Corticosterone regulation of ovarian follicular development is dependent on the energy status of laying hens

Xiao-Juan Wang, Yan Li, Qun-Qing Song, Ying-Ying Guo, Hong-Chao Jiao, Zhi-Gang Song, and Hai Lin

Department of Animal Science, Shandong Agricultural University, Taian, Shandong 271018, People’s Republic of China

Abstract Glucocorticoids participate in the arousal of stress responses and trigger physiological adjustments that shift energy away from reproduction toward survival. Ovarian follicular development in avians is accompanied by the supply of yolk precursors, which are mainly synthesized in the liver. Therefore, we hypothesized energy status and hepatic lipogenesis are involved in the induction of reproductive disorders by glucocorticoids in laying hens. The results show that corticosterone decreased the laying performance by suppressing follicular development in energy-deficit state, rather than in energy-sufficient state. In corticosterone-treated hens, the suppressed follicular development was associated with the reduced availability of yolk precursor, indicated by the plasma concentration of VLDL and vitellogenin and the decreased proportion of yolk-targeted VLDL (VLDLy). Corticosterone decreased the expression of apolipoprotein B and apolipoprotein VLH-II in the liver. A drop in VLDL receptor content and an increase in the expression of tight junction proteins occludin and claudin1 were also observed in hierarchical follicles. The results suggest corticosterone-suppressed follicular development is energy dependent. The decreased apolipoprotein synthesis and VLDLy secretion by liver are responsible for the decreased availability of circulating yolk precursor, and the upregulation of occludin and claudin expression further prevents yolk deposition into oocytes.—Wang, X-J., Y. Li, Q-Q. Song, Y-Y. Guo, H-C. Jiao, Z-G. Song, and H. Lin. Corticosterone regulation of ovarian follicular development is dependent on the energy status of laying hens. J. Lipid Res. 2013. 54: 1860–1876.

Supplementary key words glucocorticoids • ovarian development • energy state • apolipoprotein synthesis • tight junction proteins

In birds, the onset of breeding involves the activation of the hypothalamic-pituitary-gonadal (HPG) axis. The secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), which in turn activate gonadal development and release of sex steroids, including estradiol (E2) and testosterone. Stress is a common problem disrupting breeding in either wild birds or domestic chickens (1). Glucocorticoids (GC), as the final effectors of the hypothalamic-pituitary-adrenal axis, participate in the control of whole body homeostasis and the arousal of stress responses. GCs transmit information about environmental conditions to the HPG axis, which ultimately influences the timing of breeding.

Female kittiwakes with low baseline LH levels and elevated levels of baseline corticosterone (CORT), the main form of GCs in avians, were more likely to skip breeding (2). An experimental reduction of CORT release during the prelaying period was associated with an advancement of egg laying in female kittiwakes (3). In domestic chickens, an acute infusion of CORT resulted in a pause in laying and a severely reduced ovarian weight (4). Chronic and repeated exposure to CORT during the rearing phrase suppressed reproductive performance, resulting in a delay of first egg laid and a reduction of egg production (5).

In avians, the development of ovarian follicles is accompanied by the deposition of a large amount of yolk. During a laying cycle, follicular development is matched with a supply of yolk precursors. Triacylglycerols (TG), the main
components of yolk lipid (6), are mainly synthesized in the liver and secreted in the form of yolk-targeted very-low density lipoprotein (VLDLy). An elevated circulating VLDLy concentration is coincident with rapid yolk development and matched variation in total yolky follicle mass in European starlings (7). VLDLy has two associated apolipoproteins, apolipoprotein B (apoB) and apolipoprotein VLDD-II (apoVLDD-II), which are involved in the assembly of TG-rich lipoprotein particles. Avian apoB is a component of specialized VLDL particles that are produced by the liver in response to estrogen (8). apoVLDD-II mRNA was specifically expressed in the liver and upregulated after laying (9, 10).

When domestic laying hens were infused with CORT, food consumption remained high and body weight remained unchanged, although the liver weight doubled (4). In our previous work on immature chickens, GCs stimulated energy intake (11) and enhanced hepatic lipogenesis and fat deposition in adipose tissues, indicating the redistribution of energy stores in CORT-challenged chickens (12–14). GCs trigger physiological and behavioral adjustments that shift energy investment away from reproduction and toward survival (15). CORT secretion during the prelaying period mediates the timing of breeding in kittiwakes, possibly through the dynamics of energy reserves (3). High-level CORT exposure does not affect the timing of breeding in Florida scrub-jays under normal feeding conditions (16). Hence, we hypothesized that energy status and hepatic lipogenesis are involved in the physiological responses of the reproductive system to stress perturbations.

In the present study, two experiments were conducted to evaluate the effect of GCs on ovarian function in laying hens (Gallus gallus domesticus). In Experiment 1 and Experiment 2, the effects of CORT on laying performance, follicular development and hepatic lipogenesis were evaluated for two feeding states (fasting and feeding) and for diets with two different energy levels, respectively. The present study suggests that exogenous GCs perturbed the reproduction of laying hens in an energy-dependent manner.

METHODS

Ethics statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University and performed in accordance with the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, China). Animal suffering was minimized as much as possible.

Birds and treatments

Hy-Line Brown laying hens were housed in an environmentally controlled room. The hens were fed a layer diet based on a corn-soybean meal diet (containing 16% crude protein, 2,702 kcal/kg metabolizable energy, 3.6% calcium, and 0.4% available phosphorus). The lighting regime was 16 h light (from 05:00 to 21:00) and 8 h dark. All birds had free access to feed and water during the rearing period.

Experiment 1. At 26 weeks of age, 48 hens with similar body mass (BM) and egg production were randomly divided into eight groups of six chickens and subjected to one of the two following treatments (Fig. 1A): subcutaneous injection of corticosterone dissolved in corn oil at a dose of 2 mg/kg/day BM for 7 days (CORT) or sham injection of the same volume of vehicle-corn oil (Control). BM, feed intake, egg number, and egg mass were recorded daily for each group. Hen-day egg production was calculated. Eggs laid were counted daily and laying rate was expressed as a percentage of total hen amounts for each group. Before the sampling day (day 7), half of the hens from each group were randomly selected with or without 12 h overnight feed withdrawal. On day 7, eight chickens from each treatment were selected for a blood sample. The sample was obtained within 30 s with a heparinized syringe from a wing vein and collected in iced tubes. Plasma was obtained after centrifugation at 400 g for 10 min at 4°C and was stored at −20°C for further analysis. Immediately after blood sampling, the hens were sacrificed by cervical dislocation and exsanguination (17). Thereafter, the liver, ovaries and follicles, and abdominal fat were harvested and weighed. The number and weight of hierarchical follicles (HF, >12 mm) and small yellow follicles (SYF, 5–12 mm) were recorded. The liver and the follicular membranes of HF were sampled and washed with ice-cold sterilized saline, cooled down in liquid nitrogen, and stored at −80°C for further analysis.

Experiment 2. Forty-eight 30-week-old hens with similar BM and egg production were randomly divided into eight groups of six chickens and given ad libitum access to either a normal diet (2,654 kcal/kg) or a high-energy diet (2,917 kcal/kg). After a three-week dietary treatment period, half of the hens in each

Fig. 1. Schematic diagrams of design of Experiment 1 (A) and Experiment 2 (B).
group were randomly assigned to either CORT administration or sham treatment for 7 days as in Experiment 1 (Fig. 1B). At the end of the experiment, eight chickens from each treatment were selected and sampled after a 12 h feed withdrawal. The measurements were performed in the same way as in Experiment 1.

**Measurements**

**Histological analysis.** The degrees of hepatic steatosis were observed with hematoxylin and eosin staining as described in Wang et al. (18). Briefly, the liver tissue was fixed conventionally in 4% formaldehyde, dehydrated, and embedded in paraffin. De-paraffinized sections with a thickness of 4 μm were stained with Harris hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO). Sections were examined under an Olympus CX-41 phase contrast microscope (Olympus, Tokyo, Japan).

The accumulation of cytoplasmic lipid droplets in liver was visualized by Oil Red O staining according to the protocol of Lillie and Fullmer (19). Briefly, tissues were immediately frozen in liquid nitrogen and cut in a Leica CM-1850 cryostat microtome (Leica, Wetzlar, Germany). Then 18 μm thick sections were fixed in 4% formaldehyde for 60 min and stained with filtered 0.5% Oil Red O (Sigma-Aldrich, St. Louis, MO), which was made by dissolving in isopropyl alcohol for 15 min at room temperature. Sections were photographed using an Olympus CX-41 phase contrast microscope (Olympus, Tokyo, Japan).

**Hepatic TG content.** Total lipids were extracted according to the method used by Folch et al. (20). A 100 mg liver sample was homogenized with 2 ml of chloroform-methanol (2:1, v/v). After a centrifugation at 400 g for 10 min, the supernatant was obtained, and then saline (4:1, v/v) was added and vortexed. The organic phase was isolated by a centrifugation at 400 g for 10 min, then transferred to a new tube and evaporated under a stream of nitrogen. The extracted lipids were then dissolved with 1 ml isopropyl alcohol, and the levels of hepatic TG content were determined spectrophotometrically using commercial kits (GPO-PAP) purchased from the Jiancheng Bioengineering Institute (Nanjing, China).

**In vivo hepatic VLDL secretion.** VLDL secretion was measured according to Jong et al. (21). The laying hens that underwent Experiment 1 were injected intravenously with 15% (w/v) Triton WR1339 (Sigma, St. Louis, MO) (500 mg/kg BM) dissolved in 0.9% NaCl. Plasma VLDL clearance is virtually completely inhibited under these circumstances (22). Immediately before Triton administration and 45 and 90 min after Triton administration, blood samples were drawn, and TG content was measured using commercially available colorimetric assays. The TG accumulation in plasma was linear during this time period. Because the vast majority of TG is associated with the apoB-containing VLDL particles, we refer to these TG measurements as VLDL-TG. Hepatic VLDL-TG secretion rate was calculated from the slope of the line and expressed as μmol/l per kg BM.

**Determination of VLDL particle size distribution.** Plasma VLDL was isolated by centrifugation to determine particle size distributions by dynamic laser light scattering as described below. Lipoproteins were isolated from 1 ml plasma using sequential density gradient centrifugation (23). The plasma activity of lecithin:cholesterol acyltransferase was inhibited by the addition of 15 μl dithionitrobenzoic acid/ml plasma. Density solutions were prepared by adding increasing amounts of NaBr to a basal NaCl solution (density = 1.0063 g/ml) containing 0.01% EDTA and 50 KU/l each of streptomycin and penicillin. VLDL was isolated as the plasma fraction of density <1.019 g/ml by aspiration with a narrow-bore pipette after centrifugation at 148,600 g for 18 h at 14°C in a Sorvall 45.6 TFT rotor (Sorvall-DuPont, Wilmington, DE). The VLDL diameters were determined optically by dynamic light scattering using a Wyatt DynaPro Nanostar instrument (Santa Barbara, CA) at 22°C, according to a modification of the method from Walzem et al. (24). Centrifugally isolated VLDLs were suspended as a 1:30 dilution in a NaCl solution (density = 1.0063 g/ml) and placed into the sample well. The system software and 3 mW, λ = 658 nm laser beam were activated. Light scattering was recorded from the lipoprotein particles for 4 min. The data were analyzed using Dynamics 6.0 software, which automatically calculates the particle size distribution according to the characteristics of the scattered light within the measurement region. VLDL particle diameters of 25–44 nm were positively associated with egg production (25, 26).

**Plasma variables.** The concentration of glucose (no. F006), nonesterified fatty acids (NEFA, no. A042), and TG (no. F001) were measured spectrophotometrically using commercial diagnostic kits (Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of VLDL were determined as previously described (27).

Plasma insulin was measured by radioimmunoassay with a guinea pig anti-porcine insulin serum (3 V Biochemical Engineering Co., Weifang, China). A large cross-reaction was observed between chicken insulin and this anti-serum (porcine) (28). The insulin in this study is referred to as immunoreactive insulin. The sensitivity of the assay was 6.9 pmol/l, and all samples were included in the same assay to avoid interassay variability. The intraassay coefficient of variation was 6.9%. FSH, LH, and E2 levels were measured by radioimmunoassay with a rabbit anti-human serum (Jiuding Bioproduction Co., Tianjin, China). The sensitivities of the assays for FSH, LH, and E2 were 0.9 IU/l, 1.0 IU/l, and 2.1 ng/l, respectively. All the plasma samples were included in the same assay to avoid interassay variability. The intraassay coefficients of variation were 5.5, 5.4, and 7.7% for FSH, LH, and E2, respectively.

**FAS and ME activities and OVR protein content.** The activities of fatty acid synthase (FAS) and malic enzyme (ME) in the liver were measured. The liver samples were homogenized in ice-cold 0.25 mol/l sucrose, 1 mmol/l dithiothreitol, and 1 mmol/l EDTA at pH 7.4. The cytosolic fractions were obtained by centrifugation at 100,000 g for 1 h at 48°C and used for the enzymatic assays. The FAS activity was measured according to the method of Halestrap and Denton (29). One unit of FAS is defined as 1 nmol reduced NADPH consumed by 1 mg protein in 1 min. ME activity was determined by a method modified from Hsu and Lardy (30). One unit of ME is defined as 1 μmol NADPH produced by 1 mg protein in 1 min. The VLDL/vitellogenin receptor (OVR) protein content in the follicular membranes of HF was measured using an ELISA method (Cusabio Biotech Co.). The sensitivity of the assay for OVR protein content was 3.9 pg/ml. All the samples were included in the same assay to avoid interassay variability. The intraassay coefficients of variation were less than 8%.

**mRNA expression.** Gene expression was measured using real-time RT-PCR. Briefly, total RNA was extracted from the liver tissue and the HF membranes using Trizol (Invitrogen, San Diego, CA). The quantity and quality of the isolated RNA were determined using a biophotometer (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis, respectively. Reverse transcription was performed in RT reactions (10 μl) consisting of 500 ng total RNA, 5 mmol/l MgCl₂, 1 μl RT buffer, 1 mmol/l dNTP, 2.5 U AMV, 0.7 mmol/l oligo d(T), and 10 U RNase inhibitor (TaKaRa, Dalian, China). mRNA was amplified using cDNA as template in a 20 μl
PCR reaction containing 0.2 µmol/l of each primer (Sangon, Shanghai, China) and SYBR green master mix (TaKaRa, Dalian, China). Real-time PCR was performed at 95°C for 10 s of predenaturisation, followed by 40 cycles, with each cycle consisting of denaturisation at 95°C for 5 s and annealing and extension at 60°C for 40 s. mRNA expression was quantified in comparison to the comparative CT method (2^-ΔΔCT) (31). The mRNA levels of target genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, 18S rRNA (18SrRNA), and β-actin (ΔCT). The ΔCT was calibrated against the average of the control chickens. The number of target molecules relative to the control was calculated using 2^-ΔΔCT. Therefore, all gene transcription results are reported as the n-fold difference relative to the control. The primer sequences are listed in Table 1. The PCR products were verified by electrophoresis on a 0.8% agarose gel and by DNA sequencing. Standard curves were generated using pooled cDNA from the samples that were assayed. All samples were run in duplicate, and primers were designed to span an intron to avoid contamination from genomic DNA.

Western blotting analysis. Samples from the liver tissue and the HF membranes were homogenized on ice in radioimmuno-precipitation assay buffer (50 mmol/l Tris-HCl at pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mmol/l sodium orthovanadate, 150 mmol/l sodium fluoride) and centrifuged at 12,000 g for 5 min at 4°C. The protein concentration was determined using the BCA assay kit (Beyotime, Jiangsu, China). Western blots were developed and quantified using BioSpectrum 810 with VisionWorksLS 7.1 software (UVP LLC, California).

Statistical analysis. All the data were subjected to one-way ANOVA analysis with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC), and the main effect of CORT treatment was evaluated. Multiple comparisons between means were conducted using the Tukey’s honestly significant difference test. Means were considered significantly different at P < 0.05.

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RESULTS

Experiment 1

Laying performance. We investigated the effect of CORT treatment on follicular development and the involvement of energy state in Experiment 1. Table 2 shows that CORT decreased BM (P < 0.001), laying rate (P < 0.05), egg production (P < 0.05), and egg weight (P < 0.05). Feed intake, however, was not affected by CORT administration (P > 0.05).

Ovarian and follicular development. In the fasting state, CORT treatment decreased ovarian mass (P < 0.001), HF number and mass (P < 0.001), and SYF number and mass (P < 0.05, Fig. 2A, C, E). In contrast, none of these variables were altered in the feeding state (P > 0.05, Fig. 2B, D, F).

Liver and abdominal fat accumulation. The liver fat accumulation was measured to evaluate the hepatic lipogenesis. CORT-treated hens had a higher liver mass compared with control hens in either the fasting (P < 0.0001) or feeding state (P < 0.01). In contrast, CORT only decreased abdominal fat weight in the feeding state (P < 0.05, Fig. 2G, H). CORT treatment increased lipid deposition in the liver, as indicated by both histological analysis (Fig. 3A–D) and the hepatic TG content (P < 0.01, Fig. 3E).

Plasma variables. We checked the availability of yolk precursors by testing the circulating VLDL content and diameter of VLDL particles. In the fasting state, CORT administration significantly increased the plasma concentrations of FSH (P < 0.001), LH (P < 0.01), and insulin (P < 0.001), while the E2 level tended to decrease (P = 0.075, Fig. 4A). In the feeding state, CORT treatment also increased LH (P < 0.05) and FSH concentrations (P < 0.01). However, CORT administration did not significantly affect E2 and insulin levels (P > 0.05, Fig. 4B). Plasma concentrations of glucose were significantly increased in the feeding state (P < 0.01) but not in the fasting state (P > 0.05, Fig. 4C, D). The reverse is true for the NEFA level, which was only decreased by CORT in the fasting state (P < 0.01). Plasma concentrations of TG and VLDL were decreased by CORT administration in both the fasting (P < 0.001) and feeding states (P < 0.05). For small diameter VLDL particles (< 100 nm), CORT treatment significantly increased their diameter (P < 0.05) and decreased their proportion (P < 0.05) compared with control hens. In contrast, the proportion of VLDL particles with a large diameter (>100 nm) was increased by CORT (P < 0.05), while their diameter was not significantly changed (P > 0.05, Fig. 4E, F).

mRNA and protein expression of genes related to yolk deposition in HF membranes. In the fasting state, there were remarkable increases in the mRNA levels of estrogen receptor (ESR, P < 0.01), OVR (P < 0.01), claudin1 (P < 0.05), occludin (P < 0.01), and lipoprotein lipase (LPL, P < 0.001) in CORT-treated hens compared with control hens, while no significant changes were observed in the mRNA levels of FSH receptor (FSHR) and LH receptor (LHR) (P > 0.05, Fig. 5A). In the feeding state, however, the expression levels of all these genes were not significantly influenced by CORT treatment (P > 0.05, Fig. 5B). The protein expression of occludin was decreased by CORT (P < 0.01) independent of the feeding state (Fig. 5C). CORT-treated fasting hens exhibited higher claudin1 protein expression (P < 0.001, Fig. 5D) and lower OVR protein content (P < 0.05, Fig. 5E).

mRNA and protein expression and enzymatic activity of genes related to VLDL synthesis in liver. FAS activity was higher in CORT-treated hens than in control hens in both the feeding (P < 0.0001) and fasting states (P < 0.01). In contrast, ME activity was only increased by CORT in the fasting state (P < 0.01, Fig. 6A, B). The mRNA levels of sterol response element-binding protein-1c (SREBP-1c, P < 0.05), acetyl CoA carboxylase 1 (ACCI, P < 0.001), ME (P < 0.001), stearoyl-CoA desaturase 1 (SCD1, P < 0.05), and microsomal triglyceride transfer protein (MTP, P < 0.001) were upregulated by CORT in the fasting state, but they were not significantly changed in the feeding state (P > 0.05). The gene expression of liver X receptor α (LXRA) was not altered by CORT in either the feeding or fasting state (P > 0.05). In contrast, the mRNA levels of apoB100 (P < 0.001) and apoVLDL-II (P < 0.01) were downregulated, and FAS was upregulated (P < 0.001) by CORT treatment in both the feeding and fasting states (Fig. 6C, D). In the fasting state, the protein expression of apoB100 was decreased in CORT-treated hens compared with control hens (P < 0.05), but no effect was observed in the feeding state (P > 0.05, Fig. 6E). The hepatic VLDL secretion rate was reduced by CORT in both the fasting and feeding states (P < 0.05, Fig. 6F).

Experiment 2

Laying performance. We further investigated the effect of energy state on follicular development by administering

| TABLE 2. Effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days) on laying performances of hens (Experiment 1) |
|------------------|------------------|------------------|------------------|
| Item             | CORT            | Control          | Probability      |
| BM, kg           | 1.65 ± 0.02a     | 1.72 ± 0.01a     | < 0.001          |
| Feed intake, kcal/day | 288.3 ± 13.8     | 295.1 ± 30.3     | NS              |
| Laying rate, %   | 53.6 ± 12.8a     | 95.8 ± 2.0a      | 0.017            |
| Egg production, g/day/hen | 28.6 ± 6.8a     | 58.7 ± 0.8a     | 0.011            |
| Egg weight, g    | 53.5 ± 0.3a      | 56.1 ± 0.9a      | 0.034            |

Mean ± SE (n = 4). NS, not significant.

*a,b* Means within the same item with different superscript are significantly different, P < 0.05.
Corticosterone suppresses follicular growth of hen

Corticosterone (CORT) to hens that were fed a high-energy diet in Experiment 2. As shown in Table 3, the laying rate was decreased by CORT treatment (P < 0.05). In contrast, egg production tended to be lower in CORT-treated hens (P = 0.056), while egg weight remained unchanged (P > 0.05). The interaction of diet treatment and CORT affected feed intake (P < 0.05). CORT treatment increased the feed intake of hens that were fed a normal diet but did not affect hens that were fed a high-energy diet.

Ovarian and follicular development. The ovarian mass was decreased by CORT administration in hens that were fed a normal diet (P < 0.001) but not in those that were fed a high-energy diet (P > 0.05, Fig. 7A, B). Similarly, the CORT treatment decreased HF number (P < 0.001) and HF mass (P < 0.001) in hens that were fed a normal diet (Fig. 7C). For the hens that were fed a high-energy diet, however, the suppressive effect of CORT was only detected in the absolute mass of HF (P < 0.05, Fig. 7D). The SYF number and mass were not significantly changed (P > 0.05) by CORT or by diet (Fig. 7E, F).

Liver and abdominal fat accumulation. In Experiment 2, liver weight was higher in CORT-treated hens fed either a normal diet (P < 0.0001) or a high-energy diet (P < 0.01), while abdominal fat weight was only increased by CORT when hens were fed a normal diet (P < 0.05, Fig. 7G, H).

Plasma variables. When hens were fed a normal diet, plasma levels of insulin (P < 0.001) and glucose (P < 0.001) were increased by CORT administration, whereas E2 (P < 0.01), VLDL (P < 0.001), vitellogenin (VTG, P < 0.01), TG (P < 0.001), and NEFA (P < 0.01) were decreased in CORT-treated hens (Fig. 8A). For the hens that were fed a high-energy diet, the only significant change in plasma variables was an increase in insulin in CORT-treated hens (P < 0.001, Fig. 8B).

Table 3. Effect of corticosterone on organ and tissue development in Experiment 1. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days, n = 8) on the development of ovaries (A, B), hierarchical follicles (C, D), small yellow follicles (E, F), and liver and abdominal fat (G, H) of laying hens under fasting or feeding conditions. Mass (g); fractional mass (g/kg BM). a, b Means with different letters are significantly different, P < 0.05.

Fig. 2. Effect of corticosterone on organ and tissue development in Experiment 1. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days, n = 8) on the development of ovaries (A, B), hierarchical follicles (C, D), small yellow follicles (E, F), and liver and abdominal fat (G, H) of laying hens under fasting or feeding conditions. Mass (g); fractional mass (g/kg BM). a, b Means with different letters are significantly different, P < 0.05.
mRNA and protein expression of genes related to yolk deposition in HF membranes. In the laying hens that were fed a normal diet, CORT treatment induced an increase in the mRNA levels of OVR ($P < 0.05$) and occludin ($P < 0.001$), but it had no significant influence on the gene expression of ESR and LPL ($P > 0.05$, Fig. 9A). In hens that were fed a high-energy diet, CORT treatment upregulated the gene expression of ESR and OVR ($P < 0.05$), whereas the mRNA levels of occludin and LPL were not significantly affected ($P > 0.05$, Fig. 9B).

**DISCUSSION**

**GC-induced decrease in laying performance resulting from the suppression of follicular development is energy dependent**

The present result of laying performance indicated that GCs suppressed the reproductive performance of hens, in accordance with the previous work of Petite and Etches (33), who reported that continuous infusion of CORT resulted in the cessation of ovulation and in ovarian and oviductal regression in domestic hens. In zebra finches, chronically elevated levels of plasma CORT suppressed or delay reproduction (34). Conversely, CORT secretion during the prelaying period mediates the timing of breeding in kittiwakes, with the experimental reduction of CORT release leading to a significant advancement of egg laying in females (3). In the present study, the ovarian weight and the numbers and masses of HF and SYF were all significantly reduced in CORT-treated hens in the fasting state. CORT treatment was previously shown to increase the number of atretic follicles and reduce the ovarian weight (4). Therefore, this result indicates that CORT decreased laying performance by suppressing follicular development.

Although the decreased laying rate in CORT-treated hens was detected in both experiments, the decreased egg weight was only observed in Experiment 1, indicating that egg weight or egg size was not consistently decreased by CORT administration. Salvante and Williams (34) reported that chronically elevated plasma CORT in female zebra finches had no effect on egg size or clutch size. This result suggests that the effect of CORT on egg weight is independent of laying rate or clutch size.

However, the decrease in follicular development induced by CORT in the fasting state was not detected in the
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Feeding state. In mammals, many researchers have demonstrated the suppressive effect of fasting or food deprivation. A five-day fast during the luteal phase reduced the total number of follicles, causing a delay in the development of ovarian follicles in ewes (35). Food deprivation also led to the death of oocytes and attenuated glucose consumption by cumulus-oocyte complexes (36). In contrast, the blockage of mouse ovulation induced by food deprivation could be reversed by glucose, oil, or short-term feeding (36). We found that the decrease in ovarian and HF development in CORT-treated hens fed the control diet was restored by the high-energy diet. Similarly, when supplemented with food, exposure to high circulating CORT levels alone did not affect the timing of breeding in Florida scrub-jays (16). Moreover, as CORT release was experimentally reduced during the prelaying period, the increase in mass in female kittiwakes was suggested as a mechanism by which CORT mediates the timing of breeding in the seabird (3). In mammals, a short-term intermittent nutritional stimulus (high-energy diet) in the luteal phase increased the total number of ovulatory follicles and the ovulation rate in goats (37). GCs shift energy away from reproduction toward survival (15). These results together demonstrated that the effects of GC on follicular development are energy-state dependent and that the unlimited availability of food or energy intake attenuates the

**Fig. 3.** Effect of corticosterone on lipid accumulation of livers in Experiment 1. (A, B) Representative liver sections stained with hematoxylin and eosin (400×). Enlarged lipid-containing vacuoles in degenerating hepatocytes are more evident in hens treated with corticosterone (CORT, 2 mg/kg BM for 7 days, n = 8). (C, D) Oil Red O staining of liver sections for cytoplasmic lipid droplets (indicated by arrows, 400×) showing effect of corticosterone treatment on lipid accumulation in liver. (E) The effect of corticosterone treatment on the TG content in the livers of hens. *a, b* Means with different letters are significantly different, *P* < 0.05.
The absence of insulin (38). It is known that insulin plays an important role during follicular development. Retarded follicular development and ovarian dysfunction were associated with insulin resistance and compensatory hyperinsulinemia.

Fig. 4. Effect of corticosterone on plasma parameters in Experiment 1. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days) on the plasma concentrations of hormones (A, B, n = 8) and metabolites (C, D, n = 8) and VLDL particle distributions (E, F, n = 5) of laying hens under fasting or feeding conditions. Hormones: LH, FSH, E2, and insulin. Metabolites: glucose, VLDL, TG, and NEFA. 

Means with different letters are significantly different, $P < 0.05$. 

The intensity of communication about environmental conditions by CORT to the HPG.

CORT in concert with insulin is believed to be essential for the redistribution of energy stores, which does not occur in the absence of insulin (38). It is known that insulin plays an important role during follicular development. Retarded follicular development and ovarian dysfunction were associated with insulin resistance and compensatory hyperinsulinemia.
Fig. 5. Effect of corticosterone on the expression of genes related to yolk deposition of hierarchical follicles in Experiment 1. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days) on the mRNA expression of yolk deposition-related genes (A, B, n = 8) and protein expressions of occludin (C, n = 4) and claudin1 (D, n = 4) and OVR protein content (E, n = 4) under fasting or feeding conditions. Means with different letters are significantly different, *P* < 0.05.

(39, 40). The hyperinsulinemia might disturb the release of sex steroids, leading to an abnormal follicular development (41). For fasted hens in Experiment 1, circulating insulin was elevated, while estrogen level and follicular development were suppressed after CORT treatment, and all these changes simultaneously became milder in the feeding state. These results suggest that insulin might be involved in the suppressive effect of CORT on follicular development.

For control (non-CORT) hens in Experiment 1, plasma insulin level was significantly increased after feeding compared
Fig. 6. Effect of corticosterone on enzyme activity and expression of genes related to VLDL synthesis of liver in Experiment 1. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days) on the activities of FAS (nmol/min/mg protein) and ME (μmol/min/mg protein) (A, B, n = 8); the mRNA expression of LXRα, SREBP-1c, ACC1, FAS, ME, SCD1, apoB100, apoVLDL-II, and MTP (C, D, n = 8); the protein expression of apoB100 (E, n = 4); and the hepatic VLDL secretion rate (F, n = 8) of laying hens under fasting or feeding conditions. a, b Means with different letters are significantly different, P<0.05.

with fasting, in line with previous studies in chickens as well as that in mammals (42, 43). Under the physiological regulation of insulin, control (non-CORT) hens remained at a stable blood glucose level following feeding compared with fasting; For CORT hens, however, the stimulative effect of feeding on insulin release was concealed by the effect of CORT, resulting in the unobvious changed insulin level for CORT hens between two feeding states. In contrast,
blood glucose of CORT hens was elevated following feeding compared with fasting or compared with control hens of feeding state, indicating that insulin-dependent glucose utilization was impaired. These results suggest that CORT disturbed the insulin-dependent regulation of glucose utilization, in accordance with our previous work (13).

GC-suppressed follicular development is associated with reduced availability of yolk precursors

The growth of chicken oocytes can be divided into three phases: the growth of young oocytes (white follicles), the slow growth phase (SYF), and the rapid growth phase (HF) (44). During the last stage before oviposition, a large amount of the yolk precursors VLDL and VTG are taken up from the circulation each day. The decreased plasma levels of VLDL and TG in either the feeding or fasting state indicated a decreased availability of yolk precursor for the growth of oocytes. This result was consistent with previous studies in birds. In female zebra finches, CORT treatment resulted in high plasma levels of VLDL (indicated by the concentration of TG) but low levels of plasma VTG seven days after implantation, suggesting that CORT inhibited yolk precursor production and perhaps shifted lipid metabolism away from the production of VLDL and toward the production of generic (non-yolk) VLDL (34). However, the VLDL and TG levels reduced by CORT were restored when chickens were fed a high-energy diet in Experiment 2, suggesting an energy-dependent mode of action.

In laying hens, VLDL and VTG are taken up by the growing oocytes via receptor-mediated endocytosis, and both macromolecules bind to the OVR, localized on the oocyte plasma membrane (45). It is possible that OVR, as a key component in yolk precursor import, may play a significant role in the CORT-induced suppression of oocyte growth. At the onset of the rapid growth phase of oocytes, the redistribution of OVR from the preexisting pool to the peripheral region plays a crucial role in the regulation of yolk formation and oocyte growth (46). In Experiment 1, OVR protein content was decreased by CORT in the fasting state rather than in the feeding state, indicating that CORT suppressed yolk formation when insufficient energy was available. The results from Experiment 1 and Experiment 2 consistently show that the mRNA level of OVR was increased by CORT during the fasting state. The diverse change of OVR mRNA and protein expression may be related to the posttranscriptional control of gene expression. The regulation mechanism of CORT on OVR needs further investigation.

The transport of yolk precursors from the circulation to the oocytes consists of five components (47): i) vascularity, connective tissue representing an extension of the follicle stalk, ii) the thecal cell layer, iii) the basement membrane, iv) an epitheloid monolayer of granulosa cells, and v) the zona pellucida. During follicular development, there is a development-state-specific expression of occludin in granular cells (48). Claudin-based tight junctions are crucial for the barrier function in mammalian granular cells (49). The CORT-induced upregulation of occludin and claudin1 expression in the present study indicates an increase in tight junctions. These results may imply that CORT makes the granulosa cell layer more condensed, preventing the paracellular transport of yolk precursors into the oocytes. Moreover, the overexpression of occludin and claudin1 was less significant in the energy-sufficient state. These results together suggest that CORT exerts a more detrimental effect on follicle development when there is an energy deficit.

GCs decrease yolk precursor secretion by suppressing apolipoprotein synthesis

It is well known that estrogens stimulate the liver to produce the egg-yolk precursors VLDLy and VTG at the onset of sexual maturity. The shift of VLDL particle diameter is directed to be capable of being incorporated into newly forming egg yolks in birds and domestic chickens (25). According to Walzenmann (26) and Salvante et al. (25), small

### Table 3. Effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days) on laying performances of hens feeding with normal diet or high-energy diet (Experiment 2)

<table>
<thead>
<tr>
<th>Item</th>
<th>CORT</th>
<th>Control</th>
<th>Mean</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed intake, kcal/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>298.6 ± 14.1*</td>
<td>270.7 ± 6.1*</td>
<td>284.8*</td>
<td>CORT: NS</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>249.1 ± 5.8*</td>
<td>257.9 ± 3.8*</td>
<td>253.5*</td>
<td>Diet: &lt; 0.001</td>
</tr>
<tr>
<td>Mean</td>
<td>273.9</td>
<td>264.3</td>
<td></td>
<td>CORT* Diet: 0.032</td>
</tr>
<tr>
<td>Laying rate, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>54.8 ± 6.3</td>
<td>83.3 ± 9.7</td>
<td>69.1</td>
<td>CORT: 0.046</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>68.3 ± 12.8</td>
<td>82.9 ± 6.2</td>
<td>75.6</td>
<td>Diet: NS</td>
</tr>
<tr>
<td>Mean</td>
<td>61.5*</td>
<td>83.1*</td>
<td></td>
<td>CORT* Diet: NS</td>
</tr>
<tr>
<td>Egg production, g/day/hen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>31.9 ± 3.8</td>
<td>47.9 ± 6.9</td>
<td>39.9</td>
<td>CORT: 0.056</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>38.9 ± 7.2</td>
<td>48.3 ± 4.0</td>
<td>43.6</td>
<td>Diet: NS</td>
</tr>
<tr>
<td>Mean</td>
<td>35.4</td>
<td>48.1</td>
<td></td>
<td>CORT* Diet: NS</td>
</tr>
<tr>
<td>Egg weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal diet</td>
<td>58.2 ± 0.3</td>
<td>58.1 ± 0.7</td>
<td>58.2</td>
<td>CORT: NS</td>
</tr>
<tr>
<td>High-energy diet</td>
<td>57.0 ± 0.5</td>
<td>56.2 ± 0.5</td>
<td>56.6</td>
<td>Diet: NS</td>
</tr>
<tr>
<td>Mean</td>
<td>57.6</td>
<td>57.1</td>
<td></td>
<td>CORT* Diet: NS</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 3). NS, not significant.

Means with different superscript letters are significantly different, *P* < 0.05.

Means with different superscript letters are significantly different, **P** < 0.005.

Means with different superscript letters are significantly different, ***P*** < 0.001.
Fig. 7. Effect of corticosterone on organ and tissue development in Experiment 2. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days, n = 8) on the development of ovaries (A, B), hierarchical follicles (C, D), small yellow follicles (E, F), and liver and abdominal fat (G, H) of laying hens fed a normal diet (2,654 kcal/kg) or a high-energy diet (2,917 kcal/kg). Mass (g); fractional mass (g/kg BM). a, b Means with different letters are significantly different, \( P<0.05 \).
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mRNA expression of genes related to hepatic de novo fatty acid synthesis, such as FAS, ACC1, ME, and SCD1, was further analyzed, and the results demonstrate an increase in de novo hepatic lipogenesis by CORT, in agreement with the previous studies of broiler chickens (13, 50, 51). SREBP-1 is an accessory transcription factor that plays an active and central role in the hormonal and nutritional regulation of lipogenesis in mammals (52) as well as in avian species (53). LXRs, the nuclear hormone receptors that form active heterodimers with retinoid X receptors, are known to regulate SREBP-1c gene expression (54, 55). In rats, GCs are involved in the regulation of the expression of LXRα and SREBP-1c (56, 57). In the fasting state, however, the SREBP-1c mRNA level was upregulated following CORT administration without any measurable change in the expression of LXRα, indicating the different mechanisms of regulation in chickens. These results suggest that SREBP-1c is associated with enhanced gene expression for hepatic lipogenesis by CORT, in agreement with our previous observation in broilers (13). These results imply that CORT improves hepatic lipogenesis via the SREBP pathway.

diameter VLDL particles (25–44 nm) were positively associated with egg production. In Experiment 1, the increased proportion of large diameter VLDL particles and the decreased proportion of small diameter particles by CORT demonstrated the decreased circulating concentrations of VLDL-Y. This result implies two potential problems in CORT-challenged hens: suppressed hepatic lipogenesis and impaired hepatic VLDL-Y assembly and secretion.

In our previous studies of broiler chickens, GCs in concert with the high circulating concentration of insulin stimulated de novo hepatic lipogenesis, which in turn augmented blood lipid flux and peripheral fat deposition (12, 13). In the present study, the circulating insulin level was significantly increased in CORT-treated hens. Moreover, liver weight and liver lipid accumulation and hepatic TG content were increased by CORT, implying that CORT induces an augmented hepatic lipogenesis in laying hens similar to the effect in broiler chickens. This result was consistent with the elevated FAS and ME activities. The

Fig. 8. Effect of corticosterone on plasma parameters in Experiment 2. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days, n = 8) on the plasma concentrations of hormones and metabolites of laying hens fed with a normal diet (A, 2,654 kcal/kg) or a high-energy diet (B, 2,917 kcal/kg). Hormones: E2 and insulin. Metabolites: glucose, VLDL, VTG, TG, and NEFA. a,b Means with different letters are significantly different, P < 0.05.

Fig. 9. Effect of corticosterone on the expression of genes related to yolk deposition of hierarchical follicles in Experiment 2. The effect of corticosterone treatment (CORT, 2 mg/kg BM for 7 days, n = 8) on the mRNA expression of yolk deposition-related genes of laying hens fed with a normal diet (A, 2,654 kcal/kg) or a high-energy diet (B, 2,917 kcal/kg). a,b Means with different letters are significantly different, P < 0.05.
As hepatic lipogenesis remained at a higher level in CORT-treated hens, we further investigated whether CORT affected hepatic VLDL assembly and secretion. A reduction in the VLDL secretion rate in Experiment 1 implies that CORT decreased circulating VLDL content by inhibiting the assembly of lipoprotein by the liver. ApoB and apoVLDL-II are required for VLDL assembly in the liver, and they provide structural support that accommodates greater amounts of TG on the surface of the colloidal particles (58). The expressions of apoVLDL-II and apoB100 were downregulated by CORT in Experiment 1. Successful assembly of VLDL requires the cotranslational association of apoB100 with polar and neutral lipids (59). ApoVLDL-II functions as an inhibitor of LPL to ensure the delivery of VLDL to the oocyte membranes without substantial lipolysis (60). These results suggest that decreased expression of apolipoproteins could be responsible for the suppressed VLDL secretion and in CORT-treated hens.

MTP is involved in delivering TG to nascent apoB molecules during the assembly of lipoprotein particles (61, 62). Experiment 1 shows that CORT treatment elevated MTP mRNA expression. The accumulated TG in the liver may play a positive regulatory role in MTP activity (63). This result may imply that MTP-mediated lipid transfer is not the main factor responsible for decreased VLDL secretion.

ApoVLDL-II is responsible for the decrease in VLDL diameter (64). The present result suggests that the downregulated gene expression for apoVLDL-II is associated with the altered VLDL particle diameter by CORT. Moreover, apoVLDL-II is well known to confer LPL resistance to VLDL (60, 65). This characteristic enables VLDL to resist the lipolytic activity of LPL, thereby securing a high TG content for the egg yolk. In the present study, an increase in LPL mRNA expression was observed in HF upon CORT treatment in the fasting state. Further investigation is required to determine whether upregulated LPL expression and increased VLDL particle diameter are involved in the suppression of follicular development.

GC-induced decrease in estrogen level is energy dependent

Estrogen is recognized as playing the primary role in the conversion of carbohydrate to TG and in the production of apoB and apoVLDL-II, leading to the hypersecretion of these lipoproteins into the circulation (8, 66). ApoVLDL-II and apoB were shown to be rapidly induced by exogenous estrogen in the rooster liver and to accumulate within the VLDL fraction (67). With the onset of egg production, estrogen shifts hepatic lipoprotein production from generic VLDL to VLDLy, which is resistant to LPL and to intact uptake by oocyte receptors (64). This dramatic change occurs in both domestic laying hens selected for high egg production (68) and wild birds [Passerine bird (25)].

Estrogen is synthesized in the granulosa cells of follicles and exerts regulatory activity by binding to the ESR. In the present study, the circulating concentration of estrogen was decreased in the fasting state but not in the feeding state or the high-energy diet state, suggesting that the effect of CORT on the release of estrogen is energy dependent. Taken together, the decreased estrogen level, downregulated apolipoprotein expression, and reduced VLDLy proportion raise the possibility that impaired VLDLy secretion is related to the reduced induction by estrogen.

It is generally known that GCs regulate the secretion of sex steroids via indirect or/and direct mechanisms (69): The indirect mechanism is by inhibition at the supragonadal level. GCs induce an interruption of hypothalamic GnRH release and in turn pituitary gonadotropins secretion, which suppress sex steroids synthesis. An endocrine feedback loop is present in this HPG axis, as high levels of sex steroids suppress the release of GnRH through its return to the hypothalamus. The direct mechanism is at the gonadal level by inhibiting sex steroids production in granulosa cells. In the present study, estrogen secretion was reduced by CORT, while the blood concentrations of FSH and LH remained higher and the amounts of FSHR and LHR in HF were not affected. According to the previous studies (70–72), the increased plasma concentration of FSH and LH may be feedback compensation in response to low estrogen secretion. Another possibility is that CORT directly affected estrogen production in granulosa cells in a gonadotropin-independent manner. This speculation is supported by several studies. Heat stress exerted a direct effect on ovarian tissue, which was suggested as a factor contributing to the decline in egg production (73, 74). CORT altered the responsiveness of ovarian tissues to gonadotropins (75). In rats, GCs inhibited FSH-induced estrogen production in cultured rat granulosa cells (76).

In conclusion, exogenous GC administration significantly decreased the laying performance of hens in an energy-dependent manner. These results suggest that the decreased apolipoprotein synthesis and VLDL secretion by the liver might be responsible for the decreased availability of circulating yolk precursor in CORT-treated hens. In HF, the upregulated expression of occludin and claudin in granulosa cells further prevented yolk deposition into oocytes.

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