

# Gonadal Development and Associated Changes in Estradiol, Thyroid Hormones, and Sex-Related Genes During Different Growth Stages in Cultured Female Rainbow Trout (*Oncorhynchus Mykiss*) in Yunnan

**Lingfu Kong**

Yunnan Agricultural University

**Baoliang Bi**

Yunnan Agricultural University

**Yanhua Su**

Yunnan Agricultural University

**Hua Rong**

Yunnan Agricultural University

**Qing Hu** (✉ [huqinggw@126.com](mailto:huqinggw@126.com))

Yunnan Agricultural University

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## Research article

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# Abstract

**Background:** Rainbow trout (*Oncorhynchus mykiss*) is an important fishing resource in Yunnan. However, the oocyte quality and fecundity are particularly affected by environmental factors when fish are undergoing sexual maturation, which hinders the development of the *O. mykiss* industry.

**Results:** Estradiol ( $E_2$ ), thyroid hormones (THs), and sex-related genes transcription associated with gonadal development were analyzed in different growth stages of *O. mykiss* cultured in Yunnan. Females were classified into four growth stages: growth stage I, growth stage II, growth stage III, and growth stage IV. Histological analysis found a correlated relationship between body growth and ovary development. Serum levels of  $E_2$  and THs increased during ovarian development and reached the highest level at growth stage III indicated that  $E_2$  and THs play important roles in the development and maturation of oocytes in *O. mykiss*. Furthermore, real-time PCR analysis showed that the vitellogenin mRNA (*vtg*) transcription level increased significantly both in ovaries and liver during ovarian development, then reaching its peak at the post-vitellogenetic (growth stage IV) period. The same transcription trend was detected in the levels of ovarian estrogen receptors (*ers*).

**Conclusions:** These results indicated that the high level of  $E_2$  promotes the transcription of the *vtg* mediated by different types of *ers*, and then accelerates the maturation of oocytes, and THs also participate in the regulation of maturation of oocytes in cultured *O. mykiss* during the individual development in Yunnan.

## Background

Yunnan located in the Yunnan-Guizhou Plateau are topographically and climatically diverse. Yunnan is in a subtropical humid monsoon climate zone that includes tropical, subtropical, temperate and boreal climates [1]. Environmental conditions such as water temperature, water quality, lighting, and dietary nutrition, which in turn affect production traits including gonads development, reproduction, and growth in fish [2–4]. Rainbow trout (*Oncorhynchus mykiss*) is an important fishing resource in Yunnan, and it is also an expanding industry in several regions. Because of the oocyte quality and fecundity are particularly affected by environmental factors when fish are undergoing sexual maturation, it is important to attain knowledge about the oocyte development and how factors such as serum hormones and sex-related genes involved in growth changed in cultured *O. mykiss* in Yunnan.

In teleost, it is well known that the hypothalamic-pituitary-gonadal (HPG) axis regulates the vitellogenesis and the final oocyte maturation during individual growing that mediate gonadotropic hormones (GtHs) actions via steroids secreted by the follicular cells surrounding the oocyte [5]. Correlation between changes in levels of sexual steroids (testosterone and  $17\beta$ -estradiol ( $E_2$ )) and oocyte development have been well documented in fish [6–8]. Moreover, studies showed that the hypothalamus-pituitary-thyroid (HPT) axis is also involved in gonad development [9]. For instance, thyroid hormones (THs) amplified the effects of GtH on  $E_2$  secretion by isolated ovarian follicles in rainbow trout (*Salmo gairdneri*) [10]. In

Atlantic salmon (*Salmo salar*), THs were elevated before vitellogenesis and then decreased around the time when E<sub>2</sub> and vitellogenin (VTG) levels were known to rise [11]. However, in female brown trout (*Salmo trutta*), the discrepancies of plasma sex steroids observe between wild and cultured females may be due to differences in stress susceptibility, environmental conditions, life cycles, or to genetic divergence between the strains [12]. Therefore, changes in serum hormones during individual growth are important to be characterized and it will broaden the understanding of the relationship with the oocyte development and serum hormones in cultured *O. mykiss* in Yunnan.

VTG is a glycolipophosphoprotein produced in all oviparous species, which is biosynthesized in the liver under estrogen regulation and transported to the growing oocytes participating in the process of yolk formation [13, 14]. In the liver of mature females teleost, *vtg* transcription is stimulated via estrogen receptors (*ers*) signaling pathways due to the simultaneous increase of estrogen concentration [15]. Matured VTG produced in the liver was then transported to the ovary through the circulatory system. Thus, the amounts of *vtg* transcription and translation have been widely used as biomarkers during oocyte development in certain teleost. In addition, *ers* play a crucial role in the mediation of estrogen activities. Four *er* isoforms (*era1*, *era2*, *erβ1*, and *erβ2*) have been detected and dominantly expressed in the liver of *O. mykiss* [16]. These receptors may be important for estrogen signal conduction that initiates the next cycle of germ cell development in the ovary [17]. Therefore, understanding the function of *ers* in oocyte development during growing processes will be beneficial for improving the quality of cultured *O. mykiss* oocytes in the future.

Although the endocrine system of *Oncorhynchus* species has been relatively well studied, limited information is available about the relationship among oocyte development, serum hormones and sex-related genes related to different growth stages. In the present study, we described the characteristics of oocyte development and analyzed the regulatory relationship among E<sub>2</sub>, THs, and sex-related genes during different growth stages of *O. mykiss*. This study aims to clarify the regulation of oocyte development of *O. mykiss* cultured in plateau of Yunnan.

## Results

### Histology

Different growth stages of oocytes observed in the ovaries of *O. mykiss* were shown in Fig. 1. On growth stage Ⅰ and growth stage Ⅱ, oocytes at primary yolk stage appeared and showed a higher percentage among other oocyte groups. On growth stage Ⅲ, ovaries were mostly occupied by filling yolk stage oocytes. On growth stage Ⅳ, mature oocytes filled the whole ovary (Fig. 1).

**Figure 1** Histological sections of the ovaries of *O. mykiss* in different growth stages

(a) Oocyte at growth stage Ⅰ, (b) Oocyte at growth stage Ⅱ, (c) Oocyte at growth stage Ⅲ, (d) Oocyte at growth stage Ⅳ, O: Oocyte. The scale is 50 μm.

## Serum hormones profile in female *O. mykiss*

$E_2$  levels in female *O. mykiss* ranged from 5.958 pg/mL to 1496.072 pg/mL in Fig. 2.  $E_2$  content was positively correlated with individual growth and oocyte development. There was no significant difference between growth stage I and growth stage II. However, as the growth of *O. mykiss*, significantly elevated of  $E_2$  level has been detected in growth stage III ( $p < 0.05$ ). Moreover, the highest value was recorded in growth stage III when the oocytes were matured ( $p < 0.05$ ).

The levels of THs ( $T_4$ ,  $FT_4$ ,  $T_3$ ,  $FT_3$ ) peaked in growth stage III ( $p < 0.05$ ) and were correlated with *O. mykiss* growth and oocyte development. No significant difference in  $T_4$  was detected among growth stage I, growth stage II, and growth stage III. But the highest level of  $T_4$  was showed in growth stage III.  $T_3$  showed an increasing trend during *O. mykiss* growing and reached the highest level in growth stage III. The trend of  $FT_4$  during *O. mykiss* growing is similar to that of  $T_4$ , while a higher level was also found in growth stage III.  $FT_3$  started to increase from growth stage I and maintain high levels during the mature stage (Fig. 3).

### Figure 2 Changes of serum $E_2$ at different growth stages in *O. mykiss*

The  $E_2$  levels in each growth stage is presented as mean  $\pm$  SD ( $n = 3$ ). Different superscript letters indicate significant differences among different growth stages ( $p < 0.05$ ).

### Figure 3 Changes of serum THs at different growth stages in *O. mykiss*

(a) Changes of serum  $T_4$ ; (b) Changes of serum  $T_3$ ; (c) Changes of serum  $FT_4$ ; (d) Changes of serum  $FT_3$ . The THs in each growth stage is presented as mean  $\pm$  SD ( $n = 3$ ). Different superscript letters indicate significant differences among different growth stages ( $p < 0.05$ ).

## Transcription profiles of sex-related genes during different growth stages in *O. mykiss*

Variations of the *ers* transcription during the different growth stages were analyzed in this study (Fig. 4a). The *ers* showed an irregular trend throughout the different growth stages, and on significant differences have been detected in the liver. In contrast, significant increases of *era1* and *er $\beta$ 1* transcription have been detected in the gonad and the highest level was shown in growth stage III ( $p < 0.05$ ), while *er $\beta$ 2* did not fluctuate significantly during the different growth stages.

*vtg* transcription was also continuously detected in all the analyzed individuals from 80.0 to 1200.0 g. The relative *vtg* transcription in the liver was low of all detected individuals at growth stage I. However, when the individuals continue to grow, *vtg* transcription is gradually upregulated, and the highest level was detected in growth stage III. Similarly, the transcription of *vtg* in gonads was low of all detected individuals at growth stage I, growth stage II, and growth stage III. However, when the individuals growing enter into stage III, *vtg* transcription showed a big fluctuation and significantly increased (Fig. 4b).

#### Figure 4 Relative transcriptions of sex-related genes of *O. mykiss* at different growth stages

(a) heatmap of *ers* mRNA transcription in liver and gonad, red represents a higher transcription and purple represent a lower transcription. (b) *vtg* transcription level in liver and gonad. Values significantly difference are indicated by asterisks (\* $p < 0.05$ , \*\*  $p < 0.01$ ).

## Discussion

In this paper, we illustrated the relationship among the oocyte development,  $E_2$ , THs, and sex-related genes in *O. mykiss* during the different growth stages. The results showed that these indicators have an increasing trend among different growth stages associated with the oocyte development of cultured *O. mykiss* in Yunnan.

Investigating the developmental period of the oocytes during growing can reduce the time and cost to determine whether the cultured *O. mykiss* has reached sexual maturity. The result showed that oocytes at the primary yolk stage appeared and showed a higher percentage among other oocyte groups during 80–180 g of body weight. When the body weight above 700–1200 g, mature oocytes filled the whole ovary and can be ready for spawning. Study in sequential hermaphroditism species marbled swamp eel (*Synbranchus marmoratus*) showed that the females were sexually active with the mean total body length of 43.5 cm while the secondary males with about 58.5 cm [18]. Moreover, year 2–3 ovaries of 0.85–1.63 kg body weight were at stage I and year 4–5 ovaries of 3.67–6.30 kg body weight were at stage II in different ages of Amur sturgeon (*Acipenser schrenckii*) [19]. Hence, there is a correlated relationship between body growth and ovary development. On the other hand, as a key hormone to promote the development of gonad in fish, the serum concentrations of  $E_2$  significantly increased during the body growth of *O. mykiss* in this study, which corresponded with the development of ovarian histology. This result is consistent with the study of *S. trutta* in which the peak level of plasma  $E_2$  were observed at 30 d before ovulation in the wild strain [12]. In cultured strain of *S. trutta*, the  $E_2$  levels were the highest at the beginning and declined throughout two weeks before ovulation [12]. In female *S. salar*,  $E_2$  were low from December to July and increased to peak levels in September and October during reproductively maturing period, and then decreased significantly during the period of ovulation in November [11]. In contrast, the levels of  $E_2$  were not significantly different among different gonadal development stages in Sterlet (*Acipenser ruthenus*) [20]. These results indicated that  $E_2$  has different influences on different fish species and strains. Therefore, high level of  $E_2$  contribute to the development and maturation of oocytes of cultured *O. mykiss* in this study.

THs also play important roles in the regulation of many biological processes in vertebrates, such as growth, metabolism, morphogenesis, and reproduction [21]. An early study demonstrated that THs can promote all stages of reproduction including the early stages gonadal (oocyte) development, vitellogenesis, and maturation leading to successful ovulation and spawning success [22]. Similarly, a recent study showed that the treatment to female goldfish (*Carassius auratus*) with  $T_3$  resulted in an

increase of VTG [9]. In contrast, rabbitfish (*Siganus guttatus*) fed  $T_4$  during vitellogenesis did not advance oocyte maturation, but could induce spawning [23]. Moreover, in female *S. trutta* from a wild strain, the increase in plasma THs around the time of ovulation had an influence on final maturation of oocytes, while the absence of any appreciable change in profile of  $T_4$  indicated that the increase of THs are not necessary for ovarian maturation in cultured strain [12]. In our study, THs ( $T_3$ ,  $T_4$ ,  $FT_3$ ,  $FT_4$ ) all peaked when the *O. mykiss* growing entered into stage Ⅱ, which was correlated with ovarian development. Similar to  $E_2$ , elevated levels of THs contribute to the growing and final maturation of oocytes in cultured *O. mykiss*. Comparison of these results are complicated by several factors including different species and stock.

$E_2$  is synthesized by the cooperation of the theca and granulosa cell layers surrounding the oocytes, and secreted into the blood subsequently. VTG is produced by the liver in an estrogen-dependent manner by entering through diffusion, binding and activating the ERs [24]. Only three subtypes of *ers* (*era1*, *erβ1*, and *erβ2*) can be detected in our study. In teleost, *ers* expressed early during embryonic development and gonadal differentiation, suggesting the important role for estrogen in sexual differentiation in the early stage [25]. Estrogen receptor genes, such as *era* and *erβ2*, are involved in  $E_2$ -induced liver vitellogenesis in medaka (*Oryzias latipes*), especially *erβ2* [26]. In female zebrafish (*Danio rerio*), higher transcription levels of *era* and *erβ2* were detected in the liver than *erβ1* [27]. Similarly, as an important estrogen receptor gene subtype, *era* is also participated in the synthesis of liver vitellogenin induced by  $E_2$  in several teleost fish [28]. Moreover, the up-regulation of *era* mediated by normal  $E_2$  is regulated by *erβ1* and *erβ2* [29]. In this study, as the growth of *O. mykiss*, there was no significant correlation among the transcriptions of *ers* in the liver with the ovarian development. However, the transcription of *vtg* in the liver gradually raised and reached its peak at growth stage Ⅱ indicated that significantly increasing of *vtg* transcription in the liver is stimulated by high levels of  $E_2$  instead of *ers* in the later stage of vitellogenesis. Furthermore, the study of female Korean rockfish (*Sebastes schlegeli*) showed *erβ1* and *erβ2* highly expressed in the ovary at the vitellogenetic stage, which indicated that *ers* might play an important role in gonadal development [30]. In orange-spotted grouper (*Epinephelus coioides*), *era* highly expressed in mature ovaries, *erβ1* mainly expressed in immature ovaries and *erβ2* varied greatly during ovarian development. It speculated the potential roles of *ers* during female maturation [31]. The highest levels of *era* mRNA were found in late vitellogenetic ovaries of *O. mykiss* [32]. Similarly, in the present study, it was shown that ovarian *ers* (including *era* and *erβ1*) were sharply increased during *O. mykiss* growing and oocyte maturation. This result revealed an important role of ovarian *ers* in the maturation of *O. mykiss* oocyte cultured in Yunnan.

The *vtg* gene, which encodes VTG, is a precursor of lipo- and phospho-proteins involved in the formation of the yolk during oogenesis in the majority of vertebrates [33]. The previous study reported that *vtg* is mainly expressed in the liver, but also various extrahepatic *vtg* transcription was observed in fishes [34]. In female *A. ruthenus*, *vtg* transcription has been detected both in the liver and ovary, and they both reached its peak at the late vitellogenetic stage [35]. During the *O. mykiss* growing and ovary development, *vtg* transcription in this study exhibited an increasing trend both in the liver and ovaries, and reached its peak at the late growth and vitellogenetic stage (growth stage Ⅱ). In general, the rise in *vtg*

level in both liver and ovaries during vitellogenesis period, which correspond with the VTG and eggshell proteins incorporation into the oocyte, and the accumulation of enough nutrients for developing embryos [36]. We also propose that the liver and ovaries are both the sites of VTG synthesis in *O. mykiss*. Moreover, the transcription of *vtg* was in parallel with the level of serum  $E_2$ , indicated that  $E_2$  plays an important role in inducing the transcription of *vtg*. Summarizing the results of *ers* transcription, it demonstrated that the high level of  $E_2$  promotes the transcription of the *vtg* mediated by different types of *ers*, and then accelerates the maturation of oocytes in cultured *O. mykiss*.

## Conclusions

In conclusion, it was found that oocyte development,  $E_2$ , THs, and sex-related genes all significantly increased during the growth of *O. mykiss*. The serum concentrations of  $E_2$  corresponded with the development of ovary during *O. mykiss* growing indicated that  $E_2$  plays an important role in the development and maturation of oocytes. Similarly, THs ( $T_3$ ,  $T_4$ ,  $FT_3$ ,  $FT_4$ ) peaked at the highest level in growth stage Ⅳ reflected that THs contribute to the *O. mykiss* growing and final maturation of oocytes. Increasing levels of *ers* and *vtg* transcription during *O. mykiss* growing demonstrated an important role of *ers* in the maturation of oocytes. Therefore, the high level of  $E_2$  promotes the transcription of the *vtg* mediated by different types of *ers*, and then accelerates the maturation of oocytes in cultured *O. mykiss*.

## Methods

### Fish sampling

The cultured *O. mykiss* was obtained from Tanghao Aquaculture Company located in Kunming, China. Sampling permissions were also obtained for the Tanghao Aquaculture Company. Sampling was carried out in July when fish were in the peak of growth and development. The bodyweight of *O. mykiss* ranged from 80.0 g to 1200.0 g ( $n = 12$ ). They were divided into four different growth stages, which are growth stage Ⅰ (80–120 g), growth stage Ⅱ (160–180 g), growth stage Ⅲ (280–400 g), growth stage Ⅳ (700–1200 g), respectively. Fish were fasted for 24 h and then anesthetized by 100 mg/L tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO) till the cessation of opercular movements for about 5 min before sampling. Blood samples were withdrawn from the fish caudal vein with a syringe and centrifuged at 1,500 *g* for 30 min. The serum of fishes in each growth stage was pooled and then quickly frozen at  $-80\text{ }^\circ\text{C}$  for sequential detect of  $E_2$ , and THs ( $T_3$ ,  $T_4$ ,  $FT_3$ ,  $FT_4$ ). Then, the ovaries were rapidly excised and fixed in Bouin's fluid for determined the histological characteristics. Furthermore, the ovaries and liver of each fish were frozen at  $-80\text{ }^\circ\text{C}$  for the determination of gene transcription. The usage of fish was in strict accordance with the recommendations of the Guidelines for the Use of Experimental Animals of Yunnan Agricultural University. The protocol for animal care and handling used in this study was approved by the Committee on the Ethics of Animal Experiments of Yunnan Agricultural University.

### Histology

For histological studies, ovaries of each growth stage were embedded in paraffin and sectioned at 5–6 µm. Sections were stained with hematoxylin and eosin. After staining, the slides were allowed to dry and then examined with a light microscope (Olympus BX51, Japan).

## **E<sub>2</sub> analyses**

The levels of E<sub>2</sub> were measured by using commercially available <sup>125</sup>I-RIAs assay (Beijing North Institute of Biotechnology Co., Ltd., Beijing, China) in serum according to the manufacturer's instruction. The kit uses competitive radioimmunoassay to simultaneously compete for binding of radiolabeled antigens and non-labeled antigens to a limited amount of specific antibodies. The radioactive count of the labeled antigen-antibody complex is determined by separating the unbound labeled antigen. The content of E<sub>2</sub> in the samples was calculated using the standard curve and the mathematical model of the RIAs. All samples were analyzed in five duplicates. The RIAs for E<sub>2</sub> in the *O. mykiss* serum samples were validated by demonstrating parallelism between a series of diluted and spiked samples to the standard curve.

## **Thyroid hormones analyses**

THs in serum were measured using commercial ELISA kits purchased from Beijing North Institute of Biotechnology Co., Ltd., Beijing, China, following the manufacturer's instructions. All samples were analyzed in three duplicates and a separate standard curve was run for each ELISA plate. The assay sensitivities were 10 ng/mL, 0.25 ng/mL, 1.08 pmol/L, and 0.38 pmol/L, for T<sub>4</sub>, T<sub>3</sub>, FT<sub>4</sub>, and FT<sub>3</sub>, respectively.

## **RNA extraction and reverse transcription-PCR**

Total RNA in ovaries and liver of each fish was extracted using TRIpure reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the manufacturer's instructions. The concentration of RNA was measured using NanoDrop 2000c (Thermo Scientific, USA). Total RNA with the amount of 1 µg of each sample was used for reverse transcription with TRUEScript 1st Strand cDNA Synthesis Kit with gDNA Eraser (Aidlab Biotechnologies Co., Ltd., Beijing, China) in a final reaction volume of 20 µL.

## **Transcription of sex-related genes**

Relative quantitative real-time PCR was used to detect the transcription of sex-related genes. The primers for genes used in this study were shown in Table 1. Elongation factor 1 $\alpha$  (*ef1a*) was used as the reference gene. The specificity of each pair of primers was verified via the only peak of the melting curve. PCR reactions (20 µL) contained 1 µL of cDNA diluted five times, 0.5 µL of 10 mM of each primer, and 10 µL of 2 × SYBR qPCR Mix (Aidlab Biotechnologies Co., Ltd., Beijing, China). Amplification of these genes was: pre-heating at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and a final extension step at 72 °C for 30 s. The samples were analyzed in triplicate in a Light Cycler 480II/96 (Roche Diagnostics International Ltd, Switzerland). Negative control was included in each assay without cDNA. The mRNA transcription level of each gene was calculated by the  $2^{-\Delta\Delta C_t}$  method [37].

Table 1  
Nucleotide primers used in Real-time PCR

Gene	Primer name	Sequence (5' - 3')	GenBank No. or Article source
<i>vtg</i>	<i>vtg</i> -F	GTGGACTGGATGAAGGGACA	AY049952.1
	<i>vtg</i> -R	AGAGCGGCTCAGGTTGGAAT	
<i>era1</i>	<i>era1</i> -F	CCCTGCTGGTGACAGAGAGAA	[16]
	<i>era1</i> -R	ATCCTCCACCACCATTGAGACT	
<i>erβ1</i>	<i>erβ1</i> -F	CCCAAGCGGGTCCTAGCT	[16]
	<i>erβ1</i> -R	TCCTCATGTCCTTCTGGAGGAA	
<i>erβ2</i>	<i>erβ2</i> -F	CTGACCCCAGAACAGCTGATC	[16]
	<i>erβ2</i> -R	TCGGCCAGGTTGGTAAGTG	
<i>ef1α</i>	<i>ef1α</i> -F	AGGCCATCTGATCTACAAGTGC	AF498320.1
	<i>ef1α</i> -R	GGTGATACCACGCTCCCTCT	

[Table 1]

## Statistical analysis

Data analysis was performed using SPSS 16.0 software (IBM, USA). The differences among each stage were evaluated by one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) post-hoc test. All the data were expressed as mean  $\pm$  standard deviation (SD).

## Abbreviations

$E_2$ : estradiol; *ef1α*: elongation factor 1  $\alpha$ ; *ers*: estrogen receptors; GtHs: gonadotropic hormones; THs: thyroid hormones ( $T_3$ : triiodothyronine;  $T_4$ : thyroxine;  $FT_3$ : free triiodothyronine;  $FT_4$ : free thyroxine); VTG: vitellogenin; *vtg*: vitellogenin mRNA

## Declarations

### Ethics approval and consent to participate

The usage of fish was in strict accordance with the recommendations of the Guidelines for the Use of Experimental Animals of Yunnan Agricultural University. The protocol for animal care and handling used in this study was approved by the Committee on the Ethics of Animal Experiments of Yunnan Agricultural University.

### Consent for publication

Not applicable.

### **Availability of data and materials**

Not applicable. All data generated or analyzed during this study are included in this article.

### **Competing interests**

The authors declare that they have no conflict of interest.

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### **Authors' contributions**

QH designed the experiments and analyzed the experimental results. LFK and BLB carried out the experiments and wrote the manuscript. YHS, HR and QH helped to finish the experiments and the manuscript. All authors read and approved the final manuscript.

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### **Authors' Information**

1. Lingfu Kong

E-mail Address: 309011288@qq.com

Institution: Yunnan Agricultural University

2. Baoliang Bi

E-mail Address: 1448151770@qq.com

Institution: Yunnan Agricultural University

3. Yanhua Su

E-mail Address: 522852288@qq.com

Institution: Yunnan Agricultural University

4. Hua Rong

E-mail Address: 418516991@qq.com

Institution: Yunnan Agricultural University

5. Qing Hu

E-mail Address: huqinggw@126.com

Institution: Yunnan Agricultural University

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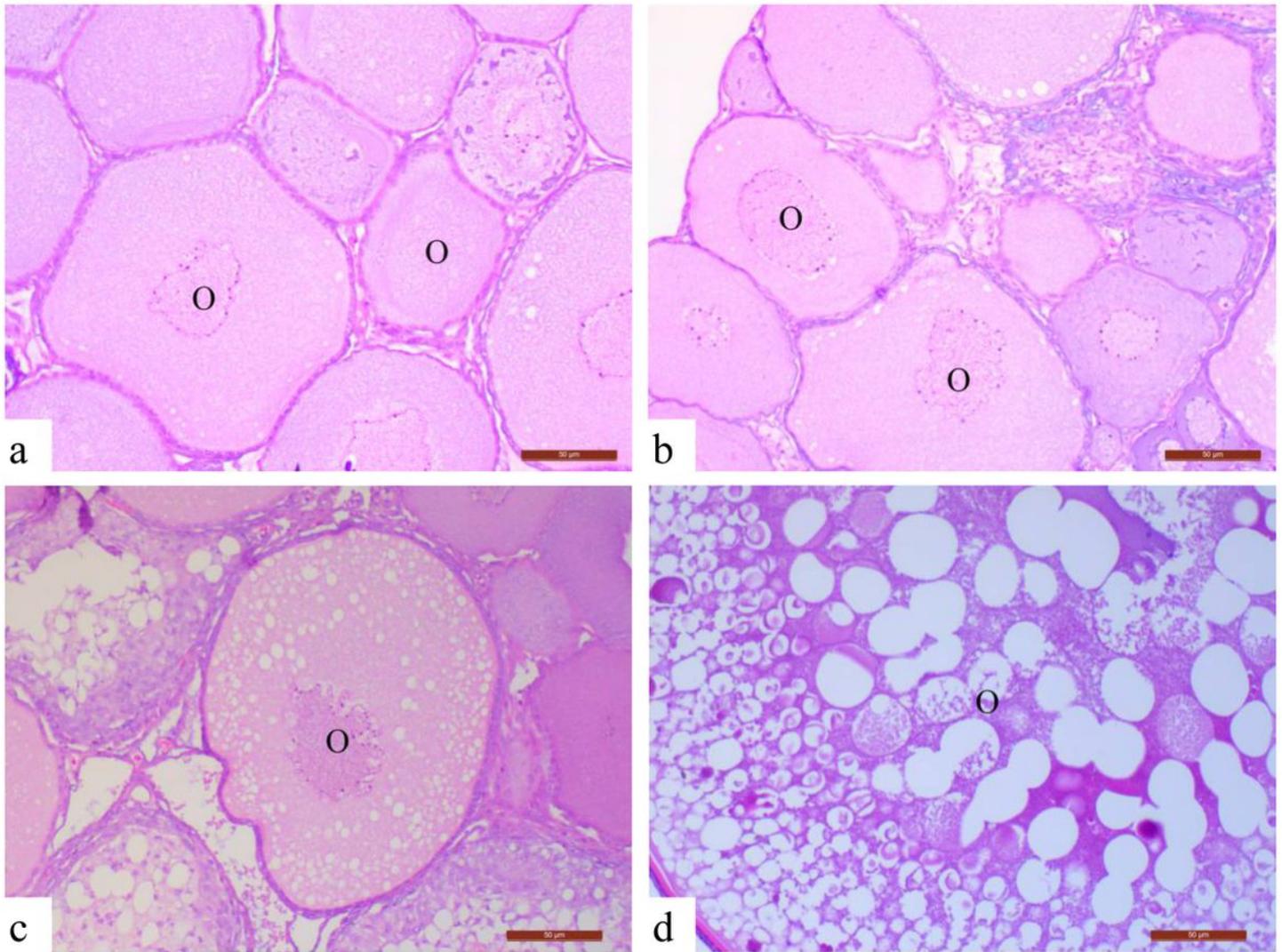
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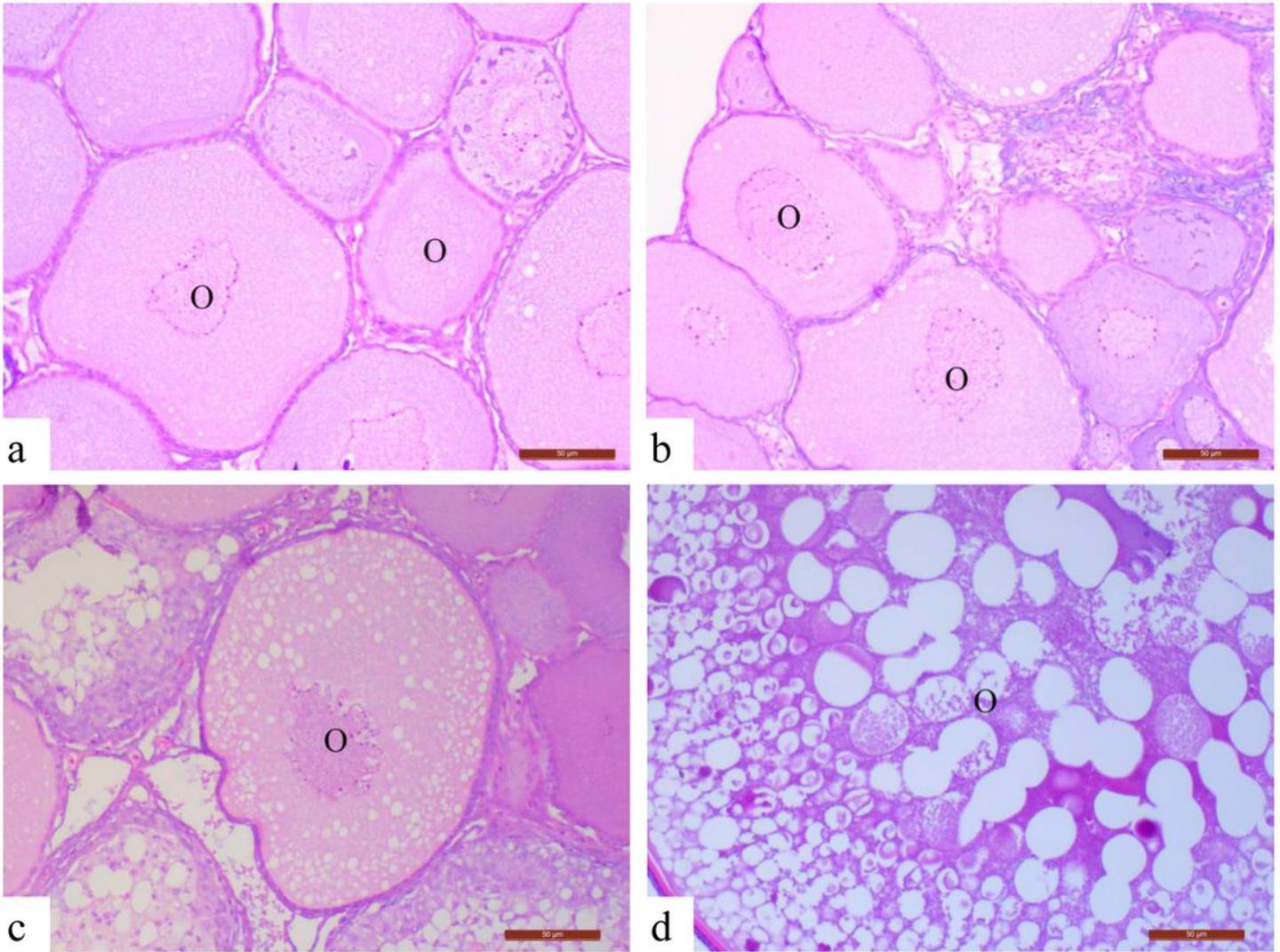
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## Figures



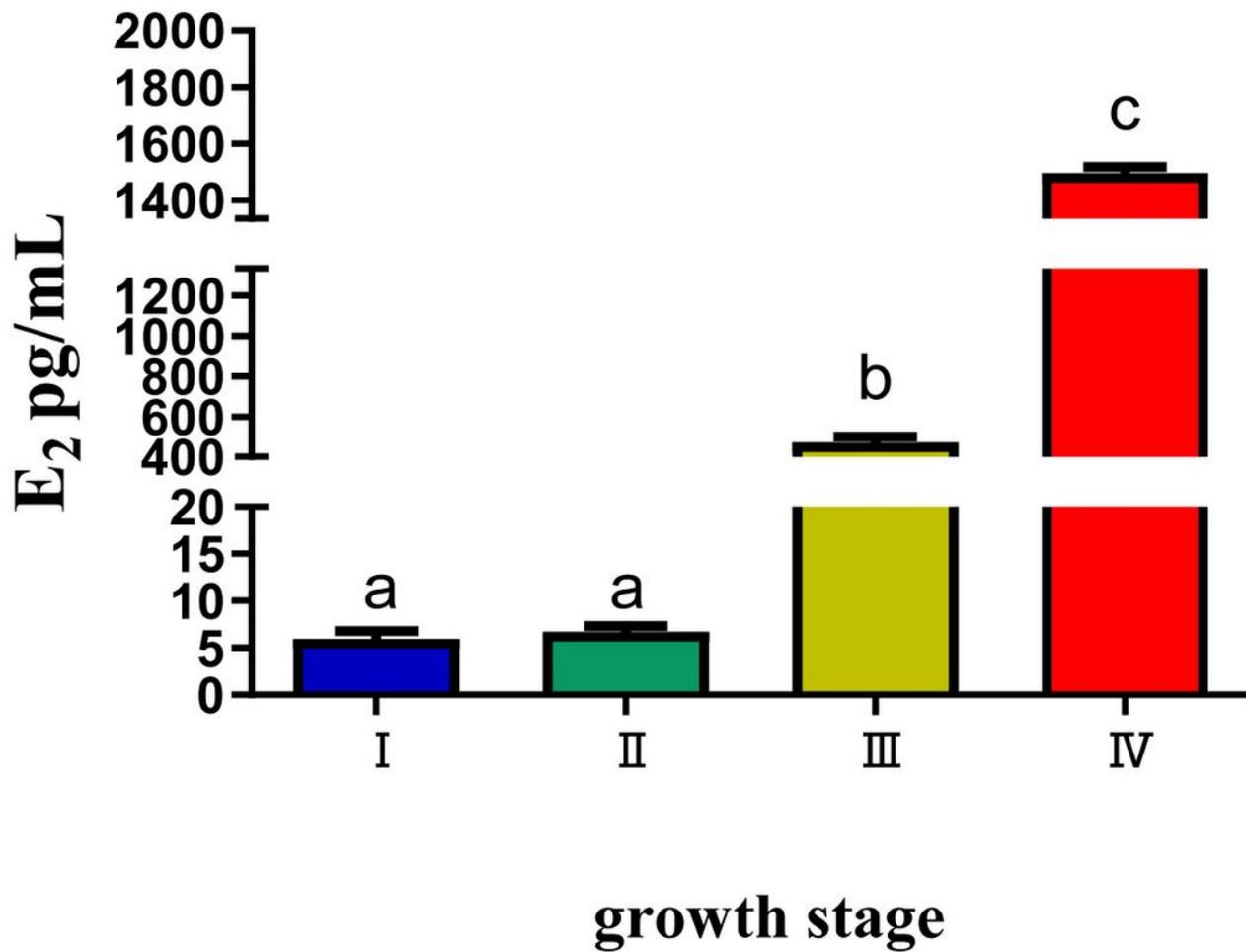
**Figure 1**

Histological sections of the ovaries of *O. mykiss* in different growth stages (a) Oocyte at growth stage I, (b) Oocyte at growth stage II, (c) Oocyte at growth stage III, (d) Oocyte at growth stage IV, O: Oocyte. The scale is 50 µm.



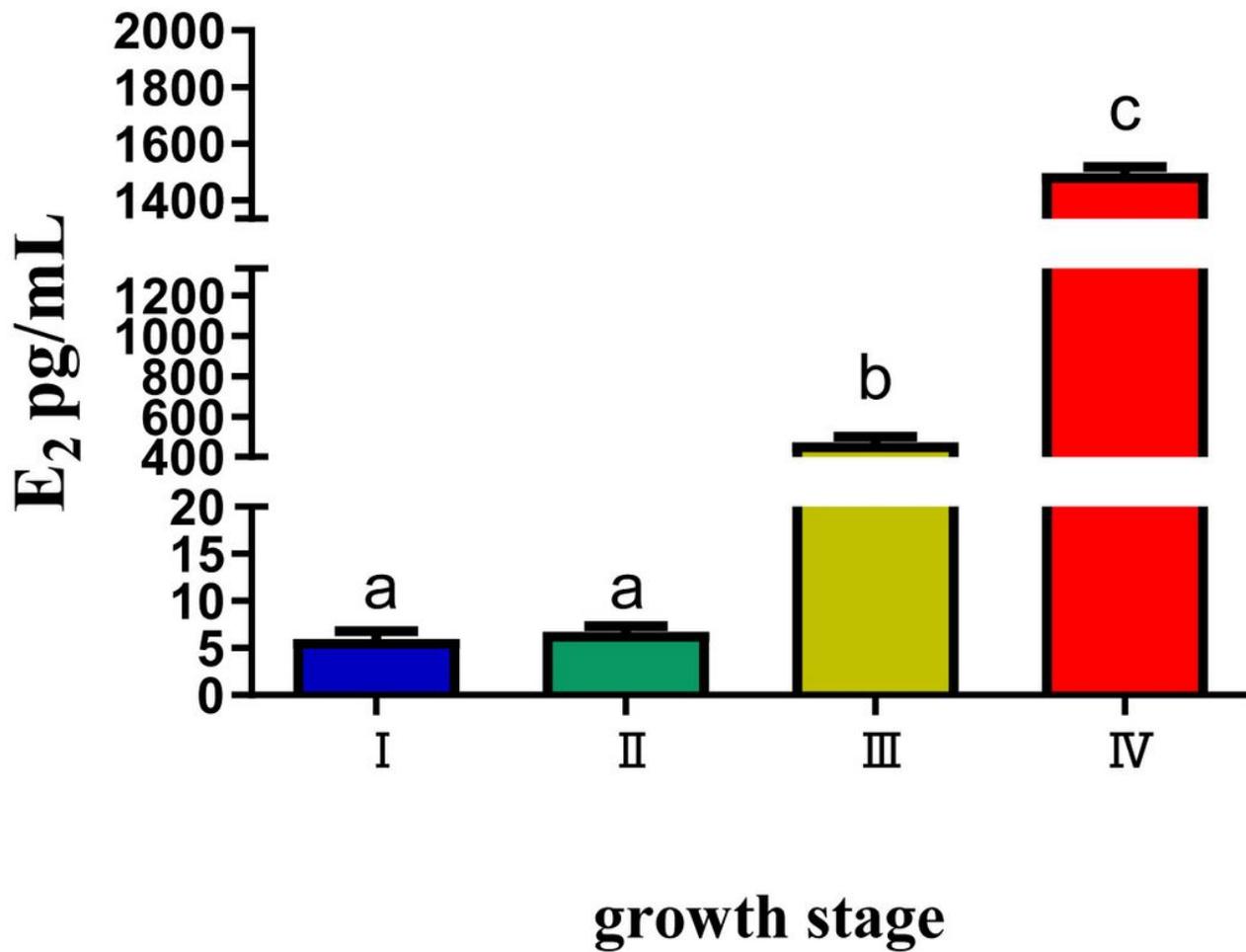
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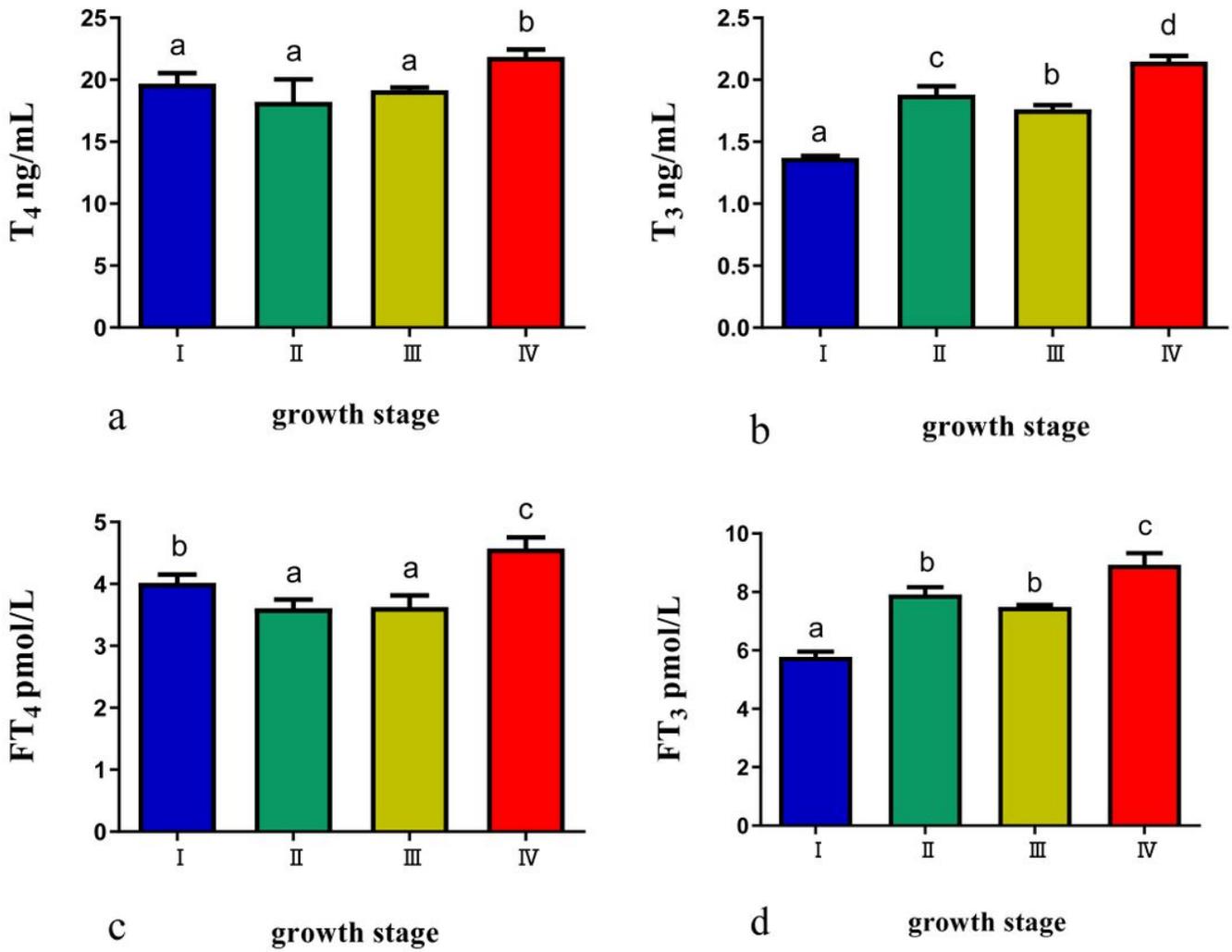
**Figure 2**

Changes of serum E2 at different growth stages in *O. mykiss*. The E2 levels in each growth stage is presented as mean  $\pm$  SD (n = 3). Different superscript letters indicate significant differences among different growth stages (p < 0.05).



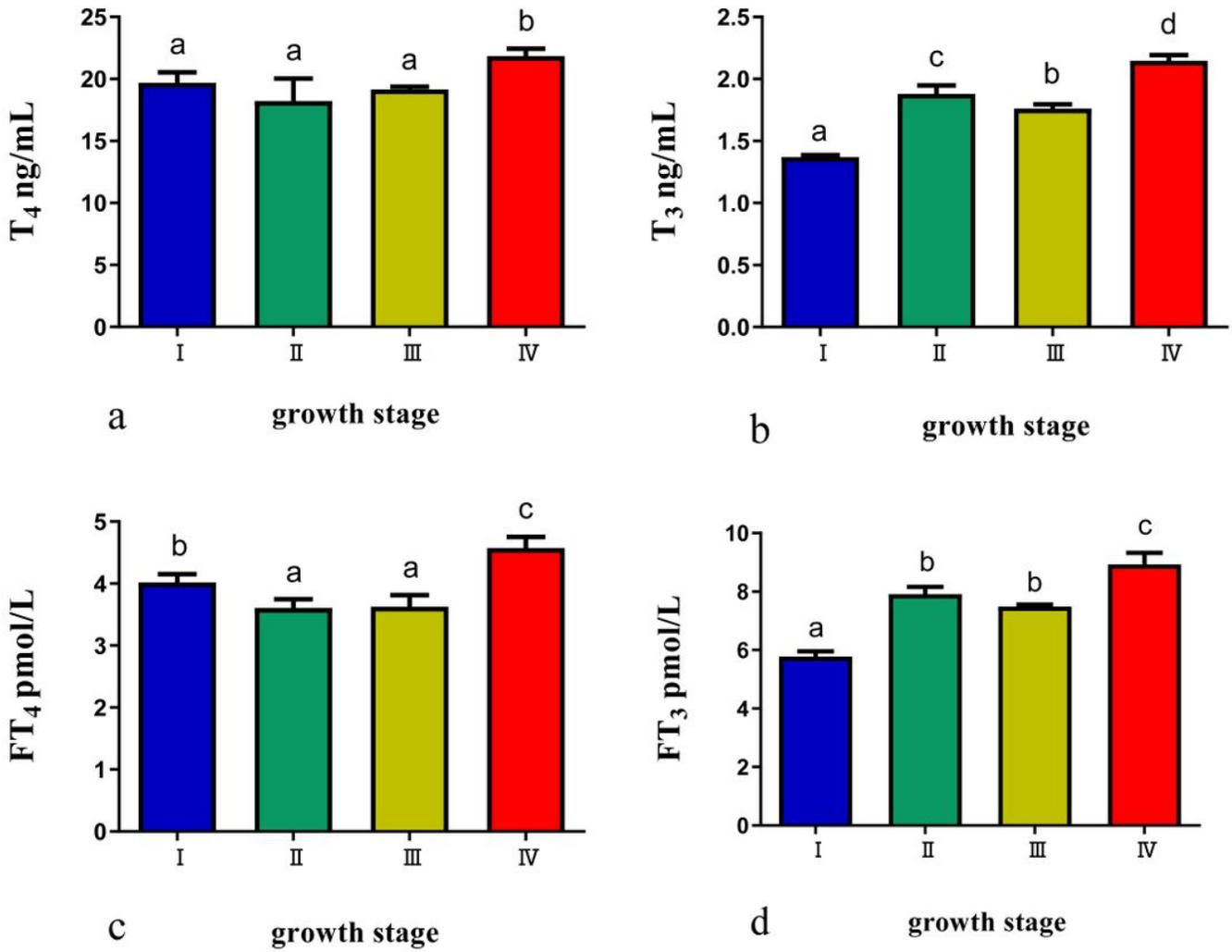
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Changes of serum E2 at different growth stages in *O. mykiss*. The E2 levels in each growth stage is presented as mean  $\pm$  SD (n = 3). Different superscript letters indicate significant differences among different growth stages ( $p < 0.05$ ).



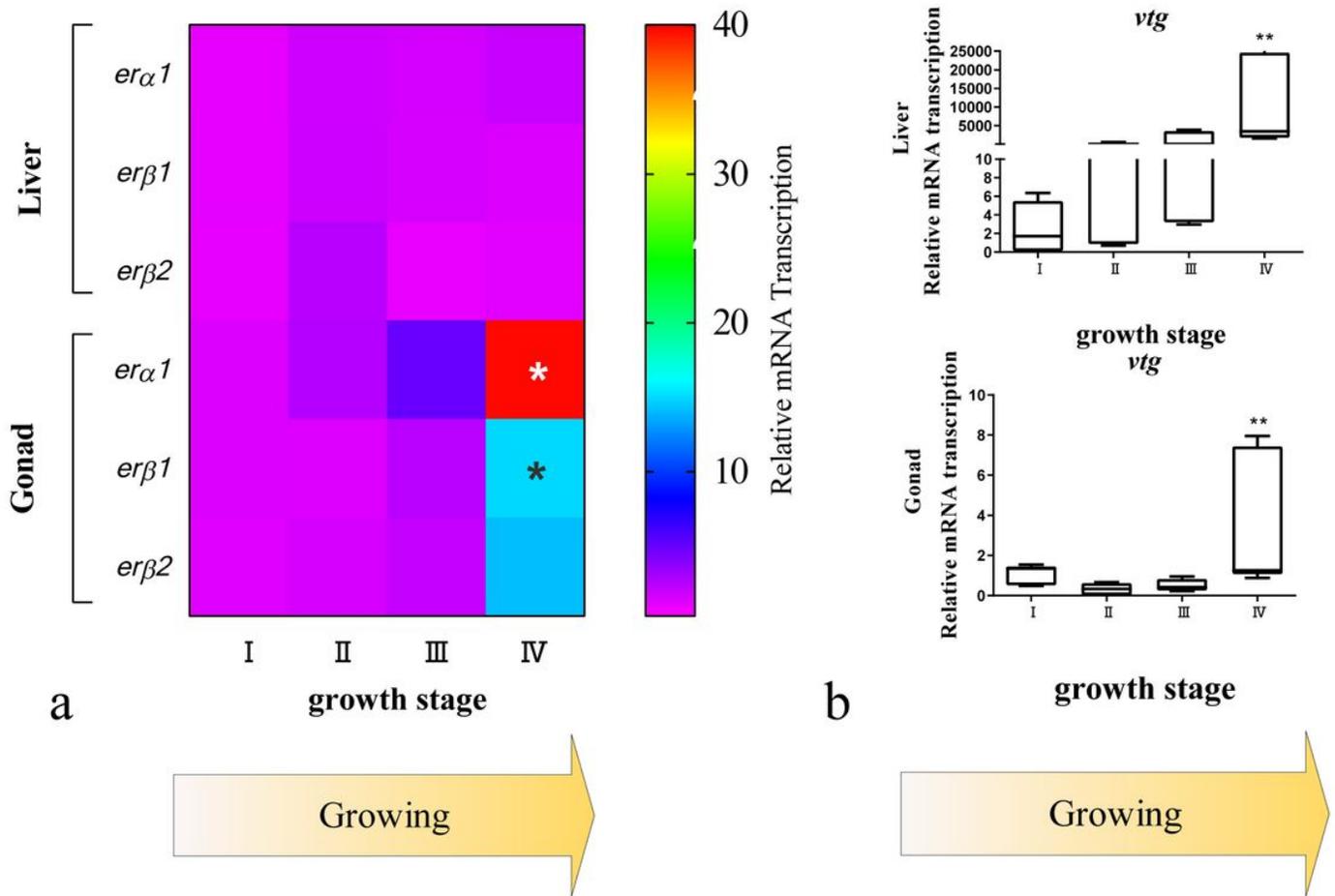
**Figure 3**

Changes of serum THs at different growth stages in *O. mykiss* (a) Changes of serum T<sub>4</sub>; (b) Changes of serum T<sub>3</sub>; (c) Changes of serum FT<sub>4</sub>; (d) Changes of serum FT<sub>3</sub>. The THs in each growth stage is presented as mean ± SD (n = 3). Different superscript letters indicate significant differences among different growth stages (p < 0.05).



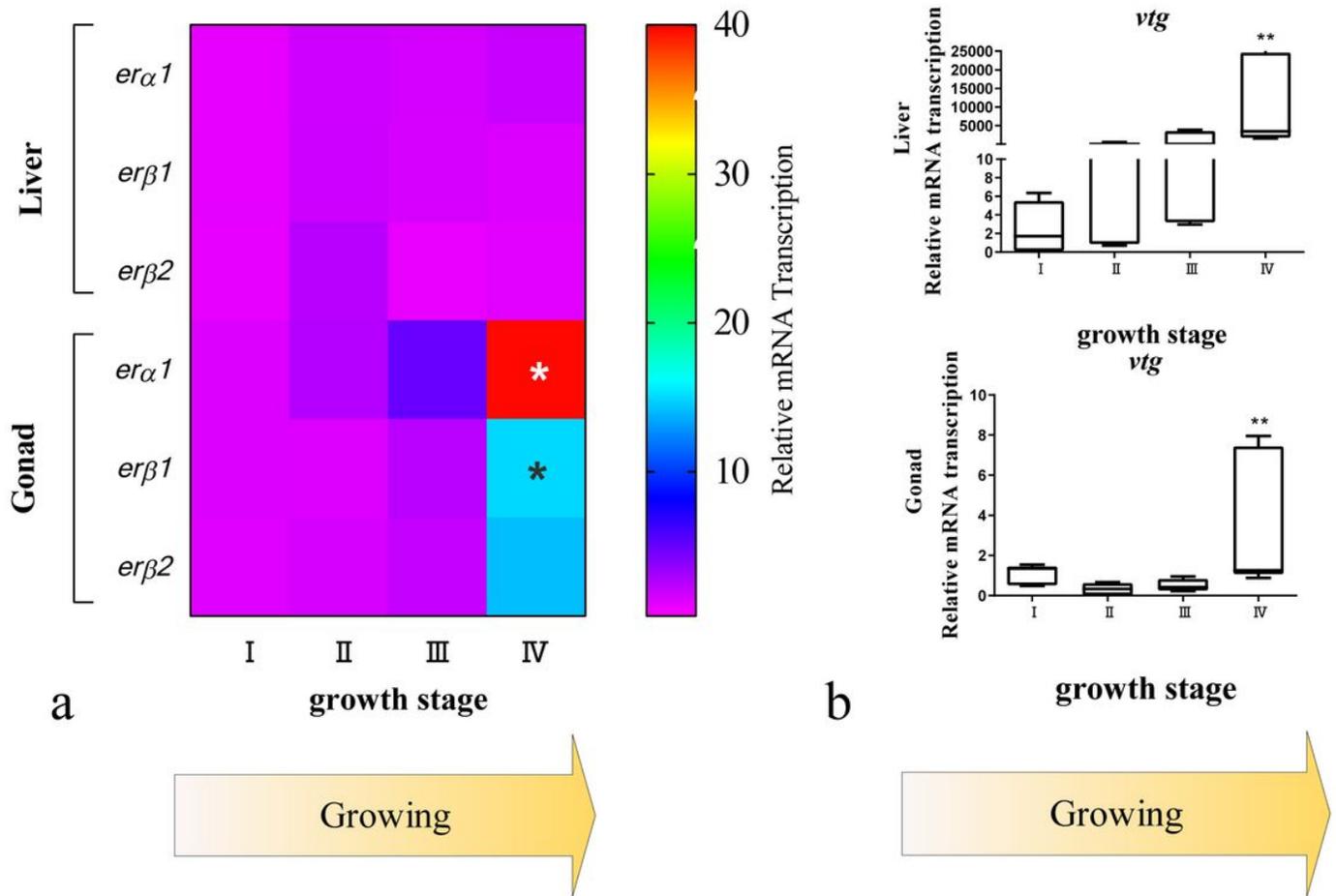
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**Figure 4**

Relative transcriptions of sex-related genes of *O. mykiss* at different growth stages (a) heatmap of *ers* mRNA transcription in liver and gonad, red represents a higher transcription and purple represent a lower transcription. (b) *vtg* transcription level in liver and gonad. Values significantly difference are indicated by asterisks (\* $p < 0.05$ , \*\*  $p < 0.01$ ).



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