

Research Note: Effects of dietary L-arginine on the production performance and gene expression of reproductive hormones in laying hens fed low crude protein diets

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ABSTRACT The present study was designed to investigate whether L-arginine (**Arg**) supplementation would influence the production performance and reproductive traits of laying hens fed low crude protein (**LCP**) diets. Hy-Line brown laying hens were randomly assigned to dietary treatments of control, LCP, and LCP supplemented with 0.05, 0.10, 0.15, or 0.20% Arg for 7 wk. The results showed no significant variations on the production performance, and relative organ indexes of hens. However, significant transcriptional and structural changes were evident along the HPG axis of hens. Arg supplementation linearly upregulated ($P < 0.05$) the gonadotropin-releasing hormone 1 (**GnRH1**), and gonadotropin inhibitory hormone in the hypothalamus.

The pituitary growth hormone, GnRH receptor, and follicle-stimulating hormone (**FSH β**) were also increased ($P < 0.05$). In the ovary, GnRH1, and estrogen receptor β were linearly increased by Arg, and the ovarian morphology revealed that LCP induced structural alterations which were minimally recovered by Arg supplementation. Serum insulin-like growth factor 1 (**IGF-1**) and nitric oxide (**NO**) were increased ($P < 0.05$) at higher levels of Arg supplementation. Therefore, supplementing high Arg (0.20%) to LCP hens influenced the ovarian morphology and modulated the gene expression of reproductive hormones in the hypothalamic-pituitary-gonadal axis of laying hens via actions that may be related to NO and IGF-1 activity.

Key words: arginine, hypothalamus, HPG axis, nitric oxide, reproduction

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INTRODUCTION

In poultry diets, protein sources are the most expensive feedstuffs, therefore, feeding strategies that would minimize production cost, while achieving optimum or maximum production performance are desirable. The utilization of low crude protein (**LCP**) diets in poultry nutrition has gained considerable interest in recent times. However, it has been realized that significant reductions in dietary crude protein (**CP**) would compromise the production performance and feed efficiency of birds (Chrystal et al., 2020). To address this, it was reported that the supplementation of crystalline AAs can relatively compensate for partial CP reduction in poultry

diets and prevent the overutilization of dietary CP while optimizing the production performance of birds (Awad et al., 2014). Therefore, it becomes pertinent to investigate the fortification of LCP with essential AAs, and/or NEAAs on the growth, production performance, and other physiological responses of poultry.

Arginine (Arg) is an essential amino acid in poultry, which is crucial for body growth, immunity, and reproduction. In chickens, dietary Arg serves as a precursor for nitric oxide, creatine, proline, glutamate, glutamine, and polyamine synthesis; and also functions as a secretagogue to stimulate growth hormone (**GH**) and insulin-like growth factor (**IGF**) production (Castro et al., 2019). However, chickens cannot synthesize de novo arginine due to their lack of a complete urea cycle and the limited activity of key enzymes required for Arg synthesis. Therefore, chickens have a high requirement for Arg and they depend largely on dietary supply to meet the body's needs.

Reproduction in poultry is under the coordinated influence of neuroendocrine mechanisms, collectively

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termed the hypothalamic-pituitary-gonadal (HPG) axis (that is, the hypothalamus, anterior pituitary gland, and the gonads). The stimulatory action of gonadotropin-releasing hormone (GnRH) allows for the synthesis and release of gonadotropins, and this is regulated by its inhibitory counterpart (gonadotropin-inhibitory hormone, GnIH) (Bedecarrats, 2015). These peptides coordinate the release of luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) from the anterior pituitary into the peripheral circulation. A synergistic activity has been identified between reproductive hormones secretion and the gene expressions of their respective receptors in chickens. However, despite the crucial role of the HPG axis in egg production, there is little information regarding how they are influenced during LCP conditions in poultry. Therefore, the present study aimed to investigate whether L-Arg supplementation would influence the growth performance and reproductive traits of laying hens fed LCP diets.

MATERIALS AND METHODS

Experimental Birds and Management

A total of 720 Hy-Line Brown laying hens, at 24 wk of age were randomly allotted into 6 dietary treatments, 8 replicates, and 15 hens each. Hens were acclimatized for 1 wk and then fed dietary treatments for a period of 7 wk which consisted of control (16% CP, 2,643 kcal/kg ME); low crude protein (LCP; 14% CP, 2,643 kcal/kg ME), LCP + 0.05% Arg, LCP + 0.10% Arg, LCP + 0.15% Arg and LCP + 0.20% Arg dietary treatments. Birds were managed under standard conditions of 16-h light and 8-h dark lighting regime with ad libitum supply of feed and water. Laying hens were housed in battery cages (60 cm × 46 cm × 44 cm) having 3 hens per cage, with a total of 5 cages constituting 1 replicate.

Performance Parameters and Tissue Collection

The egg weight and egg production were recorded daily, while the body weight and feed intake were recorded every week. Data obtained were used to compute final body weight, average daily feed intake, egg weight, laying rate, egg mass, and feed to egg ratio. At 31-wk-old, the feed was withdrawn overnight prior to blood collection to avoid interference. A total of 8 hens per treatment (1 bird per replicate) were randomly selected for blood collection. Blood was obtained from the wing vein using sterilized needles and syringes into non-heparinized tubes and centrifuged at 3,000 *g* at 4°C for 15 mins for serum collection. After blood collection, hens were euthanized by decapitation and reproductive organs were isolated, weighed immediately, and expressed as the relative percentage of the live body weight. Isolated

tissues were flash-frozen in liquid nitrogen, and stored at −80°C until molecular analysis.

Histological Observation

The ovaries were collected and fixed in 4% neutral buffered formalin. Formalin-fixed tissues were routinely processed for dehydration, and embedded in paraffin wax. Paraffin samples were sliced into 4- μ m sections and stained with Harris hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO). Stained slides were examined by light microscopy for histological characteristics. Sections were examined under an Olympus CX-41 phase contrast microscope (Olympus, Tokyo, Japan).

RNA Extraction and Real-Time Polymerase Chain Reaction

The total RNA from isolated tissues was extracted using NcmZol reagent (NCM Biotech, China) according to the manufacturer's guidelines. The RNA concentration and purity were detected using the DS-11 spectrophotometer (Denovix Incorporated, Wilmington, DE), and the total RNA was reverse transcribed with HiFi-Script cDNA synthesis kit (CW BIO, China) according to the manufacturer's instructions. After reverse transcription, the cDNA target sequence was quantified using MagicSYBR Mixture (CW BIO, Beijing, China). The gene-specific primers were adapted from earlier published studies (Jiang et al., 2020). The real-time RT-qPCR was performed on ABI QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, Carlsbad, CA, ThermoFisher Scientific, Carlsbad, CA). The tested primers were normalized against β -actin as the housekeeping gene, while the control diet was used as the calibrator. The relative expression of the target genes was analyzed using the $2^{-\Delta\Delta CT}$ method.

Determination of Nitric Oxide and Insulin-Like Growth Factor 1 Concentration

The NO concentration in serum was detected using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), as previously reported (Uyanga et al., 2021). The absorbance was detected at 540 nm using a microplate reader (Elx808, Bio-Tek Winooski, VT). The serum Insulin-like growth factor 1 (IGF-1) concentration was determined according to the manufacturer's instructions using the chicken IGF-1 ELISA kit (CSB-E09867Ch; CUSABIO, China) with an intra-assay CV of <15% and inter-assay CV of CV <15%.

Statistical Analysis

Data collected were analyzed using One-way ANOVA and expressed as mean \pm SEM using Statistical Analysis Software (SAS version 8.1; SAS Institute Inc., Cary,

NC). Means were compared using Duncan's multiple range tests where significant ($P < 0.05$). Orthogonal polynomial contrasts were performed to determine the linear and quadratic effects of treatments.

RESULTS AND DISCUSSION

Production Performance and Organ Weights of Laying Hens

Contrary to our earlier hypothesis that LCP diets may cause adverse effects on the reproductive functions of laying hens, this study demonstrated that the growth and laying performance of LCP fed hens did not differ from the control fed hens. More so, Arg supplementation to LCP diets did not evoke pronounced changes in the production indices, laying performance, and relative weights of reproductive organs in laying hens. Table 1 shows that the final body weight ranged from 1,600 to 2,350 g/bird, with an average of 2,018.75 g/bird. The laying rate tended to be influenced by a quadratic response ($P = 0.088$), and ranged from 72.2 to 97.6%, with an average of 87.29%. The egg mass also tended to be influenced by a quadratic effect ($P = 0.064$), and the values were between 46.25 and 61.58 g/hen/d, with an average of 54.44 g/hen/d. Similarly, the feed intake also showed a tendency for a quadratic response ($P = 0.072$), and was between 125.17 and 145.65 g/d, with an average of 135.80 g/d. The egg weight was 59.53 to 64.68 g, having an average of 62.36 g; while the feed to egg ratio was about 2.25 to 2.95, with an average of 2.51 g/g. However, it was observed that LCP + 0.20% Arg had the lowest mean values for final body weight (1943.75 ± 90.85); laying rate (85.07 ± 1.06); egg mass (52.75 ± 0.98), and highest feed to egg ratio (2.58 ± 0.06) compared to other groups, although without significant differences ($P > 0.05$). The relative organ weights of the ovary, shell gland, and follicles (POF, PHF, and

dominant follicles) were not significantly affected by dietary treatments ($P > 0.05$). Although unexpected, these findings corresponded with the report that reduction in dietary CP did not affect the weight gain, feed intake, and carcass weight in ducks (Xie et al., 2017). In another report, arginine supplementation (0.35% Arg) to laying hens fed with an LCP diet (13% CP) did not improve the egg quality traits or performance indices such as egg mass, egg weight, egg production, and feed intake (Dao et al., 2021). Notwithstanding, the insignificant finding in this study does not preclude that low dietary crude protein may influence the growth and performance of poultry as earlier reviewed. Further studies may adopt a longer study period to incorporate the phases of egg production and different arginine supplementation levels.

Serum nitric Oxide and IGF-1 Concentration

Since arginine is a potent NO precursor, and NO is implicated in various biological roles including growth and reproduction, we examined whether Arg supplementation to LCP diets would influence the peripheral NO levels and IGF-1 concentration. Figure 1 A shows that the serum NO content had a linear response ($P = 0.0511$) with arginine supplementation. The LCP + 0.20% Arg diet significantly increased the serum NO levels compared to other groups, whereas, the control and LCP groups did not differ in their serum NO levels. Arginine can alter the pituitary function and facilitate blood flow to the reproductive tract, likely due to the vasodilative effects of NO in the tissues. It is known that NO acts on the HPG axis to influence reproduction by inducing gonadotropin secretion and regulating ovarian function (Shit et al., 2014). Thus, Arg may probably influence reproductive processes in laying hens via promoting NO generation.

Table 1. Effects of dietary L-arginine supplementation on the production performance and organ index of laying hens fed low crude protein diets.

	Control	LCP diets					P values			
		0.00% Arg	0.05% Arg	0.10% Arg	0.15% Arg	0.20% Arg	^a Control vs LCP	^b Main effect	^c Linear effect	^d Quadratic effect
Final BW (g)	2,075.00 ± 32.73	2,031.25 ± 53.40	2,043.75 ± 72.23	1,968.75 ± 45.25	2,050.00 ± 58.25	1,943.75 ± 90.85	0.4963	0.637	0.435	0.749
	87.72 ± 1.61	85.90 ± 2.92	88.80 ± 1.54	88.68 ± 1.61	87.58 ± 1.91	85.07 ± 1.06	0.5948	0.637	0.611	0.088
Laying rate (%)										
Egg mass (g/hen/d)	54.69 ± 1.05	53.48 ± 1.93	55.57 ± 0.86	55.53 ± 0.99	54.58 ± 1.47	52.75 ± 0.98	0.5913	0.661	0.527	0.064
Feed intake (g/d)	133.55 ± 1.26	136.03 ± 1.25	137.97 ± 2.27	136.05 ± 1.08	136.60 ± 1.74	134.57 ± 1.11	0.1846	0.567	0.382	0.346
Feed to egg ratio	2.46 ± 0.04	2.57 ± 0.08	2.50 ± 0.03	2.45 ± 0.05	2.51 ± 0.05	2.58 ± 0.06	0.2063	0.401	0.898	0.072
Egg weight (g)	62.35 ± 0.33	62.25 ± 0.44	62.60 ± 0.35	62.64 ± 0.32	62.30 ± 0.53	62.00 ± 0.49	0.851	0.391	0.5164	0.2554
Relative organ weights (%)										
Ovary	0.22 ± 0.01	0.25 ± 0.02	0.24 ± 0.03	0.22 ± 0.02	0.24 ± 0.02	0.27 ± 0.03	0.317	0.608	0.548	0.194
SHG	1.00 ± 0.02	1.08 ± 0.04	1.12 ± 0.07	1.18 ± 0.03	1.06 ± 0.06	1.19 ± 0.06	0.103	0.081	0.368	0.879
DF	0.70 ± 0.03	0.71 ± 0.05	0.70 ± 0.07	0.69 ± 0.03	0.69 ± 0.03	0.71 ± 0.03	0.617	0.997	0.973	0.666
POF	1.52 ± 0.22	1.36 ± 0.14	1.28 ± 0.21	1.32 ± 0.06	1.24 ± 0.14	1.29 ± 0.07	0.562	0.842	0.646	0.768
PHF	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.14 ± 0.02	0.898	0.718	0.854	0.483

Abbreviations: DF, dominant follicle; LCP, low crude protein; PHF, pre-hierarchical follicle; POF, pre-ovulatory follicle; SHG, shell gland.

^aStatistical comparison of means between control and LCP diet (0.00% Arg).

^bStatistical comparison of means between all dietary treatments.

^cLinear contrast between LCP and LCP-Arginine supplemented groups.

^dQuadratic contrast between LCP and LCP-Arginine supplemented groups.

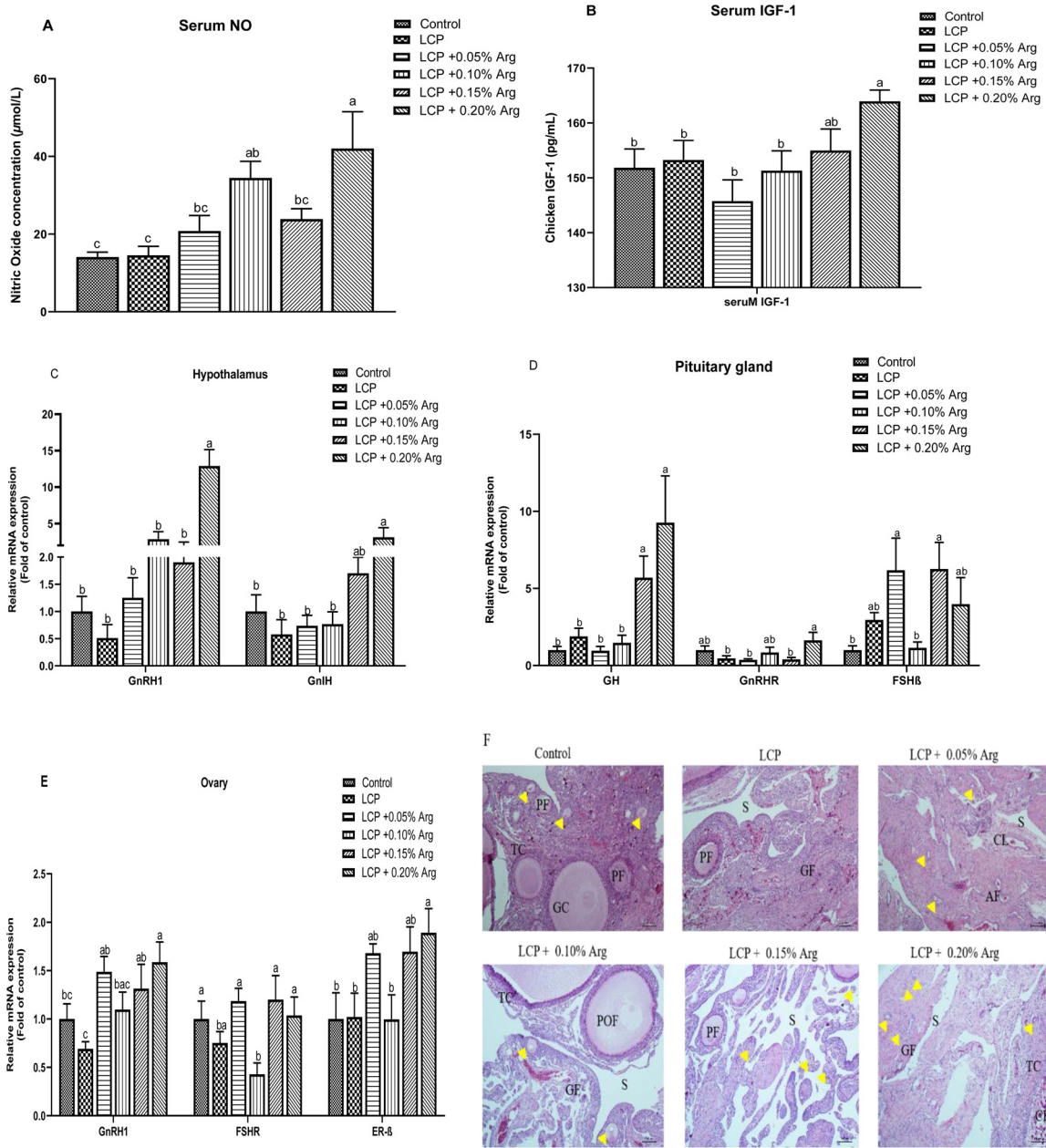


Figure 1. Effects of arginine supplementation to low crude protein diets on the serum metabolites and gene expression of reproductive hormones (A) Serum nitric oxide content (B) Serum IGF-1 content (C) Hypothalamic expression of gonadotropins (D) Ovarian expression of gonadotropins (E) Ovarian histology. Values are means \pm SEM represented by vertical bars. Means with different superscripts differ significantly ($P < 0.05$) ($n = 8$). Abbreviations: AF, atresia follicle; CL, corpus luteum; GC, granulosa cells; PF, primary follicles; POF, preovulatory follicle; S, stroma; SWF, small white follicles; TC, theca cells. Primordial follicle (PrF; Yellow triangles); . Magnification $\times 100$.

In line with this, IGFs exert biological effects on key metabolic organs to promote growth and development. In the chicken ovary, IGFs have been implicated in the regulation of steroidogenesis, follicle selection, cell proliferation, and cell differentiation (Hrabia, 2015). In the present study, arginine supplementation had a significant linear ($P = 0.0124$) and quadratic ($P = 0.0199$) effect on serum IGF-1 level (Figure 1B). The LCP + 0.20% Arg significantly increased the serum IGF-1 concentration compared to the control, LCP, LCP + 0.05% Arg and LCP + 0.10% Arg groups. These results suggest that high levels of arginine to LCP diets would act to stimulate growth and reproductive processes probably via NO and IGF-1 mediated actions.

Gene Expression of Reproductive Hormones in the Hypothalamus of Laying Hens

The HPG axis functions in regulating the development of reproductive organs and ovarian follicles through the synthesis and release of various hormones from the hypothalamus, anterior pituitary, and gonads. Thus, we investigated whether Arg supplementation to LCP diets would influence the transcriptional responses of HPG axis-related hormones (Figure 1C-E). Arg supplementation to LCP diets by 0.20% increased the hypothalamic *GnRH1* expression by 92.24% and 96.12% compared to the control and LCP groups. The hypothalamic *GnRH1* expression was significantly influenced by

both linear ($P = <0.0001$) and quadratic responses ($P = 0.0030$). An increase in the release of the hypothalamic gonadotropin-releasing hormone (GnRH) allows for the secretion of downstream gonadotropins (Bedecarrats, 2015). Thus, Arg supplementation allowed for the hypophysiotropic influence of GnRH, which would stimulate the release of pituitary gonadotropin, and the positive regulation of growth, maturation, and endocrine responses in the reproductive axis.

Similar to the changes in *GnRH1* expression, the hypothalamic *GnIH* expression was significantly influenced by a linear response ($P = 0.0076$), as it increased with higher levels of Arg supplementation (Figure 1C). *GnIH* expression was highest in laying hens fed LCP + 0.20% Arg and it was increased by 67.95% and 81.41% compared to the control and LCP group (Figure 1C). The hypothalamus acts to regulate the functions of pituitary gonadotropes by changing the ratio of stimulatory (GnRH-I) to inhibitory (GnIH) neuropeptides which are secreted into the portal system (Bedecarrats, 2015). Therefore, despite the simultaneous upregulation of *GnRH1* expression along with *GnIH*, the latter was higher by 75.81% in the LCP + 0.20% Arg group, permitting GnRH actions in stimulating gonadotropin production by binding to the *GnRH* receptor (*GnRHR*) located on the pituitary gonadotrophs.

Gene Expression of Reproductive Hormones in the Pituitary Gland of Laying Hens

Figure 1D shows that the pituitary *GH* expression was upregulated by the LCP + 0.15% Arg and LCP + 0.20% Arg groups, significantly higher than the control, LCP, LCP + 0.05% Arg and LCP + 0.10% Arg groups. The LCP + 0.20% Arg increased *GH* expression by 89.21% and 79.66%; whereas LCP + 0.15% Arg increased *GH* expression by 82.45% and 66.92% compared to the control and LCP diets respectively. There was a significant linear ($P = 0.0006$) and quadratic response ($P = 0.0177$) of Arg on pituitary *GH* expression. The pituitary *GnRHR* expression was linearly increased by arginine supplementation ($P = <0.0001$). The *GnRHR* expression was higher in the LCP + 0.20% Arg compared to LCP, LCP + 0.05% Arg and LCP + 0.15% Arg groups. Also, *FSH β* was upregulated by LCP + 0.15% Arg and LCP + 0.05% Arg, significantly higher than the control and LCP + 0.10% Arg groups (Figure 1D). Thus, at the pituitary level, the *GH*, *GnRHR*, and *FSH β* were modulated by high levels of Arg supplementation to the LCP diet. An increase in the expression of these hormones can be attributed to the upstream release of the hypothalamic GnRH. This suggests that the hypophysiotropic influence of GnRH was more evident on pituitary gonadotropin release than the inhibitory action of GnIH. Furthermore, Arg modulation of pituitary *GH* expression portrays positive regulation in the growth, maturation, and endocrine responses of ovarian follicles including steroidogenesis, cellular proliferation, and apoptosis (Hrabia, 2015).

Gene Expression of Reproductive Hormones and the Morphology of the Ovary

In the ovary, Arg supplementation to LCP diets at 0.20% significantly upregulated the *GnRH1*, and *FSHR*, consequently inducing *ER β* expression. The *GnRH1* expression was linearly increased ($P = 0.0042$) with increased arginine addition to LCP diets (Figure 1E). Feeding of LCP + 0.20% Arg modulated *GnRH1* by 36.98 and 56.36% compared to the control, and LCP groups respectively. Meanwhile, *FSHR* expression was significantly downregulated by LCP + 0.10% Arg compared to other dietary treatments, except the LCP group (Figure 1E). The expression of *ER β* was upregulated in a linear ($P = 0.0393$) fashion with arginine supplementation (Figure 1E). The LCP + 0.20% Arg diet significantly modulated ovarian *ER β* mRNA expression by 47.13, 46.03, and 47.32% compared to the control, LCP, and the LCP + 0.10% Arg group.

It is understood that the release of FSH is positively associated with the development and maintenance of follicular hierarchy in laying hens. On the other hand, estrogen is an important hormone that serves to regulate ovarian function during the reproductive period. In this study, high levels of Arg supplementation to LCP diets promoted estrogen receptor expression in response to higher FSH release in the pituitary, and gonads of laying hens. This suggest that the mRNA expression of genes related to central control of reproduction (*GnRH*, *GnIH*, *GH*, *GnRHR*) and follicle development (*FSH β* , *ER β*) within the HPG axis were modulated by Arg supplementation to LCP diets. Representative images of ovarian tissues are shown in Figure 1F. The control group displayed a normal ovarian architecture with an abundance of follicles at varying stages. However, the LCP group had fewer follicular structures and this was minimally restored with increasing levels of Arg supplementation, as evidenced by the presence of primordial follicles. Summarily, L-arginine supplementation to LCP fed hens significantly influenced transcriptional responses in the HPG axis of laying hens. It was evident that LCP fed hens were highly responsive to arginine supplementation in a dose-dependent manner. High levels (0.20%) of arginine supplementation positively stimulated the expression of gonadotropins, NO, and IGF-1 in laying hens fed LCP diets. However, the effects of dietary arginine on reproductive traits were quite variable and may be subject to dietary formulations, age of hens, and stages of egg production.

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Ethics approval: All experimental procedures were conducted in compliance with the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, P. R. China), and the study was approved by the Ethics Committee of Shandong Agricultural University, China.

DISCLOSURES

The authors declare that they have no conflicts of interest.

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