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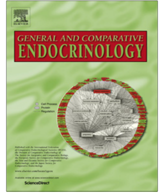

Research paper

Hypothalamus-pituitary-gonad axis of rainbow trout (*Oncorhynchus mykiss*) during early ovarian development and under dense rearing condition

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ABSTRACT

The objective of this study was to determine the hypothalamus-pituitary-gonad (HPG) axis of female rainbow trout (*Oncorhynchus mykiss*) during early ovarian development and under high rearing density. Trouts were sampled from 240 (ovarian stage II) to 540 (ovarian stage IV) days following hatching (DFH) as control group (Ctrl, 4.6–31.1 kg/m^3) to determine HPG axis during early ovarian development. Trouts from the same batch of fertilized eggs were reared in two higher densities during 240–540 DFH as stocking density 1 and 2 (SD1, 6.6–40.6 kg/m^3; SD2, 8.6–49.3 kg/m^3) to elucidate effects of high density on reproductive parameters. Dopamine, E_2 (estradiol), 17α,20β-P (17α,20β-dihydroxy4-pregnen-3-one) and P4 (progesterone) were evaluated by radioimmunoassay or ELISA. mRNA expression of hypothalamic *gnrh-1, -2* (gonadotropin-releasing hormone-1, -2), pituitary gonadotropins (*fsh/lh*, follicle-stimulating hormone/luteinizing hormone) and their cognate receptors (*fshr/lhr*) in ovaries were examined by qRT-PCR. Our findings demonstrated mRNA expression of hypothalamic *sgnrh-1*, pituitary *fsh* and ovarian *fshr* increased in early ovarian development (360 DFH). Serum 17α,20β-P and pituitary *lh* mRNA expression first increased when trouts were in ovarian stage III (420 DFH). Ovaries were at different stages when reared in different densities. Long-term high density treatment (over 31.7 kg/m^3) resulted in decreased hypothalamic *sgnrh-1*, pituitary *fsh*, ovarian *fshr*, serum E_2, and increased hypothalamus *gnrh-2* and serum dopamine during vitellogenin synthesis, suggesting HPG of rainbow trout might be retarded under dense rearing condition.

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1. Introduction

As in higher vertebrates, various reproductive events of teleost, such as gonadal development and the synthesis and release of steroid hormones (E_2, estrogen; T, testosterone; 17α,20β-P, 17α,20βdi hydroxy4-pregnen-3-one) are primarily under the control of two distinct gonadotropins (Swanson et al., 2003; Pramanick et al., 2013): follicle-stimulating hormone, or FSH, (previously called GtH-I in fish) and luteinizing hormone, or LH, (previously called GtH-II in fish) (Suzuki et al., 1988). FSH primarily regulates oocyte growth, folliculogenesis, and other developmental phenomena via its effects on steroid biosynthesis of hormones such as E_2, T, 17α,20β-P and P4 (progesterone, a precursor of 17α,20β-P), whereas the main physiological function of LH occurs in dynamic events such as final maturation and ovulation (Swanson et al., 1991; Murata et al., 2012). Studies on salmonid and non-salmonid fishes both have reported that maximum LH reached when fish reached the final stages of oocyte maturation or ovulation (Goméz et al., 1999 (rainbow trout); Kumar and Trant, 2004 (catfish (*Ictalurus punctatus*); Levavi-Sivan et al., 2010 (summarize); Taranger et al., 2010; Molés et al., 2012 (*Dicentrarchus labrax*)).

Both FSH and LH must bind to their receptors (FSHR or LHR), which are distributed in the target cell, in order to fulfill their physiological function. FSHR and LHR are G protein-coupled receptors from the family of the rhodopsin-like receptors (Gether, 2000). Together with the TSHRs, they constitute the subfamily of glycoprotein hormone receptors with a large extracellular domain,

Abbreviations: DFD, days following density treatment; DFH, days following hatching; SD1, 2, stocking density 1, 2; Ctrl, control group; HPG axis, hypothalamus-pituitary-gonad axis; fsh/lh, gene of follicle-stimulating hormone/luteinizing hormone; fshr/lhr, fsh/lh receptor; sgnrh-1, salmon gonadotropin-releasing hormone-1; cgnrh-2, chicken gonadotropin-releasing hormone-2; E_2, estradiol; 17α,20β-P, 17α,20βdihydroxy4-pregnen-3-one; P4, progesterone; RIA, radioimmunoassay; ELISA, enzyme-linked immuno sorbent assay.

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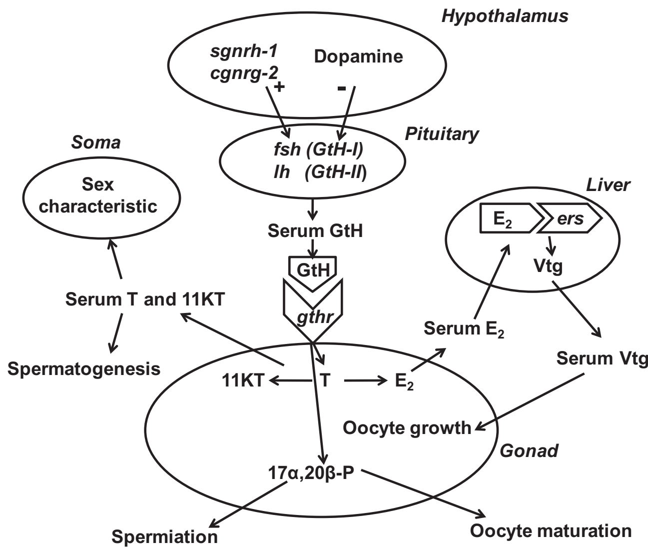

Fig. 1. Summary of the reproductive endocrine system of teleost (from Fish Stress and Health in Aquaculture, Cambridge University Press, Pankhurst and Van der Kraak, 2011).

followed by seven transmembrane helices and a carboxy-terminal intracellular tail (Vassart et al., 2004; Levavi-Sivan et al., 2010). The FSHR/LHR mediate the effects of gonadotropins on steroidogenesis in granulosa and theca cells (Miwa et al., 1994; Nyuji et al., 2013). Variation of mRNA expression in *fshr* and *lhr* were correlated with the development of ovarian follicles (Kwok et al., 2005).

FSH and LH release is stimulated by gonadotropin-releasing hormone (GnRH) and inhibited by dopamine released from hypothalamus (Yada, 2012; Levavi-Sivan et al., 2010). In salmonid fishes, two forms of GnRH, sGnRH-1 and cGnRH-2, have been identified (Okuzawa et al., 1990). FSH and LH are also regulated by peripheral hormones (E_2, P4 and 17α,20β-P) from the gonads, paracrine factors within the pituitary, and multiple environmental changes (Nagahama, 1994; Lin and Ge, 2009; Andersson et al., 2013). Environmental changes may result in stress to teleost and stress has inhibitory effects on reproduction and can be manifested at various levels of the reproductive endocrine axis (Pankhurst and Van der Kraak, 2011). But data regarding the connections among early ovarian development, reproductive hormone and rearing density in fish is limited. In the present study, we aimed to clarify the mechanism of HPG axis (Fig. 1) in female rainbow trout (*Oncorhynchus mykiss*) during the early stages of ovarian development from 240 to 540 days following hatching and with different stocking density.

2. Materials and methods

2.1. Fish and experimental design

2.1.1. Experiment 1. HPG axis of trout during early ovarian development (240 (ovarian stage II) to 540 (ovarian stage IV) days following hatching)

All procedures were approved by the Academic Council of Ocean University of China and in accordance with the Chinese Association for Laboratory Animal Sciences (CALAS). Immature 240 day-old rainbow trout (114.44 ± 5.21 g, 19.69 ± 0.31 cm) were cultured outdoors in the Liujia Gorge Fish Farm, National Excellent Rainbow Trout Seed Station of China. The trout were stocked in pen-reared cages (3 m * 3 m * 3 m) for 300 days (from May 2011 to March 2012, and growth performance is illustrated in Supplementary Table 1). Three experimental replicates and one supplemental group (to maintain density after sampling) were set. Trout were reared at initial density of 4.6 kg/m^3 and final density of 31.1 kg/m^3 (40 fish/m^3 and 1080 fish/cage) as the control group (Ctrl).

Twelve fish of the total 1080 fish in each cage were sampled every 60 days to evaluate ovarian development. And twelve trouts of similar size in supplemental group were added to each cage to maintain density after sampling. All trout (groups of immature, before 1st maturation) were spawned on the same day and therefore all trout were synchronized in terms of reproductive development. Trout were fed twice a day, corresponding to 2–5% (per day) of the estimated biomass and feeding behavior using pellet diet consisting of 40% protein, 26% fat and 14% carbohydrate. We stopped feeding when trout were eating sluggishly. Water quality (temperature, ammonia nitrogen and dissolved oxygen) were monitored daily. During the experimental period, mean temperature was 8.51–18.2 °C and under natural photoperiod. Maximum and mean NH_4^+-N were 0.53 ± 0.05 and 0.38 ± 0.07 mg/L respectively. Minimum and mean dissolved oxygen were 6.5 ± 0.22 and 6.93 ± 0.36 mg/L respectively, as detailed in Supplementary Table 2.

2.1.2. Experiment 2. Effects of density on trout HPG axis

Rainbow trout from the same batch of fertilized eggs with Ctrl were distributed into pen-reared cages in two higher densities for 300 days, SD1: initial density of 6.6 kg/m^3 and final density of 40.6 kg/m^3 (60 fish/m^3, 1620 fish/cage) and SD2: initial density of 8.6 kg/m^3 and final density of 49.3 kg/m^3 (80 fish/m^3, 2160 fish/cage). Changes of density is illustrated in Table 1, relationship between days following density treatment and hatching is illustrated in Table 2, and growth performance is illustrated in Supplementary Table 1. Three experimental replicates and one supplemental group (to maintain density after sampling) were set in each density. Trout in Ctrl were regarded as Ctrl. Density of 4.6 and 8.6 kg/m^3 is considered standard and high initial stocking density respectively, and 30 kg/m^3 is considered a marginally high density, when overall welfare of trout was not impaired according to the standards of aquaculture industry (Standard of National Excellent Rainbow Trout Seed Station of China; Farm Animal Welfare Council (FAWC) of UK; Anon, 1996; Aksakal et al., 2011).

Twelve fish were sampled from SD1 and SD2 bimonthly as described in Experiment 1. And twelve trouts of similar size in supplemental group were added to each cage to maintain density after sampling. Maximum NH_4^+-N in SD1 and SD2 were 0.55 ± 0.01 and 0.60 ± 0.06 mg/L respectively; mean NH_4^+-N in SD1 and SD2 were 0.40 ± 0.08 and 0.45 ± 0.10 mg/L, respectively. Minimum dissolved oxygen in SD1 and SD2 were 6.3 ± 0.35 and 5.4 ± 0.31 mg/L, respectively; mean dissolved oxygen in SD1 and SD2 were 6.71 ± 0.50 and 6.61 ± 0.75 mg/L respectively, as detailed in Supplementary Table 2.

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

Days following density treatment (Days following hatching)	0 (240)	60 (300)	120 (360)	180 (420)	240 (480)	300 (540)
Ctrl (40 fishes/m^3)	4.6 kg/m^3	8.9 kg/m^3	12 kg/m^3	22.6 kg/m^3	26.3 kg/m^3	31.1 kg/m^3
SD1(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 kg/m^3	40.6 kg/m^3
SD2(80 fishes/m^3)	8.6 kg/m^3	15.9 kg/m^3	20 kg/m^3	40.6 kg/m^3	45 kg/m^3	49.3 kg/m^3

* Ctrl: control group; SD1: stocking density 1; SD2: stocking density.

Table 2
Relationship between days following density treatment (DFD) and days following hatching (DFH).

Days Following Density Treatment (DFD)	0 DFD	60 DFD	120 DFD	180 DFD	240 DFD	300 DFD
Days Following Hatching (DFH)	240 DFH	300 DFH	360 DFH	420 DFH	480 DFH	540 DFH

2.2. Sampling

At each sampling point, all trout were individually sampled under anaesthesia (MS222, tricaine methane sulphonate, 35–45 mg/L (35–45 ppm)). Body weight (g), length (cm), ovarian weight (g) were recorded to calculate the GSI (gonad somatic index; GSI = ovarian weight/eviscerated weight). A few ovarian lamellae from each female were isolated by transversal cuts with a scalpel blade. Pieces of ovary were fixed in Bouin's fluid for histological analysis. Ovary, pituitary and hypothalamus were rapidly sampled and approximately 200 mg of ovary and pituitary tissues were placed in frozen 1.5 ml tube (RNase-free) and stored at −80 °C until analyzed for mRNA expression and blood was sampled by using a 1.5 ml syringe from caudal vessels and the serum was separated from the blood by centrifugation and frozen at −80 °C for RIA (radioimmunoassay) or ELISA (enzyme-linked immunosorbent assay) analysis.

2.3. Histology observation

Ovarian lamellae were fixed in Bouin's fluid (75:25:5 of picric acid:formalin:acetic acid) for 24 h and then transferred to 70% ethanol and dehydrated through a graded series of ethanol (70% ethanol 25 min; 80% ethanol 25 min; 95% ethanol 30 min; 95% ethanol 30 min; 100% ethanol 25 min twice), and cleared in 100% xylene, and embedded in paraffin melting in 54–56 °C. Ovarian lamellae were cut in cross-section at 5–8 μm thickness by microtome (Leica-RM2016) and then placed into a pure xylene solution for deparaffinage twice, for 15 min each. Then ovarian lamellae were rehydrated through a graded series of ethanol (100% ethanol 2 min; 95% ethanol 2 min; 80% ethanol 2 min; 50% ethanol 2 min; 30% ethanol 2 min) and finally stained with hematoxylin for 90 s and eosin for 25 s for histological observation under light microscope (Nikon-E200, Japan).

Ovaries were staged according to previous report (Sharma and Bhat, 2013). Briefly, ovaries were staged as ovarian stage I (chromatin-nucleolus stage, Cn), ovarian stage II (perinucleolar stage, Pn), ovarian stage III (cortical alveolus stage, Ca), ovarian stage IV (primary vitellogenic stage, Pv), ovarian stage V (secondary vitellogenic stage, Sv), ovarian stage VI (maturation phase, M) and ovarian stage VII (spent phase, S). In this experiment, ovaries were primarily at ovarian stage II (perinucleolar stage), ovarian stage III (cortical alveolus stage), and ovarian stage IV (primary vitellogenic stage).

2.4. Serum E_2, P4, 17α,20β-P and dopamine

Serum P4 was analyzed by RIA using a commercially available kit (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Tianjin, China), validated previously by us (Ding et al., 2012). Specificity, cross reactivity, and non-specific binding were: 95–104%, <0.09%, <3% respectively. Serum 17α,20β-P, E_2 and dopamine content were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (RD Biosciences, San Diego, CA, USA) for rainbow trout. This assay is based on competition between free 17α,20β-P, E_2 and dopamine tracer for a limited number of 17α,20β-P, E_2 and dopamine-specific antibody binding sites. 17α,20β-P, E_2 and dopamine in the samples is determined by comparing the O.D. to the standard curve (details shown in Supplementary materials).

2.5. RNA isolation and real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Takara, Dalian, China) according to the manufacturer's protocol. The quantity and quality of total RNA was determined using micro-ultraviolet spectrophotometry at 260 and 280 nm and visualization on 1.5% agarose gels. One μg of total RNA was reverse transcribed using the rapid reverse transcriptase kit (Takara, DNase used for RNA treatment and primers chosen for reverse transcription were included in the kit (Oligo dT Primer)). The cDNA was diluted and stored at −20 °C until used in real-time PCR.

Information of primers (including GenBank accession numbers, primer sequences, Tm (°C), product size and PCR efficiency) are given in Table 3. PCR primers were designed by Primer Premier 6.0. Transcript levels were determined by SYBR quantitative real-time PCR (Takara). Preliminary experiments showed that *β-actin* was an appropriate reference gene for providing an internal control for gene expression. All qPCR assays were performed in duplicate, using 96-well optical plates. Each run for *β-actin* gene included the cDNA control, negative controls (no-reverse transcription of DNase I-treated RNA) and blank controls (water). PCR efficiency was determined for each primer pair by performing standard curves from serial dilutions (4-, 16-, 64-, 256-, and 1024-fold dilution) to ensure PCR efficiency ranged from 90% to 100% ($R^2 > 0.99$). Twenty μl reactions contained 10 μl SYBR Premix Ex Taq, 0.5 μl primer pairs (F and R each), 2 μl cDNA (4-fold dilution) or no-RT controls and 7 μl ddH_2O in an Applied Biosystems ABI real-time PCR system (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). Melting curve was 50 °C (+1 °C/30 s) to 95 °C. Results were calculated with the $2^{-\Delta\Delta CT}$ method (Willems et al., 2008).

Table 3
Primers used in qRT-PCR assays in rainbow trout tissues.

Gene	GenBank accession number	Sequence (5′-3′)	Tm (°C)	Production Size	PCR efficiency
fsh	AB050835.1	F:GCGAAACAACGGACCTGAACTAT R:GGACCACTCCTTGAAGTTACACA	52	118	96.7
lh	AB050836.1	F:CTGCGTCACCAAGGAGCCGGTTT R:GACAGTCAGGTAGGCGGATCGTT	52	107	98.8
fshr	AF439405.1	F:TCAGTCACCTGACGATCTGCAA R:TCCTGCAGGTCCAGCAGAAACG	52	98	98.2
lhr	AF439404.1	F:CTTCTCAACCTCAATGAAATCTTC R:GGATATACTCAGATAACGCAGCTT	52	105	99.1
sgnrh-1	AF110992.1	F: GGTGGTGTAATGGCTCTT R: CATCATCGCTGTCTTCTTG	51.5	147	97.8
cgnrh-2	AF125973.1	F:CTGTGAGGCAGGAGAATG R:ACGGTTGATAGGTTGTCTAA	53	125	98.2
β-actin	AF157514.1	F:ATCCTGACGGAGCGCGGTTACAGC R:TGCCCATCTCCTGCTCAAAGTCCA	52	111	99.8

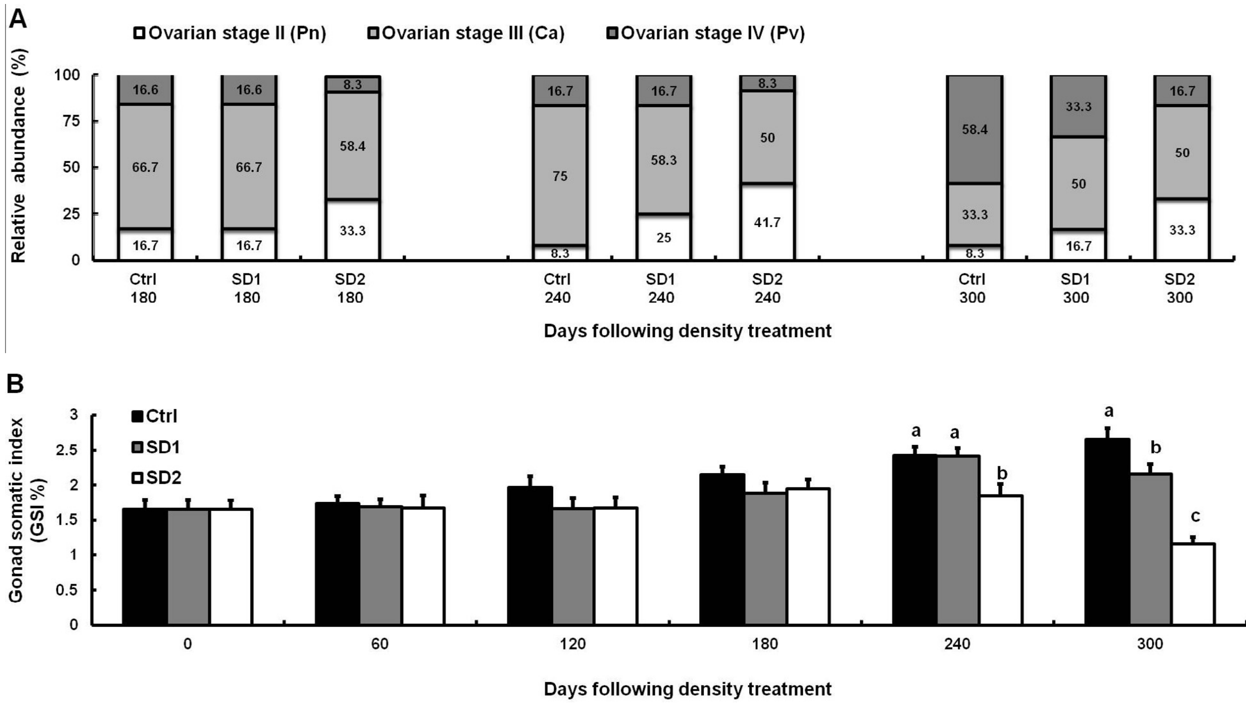

Fig. 2. Ovarian histology in female rainbow trout under different densities (A) and changes of GSI under different densities (B). Relationship between days following density treatment and hatching is illustrated in Table 2. Data are presented as means ± S.E., N = 12. Different letters within one sample time indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's Multiple Range test). Fig. 2 is firstly displayed in our previous article in Chinese Journal of Oceanology and Limnology (Hou et al., 2016).

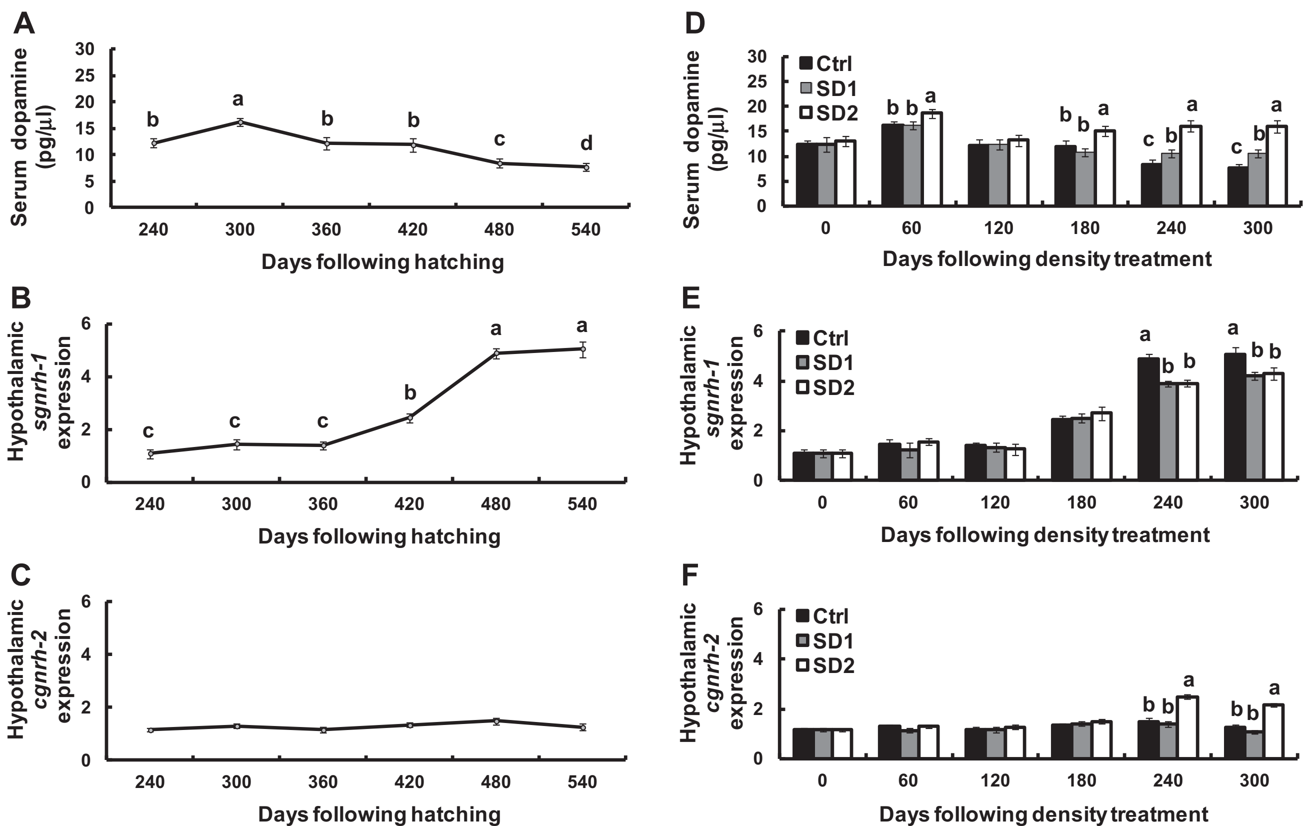

Fig. 3. Changes of serum dopamine (A), *sgnrh-1* mRNA expression (B) and *cgnrh-2* mRNA expression (C) of female rainbow trout during experiments in control group. Changes of serum dopamine (D), hypothalamic *sgnrh-1* mRNA expression (E) and hypothalamic *cgnrh-2* mRNA expression (F) under different densities. Relationship between days following density treatment and hatching is illustrated in Table 2. Data are presented as means ± S.E., N = 4. Different letters within one sample time indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's Multiple Range test).

2.6. Statistical analysis

Data (GSI, serum dopamine/steroid hormone, and gene expression) of Ctrl, SD1 and SD2 were subjected to one-way analysis of variance (ANOVA) and differences among means were analyzed by Duncan's Multiple Range test, and significance was set at $P < 0.05$. Data are presented as means ± standard error (means ± S.E.). All data were analyzed using the SPSS17 program.

3. Results

3.1. Ovarian development and GSI patterns

Summary of the ovarian histological observation is given in Fig. 2A. There was considerable heterogeneity in ovarian development under different rearing densities. At 180 DFD (420 DFH), most ovaries of Ctrl (**66.7%**), SD1 (**66.7%**) and SD2 (**58.4%**) groups had reached ovarian stage III (cortical alveolus stage). At 300 DFD (540 DFH), the majority of trout (**58.4%**) from Ctrl group had reached ovarian stage IV (primary vitellogenic stage), whereas oocytes in ovarian stage II (perinucleolar stage) occupied 33.3% females in SD2.

As shown in Fig. 2B, GSI showed significant difference between the first two densities (Ctrl and SD1) and SD2 at 240 DFD (480 DFH). Relative to SD2 at 300 DFD (540 DFH), GSI were **2.27-** and **1.84-**fold higher in Ctrl and SD1.

3.2. Changes of serum dopamine and hypothalamic sgnrh-1/cgnrh-2 mRNA expression

Serum dopamine level of Ctrl group displayed a transient increase at 300 DFH, followed by significantly lower dopamine levels at sampling times of 420, 480 and 540 DFH, respectively, with **1.4-**, **1.9-** and **2.1**-fold lower relative to the dopamine level at 300 DFH (Fig. 3A). *sgnrh-1* mRNA showed a significant peak in expression at 480 and 540 DFH, whereas *cgnrh-2* mRNA expression level did not differ in Ctrl at the different sampling times (Fig. 3B, C).

Compared to the serum dopamine response of Ctrl and SD1, serum dopamine of SD2 was significantly higher throughout the experiment except at 120 DFD (Fig. 3D). *sgnrh-1* and *cgnrh-2* mRNA displayed opposite expression patterns among densities at 240 and 300 DFD, significantly decreased *sgnrh-1* and significantly increased *cgnrh-2* mRNA expression in SD2 in comparison to those of Ctrl and SD1 (Fig. 3E, F).

3.3. Changes of E_2, P4 and 17α,20β-P

Although a transient decrease of E_2 was seen at 420 DFH, trout increased E_2 gradually throughout the experiment (Fig. 4A). Serum P4 level was at the lowest level at 240 DFH and remained low until 420 DFH when it increased dramatically at 540 DFH (Fig. 4B). 17α,20β-P significantly increased from 360 to 540 DFH (Fig. 4C). Serum contents of E_2, P4 and 17α,20β-P at 540 DFH were **2.9-**, **6.4-** and **6.8**-fold higher, respectively, than the basal levels.

E_2 in Ctrl was significantly higher than that of both SD1 and SD2 after 120 days' density treatment (Fig. 4D). In general, density treatment had similar effects on P4 and 17α,20β-P. Relative to Ctrl, the higher density treatments were associated with significantly lower serum P4 and 17α,20β-P levels at 300 DFD (Fig. 4E, F).

3.4. Changes of fsh and lh mRNA expression in pituitary

Significant increase of pituitary *fsh* mRNA *ex*pression was recorded from 240 to 480 DFH (Fig. 5A). The pattern of pituitary

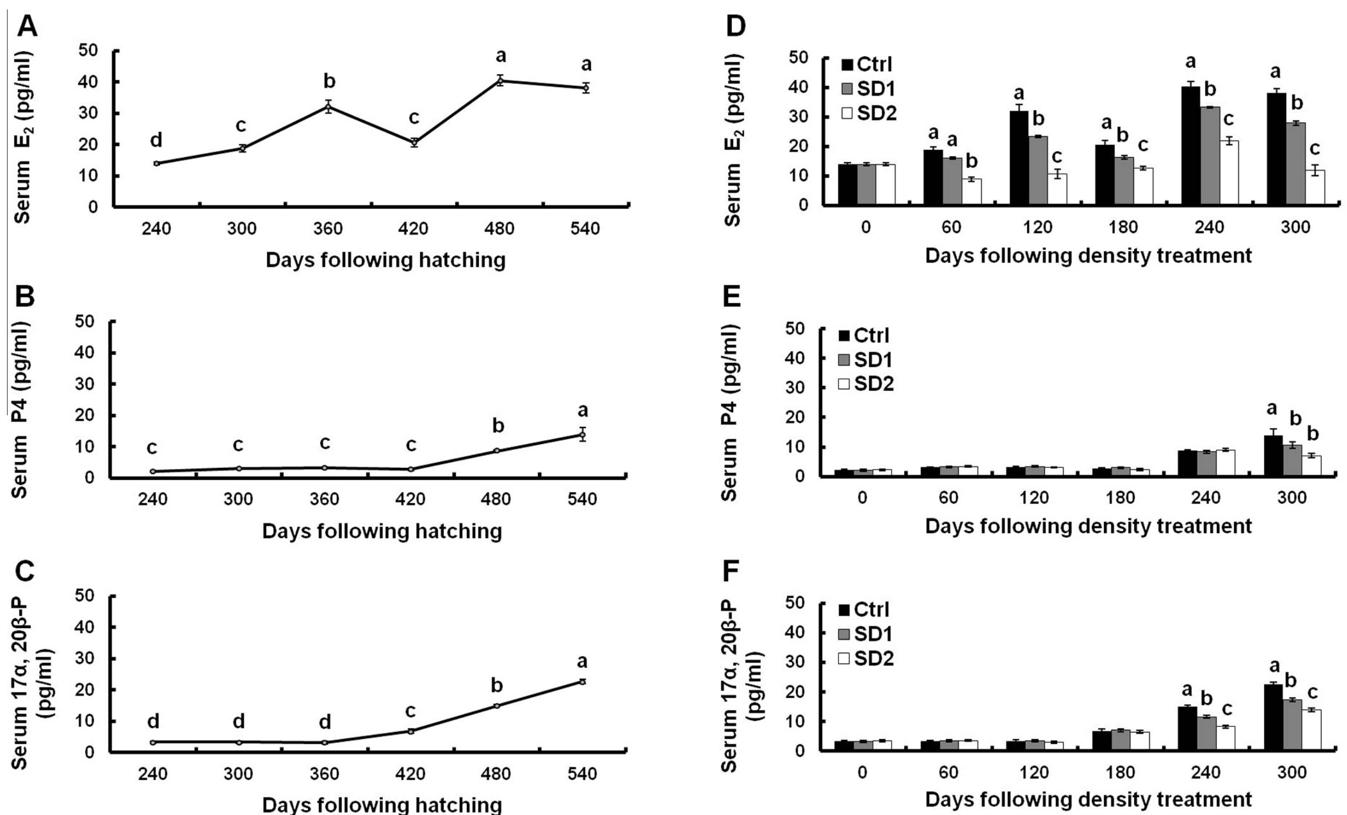

Fig. 4. Changes of E_2 (A), P4 (B) and 17α,20β-P (C) of female rainbow trout during experiments in control group. Changes of E_2 (D), P4 (E) and 17α,20β-P (F) in serum (1000-fold dilution) under different densities. Relationship between days following density treatment and hatching is illustrated in Table 2. Data are presented as means ± S.E., N = 12. Different letters within one sample time indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's Multiple Range test). Fig. 4F is firstly displayed in our previous article in Chinese Journal of Oceanology and Limnology (Hou et al., 2016).

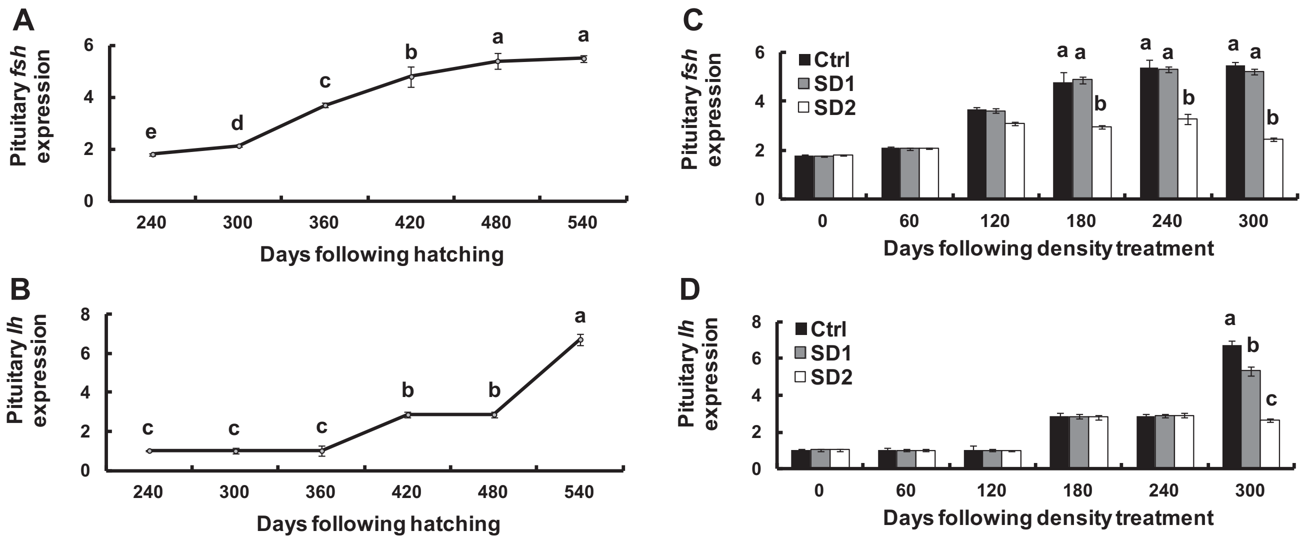

Fig. 5. Changes of pituitary *fsh* mRNA (A) and *lh* mRNA (B) expression of female rainbow trout in control group. Changes of pituitary *fsh* mRNA (C) and *lh* mRNA (D) expression under different densities. Relationship between days following density treatment and hatching is illustrated in Table 2. Data are presented as means ± S.E., N = 4. Different letters within one sample time indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's Multiple Range test).

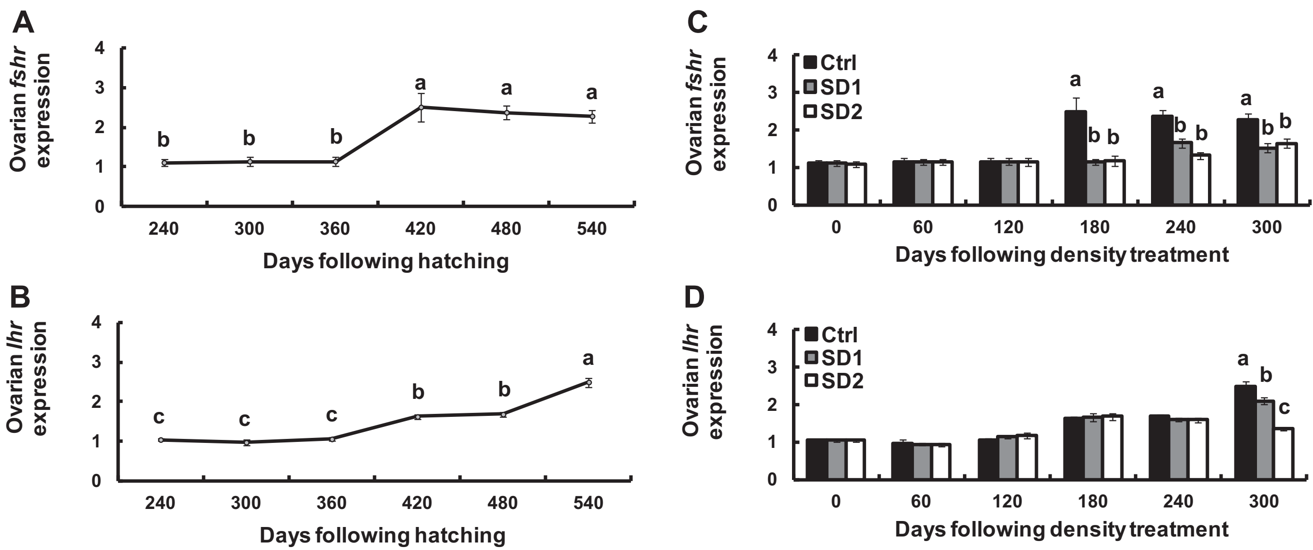

Fig. 6. Changes of ovarian *fshr* (A) and *lhr* (B) mRNA expression level of female rainbow trout in control group. Changes of ovarian *fshr* (C) and *lhr* (D) under different densities. Relationship between days following density treatment and hatching is illustrated in Table 2. Data are presented as means ± S.E., N = 4. Different letters within one sample time indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's Multiple Range test).

lh mRNA expression started with a low level, which remained fairly static until 360 DFH when a significant increase at 540 DFH occurred (Fig. 5B).

Compared to that in Ctrl, pituitary *fsh* mRNA expressions of SD2 decreased by **1.6-** to **2.2**-fold from 180 to 300 DFD (Fig. 5C). Pituitary *lh* mRNA expression in SD2 group was significantly decreased at 300 DFD when compared to Ctrl or SD1 (Fig. 5D).

3.5. Changes of fshr and lhr mRNA expression in ovary

Ovarian *fshr* showed a significant increase in mRNA expression at 420 DFH (Fig. 6A). *lhr* mRNA expressions at 420 and 540 DFH were **1.6-** and **2.4**-fold significantly higher than the basal mRNA expression at 240 DFH (Fig. 6B).

Ovarian *fshr* mRNA expression of SD2 was significantly lower than that of Ctrl at 180 (**2.1-fold lower**), 240 (**1.8-fold lower**) and 300 (**1.4-fold lower**) DFD (Fig. 6C). Compared to that of Ctrl, SD2 showed **1.8-fold** significantly lower ovarian *lhr* mRNA expression at 300 DFD (Fig. 6D).

3.6. Heatmap of mRNA expression of female rainbow trout during first ovarian development

The mRNA expression for given genes in HPG axis were present in clearly different amounts when the genes from different ovarian stages and density treatment were compared (Fig. 7). mRNA expression of genes that were differently expressed among density treatment were hypothalamic *sgnrh-2* and pituitary *fsh*.

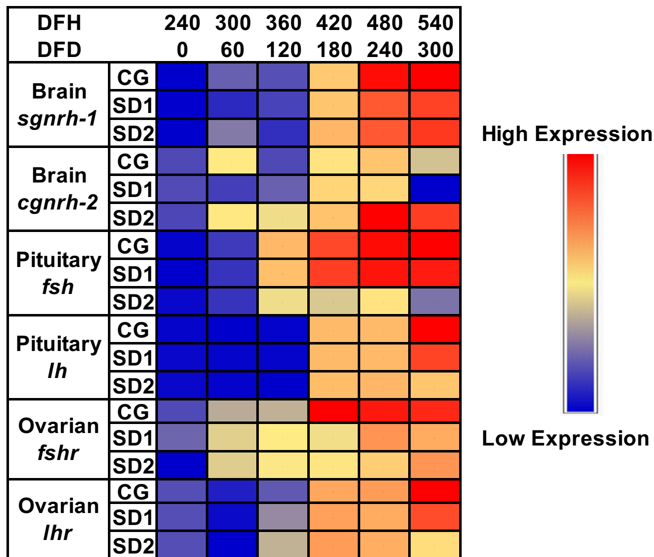

Fig. 7. Heatmap of hypothalamic *cgnrh-1/sgnrh-2*, pituitary *fsh/lh*, ovarian *fshr/lhr* mRNA expression of female rainbow trout during first ovarian development. Red represent higher expression and blue represent lower expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In several teleost species, serum E_2 could induce the dopaminergic inhibitory tone during vitellogenin synthesis (Levavi-Sivan et al., 2010). With regard to our results, serum E_2 and dopamine displayed opposite patterns during ovarian maturation. In shad (*Tenualosa ilisha*) and Atlantic salmon (*Salmo salar*), an elevated E_2 level was observed during vitellogenin synthesis (Andersson et al., 2013; Pramanick et al., 2013). In line with these studies, serum E_2 continuously increased in Ctrl with a rising GSI and vitellogenin synthesis. E_2 is produced during ovarian growth, whereas the maturation inducing hormone (MIH), such as 17α,20β-P, is produced during ovarian maturation. A shift in the steroidogenic pathway from E_2 to 17α,20β-P occurs in fish during oocyte maturation (Nagahama and Yamashita, 2008; Levavi-Sivan et al., 2010). In this study, serum 17α,20β-P began to increase significantly when most oocytes were in the process of maturation as vitellogenin accumulated.

In Ctrl, pituitary *fsh* mRNA expressions were increased at the early phases of ovarian development. The profile of pituitary *fsh* mRNA expression was in accordance with the previous result, and confirms the important roles of FSH in both initiation and maintenance of vitellogenin synthesis (Goméz et al., 1999; Andersson et al., 2013). Studies of carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) displayed sensitive responses of pituitary *lh* mRNA expression after GnRH implantation (Klausen et al., 2001; Kandel-Kfir et al., 2002), and in this study, elevated pituitary *lh* mRNA expression following a significant increase in hypothalamic *sgnrh-1* mRNA expression in Ctrl. LH regulates the final stages of oocyte maturation, and pituitary *lh* mRNA expression of Ctrl increased continuously from a relatively early stage of oocyte maturation in this study. This result suggested that endocrine function of LH also plays a vital role in vitellogenin synthesis and is complementary to the previous conclusion that LH stimulates MIH during the final maturation stages (Planas and Swanson, 1995).*fshr*/*lhr* expression in the ovary ensured the important physiologic function of FSH/LH during the time window of recruitment into puberty or the time window of vitellogenin synthesis (Andersson et al., 2013). As LH is mainly involved in the final stages of maturation and more potent in stimulating MIHs such as 17α,20β-P (Levavi-Sivan et al., 2010; Pramanick et al., 2013), a further increase in ovarian *lhr* mRNA expression was observed at 540 DFH in Ctrl when most females had reached the ovarian stage IV.

High stocking density is regarded as an environmental factor that drives fish metabolism to divert energy from normal metabolic processes (i.e. growth or gonad development) towards that associated with stress coping mechanisms (Mommsen et al., 1999; Andrade et al., 2015). In this study, we found that HPG axis of rainbow trout appeared to be retarded in higher densities. Higher densities showed 16.7% of females in ovarian stage IV only, and at 300 DFD, serum E_2 and GSI levels of higher densities were significantly lower than that of Ctrl. These results suggested that when most oocytes were in vitellogenin synthesis, the density-related chronic stressors impede the sustained development through vitellogenesis of trout in the higher density group.

Previous studies reported that hypothalamic *cgnrh-2* mRNA expression might mainly work as the neurotransmitter and neuromodulator and might regulate hypothalamic *sgnrh-1* mRNA expression (Li et al., 2004). We showed that significantly increased hypothalamic *cgnrh-2* mRNA expression was observed in higher densities when mRNA expression of hypothalamic *sgnrh-1* decreased. Dopamine released from hypothalamus played an inhibitory role on pituitary FSH release in the rainbow trout (Vacher et al., 2000), and in this study, pituitary *fsh* mRNA expression of higher densities showed significant declines followed by marked increases in circulating dopamine after 180 DFD.

Salmonidae have been particularly interesting research models of FSH/LH endocrine systems, which integrates feedback signals on the environmental changes (Watts et al., 2004; Taranger et al., 2010). Continuous light can interrupt recruitment into ovarian maturation of Atlantic salmon with collapsed pituitary *fsh* mRNA expression (Andersson et al., 2013). In this study, rearing density is selected as the environmental changes and pituitary *fsh/lh* mRNA expressions of higher densities were significantly decreased. These results were in accordance with previous studies of cod (*Gadus morhua*) and grouper (*Epinephelus malabaricus*) (De Almeida et al., 2011; Murata et al., 2012), suggesting that feedback signals of environment such as density variation and photoperiod modulation may affect pituitary FSH/LH endocrine systems in teleost.

Significant decrease of ovarian *fshr* in the higher densities was seen as early as at 180 days under dense rearing condition, and these results (viz. serum E_2, pituitary *fsh* and ovarian *fshr* of SD2 showed significant decline when compared to those of Ctrl) highlight ovarian FSHR-related regulation of ovarian development was disturbed in high density when it reached 31.7 kg/m^3. Obvious reduction of ovarian *lhr* mRNA expression of higher densities was observed later than ovarian *fshr* in our experiment, consistent with the previous study that LH and FSH are differentially expressed during gametogenesis, with an early increase in *fsh* expression and a late increase in *lh* expression (Schmitz et al., 2005).

5. Conclusion

This paper presented the overall profiles of HPG axis of female rainbow trout during early ovarian development and under high rearing density. At 420 DFH, when most trout of Ctrl were in vitellogenin accumulation stage, mRNA expression of hypothalamic *sgnrh-1* and pituitary *fsh* increased significantly and *cgnrh-2* displayed no change. Pituitary *lh* mRNA expression showed an obvious increase in rapid vitellogenin accumulation stage with high GSI. Serum E_2, P4, 17α,20β-P increased and dopamine decreased. Trout reared in higher densities displayed increased hypothalamic *cgnrh-2*, serum dopamine and decreased hypothalamic *sgnrh-1*,

pituitary *fsh*/*lh*, ovarian *fshr*/*lhr*, serum E_2, P4, and 17α,20β-P. Based on our findings, HPG axis of female rainbow trout may be retarded in higher densities and we recommend that rainbow trout (114.44 ± 6.21 g, 19.69 ± 0.31 cm) initially stocked in 6.6 or 8.6 kg/m^3 (60 or 80 $fish/m^3$) should be subdivided into lower densities when densities are over 30 kg/m^3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.07.011.

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