Animal Nutrition 20 (2025) 66–79

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

# Integrated transcriptomic hypothalamic-pituitary-ovarian axis network analysis reveals the role of energy availability on egg production in layers

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ARTICLE INFO

*Article history:*
Received 3 May 2024
Received in revised form
16 August 2024
Accepted 22 August 2024
Available online 6 November 2024

*Keywords:*
Energy deprivation
Refeeding
Transcriptome
Hypothalamic-pituitary-ovarian axis
Egg production
Layer

ABSTRACT

Energy is a crucial component for maintaining egg production in layers. The hypothalamic-pituitary-ovarian (HPO) axis is an energy-sensitive functional axis for follicle development, synthesis, and secretion of reproductive hormones, and plays a key role in modulating sustained ovulation in layers. To investigate the mechanism of integrated network regulation of the HPO axis under energy fluctuation, ninety Hy-line brown layers (265-day-old, 1.92 ± 0.02 kg) were randomly divided into three groups for a 17-day experiment: a control group (Con group) fed ad libitum from days 1 to 17, an energy-deprived group (ED group) that was fed ad libitum from days 1 to 12 and then underwent a fasting period from days 13 to 17 to induce a pause in laying, and a re-fed group (Rf group) that fasted for seven days (specifically, days 1 to 5, day 7, and day 9), had ad libitum access to feed on days 6 and 8, and was continuously fed from days 10 to 17. Each treatment consisted of 10 replicates with 3 birds per replicate. The study found that energy deprivation significantly decreased reproductive performance such as egg laying rate, ovarian index, number of small yellow follicles (SYF), and normal hierarchical follicles (NHIE) ($P < 0.05$), which recovered after refeeding, indicating the importance of energy availability for sustained ovulation in layers. In addition, estradiol (E2), estradiol to progesterone (E2/P4) ratio, and luteinizing hormone (LH) displayed changes similar to follicle number, whereas follicle-stimulating hormone (FSH) exhibited a contrasting pattern. Transcriptome analysis revealed that energy deprivation downregulated genes related to energy and appetite-regulated neurotransmitter receptors and neuropeptides in the hypothalamus. These signals combined to inhibit gonadotropin-releasing hormone (GnRH) secretion and subsequently downregulated the crucial genes responsible for synthesizing gonadotropins, gonadotropin-releasing hormone receptor (*GnRHR*), and glycoprotein hormones alpha chain (*CGA*). Consequently, this suppression of the hypothalamus and pituitary affected ovarian function through ovarian steroidogenesis and the extracellular matrix (ECM)-receptor interaction. These findings suggest that energy deprivation inhibits the function of the HPO axis, leading to impaired follicle development and reduced egg production, and that refeeding can partially restore these indicators.


## 1. Introduction

Energy acquisition and consumption are essential for animal survival and successful reproduction. Previous studies have indicated that wild birds experience starvation during migration periods, and the allocation of energy can impact reproductive events, specifically laying (Lamarre et al., 2017). In avian species, growing evidence has demonstrated that energy availability is vital for egg

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Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.08.009

production and reproduction (Mishra et al., 2020; Rama et al., 2023). A recent study reported that supplying Rugao layers with energy-restricted diets resulted in delayed ovarian development, a decrease in the number of large yellow follicles, and a reduction in egg production (Lu et al., 2021). When dietary energy is severely deficient, the energy requirements for maintaining basic metabolism and laying eggs of hens cannot be met, resulting in reduced egg production including egg mass and egg weight (Mikulski et al., 2020). Previous research has also shown that energy deprivation has a suppressive effect on egg production in high-yielding layers, ultimately resulting in a complete cessation of laying (Sirotkin and Grossmann, 2015; Socha and Hrabia, 2019). Furthermore, hunger during long-distance transportation has been identified as a significant factor leading to reduced body weight and increased mortality rates in layers (Vecerkova et al., 2019).

It has been well-established that egg production is highly dependent on the hypothalamic-pituitary-ovarian (HPO) axis, which regulates sex hormone synthesis and secretion. The hypothalamic pulsatile secretion of gonadotropin-releasing hormone (GnRH) mediates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary, and these gonadotropins ultimately act on the ovary to secrete estradiol (E2) and progesterone (P4) (Joseph et al., 2013). Furthermore, follicle development in chickens is fundamental to egg production, which is activated by hormones produced in the hypothalamus and pituitary gland (Hanlon et al., 2021). Studies have shown that the HPO axis is sensitive to various metabolic cues and is gated by energy homeostasis (Caprio et al., 2001; Roa et al., 2010). The GnRH neuron is regulated by a range of neurotransmitters and neuropeptides including agouti-related peptide (AgRP), pro-opiomelanocortin (POMC) (Roa and Herbison, 2012), dopamine (DA) (Liu and Herbison, 2013), glutamate (Glu) (Iremonger et al., 2010), gamma-aminobutyric acid (GABA) (Watanabe et al., 2014), serotonin (5-HT) (Bhattarai et al., 2014), and acetylcholine (Ach) (Arai et al., 2017), eventually affecting the HPO axis. Alterations in nutrient availability can trigger significant changes in these hypothalamic neurotransmitters and neuropeptides (Bruning and Fenselau, 2023), affecting GnRH pulse secretion. Therefore, investigating the neural mechanisms that influence GnRH secretion due to changes in poultry neuropeptides and neurotransmitters induced by energy deprivation holds significant importance. The regulatory mechanisms of the hypothalamic GnRH-involved HPO axis during energy deficiency in layers have also received extensive attention. Jehl et al. (2019) reported that chickens fed a low-energy diet exhibited upregulation of genes associated with the complement system and endocannabinoid signaling in their hypothalamus. Zhang et al. (2021) performed the hypothalamic and ovarian transcriptome analysis between natural and forced moulting in layers, revealing massive genes related to anti-aging and disease resistance that promotes reproductive system function during forced moulting. Although Xin et al. (2022) investigated the effects of dietary energy and protein alterations on reproductive hormones and HPO axis-related genes in layers, the complete transcriptional regulatory mechanism of the HPO axis in energy-deprived and re-fed layers are still unknown. Taken together, there is a shortage of comprehensive studies on the association between energy and the HPO axis in poultry.

Based on the above information, we hypothesized that the comprehensive network of reproductive hormones and genes in the HPO axis would be affected after energy deprivation and refeeding. In this work, we investigated the transcriptomic dynamics of the HPO axis across the control, ad libitum fed (Con) group, energy-deprived (ED) group, and re-fed (Rf) group, which is aimed to elucidate the interactive network regulation mechanism of the HPO axis. Our study seeks to elucidate gene expression patterns and important pathways during the energy change period, providing a better insight into the adaptive response to energy deficiency in layers.

## 2. Materials and methods

### 2.1. Animal ethics statement

The study was conducted according to the research protocol approved by the Institutional Animal Care and Use Committee (Jiangxi Agricultural University, Nanchang, Jiangxi, China, JXAULL-2024-05-01).

### 2.2. Animal and sample collection

A total of 90 Hy-line brown layers, all weighing approximately the same and aged 36 weeks, were procured from a local farm. They were provided ad libitum basal diet and water with a 16L:8D photoperiod. At 265 days of age, the layers (1.92 ± 0.02 kg) were randomly divided into three treatment groups, each consisting of 10 replications with 3 birds per replication. The treatments included a Con group that was fed ad libitum from days 1 to 17; an ED group that received ad libitum feeding from days 1 to 12, followed by a fasting period from days 13 to 17 to induce a cessation in laying; and a Rf group that fasted for seven days (from days 1 to 5, day 7, and day 9), had ad libitum access to feed on days 6 and 8, and was continuously fed from days 10 to 17. Samples were collected by sacrificing 90 birds on day 18 of the experiment. Feed consumption was recorded daily. To determine egg quality, a total of 40, 39, and 77 eggs were collected from the Con group, ED group, and Rf group, respectively (the collection time was during days 3 to 5 and days 15 to 17 of the experiment). Blood samples were taken from the wing veins of birds and placed in sterilized tubes, then centrifuged at 1006.2 × *g* for 10 min at 4 °C to obtain serum, which was stored at -80 °C for sex hormone determination. After decapitation, the whole ovary was extracted and weighed. The following ovarian components were isolated and accurately counted: small white follicles (SWF, 1 to 2 mm in diameter), large white follicles (LWF, 3 to 5 mm in diameter), small yellow follicles (SYF, 6 to 8 mm in diameter), and normal hierarchical follicles (NHIE, 9 to 40 mm in diameter). The separated hypothalamus, pituitary gland, and ovarian tissues (containing follicles <2 mm) were promptly frozen in liquid nitrogen for storage at - 80 °C.

### 2.3. Feed chemical analyses

The corn-soybean meal basal diet was formulated based on the guidelines provided by the National Research Council (NRC, 1994). Table 1 displays the ingredients and nutrient composition of the diet. The metabolizable energy was computed using information from the China feed database (2020). The determination of crude protein content utilized the Kjeldahl method as outlined in the China National Standard (GB/T 6432-2018), while ether extract was assessed using a Soxhlet extractor in accordance with the China National Standard (GB/T 6433-2006). Ash content was measured following the methodology specified in the China National Standard (GB/T 6438-2007). Amino acids were detected by the China National Standard (GB/T 18246-2019). Total phosphorus and calcium were measured following the methods (GB/T 6437-2018 and GB/T 6436-2018).

### 2.4. Egg production performance

Eggs were collected daily to measure the egg laying rate and assess egg quality. After being weighed on a digital analytical scale

**Table 1**
Ingredients and nutrient levels of the basal diet (as-fed basis, %).

| Item | Content |
|---|---|
| **Ingredients** | |
| Corn | 64.50 |
| Soybean meal | 23.50 |
| Fish meal | 1.50 |
| $CaHPO_4$ | 1.50 |
| $CaCO_3$ | 8.00 |
| Salt | 0.30 |
| L-Lysine monohydrochloride (L-lysine ≥78.8%) | 0.10 |
| Feed grade DL-methionine (DL-methionine ≥98.5%) | 0.20 |
| Choline chloride (choline ≥50%) | 0.10 |
| Premix[1] | 0.30 |
| Total | 100.00 |
| Nutrient levels[2] | |
| Metabolizable energy, MJ/kg | 11.56 |
| Crude protein | 16.35 |
| Lysine | 0.91 |
| Methionine | 0.51 |
| Threonine | 0.57 |
| Ether extract | 3.08 |
| Ash | 3.16 |
| Calcium | 3.49 |
| Total phosphorus | 0.58 |
| Available phosphorus | 0.39 |

[1] Provided the following per kilogram of diet: chromium 0.2 mg, manganese 25 mg, iodine 1.0 mg, copper 2.5 mg, selenium 0.35 mg, cobalt 0.48 mg, zinc 115 mg, iron 75 mg, VA 12,000 IU, $VD_3$ 4400 IU, VE, 85 mg, $VK_3$ 5 mg, thiamine 4 mg, riboflavin 15 mg, niacin 65 mg, pantothenic acid 21 mg, pyridoxine 7 mg, biotin 0.35 mg, folic acid 3 mg, cobalamine 0.035 mg.
[2] Metabolizable energy was calculated from data provided by the Feed Database in China (2020) and the rest were measured values.

(0.01 g precision), each egg was measured with a vernier caliper for egg shape index and broken to determine eggshell strength using an eggshell strength gauge (Tenovo Technology, Beijing, China). Albumen height was measured by a digital Haugh tester (Tenovo Technology, Beijing, China) to compute the Haugh unit. Following the assessment and scoring of yolk color using the yolk color chart (Robotmatio, Tokyo, Japan), the yolk was subsequently separated and weighed for the percentage of yolk. The membrane of the eggshell was removed to determine eggshell thickness at three distinct points (sharp, equator, and blunt ends) by digital micrometer gauge.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of FSH and P4 in serum were measured by ELISA kits (Shanghai meilian, Shanghai, China), with catalog numbers ml093521-J and ml059935, respectively. Similarly, the concentrations of LH and E2 were detected using ELISA kits purchased from Jianglai (Jianglai, Shanghai, China), with catalog numbers JL13001 and JL15972, respectively.

### 2.6. RNA sequencing

To enhance the accuracy of RNA-Seq analysis, we used the hypothalamic tissue from six Hy-line brown layers as one pool, with each group consisting of four pools. Similarly, samples from the pituitary and ovary were also processed. mRNA with Poly(A) tails was extracted through the utilization of Oligo (dT) beads followed by the random fragmentation of mRNA into short fragments using a Frag/Prime buffer. NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was then employed to reverse transcribe the generated RNA fragments into cDNA. The paired cDNA fragments were ligated with an additional A base and were purified using AMPure XP Beads at a 1.0X bead-to-sample ratio before Illumina sequencing. Following purification, the cDNA library underwent amplification through PCR. Ultimately, the cDNA libraries were subjected to sequencing on the Illumina Novaseq 6000 platform at Genedenovo Biotechnology Co., Ltd. in Guangzhou, China.

### 2.7. RNA-Seq analysis

Raw data of samples, including read number, GC content percentage, and Q30 were calculated. High-quality clean reads were generated by removing low-quality reads with over 50% of bases (Q-value ≤20), as well as reads containing adapters and more than 10% of unknown nucleotides (N). Clean reads were mapped to the reference genome of *Gallus gallus* (Gallus_gallus - Ensembl genome browser 106) utilizing HISAT2.2.4.

The aligned reads for individual samples were assembled based on the references by StringTie v1.3.1 (Pertea et al., 2016), utilizing a reference-based method. Expression abundance and variation within each transcript region were homogenized by determining the Fragment per kilobase million (FPKM) value. Differential expression analysis across different groups was conducted employing the DESeq2 software (Love et al., 2014). The differentially expressed genes (DEGs) were identified by the threshold: $|\text{Log2 fold change (FC)}| > 1$ and false discovery rate (FDR) $< 0.05$.

### 2.8. Bioinformatics analysis

In order to elucidate the roles of DEGs, an analysis of gene ontology (GO) enrichment was performed utilizing the GO database (http://www.geneontology.org/). Three ontologies of GO including molecular function, cellular component, and biological process were used to annotate the biological functions of DEGs. Pathway enrichment analysis was carried out by referencing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to gain further insights into the functions of DEGs. The threshold (corrected *P*-value <0.05) was used to identify significant GO terms and KEGG pathways. The DEGs exhibiting dynamic expression profiles were examined using Short-Term series Expression Miner (STEM).

### 2.9. RNA isolation and real-time qPCR (RT-qPCR)

Following the provided instructions in the Trizol reagent kit (Invitrogen, Carlsbal, CA, USA), total RNA was extracted from the hypothalamus, pituitary, and ovaries. The quality of RNA samples was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The relative expression of DEGs was determined using RT-qPCR, with β-actin serving as the housekeeping gene. The experiment was performed using TB Green Premix Ex Taq II (TaKaRa, Beijing, China) and StepOne Real-time PCR system (Applied Biosystems, Waltham, MA, USA). Each 20 μL reaction contained 10 μL 2 × TB Green Premix Ex, 8 μL sterile distilled $H_2O$, 1 μL cDNA, 0.5 μL upstream primer (10 μmol/L), and 0.5 μL downstream primer (10 μmol/L). The reaction protocol was conducted as follows: denaturation at 95 °C for 30 s, followed by 40 cycles consisting of 15 s at 95 °C and 30 s at 60 °C. The primers of 17 genes were designed using Primer-Blast of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China) (Table 2). Gene expression levels were quantified using the $2^{-\triangle\triangle Ct}$ method after normalization to the expression levels of β-actin (Annaratone et al., 2013).

**Table 2**
List of genes and their primer sequences.

| Gene | Forward primer (5′—3′) | Reverse primer (3′—5′) | GenBank number |
|---|---|---|---|
| β-Actin | ACCCCAAAGCCAACAGAGAGAAG | TAACACCATCACCAGAGTCCATCAC | NM_205518.2 |
| *AgRP* | TGGAACCGCAGGCATTGTCC | GGCAGTAGCAGGTGGCACAG | NM_001398243.1 |
| *ESR2* | AAGAAGAGAACGCTGTGGGTATCG | GACTGACTGTGCTGAGGAGGATC | NM_001396358.1 |
| *LHCGR* | CTCGTCCTCATAACCAGCCACTAC | TTGTCTGAGCATCCACCGAAGC | NM_204936.2 |
| *AQP1* | GGCGGTGGTGGCTGAGTTC | CGTTGTCCTGAGTTGCTGATGTC | NM_001039453.2 |
| *PGR* | TGGTGAAGGCCATTGGTTTGC | AGGGAATTCAACGCTCAGTGC | NM_205262.2 |
| *SNAP25* | TTGTAGATGAACGGGAGCAGATGG | GTTGCCAATGATGCCGCTGAC | NM_001398210.1 |
| *GnRHR* | CTGGTGACGGTGGTGGTGATG | CTGATGACGACGGTGACGAAGG | NM_001012609.1 |
| *CGA* | GTGCAAGCTAGGGGAGAACAGG | GCATGGTCTTCTTGGATCTCATTGG | NM_001278021.1 |
| *LPAR1* | TGTGGTCATGGTGGTCCTATATGC | TTCAGGAGGCTCATCATCGTGTC | NM_001115082.2 |
| *LIPG* | TGTTTGTGGACTCCCTCGTGAAC | CGTGTCCTCCTGGCGTTGTAG | XM_046936191.1 |
| *LCAT* | ACCTGGTGAACAACGGCTACG | AAGACACGCTGCTGGTACTCATC | NM_001293094.2 |
| *HSD3B1* | CCTCAACCAACCGCCACCTG | CTAGTGTACCTCTTTGCCTCTTCCC | NM_205118.2 |
| *COL6A1* | GCAGGCTGGTATTGAGGTCTACG | GAGGTATAGTCAGGCACACGGAAC | NM_205107.2 |
| *TFPI2* | ACGACGACTGCGAGAAGAACTG | CCGTAGATGAACTCCTCACACCTC | XM_418662.8 |
| *CTSD* | ACGGGCTCCTCCAACCTCTG | AACTCAGTGCCATTCTCCACATAGG | NM_205177.2 |
| *PCK1* | TGCCTTCACTCGGTTGGATGTC | AGCCGCTGCCAAATGAAATGATC | NM_205471.2 |
| *CAV1* | GTGGACTCGGAGGGCTTTCTG | GCACCGCCTTCTCGCTCAG | NM_001105664.3 |

*AgRP* = agouti-related neuropeptide; *ESR2* = estrogen receptor 2; *LHCGR* = luteinizing hormone/choriogonadotropin receptor; *AQP1* = aquaporin 1; *PGR* = progesterone receptor; *SNAP25* = synaptosome-associated protein 25; *GnRHR* = gonadotropin-releasing hormone receptor; *CGA* = glycoprotein hormones alpha chain; *LPAR1* = lysophosphatidic acid receptor 1; *LIPG* = lipase G, endothelial type; *LCAT* = lecithin-cholesterol acyltransferase; *HSD3B1* = hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; *COL6A1* = collagen type VI, alpha 1 chain; *TFPI2* = tissue factor pathway inhibitor 2; *CTSD* = cathepsin D; *PCK1* = phosphoenolpyruvate carboxykinase 1; *CAV1* = caveolin 1.

### 2.10. Statistical analysis

The statistical analysis was conducted using SPSS software version 26.0 (IBM Corp, Armonk, New York). The statistical model was as follows:

$$Y_{ij} = \mu + T_i + B_j + \varepsilon_{ij},$$

where $Y_{ij}$ denotes the observation of the dependent variable, $\mu$ signifies the overall mean, $T_i$ represents the fixed effect associated with the treatment, $B_j$ indicates the random effect attributable to the individual bird, and $\varepsilon_{ij}$ corresponds to the residual error associated with the observation. The data including egg quality, ovarian index, and the number of follicles were subjected to one-way ANOVA and then mean values were compared using the Duncan's multiple range test. All results were expressed as mean and pooled standard error of the mean (SEM), with statistical significance defined as $P < 0.05$.

## 3. Result

### 3.1. Egg production performance and morphological characteristics of ovaries

The average daily feed intake (ADFI) of layers during the experimental period is shown in Table 3. Following a 5-day fasting period, the egg laying rates of the ED and Rf groups were 10.00% and 13.33%, respectively. Subsequently, on the 17th day of the experiment, the egg laying rate in the Rf group recovered to 76.67%, representing a decrease of 16.66% compared to the egg laying rate of the Con group (Table 4). We analyzed the egg quality of the eggs collected during the ad libitum feeding, energy deprivation, and

**Table 3**
The average daily feed intake (ADFI, g/bird per day) of layers during the experimental period.[1]

| Experimental period | Con | ED | Rf |
|---|---|---|---|
| Day 1 | 113.65 | 113.75 | 0 |
| Day 2 | 111.78 | 112.84 | 0 |
| Day 3 | 112.94 | 110.52 | 0 |
| Day 4 | 110.79 | 113.67 | 0 |
| Day 5 | 111.89 | 110.98 | 0 |
| Day 6 | 113.64 | 112.32 | 112.73 |
| Day 7 | 112.23 | 111.83 | 0 |
| Day 8 | 114.03 | 113.24 | 113.33 |
| Day 9 | 110.74 | 109.80 | 0 |
| Day 10 | 111.90 | 114.09 | 112.92 |
| Day 11 | 113.71 | 112.73 | 113.56 |
| Day 12 | 112.88 | 113.05 | 112.96 |
| Day13 | 112.34 | 0 | 112.45 |
| Day 14 | 111.55 | 0 | 111.23 |
| Day 15 | 110.72 | 0 | 113.35 |
| Day 16 | 112.39 | 0 | 112.87 |
| Day 17 | 111.49 | 0 | 111.15 |

[1] The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. Each value represents the mean of each group of 30 layers and 0 means that the group is in a fasting state.

**Table 4**
The average laying rate (%) of layers during the experimental period.[1]

| Experimental period | Con | ED | Rf |
|---|---|---|---|
| Day 1 | 100.00 | 86.67 | 100.00 |
| Day 2 | 86.67 | 86.67 | 88.24 |
| Day 3 | 100.00 | 100.00 | 76.47 |
| Day 4 | 100.00 | 100.00 | 46.67 |
| Day 5 | 106.67 | 100.00 | 13.33 |
| Day 6 | 93.33 | 93.33 | 29.41 |
| Day 7 | 93.33 | 86.67 | 0 |
| Day 8 | 93.33 | 93.33 | 0 |
| Day 9 | 106.67 | 86.67 | 23.53 |
| Day 10 | 86.67 | 100.00 | 17.65 |
| Day 11 | 100.00 | 100.00 | 5.88 |
| Day 12 | 93.33 | 100.00 | 52.94 |
| Day 13 | 93.33 | 100.00 | 52.94 |
| Day 14 | 100.00 | 93.33 | 70.59 |
| Day 15 | 93.33 | 86.67 | 52.94 |
| Day 16 | 106.67 | 33.33 | 70.00 |
| Day 17 | 93.33 | 10.00 | 76.67 |

[1] The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. Each group contains 30 Hy-line brown layers. Each value represents the mean of each group of 30 layers.

**Table 5**
Effects of energy deprivation and refeeding on egg quality of Hy-line brown layers.[1,2]

| Item | Con | ED | Rf | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| Egg weight, g | 60.30 | 58.81 | 58.73 | 0.614 | 0.110 |
| Egg shape index | 1.27 | 1.28 | 1.26 | 0.007 | 0.106 |
| Shell strength, N | 53.74$^{a}$ | 28.61$^{b}$ | 55.09$^{a}$ | 1.535 | <0.001 |
| Albumen height, mm | 6.53$^{b}$ | 7.87$^{a}$ | 7.52$^{a}$ | 0.165 | <0.001 |
| Haugh unit | 79.86$^{b}$ | 88.59$^{a}$ | 86.95$^{a}$ | 1.076 | <0.001 |
| Yolk color | 7.37$^{a}$ | 6.60$^{b}$ | 6.84$^{b}$ | 0.137 | <0.001 |
| Yolk weight, g | 14.96$^{ab}$ | 15.19$^{a}$ | 14.38$^{b}$ | 0.230 | 0.035 |
| Yolk percentage, % | 0.249$^{b}$ | 0.258$^{a}$ | 0.250$^{a}$ | 0.0030 | 0.017 |
| Shell thickness, mm | 0.40$^{a}$ | 0.25$^{c}$ | 0.37$^{b}$ | 0.006 | <0.001 |

$^{a,b}$ Means with different letters indicate significant differences ($P < 0.05$).
[1] The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17.
[2] A total of 40 eggs were obtained from the Con group layers during the ad libitum feeding phase, specifically on days 3 to 5 and days 15 to 17 of the experimental period. Seventy-one eggs were collected in the energy deprivation period (from ED group layers on days 15 to 17 and from Rf group layers on days 3 to 5). Forty-four eggs were collected in the refeeding period (from Rf group layers on days 16 to 17).

**Table 6**
Effects of energy deprivation and refeeding on ovarian indicators and reproductive hormone levels in serum.[1]

| Item | Con | ED | Rf | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| Ovarian index, % | 2.55$^{a}$ | 1.71$^{b}$ | 2.71$^{a}$ | 0.145 | <0.001 |
| **The number of follicles** | | | | | |
| SWF | 15.78$^{ab}$ | 10.11$^{b}$ | 21.75$^{a}$ | 2.298 | 0.007 |
| LWF | 19.13 | 22.13 | 21.71 | 1.883 | 0.117 |
| SYF | 15.53$^{a}$ | 9.53$^{b}$ | 15.07$^{a}$ | 1.321 | 0.004 |
| NHIE | 7.33$^{a}$ | 1.53$^{b}$ | 8.87$^{a}$ | 0.466 | <0.001 |
| **Serum hormone levels** | | | | | |
| LH, IU/L | 1.10$^{a}$ | 0.43$^{b}$ | 0.87$^{ab}$ | 0.145 | 0.018 |
| FSH, IU/L | 0.90$^{b}$ | 1.54$^{a}$ | 0.86$^{b}$ | 0.039 | <0.001 |
| E2, pg/mL | 502.02$^{b}$ | 79.39$^{c}$ | 738.48$^{a}$ | 34.725 | <0.001 |
| P4, ng/mL | 14.58 | 16.89 | 16.59 | 1.141 | 0.345 |
| E2/P4 | 0.032$^{a}$ | 0.0043$^{b}$ | 0.0720$^{a}$ | 0.00595 | <0.001 |

SWF = small white follicles; LWF = large white follicles; SYF = small yellow follicles; NHIE = normal hierarchical follicles; LH = luteinizing hormone; FSH = follicle-stimulating hormone; E2 = estradiol; P4 = progesterone; E2/P4 = estradiol/progesterone.
$^{a-c}$ Means with different letters indicate significant differences ($P < 0.05$). $n = 6$.
[1] The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17.

refeeding periods. The results are shown in Table 5, with more detailed results available in Table S1. Significant reductions were observed in eggshell strength, eggshell thickness, and yolk color in the ED group compared with the Con group ($P < 0.05$). After refeeding, only the shell strength in the ED group significantly increased to the level of the Con group ($P < 0.001$). The albumen height ($P < 0.001$), Haugh unit ($P < 0.001$), and yolk percentage ($P = 0.017$) in the ED group were significantly higher compared to the Con group, and they did not show significant differences as compared to the Rf group. Additionally, ovarian morphology in the ED group differed greatly from that of the Con and Rf groups, with the hierarchical follicles being hemorrhagic, atretic, and grossly regressed, containing less yolk (Fig. 1). The ovarian index was decreased by 0.84% and 1.00% in the ED group compared to the Con and Rf groups ($P < 0.001$), respectively (Table 6). The number of SWF in the ED group tended to be lower than that in the Con group ($P = 0.052$) and significantly lower than that in the Rf group ($P = 0.007$). The variations in the number of SYF ($P = 0.004$) and NHIE ($P < 0.001$) were consistent with SWF and both showed statistical differences (Table 6).

### 3.2. Reproductive hormone levels in serum

Table 6 presents the significant impact of energy deprivation on the levels of reproductive hormones, which largely recovered after refeeding. LH levels were significantly reduced in the ED group compared to the Con group ($P = 0.018$). FSH levels were significantly higher in the ED group compared to both the Con and Rf groups ($P < 0.001$). A significant decline in E2 levels was observed in the ED group in comparison to the Con and Rf groups ($P < 0.001$). Additionally, E2 levels in the Rf group exhibited a statistically significant increase compared to those in the Con group ($P < 0.001$). While serum P4 concentration did not differ significantly among the three groups ($P = 0.345$), it is noteworthy that the ratio of E2/P4 was significantly lower in the ED group in comparison to the Con and Rf groups ($P < 0.001$).

### 3.3. Dynamic transcriptome of the HPO axis in layers after energy deprivation and refeeding

We demonstrated transcriptome dynamics in the HPO of layers after energy deprivation and refeeding, with four biological replicates in each group. The clean data of 36 samples were considered high quality, with Q30 > 91.62%. A total of 19,852 genes were identified in the hypothalamus, pituitaries, and ovaries. Every group was separated from the other two by the first principal component 1 (PC1) and the second principal component 2 (PC2) in three tissues (Figs. S1A, C, E). Pairwise differential expression was analyzed in three groups for each tissue. Overall, a greater number of genes were found to be downregulated compared to those that

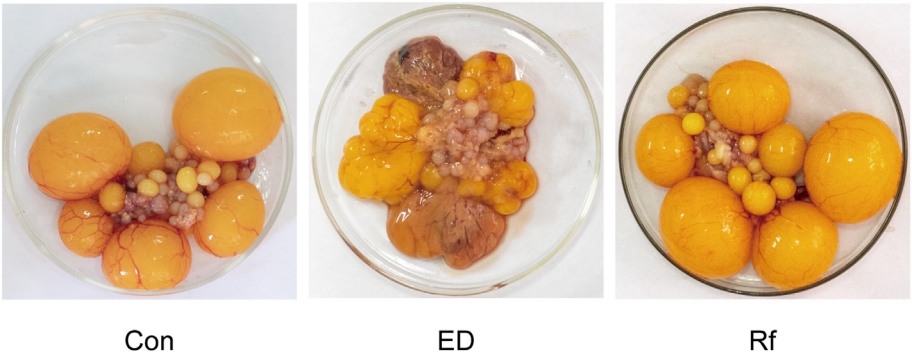


**Fig. 1.** The morphological characteristics of ovaries of layers. The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17.

were upregulated in the DEGs of Con vs ED in the three tissues (Figs. S1B, D, and F). Conversely, in the DEGs of ED vs Rf, a higher proportion of upregulated genes were observed in the pituitary and ovary compared to downregulated genes. The heatmap of DEGs in the HPO axis is shown in Fig. 2. Subsequently, trend analysis was conducted to explore potential adaptive patterns of DEGs in the HPO axis following energy deprivation and refeeding. Four main clusters were identified.

A total of 1152 DEGs were detected in the hypothalamus based on the criteria (|Log2FC| > 1 and FDR <0.05). Among these DEGs, 86.82% of the 971 DEGs in Con vs ED were downregulated, while 85.80% of the 641 DEGs in Con vs Rf were downregulated (Fig. S1B). In the Rf group, 38 genes were upregulated and 12 genes were downregulated in comparison to the ED group. To characterize the differential expression profiles in the hypothalamus of groups Con, ED, and Rf, we used STEM software to classify DEGs into different clusters and performed functional enrichment analysis accordingly.

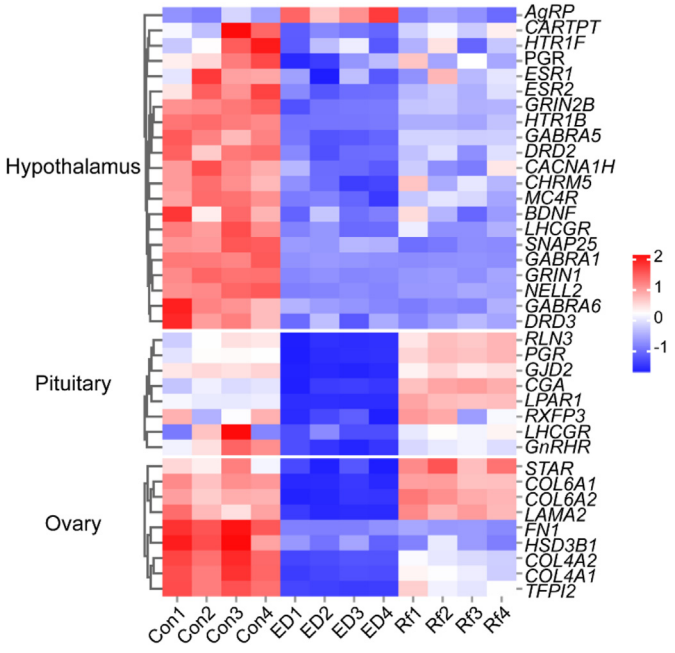


**Fig. 2.** The heatmap of differentially expressed genes (DEGs) in the hypothalamic-pituitary-ovarian (HPO) axis ($n$ = 4). The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. *AgRP* = agouti-related neuropeptide; *CARTPT* = cocaine-and amphetamine-regulated transcript protein; *HTR1F* = 5-hydroxytryptamine receptor 1F; *PGR* = progesterone receptor; *ESR1* = estrogen receptor 1; *ESR2* = estrogen receptor 2; *GRIN2B* = glutamate ionotropic receptor N-methyl-D-aspartate type subunit 2B; *HTR1B* = 5-hydroxytryptamine receptor 1B; *GABRA5* = gamma-aminobutyric acid type A receptor subunit alpha 5; *DRD2* = dopamine receptor D2; *CACNA1H* = calcium voltage-gated channel subunit alpha 1H; *CHRM5* = cholinergic receptor muscarinic 5; *MC4R* = melanocortin 4 receptor; *BDNF* = brain-derived neurotrophic factor; *LHCGR* = luteinizing hormone/choriogonadotropin receptor; *SNAP25* = synaptosome-associated protein 25; *GABRA1* = gamma-aminobutyric acid type A receptor subunit alpha 1; *GRIN1* = glutamate ionotropic receptor N-methyl-D-aspartate type subunit 1; *NELL2* = neural epidermal growth factor-like 2; *GABRA6* = gamma-aminobutyric acid type a receptor subunit alpha 6; *DRD3* = dopamine receptor D3; *RLN3* = relaxin 3; *GJD2* = gap junction protein delta 2; *CGA* = glycoprotein hormones alpha chain; *LPAR1* = lysophosphatidic acid receptor 1; *RXFP3* = relaxin family peptide receptor 3; *GnRHR* = gonadotropin-releasing hormone receptor; *STAR* = steroidogenic acute regulatory protein; *COL6A1* = collagen type VI alpha 1 chain; *COL6A2* = collagen type VI alpha 2 chain; *LAMA2* = laminin subunit alpha 2; *FN1* = fibronectin 1; *HSD3B1* = hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; *COL4A2* = collagen type IV alpha 2 chain; *COL4A1* = collagen type IV alpha 1 chain; *TFPI2* = tissue factor pathway inhibitor 2.

Out of the 8 identified clusters, we focused on cluster 2, cluster 3, cluster 6, and cluster 7 (Fig. 3). Cluster 2 and cluster 3 comprised a total of 877 genes, with 745 genes in cluster 2 and 132 genes in cluster 3. These genes were downregulated after energy deprivation, but after refeeding, the gene expression of cluster 2 remained in a platform stage and that of cluster 3 returned to the Con level. GO results showed that genes in cluster 2 primarily participated in synaptic function, and genes in cluster 3 were mainly about G-protein coupled receptor activity. In contrast, the expression of 118 genes in cluster 7 mainly related to nephron development and germ cell proliferation was specifically upregulated in Con vs ED, and that of 25 genes in cluster 6 enriched to positive regulation of feeding behavior and type 1 melanocortin receptor binding was peaked in the ED group.

Compared to the hypothalamus, the pituitary gland exhibited a lower number of DEGs, with a total of 918 DEGs detected. The number of DEGs in Con vs ED, ED vs Rf, and Con vs Rf was 667 (236 up, 431 down), 665 (348 up, 317 down), and 147 (33 up, 114 down), respectively (Fig. S1D). DEGs in cluster 3 and cluster 6 were statistically significant ($P$ < 0.001), and showed opposite trends in gene expression (Fig. 4). The 384 genes in cluster 3 were primarily associated with nucleosome assembly, while 276 genes in cluster 6 were enriched in the high-density lipoprotein (HDL) particle and detection of mechanical stimulus. The genes in cluster 2 were mainly annotated to extracellular region functions, and the genes in cluster 7 were assigned to myelinization.

The target organ of the HPO axis, the ovary, exhibited a total of 1080 DEGs, with 518 DEGs (157 up, 361down), 552 DEGs (425 up, 127 down), and 595 DEGs (367 up, 228 down) in Con vs ED, ED vs Rf, and Con vs Rf, respectively (Fig. S1F). We identified significant enrichment of DEGs in cluster 1, cluster 3, cluster 5, and cluster 8 (Fig. 5). Within cluster 5 and cluster 8, 341 genes were upregulated after energy deprivation or refeeding, and these genes were primarily associated with cilium and microtubule-related processes. The genes in cluster 3 were primarily involved in blood circulation and contractile fiber, and genes continuously downregulated in cluster 1 were assigned to blood coagulation and hemostasis.

### 3.4. Reproduction-related pathways in the HPO axis

To fully comprehend the transcriptional changes in the HPO axis after energy deprivation and refeeding, KEGG pathway enrichment analysis was performed. Figure 6 shows several potential pathways that are closely related to egg production performance in the HPO axis. Neuroactive ligand–receptor interaction was common and significantly enriched in HPO axis-related tissues during the experimental period. The DEGs from the comparative group Con vs ED and Con vs Rf in the hypothalamus were significantly enriched in pathways associated with synaptic function, such as synaptic vesicle cycle, glutamatergic synapse, GABAergic synapse, cholinergic synapse, dopaminergic synapse, GnRH secretion. In the pituitary, five pathways were found to be involved in organismal systems (cholesterol metabolism, GABAergic synapse, and GnRH signaling pathway), environmental information processing (cyclic adenosine monophosphate [cAMP] signaling pathway), and metabolism (steroid biosynthesis) when comparing groups Con vs ED and ED vs Rf. In the ovary, there were seven pathways involved in environmental information processing (extracellular matrix [ECM]-receptor interaction, phosphatidylinositol-3-kinase [PI3K]-protein kinase B [Akt] signaling pathway), cellular processes (focal adhesion), and organismal systems (peroxisome proliferator-activated receptor [PPAR] signaling pathway, serotonergic synapse, mineral absorption, and ovarian steroidogenesis) and these pathways were significantly enriched in Con vs ED. The five pathways, which include neuroactive ligand–receptor interaction, GnRH secretion,

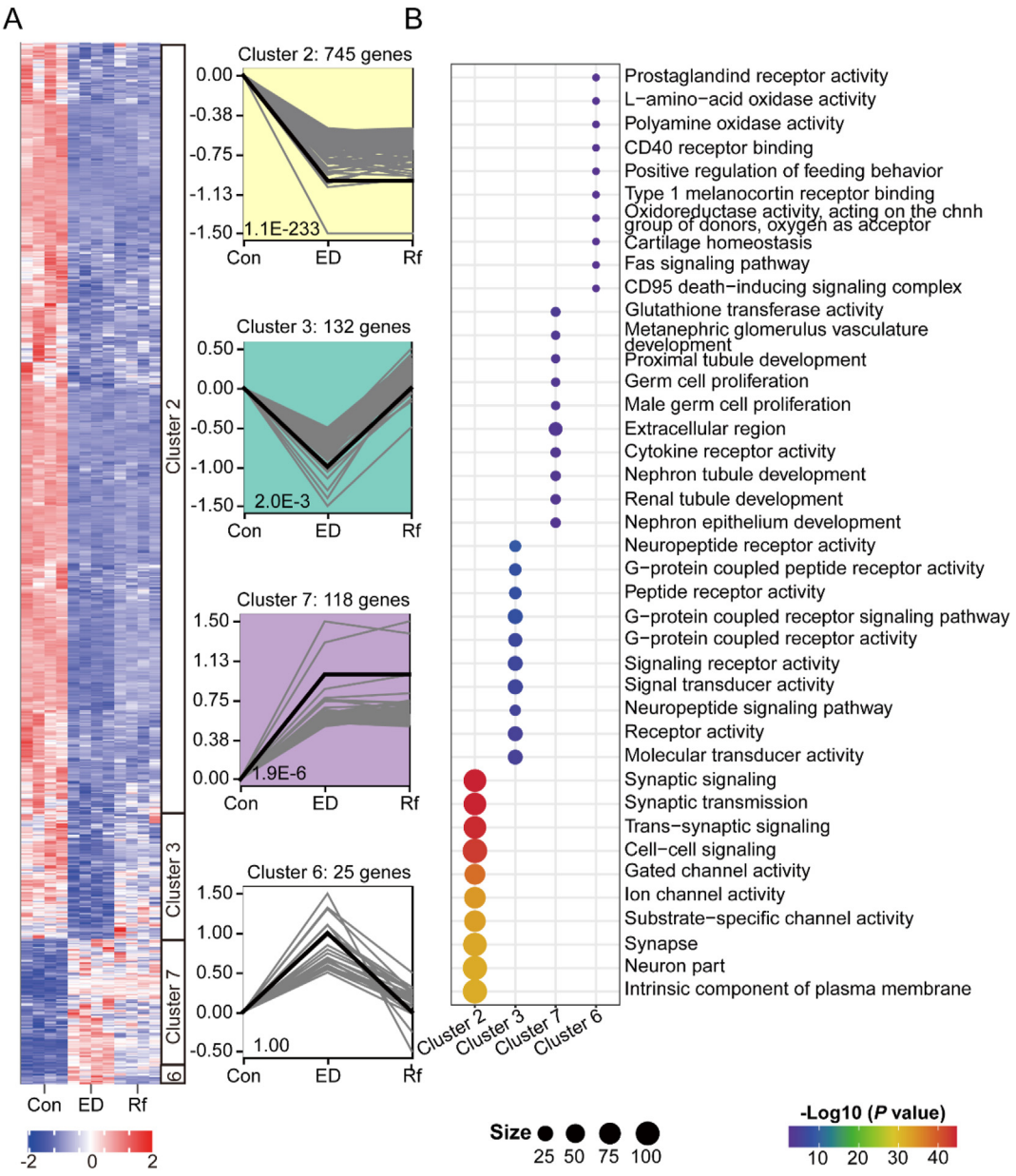


**Fig. 3.** The dynamic trend analysis and gene ontology (GO) terms of the hypothalamus after energy deprivation and refeeding ($n = 4$). (A) The heatmap and their corresponding clusters, colored represent significant concentrations. (B) The top ten significantly enriched GO Terms for genes in each cluster. The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. CD40 = cluster of differentiation 40; Fas (CD95) = factor-related apoptosis.

GnRH signaling, ovarian steroidogenesis, and ECM-receptor interaction, play a vital role in the optimal operation of the HPO axis.

### 3.5. Validation of gene expression by RT-qPCR

Based on the analysis above, we selected the six most relevant or highly expressed genes in each tissue for RT-qPCR. These genes include synaptosome-associated protein 25 (*SNAP25*), *AgRP*, aquaporin 1 (*APQ1*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), estrogen receptor 2 (*ESR2*), and progesterone receptor (*PGR*) in the hypothalamus; lecithin-cholesterol acyltransferase (*LCAT*), lipase G, endothelial type (*LIPG*), gonadotropin-releasing hormone receptor (*GnRHR*), lysophosphatidic acid receptor 1 (*LPAR1*), glycoprotein hormones alpha chain (*CGA*), and *PGR* in the pituitary gland; and caveolin 1 (*CAV1*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), cathepsin D (*CTSD*), tissue factor pathway inhibitor 2 (*TFPI2*), collagen type VI alpha 1 chain (*COL6A1*), and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*) in the ovary. The results from the study demonstrated that the expression patterns of these DEGs were aligned with the RNA-seq, thereby confirming the reliability of the transcriptomic data (Fig. S2).

## 4. Discussion

Energy is a crucial factor in maintaining egg production in layers. The mechanisms by which energy deprivation affects laying performance and follicle development via the HPO axis are not yet fully understood. This study investigated the egg production performance, ovarian morphology, follicle development, serum sex

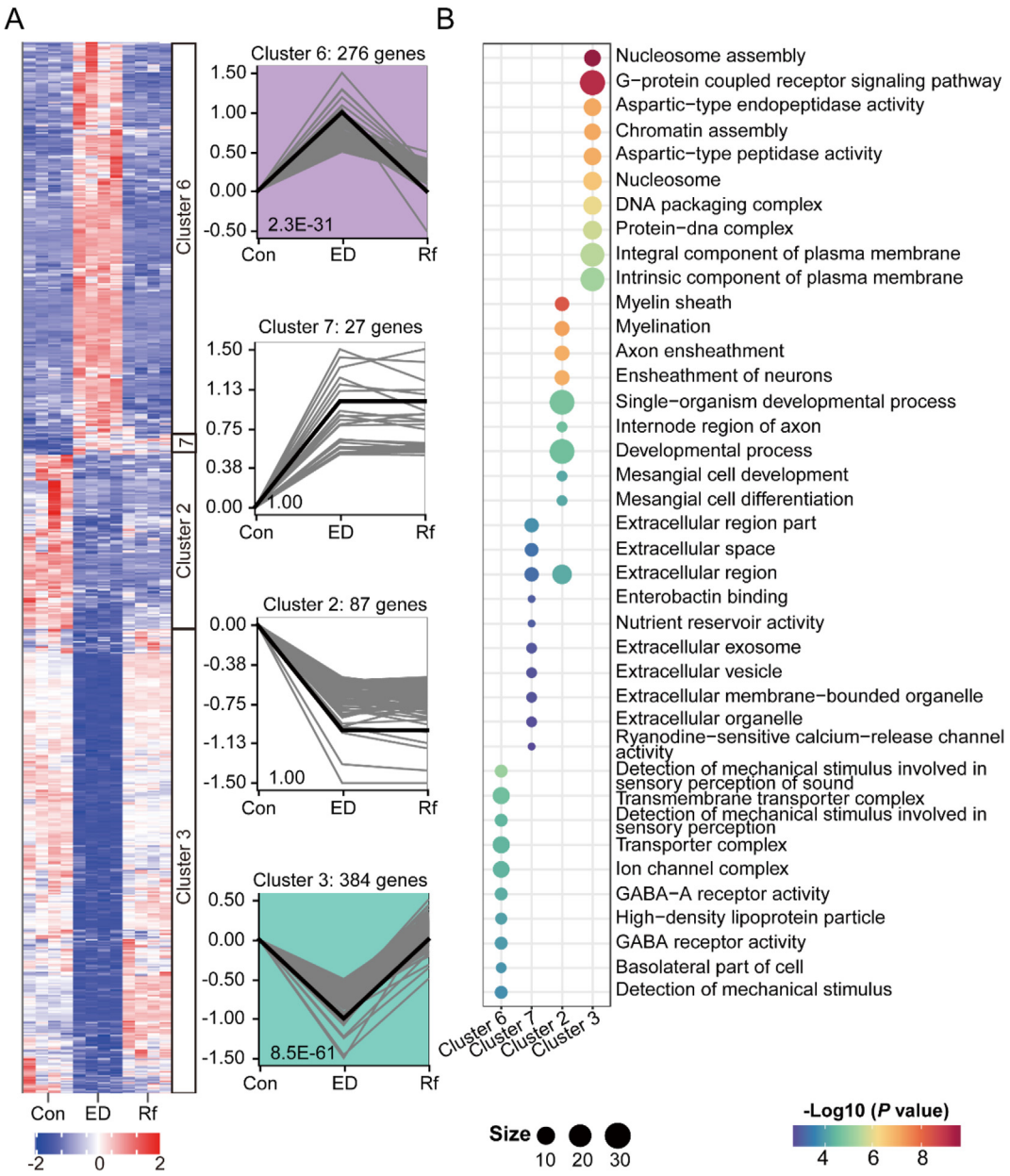


**Fig. 4.** The dynamic trend analysis and gene ontology (GO) terms of the pituitary after energy deprivation and refeeding ($n = 4$). (A) The heatmap and their corresponding clusters, colored represent significant concentrations. (B) The top ten significantly enriched GO Terms for genes in each cluster. The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. DNA = deoxyribonucleic acid; GABA = gamma-aminobutyric acid.

hormone levels, and HPO axis transcriptome analysis in 265-day-old Hy-line brown layers after energy deprivation and refeeding, to construct a comprehensive molecular network map of the HPO axis.

After five days of energy deprivation, the layers experienced a cessation of egg production, ovarian atrophy, and a reduction in follicles (including SWF and SYF), as well as hemorrhagic atresia of hierarchical follicles. These effects were reversed after refeeding, which is consistent with the findings of Socha (Socha and Hrabia, 2019) and Wolak (Wolak and Hrabia, 2021). Next, a serological test was conducted to measure the concentrations of reproductive hormones (LH, FSH, E2, and P4) in the serum. Studies revealed that LH surge drives the successful ovulation of preovulatory follicle F1 (Tischkau et al., 2011). In the current study, we observed that energy deprivation led to a significant decrease in the number of healthy hierarchal follicles and the serum LH concentration. Additionally, there was a substantial decline in the egg laying rate, indicating that energy deprivation can inhibit ovulation by reducing LH levels. Another gonadotropin, FSH, can stimulate the growth of early follicles, promoting the activation of primordial follicles (Guo et al., 2019), the proliferation of granulosa cells in prehierarchal follicles (Kim and Johnson, 2018), and follicle selection (Johnson, 2015) during follicle development in layers. However, other studies reported that excessive FSH dose inversely decreased oocyte maturation and cumulus cell function in mammals, and even induced premature luteinization with a lower E2 to P4 ratio (Clark et al., 2022). In the current study, FSH levels increased significantly after energy deprivation and were higher in

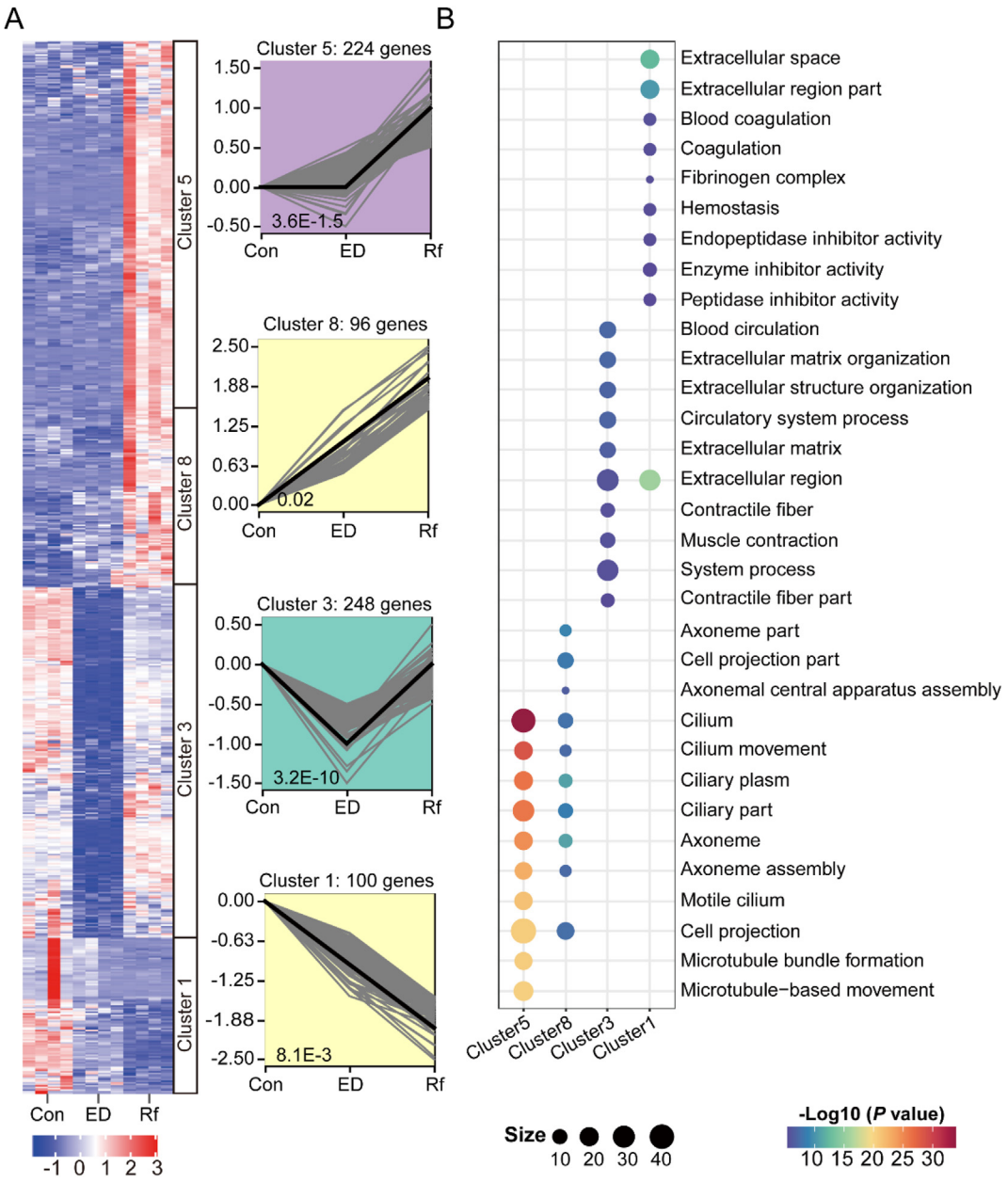


**Fig. 5.** The dynamic trend analysis and gene ontology (GO) terms of the ovary after energy deprivation and refeeding ($n = 4$). (A) The heatmap and their corresponding clusters, colored represent significant concentrations. (B) The top ten significantly enriched GO Terms for genes in each cluster. The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17.

the ED group than those in the Con and Rf groups. Based on these findings, we hypothesized that energy deprivation might affect the development of oocytes by increasing FSH. As is well known, it has been reported that steroid hormones such as E2 and P4 can stimulate granulosa cell mitosis and follicle growth (Bendell and Dorrington, 1991). In this study, the concentration of E2 in the ED group was significantly lower than that in the Con group and the Rf group, while the P4 concentration did not show a significant change. As a result, the E2 to P4 ratio in the ED group was significantly lower than that in the Con group and the Rf group. The follicle atresia and decreased ovulation in the ED group may be associated with the reduction in E2 concentration and E2to P4 ratio, which aligns with the hypothesis proposed by Clark et al. (2022). Compared to the Con group, the decrease in E2 concentration in the ED group may also be related to a decrease in the number of SWF, further leading to an increase in FSH levels in the ED group. Overall, our findings suggested that energy deprivation suppressed folliculogenesis and the ovulatory process in layers through the disruption of reproductive hormones. These disruptions could be largely reversed once the layers were re-fed, restoring normal hormone levels and functioning.

To further investigate the precise molecular mechanisms underlying the effects of energy deprivation and refeeding on egg production in layers, we conducted transcriptome analyses of the hypothalamus, pituitary, and ovaries. We applied clustering to identify gene expression patterns using all DEGs in the tissues related to the HPO axis. Furthermore, we performed GO enrichment analysis based on these gene clusters. Additionally, we

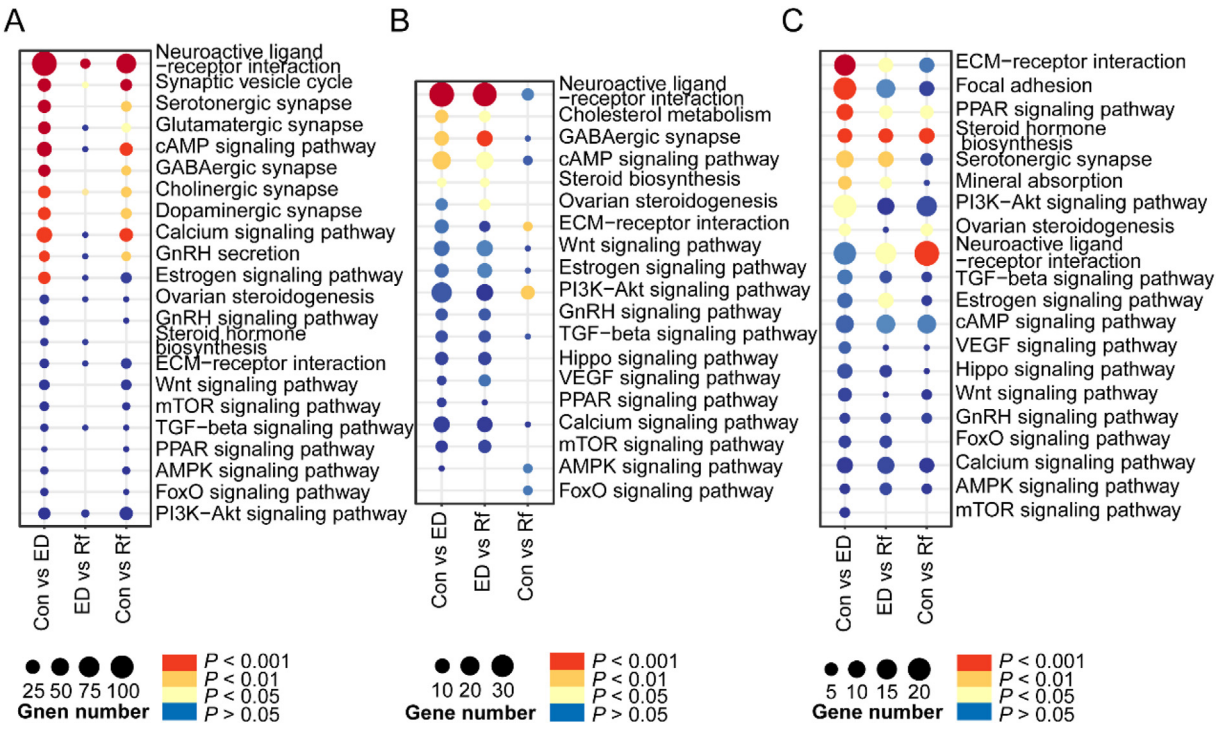


**Fig. 6.** Reproduction-related pathways in the hypothalamic-pituitary-ovarian (HPO) axis ($n = 4$). (A) Hypothalamus. (B) Pituitary. (C) Ovary. The Con group fed ad libitum from days 1 to 17, the ED group fed ad libitum from days 1 to 12 and then fasted from days 13 to 17, and the Rf group fasted for seven days (specifically, days 1 to 5, day 7, and day 9), and refed on days 6 and 8, and days 10 to 17. cAMP = cyclic adenosine monophosphate; GABA = gamma-aminobutyric acid; GnRH = gonadotropin-releasing hormone; ECM = extracellular matrix; Wnt = wingless/integrated; mTOR = mammalian target of rapamycin; TGF-beta = transforming growth factor beta; PPAR = peroxisome proliferator-activated receptor; AMPK = adenosine 5′-monophosphate (AMP)-activated protein kinase; FoxO = forkhead box O; PI3K = phosphatidylinositol-3-kinase; Akt (PKB) = protein kinase B; VEGF = vascular endothelial growth factor.

specifically examined pathways related to reproduction in the HPO axis.

In the hypothalamus, we observed a significant number of DEGs after energy deprivation, most of which did not return to their normal expression levels after refeeding. These DEGs were primarily associated with synaptic signaling, suggesting that energy deprivation could result in irreversible damage to synaptic function. Since communication between neurons relies on robust exocytosis of neurotransmitters from synaptic vesicles at synapses (Sansevrino et al., 2023), two types of neurons in the hypothalamus that play crucial roles in regulating fuel metabolism and feeding behavior may be affected. AgRP and neuropeptide Y (NPY) neurons promote foraging and food consumption (Morton and Schwartz, 2001), while POMC and cocaine-and amphetamine-regulated transcript (CART) neurons increase energy expenditure and reduce food intake (Hill, 2010). Specifically, in this study, we observed an increase in *AgRP* expression and a decrease in CART prepropeptide (*CARTPT*) and melanocortin 4 receptor (*MC4R*) expressions after energy deprivation, which ties well with previous research that food deprivation leads to changes in appetite-regulating signals in the hypothalamus of layers (Tachibana et al., 2003; Zhang et al., 2017). Additionally, various literature suggested a strong association between neurotransmitters such as GABA, Glu, Ach, DA, 5-HT, and energy homeostasis (Andersen et al., 2022; Maresca and Supuran, 2008; Volkow et al., 2011). Our findings indicated that the expression levels of several neurotransmitter receptors, specifically dopamine receptor D3 (*DRD3*), gamma-aminobutyric acid type A receptor subunit alpha 1 (*GABRA1*), glutamate ionotropic receptor N-methyl-D-aspartate type subunit 2B (*GRIN2B*), cholinergic receptor muscarinic 5 (*CHRM5*), and 5-hydroxytryptamine receptor 1B (*HTR1B*), were significantly downregulated in response to energy deprivation. These expression levels did not return to normal levels after refeeding, which may be related to inadequate refeeding and the challenge of repairing brain damage.

Interestingly, these neuropeptides and neurotransmitter receptor-related genes affected by energy deprivation in the hypothalamus have been reported to be closely associated with GnRH neurons, thus we explored their potential connection to the reproductive axis function. In our study, DEGs identified in the hypothalamus of Con vs ED and Con vs Rf were significantly enriched in GnRH secretion which included the two downregulated genes —*ESR2* and calcium voltage-gated channel subunit alpha 1H (*CACNA1H*). E2 regulates the surge of hypothalamic GnRH through estrogen receptors; however, caution should be exercised when discussing the specific role of E2 on GnRH due to the differential biological functions of estrogen receptor 1 (*ESR1*) and *ESR2* (Zhao et al., 2008). Research has shown that beta-estradiol modulates the expression of *ESR1* and *ESR2* in telocytes from human myometrium (Banciu et al., 2018). Furthermore, it can directly modulate the expression of the tachykinin receptor 3 (*TAC3*) and prodynorphin (*PDYN*) in the hypothalamus of layers via ESR1, consequently impacting GnRH secretion (Wu et al., 2024). Based on these, we speculated that energy deprivation inhibited the GnRH secretion signaling pathway by affecting the expression of neuropeptides and neurotransmitter receptor-related genes. Furthermore, some presynaptic plasma membrane proteins that promote neurotransmitter release and secretory proteins that facilitate neural synapse growth may also be involved in the secretion of GnRH (Ha et al., 2008; Przybył et al., 2021; Rothman, 1996). In the present study, we found that the genes *SNAP25*, brain-derived neurotrophic factor (*BDNF*), and neural epidermal growth factor-like 2 (*NELL2*) were downregulated after energy deprivation, which may further

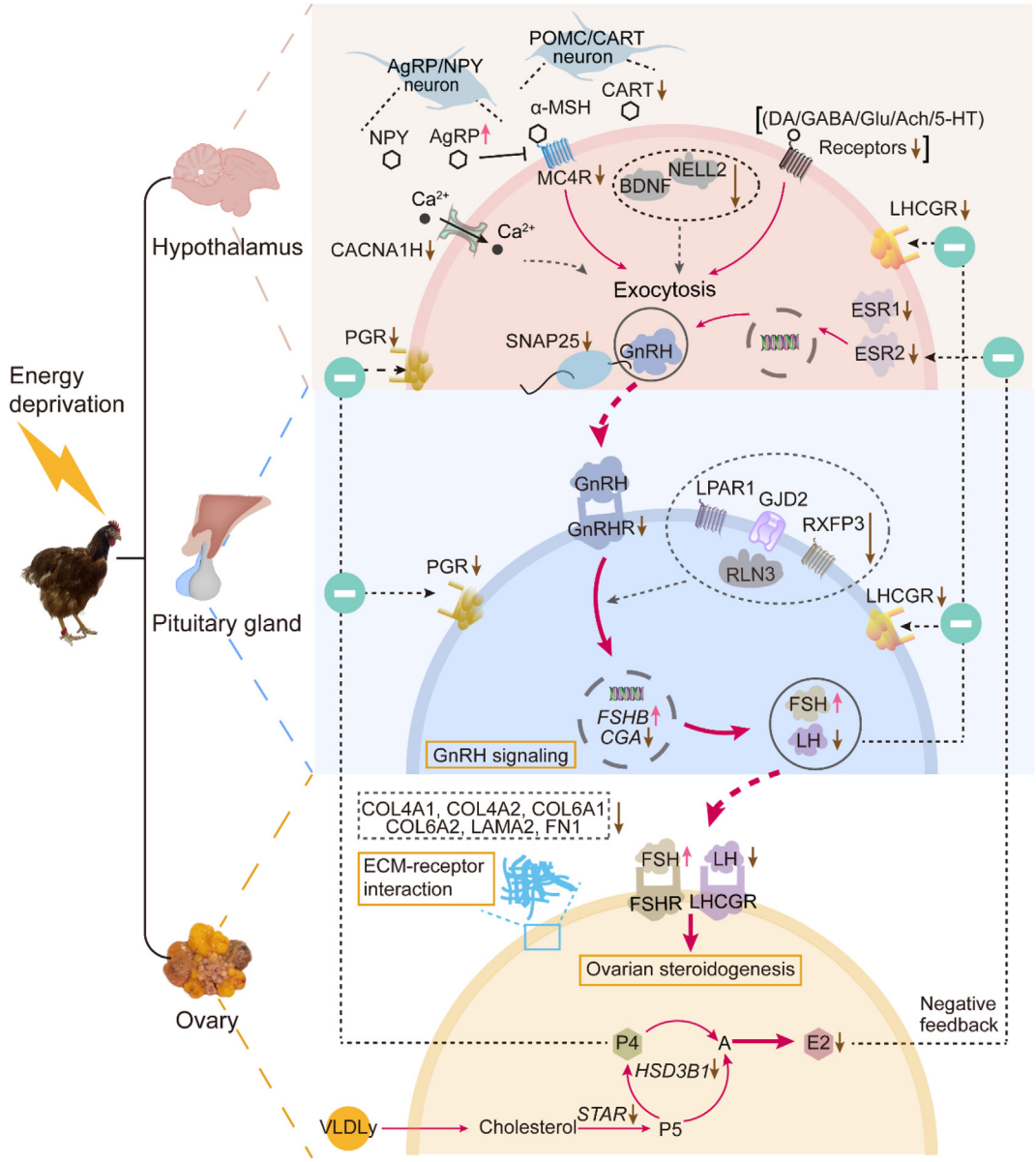


**Fig. 7.** Potential integrated hypothalamic-pituitary-ovarian (HPO) axis network response to energy deprivation in layers ($n = 4$). The upward and downward arrows in the image represent the upregulation and downregulation of expression, respectively. NPY = neuropeptide Y; AgRP = agouti-related peptide; α-MSH = α-melanocyte-stimulating hormone; POMC = pro-opiomelanocortin; CART = cocaine-and amphetamine-regulated transcript; DA = dopamine; GABA = gamma-aminobutyric acid; Glu = glutamate; Ach = acetylcholine; 5-HT = serotonin; CACNA1H = calcium voltage-gated channel subunit alpha 1H; MC4R = melanocortin 4 receptor; BDNF = brain-derived neurotrophic factor; NELL2 = neural epidermal growth factor-like 2; LHCGR = luteinizing hormone/choriogonadotropin receptor; PGR = progesterone receptor; SNAP25 = synaptosome-associated protein 25; GnRH = gonadotropin-releasing hormone; ESR1 = estrogen receptor 1; ESR2 = estrogen receptor 2; GnRHR = gonadotropin-releasing hormone receptor; LPAR1 = lysophosphatidic acid receptor 1; GJD2 = gap junction protein delta 2; RLN3 = relaxin 3; RXFP3 = relaxin family peptide receptor 3; *CGA* = glycoprotein hormones alpha chain; *FSHB* = follicle-stimulating hormone subunit beta; FSH = follicle-stimulating hormone; LH = luteinizing hormone; FSHR = follicle-stimulating hormone receptor; COL4A1 = collagen type IV alpha 1 chain; COL4A2 = collagen type IV alpha 2 chain; COL6A1 = collagen type VI alpha 1 chain; COL6A2 = collagen type VI alpha 2 chain; LAMA2 = laminin subunit alpha 2; FN1 = fibronectin 1; ECM = extracellular matrix; P4 = progesterone; P5 = pregnenolone; A = androstenedione; E2 = estradiol; VLDLy = very low density lipoprotein yolk targeted; *HSD3B1* = hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; *STAR* = steroidogenic acute regulatory protein.

support the hypothesis that GnRH secretion was inhibited. In summary, energy deprivation might hamper the function of hypothalamic GnRH neurons, resulting in a reduction in GnRH secretion.

Pituitary functions are regulated by the hypothalamus and its secretion of the gonadotropins LH and FSH plays vital roles in controlling ovarian steroidogenesis, folliculogenesis, and ovulation (Kaiser, 2011). GnRH pulses to the anterior pituitary through the hypophyseal portal circulation and targets GnRHR on the membrane of gonadotropes, activating signal transduction events that ultimately regulate the transcription of CGA, luteinizing hormone subunit beta (LHB), and follicle-stimulating hormone subunit beta (FSHB) (Bliss et al., 2010). LH and FSH consist of a common α-subunit (encoded by CGA) and different β-subunits (encoded by LHB or FSHB). In this study, CGA and GnRHR in cluster 3 were annotated to the GnRH signaling pathway, suggesting that the decrease in GnRH secretion induced by energy deprivation led to the downregulation of *GNRHR* and *CGA* in the pituitary, ultimately resulting in a decrease in circulating LH concentration. Ciechanowska et al. (2016) reported that the levels of GnRH in the hypothalamus and

GnRHR in the anterior pituitary of ewes subjected to short-term and prolonged stress fluctuate in sync with the release of LH, which is consistent with our results. Additionally, while gene *FSHB* did not meet the criteria for DEGs, compared to the Con group, the fold change of *FSHB* was 1.996 in the ED group and 1.421 in the Rf group. In our study, the serum levels of FSH and LH in the ED group exhibited significant increases and decreases, respectively, in comparison with the Con group. Following refeeding, these levels were essentially restored, aligning with the observed trends in *FSHB* and *CGA* gene expression. Several studies have also reported some genes potentially involved in the process of gonadotropin synthesis. Relaxin-3 (RLN3) and relaxin-3 receptor (RXFP3) are involved in appetite regulation, motivated behavior (Smith et al., 2014), and regulation of the hypothalamic-pituitary-gonadal (HPG) axis (Calvez et al., 2016) in mammals. Lv et al. (2022) confirmed that *cRLN3* is expressed at high levels in the chicken pituitary gland, shared location with *FSHB* and *LHB* genes, and its expression is modulated by E2 and GnRH. Similarly, the *LPAR1* gene co-localizes with the expression of *CGA* and *LHB* in the anterior pituitary of mice (Moriyama and Fukushima, 2021). Its expression rises after ovariectomy and is induced following E2 treatment, suggesting its potential participation in gonadotropin-related physiological effects. Göngrich et al. (2016) found that the coupling between gonadotropins mediated by gap junction protein delta 2 (GJD2) in the anterior pituitary contributed to the LH secretion stimulated by GnRH pulses and is subject to negative feedback regulated by E2. In our study, we observed that *RLN3*, *RXFP3*, *LPAR1*, and *GJD2* in cluster 3 exhibited downregulated expression after energy deprivation and recovered to Con group levels after refeeding, being consistent with the trend in LH changes. Therefore, these genes may act as regulators of gonadotropins other than GnRH stimulation in the hypothalamus. Based on these results, it is clear that the synthesis and secretion of gonadotropins in the pituitary were disturbed after energy deprivation but essentially recovered to Con levels after refeeding.

We are primarily interested in the ovarian response to energy challenges because it is a target organ of the HPO axis and tightly correlates with follicle development and egg production. The hormone signals from the pituitary gland regulate the biosynthesis of E2 and P4 in the ovary thereby mediating follicle growth, follicle selection, and ovulation induction (Rangel and Gutierrez, 2014). Previous studies indicated that chickens synthesize P4 and E2 via the delta 4 and delta 5 pathways, both being dependent on the involvement of HSD3B1 (Lee et al., 1998). Accumulating evidence suggested that the steroidogenic acute regulatory protein (*STAR*) gene encodes StAR responsible for facilitating the transportation of cholesterol from the outer to the inner mitochondrial membrane, and it is the rate-limiting enzyme of the steroidogenic reaction. Our observations exhibited that HSD3B1 and STAR were enriched in ovarian steroidogenesis. Their expressions difference of *STAR* and *HSD3B1* in cluster 3 were confirmed by serologic tests, which show similar dynamics in circulating E2 levels. Energy deprivation caused inhibition of GnRH secretion in the hypothalamus and LH secretion in the pituitary, resulting in decreased production of ovarian E2. Additionally, ovarian function is also regulated by the ECM except for steroid hormones. The ECM acts as a natural substrate supporting the highly dynamic processes of follicle growth, ovulation, and atresia in the ovary, which is shaped by the integration of mechanical forces and chemical signals (Fiorentino et al., 2023). As the follicles grow and develop, their diameter increase and ovarian morphology changes, and the ECM composition undergoes remodeling. The ECM primarily consists of collagens (encoded by collagen type VI alpha 1 chain [COL6A1], collagen type VI alpha 2 chain [CLO6A2], collagen type IV alpha 1 chain [COL4A1], and collagen type IV alpha 2 chain [COL4A2]), laminin (encoded by laminin subunit alpha 2 [LAMA2]), and fibronectin (encoded by fibronectin 1 [FN1]). In the present study, these ECM-related genes were primarily found in cluster 3, and the ECM-receptor interaction was the top 1 significant pathway in the comparison between the Con group and the ED group, indicating dysregulation of ECM turnover and ovarian function. The above analysis identified potential causes of ovarian atrophy and cessation of egg production in layers after energy deprivation and enriched the present knowledge on the mechanism of how the ovary is regulated by the HPO axis. In the HPO axis, regulation and feedback of the HPO axis are complex processes involving long feedback from the ovary to the hypothalamus and pituitary, short feedback from the pituitary to the hypothalamus, and ultra-short feedback from the pituitary to regulate itself (Padmanabhan and Cardoso, 2020; Sawyer, 1975). In this study, key reproductive hormone receptors were downregulated by energy deprivation, including *ESR1*, *ESR2*, *PGR*, and *LHCGR* in the hypothalamus, and *PGR*, *LHCGR*, and *GnRHR* in the pituitary, highlighting the negative feedback in the HPO axis. A mechanistic integration map illustrating the interactions and crosstalk among the HPO axis components, including hypothalamic GnRH, pituitary gonadotropins, and ovarian steroid hormones, after energy deprivation, is summarized and presented in Fig. 7.

## 5. Conclusion

In this study, we investigated reproductive traits, characterized the dynamic expression of key genes, and performed global integration based on the HPO axis. Our results suggested that energy deprivation could initiate functional inhibition of the HPO axis and result in impaired follicle development and laying pause, which is largely recovered after refeeding. This work will enrich the knowledge of the signal transduction mechanisms of the hypothalamus, pituitary, and ovarian tissues in response to energy alterations in layers, contributing to the understanding of the reproductive axis, improvement of laying performance, and energy supply strategies in chickens.

## CRediT authorship contribution statement

**Jianling Peng:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Yong Cui:** Visualization, Software, Formal analysis. **Haiping Liang:** Visualization, Conceptualization. **Shenyijun Xu:** Methodology, Data curation. **Linjian Weng:** Resources, Investigation. **Meng Ru:** Formal analysis, Data curation. **Ramlat Ali:** Writing – review & editing. **Qing Wei:** Formal analysis, Conceptualization. **Jiming Ruan:** Writing – review & editing, Supervision. **Jianzhen Huang:** Writing – review & editing, Resources, Project administration, Funding acquisition.

## Credit Author Statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

## Acknowledgments

This study was financially supported by the grant of the National Natural Science Foundation of China (32360867, 31960690). We extend our sincere gratitude to our colleagues at the College of Animal Science and Technology, Jiangxi Agricultural University, for their invaluable assistance in collecting the samples.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.08.009.

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