Effects of rearing systems on reproductive hormones secretion and their receptors gene expression in Xianju chickens under summer conditions

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ABSTRACT Previous study in our lab showed that indigenous Xianju chickens from free-range system (FRS) under summer conditions had lower egg production than those from conventional cage rearing system (CRS). The objective of this study was to preliminarily determine the FRS-dependent mechanism of depressing laying performance according to determining the effect of rearing systems on reproductive hormones secretion and their receptors mRNA expression in Xianju chickens reared under summer conditions. A total of 360 indigenous Xianju chickens were randomly allocated to CRS and FRS groups, each of which included 5 replicates of 36 hens. The experiment lasted between 21 and 29 wk of age. We found that the ovarian weight, numbers of small yellow follicles, and large white follicles in the FRS group were lower than those in the CRS group ($P < 0.05$). Changing from CRS to FRS increased serum concentrations of prolactin and decreased serum-luteinizing hormone and progesterone levels ($P < 0.05$). Gene expressions in the preovulatory follicles from FRS hens were upregulated for prolactin receptor and downregulated for luteinizing hormone receptor and progesterone receptor, compared to those from CRS hens ($P < 0.05$). It can be concluded that changing from CRS to FRS in the current experimental conditions depressed egg production traits in Xianju chickens by inducing a synergistic activity of reproductive hormones and the gene expressions of their receptors.

Key words: Xianju chicken, reproductive hormone, gene expression, rearing system, summer conditions

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INTRODUCTION

Alternative rearing systems such as enriched cage, organic system, and free-range system (**FRS**) have been shown to have a significant effect on production traits in laying hens (Tauson et al., 1999; Mugnai et al., 2009; Neijat et al., 2011; Ahammed et al., 2014). The general trend in layer strains showed that egg production was higher in conventional cage systems than in alternative rearing systems (Yakubu et al., 2007; Mugnai et al., 2009; Anderson, 2010). However, Küçükyılmaz et al. (2012) found that White Lohmann LSL layers produced more eggs in the cage system than in the organic system, while brown ATAK-S laying hens showed lower laying performance in the cage system than in the organic system, indicating that some genotypes will show better performance under conventional cage systems, whereas some genotypes will respond positively when allowed fresh air and freedom of movement. Furthermore, compared to the conventional cage systems, the egg yield from alternative rearing systems was more affected by seasonal conditions (temperature and photoperiod) as described by Mugnai et al. (2009). Thus, studies on the effect of alternative rearing systems on production traits are continuing, especially in relation to the ability of native pure breeds to adapt to these alternate systems under certain climates. Xianju chicken, originated from humid subtropical areas in Zhejiang provinces, is considered to be one of the most famous indigenous layer breeds in China due to its high egg production (180 to 200/yr) and adaptability (Tang et al., 2009). A recently study in our lab showed that Xianju chickens from FRS under summer conditions had lower egg production than those from conventional cage system (Dong et al., 2017). The debilitating effect on egg production is well recognized, but the mechanism involved is not clearly understood.

Egg production is under the control of hormonal status of hen (Pirsaraei et al., 2008). It has been shown that changes of reproductive hormones such as follicle stimulating hormone (**FSH**), luteinizing hormone (**LH**), progesterone (**P4**), and prolactin (**PRL**) may induce different performance traits in layers (Onagbesan et al., 2006; Li et al., 2011). We hypothesized that the regulatory mechanisms for the reduced egg production in the hens from FRS under summer conditions might be modulated at the level of the reproductive hormone secretion. Hence, the present study

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was planned to investigate the effects of alternative rearing systems on reproductive hormones response in indigenous Xianju chickens under summer conditions.

MATERIAL AND METHODS

The experiment was conducted in accordance with the Chinese guidelines for animal welfare and was approved by the animal welfare committee of the Animal Science College, Zhejiang University.

Birds, Feeding, and Rearing Systems

A total of 360 indigenous Xianju chickens, at the start of laying (21 wk of age), were randomly allocated to 2 treatment groups, each of which included 5 replicates of 36 hens. The conventional cage rearing system (**CRS**) and FRS were located on the same field in Jinshanjiao layer farm (Taishun within Zhejiang province, China). We conducted a 9-wk trial (with a 1-wk acclimation period) to make sure that the chickens were reared under summer conditions (from June to August) throughout the study period.

For CRS, hens were reared with the 4-tiered rearing cages fitted with linear feeders and nipple drinkers (2 nipple drinkers per cage). Each replication consisted of 12 adjacent cages. Hens were kept in cages (47 cm length, 37 cm width, and 50 cm height) with 3 hens per cage providing approximately 579 cm^2 floor space per bird, and housed in a windowed layer house with a controlled ventilation regime and a wet curtain cooling system. The FRS had an indoor and paddock area. The indoor area was an open-sided naturally ventilated layer house and its floor was covered with wood shavings. A total 6 hens per m^2 was provided in the FRS indoor area. The paddock area was located in front of the open-sided house, covered with alfalfa and trifolium mixture, providing stocking density of 0.25 birds/m^2. Inside the paddocks area, there was a small hut with nests (6 hens per nest) and perches (18 cm of area per hen). Replication in FRS was done with the help of fish-net. The paddock area was enclosed by wire fences to keep out predators. The circular feeders and plastic drinkers were used in the indoor and paddock areas.

Natural light, supplemented with artificial light to achieve 16L:8D was used in all of the systems. Hens in all rearing systems were fed the same complete diet based on corn and soybean meal with 17% CP and 2750 kcal/kg ME. Feed and drinking water were offered ad libitum to all hens. The mean daily temperature was 31°C (range 26 to 35°C) and ambient RH was 75% (range 60 to 90%) in the FRS throughout the study period. The mean daily temperature and RH inside the barn from CRS was 27°C (range 24 to 30°C) and 80% (range 70 to 90%), respectively. The mean mortalities in CRS and FRS were 0.00 and 2.22%, respectively.

Blood sampling and analysis

At the end of wk 29, 12 h after feed withdrawal, 2 birds were randomly selected from each replicate and blood samples were collected from the axillary vein. Serum was isolated by centrifugation of blood at 3000× *g* for 10 min, and was stored in 1.5-mL Eppendorf tubes at −70°C until analyses. Serum FSH, P4, LH, and PRL levels were analyzed using RIA kits (Hengyuan Biological Technology Co., Ltd, Shanghai, China) by radio immunoassay, according to the recommendations of the manufacturer. For measurement of FSH concentration, the assay sensitivity, measuring range, intra- and interassay CVs were 0.4 mIU/mL, 1.5 mIU/mL to 100mIU/mL, <10% and <15%, respectively. For measurement of P4 concentration, the assay sensitivity was 0.2 ng/mL, measuring range was 0.5 to 80 ng/mL, and the intra- and interassay CVs were <10%. For measurement of LH concentration, the assay sensitivity, measuring range, intra- and interassay CVs were 0.02, 0.5 to 20 ng/mL, <10% and <15%, respectively. For measurement of PRL concentration, the assay sensitivity was 0.5 ng/mL, measuring range was 5.0 to 100 ng/mL, and the intra- and interassay CVs were <10%. All the samples were measured in one single assay.

Quantification of reproductive hormone receptors mRNA with real-time PCR

The hens selected for blood sampling were then slaughtered by cervical dislocation and the ovary were quickly removed and weighed. The numbers of preovulatory follicles (**POFs**) [**F1** (the first largest one of POFs; about 34 mm in diameter), **F2** (the second largest one of POFs; 25 to 33 mm in diameter), **F3** (the third largest one of POFs; 15 to 24 mm in diameter)], small yellow follicles (**SYFs**) (4 to 10 mm in diameter), and large white follicles (**LWFs**) (2 to 4 mm in diameter) of every chicken were counted. Then, the granulosa layers of the POFs, SYFs, and LWFs were separated, minced with a razor blade, frozen as aliquots in liquid nitrogen, and stored at –70°C for gene expression analysis.

Total RNA was isolated from the granulosa layers of the POFs, SYFs, and LWFs using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of total RNA was checked by both native RNA electrophoresis on 1.0% agarose gel and the UV absorbance ratio at 260 and 280 nm. The cDNA was synthesized from total RNA by a reverse transcriptase (M-MLV; Takara, Dalian, China) at 42°C for 60 min with oligo dT-adaptor primer using the protocol of the manufacturer.

The primer sequences of follicle-stimulating hormone receptor, luteinizing hormone receptor (***LHR***), progesterone receptor (***PR***), and prolactin receptor (***PRLR***) used for quantitative realtime PCR were synthesized by Generay Biotech Co., Ltd (Shanghai, China) and shown

Table 1. Primers used in real-time PCR.

Gene[1]	Primers sequences (5′-3′)	Reference
FSHR	TCAGCAGCTACATGAAGGT AAGGCAAGTACATTCAACACTA	Long et al., 2017
LHR	GCTGATCTTAATGCTCAACG TTGGCAATCTTGGTGTCTTTAT	
PRLR	CAGATTCACGAGTTCCGCA GGCATAAATGAGGATGGGT	
PR	ATCATCGTTCTATTCACTGT CTCGTTCTCATCTCATCAA	Liu et al., 2015
18S rRNA	ATTCCGATAACGAACGAGACT GGACATCTAAGGGCATCACA	Deng et al., 2014

[1]*FSHR*, follicle-stimulating hormone receptor; *LHR*, luteinizing hormone receptor; *PR*, progesterone receptor; *PRLR*, prolactin receptor; *18S rRNA* served as endogenous reference gene.

in Table 1. The abundance of mRNA was determined on a StepOne Plus Real-Time PCR system (ABI 7500, Applied Biosystems, Foster City, CA) using SYBR Premix PCR kit (Takara, Dalian, China). The PCR program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Efficiency of the real-time PCR primers for all the examined genes was calculated from standard curves. Each sample was run in triplicate and no template control was included. Specificity of the amplification was verified at the end of PCR run by melting curve analysis. Specificity of the product was also confirmed by running samples on a 1.2% agarose gel, excising for purification using a DNA purification kit (Takara, Dalian, China) and sequencing (Shanghai Sangon Biotech Co. Ltd., Shanghai, China). The difference of Ct (cycle threshold) value for 18S rRNA was less than 0.5 between the CRS and FRS groups, and, therefore, was considered to be an appropriate endogenous control. Average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The calibrator was the average ΔCt value of each gene in LWF of the CRS group.

Statistical analysis

The data were expressed as means ± SE and analyzed statistically by two-tailed Student's *t*-test, using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was considered at $P < 0.05$. An individual hen served as the experimental unit for all data.

RESULTS AND DISCUSSION

A previous study in our lab showed that changing from CRS to FRS resulted in a 23.20% decrease of hen-day egg production (74.34 vs. 57.09%) in Xianju chickens under summer conditions (Dong et al., 2017). As the serum hormone level has been considered to be a sensitive indicator of laying performance (Mohammadi and Ansari-Pirsaraei, 2014), we determined the effects of rearing systems on serum hormone profile in the current study by using the same pure native breeds under summer conditions. We found that rearing systems had

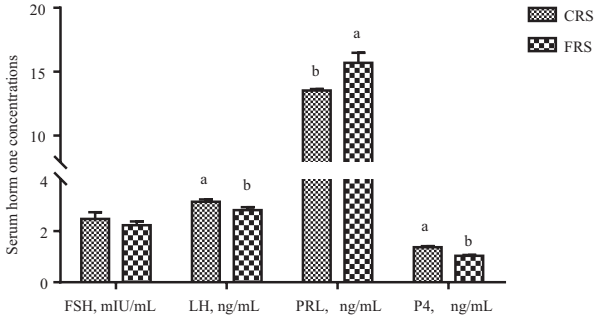

Figure 1. Effects of conventional cage rearing system (CRS) and free-range system (FRS) on serum follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P4), and prolactin (PRL) concentrations in Xianju chickens reared under summer conditions. Data shown as means ± SE (n = 10). Means on each bar with no common letter differ significantly at $P < 0.05$.

no significant effect on FSH secretion; however, changing from CRS to FRS increased serum concentrations of PRL and decreased serum LH and P4 levels ($P < 0.05$; Figure 1). Prolactin, secreted by the pituitary gland, has been considered as a negative regulator of bird reproductive activities by inducing expression of incubating behavior, suppressing secretion of gonadotropins (e.g., FSH and LH), and causing atresia of ovarian follicles (El Halawani and Rozenboim, 1993; Chaiseha and El Halawani, 2005). LH, as a kind of gonadotropins, plays an important role in the course of follicular development and promotes the secretion of P4 in the POFs (Palermo, 2007; Thompson and Kaiser, 2014). Regarding to P4, it is demonstrated that more P4 production could induce ovulation by stimulating the synthesis of protein-decomposing enzymes and collagen hydrolase in the follicular cells (Donoghue, 1989). Accordingly, the diminished egg production in FRS chickens may, in part, be related to increased PRL secretion and decreased LH and P4 secretion. The altered PRL and LH secretion might be partly due to the rearing system but mostly to the environmental factors in FRS, which provide more natural but poorer living conditions as reflected by higher temperature than CRS throughout the current study. Previous studies have been demonstrated that stress, such as heat stress, increased circulating PRL and decreased circulating gonadotropins, respectively, in cows and goats (Johke, 1970), turkey poults (Opel and Proudman, 1982), turkey hens (El Halawani et al., 1985; Rozenboim et al., 2004), and laying hens (Johnson, 1981). The depressed P4 secretion of hens reared under FRS might be associated with the reduction of serum cholesterol concentrations, because steroid hormones including the reproductive hormones P4 are derived from cholesterol (Alodan, 2001; Yoder et al., 2004). Our previous study had confirmed that hens from FRS exhibited lower serum concentration of cholesterol than that in hens reared under CRS (6.44 vs. 10.47 mmol/L; Dong et al., 2017).

The biological effect of FSH, LH, P4, and PRL on target cells is elicited through their receptors (Kobayashi et al., 2008; Li et al., 2011; Liu et al., 2015;

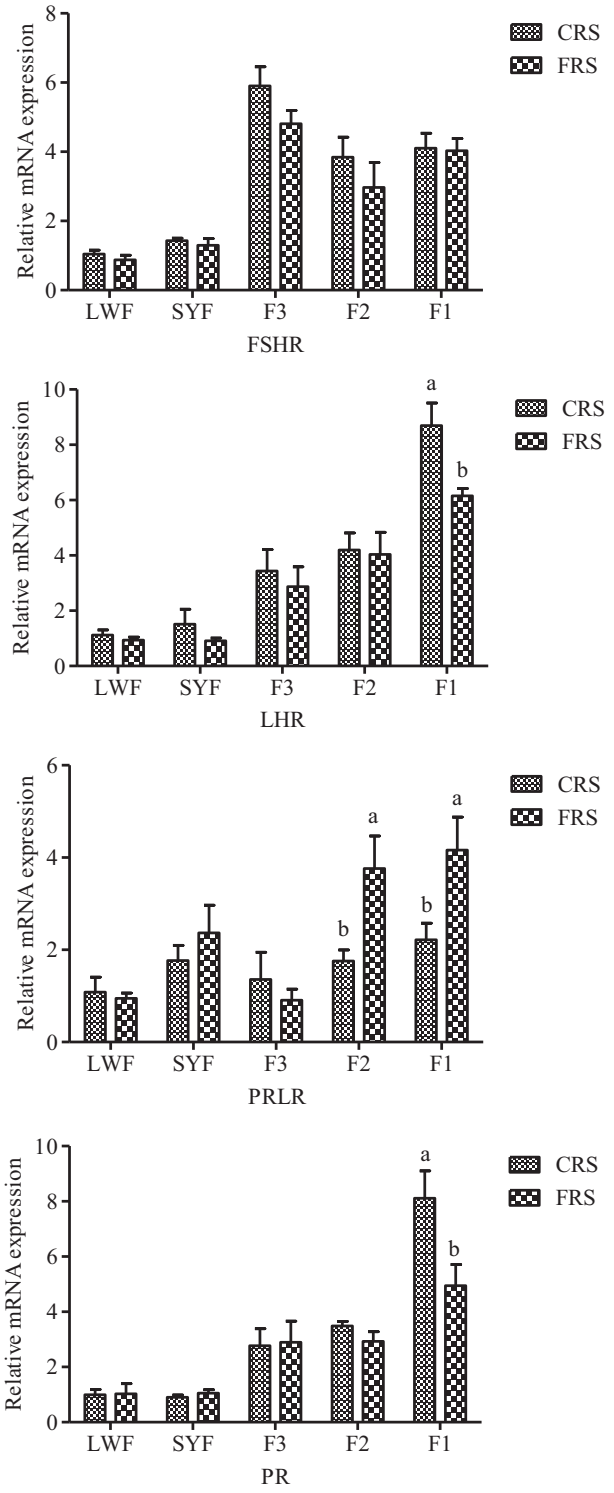

Figure 2. Effects of conventional cage rearing system (CRS) and free-range system (FRS) on the mRNA expression of follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), progesterone receptor (PR), and prolactin receptor (PRLR) in follicles of different size from Xianju chickens reared under summer conditions. F1, F2, F3, preovulatory follicles; SYFs, small yellow follicles; LWFs, large white follicles. Relative gene expression ± SE (n = 10) was calculated using the $2^{-\Delta\Delta Ct}$ method with 18S rRNA as the endogenous control and the average ΔCt value of each gene in LWF of CRS group as the calibrator. Different letters on each bar indicate a significant difference ($P < 0.05$) at different follicles between CRS and FRS groups.

Table 2. Effects of rearing systems on ovarian weight and number of different-sized follicles in Xianju chickens.[1]

Item[2]	Rearing systems[3] CRS	FRS	*P*-value
Ovarian weight	40.83 ± 1.41[a]	36.90 ± 1.92[b]	0.03
Number of POFs	5.33 ± 0.29	5.00 ± 0.24	0.38
Number of SYFs	19.20 ± 0.86[a]	16.20 ± 0.58[b]	0.02
Number of LWFs	28.50 ± 1.53[a]	22.85 ± 1.28[b]	0.02

[1]n = 10 hens per group.
[2]POFs, preovulatory follicles; SYFs, small yellow follicles; LWFs, large white follicles.
[3]Rearing systems: CRS, conventional cage rearing system; FRS, free-range system.
[a,b]Means within a row with different superscripts differ significantly ($P < 0.05$).

Long et al., 2017). In the current study, FSH, LH, P4, and PRL receptor gene expressions were furtherly investigated. Consistent with previous studies (LaBarbera, 1994; Seekallu et al., 2010; Liu et al., 2015), the mRNA expression of follicle-stimulating hormone receptor was greatest in the granulosa layer of small POFs (F3), while greatest expression of *LHR*, *PRLR*, and *PR* was found in large POFs (F1) (Figure 2). Regarding to the effects of rearing systems on those receptors expression, we found that mRNA expressions in the granulosa layer of POFs from FRS hens were upregulated for *PRLR* and downregulated for *LHR* and *PR*, compared to those from CRS hens ($P < 0.05$; Figure 2). Our results showed that the significant alteration of circulating reproductive hormone levels of hens reared under different rearing systems was associated with the alteration of their receptors mRNA expressions in the POFs. This synergistic reduction between reproductive hormones and their receptors caused by FRS allows for the decrease of ovarian weight and follicular numbers ($P < 0.05$; Table 2), as well as follicle growth, development, maturation, and ovulation in the ovary, and thus eventually debilitate egg production of laying hens. Convincing evidence implicating the synergistic change between reproductive hormones and their receptors secretion as a causative factor for managing productivity traits in laying hens has been presented by Long et al. (2017).

In general, we presented the first data referring to the effect of rearing systems on circulating reproductive hormones and mRNA expression of their receptors in follicles of different size by using indigenous Xianju chickens reared under summer conditions. It can be concluded that changing from CRS to FRS in the current experimental conditions depressed egg production traits in Xianju chickens by inducing a synergistic activity of reproductive hormones and the gene expressions of their receptors.

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