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Abstract

11-ketotestosterone (11-KT) has previously been shown to promote previtellogenic ovarian development and vitellogenin-related hepatic responses in sterlet (*Acipenser ruthenus*), but the endocrine genes involved in this process remain unclear. In this study, previtellogenic female sterlets were implanted with slow-release 11-KT strips at 5 or 25 mg/kg for 30 days, and transcriptional responses of HPG-axis-related genes, including GnRH paralogs, gonadotropins, and gonadotropin receptors, were examined in the brain-pituitary complex, liver, and ovary. In the brain-pituitary complex, 11-KT increased *fsh* expression in the 25 mg/kg group and upregulated *fshr* in both treatment groups, whereas *gnrh* paralogs showed limited responses. In the liver, most GnRH- and gonadotropin-related genes were not significantly altered, suggesting that hepatic expression of upstream reproductive-axis genes was not a major *in vivo* target of 11-KT feedback. The ovary exhibited the strongest response, with increased *gnrh2* and *fsh* expression and reduced *gnrh3*, *fshr*, and *lhr* expression, especially at the higher dose. To further examine receptor-related regulation of hepatic *vtg* expression, liver tissue was incubated *in vitro* with 11-KT alone or in combination with the androgen receptor antagonist flutamide or the estrogen receptor antagonist fulvestrant. Flutamide did not abolish 11-KT-induced *vtg* expression, whereas high-dose fulvestrant reduced both *era* and *vtg* expression relative to 11-KT treatment alone. Overall, 11-KT elicited tissue-specific transcriptional responses in HPG-axis-related genes, particularly involving ovarian *gnrh2*, *fsh*, *fshr*, and *lhr*. The differential antagonist responses observed in liver explants provide *in vitro* evidence consistent with the involvement of ER-associated signaling in hepatic *vtg* expression, but do not establish direct receptor-mediated regulation.

Keywords: gonadotropin receptor; ovarian development; androgen feedback; fulvestrant; flutamide; vitellogenesis



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Key Contribution: This study characterizes tissue-specific transcriptional responses of HPG-axis-related genes to 11-ketotestosterone in previtellogenic sterlet. The principal responses involved *fsh/fshr* in the brain-pituitary complex and *gnrh2*, *fsh*, *fshr*, and *lhr* in the ovary, whereas hepatic HPG-axis-related genes showed limited changes. Liver explant experiments further showed differential sensitivity of 11-KT-induced *vtg* expression to flutamide and fulvestrant, providing preliminary pharmacological evidence consistent with ER-associated involvement without establishing direct receptor-mediated regulation.

1. Introduction

The previtellogenic stage is a critical period in female fish reproductive cycle, during which oocytes undergo active primary growth and gradually acquire the endocrine and metabolic competence required for the initiation of vitellogenesis [1,2]. This transition is regulated by interactions among gonadotropin signaling, ovarian steroidogenesis, hepatic vitellogenin synthesis, and local intraovarian factors [3–6]. In the classical hypothalamic-pituitary-gonadal axis, gonadotropin-releasing hormones regulate pituitary gonadotropins, which in turn modulate ovarian steroid production and follicular development [3,4,6–8]. Steroid signals then act on both the ovary and liver, thereby linking ovarian growth with hepatic production of yolk precursors [1,5,9]. Recent studies in eel models have further emphasized this endocrine sequence, in which FSH-Fshr signaling promotes ovarian E2 production and E2 subsequently activates hepatic estrogen receptor-dependent vitellogenin synthesis [10]. However, the relative contribution of systemic endocrine signals and local ovarian or hepatic responses may vary among species and reproductive stages [6,11].

11-ketotestosterone (11-KT) is generally regarded as a major non-aromatizable androgen in fishes and is best known for its roles in male reproductive physiology, including spermatogenesis, secondary sexual characteristics, and reproductive behavior [12–14]. Increasing evidence, however, indicates that 11-KT also participates in female reproductive development, particularly during previtellogenic ovarian growth [15–19]. In several teleost species, including anguillid eels, Atlantic cod (*Gadus morhua*), and salmonids, 11-KT has been associated with oocyte growth, lipid accumulation, follicular development, and preparation for vitellogenesis [1,16–18,20–22]. More recently, transcriptomic analysis in coho salmon (*Oncorhynchus kisutch*) showed that in vivo 11-KT treatment rapidly altered the ovarian transcriptome during previtellogenic secondary growth and affected genes associated with the transition toward a secondary-growth phenotype [23]. In the shortfinned eel (*Anguilla australis*), androgen treatment has been shown to regulate follicle-stimulating hormone-related signaling in the previtellogenic ovary and pituitary, suggesting that 11-KT may enhance ovarian responsiveness before full vitellogenic activation [24]. Similar androgen-related responses have also been reported in sturgeons, including sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*), indicating that the role of 11-KT in female reproductive development may be conserved, although the underlying mechanisms remain incompletely understood [25,26].

Several mechanisms may explain how androgens promote vitellogenin-related hepatic responses. Some androgens may be converted into estrogens and then stimulate vitellogenin production through canonical estrogen receptor signaling [9,27,28]. Alternatively, androgens or androgen-derived metabolites may interact directly or indirectly with estrogen receptor pathways, thereby producing estrogen-like effects in hepatic tissue [29–32]. A third possibility is that androgen receptor signaling contributes to vitellogenin induction, either independently or in coordination with estrogen receptor-mediated pathways [33,34]. These possibilities are particularly relevant for 11-KT because it is usually considered a non-aromatizable androgen [12,19]. Therefore, 11-KT-induced vitellogenic responses should not be assumed to result from simple aromatization to estradiol. Instead, they may involve more complex interactions among androgen receptor signaling, estrogen receptor signaling, and steroid metabolic pathways [27,35–41].

Sturgeons are ancient actinopterygian fishes with long reproductive cycles, delayed female maturation, and high aquaculture value [42]. The prolonged period required for ovarian development increases production costs and complicates broodstock management. Sterlet is one of the smallest and earliest-maturing sturgeon species and is therefore a useful model for investigating endocrine regulation of ovarian development in sturgeons [42]. Previous work in sterlet showed that 11-KT implantation can promote previtellogenic

ovarian development, increase oocyte size, and affect expression of genes related to steroid signaling and vitellogenin synthesis [26]. Recent work in Chinese sturgeon (*A. sinensis*) also indicates that nutritional modulation can alter 11-KT levels and HPG-axis-related genes such as *gnrh2*, *ar*, and *fshr* during early gonadal development, further supporting the sensitivity of acipenserid reproductive development to endocrine-axis regulation [43]. However, previous studies in sturgeons have mainly focused on ovarian phenotypes, steroid profiles, or selected genes in individual tissues [25,26]. A systematic analysis linking central HPG-axis-related endocrine nodes with peripheral hepatic receptor-associated responses is still lacking. In particular, it remains unclear whether 11-KT acts primarily through gonadotropin-related feedback, local ovarian endocrine responses, hepatic receptor-mediated pathways, or a coordinated interaction among these components.

In the present study, we tested the hypotheses that 11-KT elicits tissue-specific transcriptional responses in HPG-axis-related genes across the brain-pituitary complex, ovary, and liver, and that 11-KT-induced hepatic vtg expression is differentially sensitive to AR and ER antagonism. Previtellogenic female sterlets were implanted with slow-release 11-KT strips for 30 days to examine transcriptional responses in brain-pituitary, liver, and ovary. We primarily analyzed HPG-axis-related genes, including GnRH paralogs, gonadotropins, and gonadotropin receptors, to evaluate tissue-specific endocrine responses to 11-KT. Steroid receptor genes were included as contextual markers, whereas the receptor-associated regulation of hepatic vtg expression was further tested using an in vitro liver incubation system with androgen and estrogen receptor antagonists. By combining in vivo implantation with in vitro receptor-antagonist treatment, this study aimed to clarify the potential endocrine and receptor-related pathways through which 11-KT contributes to previtellogenic ovarian development and hepatic competence for vitellogenin induction in sterlet.

2. Materials and Methods

2.1. Preparation of 11-KT and Slow-Release Implants

11-KT powder was synthesized by reducing the C17 ketone group of adrenosterone (284998, Sigma, Saint Louis, MO, USA) with sodium borohydride [44]. Using standard 11-KT (K8250, Sigma, Saint Louis, USA) as a control, the synthesized product was verified by high-performance liquid chromatography (HPLC) and showed a purity of 99.9%. It was then stored at 4 °C. Slow-release silastic strips containing 11-KT were prepared according to a previously published protocol [26].

2.2. In Vivo Implantation Experiment

Eighteen thirty-month-old previtellogenic female sterlets were selected after endoscopic examination and allocated to three balanced groups under anesthesia: a control group (368.30 ± 25.64 g, $N = 6$), a low-dose 11-KT group (T1, 5 mg/kg, 377.42 ± 36.67 g, $N = 6$), and a high-dose 11-KT group (T2, 25 mg/kg, 397.68 ± 36.57 g, $N = 6$). The 5 and 25 mg/kg doses were selected based on the previous sterlet implantation study [26], allowing the present transcriptional responses to be compared with the previously characterized ovarian and hepatic responses under the same lower- and higher-dose conditions. Body weight did not differ significantly among groups ($p = 0.135$). After anesthesia with 400 ppm MS-222, fish received a small ventral incision. The appropriate length of silastic strip was implanted to deliver the designated 11-KT dose in the treatment groups, whereas blank strips were implanted in the control group. Incisions were treated with erythromycin ointment after surgery. Fish were reared in indoor cylindrical tanks with a flowing-through water system for 30 days, fed a commercial diet daily. Water temperature ranged from 16 to 22 °C. Fish were fasted for 24 h before sampling. At the end of the treatment period,

fish were anesthetized again and the brain-pituitary complex (including olfactory bulb, telencephalon, diencephalon, hypothalamus, midbrain, and pituitary), liver, and ovary were collected. Tissues were snap-frozen in liquid nitrogen and stored at -80°C . A small portion of gonad tissue was fixed for histological examination. The *in vivo* transcriptional analysis focused primarily on HPG-axis-related genes, including *gnrh1*, *gnrh2*, *gnrh3*, *fsh*, *lh*, *fshr*, and *lhr*. Steroid receptor genes were retained as receptor-context markers; hepatic and ovarian *ar*, *era*, and *erb* data are presented in Supplementary Figure S1 because comparable hepatic and ovarian steroid receptor responses to 11-KT have been reported previously in sterlet.

2.3. In Vitro Liver Incubation Experiment

Fresh liver tissue from three previtellogenic female sterlets was rinsed in precooled $1\times$ phosphate-buffered saline and transferred into chilled Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, 1:1; Paisley, Scotland, UK), a basal medium commonly used for tissue and cell culture. The liver was minced into small fragments, and 100 mg tissue was placed into each well of a six-well culture plate. The experiment included one control group and seven treatment groups. Liver tissue from each of the three fish was processed independently. For each fish, liver fragments were distributed across all treatments, and each treatment was performed in technical triplicate wells. The mean value of technical triplicates from each fish was used as one biological replicate. Liver tissue from each donor fish was distributed across all experimental treatments. Technical triplicates were averaged before statistical analysis, and the resulting mean for each donor and treatment was treated as one biological replicate. After tissue attachment, four treatment groups received DMEM/F12 supplemented with 1% penicillin-streptomycin, 20% hormone-free serum (Gibco, USA), 100 μM 11-KT, and either 10 μM or 100 μM flutamide (FLU) or fulvestrant (FUL), for a final volume of 2.5 mL. Three additional groups received 100 μM 11-KT alone, 100 μM FLU alone, or 100 μM FUL alone. The control group received no receptor antagonist and contained 0.08% ethanol as vehicle. Stock solutions of 11-KT, FLU, and FUL were prepared in ethanol, and the final ethanol concentration was adjusted to 0.08% in all treatment and control wells. Cultures were maintained at 24°C for 72 h. At the end of incubation, both tissue fragments and conditioned medium were collected and stored at -80°C . Media were used for testosterone (T), estradiol (E2), and tissues were used for total RNA extraction and quantitative real-time PCR (qPCR) analysis of *vtg*, *era*, *erb*, and *ar*.

2.4. Histology

Gonads were fixed in Bouin's solution for 24 h and then transferred to 70% ethanol. Samples were dehydrated, paraffin embedded, sectioned at 6–8 μm , stained with hematoxylin and eosin, and examined microscopically to confirm ovarian developmental stage.

2.5. RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and genomic DNA was removed with the RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA concentration and purity were measured with a NanoDrop 2000 (Wilmington, DE, USA), and integrity was checked by agarose gel electrophoresis, Agilent 2100 analysis (Waldbronn, Germany), and Qubit 2.0 assessment (Singapore).

Primers were designed using Primer 3, and primer specificity was confirmed by cloning and sequencing. Primer efficiency was assessed using standard curves [45]. Complementary DNA (cDNA) was synthesized from 4 μg total RNA using oligo (dT) primers and SuperScript III First-Strand Synthesis System. qPCR was performed with SYBR Green chemistry on a QuantStudio 3 instrument (Singapore). The final qPCR reaction volume of

20 μ L, contained 1 μ L of 5-fold diluted cDNA template, 10 μ L $2 \times$ SYBR green real-time PCR master mix, 0.2 μ L of each primer (10 μ M) and 8.6 μ L double-distilled H₂O. The amplification program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, followed by dissociation curve analysis. R^2 values and efficiency for all standard curves were >0.99 and 90–110%, respectively (Table 1). Negative controls were prepared with sterile deionized water instead of cDNA template. All reactions were run in triplicate. For each biological sample, the mean Ct value of the three technical replicate wells was used for relative-expression calculation. Technical replicates were averaged before statistical analysis and were not treated as independent observations. For the in vivo implantation experiment, qPCR analysis focused on HPG-axis-related genes, including *gnrh1*, *gnrh2*, *gnrh3*, *fsh*, *lh*, *fshr*, and *lhr*, in the brain-pituitary complex, liver, and ovary. Steroid receptor genes (*ar*, *era*, and *erb*) were also measured to provide receptor-related context; however, hepatic and ovarian receptor data are shown in Supplementary Figure S1 because comparable receptor responses have been reported previously. For the in vitro liver incubation experiment, *vtg*, *ar*, *era*, and *erb* were analyzed to evaluate receptor-associated regulation of 11-KT-induced hepatic *vtg* expression.

Table 1. Primer sequences and qPCR performance parameters for target and reference genes analyzed in the brain-pituitary complex, liver, and ovary of sterlet. The table presents forward and reverse primer sequences, amplicon sizes, coefficients of determination (R^2), amplification efficiencies, and GenBank accession numbers.

Gene	Primers Sequence (5'-3')	Amplicon Size	R^2	Eff%	Accession No
<i>ar</i>	F: AAGCCAATCTGTTTCACAA R: CACTGCCTGTTGACTAAATG	121	0.999	99.56	KP996194
<i>era</i>	F: GTGCCTTTCCCTTTCTCTCC R: GGCTTGCTTCTGGAAGTGCT	146	0.999	96.40	KP996192
<i>erb</i>	F: ATTGCTGCTGGAGATGCTG R: TTCTGGCTTTGAACAGGTGA	121	0.998	99.41	KP996193
<i>fsh</i>	F: GGCTGCGGAACTGTGTATC R: CCACGGGGTAGGTGTAAAAA	189	0.993	103.75	KX247394
<i>fshr</i>	F: TACTCAGTGCTACCTCGGCT R: TGGCGTAGGTCAGGTTGGCT	90	0.992	99.99	KU517164
<i>lh</i>	F: AGAGAGAGACGCTGCCTGAG R: GATGAGGAGGCACTTTGGAC	190	0.992	90	AY333426
<i>lhr</i>	F: GTTTCAGACATGCTGCCATC R: TCTTGTAGCTGCTCACTCC	88	0.994	94.35	XM_034018708
<i>gnrh1</i>	F: CTGGACACCCTGCAAGAGATT R: CGTGCCAGCACCCCTTTA	115	0.995	103.05	XM_033996722.3
<i>gnrh2</i>	F: GGAGACGAGTGCAGCTATCT R: GGAGGGGAGGTCACCTCTTC	103	0.996	98.37	XM_034917046.1
<i>gnrh3</i>	F: AGTCAGCTGCTCCGAAGAAC R: TCTTACCCCTGTATCTGATAACTCA	82	0.991	103.14	XM_058985206
<i>vtg</i>	F: GGAAGACCATCACCTGGA R: ACAGCGTGCGACCATCTT	130	0.996	101.65	KF918450
<i>bactin</i>	F: TGCCATCCAGGCTGTGCT R: TCTCGGCTGTGGTGGTGAAG	215	0.998	90.77	AY878120.1
<i>efla</i>	F: GGAATCCACTGAGCCACCT R: GGGTTGTAGCCGATCTTCTTG	89	0.989	108.07	XM_034004589.3
<i>rpl6</i>	F: GTGGTCAAACCTCCGCAAGA R: GCCAGTAAGGAGGATGAGGA	149	0.995	90.06	HQ449564.1
<i>rpl13</i>	F: GGACGTGGTTTACCCCTTG R: GGGAAGAGGATGAGTTTGA	166	0.998	96.78	XM_034042120.3

Gene expression was normalized to the geometric mean of two tissue-specific reference genes: *rpl13* and *bactin* for brain-pituitary complex, *rpl13* and *ef1a* for liver, *rpl6* and *bactin* for ovary [46]. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method [47].

2.6. Measurement of T and 17 β -Estradiol

Conditioned culture medium was used to determine T and E2 concentrations, with approximately 0.2 mL analyzed for each sample. T levels were quantified using a Testosterone Radioimmunoassay Kit (Cat. No. B10PZA; Beijing North Biotechnology Institute, Beijing, China), following the manufacturer's instructions. The analytical sensitivity of the assay was 0.02 ng/mL. According to the manufacturer, the antibody showed low cross-reactivity with structurally related steroids, including dihydrotestosterone ($1.1 \times 10^{-2}\%$), 17 β -estradiol ($2.1 \times 10^{-2}\%$), estriol ($6.2 \times 10^{-15}\%$), progesterone ($3.2 \times 10^{-2}\%$), and 11-KT ($1.2 \times 10^{-2}\%$).

E2 was quantified using a chemiluminescent immunoassay kit for 17 β -estradiol (Cat. No. YZB0051-2006; Beijing North Biotechnology Institute, Beijing, China). The assay sensitivity was <4.0 pg/mL. The manufacturer reported cross-reactivity values of <0.5% for estriol, <1.5% for progesterone, and <0.01% for testosterone. Calibration curves were constructed using a logit (%B/B₀) transformation against log-transformed standard concentrations. Each sample was measured in duplicate, and the average intra-assay coefficients of variation were 6.5% and 7.2% for T and E2, respectively.

2.7. Statistical Analysis

Steroid hormone concentrations and relative mRNA expression levels are expressed as mean $\pm$ SEM. Statistical analyses were performed with SPSS Statistics 22.0. Normality and homogeneity of variance were checked using the Shapiro–Wilk and Levene's tests, respectively. Data meeting the assumptions for parametric analysis were compared by one-way ANOVA, and Tukey's post hoc test was applied for multiple comparisons. For variables that were not normally distributed, log transformation was performed before statistical testing. When transformed data still did not satisfy the assumptions of parametric analysis, group differences were analyzed using the Kruskal–Wallis test, with all pairwise comparisons performed. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects of 11-KT on Gene Expression in the Brain-Pituitary Complex

As shown in Figure 1, most *gnrh* transcripts in the brain-pituitary responded only modestly to 11-KT implantation. Expression of *gnrh1*, *gnrh2*, and *gnrh3* did not differ significantly among treatment groups (all $p > 0.05$). By contrast, the FSH-related markers showed clearer responses. Expression of *fsh* increased progressively from the control to the low-dose and high-dose groups, and the 25 mg/kg group was significantly higher than the control ($p = 0.027$, Figure 1D). Likewise, *fshr* expression was significantly elevated in both 11-KT-treated groups relative to the control ($p = 0.015$ and $p = 0.004$, respectively), although the two treatment groups did not differ from each other ($p = 0.626$ Figure 1E). Expression of *lh* and *lhr* showed no significant differences among groups ($p = 0.372$ and $p = 0.078$, respectively). Steroid receptor genes in the brain-pituitary complex showed no significant changes (Figure 1). Together, these results suggest that exogenous 11-KT preferentially affected the FSH-related component of the brain-pituitary complex, whereas GnRH paralogs and steroid receptor genes showed limited transcriptional responses.

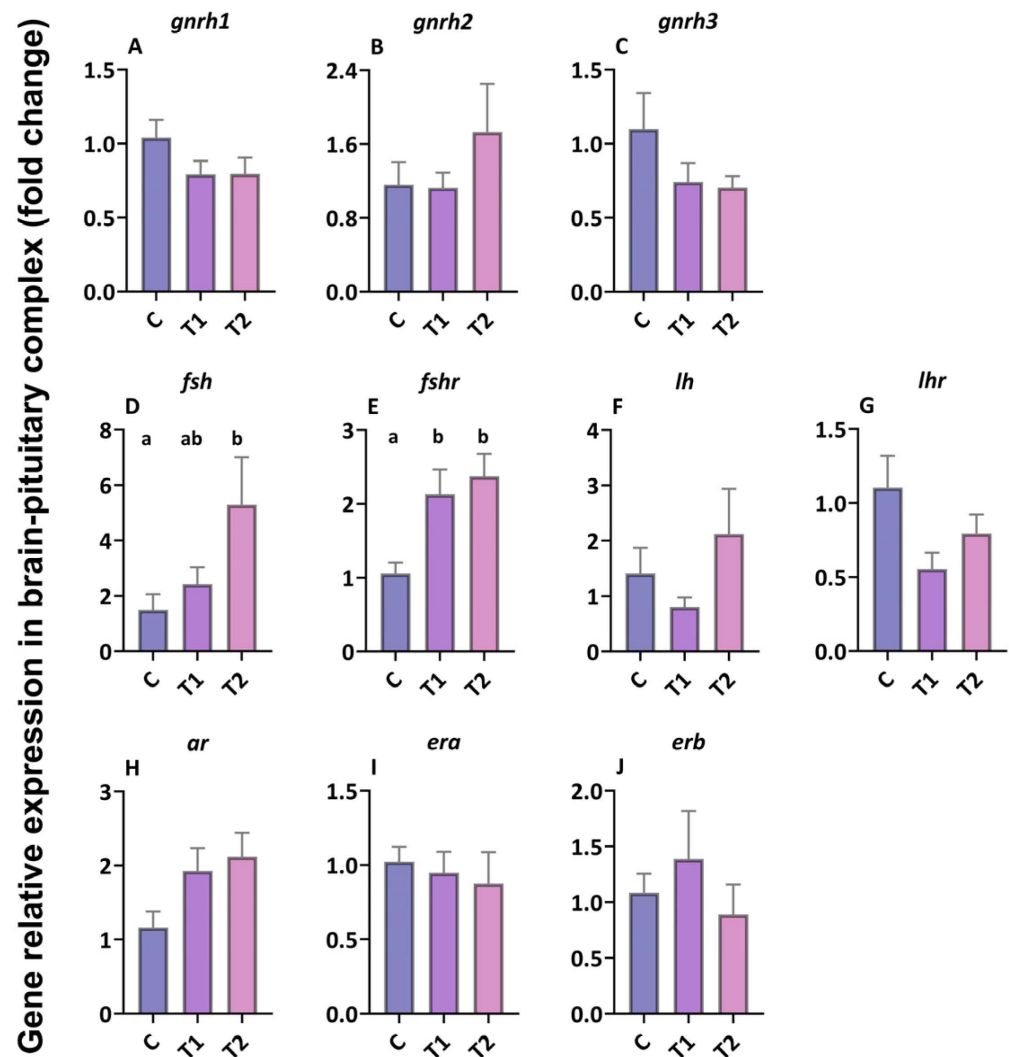


Figure 1. Effects of 30-day slow-release 11-KT implantation on HPG-axis-related and steroid receptor gene expression in the brain-pituitary complex of previtellogenic female sterlet. Fish were implanted with blank silastic strips (control), 5 mg/kg 11-KT, or 25 mg/kg 11-KT ($n = 6$ fish per group). Relative mRNA levels of *gnrh1* (A), *gnrh2* (B), *gnrh3* (C), *fsh* (D), *fshr* (E), *lh* (F), *lhr* (G), *ar* (H), *era* (I), and *erb* (J) were determined by qPCR. Data are presented as mean $\pm$ SEM. Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test, except for *fshr* and *lh* were analyzed using the Kruskal–Wallis test followed by all pairwise comparisons.

3.2. Effects of 11-KT on Gene Expression in the Liver

Figure 2 shows the hepatic expression of HPG-axis-related genes after 11-KT implantation. None of the examined hepatic HPG-axis-related genes differed significantly among treatment groups (all $p > 0.05$, Figure 2). These results suggest that hepatic expression of GnRH- and gonadotropin-related genes was not a major *in vivo* transcriptional target of 11-KT treatment. Hepatic steroid receptor genes are provided in Supplementary Figure S1 as receptor-context information.

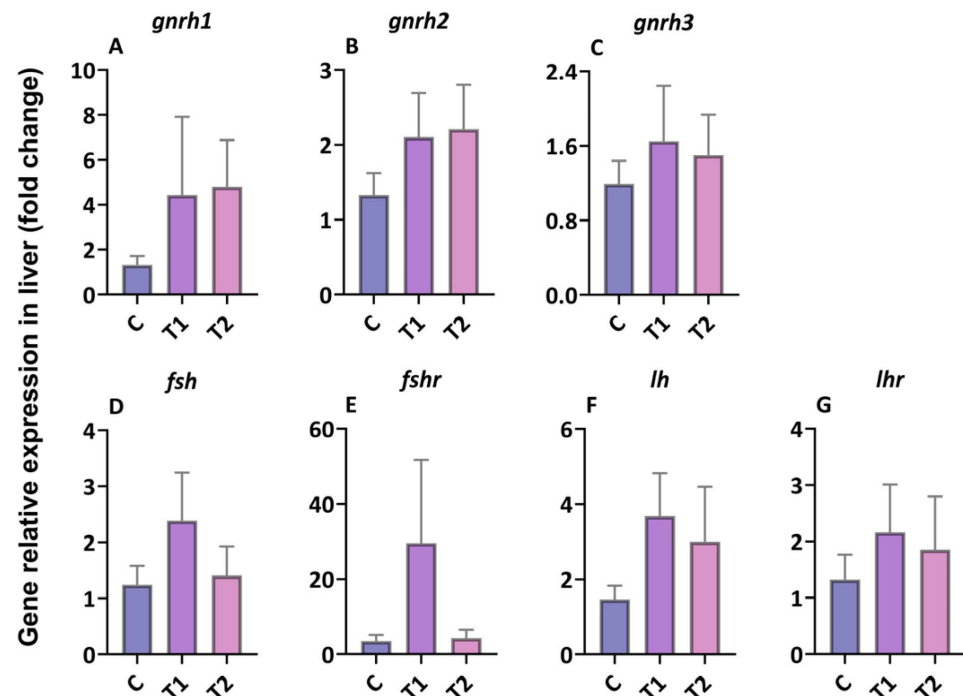


Figure 2. Effects of 30-day slow-release 11-KT implantation on hepatic HPG-axis-related gene expression in previtellogenic female sterlet. Fish were implanted with blank silastic strips (control), 5 mg/kg 11-KT, or 25 mg/kg 11-KT ($n = 6$ fish per group). Relative mRNA levels of *gnrh1* (A), *gnrh2* (B), *gnrh3* (C), *fsh* (D), *fshr* (E), *lh* (F), and *lhr* (G) were determined by qPCR. Data are presented as mean $\pm$ SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test, except for *gnrh1*, *fshr*, *lh*, and *lhr*, which were analyzed using the Kruskal–Wallis test followed by all pairwise comparisons.

3.3. Effects of 11-KT on Gene Expression in the Ovary

The ovary exhibited the strongest transcriptional response to 11-KT implantation (Figure 3). Ovarian *gnrh1* and *lh* did not differ significantly among groups ($p = 0.682$, Figure 3F and $p = 0.331$, Figure 3F). By contrast, *gnrh2* was significantly higher in both treatment groups than in the control ($p = 0.018$, Figure 3B), with the 5 mg/kg group showing the greatest mean expression. Ovarian *gnrh3* displayed the opposite pattern: the 25 mg/kg group was significantly lower than both the control and 5 mg/kg groups ($p = 0.007$, $p = 0.016$, respectively), whereas the latter two groups did not differ ($p = 0.922$) (Figure 3C). Genes associated with gonadotropin signaling also responded markedly. The 25 mg/kg group was significantly higher than the control ($p = 0.011$), whereas the 5 mg/kg group did not differ significantly from either group (Figure 3D). In contrast, *fshr* decreased with increasing 11-KT dose and was significantly lower in the 25 mg/kg group than in the control and 5 mg/kg groups ($p \leq 0.001$, $p = 0.001$, respectively, Figure 3E). A similar inhibitory pattern was observed for *lhr*, which was highest in the control ($p = 0.024$, Figure 3G). Together, these results suggest that ovarian responses to 11-KT were characterized by selective activation of *gnrh2* and *fsh*, together with suppression of gonadotropin receptor genes at the higher dose, suggesting a dose-dependent feedback pattern in the previtellogenic ovary. Ovarian steroid receptor genes are provided in Supplementary Figure S1.

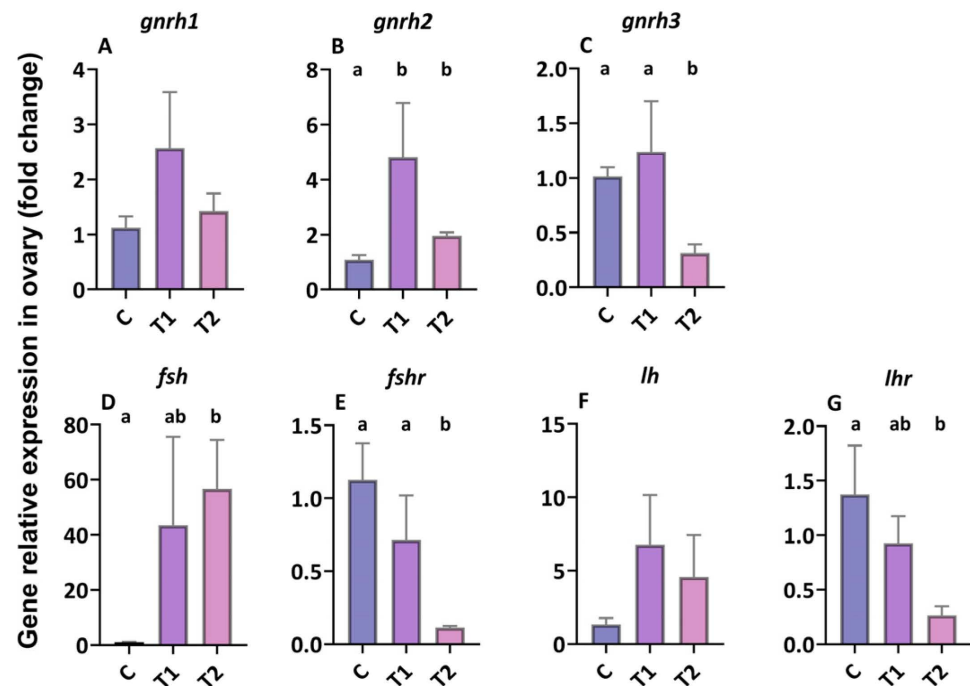


Figure 3. Effects of 30-day slow-release 11-KT implantation on ovarian HPG-axis-related gene expression in previtellogenic female sterlet. Fish were implanted with blank silastic strips (control), 5 mg/kg 11-KT, or 25 mg/kg 11-KT ($n = 6$ fish per group). Relative mRNA levels of *gnrh1* (A), *gnrh2* (B), *gnrh3* (C), *fsh* (D), *fshr* (E), *lh* (F), and *lhr* (G) were determined by qPCR. Data are presented as mean $\pm$ SEM. Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test, except for *gnrh2*, *fsh*, *lh*, and *lhr*, which were analyzed using the Kruskal–Wallis test followed by all pairwise comparisons.

3.4. Histological Confirmation of Ovarian Developmental Stage

Histological examination confirmed that ovaries from all experimental groups were at the previtellogenic stage. Oocytes displayed typical stage II characteristics, including a distinct germinal vesicle, oocyte, and oogonia. These observations confirmed that the transcriptional responses analyzed in this study occurred within the intended previtellogenic developmental stage.

3.5. Effects of Receptor Antagonists on Hepatic Vtg Expression and Steroid Levels In Vitro

Gene-expression responses in the in vitro liver were shown in Figure 4. Expression of *ar* did not differ significantly among treatments ($p = 0.509$, Figure 4A). By contrast, *era* showed marked treatment-dependent response: expression remained relatively high in 11-KT + FLU groups but was strongly reduced in the 11-KT + FUL groups, especially at 100 μ M FUL ($p \leq 0.001$ and $p \leq 0.001$, respectively, Figure 4B). Expression of *erb* showed more moderate variation ($p = 0.452$, Figure 4C). Expression of *vtg* was significantly induced by 11-KT, 11-KT + 100 μ M FLU, and 11-KT + 10 μ M FUL, with the strongest expression observed in the 11-KT + 100 μ M FLU group. In contrast, *vtg* expression was comparatively low in the FUL-only and 11-KT + 100 μ M FUL groups ($p = 0.011$, Figure 4D). These results show that 11-KT-induced hepatic *vtg* expression persisted in FLU-containing treatments but was reduced in the presence of 100 μ M FUL.

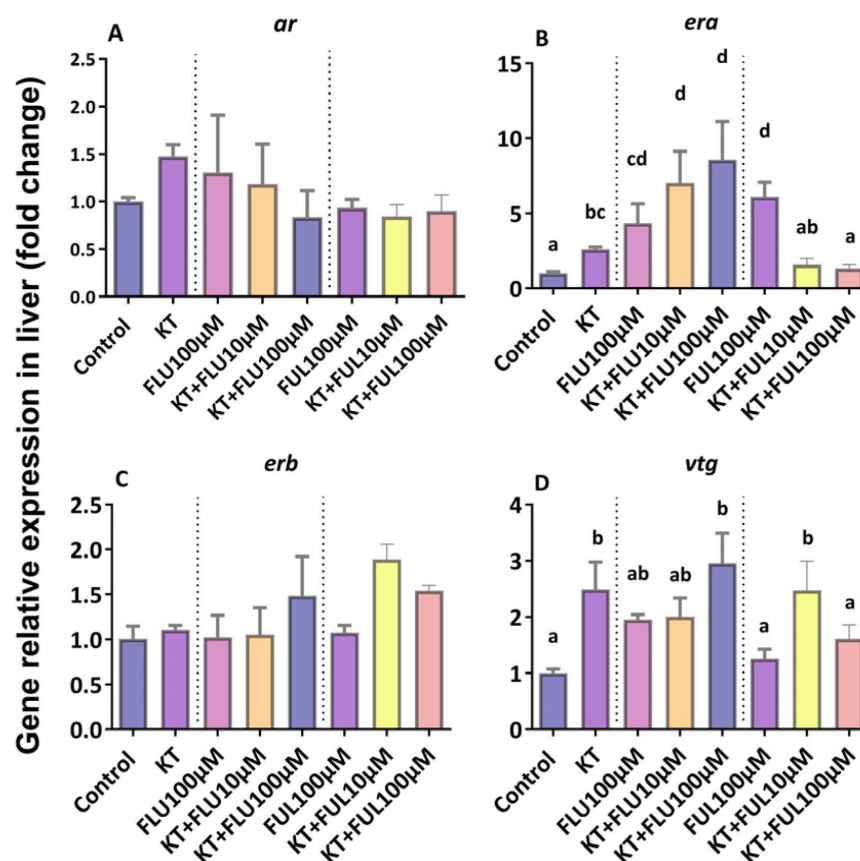


Figure 4. Effects of androgen and estrogen receptor antagonists on 11-KT-induced gene expression in liver explants from previtellogenic female sterlet. Liver explants were incubated for 72 h with vehicle control, 100 µM 11-KT alone, 100 µM flutamide (FLU) alone, 100 µM fulvestrant (FUL) alone, or 100 µM 11-KT combined with 10 or 100 µM FLU or FUL. Relative mRNA levels of *ar* (A), *era* (B), *erb* (C), and *vtg* (D) were determined by qPCR. Liver tissue from three donor fish was processed independently, and the mean of the technical triplicates for each fish was treated as one biological replicate ($n = 3$ biological replicates). Data are presented as mean $\pm$ SEM. Different lowercase letters indicate significant differences among treatments ($p < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test, except for *erb*, which was analyzed with the Kruskal–Wallis test followed by all pairwise comparisons.

For steroid concentrations (Figure 5), testosterone remained low in the control, FLU-only, and FUL-only groups, but was significantly elevated in the 11-KT group and in all 11-KT co-incubation groups. These 11-KT-containing treatments did not differ significantly from one another, indicating that the receptor antagonists did not abolish the effect of 11-KT on T accumulation in the culture medium. Estradiol showed a different pattern. E2 remained low in the control and FLU-only groups, increased slightly in the 11-KT group and 11-KT + FLU groups, and accumulated markedly in FUL-containing treatments, especially in the 11-KT + 100 µM FUL group ($p \leq 0.001$, Figure 5B). Thus, the E2 assay detected markedly increased immunoreactivity in FUL-containing treatments particularly in the 11-KT + 100 µM FUL group. This increase occurred despite reduced *era* and *vtg* transcript abundance under high-dose FUL treatment.

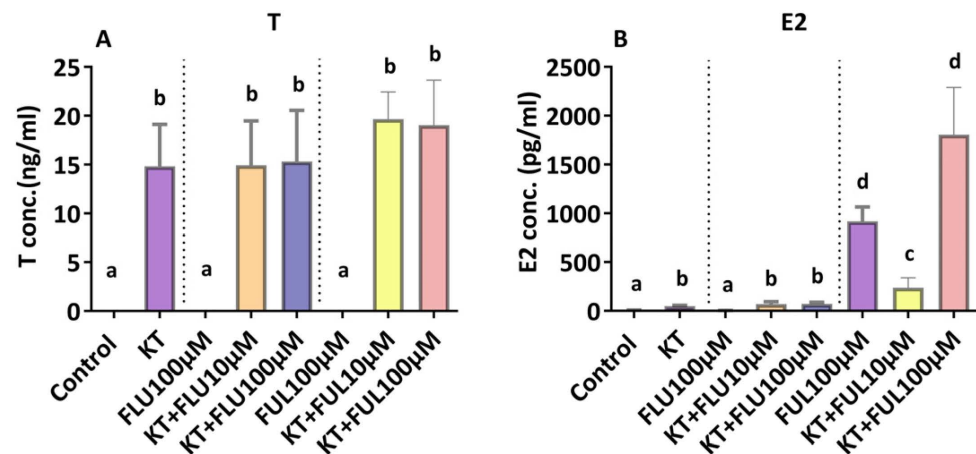


Figure 5. Effects of androgen and estrogen receptor antagonists on testosterone (T) and 17 β -estradiol (E2) concentrations in conditioned medium from sterlet liver explants. Liver explants from previtellogenic female sterlet were incubated for 72 h with vehicle control, 100 μ M 11-KT alone, 100 μ M flutamide (FLU) alone, 100 μ M fulvestrant (FUL) alone, or 100 μ M 11-KT combined with 10 or 100 μ M FLU or FUL. Concentrations of T (**A**) and E2 (**B**) were measured in the conditioned medium. Liver tissue from three donor fish was processed independently ($n = 3$ biological replicates). Data are presented as mean $\pm$ SEM. Different lowercase letters indicate significant differences among treatments ($p < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test.

4. Discussion

The present results indicate that exogenous 11-KT does not broadly activate all measured components of the reproductive endocrine axis in previtellogenic sterlet. Instead, its effects were tissue-specific and concentrated in 11-KT-responsive transcripts. In the brain-pituitary complex, the main response involved *fsh* and *fshr* rather than *gnrh* paralogs. In the ovary, 11-KT strongly affected *gnrh2*, *fsh*, *fshr*, and *lhr*, indicating that local ovarian GnRH- and gonadotropin-related pathways are important targets of androgenic stimulation. In contrast, hepatic GnRH- and gonadotropin-related genes showed only limited responses *in vivo*. These patterns suggest that the biological action of 11-KT is shaped by feedback regulation within the HPG axis, rather than by a simple linear stimulation of ovarian development. This dissociation between *fsh* responsiveness and limited *gnrh* transcriptional changes is consistent with emerging evidence that FSH regulation in teleosts may involve GnRH-independent or GnRH-complementary mechanisms [48].

The dissociation between *fsh/fshr* upregulation and the absence of significant changes in *gnrh1*, *gnrh2*, and *gnrh3* suggests that androgenic feedback on the FSH-related component of the brain-pituitary complex may not depend solely on increased transcription of classical *gnrh* ligands. One possibility is that 11-KT acts directly or indirectly at the pituitary level to enhance *fsh* expression or FSH-cell responsiveness. Another possibility is that GnRH-complementary neuroendocrine systems participate in this response. Recent work has identified cholecystokinin as an FSH-releasing hormone in teleosts [49]. However, CCK and other upstream neuropeptides were not examined here, and their possible involvement remains a hypothesis for future investigation.

A key issue raised by the present data is the role of FSHR signaling in early ovarian development. In teleosts, FSHR is widely regarded as a major regulator of follicle growth, steroidogenesis, and the transition into vitellogenesis, and genetic evidence in zebrafish (*Danio rerio*) has shown that disruption of gonadotropin signaling severely impairs ovarian development [50,51]. Stage-dependent involvement of gonadotropin receptors is also supported by recent work in cloudy catshark (*Scyliorhinus torazame*), in which FSHR was abundant in previtellogenic follicles, whereas LHR increased during vitellogenic and ma-

turing stages [52]. In anguillid models, this pathway appears especially relevant. Setiawan et al. (2012) reported androgen-specific regulation of FSH signaling in the previtellogenic ovary and pituitary of shortfinned eel, showing that 11-KT enhances ovarian responsiveness during early development [24]. More recent work in eel has likewise emphasized that initiation of vitellogenesis involves a coordinated endocrine network in which gonadotropin responsiveness, hepatic vitellogenin production, and steroid feedback are tightly linked [53].

In the present study, this stage-dependent framework was reflected in a dose-dependent ovarian response to 11-KT. The lower-dose treatment was associated with altered expression of selected ovarian endocrine-related genes, whereas the decline of *fshr* and *lhr* at the higher dose suggests that excessive androgen exposure may trigger receptor-level feedback or reduce ovarian sensitivity to gonadotropin signals. Thus, the action of exogenous 11-KT appears to be modulated by the HPG axis rather than acting as an unrestricted stimulatory signal. A similar trend has also been reported in cultured beluga, where 11-KT altered ovarian gonadotropin receptor transcripts during the previtellogenic period [25]. The simultaneous increase in ovarian *fsh* and decrease in *fshr* and *lhr* at the high dose is particularly relevant for practical application. It suggests that excessive androgenic stimulation may reduce ovarian responsiveness to gonadotropin signals even when endocrine ligand expression is elevated. Therefore, 11-KT-based intervention should be considered as a dose-sensitive strategy rather than a simple acceleration treatment.

The present findings also suggest that the ovarian GnRH system may participate in the local response to 11-KT, although this response was selective rather than generalized. Among the three GnRH paralogs examined, ovarian *gnrh2* was upregulated after 11-KT treatment, whereas *gnrh3* was reduced at the higher dose and *gnrh1* showed no significant change. This divergent pattern is consistent with the view that GnRH ligands in fishes are not functionally identical. Previous studies have shown that GnRH paralogs may differ in hypophysiotropic potency, tissue distribution, local expression patterns, and reproductive functions [11,54,55]. In Chinese sturgeon, different *gnrh* genes also show distinct tissue expression and steroid responsiveness, supporting the possibility that GnRH paralogs may have species- and tissue-specific roles in acipenserids [56]. At the same time, genetic studies in zebrafish have shown that disruption of individual or even multiple GnRH ligands can produce limited reproductive phenotypes, likely because of compensatory regulation by other neuroendocrine pathways [54,57]. Therefore, the opposite responses of ovarian *gnrh2* and *gnrh3* in the present study should not be interpreted as evidence that GnRH signaling is the primary mediator of 11-KT action. Rather, these changes suggest that local GnRH paralogs may contribute to fine regulation of ovarian endocrine responses during the previtellogenic stage. Because cell-specific localization, GnRH receptor expression, and functional activation were not examined, *gnrh2* and *gnrh3* should be regarded as tissue-specific transcriptional responses that require further validation.

Hepatic and ovarian steroid receptor genes were retained as contextual markers and are presented in Supplementary Figure S1 because comparable in vivo receptor responses to 11-KT have been reported previously in sterlet [26]. Therefore, the present study examined receptor-associated regulation of hepatic *vtg* expression more directly using the in vitro antagonist experiment. The differential responses to FLU and FUL suggest that 11-KT-induced hepatic *vtg* expression is more sensitive to ER antagonism than to AR antagonism. Blocking AR with FLU did not abolish 11-KT-induced *vtg* expression, whereas high-dose FUL reduced *vtg* expression and suppressed *era*. This pattern agrees with earlier work in shortfinned eel showing synergistic effects of estradiol and 11-KT on vitellogenin physiology [53], and with previous evidence that 11-KT promotes previtellogenic ovarian development and hepatic vitellogenin-related responses in sterlet [26]. Comparative stud-

ies in anguillid eels have likewise suggested that the onset of vitellogenesis depends on interactions among steroid hormones, hepatic responsiveness, and gonadotropin signaling rather than on a single linear pathway [58]. In this context, the parallel reduction in *era* and *vtg* expression under high-dose FUL treatment suggests that *era*-associated ER signaling may participate in 11-KT-induced hepatic *vtg* expression. However, because receptor protein abundance, receptor binding activity, and loss-of-function validation were not examined, the present data should be regarded as pharmacological evidence consistent with the possible involvement of *era*-associated signaling rather than definitive proof of direct mediation by ER α .

An important related question is why the liver showed only limited *in vivo* responses for most HPG-axis-related genes. Physiologically, this is not unexpected. In fish, liver is not a primary site for strong transcriptional regulation of upstream reproductive regulators such as GnRH or gonadotropin subunits; rather, its major reproductive role is to function as an effector organ that integrates endocrine input and produces yolk precursors such as VTG. The weak hepatic response of *gnrh1*, *gnrh2*, *gnrh3*, *fsh*, and *lh* in the present implantation experiment therefore likely reflects tissue specialization: liver is downstream of the hypothalamic-pituitary system and should be more responsive to steroidal and receptor-mediated signals than to broad activation of central reproductive genes. This interpretation is consistent with the present result that hepatic HPG-axis-related genes showed limited responses *in vivo*, and with previous studies showing that liver primarily acts as a steroid-responsive effector organ for vitellogenin production. Thus, under previtellogenic *in vivo* conditions, hepatic responsiveness appears to be constrained until an appropriate steroid-receptor context is reached, whereas direct *in vitro* exposure can unmask the hepatic vitellogenic capacity [53,58,59].

The E2 assay detected markedly increased immunoreactivity in FUL-containing media, particularly in the 11-KT + 100 μ M FUL treatment. The intra-assay variation was acceptable, and the manufacturer reported low cross-reactivity of the E2 assay with estriol, progesterone, and testosterone. However, cross-reactivity with FUL, 11-KT, FLU, or their metabolites was not evaluated. Therefore, analytical interference or detection of unidentified E2-like immunoreactive compounds cannot be excluded. The present experiment does not establish whether the increased signal reflects authentic E2 immunoreactivity, altered steroid metabolism, or assay-related interference. 11-KT is generally considered a non-aromatizable androgen and therefore cannot be directly converted into E2. Fulvestrant is a selective estrogen receptor downregulator that binds ER with high affinity, inhibits ER signaling, and promotes receptor downregulation [36], in contrast to antiestrogens with partial agonist activity [60]. At the same time, high-dose FUL reduced both *era* and *vtg* abundance. Thus, the increased E2 immunoreactivity was not accompanied by an enhanced *era/vtg* transcriptional response. The present experiment did not determine the origin of this increased signal. Possible explanations include the release of residual endogenous E2 or aromatizable precursors from the liver explants, altered hepatic steroid binding, conjugation, clearance, or metabolism under FUL treatment, and analytical interference caused by FUL or unidentified steroid metabolites. Although basal aromatase- or hydroxysteroid dehydrogenase-related activity could theoretically act on endogenous steroid precursors, these enzymes and metabolic pathways were not examined in the present study. Therefore, these explanations remain speculative and require validation using medium-only controls, independent steroid analysis such as LC-MS/MS, and measurements of steroid-metabolizing enzymes [61].

Among the proposed mechanisms for androgen-induced VTG synthesis, three main hypotheses remain relevant to the present study. The first is the aromatization hypothesis, in which androgens are converted to estrogens and then act through canonical ER

signaling [9,27,28]. This mechanism is unlikely to fully explain the present results because 11-KT is generally considered non-aromatizable. The second is the ER-mediated hypothesis, in which androgens or androgen-derived metabolites activate ER signaling directly or indirectly without requiring simple aromatization to E2 [9,29–32,36,53]. The third is the AR-mediated hypothesis, which predicts that androgen-induced hepatic *vtg* expression should be suppressed by AR antagonism [34]. The present data do not fit a purely AR-mediated model: flutamide did not abolish *vtg* expression, whereas high-dose fulvestrant reduced *vtg* expression and suppressed *era* expression. Although FUL-containing treatments caused marked E2 immunoreactivity, this increase occurred together with reduced ER-dependent transcriptional output and therefore should not be interpreted as evidence of enhanced estrogenic signaling. Taken together, the present results favor an ER-related interpretation while indicating that the metabolic and signaling relationships among 11-KT, endogenous steroids, and hepatic ER pathways remain incompletely resolved.

Taken together, the present results support a model in which exogenous 11-KT modulates selected HPG-axis and receptor-related response during previtellogenic ovarian development in sterlet. In the brain-pituitary complex, 11-KT mainly affected *fsh/fshr* signaling, whereas in the ovary it altered *gnrh2*, *fsh*, *fshr*, and *lhr* expression. In the liver, the in vivo response of HPG-axis-related genes was limited, but in vitro antagonist experiments suggest that 11-KT-induced hepatic *vtg* expression involves ER-related signaling, particularly the *era*-associated pathway. These findings are relevant to the commonly observed delay or arrest of ovarian development around the onset of vitellogenic deposition in farmed female sturgeons [62,63]. Recent work in lumpfish (*Cyclopterus lumpus*), for example, showed that integrated photoperiod and temperature manipulation can advance ovarian development and spawning while altering circulating T, 11-KT, and E2 levels, illustrating the practical value of endocrine-informed reproductive management [64]. From an aquaculture perspective, this dose-sensitive pattern is particularly relevant to the prolonged arrest or delay often observed around the transition from previtellogenic growth to vitellogenic deposition in farmed female sturgeons [26,63,65,66]. The marked suppression of ovarian *fshr* and *lhr* in the 25 mg/kg group suggests that high-dose androgen exposure may reduce ovarian responsiveness to gonadotropins, even when ovarian *fsh* expression is elevated. Therefore, practical application should not simply aim to maximize 11-KT levels. Instead, lower-dose or lower-release-rate regimens, with treatment timing and duration optimized empirically may be more appropriate for stimulating early ovarian endocrine activity while minimizing the suppression of ovarian gonadotropin receptor transcripts observed at the higher dose. These strategies should be evaluated together with endocrine monitoring, ovarian growth, vitellogenic progression, spawning performance, and egg quality.

5. Conclusions

Exogenous 11-KT selectively regulated HPG-axis-related genes in a tissue-dependent manner in previtellogenic sterlet. The main in vivo responses included increased *fsh/fshr* expression in the brain-pituitary complex and altered ovarian *gnrh2*, *fsh*, *fshr*, and *lhr* expression, whereas hepatic HPG-axis-related genes showed limited transcriptional changes. The reduction in ovarian *fshr* and *lhr* at 25 mg/kg suggests a dose-dependent feedback response that may reduce ovarian sensitivity to gonadotropin signals under excessive androgen exposure.

In liver explants, flutamide did not abolish 11-KT-induced *vtg* expression, whereas high-dose fulvestrant reduced both *era* and *vtg*. These differential antagonist responses provide preliminary in vitro pharmacological evidence consistent with ER-associated involvement, but direct receptor-mediated regulation was not demonstrated and requires

further functional validation. The increased E2 immunoreactivity detected in fulvestrant-containing media remains unresolved and should be verified using an independent analytical method.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes11080447/s1>, Figure S1. Effects of 30-day slow-release 11-ketotestosterone implantation on steroid receptor gene expression in the liver and ovary of previtellogenic female sterlet (*Acipenser ruthenus*). Fish were implanted with blank silastic strips (control), 5 mg/kg 11-KT, or 25 mg/kg 11-KT ($n = 6$ fish per group). Relative mRNA levels of *ar*, *era*, and *erb* were determined by qPCR and are presented as receptor-context information. Data are shown as mean $\pm$ SEM. Different lowercase letters indicate significant differences among groups ($p < 0.05$). Figure S2. Histological confirmation of the previtellogenic ovarian stage in female sterlet following 30-day implantation with blank silastic strips (control), 5 mg/kg 11-KT, or 25 mg/kg 11-KT. Representative ovarian sections from the control (A), 5 mg/kg 11-KT (B), and 25 mg/kg 11-KT (C) groups show different ovarian stage characterized with a distinct follicular layer, germinal vesicle, nucleoli, and oogonia. Abbreviations: GV, germinal vesicle; NU, nucleolus; OC, oocyte; OG, oogonia. Scale bars = 50 μ M. Scale bars = 400 μ m.

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Abbreviations

The following abbreviations are used in this manuscript:

11-KT	11-Ketotestosterone
T	Testosterone
E2	17 β -Estradiol
HPG-axis	Hypothalamic-Pituitary-Gonadal axis
GnRH1	Gonadotropin-Releasing Hormone 1
GnRH2	Gonadotropin-Releasing Hormone 2
GnRH3	Gonadotropin-Releasing Hormone 3
FSH	Follicle-Stimulating Hormone

FSHR	Follicle-Stimulating Hormone Receptor
LH	Luteinizing Hormone
LHR	Luteinizing Hormone Receptor
FUL	Fulvestrant
FLU	Flutamide
ar	Androgen Receptor
er	Estrogen Receptor
vtg	Vitellogenin
bactin	β -actin
ef1a	Elongation Factor 1 Alpha
rpl6	Ribosomal Protein L6
rpl13	Ribosomal Protein L13

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