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The effect of induced molting on the testicular physiological remodeling in no-semen roosters

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Abstract

Background The fertility of roosters significantly impacts the economic outcome of the poultry industry. However, it is common for some roosters to fail to produce semen during production, and the underlying reasons remain largely unclear.

Results To investigate a solution to this problem, induced molting (IM) was performed on no-semen (NS) roosters. Remarkably, the NS roosters recovered and began producing semen on 30 d after recovery feeding (R30), with semen quality and ejaculation volume returning to normal levels by 39 days after recovery feeding (R39). The difference in testicular weight between the NS and healthy roosters was significant on one day before fasting (F0) ($P < 0.05$). Meanwhile, morphological analysis of NS roosters' testicular seminiferous tubules suggested that Sertoli cells (SCs), which form the scaffold in the testicular microenvironment, were severely damaged in NS roosters. Their spermatogenic cells were disordered and fewer, suggesting abnormal testicular function in NS roosters. Following induced molting, the epithelial structure of seminiferous tubules in the testes of NS roosters was restored, and follicle-stimulating hormone (FSH) levels in both serum and testicular were significantly higher ($P < 0.05$). To further elucidate the mechanisms, transcriptome analysis was conducted to uncover dynamic gene expression changes in testicular tissues at two time points: F0 and R39. Results indicated that *ALDH1A1* levels in the testes of NS roosters were 16.0-fold lower than those in healthy roosters at F0 but significantly increased by R39, suggesting that the *ALDH1A1* gene may be closely related to testicular failure in NS roosters. Pathway-enrichment analyses revealed that IM significantly activated the phagosome pathway in the testes of NS roosters, and the genes *ATP6VOD2,ATP6V1A,Ighm* and *MHCY2B1* were involved in this pathway, associated with autophagy. We hypothesize that in response to nutrient deprivation, autophagy is initiated to degrade damaged components in the seminiferous tubules of NS roosters, leading to testicular physiological remodeling and resumption of semen production.

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Conclusions This report identifies critical pathway and molecular markers related to testicular failure and physiological remodeling in NS roosters caused by induced molting, offering an essential reference for accelerating genetic selection.

Keywords Induced molting, No-semen rooster, Testicular physiological remodeling, Autophagy

Introduction

The reproductive performance of roosters is a crucial factor influencing the economic outcomes in the poultry industry. However, there are a considerable number of roosters are unable to produce semen during production, which we define as no-semen (NS) roosters, and this problem for the roosters is undoubtedly fatal [1]. Reproductive disorders in cocks directly affects the production performance of hens and may even endanger poultry germplasm resources [2]. Due to this issue, approximately 10–20% of breeding roosters are culled annually. Therefore, conducting an etiological analysis of NS in breeding roosters and identifying effective methods to alleviate this issue is imperative. This dramatically improves economic benefits and has significant implications for the protection of poultry germplasm resources, accelerating the improvement of local chicken breeds.

To maximize economic gains, the poultry industry always expects roosters to maintain high levels of semen quality for longer. However, several issues can lead to decreased fertility in roosters, including increased body weight, decreased circulating testosterone levels, decreased semen quality, and dysfunction of Sertoli cells (SCs) [3, 4]. Over the past decades, this issue has attracted significant attention from researchers seeking effective strategies to improve fertility in roosters with reproductive disorders. Most relevant studies have focused on nutritional regulation [5, 6]. However, many of these approaches are uneconomical and challenging to implement in practice. Induced molting through feed withdrawal is a well-known and widely used technique in the poultry industry [7, 8]. Research has shown that induced moulting (IM) can effectively enhance poultry production performance [9] and reproductive performance [10]. Furthermore, our previous study has confirmed that induced molting can effectively improve the semen quality of aging roosters, resulting in the testicular physiological rejuvenation [11]. However, it remains indeterminate whether this model can alleviate the symptoms of NS roosters, and further research is needed.

No semen is analogous to azoospermia or oligospermia in humans, representing a complex reproductive system disease influenced by multiple factors, with most causes linked to primary or secondary testicular failure [12–14]. Studies suggest that 40% of testicular failures are idiopathic, with the pathogenesis linked to new gene mutations [15]. Although the adverse consequences of this disease are enormous, few studies have been conducted in poultry to date. Moreover, with the rapid development of sequencing technology in recent years, it has provided great convenience for researchers in identifying essential genes. In Beijing You chickens, a high genetic correlation was found between semen volume, abnormal sperm rate, and sperm density ($r = 0.47, 0.68$) [16]. Avian spermatogenesis is closely related to gene expression levels [17]. However, only a few of genes have been characterized in terms of function. For instance, disrupting the genes in the PIWI-interacting RNAs (piRNAs) pathway would cause spermatogenic failure [18]. Spermatogenesis is a complex orderly process, involving various genes interactions [19], necessitating further exploration.

Most prior studies on decreased fertility in roosters primarily focused on asthenospermia, and the improving methods were based on nutritional regulation. In this study, we innovatively investigated NS. We successfully established a physiological remodeling model through induced molting, a more economical and convenient approach, enabling NS roosters, which initially produced no semen, to resume semen production. Additionally, RNA-seq was conducted to identify critical molecular markers related to NS and the restoration of reproductive performance due to induced molting, providing an essential reference for accelerating genetic selection. Our findings offer new insights into extending the service lifespan of roosters and elucidate the biological functions of physiological remodeling in spermatogenesis.

Materials and methods

Ethics statement

The handling of experimental roosters adhered strictly to humane euthanasia procedures outlined in accordance with the Laboratory Animal Guidelines for Euthanasia (T/CALAS 31-2017; Chinese Association for Laboratory Animal Sciences). Chickens were euthanasia with artificial cervical dislocation before bled. All efforts were made to minimize animal suffering. All protocols concerning animals adhered to the regulations outlined by the Ministry of Agriculture of China and obtained consent from the ethics committee at Henan Agricultural University (Zhengzhou, Henan, China), under approval number 11–0085.

Animal experimental design

"Yufen 1"H Line roosters (58 weeks of age) were obtained from the Animal Center of Henan Agricultural University and divided into two experimental groups. Seven NS

roosters that could not produce semen, regardless using any stimulation method, were selected, and the healthy roosters ($n=7$) were used as a negative control (NC). Roosters that inability to produce semen following two consecutive weeks of artificial semen collection training are classified as NS roosters. Roosters with ejaculation volume > 0.2 mL per collection and semen color appearing milky white are classified as healthy roosters. The two groups were both performed IM. The IM process of the roosters is designed by referring to our previous studies [11]. Some key time points in this experiment were showed in Table 1 (namely, F0 [the day before the first day of feed breaking]; F15 [the 15th day of feed breaking]; and R39 [the 39th day of feed resuming]). Euthanasia and sample collection were performed at two critical periods (F0 and R39). The testis samples were collected for tissue histology study and transcriptome sequencing. Hormone assays were also carried out.

Sample collection

The body weight and loss of weight rate of the experimental chickens were gained every 3 d during the experimental period. Semen samples of experimental roosters were collected every 3 d from R15 to R39 using the technique described by Burrows and Quinn (1937) [20]. The collected semen was placed in a 37℃ water bath for semen quality assessment [21]. Three roosters with a uniform weight selected from each group were humanely euthanized at F0 and R39 periods, respectively. A total of 12 samples (testes) were collected and weighed [22]. The right testis was homogenized into a pellet, placed in a 1.5 mL sterile enzyme-free centrifuge tube, and stored in an − 80℃ freezer for subsequent RNA extraction and hormone assays. Meanwhile, the left testes were fixed in a formaldehyde fixation solution for preservation.

Table 1 IM program induced by starvation

	Treatment				
Test period	Feed	Water	Light	Processing time for each stage	Semen state
F0	Normol Feed	Normal drinking water	16 h	On the day before the test	semen cannot be collected
F15	No Feed	Water cut off	8 h	On the 15th day of fasting	semen cannot be collected
R15	Gradually resuming feeding	Normal drinking water	8 h + 0.5 h per day (15.5 h)	On the 15th day of recovery	semen cannot be collected
R39	Normal feed	Normal drinking water	16 h	On the 39nd day of recovery	Ejaculation volume > 0.2 mL, semen color completely recovered to milky white

Semen quality determination

The collected semen was diluted 1:500 with 0.9% sodium chloride solution [23]. To assess the motility characteristics of sperm, 3 μL of diluted semen were placed on the prewarmed (38℃) chamber slide (Leja, Nieuw-Vannep, Netherlands), and covered with a coverslip. Spermatozoa were counted under a phase-contrast microscope at 40x microscope in the minimum of five fields including the four corners and the central square of the counting chamber. Sperms on square boundaries were counted on adjacent sides and angles. Then we counted the number of motile sperm moving forward to evaluate the parameters including sperm motility and sperm density. The above counting was done by the same person. Sperm motility was expressed as the percentage of spermatozoa showing moderate or progressive forward movement to the total counted sperms. Sperm density = total sperm count / $80 \times 500 \times 400 \times 10{,}000$ (80 represents the number of lattices; 500 represents the dilution factor; 400 represents the magnification of the microscope; 10000 represents the conversion factor).

Statistics of fertilization and hatching rates

Forty 67-wk-old "Yufen 1"H Line hens were divided into 2 groups (20 hens per group), which were fed normally in the same batch. On the 50th day after the resumption of feeding, semen samples collected from five NS roosters with induced molting and five healthy breeding roosters without induced molting were used for artificial insemination. The semen samples pooled and diluted to a concentration of 400×10^6 spermatozoa/mL [24]. The first round of artificial insemination was carried out for two consecutive days. Each hen was inseminated with 20 mL diluted semen. Then, artificial insemination was conducted every 3 d. At 2 to 14 d after the first insemination, eggs were collected daily and kept at a temperature of 18 ℃ and a relative humidity of 75% until incubation [25]. Eggs that did not meet hatching criteria such as sand shell eggs were eliminated before hatching. The sixty eggs (30 eggs per group) were then incubated at 37.5 ℃ and 55% RH. On Day 7 of incubation the eggs were candled for viable embryos, and the percent fertility was expressed as the fertilization rate (number of fertilized eggs / number of incubated eggs × 100). On Day 21 of the incubation, by breaking unhatched eggs, hatchability (number of healthy chickens / number of fertilized eggs × 100) and stillbirth rates (number of dead chickens / number of fertilized eggs × 100) were calculated.

Testis histology study

On F0 and R39 of the experiment, three roosters per group were randomly selected and euthanized for necropsy evaluation. Testes were dissected from experimental animals, fixed in 4% neutral buffered formalin, dehydrated in ethyl alcohol, cleared in xylene, and finally embedded in paraffin. Testicular Sect. 7 mm thick were used and stained with hematoxylin and eosin (H&E). For histological observation, slides were investigated by optical microscopy at a final magnification of ×100. Setting scale and measurements were performed using Imagepro Plus software. We measured the diameter of the seminiferous tube of the seminiferous epithelium on at least ten random sections of seminiferous tubules.

RNA-Seq analysis and validation

Total RNA was extracted from the testicular tissues using the Total RNA Extraction Kit (Sangong, Shanghai, China) according to the manufacturer's instructions. The concentration, quality and integrity of all the RNA tissues were determined using a NanoDrop spectrophotometer (Thermo Scientific, Shanghai, China). Subsequently, equal amounts of total RNA of each group were pooled for testes tissues and used to construct the libraries for transcriptome analysis. RNA-seq of these tissues was conducted by Genedenovo Biotech (Guangzhou, China). FASTP was used for quality control processing to filter the low-quality data, and HISAT2 was used to align clean data to the chicken reference genome. DESeq2 was used to analyze differential expression among the tissues at FDR < 0.05 and |log2FC|>1. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to determine the biological functions of the differentially expressed genes (DEGs).

Real-time quantitative PCR (RT-qPCR) was used to verify the accuracy of the transcriptome data. Total RNA from testis tissues was reverse transcribed to cDNA using the Evo M-MLV Reverse Transcriptase Kit (Ruizhen, Guangzhou, China) according to the manufacturer's instructions. Gene primer sequences were showed in Table 2, and qPCR were performed in a 10 mL volume containing 1.0 mL of cDNA, 5.0 mL of SYBR Premix Ex Taq II (TaKaRa, Dalian, China), 0.5 mL of each primer, and 3 mL of RNase-free water. A LightCycler96 real time PCR system was used (Roche Applied Science, Indianapolis, IN), and the amplification conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final 10-min extension at 72 °C. All the reactions were performed in triplicate. All the primers used in this study were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The GAPDH mRNA was used as an internal control, and the relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method [26].

Table 2 Effect of induced molting on semen quality of NS and NC roosters in different periods

Time	Group	Semen volume(mL)	Sperm concentration (1×10^9/mL)	Sperm motility	Abnormal sperm (%)
F0	NS	0	0	0	0
	NC	0.36 ± 0.08	6.10 ± 1.57	0.67 ± 0.06	12.33 ± 2.41
	P	0.013	0.003	0.002	0.077
R30	NS	0.13 ± 0.01	4.30 ± 3.28	0.77 ± 0.19	9.00 ± 2.65
	NC	0.16 ± 0.02	4.20 ± 4.31	0.52 ± 0.29	4.67 ± 3.06
	P	0.074	0.976	0.282	0.137
R33	NS	0.19 ± 0.11	6.54 ± 1.74	0.80	5.33 ± 1.16
	NC	0.25 ± 0.12	5.73 ± 2.69	0.73 ± 0.08	7.67 ± 3.79
	P	0.335	0.699	0.270	0.365
R36	NS	0.27 ± 0.06	5.83 ± 1.55	0.67 ± 0.14	7.67 ± 1.53
	NC	0.27 ± 0.11	5.79 ± 0.40	0.65 ± 0.10	8.67 ± 2.52
	P	0.768	0.103	0.877	0.588
R39	NS	0.29 ± 0.46	6.73 ± 0.67	0.68 ± 0.03	5.00
	NC	0.31 ± 0.05	6.39 ± 1.19	0.63 ± 0.03	9.33 ± 2.08
	P	0.872	0.841	0.133	0.068

Statistical analysis

All experiments were performed at least in triplicate. All data were expressed as mean ± SEM. ANOVA analysis with Tukey's post-hoc test was used to compare multiple groups. P-values of less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS 22.0 (IBM, USA) and figures were generated by Prism v8.2.1 (GraphPad Software Inc.,USA). $^{*}P < 0.05$; $^{**}P < 0.01$.

Results

Effect of IM on body weight and testes weight

The effects of IM on body weight and testes weight over the experimental period are presented in Fig. 1A and C. This study found that the body weight of roosters in each group was significantly decreased ($P < 0.05$) on the 15th day of feed breaking (Fig. 1B), and then recovered to the levels before molting gradually with the development of the feed withdrawal refeeding experiment ($P > 0.05$). On F0 and R39 of the experiment, no statistically significant difference in body weight was observed between the NS and NC groups ($P > 0.05$). The testicular weight of NC roosters was significantly higher than that of NS roosters on F0 ($P < 0.05$), and the testes of NC rooster grew larger than NS roosters at F0 (Fig. 1A). However, with the resumption of feeding after IM, the difference in testicular weight between the two groups narrowed ($P > 0.05$) (Fig. 1C), and the color of NS roosters' testes changed to pink (Fig. 1A).

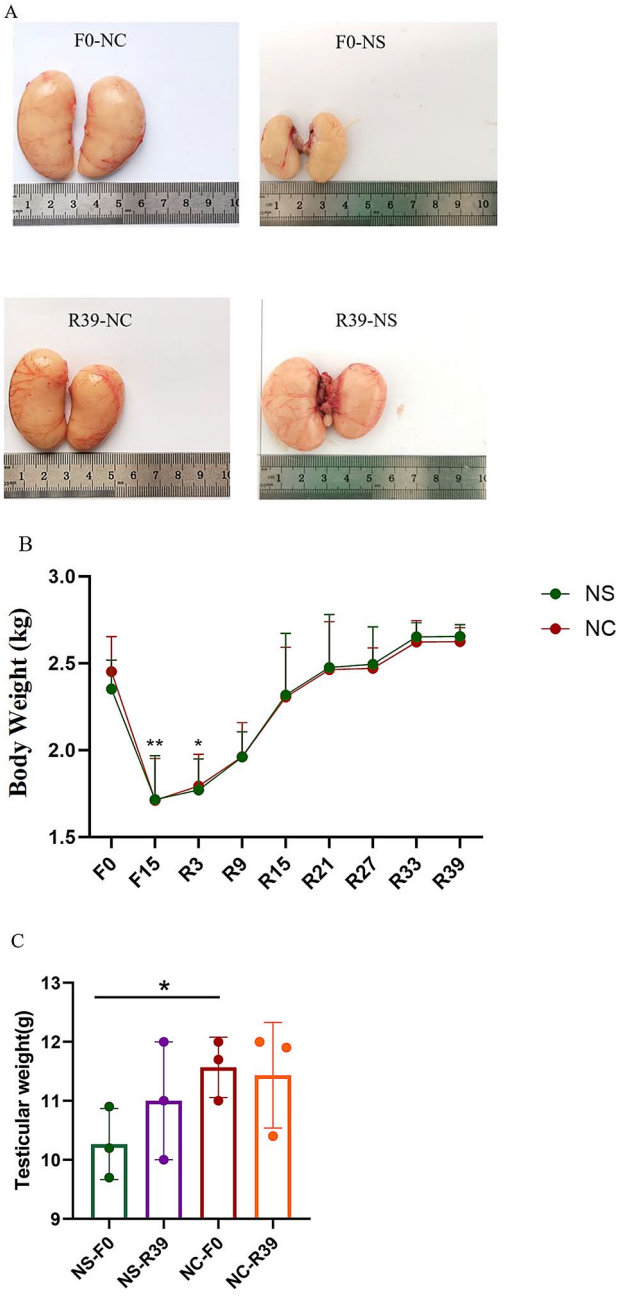


Fig. 1 Changes in appearance, weight, and weight of testes in different periods. (**A**) Photographs of testes in different periods. (**B**) Changes of chicken body weight in different periods ($n=7$ roosters per group at F0; $n=4$ roosters per group at the other periods). (**C**) Changes of testis weight in different periods. *$P<0.05$; **$P<0.01$

Effect of IM on semen quality and reproductive performance

The effects of IM on seminal characteristics over the experimental period are presented in Table 3. There was no semen collected neither from NC nor NS groups after refeeding for 15 d. However, we were surprised to find that the NS roosters recovered semen production miraculously on R30 as well as the NC roosters. In addition, the semen volume, sperm concentration, sperm motility and abnormal sperm of NS roosters had no significant difference with NC roosters until R39 ($P>0.05$) (Table 3). On the 50th day after the resumption of feeding, artificial insemination was carried out. The differences in fertilization rate, stillbirth rate, and hatchability between NS roosters and the healthy roosters were insignificant ($P>0.05$) (Fig. 2).

Testicular seminiferous tubules morphological analysis (paraffin section)

The effects of induced molting on testicular tissue morphology are presented in Fig. 3. Referencing to the Moisan's method [27], a testicular hematoxylin-eosin staining experiment was performed, we found that Sertoli cells form the scaffold in the testicular microenvironment were severely damaged in NS roosters. For NS roosters on F0, the majority of the epithelium in seminiferous tubules was exfoliated, making the tubles hollow. The seminiferous tubule of NS roosters was small or twisted as compared to healthy roosters, and their spermatogenic cells were disordered and fewer in the testis. However, after molting, a large number of mature sperms at R39 were observed, which gathered in the central area of the sperm tube lumen. Comparing the seminiferous tubule of NS roosters at the pre- and post-molt phase, we observed that the spermatogenic cells and Sertoli cells distributed more densely, and the epithelial structure of the seminiferous tubules in the testis was restored to completeness.

Effect of IM on hormone levels

To further elucidate the reasons for the resumption of semen production in NS roosters, we employed enzyme-linked immunosorbent assay (ELISA) (the kit was provided by Jiangsu Meimian Industrial Co., Ltd) to measure the levels of luteinizing hormone (LH), testosterone (T), and follicle-stimulating hormone (FSH) in the testis and serum of both NS roosters and NC roosters, during the pre- and post-moult phase, and these sex hormone content in testis and serum are shown in Fig. 4A F. For NS roosters, FSH levels in serum and testicular tissue for NS roosters were significantly lower than those in NC roosters at F0 ($P<0.05$; Fig. 4A and B). Additionally, at 39 days after resuming diet following induced molting (R39), serum and testicular FSH levels were significantly higher than those before induced molting (F0) ($P<0.05$). At the same time, LH (Fig. 4C and D) and T (Fig. 4E F) contents increased slightly, but the differences were insignificant ($P>0.05$).

Transcriptome data analysis and validation

In order to investigate the potential mechanism of oligospermia and the reasons for resuming semen production after IM for NS roosters, RNA-sequencing was

Table 3 Summary statistics for sequence quality and alignment information of 12 testicular samples

Sample	RawData(bp)	BF_Q20(%)	BF_Q30(%)	CleanData(bp)	AF_Q20(%)	AF_Q30(%)
F0NC-1	6,310,914,000	6,207,302,234 (98.36%)	6,022,904,849 (95.44%)	6,205,770,750	6,120,306,783 (98.62%)	5,949,012,649 (95.86%)
F0NC-2	6,290,483,400	6,204,851,905 (98.64%)	6,058,685,788 (96.32%)	6,186,927,206	6,119,594,830 (98.91%)	5,987,396,478 (96.77%)
F0NC-3	5,516,561,700	5,441,063,321 (98.63%)	5,313,374,964 (96.32%)	5,432,202,226	5,372,046,561 (98.89%)	5,255,766,935 (96.75%)
R39NC-1	6,488,774,100	6,408,712,296 (98.77%)	6,267,917,355 (96.60%)	6,406,797,788	6,340,752,870 (98.97%)	6,210,386,065 (96.93%)
R39NC-2	5,472,417,600	5,407,635,343 (98.82%)	5,291,831,596 (96.70%)	5,407,892,698	5,353,695,231 (99.00%)	5,246,109,385 (97.01%)
R39NC-3	5,882,029,200	5,810,507,046 (98.78%)	5,681,706,226 (96.59%)	5,817,352,574	5,756,871,427 (98.96%)	5,636,568,356 (96.89%)
F0NS-1	6,302,370,000	6,229,405,844 (98.84%)	6,097,771,671 (96.75%)	6,230,841,873	6,169,957,583 (99.02%)	6,047,593,494 (97.06%)
F0NS-2	5,885,412,300	5,810,877,151 (98.73%)	5,674,607,076 (96.42%)	5,806,544,035	5,744,340,262 (98.93%)	5,617,222,921 (96.74%)
F0NS-3	5,590,396,500	5,514,328,502 (98.64%)	5,375,619,720 (96.16%)	5,514,890,641	5,450,556,671 (98.83%)	5,321,007,631 (96.48%)
R39NS-1	6,523,815,900	6,444,961,790 (98.79%)	6,303,097,584 (96.62%)	6,452,170,485	6,385,740,999 (98.97%)	6,253,335,623 (96.92%)
R39NS-2	5,934,262,500	5,861,248,929 (98.77%)	5,727,060,206 (96.51%)	5,866,597,956	5,804,788,523 (98.95%)	5,679,360,896 (96.81%)
R39NS-3	6,605,385,000	6,519,134,534 (98.69%)	6,360,052,743 (96.29%)	6,533,752,999	6,458,876,548 (98.85%)	6,308,335,839 (96.55%)

Notes: Q20,Q30: the percentage of bases with Phred values greater than 20 and 30 in the total number of bases, respectively

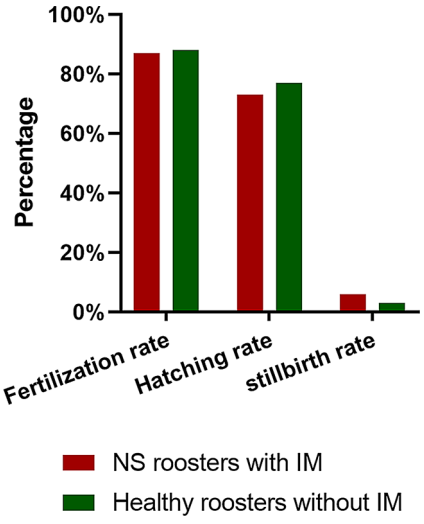


Fig. 2 Effect of induced molting on reproductive performance. Comparison of fertilization rate, stillbirth rate and hatching healthy chick rate between molting NS group and non-molting healthy rooster group (30 eggs per group)

performed to analyze the differentially expressed genes in NS and NC roosters at pre- and post-moult phase (F0 and R39). The results of the transcriptome sequencing were presented in Table 4. The Q20 value exceeded 98%, and the Q30 value exceeded 95%. No separation phenomena were observed, suggesting high-quality sequencing data that met the required standards. Therefore, the subsequent work could proceed. A principal component analysis (PCA) was conducted on the fragments per kilobase per million mapped fragments of each testis sample (Fig. 5), and it was found that all the subgroups had excellent within-group repeatability and between-group variability. We performed multi-angle comparisons of the gene expression, and the comparisons were between the groups as follows: NS and NC on F0, NS and NC at R39, and the DEGs on F0 and R39 for NS roosters. (Fig. 6) A total of 1255 DEGs were predicted that were significantly differentially expressed between the three groups (FDR < 0.05 and |log2FoldChange| >1).

In the NS vs. NC group, we identified 567 DEGs (419 upregulated and 148 downregulated genes) and 307 DEGs (143 upregulated and 164 downregulated genes) in the testis tissues of the F0 and R39 periods, respectively. We focused on the genes with significant differences between the two groups at F0, while there was no significant difference at R39. The criteria used for target genes identification was raised, which setting as Q-value < 0.05 and |log2FC|>3, and the DEGs were crossed retrieved from F0 and R39. The Wayne diagrams in Fig. 7. A total of 25 genes were identified, which might be considered fiercely related to the occurrence of NS roosters. Notably, some well-documented genes with crucial roles in spermatogenesis, such as *ALDH1A1* and *APOB*, were included in this set of 25 genes.

For NS roosters, as Fig. 6 shows, there were 381 DEGs in the testis tissues at the pre- and post-moult phase, and these DEGs were subjected to GO and KEGG enrichment analyses to determine their biological functions. GO enrichment analysis showed that the DEGs were significantly enriched in cellular processes, metabolic processes, biological regulation, regulation of biological processes, response to stimulus, multicellular organismal processes, and developmental processes and so on. (Fig. 8A). The KEGG analysis revealed 381 DEGs were mapped in 143 pathways, including 21 significantly enriched pathways including phagosome, intestinal immune network for IgA production and oxidative phosphorylation pathways, etc. (Fig. 8B). Further analysis of the DEGs on these pathways revealed that *ATP6VOD2,ATP6V1A,Ighm*, and *MHCY2B1*, associated with testicular cell functions, may play a vital role in regulating spermatogenesis during IM periods.

RT-qPCR analysis of the DEGs (*ALDH1A1,NAT10,SULT1E1,LY6E, SLC9A3R1*) from transcriptome sequencing revealed a consistency

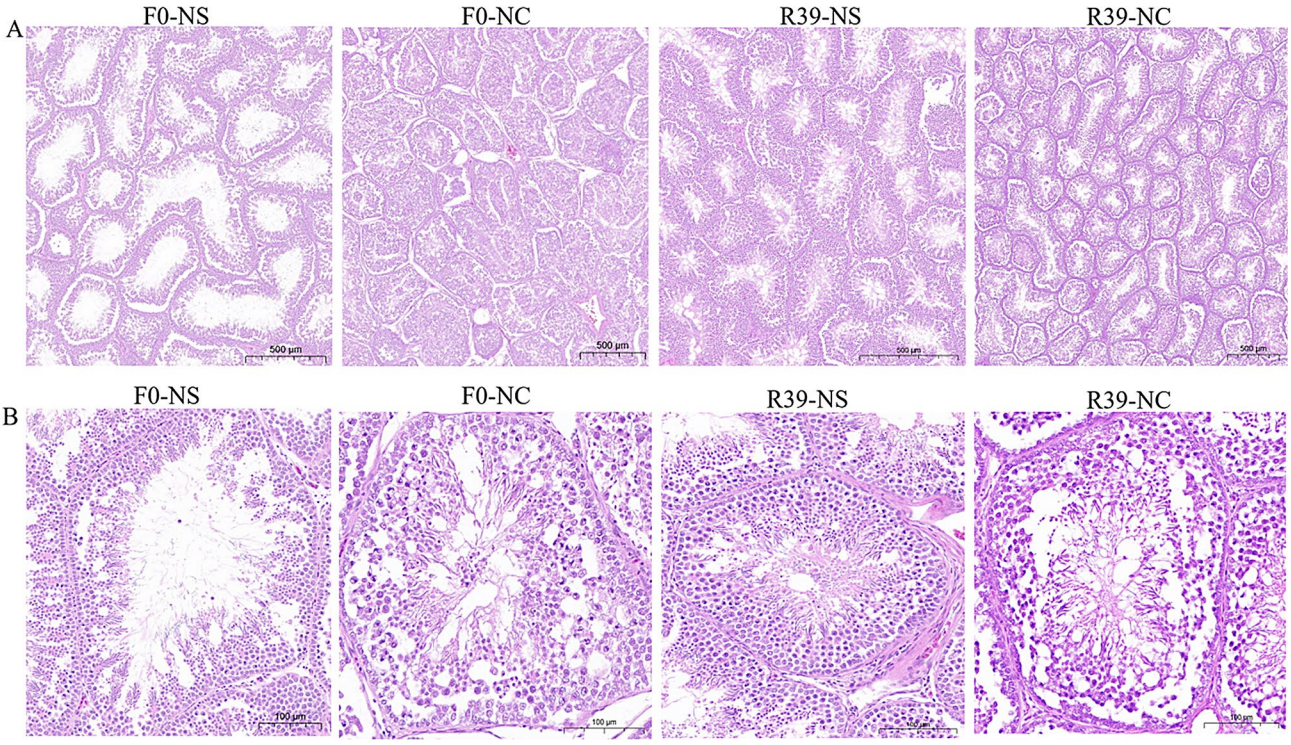


Fig. 3 The effect of induced molting on the morphology of testis tissue of NS roosters. (**A**) Slices of cock testes are 4× photographic results (scale: 500 mm) in NS and NC roosters at different stages; (**B**) Slices of cock testes are 20× photographic results (scale: 100 mm) in NS and NC roosters at different stages

between the RNA-seq and gene expression results (Fig. 9), thus proving the reliability of the results.

Discussion

In recent years, increasing attention has been focused on the reproductive performance of breeding roosters, which holds significant economic importance in the poultry industry. Reduced breeding efficiency in roosters increases breeding costs and directly reduces production profits. The testicles are the primary site of sperm production. Normal testis development is crucial for maintaining proper physiology of the reproductive axis and male fertility. Evidence suggests that reproductive performance is influenced by genetic and intrinsic factors, including testicular size [28, 29]. Additionally, testicular weight is highly correlated with sperm production [30], with correlations ranging from 0.50 to 0.65 [31, 32] and even 0.90 [33]. Selecting for testis size is an effective strategy for enhancing spermatogenesis [34, 35]. In this study, we collected testicular tissue from the experimental subjects. At F0, the testicular size and testicular weight in NS roosters were significantly lower than those in NC roosters, which might suggest the dysfunction of testicles in the NS roosters, affecting semen production. However, these parameters improved significantly following induced molting (IM), allowing NS roosters, which initially produced no semen, to unexpectedly resume semen production, with quality returning to normal. Semen quality reflects the production of spermatozoa in the testes, the patency of the duct system and the glandular secretory activity [36]. Our experimental results demonstrated that IM successfully induced physiological remodeling in the testes of NS roosters, thereby restoring semen production, and the recovered produced semen was capable of fertilization.

To investigate the effects of IM on spermatogenesis and the pathogenesis in NS roosters, we conducted a morphological analysis of testicular seminiferous tubules. Spermatogenesis depends on the complete maturation of the somatic microenvironment [37]. This process commences with the differentiation of germ cells and culminates in the generation of fully developed spermatozoa, occurring within the seminiferous tubules of the testes [38]. Consistent with our hypothesis, we found that Sertoli cells (SCs), which provide structural support in the testicular microenvironment, were severely damaged in NS roosters, resulting in disorganized and fewer spermatogenic cells. Previous studies indicate that proper interactions between SCs and germ cells within seminiferous tubules are essential for healthy spermatogenesis [39]. In fact, the tight connection between SCs and the blood-testis barrier assists SCs in providing nourishing and spatial support [40, 41]. Timely cell proliferation and differentiation in somatic and germinal testicular cells are crucial for normal testis development and spermatogenesis [42]. Disruption of these processes can significantly

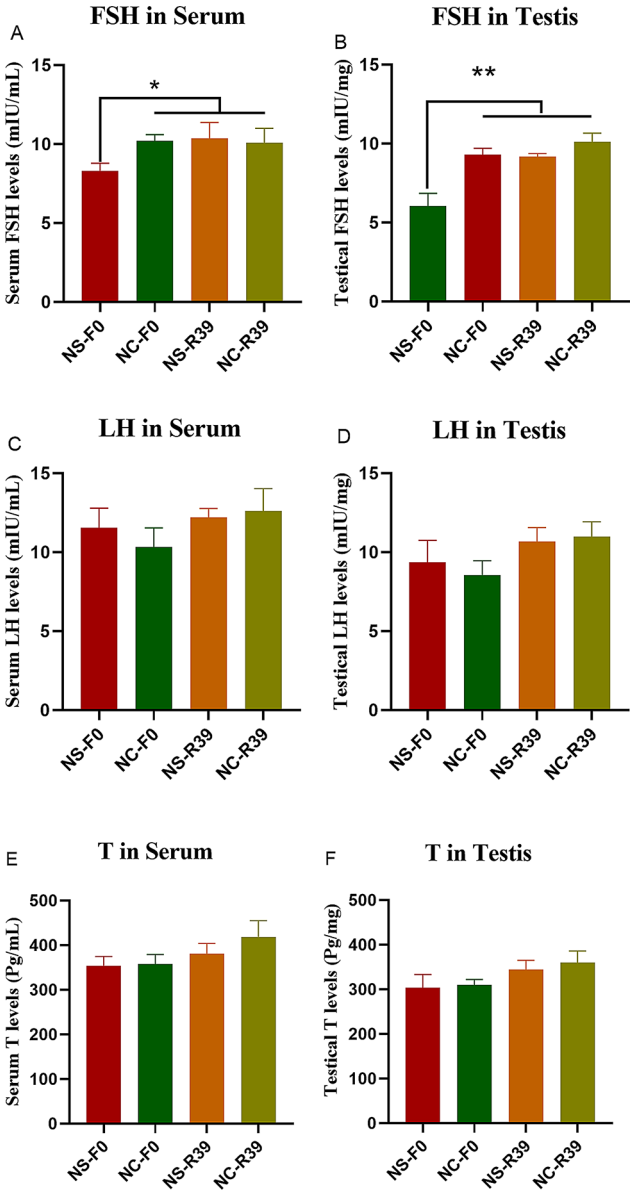


Fig. 4 Sex hormone content in testis and serum. (**A-B**) The levels of FSH in serum and testis of NS and NC roosters at F0 and R39 periods; (**C-D**) The levels of LH in serum and testis of NS and NC roosters at F0 and R39 periods; (**E-F**) The levels of T in serum and testis of NS and NC roosters at F0 and R39 periods. *$P < 0.05$; **$P < 0.01$

Table 4 Gene primer sequences

Gene	Primer sequence	Gene	Primer sequence
NAT10	F: GCCACAAACATTCGCTAC	SLC9A3R1	F: AGGTGAATGGGATGTCCGTG
	R: TACTACAATTCCACCCCC		R: GGCTCTCCACCGTTTTCCTT
SULT1E1	F: CATGGATGGGAAAGTGGCCT	LY6E	F: CATCTGCTTTTCGTGCTCGG
	R: GTCCATCAGTTCAGTGGGCA		R: ACTCCGACGTACGTTGTCAC
ALDH1A1	F: GCAGCAGGGAAGACCAATCT		
	R: TCCGGCGAACAAACTCATCA		

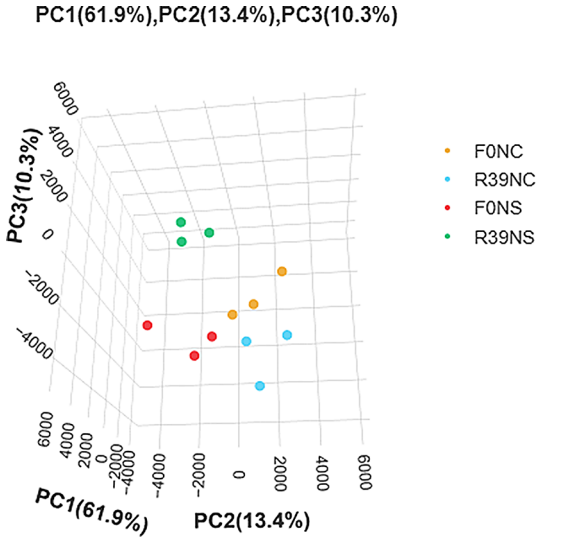


Fig. 5 Three-dimensional principal component analysis (PCA) of gene expression data across different samples at different stages. The numbers within parentheses represent the variance explained by each principal component. Red, yellow, green, and blue dots represent NS and NC group samples from the F0 and R39 periods, respectively

impair testis growth, sperm production, and reproductive functions. In this study, the inability of NS roosters to produce semen before induced molting may result from abnormal testicular function triggered by the damage in Sertoli cells. The testicular somatic microenvironment plays a role analogous to soil for sprouting seeds, supplying essential nutrients to germ cells. Sertoli cells provide structural and functional support to germ cells. These testicular functions are dependent on the hypothalamic-pituitary-testicular (HPT) axis [43]. The pulsatile secretion of gonadotropin-releasing hormone (GnRH) stimulates the release of FSH and LH from the anterior pituitary. Hyposecretion of LH and FSH compromises normal spermatogenesis and production of testosterone, and is responsible for infertility [44, 45]. FSH enhances Sertoli cell function and spermatogenesis [46]. Similarly, our results showed that FSH levels in NS roosters were significantly lower than those in NC roosters at F0. On the 39th day of feed resuming after induced molting, the spermatogenic cells and Sertoli cells in the seminiferous tubule of NS roosters distributed became denser, and the epithelial structure of the seminiferous tubules in the testis restored to completeness. Concurrently, serum and testicular FSH levels were significantly elevated, and NS roosters resumed semen production. These results

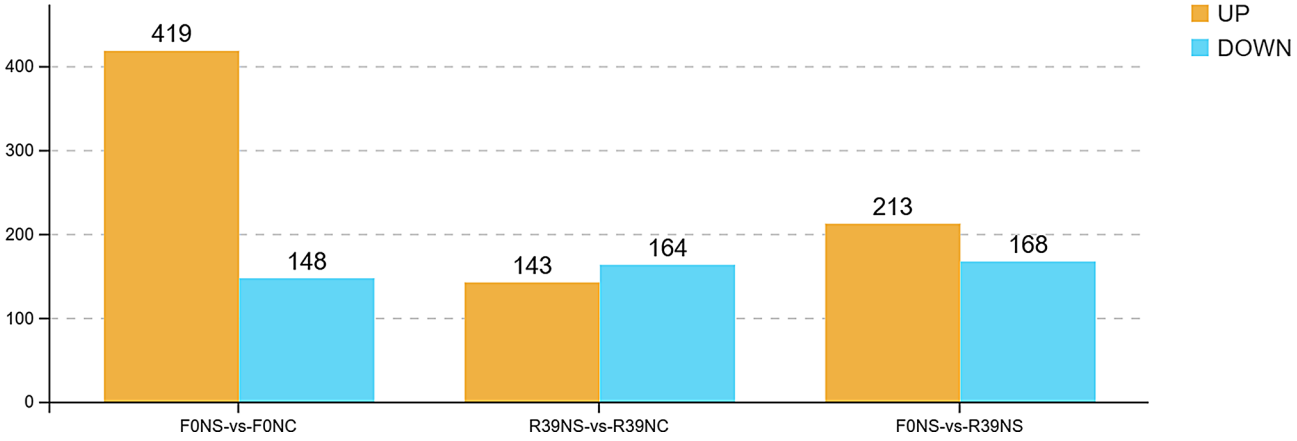


Fig. 6 Differentially expressed genes in ns and nc roosters in different grouping scheme. The number of differentially expressed genes at F0 and R39, the X-axis represents 3 grouping scheme: NS vs. NC at F0; NS vs. NC at R39; NS at F0 vs. NS at R39. Yellow represents transcripts that were significantly upregulated, and blue indicates that those transcripts were significantly downregulated. The parameters FDR < 0.05 and |Log FC| ≥ 1 were used as the thresholds to judge the significance of gene expression differences

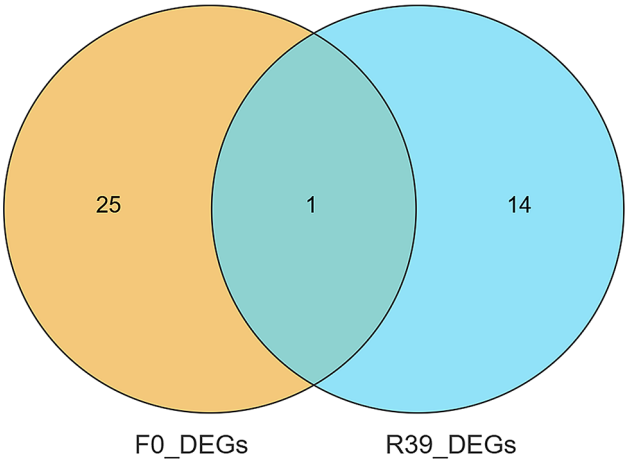
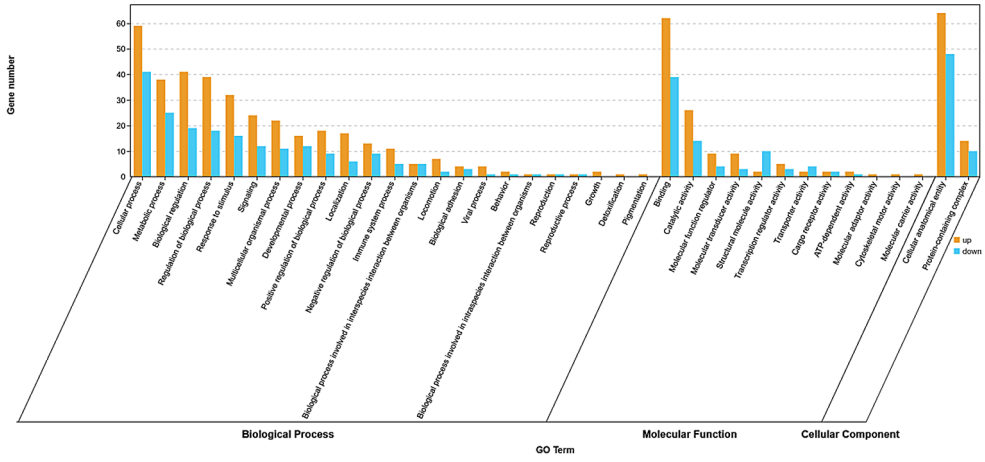


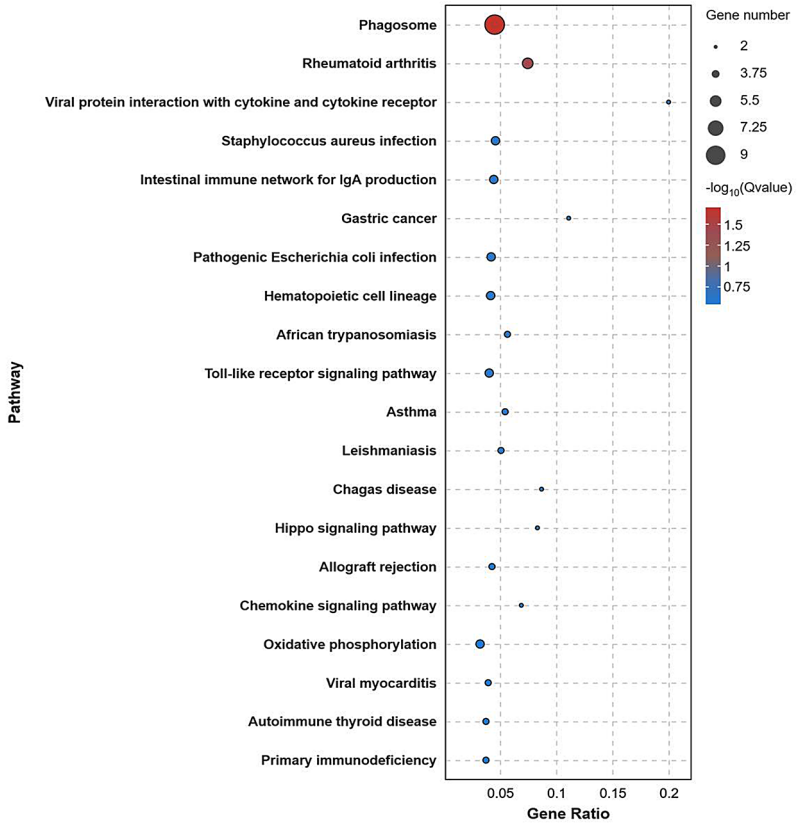
Fig. 7 Differentially expressed genes in different stages. Yellow represents DEGs that were specifically different at F0, and blue indicates that DEGs that were specifically different at R39. The parameters Q-value < 0.05 and |Log FC| ≥ 3 were used as the thresholds to judge the significance of gene expression differences in NS vs. NC group at F0 and R39

suggest that the cause of the inability of NS roosters to produce semen may be linked to primary or secondary testicular failure. The improvement in productive performance is likely due to significantly increased levels of FSH steroid hormones following induced molting, which restored the functions of spermatogenic and Sertoli cells in the seminiferous tubules of NS roosters.

What are the causes of testicular dysfunction in NS roosters? What causes the elevation of FSH levels in NS roosters after induced molting? In the past decade, several genetic, environmental, and lifestyle factors contributing to testicular failure have been identified. Nevertheless, 40% of cases remain idiopathic, with novel monogenic genes linked in the etiopathogenesis [15]. Another study demonstrated a close association between avian spermatogenesis and gene expression levels [17]. To further investigate the mechanisms underlying testicular physiological remodeling in NS roosters, we conducted RNA-seq analysis of testicular tissues. Comparing the DEGs between NS and NC roosters at F0 and R39 periods revealed 25 significant genes between the two groups at F0, while no significant differences were observed at R39, among these, the ALDH1A1 gene has been well-documented for its crucial role in spermatogenesis [47, 48]. ALDH1A1 is one of three retinal dehydrogenases that irreversibly oxidizes retinal to retinoic acid (RA) [49–51]. Inhibitors of ALDH1A1 adversely affect spermatogenesis [52]. Research indicates that RA is essential for the transition from undifferentiated spermatogonia A to differentiating spermatogonia A1, a necessary step for entering spermatogenesis [53]. RA signaling is crucial for inducing the differentiation of spermatogonial stem cells (SSCs) and initiating meiosis [54], which is directly associated with adequate spermatogenesis [55, 56]. In the absence of retinoic acid, undifferentiated spermatogonia never begin this progression [57]. Consistent with this, our results show that *ALDH1A1* expression in the testes of NS roosters was 16-fold lower than in NC roosters at F0; Concurrently, sertoli cells in the spermatic tubules were severely damaged, with only a few spermatogenic cells present. However, on day 39 after induced molting, *ALDH1A1* levels in the testes of NS roosters significantly increased, eliminating the significant difference between the two groups. This suggests that the *ALDH1A1* gene may play a role in the pathogenesis of NS roosters, regulating spermatogenesis. Furthermore, the *ALDH1A1* gene is most highly expressed in Sertoli cells within testicular tissues [58]. We speculate that the testicular dysfunction and impaired spermatogenesis in NS roosters may be due to insufficient expression of the *ALDH1A1* gene in the testes, disrupting RA synthesis and the



A



B

Fig. 8 GO terms and KEGG pathways enriched by genes with nonadditive expression. (**A**) DEGs GO annotation analysis at F0 and R39 in NS roosters, and the results were showed as Go and KEGG pathways. (**B**) Top 20 KEGG pathway enrichment of DEGs at F0 and R39 in NS roosters

development of Sertoli cells, thus hindering the progression from undifferentiated spermatogonia A to differentiating spermatogonia A1. Our subsequent study will utilize single-cell sequencing technology to identify the *ALDH1A1* gene and investigate its effects on SCs. Additionally, we will investigate the signals regulating RA biosynthesis in the reproductive system in future research. Moreover, we found that the DEGs in the testis tissues of NS roosters during the pre- and post-moult phase were significantly enriched in the phagosome pathway. The genes *ATP6VOD2,ATP6V1A,Ighm*, and *MHCY2B1* are involved in the pathway, and are associated with autophagy. Autophagy is considered as a novel biomarker of sperm quality, as any alterations in the expression

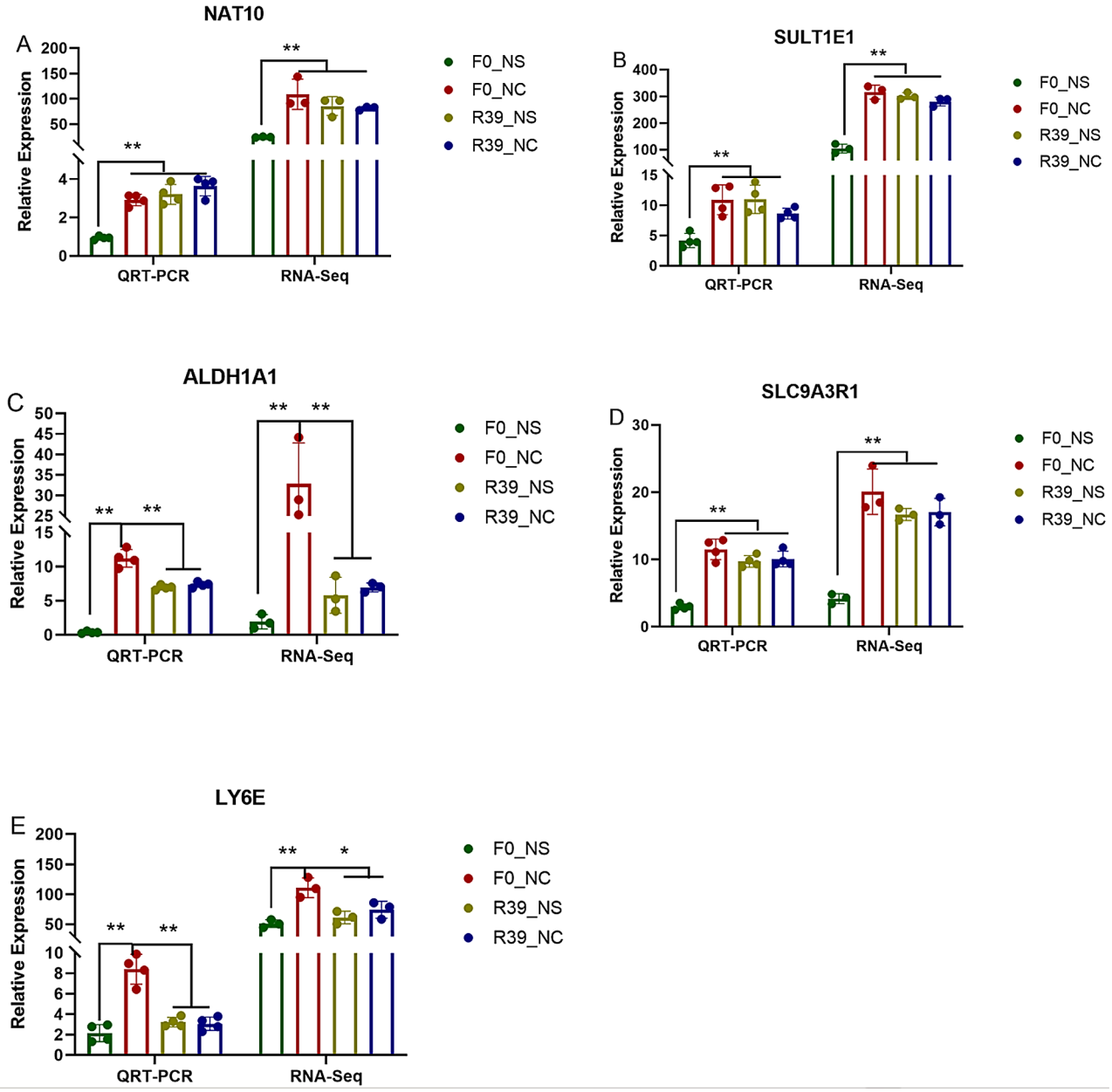


Fig. 9 Expression comparison of 5 genes by qRT-PCR and RNA-Seq in NS and NC roosters at F0 and R39 periods. (**A**) NAT10; (**B**) SULT1E1; (**C**) ALDH1A1; (**D**) SLC9A3R1; (**E**) LY6E. *$P < 0.05$; **$P < 0.01$

of related genes correlate with decreased sperm quality [59]. In response to nutrient deprivation or cellular stress signals, autophagy is initiated to maintain cellular homeostasis [60–62]. Increasing evidence suggests that fasting is an effective intervention for enhancing health, prolonging lifespan, slowing aging and improving reproductive performance [63–66]. Another study demonstrated that intermittent fasting can eliminate damaged organelles via autophagy in response to food deprivation, thereby promoting rejuvenation [67]. Furthermore, studies have demonstrated that *ATP6VOD2* is essential for efficient autophagosome-lysosome fusion and eliminating damaged organelles. Deletion of *ATP6VOD2* resulted in enhanced inflammasome activation [68]. Similarly, our results indicate that autophagy plays a crucial role in the recovery of semen production in NS roosters. After induced molting, the autophagy pathway is significantly activated in the testes of NS roosters. The main autophagic activities in SCs include the degradation of unnecessary components in seminiferous tubules, regulation of normal cytoskeletal organization, and adjustment of sex steroid biosynthesis [69]. Therefore, we hypothesize that the possible reason for the recovery of production performance in NS roosters was that roosters fasted

for 15 days, which allowed the maximum time to eliminate the damaged organelles and repaired the damaged SCs within the seminiferous tubules of the testes in NS roosters through autophagy. Upon resuming feeding, the nutrients of germline cells were restored and satisfied, and the neurological and endocrine functions were preferentially reestablished. Autophagy occured in SCs significantly increased the level of sex steroid hormone FSH in NS roosters, remodeled testicular function, thereby restored semen production.

Conclusion

In this study, we successfully established a physiological remodeling model of rooster testes by inducing molting, enabling NS roosters, which initially produced no semen, to resume semen production. Transcriptome sequencing analysis of the testes at F0 and R39 revealed a close association between the *ALDH1A1* gene and the incidence of NS roosters. Additionally, we found that the regulation of the autophagy pathway was closely related to spermatogenesis, facilitating physiological remodeling in the testes of NS roosters and restoring semen production. We hypothesized that the recovery of reproductive performance is likely due to the roosters fasting for 15 days, allowing maximum time to degrade unnecessary components in seminiferous tubules, regulate normal cytoskeletal organization, and adjust the biosynthesis of sex steroid hormone FSH through the regulation of autophagy relative genes, such as *ATP6VOD2,ATP6V1A,Ighm* and *MHCY2B1*. The verification of these findings will be the focus of our future research. This study is the first conducted in poultry, extending the lifespan of roosters, and offering new insights for reducing the economic cost of breeding. Additionally, this research has significant implications for protecting poultry germplasm resources and accelerating the improvement of local chicken breeds.

Abbreviations

IM	Induced molting
NS	No semen
NC	Negative control
R30	30 d after recovery feeding
R39	39 d after recovery feeding
F0	One day before fasting
SCs	Sertoli cells
FSH	Follicle-stimulating hormone
piRNAs	PIWI-interacting RNAs
H&E	Hematoxylin and eosin
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
DEGs	Differentially expressed genes
ELISA	Enzyme-linked immunosorbent assay
LH	Luteinizing hormone
TE	Testosterone
FSH	Follicle-stimulating hormone
PCA	Principal component analysis
HPT	Hypothalamic-pituitary-testicular
RA	Retinoic acid
SSCs	Spermatogonial stem cells

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Author contributions
W.Y.X., W.T.L., G.R.S. and X.T.K. design the experiment. M.Z.X., T.Q.Z., Y.H.H., and W.J.L. collected samples. W.Y.X. and M.Z.X. performed the experiment. W.Y.X. analysed the data and drafted the manuscript. H.Y.L. and G.R.S. provided insights into the manuscript. X.T.K. and W.T.L. conceptualised the paper, contributing to the writing. All the authors read and approved the final manuscript.

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Data availability
All the RNA-seq data associated with this article are available in the SRA database with the accession number of PRJNA1175412 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1175412).

Declarations

Ethics approval and consent to participate
The handling of experimental chickens adhered strictly to humane euthanasia procedures outlined in accordance with the Laboratory Animal Guidelines for Euthanasia (T/CALAS 31-2017; Chinese Association for Laboratory Animal Sciences). Chickens were euthanasia with artificial cervical dislocation before bled. All efforts were made to minimize animal suffering. All protocols concerning animals adhered to the regulations outlined by the Ministry of Agriculture of China and obtained consent from the ethics committee at Henan Agricultural University (Zhengzhou, Henan, China), under approval number 11–0085.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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