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Research paper

Expression of estrogen receptors in female rainbow trout (*Oncorhynchus mykiss*) during first ovarian development and under dense rearing condition

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ABSTRACT

To study the expression of four estrogen receptor genes (*erα1, erα2, erβ1, erβ2*) of female rainbow trout (*Oncorhynchus mykiss*) during first ovarian development, trouts were sampled from different ovarian stages. Serum E_2 (estradiol) was measured by ELISA and estrogen receptors mRNA expression were examined by qRT-PCR. Our results showed a close association between increased *erα1* and *vitellogenin* mRNA expression during ovarian maturation and increased *erα2* mRNA expression in mature ovarian stages. Correlation analysis revealed that a negative relationship between serum E_2 and ovarian *erβ1* (or hepatic *erβ2*), but ovarian *erβ2* mRNA expression was relatively unchanged during first ovarian development. Trout were also reared in different densities as stocking density 1, 2 and 3 (SD1, 4.6–31.1 kg/m^3; SD2, 6.6–40.6 kg/m^3; SD3, 8.6–49.3 kg/m^3) to elucidate effects of high density on estrogen receptor expression. Histology observation showed ovarian development of trout in higher densities were retard with a relatively early stage and fewer vitellogenin accumulation. Trout in high densities showed significantly decreased serum E_2, *erα* mRNA expression and increasing trends of *erβ* mRNA expression. A noticeable increase of ovarian *erβ2* mRNA expression was seen in trout when density is approaching to 50 kg/m^3. In conclusion, we may hypothesize that increased *erβ* mRNA expression triggered by high density result in decreased *erα* mRNA expression and vitellogenesis. As a result, ovarian development in higher densities was retard.

1. Introduction

Steroid hormones of teleost belong to different classes such as sex hormone (androgens, estrogens, progestins), glucocorticoids, mineralocorticoids and vitamin D derivatives. Estrogen (E_2) is an important steroid hormone in regulating sex differentiation, ovarian development and hepatic vitellogenin production of females (Lassiter et al., 2002; Nagler et al., 2012; Ni et al., 2013). Signaling activation mechanism of E_2 involves binding to their cognate estrogen receptors (ERs) within the cell or G-protein-coupled estrogen receptor (also called GPR 30) on the cell plasma membrane (Edwards 2005; Thomas et al., 2006; Levin, 2009; Nagler et al., 2010). Activation of ERs is an action of ligand-dependent way which induces migration of the ERs from cytosol to nucleus and binding to either the estrogen response element (palindromic DNA-sequence) or transcription factor complexes as dimer (Heldring et al., 2007). Apart from this ligand-dependent action, E_2 initiates a "rapid action" within seconds to minutes via kinase pathways (Cato et al., 2002; Lösel et al., 2003).

In mammals, there are two ER subtypes (ERα and ERβ) as the genes have duplicated over the course of evolution (Nagler et al., 2012). Studies of fish ER have reported an additional isoform of either (both) ERα or (and) ERβ due to an additional genome duplication (Meyer and Schartl, 1999). In rainbow trout (*Oncorhynchus mykiss*), complete ER family consists of two subtypes (ERα and ERβ), and each of this subtype has two isoforms as ERα1/α2 and

Abbreviations: erα1, erα2, erβ1, erβ2, estrogen receptors α1, α2, β1, β2; E_2, estradiol; ELISA, enzyme-linked immuno sorbent assay; FAWC, UK Farm Animal Welfare Council; GSI, gonad somatic index; HSI, hepatic somatic index; *O. mykiss*, *Oncorhynchus mykiss*; qPCR, Real-time quantitative PCR; SD1, 2, 3, stocking density 1, 2, 3; CA, cortical alveoli; LD, lipid droplets; NO, nucleolus; YG, yolk globules; ZP, zona pellucida; Cn, Chromatin-nucleolus stage; Pn, Perinucleolar stage; Ca, Cortical alveolus stage; Pv, Primary vitellogenic stage; Sv, Secondary vitellogenic stage; M, Mature phase.

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ERβ1/β2 (Boyce-Derricott et al., 2009; Nagler et al., 2007). These four isoforms of ERs are detectable in the gonads and liver of rainbow trout. Recently, several studies on ERs in rainbow trout focused on the biological function of ERs in embryonic rainbow trout, juvenile males and mature females over a complete reproductive cycle (Boyce-Derricott et al., 2010; Nagler et al., 2012; Delalande et al., 2015). Some other studies centered on the *er* gene expression patterns stimulated by exogenous hormones such as estrone and 17α-ethynylestradiol (Boyce-Derricott et al., 2009; Osachoff et al., 2016). There are studies about ERs in other teleost such as black rockfish (*Sebastes schlegeli*), loach (*Paramisgurnus dabryanus*) and Atlantic salmon (*Salmo salar*) (Shi et al., 2011; Zhang et al., 2012; Nikoleris and Hansson, 2015). However, information regarding *er* gene expression in immature female rainbow trout during first oocyte development is limited. In this study, the first purpose is to clarify the *er* gene expression patterns in the immature female rainbow trout during first oocyte development.

As an effort of making full use of rearing space and boosting the maxima fishery harvest, trout is often reared in dense condition that is much higher than the wild. Aquaculture operations such as crowding can produce undesired and unavoidable stress (Bonga, 1997). These stress from high stocking density might cause undesirable results for aquaculture productivity and growth-suppressing effects linked to actions on food intake, intermediary metabolism and muscle mass regulation of fish by disrupting the physiological balance (Madison et al., 2015). Growth reduction in rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) has been demonstrated in high stocking density (Irwin et al., 1999; Lambert and Dutil, 2001; Liu et al., 2016).

In teleost, net energy for production consists of growth, fat store and reproduction (Elliott, 1976). Growth is retarded as teleost may promote redistribution of energy in a dense rearing condition (Ellis 2001; Madison et al., 2015). However, studies regarding whether the reproduction function is also affected by dense rearing condition within this category of net energy is limited. In this study, the second purpose is to supplement the information of reproduction changes in a dense rearing condition where the growth performance is retarded (our previous studies indicated that density affects trout growth, Liu et al., 2016). According to the logistic growth model, biomass of a given species is variable during aquaculture and there are two common protocols for density study. In some studies, the total individuals in given space is constant and as the fish grow, the total weight in this given space is variable. Another protocol is to remain the total weight in a given space through the add or remove of animal individuals (Skov et al., 2011). There is no increasing of fish individuals in aquaculture and the majority of aquaculture studies about density use the constant individuals (Braun et al., 2010; Andrade et al., 2015; Millán-Cubillo et al., 2016). Also, we maintained a constant number of individuals of trout in this study.

2. Materials and methods

2.1. Experiment design and sample collection

2.1.1. Experiment 1. er mRNA expression patterns of female rainbow trout during first ovarian development

For this study, rainbow trout specimen were procured from National Excellent Rainbow Trout Seed Station of China. These trout were spawned from the same batch of fertilized eggs and reared in same rearing condition to ensure synchronized ovarian development. As no morphological difference exists in immatured female and male trout, we sampled 10–12 trout to ascertain that at least 6 female trout were sampled. Mature female trout could be distinguished from the underjaw.

Trout of different ovarian developmental stages were sampled under anaesthesia with 35–45 mg/L tricaine methane sulphonate. Morphology indices of weight (g), standard length (cm), and ovarian weight (g) were recorded to calculate the gonad somatic index (GSI = 100 * ovarian weight/eviscerated weight) and hepatic somatic index (HSI = 100 * hepatic weight/eviscerated weight). Ovarian lamellae of each ovary were transversally cut and fixed in Bouin's fluid (75:25: 5 picric acid: formalin: acetic acid) for 24 h and then transferred to 70% ethanol for histological analysis. Approximately 200 mg of ovary and liver were sampled and placed in frozen RNase-free container and stored at −80 °C until used for *er* mRNA expression analysis. Blood was sampled using autoclaved syringe from caudal vessels and frozen at −80 °C for hormone analysis.

Trout were fed twice a day (8 a.m. and 6 p.m.) with a commercial trout and sturgeon feed (40% protein, 26% fat, 14% carbohydrate) and daily diet were 2–5% of the estimated biomass. Feeding was stopped when trout were eating sluggishly. Dissolved oxygen is measured in the afternoon as oxygen concentration is higher in the morning and lower in the afternoon. During the experimental period, water temperature ranged from 5.8 to 17.6 °C and natural photoperiod was used. Maximal NH_4^+-N was 0.36 ± 0.3 mg/L and minimal dissolved oxygen was 6.8 ± 0.5 mg/L respectively.

2.1.2. Experiment 2. er and vitellogenin mRNA expression patterns of female rainbow trout under dense rearing condition

Trout in experiment 2 were 240 day-old immature rainbow trout with initial weight of 114.44 ± 5.21 g. These trout were isogenic with trout in experiment 1 and hatched from the same batch of fertilized eggs. According to the standards of National Excellent Rainbow Trout Seed Station of China, UK Farm Animal Welfare Council (FAWC) and body size, 40 individuals/m^3 (4.6 kg/m^3) and 80 individuals/m^3 (8.6 kg/m^3) were considered low and high initial stocking densities, and 30 kg/m^3 was the critical density when overall trout welfare was impaired or not (Standard of National Excellent Rainbow Trout Seed Station of China; Farm Animal Welfare Council UK, 1996; Aksakal et al., 2011). Trout were distributed into cages (3 * 3 * 3 m, volume 27 m^3) at three densities, SD1: initial density of 4.6 kg/m^3 and final density of 31.1 kg/m^3 (40 individuals/m^3, 1080 individuals/cage), SD2: initial density of 6.6 kg/m^3 and final density of 40.6 kg/m^3 (60 individuals/m^3, 1620 indivi duals/cage) and SD3: initial density of 8.6 kg/m^3 and final density of 49.3 kg/m^3 (80 individuals/m^3, 2160 individuals/cage). Changes in density is illustrated in Table 1. During the experiment, three experimental replicates and one supplemental group (to maintain density after sampling) were set in each density. This experiment lasted 300 days (from May to March of next year) and we stopped this experiment at day 300 after density treatment as the following reasons: 1. the lowest density reached 31.1 kg/m^3 in day 300 and this is the critical density for trout welfare as mentioned above; 2. according to experiment 1, immature trout reached ovarian stage Pv or Sv from December to January (next year), and reached ovarian stage M in January or February. However, the isogenic trout in Experiment 2 just reached to ovarian stage Pv in March next year. Trout starts ovarian degeneration when the water temperature is increasing. As water temperature in the rearing area started to rise in March and we deduced that trout of Experiment 2 missed the maturation stage and we stopped experiment in March.

During Experiment 2, ovarian development of trout in the three densities were observed bimonthly by sampling and the typical ovarian development stage were chosen to study serum E_2, and *vitellogenin*, *er* mRNA expression. Sampling, feed strategy, photope-

Table 1
Density variation of Ctrl, SD1 and SD2 during experiment.

Days following density treatment (Days following hatching)	0 (2 4 0)	60 (3 0 0)	120 (3 6 0)	180 (4 2 0)	240 (4 8 0)	300 (5 4 0)
SD1 (40 fishes/m^3)	4.6 kg/m^3	8.9 kg/m^3	12.0 kg/m^3	22.6 kg/m^3	26.3 kg/m^3	31.1 kg/m^3
SD2 (60 fishes/m^3)	6.6 kg/m^3	12.7 kg/m^3	16.4 kg/m^3	31.7 kg/m^3	36.0 kg/m^3	40.6 kg/m^3
SD3 (80 fishes/m^3)	8.6 kg/m^3	15.9 kg/m^3	20.0 kg/m^3	40.6 kg/m^3	45.0 kg/m^3	49.3 kg/m^3

SD1, SD2 and SD3 means stocking density 1, 2 and 3.

riod and water monitoring were similar to Experiment 1 and water condition is displayed in Supplementary Table S1.

2.2. Histology observation and E_2 enzyme-linked immunosorbent assay (ELISA)

Ovarian lamellae was fixed in Bouin's fluid for 24 h and then transferred to 70% ethanol for fixation. Ovarian stages were grouped into the following stages: Chromatin-nucleolus stage (Cn), Perinucleolar stage (Pn), Cortical alveolus stage (Ca), Primary Vitellogenic stage (Pv), Secondary Vitellogenic stage (Sv), Maturation phase (M) and Spent phase (S). E_2 was measured by ELISA kits (RD Biosciences, San Diego, CA, USA) for rainbow trout and the concentration of E_2 in the samples is then determined by comparing the O.D. to the standard curve. Protocol of histology observation and ELISA is displayed in our previous papers (Hou et al., 2016).

2.3. Gene expression

Total RNA were extracted using Trizol Reagent (Takara, China). Pellets of total RNA were re-dissolved in RNase-free water and quantitated using micro ultraviolet spectrophotometry at 260 and 280 nm. Prior to cDNA synthesis, the integrity of RNA was evaluated by analyzing in 1.5% agarose gel. One microgramme of total RNA was treated with DNase I (Takara) and then reverse transcribed to cDNA using the rapid reverse transcriptase kit (Takara) Primers in this rapid reverse transcriptase kit for reverse transcription were Oligo dT Primers. cDNA was diluted 4-fold and stored at −20 °C.

Triplicates of each cDNA sample were amplified by SYBR quantitative real-time (qPCR) and followed by a melting curve analysis to verify the specificity whether each PCR product has only a unimodal dissociation curve. qPCR in a 20 μl reaction system contained 10 μl SYBR Premix Ex Taq, 0.5 μl forward primer and 0.5 μl reverse primer, 2 μl cDNA and 7 μl ddH_2O. A program of 10 min at 95 °C; 40 cycles of 10 s at 95 °C, 40 s at Tm °C and 40 s at 72 °C; 10 min at 95 °C was performed in an Applied Biosystems ABI real-time PCR system. Melting curve was 50 °C (+1 °C/30 s) to 95 °C. Primer pairs of target genes for qPCR were designed by Primer Premier 6.0 software based on rainbow trout sequences in NCBI (Table 2). We first analyzed gene expression of the housekeeping genes of *18s* and *β-actin* through different ovarian stages, and the C_T value of *β-actin* was observed to be more consistent. As a result, *β-actin* was selected as the housekeeping gene for internal control of gene expression. All qPCR assays were performed in a repeated-measures and including the negative controls (no-reverse transcription RNA treated with DNase I) and blank controls (water). PCR efficiency of each primer pair was evaluated by standard curves from a graded series of diluted cDNA (4-fold; 16-fold; 64-fold; 256-fold; 1024-fold) to ensure that PCR efficiency of selected primer pair ranged from 90% to 100% ($R^2 > 0.99$). For comparisons among different samples, we normalized target gene expression to that of the *β-actin* gene and calculated relative expression to the control by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.4. Statistical analysis

Results are presented as mean ± S.E.M. A one-way ANOVA followed by Duncan's multiple range test with the significant level set at $P < .05$ was used to determine the effects of E_2, four *er* isoforms and vitellogenin mRNA expression in different ovarian development stages and different rearing density treatment ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test). Ovarian *erα2* and hepatic *erα2* mRNA expression were assessed by Student t test ($P < .05$ was considered statistically significant). Relationships between E_2 and *er* mRNA expression in different ovarian development stages were analyzed by Correlation test. All data were analyzed by SPSS17 program.

3. Results

3.1. Morphology of female rainbow trout during first ovarian development

Histology of ovarian differentiation is displayed in Fig. 1 and ovarian stages ranged from stage Cn to stage M. GSI and oocyte diameter in ovarian stage Cn were lowest and in ovarian stage M were highest (6.06-fold and 12.73-fold increases) (Table 3). Maximal HSI was displayed in ovarian stage Sv (2.49-fold increase) and a significantly decreased was seen subsequently in stage M (Table 3).

3.2. Serum E_2 and vitellogenin mRNA expression during first ovarian development

Serum E_2 increased significantly from stage Cn to Sv and remained unchanged in ovarian stage Sv and M (Fig. 2A). *Vitellogenin* mRNA expression displayed basal expression level until stage Pn, and then increased steadily from stage Ca to M. Relative to stage Cn, *vitellogenin* mRNA expression was maximal and 5.5-fold higher in stage M (Fig. 2B).

3.3. Hepatic ers mRNA expression during first ovarian development

Hepatic *erα1* started with a low level and then increased significantly during ovarian stages Ca and Pv. Maximal hepatic *erα1* mRNA expression in stage Sv was higher than that of stage Pn by 3.6-fold (Fig. 3A). Hepatic *erα2* mRNA expression was low and essentially unchanged during ovarian development (Fig. 3B). Both hepatic *erβ1* and *erβ2* showed a significant peak in mRNA expression in ovarian stages Cn and Pn, and decreased significantly in subsequent ovarian stages (Fig. 3C and D). Maximal *erβ1* (*erβ2*) mRNA expression in ovarian stage Pn was 2.9 (6.0)-fold higher than that of the lowest expression in ovarian stage M (Sv).

3.4. Ovarian ers mRNA expression during first ovarian development

Ovarian *erα1* mRNA expression increased significantly during ovarian stage Pn and Sv and maximal expression in stage Sv was 3.5-fold higher than the basal expression in stage Cn (Fig. 4A).

Table 2
Primers used in qRT-PCR assays in rainbow trout tissues.

Gene	GenBank accession number	Sequence (5′-3′)	Tm (°C)	Production Size	PCR efficiency
erα1	AJ242740	F: CAACTCTGGTGCCTTCTC R: GCTGATGTGGTGGATGAG	50.0	112	92.8
erα2	DQ177438	F: TCTCCTTCTGTTGTTACTCTG R: TCCTGATTGGCTGATGTTG	50.5	107	96.4
erβ1	DQ177439	F: ATGTCACTCACCAACCTG R: TAGCATCAGCACCTCCAA	50.0	138	95.2
Erβ2	DQ248229	F: CCAACATCTGCTCTAACTCT R: ATGACTGCTGCTGGAATG	50.1	138	98.7
vtg	S82450	F: ATGAACGAAGAGGAGGAAG R: GGAACTACTAGAGCGAGAG	49.3	144	96.7
β-actin	AF157514/AJ438158/NM_001124235	F: GTATGGAGTCTTGCGGTAT R: GGTACATGGTGGTTCCTC	49–51	117	97.7

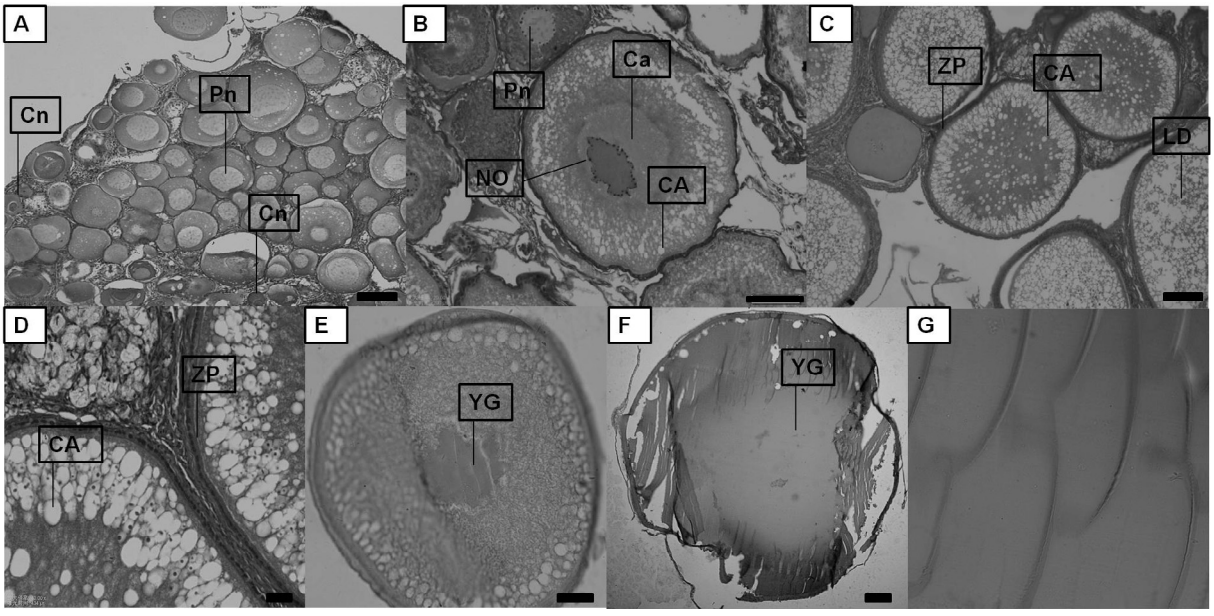


Fig. 1. Ovarian histology of female rainbow trout during first ovarian development. A, ovarian stages of chromatin-nucleolus stage (Cn) and perinucleolar stage (Pn). Bar represents 100 μm; B, ovarian stages of perinucleolar stage (Pn) and cortical alveolus stage (Ca). Bar represents 80 μm; C, ovarian stage of primary vitellogenic stage (Pv). Bar represents 100 μm; D, amplification of zona pellucida (ZP) and cortical alveoli filled in the oocyte (CA). Bar represents 20 μm; E, ovarian stage of secondary vitellogenic stage (Sv). Bar represents 100 μm; F, ovarian stage of mature phase (M). Bar represents 200 μm; G, amplification of yolk globules (YG). CA, cortical alveoli; LD, lipid droplets; NO, nucleolus; YG, yolk globules; ZP, zona pellucida.

Table 3
Morphology of female rainbow trout during first ovarian development.

Ovarian Stage	Cn	Pn	Ca	Pv	Sv	M
GSI(%)	1.70 ± 0.05^{d}	2.06 ± 0.09^{de}	2.50 ± 0.11^{d}	3.28 ± 0.05^{c}	5.40 ± 0.23^{b}	10.30 ± 0.28^{a}
HSI(%)	1.73 ± 0.04^{c}	1.76 ± 0.04^{c}	1.92 ± 0.07^{c}	1.96 ± 0.06^{c}	4.31 ± 0.14^{a}	2.34 ± 0.06^{b}
Maximal oocytediameter (mm)	164.7 ± 6.96^{d}	194.6 ± 5.67^{d}	409.5 ± 5.07^{c}	434.2 ± 6.22^{c}	627.6 ± 10.21^{b}	2097.1 ± 32.16^{a}

Data are presented as means ± S.E., n = 8. Different letters indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test).

Compared to low expression level from Cn to Pv, ovarian *erα2* displayed an approximately 4.6-fold significant up-regulations in ovarian stages Sv and M (Fig. 4B). Moreover, from ovarian stage Cn to M, ovarian *erα2* mRNA was significantly higher than that of liver (Fig. 5). Ovarian *erβ1* mRNA displayed a significant decline from Ca to M and in stage M, a 3.2-fold lower mRNA expression was seen relative to ovarian stage Cn (Fig. 4C). The pattern of *erβ2* mRNA expression was low and essentially unchanged throughout the first ovarian development (Fig. 4D).

3.5. Histological observation of female rainbow trout under dense rearing condition

Rainbow trout of SD1, SD2 and SD3 were all in ovarian stage Pn on the first day of density treatment (Fig. 6A–C). After 300 days of density treatment, trout of SD1 and SD2 reached ovarian stage Sv with obvious vitellogenin mass, but ovarian development of trout of SD3 were retarded and in the late phase of stage Ca with few vitellogenin accumulation (Fig. 6D–F).

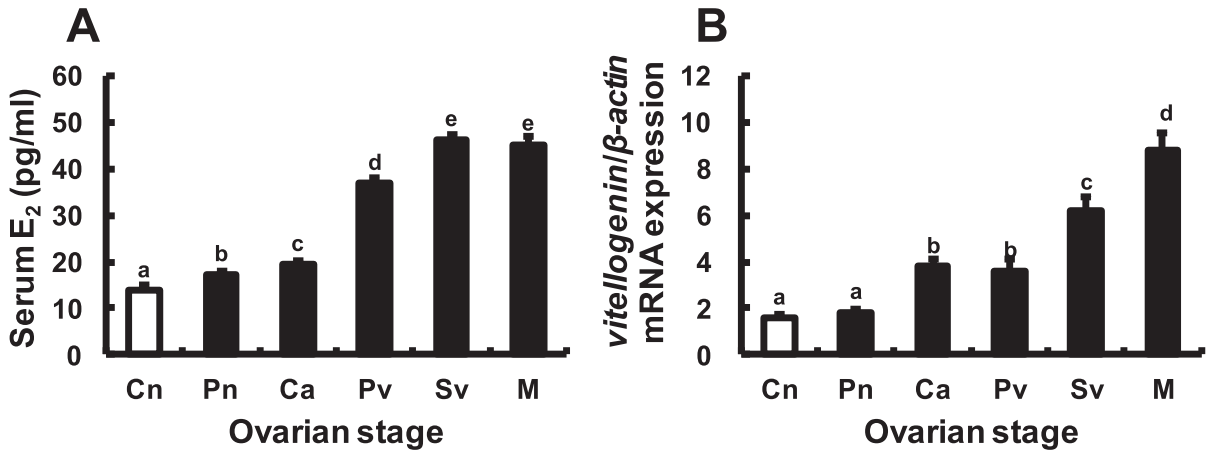


Fig. 2. Changes of E_2 (A) and *vitellogenin* mRNA expression (B) of female rainbow trout during first ovarian development. Data are presented as means ± S.E., n = 8 for serum E_2 and n = 4 for vitellogenin mRNA expression. Different letters indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test).

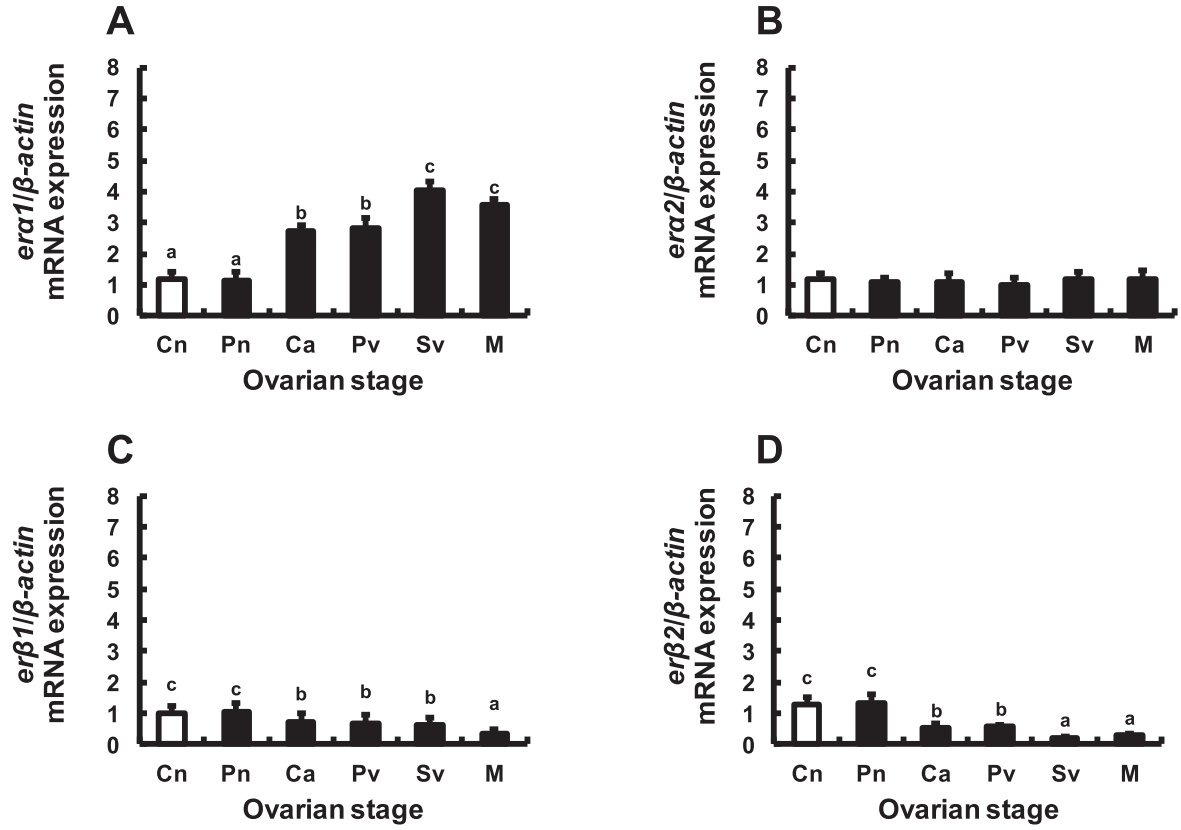


Fig. 3. Hepatic *erα1* (A), *erα2* (B), *erβ1* (C) and *erβ2* (D) mRNA expression of female rainbow trout during first ovarian development. Data are presented as means ± S.E., n = 4. Different letters indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test).

3.6. Changes of hepatic er mRNA expression under dense rearing condition

$E_2$of trouts in SD1, SD2 and SD3 fluctuated from day 0 to day 180 and reached to maximal levels on day 240, after that, E_2 levels decreased. Details are displayed in our previous paper. (Hou et al., 2016).

Hepatic *erα1* mRNA expression of SD1 started with a low level and then increased significantly, a significant decrease was seen on day 300. In SD2 and SD3, hepatic *erα1* mRNA expression decreased significantly on day 240 (Pv) and on day 120 (Ca) respectively (Fig. 7A). No obvious change of hepatic *erα2* mRNA expression was seen among the different rearing densities (Fig. 7B). In SD1, *erβ1* decreased significantly on day 240 (Pv) and day 300 (Pv), but significant up-regulation of *erβ1* was displayed in SD3 in the same time (Fig. 7C). *erβ2* mRNA expression decreased significantly in stage Ca among densities. After that, trout in SD1 displayed a relatively unchanged *erβ2* mRNA expressions, but *erβ2* mRNA expressions in SD2 and SD3 started to increase significantly on day 300 (Pv) and day 240 (Ca) respectively (Fig. 7D).

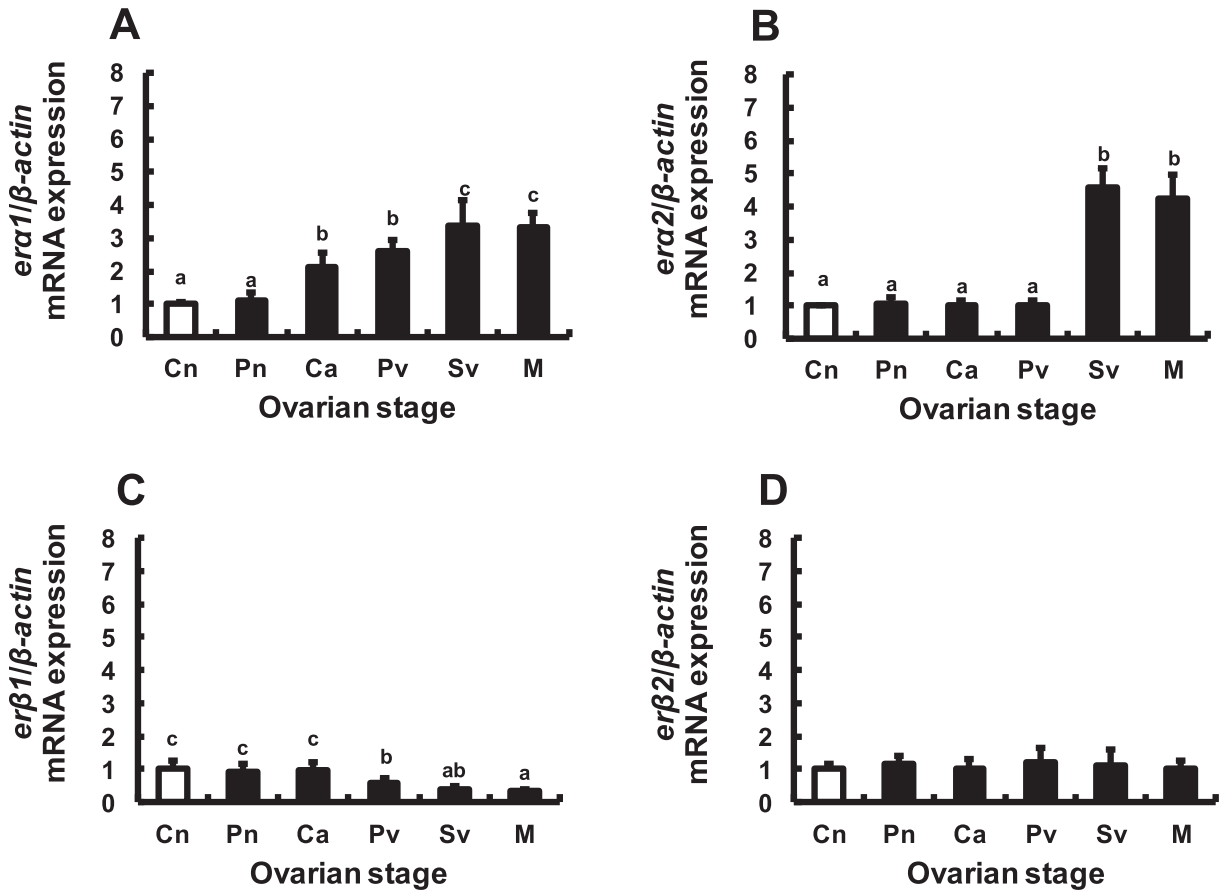


Fig. 4. Ovarian *erα1* (A), *erα2* (B), *erβ1* (C) and *erβ2* (D) mRNA expression of female rainbow trout during first ovarian development. Data are presented as means ± S.E., n = 4. Different letters indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test).

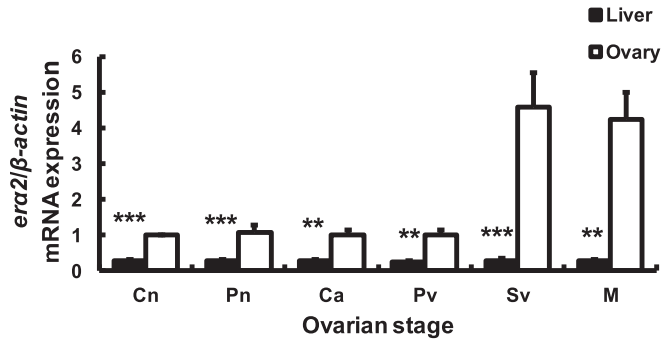


Fig. 5. Ovarian *erα2* and hepatic *erα2* mRNA expression of female rainbow trout during first ovarian development. Data are presented as means ± S.E., n = 4. Data were assessed by Student *t* test. Different asterisk as *, ** and *** in a given ovarian stage indicates $P < .05$, <.01 and <.005 respectively.

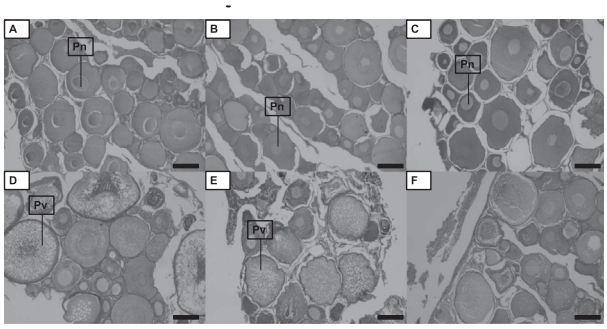


Fig. 6. Ovarian histology of female rainbow trout under dense rearing condition. A, B and C, ovarian histology of female rainbow trout at day 0 (first day of density treatment) of SD1, SD2 and SD3. Pn represents ovarian perinucleolar stage. Bar represents 100 μm; D, E and F, ovarian histology of female rainbow trout at day 300 (last day of density treatment) of SD1, SD2 and SD3. Pv represents ovarian primary vitellogenic stage. Bar represents 100 μm.

3.7. Changes of ovarian er mRNA expression under dense rearing condition

Ovarian *erα1* mRNA expression of SD1 increased significantly on day 120. After a slightly decrease on day 180, *erα1* mRNA expression on day 240 and 300 restored to same level as on day 120. In SD2 and SD3, ovarian *erα1* mRNA expression decreased significantly on day 300 (Fig. 8A). Significant up-regulation of ovarian *erα2* mRNA expression was seen in SD1 on day 300 (Fig. 8B). Ovarian *erβ1* mRNA expressions in SD1 and SD2 displayed significant down-regulation on day 240 (Pv) and day 300 (Pv), but significant up-regulation was seen in SD3 on day 240 (Ca) and day 300 (Ca, Fig. 8C). Ovarian *erβ2* mRNA expression of SD1 and SD2 was essentially unchanged during density treatment, but *erβ2* mRNA expression in ovaries of SD3 displayed significant up-regulation on day 300 (Ca, Fig. 8D).

3.8. Correlation analysis

Regression and correlation analysis were displayed in Figs. 9 and 10. Hepatic *erα1* and ovarian *erα* subtypes were positively correlated with serum E_2 and vitellogenesis, whereas hepatic *erβ* sub-

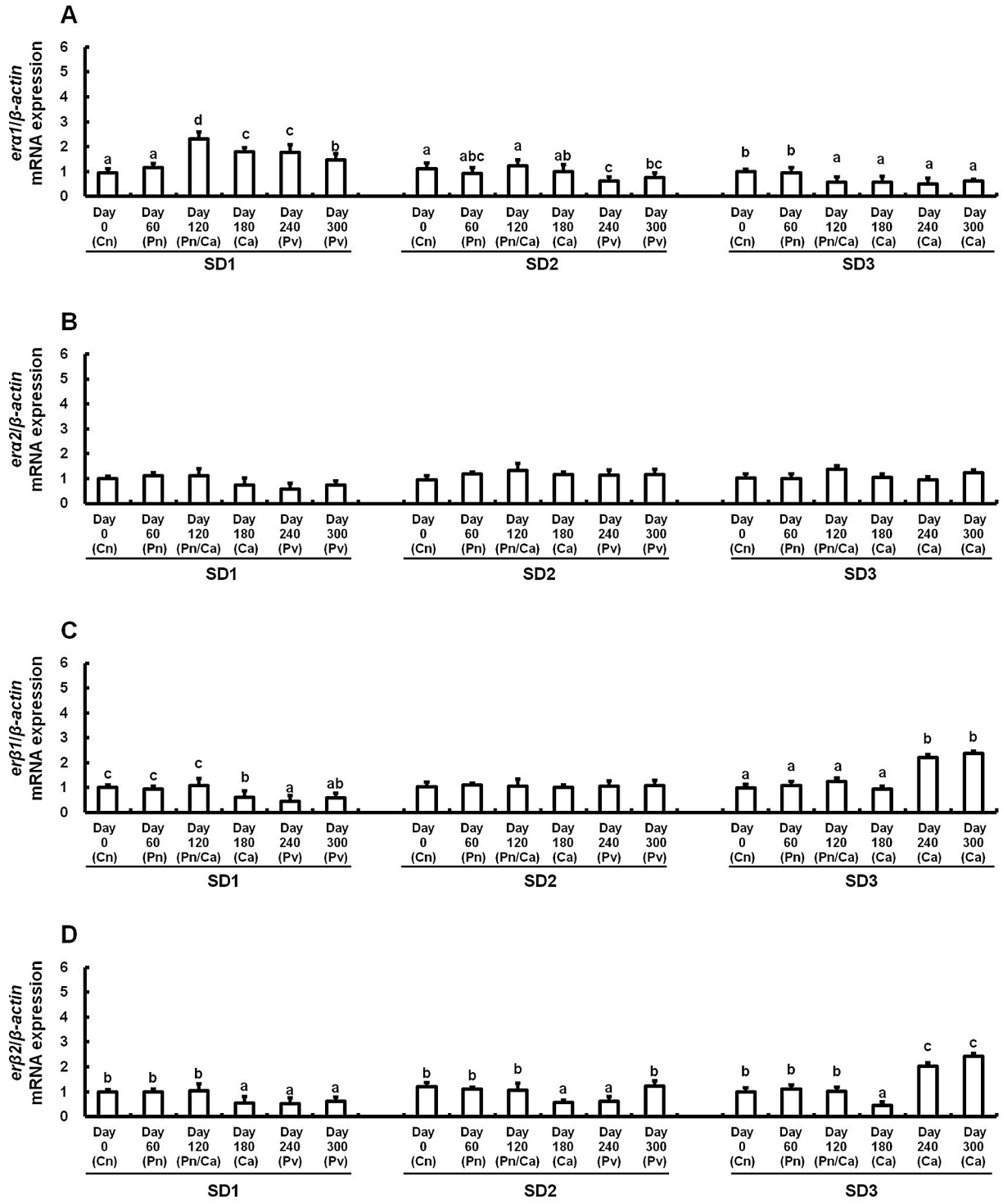


Fig. 7. Hepatic *erα1* (A), *erα2* (B), *erβ1* (C), *erβ2* (D) mRNA expression of female rainbow trout under dense rearing condition of SD1, SD2 and SD3. Data are presented as means ± S.E., n = 4 for gene expression. Different letters within given density indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test). In X axis, day 0, 60, 120, 180, 240 and 300 represents days post dense rearing and abbreviations represents ovarian stage in the given time. Cn, Chromatin-nucleolus stage; Pn, Perinucleolar stage; Ca, Cortical alveolus stage; Pv, Primary vitellogenic stage; Sv, Secondary vitellogenic stage.

types and ovarian *erβ1* mRNA expression were negatively correlated with serum E_2 and vitellogenesis. Hepatic *erα2* and ovarian *erβ2* displayed low correlation with serum E_2.

4. Discussion

ERs could transduce E_2 signals in ovary and liver to initiate or maintain ovarian development and vitellogenin synthesis, respectively. In this experiment, four *er* subtypes displayed distinct expression patterns in different ovarian stages. A previous study reported that *erα1* is affected by E_2 (Boyce-Derricott et al., 2009), and in this study, correlation analysis showed a positive relationship between increased serum E_2 and ovarian/hepatic *erα1* mRNA expression during ovarian maturation.

Significantly higher ovarian *erβ1* and hepatic *erβ1, erβ2* mRNA expressions were observed in the early stage of ovarian

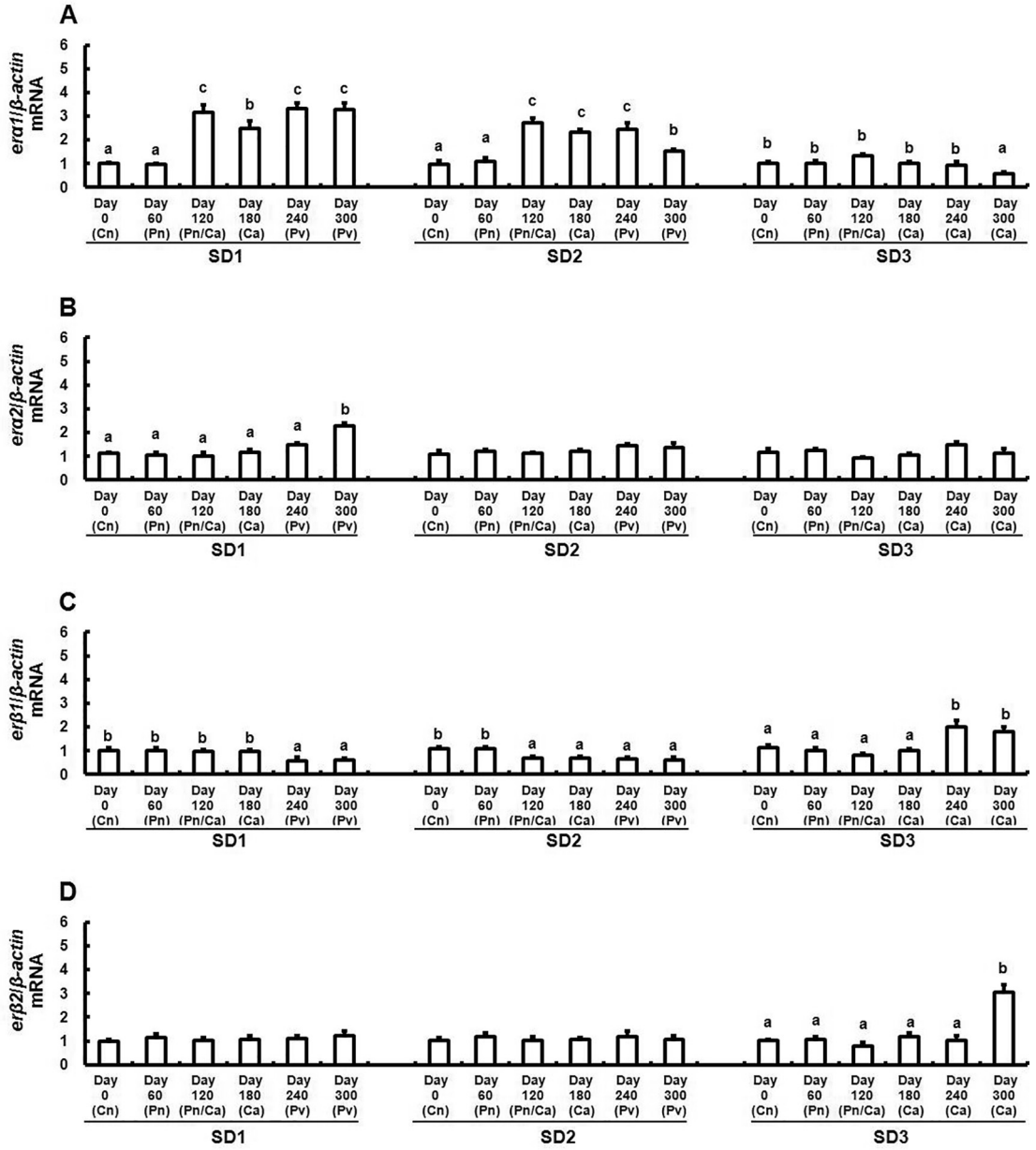
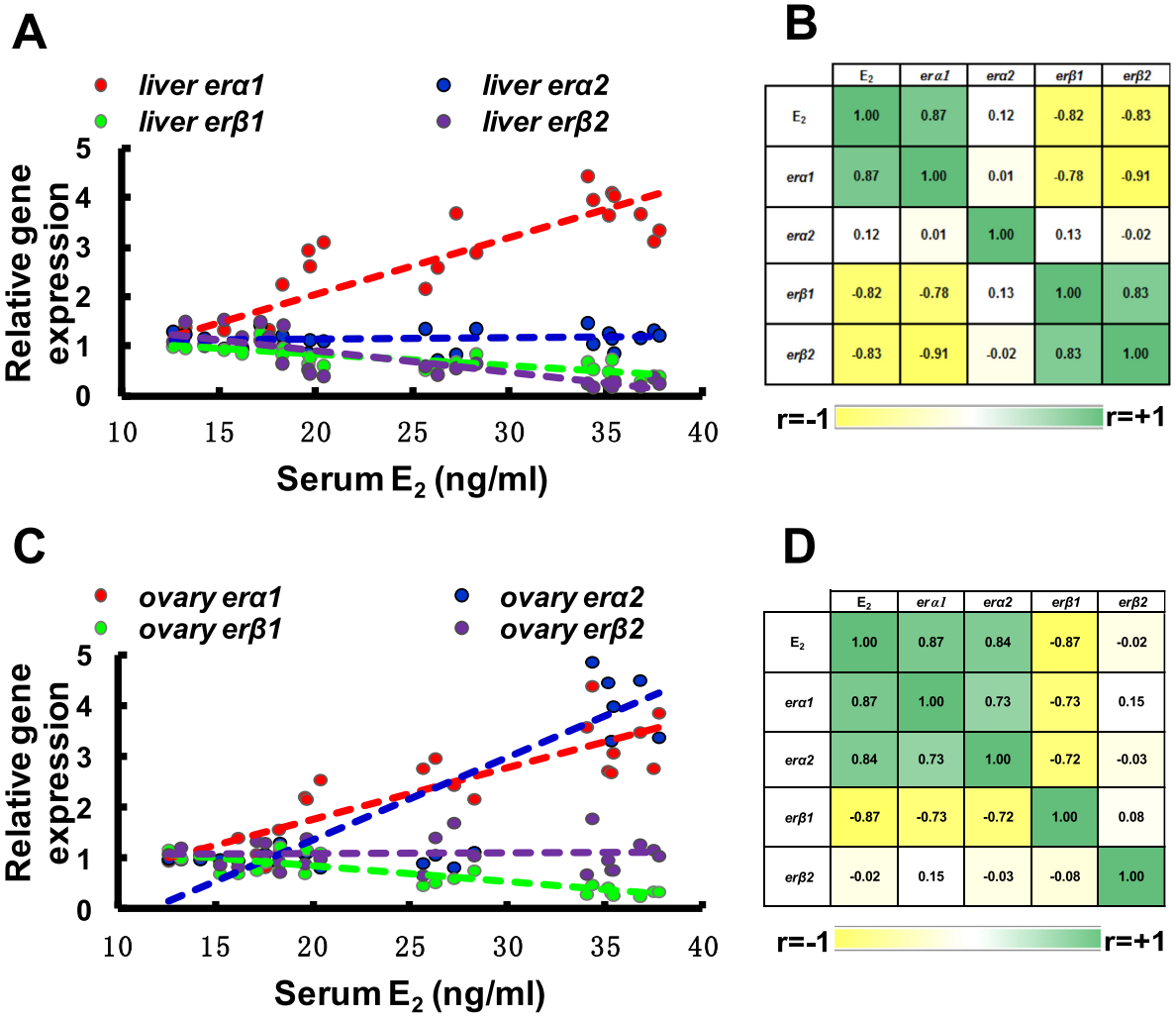


Fig. 8. Ovarian *erα1* (A), *erα2* (B), *erβ1* (C), *erβ2* (D) mRNA expression of female rainbow trout under dense rearing condition of SD1, SD2 and SD3. Data are presented as means ± S.E., n = 4 for gene expression. Different letters within given density indicate significant differences ($P < .05$, one-way ANOVA, followed by Duncan's multiple range test). In X axis, day 0, 60, 120, 180, 240 and 300 represents days post dense rearing and abbreviations represents ovarian stage in the given time. Cn, Chromatin-nucleolus stage; Pn, Perinucleolar stage; Ca, Cortical alveolus stage; Pv, Primary vitellogenic stage; Sv, Secondary vitellogenic stage.

development. In zebrafish and channel catfish, ERβs bind E_2 with higher affinity than ERα (Xia et al., 1999, 2000; Menuet et al., 2002). Nelson and Habibi proposed a model that in early recrudescent females, slightly increased E_2 levels particularly activate ERβs. High *erβs* expression results in a small up-regulation of *vitellogenin* and *era1*. Increased *era1* dramatically increases vitellogenesis (Nelson and Habibi, 2010). Our result supports this model. Strong up-regulations of *erα1* and down-regulations of *erβs* (except for ovarian *erβ2*) occurred during rapid accumulation of vitellogenin. These results indicate that ERβs is the initial mediator of E_2 induction of vitellogenin, and *erα1* plays a larger role in vitellogenesis as females proceed ovarian maturation.

ERα2 exists in a limited number of teleosts (Nagler et al., 2007). In this experiment, different expression patterns of *erα2* in liver and ovary were observed. Ovary exhibited a significantly higher *erα2* than liver, especially in mature ovaries (Sv and M stages). Trout displayed significantly higher *erα2* mRNA expressions in ovarian stages of Sv and M. Liver is the major organ of vitellogenesis, but *erα2* mRNA expression in liver is low. These results indicate that *erα2* might have a limited role in inducing hepatic vitellogenesis in the early phase of ovary development.

Previous studies indicated that exogenous environmental stimuli could affect ovarian development (Andersson et al., 2013). Stocking density is an environmental stimulus, leading to stress in fish. In this study, when vitellogenin was clearly observed in trout reared in low densities, trout in higher densities was still in a relatively immature ovarian stage (Cn) with few vitellogenin accumulation in oocytes and lower serum E_2. Fish accumulate large amounts of hepatic lipid and glycogen before reproduction. Our previous studies reported that circulating cortisol increases signif-



B	E_2	erα1	erα2	erβ1	erβ2
E_2	1.00	0.87	0.12	-0.82	-0.83
erα1	0.87	1.00	0.01	-0.78	-0.91
erα2	0.12	0.01	1.00	0.13	-0.02
erβ1	-0.82	-0.78	0.13	1.00	0.83
erβ2	-0.83	-0.91	-0.02	0.83	1.00

D	E_2	erα1	erα2	erβ1	erβ2
E_2	1.00	0.87	0.84	-0.87	-0.02
erα1	0.87	1.00	0.73	-0.73	0.15
erα2	0.84	0.73	1.00	-0.72	-0.03
erβ1	-0.87	-0.73	-0.72	1.00	0.08
erβ2	-0.02	0.15	-0.03	-0.08	1.00

Fig. 9. Regression analysis of serum E_2 and hepatic (A)/ovarian (C) mRNA expression in female rainbow trout during first ovarian development; correlation of serum E_2 and hepatic (B)/ovarian (D) mRNA expression in female rainbow trout during first ovarian development.

icantly when trout is reared in high density (Liu et al., 2016). High cortisol may mobilize these substances that are supposed to be converted into vitellogenin. High cortisol and low estrogen were observed when trout was reared in high density. Cortisol and E_2 are important steroid hormones that can drive biobehavioral adaptations of survival and propagation (Juster et al., 2016). Cortisol directly suppresses cyp19a1 and causes masculinization in Japanese flounder (*Paralichthys olivaceus*, Yamaguchi et al., 2010). In salmon and trout, cortisol inhibits E_2 secretion by suppressing either steroidogenesis-related genes or E_2 induced vitellogenesis (Berg et al., 2004; Barkataki et al., 2013). We suggest that high density might trigger adaptive mechanisms of female trout and increase circulating cortisol, thus it inhibits reproductive functions by suppressing E_2 secretion.

Overall trout welfare is impaired when rearing density is over 30 kg/m^3. (Standard of National Excellent Rainbow Trout Seed Station of China; Farm Animal Welfare Council UK, 1996; Aksakal et al., 2011). In this study, trout in high densities showed significantly decreased *erα1* mRNA expression with increasing trends of *erβ* mRNA, especially when the density was over 40 kg/m^3. It is possible that reduced *erα1* resulted from decreased serum E_2 and up-regulated *erβs*. *erα1* is affected by E_2 (Boyce-Derricott et al., 2009), thus one possible reason of down-regulated *erα1* in high density is that high density inhibited E_2 secretion. ERβ is a *trans*-dominant inhibitor of ERα activity through the formation of heterodimers (Hall and McDonnell, 1999) and studies indicated ERβ can decrease *erα* gene *trans*-activation or enhance ERα proteolytic degradation (Matthews and Gustafsson, 2003; Matthews et al., 2006; Nelson and Habibi, 2013). Negative relationships were observed between *erα1* and *erβs* in liver, and between *erαs* and *erβ1* in ovary. We hypothesize that high rearing density could induce *erβs* mRNA transcription and/or increase *erβs* stabilities, resulting in decreased *erα1* and *vitellogenin* mRNA expression. As a result, ovarian development in higher densities was retarded. Furthermore, significant increase of ovarian *erβ2* mRNA expression existed when density was approaching 50 kg/m^3, suggesting extremely high density could induce *erβ2* gene transcription or increase *erβ2* stability.

ERα2 only exists in a few teleosts and tetraploid species, thus limited information is available on its function (Nagler et al., 2007). A significant up-regulation of ovarian *erα2* was observed in trout in low density in day 300. However, trout in higher densities displayed relatively low *erα2* mRNA expression that was not changed. One possible function of *erα2*, coupled with high serum E_2, is responsible for shutting down ovarian uptake of vitellogenin when ovary is approaching maturation (Perazzolo et al., 1999; Nagler et al., 2012). Trout ovarian development was inhibited by higher densities and ovaries of these trout did not develop to

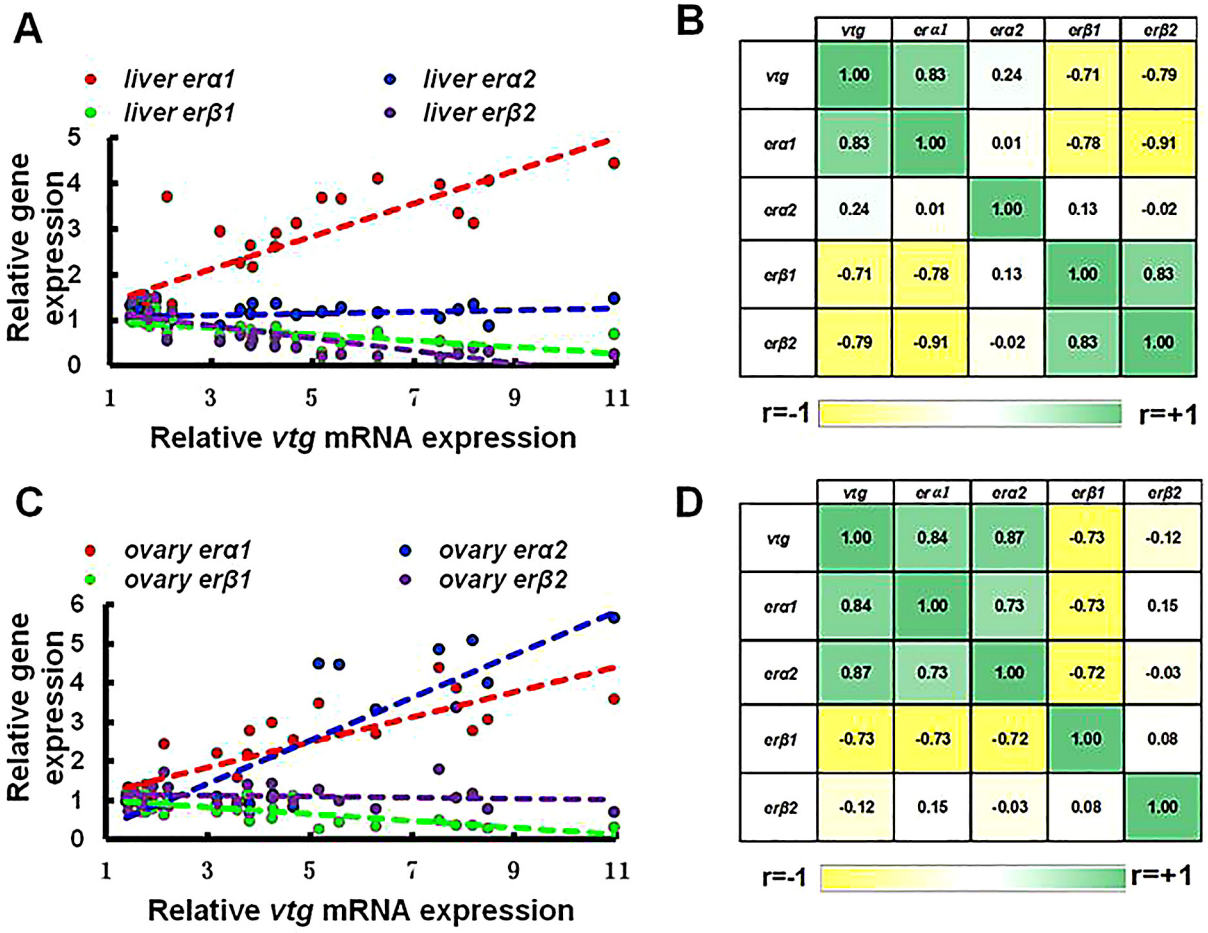


Fig. 10. Regression analysis of hepatic *vtg* and hepatic (A)/ovarian (C) mRNA expression in female rainbow trout during first ovarian development; correlation of hepatic *vtg* and hepatic (B)/ovarian (D) mRNA expression in female rainbow trout during first ovarian development.

mature stage, hence these trout displayed low *erα2* mRNA expression.

5. Conclusion

We reported herein the overall patterns of estrogen receptor expression in ovary and liver of female rainbow trout during first ovarian development and under high rearing densities. During first ovarian development, hepatic and ovarian *erα1* mRNA expression increased significantly and a close association between up-regulated *erα1* mRNA expression and increased *vitellogenin* mRNA expression existed. Ovarian *erα2* mRNA expression was important in the final phase of ovarian development. With regards to rearing densities, trout in the higher densities were in a more immature ovarian stage (Cn) with less vitellogenin accumulation and lower serum E_2. These trout showed significantly decreased *erα* mRNA expression with increased *erβ* mRNA expression. A significant increase of ovarian *erβ2* mRNA expression existed only in high densities when densities were approaching 50 kg/m^3, suggesting that stress might stimulate *erβ2* gene transcription or increase *erβ2* mRNA stability. In conclusion, we suggest that high rearing density could promote the *erβ* mRNA expression and decrease *erα* mRNA expression and vitellogenesis. As a result, ovarian development in higher densities was retarded.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.10.001.

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