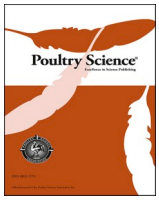
104 (2025) 104994

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj



Full-Length Article

Genome-wide association analysis identified the involvement of *MRPS22* in the regulation of Muscovy duck broodiness

Hailiang Yu [a,b], Shiqi Yu [a], Wanli Yang [a], Weihuang Lin [a], Xiaowei Yang [a], Xiao Wang [a], Cheng Zhang [a,b], Liping Guo [a,b], Xingyong Chen [a,b,*]

[a] College of Animal Science and Technology, Anhui Agricultural University, No. 130 Changjiang West Road, Hefei, 230036, PR China
[b] Anhui Province Key Laboratory of Local Livestock and Poultry Genetic Resource Conservation and Bio-breeding, Anhui Agricultural University, No. 130 Changjiang West Road, Hefei, 230036, PR China

ARTICLE INFO

Keywords:
Muscovy duck
Broodiness
Whole genome resequencing
SNPs
MRPS22

ABSTRACT

Compared to Chinese domesticated duck, Muscovy duck is the only species that retains the broodiness characteristic. Strong broodiness seriously limits its reproductive performance. In order to explore the molecular mechanisms that regulate broody behavior in Muscovy duck, this study used whole genome resequencing (WGRS) to obtain genomic variation sites of Muscovy ducks and conducted association analysis with broody traits. A total of 6,131,623 Single Nucleotide Polymorphisms (SNPs)were obtained from 295 female Muscovy ducks. After genome wide association study (GWAS) with the total broody days, average broody days, broody frequency and the first day of broodiness, 39, 130, 29 and 138 significant SNPs were obtained, respectively. The key genes annotated to these SNPs loci include *NCOA6, MRPS22, SCAP, CRY2, CK1δ* and *EZH1*, which could be candidate genes regulating the broodiness of Muscovy ducks. Functional analysis showed that over expression of *MRPS22* upregulated the expression of *CYP19A1* to promote the synthesis of intracellular estradiol, and downregulated the expression of *CYP11A1* and *3β-HSD* to inhibit the synthesis of progesterone to regulate broodiness of Muscovy ducks. The genetic polymorphism results showed that *MRPS22*: g.19000662G>A was significantly associated with average broody days. The average broody days in GA mutant ducks increased by an average of 2.23 days compared to wild GG type, which can be used for molecular marker for broody behavior selection. In conclusion, our study revealed *MRPS22* regulated the broody performance by affecting the synthesis of estradiol and progesterone, and g.19000662G>A in *MRPS22* was significantly associated with average broody days of Muscovy duck.

Introduction

Muscovy duck is an excellent meat-type duck with high lean meat rate and tender meat, which is favored by world-wide consumers. However, the strong broodiness reduced egg-laying and reproductive performance of Muscovy duck, which severely limited its industrialization (Wu et al., 2014; Ye et al., 2019). The broody behavior of poultry is influenced by many factors. Geng et al. (2014) showed that the broodiness rate of native laying hens under 16 and 18 h light was significantly lower than 14 h and suggested that the photoperiod (12L:2D:4L:6D) is optimal for the birds' performance to give the lowest broodiness rate. The broody behavior in poultry is mainly regulated by the hypothalamus-pituitary-gonadal (HPG) axis and a variety of endocrine hormones (Shen et al., 2012). Prolactin (PRL) is the most direct hormone that causes broody behavior, and females begin to exhibit broody behavior when PRL concentration is elevated in serum (Sharp et al., 1988; Proudman and Opel, 1981). Gonadotropin-releasing hormone (GnRH) binds to pituitary receptors to regulate the synthesis of PRL, thereby participating in broodiness regulation (Chi et al., 1993; Wu et al., 2015). High serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) promote egg production in poultry, and lower concentrations may lead to broody behavior (Liu et al., 2015; Cogger et al., 1979).

In addition to the above factors, avian broodiness is also mainly

Section: genetics and molecular biology.

* Corresponding author at: Anhui Province Key Laboratory of Local Livestock and Poultry Genetic Resource Conservation and Bio-breeding, Anhui Agricultural University, No. 130 Changjiang West Road, Hefei, 230036, PR China.
E-mail address: chenxingyong@ahau.edu.cn (X. Chen).

https://doi.org/10.1016/j.psj.2025.104994
Received 4 November 2024; Accepted 5 March 2025
Available online 6 March 2025

regulated by individual genetics (Romanov et al., 2002, 1999). PRL and prolactin receptor (PRLR) genes have been reported to be involved in the regulation of broody process in poultry (Jiang et al., 2005). Luan et al. (2014) conducted the gene profiling on hypothalamic tissues of laying period and ceased period Huoyan geese and showed that *AdipoR2, Nrg1* and *NCAM1* genes associated with GnRH secretion may play important roles in regulating broody behavior in goose. Moreover, up-regulation of *LC3, Beclin1*, and *Atg9* gene expression increases autophagy in granulosa cells, thereby affecting the goose broodiness (Yu et al., 2016). These work help reveal the molecular mechanisms underlying the occurrence and maintenance of broody behavior. In present study, we collected phenotypic data of broody traits from 295 Muscovy ducks during the entire laying cycle, and used genome-wide association analysis to identify genetic loci significantly associated with broody traits. Finally, we revealed the functions of the candidate genes in follicle granulosa cells. The purpose of this study is to provide theoretical support for genetic improvement and breeding of broodiness in Muscovy ducks.

Materials and methods

Ethics statement

All animal experimental procedures in this study were approved by the Animal welfare committee of Anhui Agricultural University with the assurance number SYDW-P20210823021.

Animals

A total of 295 female Muscovy ducks (28 weeks old) with similar body weight were provided by Yongqiang Agricultural Science and Technology Stock Co., Ltd., (Anqing, China). The ducks were raised in a semi-open duck house with a combination of mechanical and natural ventilation and thick bedding. Ducks were separated by small pens, with 1 male and 5 females per pen. All the ducks were free to feed and water, and provided 16-18 h of light during laying (28 weeks -56 weeks). The nutritional levels of ducks were met by the National Research Council (NRC).

Broody traits records

When the duck without egg laying for 5consecutive days, it is considered for broodiness. It is also judged by the broody behavior shown as loose and disordered feather, spread tail feather and difficult to drive away. The broody traits, including the total broody days (TBD), the average broody days (ABD), broody frequency (BF), and the first day of broodiness (FBD), were recorded by calculating days without egg laying and behavioral performance.

Blood sample collection and DNA extraction

At 51 weeks of age, the blood samples were collected from Muscovy ducks by venipuncture, and genomic DNA was extracted by phenol-chloroform method. The concentration and purity of genomic DNA were detected by NanoDrop 2000 spectrophotometer (Thermo Fisher, USA), and the integrity was detected by agarose gel electrophoresis (General Electric, USA).

Whole-genome resequencing

After the DNA test was qualified, all the samples were sequenced using Illumina NovaSeq 6000 PE150 platform to generate 150 bp paired-end fragments with an average sequencing depth of 10X. The sequencing was performed by Novogene Technology Co., Ltd. (Beijing, China).

Genomic alignment and variant calling

The raw reads were quality-controlled and filtered using NGSQCToolkit v2.3.3 (Patel and Jain, 2012) software to remove reads with adapters and low quality. Then, the clean reads were mapped to the Muscovy duck reference genome (ASM1810499v1) included in the NCBI database using BWA v0.7.17 software (Li and Durbin, 2009). The mapping results were sorted to exclude duplicate reads using Samtools (v1.9) (Daneceketal et al., 2021). SNPs and InDels were called using the Haplotype Caller module in GATK (v3.8) (McKenna et al., 2010) and were filtered with the following parameters: QD (Quality by Depth) $<$ 2.0, FS (Fisher Strand) $>$ 60.0, MQ (root mean square of Mapping Quality) $<$ 40.0, MQRankSum $<$ -12.5, ReadPosRankSum $<$ -8.0 and SOR $>$ 3.0. VCFtools v0.1.17 software (Danecek et al., 2011) was used to perform quality control filtering on the SNP data to remove the influence of false positive SNPs. After processing, all SNPs obtained were used in the subsequent genome-wide association analysis.

Genome-wide association analysis

The univariate linear mixed model (LMM) in GEMMA v0.98.4 software was used to identify SNPs associated with broody traits in Muscovy ducks (Zhou and Stephens, 2012). The statistical model is listed as follows:

$$y = W\alpha + x\beta + \mu + \varepsilon$$

Where y is the vector of individual phenotypes, W is the covariance matrix, α represents a vector of corresponding effects comprising the intercept, x is the vector of marker genotype, β denotes the estimate of the marker/SNP additive effect, μ represents the vector of random effect; ε is the vector of error. Manhattan plots and Quantile-Quantile plots were generated using QQman software in the R language package. The ENSEMBL website (http://www.ensembl.org/biomart/martview/) was used to annotate SNPs that were highly correlated with broodiness in Muscovy duck and obtain adjacent coding genes.

Enrichment analysis of annotated genes

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed on the annotated genes using the KOBAS 3.0 database (Xie et al., 2011). For GO enrichment analysis: All annotated candidate genes were mapped to each term in the Gene Ontology database, and the number of genes enriched for each term was calculated, then the significance of the GO term was calculated using hypergeometric distribution test. For KEGG pathway analysis: the pathways enriched in annotated candidate genes were determined based on the KEGG background database, and the significance of the enriched pathways was calculated using the hypergeometric distribution test. $P < 0.05$ was considered statistically significant.

Isolation and culture of follicle granulosa cells from Muscovy duck

The pre-hierarchical follicles of Muscovy duck were collected for granulosa cell culture in vitro and the isolation process was referred to the previously described method (Yang et al., 2023). Cells were cultured in M199 complete medium (containing 10 % FBS, 1 % penicillin and streptomycin) in a 37 °C, 5 % CO_2 incubator.

Plasmid construction and RNA oligonucleotides

The construction of *MRPS22* gene overexpression and control vectors were designed and synthesized by GenePharma Biotechnology (Shanghai, China). Three small interfering RNA sequences targeting the *MRPS22* gene were designed using the website http://www.invivogen.com/index.php (Table S1). Granulosa cells were transfected with lipo2000 (Vazyme Biotech, China) and the samples were collected 48 h

after transfection to screen out the siRNA with the best interference efficiency for subsequent experiments.

Cell proliferation assay

The granulosa cells were seeded in 96-well plates and grown to 40 % confluence. The si-*MRPS22*-3, siNC, pcDNA3.1-*MRPS22*, and pcDNA3.1-NC were transfected to granulosa cells, respectively. 10 μL CCK-8 reagent (Biosharp, China) was added to each well at 0, 24, 48, and 72 h after transfection. The cells were incubated in a 37 °C, 5 % CO_2 incubator for 3 h, and the D value at 450 nm was detected using a microplate reader.

Cell apoptosis assay

The granulosa cells were seeded in 6-well plates and grown to 70 % confluence. The above vectors were transfected into granulosa cells, and samples were collected 48 h after transfection for cell apoptosis detection according to the instructions of the Annexin V-PE/7-ADD Apoptosis Detection Kit (Vazyme Biotech, China). Flow cytometry (FACSCaliburc, USA) was used to analyze cell apoptosis.

The detection of Cellular steroid hormone

The granulosa cells were seeded in 24-well plates. After 72 h of transfection, the supernatants from all the samples were collected into enzyme-free EP tubes and centrifuged at 1000 r/min for 3 min to remove the precipitate. After concentrating the supernatant using a rotary evaporator (Bio-Rad, USA), the concentrations of estradiol (E2) and progesterone (P4) were detected by ELISA (Keshun Biotech, Shanghai).

Complementary DNA (cDNA) synthesis and quantitative real-time PCR (qRT-PCR)

The total RNA of tissues and ovarian granulosa cells was extracted by Trizol method and the purity and concentration of RNA were detected by Nanodrop 2000 spectrophotometer (Thermo Fisher, USA). Reverse transcription reaction was performed using cDNA synthesis kit (Yisheng, China). qRT-PCR was performed using Hieff® qPCR SYBR Green Master Mix kit according to the manufacturer's instructions. The primers were synthesized by General Biotech (General, China) and the sequences are listed in Table S2.

Analysis of MRPS22 gene polymorphism

To identify the variant Loci on the exons of the *MRPS22* gene, we used direct PCR and restriction digestion methods to amplify and sequence. PCR primers (F: AAGAACTCCAGAGACAGATG, R: CAGACACAACCACTTCATTC) were designed using Primer Premier 6.0 software based on the *MRPS22* gene sequence published in the NCBI database. PCR amplification was then performed and the product was sequenced by Tsingke Biotech (Tsingke, China). Total volume of the PCR product digestion system was 10 μL, including 1 μL PCR product, 1 μL Buffer, 0.2 μL Hpy188III restriction endonuclease, and 7.8 μL ddH2O. The digestion reaction conditions were incubation at 37 °C overnight, heat inactivation at 60 °C for 1 h, and storage at 4 °C. The digestion effect was detected by 1 % agarose gel electrophoresis.

Statistical analysis

Shapiro-Wilk was performed to analyze the normal distribution of original data. Bonferroni multiple testing was used to determine genome-wide significance thresholds in association studies. One-way ANOVA analysis was used for the difference in tissue expression profiles, proliferation, apoptosis, and steroid hormone indicators of granulosa cells among different groups. The relative expression of mRNA was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The data were analyzed using SPSS 25.0 software and presented as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Results

The broodiness characteristics of Muscovy duck

The average broody days for 295 Muscovy ducks were 37 days, and the average broody frequency was 3 (Table 1). The different broody traits showed a wide range of variation from 40.7 % to 55.5 %, indicating rich genetic variation in Muscovy duck broodiness (Table 1). The broody traits were approximately normally distributed, indicating that broodiness are quantitative traits and the phenotypic data can be used for subsequent genome-wide association analysis (Fig. 1A). In addition, the correlation of phenotypic values was analyzed for the total broody days, average broody days, broody frequency and the first day of broodiness of 295 Muscovy ducks and significant correlations were observed between different broody traits (Fig. 1B).

Genome-wide association studies for broody traits in Muscovy ducks

The whole genome of 295 F7 Y2 strain female Muscovy ducks were re-sequenced, yielding a total of 12,263,999 SNPs, and 6,131,623 SNPs were available for analysis after quality control, alignment and filtering. After association analysis with the total broody days, average broody days, broody frequency and the first day of broodiness, 39, 130, 29 and 138 significant SNPs were obtained, respectively. Specifically, SNPs significantly associated with the total broody days were mainly distributed on chromosomes 5, 7 and 28, and six genes were annotated, including *EZH1, FNBP1, CNTNAP1* and *RAMP2* genes (Fig. 2A, Table S3); SNPs significantly associated with average broody days were distributed on chromosomes 1, 5, 7, 11, 24 and sex chromosomes, with nineteen genes annotated, including *MRPS22, NCOA6, SLCO3A1* and *CK1δ* (Fig. 2B, Table S4); SNPs significantly associated with broody frequency were distributed on chromosomes 2, 18, 22, and 28, and eight genes were annotated, including *ACAP3, FNBP1*, and *EZH1* (Fig. 2C, Table S5); The SNPs significantly associated with the first day of broodiness were mainly distributed on chromosomes 2, 18, 22 and 28, and twenty genes were annotated, including *SCAP, CD40, CRY2, CK1δ* and *KIF21B* (Fig. 2D, Table S6). The above genes may serve as candidate genes for breeding of broody traits in Muscovy ducks.

Functional enrichment analysis of candidate genes located in SNPs

To predict the potential functions of these genetic variation sites, the GO terms and KEGG pathway analysis were performed on 49 candidate genes annotated in the broody traits of 295 Muscovy ducks. The GO analysis results showed that a total of 15 terms were significantly enriched, mainly including epithelial tube formation, regulation of synaptic organization, vascular processes in the circulatory system, response to nutrients, and rhythmic processes (Fig. 3A). The KEGG pathway analysis showed that 6 pathways were significantly enriched ($P < 0.05$), including one carbon pool by folate, Notch signaling pathway, lysine degradation and metabolic pathways (Fig. 3B).

Table 1
Phenotypic analysis of broody traits in Muscovy ducks.

Traits	Range	Average	CV (%)
TBD	0–99	37	51.3
ABD	0–39	12	40.7
BF	0–8	3	50
FBD	5–39	9	55.5

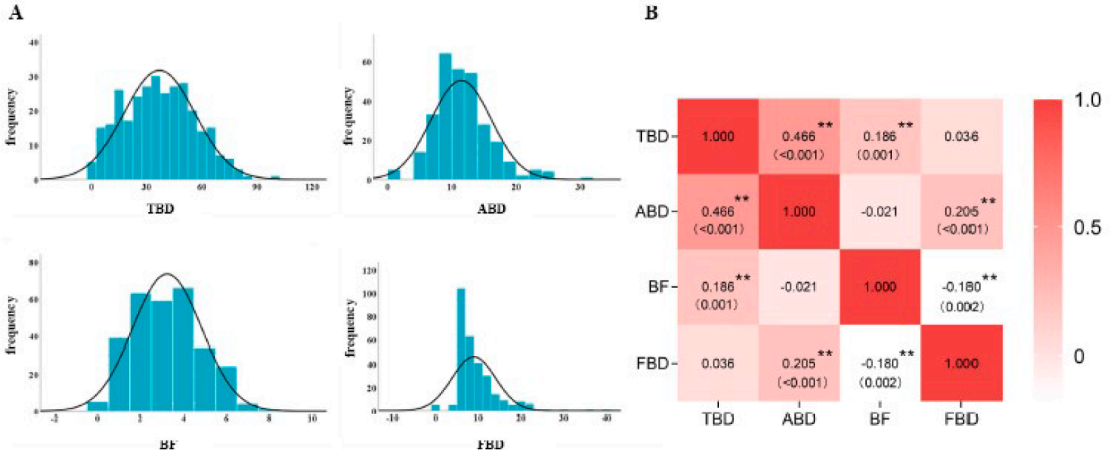


Fig. 1. Frequency distribution and correlation analysis of broody traits in 295 Muscovy ducks. (A) Frequency distribution of broody traits. (B) Pearson's Correlation coefficient among the four broody traits. The value in the box represents the Pearson's correlation coefficient between the any two broody traits. The red indicates appositive correlation, and the white shows a negative correlation. TBD: total broody days, ABD: average broody days, BF: broody frequency, FBD: The first day of broodiness.

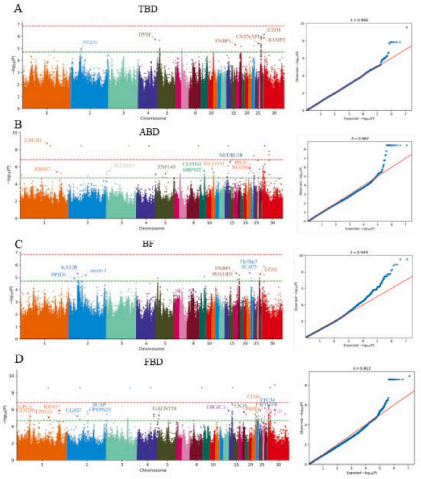


Fig. 2. The Manhattan and quantile-quantile (QQ) plots of genome-wide association analysis of broody traits. (A) Total broody days (TBD). (B) Average broody days (ABD). (C) Broody frequency (BF). (D) The first day of broodiness (FBD). For the Manhattan plot, the x-axis of each plot represents the respective positions on each chromosome of each SNP, and the y-axis displays the negative log10 of *P*-values for each SNP included in the GWAS. For the quantile-quantile plot, the x-axis shows the expected-log10-transformed *P*-values, and the y-axis represents the observed-log10-transformed *P*-values.

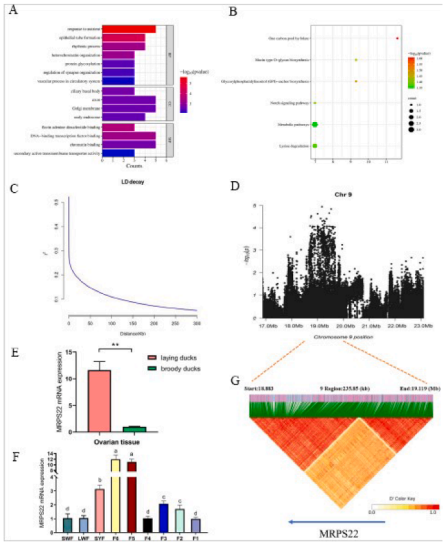


Fig. 3. The functional enrichment analysis of candidate genes related to broody traits and the linkage disequilibrium analysis of genetic loci. (A) GO terms analysis for candidate genes related to broody traits. The x-axis indicates the number of genes for each GO term; the y-axis corresponds to the GO terms. The color of the bar represents the *P*-values. (B) KEGG pathway analysis for candidate genes related to broody traits. The x-axis shows the gene ratio; the y-axis represents KEGG pathways. The dot size represents the number of genes enriched in the reference pathway, and the dot color represents the *P*-values. (C) The decay distance of the Muscovy duck population. (D) Regional plots for the loci ranging from 17.0 Mb to 23. 0 Mb associated with the average broody days. (E) Tissue expression level of *MRPS22* gene in the ovaries of laying and broody ducks. (F) Tissue expression level of *MRPS22* gene in different follicles. (G) The linkage disequilibrium analysis of genetic loci from 18.883 Mb to 19.119 Mb associated with the average broody days. * $P < 0.05$, ** $P < 0.01$. Abbreviations: SWF, small white follicle; LWF, large white follicle; SYF, small yellow follicle; F1, F1 follicle; F2, F2 follicle; F3, F3 follicle; F4, F4 follicle; F5, F5 follicle; F6, F6 follicle.

The linkage disequilibrium (LD) analysis of significant candidate regions

The population decay distance was calculated using PopLDdecay, indicating that the decay distance of the Muscovy duck population was 118 kb (Fig. 3C). According to the GWAS results, the average broody days had significant candidate regions on chromosomes 9 (Fig. 3D). Linkage disequilibrium analysis was then performed on 118 kb upstream and downstream of the key locus (g.19000662A>G) on duck chromosome 9 using Ldblockshow 1.4.2 (Fig. 3D). The g.19000662A>G locus was located in the same block as the downstream *MRPS22* gene, which suggests that strong linkage between this locus and the downstream *MRPS22* gene (Fig. 3G). *MRPS22* gene mutation causes premature ovarian failure in animals (França and Mendonca, 2022, Chen et al., 2018). Moreover, we examined the expression revel of *MRPS22* gene in the ovarian tissue of laying and broody Muscovy ducks, as well as its expression in different follicles. RT-qPCR results showed that the expression level of *MRPS22* in the ovarian tissue of laying ducks was significantly higher than that in brooding ducks ($P < 0.05$, Fig. 3E). In addition, the expression level of *MRPS22* gene in SYF (small yellow follicle), F6 (F6 follicle) and F5 (F5 follicle) was significantly higher than that in other follicles (Fig. 3F). Therefore, *MRPS22* was selected as a candidate gene related to broodiness in Muscovy ducks.

Effect of MRPS22 gene on proliferation of granulosa cells

To investigate the effect of *MRPS22* gene on granulosa cells, we constructed *MRPS22* interference and overexpression vectors. The vector transfection efficiency exceeded more than 60 % for the siNC, interference and overexpression vectors (Fig. 4A-D). qRT-PCR results showed that si-*MRPS22*-3 had the best interference effect (inhibition efficiency of about 70 %), which was lower than si-*MRPS22*-1 and si-*MRPS22*-2 (Fig. 4E), so si-*MRPS22*-3 was selected for subsequent experiments. The mRNA expression levels of *MRPS22* were significantly higher after transfection with pcDNA3.1-*MRPS22* than that in the control group (Fig. 4F).

MRPS22 gene interference and overexpression vectors were transfected to explore the effect on granulosa cell proliferation. CCK8 results showed that at 0, 24, 48, and 72 h of transfection, the OD value of the *MRPS22* interference group was not significantly different from that of the control group and siNC group ($P > 0.05$, Fig. 4G), and similar results were observed in the *MRPS22* overexpression group ($P > 0.05$, Fig. 4H). Then, qRT-qPCR was used to detect the expression levels of cell proliferation-related genes *CCND2, FOXM*, and *CDK2*. The results showed that proliferation-related genes were not significantly different between the groups ($P > 0.05$, Fig. 4I-K). Therefore, the above results indicate that the *MRPS22* gene does not affect granulosa cell proliferation.

Effect of MRPS22 gene on apoptosis of granulosa cells

Apoptosis of granulosa cells is the direct cause of follicular atresia in Muscovy duck (Tao et al., 2024). The Annexin V-PE/7-ADD staining kit were used to detect the effects of transfection of *MRPS22* overexpression and interference vectors on granulosa cell apoptosis. Flowjo analysis showed that the percentage of apoptotic cells in the *MRPS22* knockdown group was not significantly different from that in the siNC group ($P > 0.05$, Fig. 5A and 5B). The apoptotic cell percentage of pcDNA3.1-*MRPS22* groups was not significantly different from that in the pcDNA3.1-NC group ($P > 0.05$, Fig. 5C and 5D). The qRT-PCR results also found that there was no significant difference in the expression of apoptosis-related genes *Caspase3, Caspase9* and *Bcl-2* genes in each group ($P > 0.05$, Fig. 5E-G). These data suggest that*MRPS22* gene does not affect the apoptosis of granulosa cells.

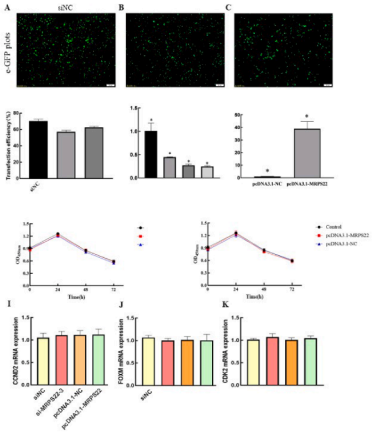


Fig. 4. Effect of *MRPS22* gene on proliferation of granulosa cells. (A-C) The e-GFP plots of cells in siNC transfection group (A), si-*MRPS22*-3 group (B) and pcDNA3.1-*MRPS22* (C). (D) Transfection efficiency plot of vectors. (E-F) Efficiency detection of interference (E) and overexpression constructs (F). (G-H) Cell growth was measured following the transfection of si-*MRPS22*-3 (G) and pcDNA3.1-*MRPS22* group (H) in granulosa cell. (I-K) Expression levels of cell proliferation-related genes *CCND2* (I), *FOXM* (J), and *CDK2* (K) after transfection with si-*MRPS22*-3 and pcDNA3.1-*MRPS22* constructs in granulosa cells.

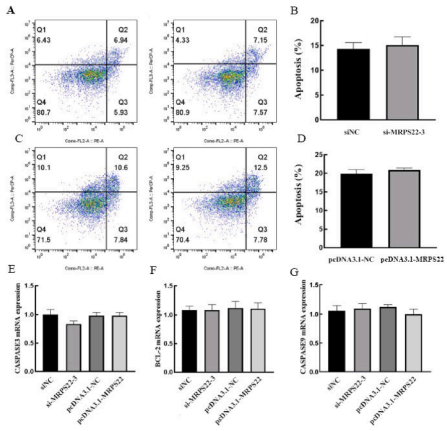


Fig. 5. Effect of *MRPS22* gene on apoptosis of granulosa cells. (A-B) The cell apoptosis rate was detected at 48 h after transfection with siNC and si-*MRPS22*-3 by the flow cytometry assay. (C-D) The cell apoptosis rate was detected at 48 h after transfection with pcDNA3.1-NC and pcDNA3.1-*MRPS22* by the flow cytometry assay. (E-G) The relative mRNA expression of *Caspase3, Bcl2*, and *Caspase9* at 48 h after transfection with si-*MRPS22*-3 and pcDNA3.1-*MRPS22* constructs in granulosa cells.

Effect of MRPS22 gene on steroid hormone secretion in granulosa cells

After 72 h of plasmid transfection in granulosa cells, the supernatant was collected to detect the expression levels of estradiol (E2) and progesterone (P4) in granulosa cells using ELISA. The intracellular E2 level in the si-*MRPS22*-3 group was significantly lower than that in the siNC group (Fig. 6A, $P < 0.05$), while the P4 content was significantly higher than that in the siNC group (Fig. 6B, $P < 0.05$). Knockdown of *MRPS22* also significantly suppressed E2/P4 levels in granulocyte supernatants (Fig. 6C, $P < 0.05$). In addition, we also detected the changes in the expression levels of steroid-related genes *StAR, 3β-HSD, CYP11A1*, and *CYP19A1*. qRT-PCR results showed that Knockdown of *MRPS22* significantly decreased *CYP19A1* expression levels (Fig. 6D, $P < 0.05$) and significantly increased *3β-HSD* and *CYP11A1* expression levels (Fig. 6E and 6G, $P < 0.05$), whereas there was no significant change in *StAR* expression levels (Fig. 6F, $P > 0.05$).

In contrast, pcDNA3.1-*MRPS22* significantly promoted the intracellular E2 level (Fig. 6A, $P < 0.05$) and significantly reduced the P4 content compared with the pcDNA3.1-NC group (Fig. 6B, $P < 0.05$). pcDNA3.1-*MRPS22* also significantly promoted E2/P4 levels (Fig. 6C, $P < 0.05$). qRT-PCR results showed that *MRPS22* overexpression significantly promoted the expression level of *CYP19A1* (Fig. 6D, $P < 0.05$) and significantly decreased the expression level of *3β-HSD* and *CYP11A1* (Fig. 6E and 6G, $P < 0.05$). Taken together, these data suggest that *MRPS22* promotes granulosa cell estradiol synthesis and inhibits progesterone synthesis.

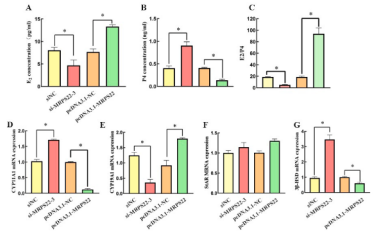


Fig. 6. Effect of *MRPS22* gene on steroid hormone secretion in granulosa cells. (A-C) The expression levels of E2 (A), P4 (B) and E2/P4 (C) in granulosa cells after transfection with si-*MRPS22*-3 and pcDNA3.1-*MRPS22* constructs. (D-G) The relative mRNA expression of *CYP11A1* (D), *CYP19A1*(E), *StAR*(F) and *3β-HSD* (G) after transfection with si-*MRPS22*-3 and pcDNA3.1-*MRPS22* constructs in granulosa cells. * $P < 0.05$, ** $P < 0.01$.

The polymorphism analysis of MRPS22 gene

The key locus (g.19000662A>G) on chromosome 9 of Muscovy duck was associated with the broody trait of Muscovy duck and annotated to the *MRPS22* gene. Functional experiments confirmed that *MRPS22* was involved in the regulation of Muscovy duck broodiness. Therefore, this study continued to verify the existence and polymorphism of this locus in the *MRPS22* gene. Restriction endonuclease typing and Sanger sequencing were used to confirm the polymorphism of the *MRPS22* gene in Muscovy duck population. The amplified product was cut into two bands of 64 bp and 196 bp after restriction endonuclease digestion (Fig. 7D). Two bands were homozygous wild GG type, with 225 individuals and a genotype frequency of 0.76; one band was homozygous mutation AA, with 4 AA type individual and a genotype frequency of 0.02; three bands were heterozygous mutation GA type, with 65 GA type individuals and a genotype frequency of 0.22. The A gene frequency was 0.13, and the G gene frequency was 0.87. Moreover, Sanger sequencing was used to verify the restriction digestion results (Fig. 7A-C), confirming the presence of polymorphism at this locus. The average broody days of Muscovy ducks of each genotype was recorded and analyzed by SPSS software. The results showed that the average broody days of the AA type (19.25 days) and GA type (13.06 days) Muscovy ducks was significantly higher than the GG type (10.83 days) (Fig. 7E, $P < 0.05$).

Discussion

Broodiness is a common characteristic of most indigenous poultry and is associated with frequent nest occupancy, turning and retrieval of eggs, aggressive or defensive behaviors, characteristic clucking, and cessation of egg production (Jiang et al., 2010). Differences in broodiness characteristics exist in different avain species. The average duration of broodiness in Chinese indigenous chickens is approximately 20 days (Jiang et al., 2005). Studies have shown that the broody duration of the Zhedong White Goose, Sichuan White Goose and Carlos Goose was more than 30 days. Among them, the average broody duration of the Sichuan White Goose (49 days) was significantly higher than that of the Zhedong White Goose (39.7 days) and the Carlos Goose (41.7 days) (Yao et al., 2019). In addition, the broody proportion (100 %) and frequency (2 times) of the Zhedong White Goose were significantly higher than those of the Sichuan White Goose (33.33 %, 1 time) and the Carlos Goose (19.4 %, 1 time) (Yao et al., 2019). Muscovy duck is the only domesticated duck species that retains the broody behavior, and strong broodiness is the main reason for reduced reproductive capacity (Wu et al., 2021). In present study, we found that the average broody frequency of Muscovy ducks was 3 times in a laying cycle, and the longest broody period was 99 days, which seriously affected the reproductive performance of Muscovy ducks.

Yuan et al. (2015) used an F2 hens from White Leghorn and Dongxiang chickens reciprocal cross in a genome-wide association analysis to detect genetic variants and candidate genes associated with daily feed intake and efficiency, showing that 20 SNPs were significantly correlated with residual feed intake, 17 SNPs were correlated with feeding efficiency, and the *CAB39L* gene was revealed as a candidate gene affecting chicken feed intake. Wang et al. (2023) estimated the genetic parameters of 399 purebred Laiwu black chickens by GWAS and found that 421 SNPs were significantly associated with chicken egg-laying performance, and *NELL2, SMYD9, SPTLC2, SMYD3* and *PLCL1* were candidate genes affecting the egg-laying traits. In domestic ducks, the *THSD1, SLC6A4* and *DGAT2* were identified as important candidate marker loci associated with the neck ring of the Chinese Nonghua mallard, which provided a reference for the genetic mechanism of duck coat color (Wang et al., 2023). Another study revealed genomic regions associated with red duck plumage by GWAS, and results showed that *ESRRG* and *SPATA5* were significantly associated with red plumage, while *GMDS, PDIA6* and *ODC1* were significantly associated with brown duck plumage (Zhang et al., 2024). However, the polymorphic loci and gene functions related to broody traits in Muscovy ducks still remains unclear. In present study, we used whole genome resequencing to obtain the genome variant loci of Muscovy ducks, and screened 336 SNPs affecting broody traits through association analysis, and annotated a total of 49 candidate genes, including *NCOA6, MRPS22, SCAP, CRY2, CK1δ* and *EZH1*genes, which provide the target for molecular marker-assisted selection for broody traits.

Based on the association analysis between WGRS and broody traits, the Manhattan plot showed a significant correlation between *MRPS22* and broody trait. Moreover, Studies have shown that deletion of *MRPS22* in Drosophila germ cells results in decreased cell viability, slowed ovarian development and sterility (Chen et al., 2018). In the review of the genetic mechanisms of primary ovarian insufficiency, França and Mendonca. (2022) showed that the *MRPS22* gene contributes to premature ovarian failure by causing mitochondrial dysfunction. Recent studies have shown that this may be due to the reduced ATP production in *MRPS22* mutants, which impairs mitochondrial function and causes severe embryonic developmental delays (Cheong et al., 2020). Therefore, this study selected *MRPS22* as a candidate gene for the regulation of broodiness in Muscovy ducks for further research.

We constructed *MRPS22* gene overexpression and interference vectors to transfect granulosa cells for functional assays, and the results showed that *MRPS22* significantly inhibited the expression of progesterone synthase encoding genes *CYP11A1* and *3β-HSD*, thereby inhibiting the secretion of P4 in Muscovy duck ovarian granulosa cells. The ovaries regulate the reproductive system of female birds and promote the production of ovarian follicles by secreting hormones. E2 and P4 are key hormones that promote the continued development of follicles and are widely expressed in the ovaries and oviducts (Taraborrelli, 2015). P4 in Muscovy ducks is mainly synthesized in the primary follicles, promoting protease and collagenase to decompose follicle wall cells and promote ovulation (Xiao et al., 2011). High doses of P4 feedback

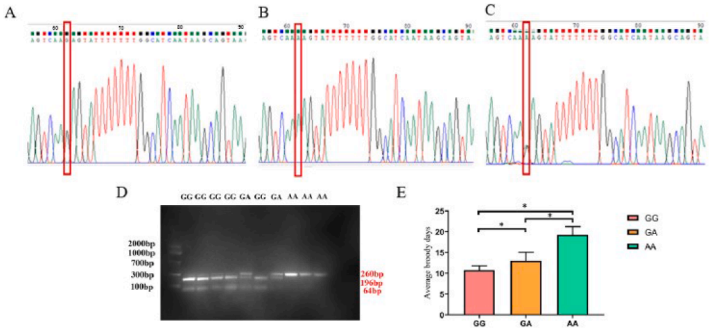


Fig. 7. The polymorphism analysis of *MRPS22* gene. (A-C) Sequencing results of wild-type GG (A), mutant-type AA (B), mutant-type GA (C). (D) The enzyme digestion results of g.19000662A>G loci in *MRPS22*. (E) The average broody days for different genotypes of g.19000662A>G loci in *MRPS22*. * $P < 0.05$, ** $P < 0.01$.

regulate the hypothalamus and inhibit follicular development and ovulation (Nakada et al., 1994). In addition, we also found that *MRPS22* significantly promoted the expression of *CYP19A1*, a gene encoding estradiol synthase, to promote estradiol secretion from ovarian granulosa cells. E2 is a steroid hormone that regulates sequence of ovarian follicle growth and maturation. E2 is produced by granulosa cells of the developing antral follicles in response to FSH to promote follicle growth and maturation (Chauvin et al., 2022). It has been shown that E2 induces dominant follicular atresia by decreasing granulosa viability and steroid secretion (Hutz et al., 1990). Overall, our study suggests that *MRPS22* is involved in the regulation of broodiness in Muscovy ducks by promoting estradiol synthesis in granulosa cells and inhibiting progesterone synthesis.

Broodiness is a polygenic control trait with low heritability. The G+123A and C+1107T in chicken *DRD1* genes were significantly associated with the broody frequency of Ningdu Sanhuang chickens, in which the broody frequency of GG type (51.69 %) was significantly higher than that of AA type (29.03 %), and the CC type (51.61 %) was significantly higher than that of TT type (15.38 %) (Xu, et al. 2010). Jiang et al. (2005) investigated the association between the polymorphism of *PRL* and *PRLR* genes and the broody characteristics of blue-shelled chickens and found that a 24 bp insertion/deletion in the promoter region of the *PRLpro2* gene significantly affected the broodiness of blue-shelled chickens. Another study reported the C+338T in chicken *VIP* gene was associated with duration of broodiness (Zhou, et al. 2010). In present study, we found that the *MRPS22* gene g.19000662G>A was significantly associated with the average broody duration of Muscovy ducks, and the AA genotype duck (19.25 days) was significantly higher than that of the GA type (13.06 days) and the GG type (10.83 days). The strong broodiness of AA type will be continuously eliminated in conventional breeding, resulting in a significant decrease in the genotype frequency. Our study showed that g.19000662A>G could be a candidate locus for molecular marker-assisted selection of broody traits in Muscovy ducks.

Conclusion

A total of 336 SNPs were identified to be significantly associated with broody traits in Muscovy ducks. The *NCOA6, MRPS22, CRY2, CK1δ, EZH1* and *SCAP* were identified to be candidate genes regulating the broodiness of Muscovy ducks. *MRPS22* regulates the broody performance through promoting the synthesis of intracellular estradiol and inhibit the synthesis of progesterone of Muscovy duck. Key genetic loci (g.19000662G>A in *MRPS22*) was revealed to be significantly associated with average broody days.

Disclosures

The authors declare no conflicts of interest.

Acknowledgements

This work was financially supported by the Key Research and Development Program of Anhui Province (2023z04020002) and the Science and Technology Major Project of Anhui Province (202203a06020008).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104994.

References

Chi, L., Zhou, W., Prikhozhan, A., Flanagan, C., Davidson, J.S., Golembo, M., Illing, N., Millar, R.P., Sealfon, S.C., 1993. Cloning and characterization of the human GnRH receptor. Mol. Cell. Endocrinol. 91, R1–R6. https://doi.org/10.1016/0303-7207(93)90278-r.

Cogger, E.A., Burke, W.H., Ogren, L.A., 1979. Serum luteinizing hormone, progesterone, and estradiol levels in relation to broodiness in the turkey (Meleagris gallapavo). Poult. Sci. 58, 1355–1360. https://doi.org/10.3382/ps.0581355.

Chauvin, S., Cohen-Tannoudji, J., Guigon, C.J., 2022. Estradiol signaling at the heart of folliculogenesis: its potential deregulation in human ovarian pathologies. Int. J. Mol. Sci. 23, 512. https://doi.org/10.3390/ijms23010512.

Chen, A.L., Tiosano, D., Guran, T., Baris, H.N., Bayram, Y., Mory, A., Shapiro-Kulnane, L., Hodges, C.A., Akdemir, Z.C., Turan, S., Jhangiani, S.N., Akker, F.V.D., Hoppel, C.L., Salz, H.K., Lupski, J.R., Buchner, D.A., 2018. Mutations in the mitochondrial ribosomal protein MRPS22 lead to primary ovarian insufficiency. Hum. Mol. Genet. 27, 1913–1926. https://doi.org/10.1093/hmg/ddy098.

Cheong, A., Archambault, D., Degani, R., Iverson, E., Tremblay, K.D., Mager, J., 2020. Nuclear-encoded mitochondrial ribosomal proteins are required to initiate gastrulation. Development 147, dev188714. https://doi.org/10.1242/dev.188714.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and BCFtools. Gigascience 10, giab008. https://doi.org/10.1093/gigascience/giab008.

Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., Sherry, S.T., McVean, G., Durbin, R., 2011. The variant call format and VCFtools. Bioinformatics 27, 2156–2158. https://doi.org/10.1093/bioinformatics/btr330.

França, M.M., Mendonca, B.B., 2022. Genetics of ovarian insufficiency and defects of folliculogenesis. Best Pract. Res. Clin. Endocrinol. Metab. 36, 101594. https://doi.org/10.1016/j.beem.2021.101594.

Geng, A.L., Xu, S.F., Zhang, Y., Zhang, J., Chu, Q., Liu, H.G., 2014. Effects of photoperiod on broodiness, egg-laying and endocrine responses in native laying hens. Br. Poult. Sci. 55, 264–269. https://doi.org/10.1080/00071668.2013.878782.

Hutz, R.J., Dierschke, D.J., Wolf, R.C., 1990. Role of estradiol in regulating ovarian follicular atresia in rhesus monkeys: a review. J. Med. Primatol. 19, 553–571.

Jiang, R.S., Xu, G.Y., Zhang, X.Q., Yang, N., 2005. Association of polymorphisms for prolactin and prolactin receptor genes with broody traits in chickens. Poult. Sci. 84, 839–845. https://doi.org/10.1093/ps/84.6.839.

Jiang, R.S., Chen, X.Y., Geng, Z.Y., 2010. Broodiness, egg production, and correlations between broody traits in an indigenous chicken breed. Poult. Sci. 89, 1094–1096. https://doi.org/10.3382/ps.2009-00621.

Luan, X.H., Cao, Z.Z., Li, R.R., Liu, M., Hu, J.M., 2014. Differential expression profiling of hypothalamus genes in laying period and ceased period Huoyan geese. Mol. Biol. Rep. 41, 3401–3411. https://doi.org/10.1007/s11033-014-3202-x.

Liu, L.B., Li, D.Y., Gilbert, E.R., Xiao, Q.H., Zhao, X.L., Wang, Y., Yin, H.D., Zhu, Q., 2015. Effect of monochromatic light on expression of estrogen receptor (ER) and progesterone receptor (PR) in ovarian follicles of chicken. PLoS One 10, e0144102. https://doi.org/10.1371/journal.pone.0144102.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303. https://doi.org/10.1101/gr.107524.110.

Nakada, T., Koja, Z., Tanaka, K., 1994. Effect of progesterone on ovulation in the hypophysectomised hen. Br. Poult. Sci. 35, 153–156. https://doi.org/10.1080/00071669408417680.

Proudman, J.A., Opel, H., 1981. Turkey prolactin: validation of a radioimmunoassay and measurement of changes associated with broodiness. Biol. Reprod. 25, 573–580. https://doi.org/10.1095/biolreprod25.3.573.

Patel, R.K., Jain, M., 2012. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7, e30619. https://doi.org/10.1371/journal.pone.0030619.

Romanov, M.N., Talbot, R.T., Wilson, P.W., Sharp, P.J., 2002. Genetic control of incubation behavior in the domestic hen. Poult. Sci. 81, 928–931. https://doi.org/10.1093/ps/81.7.928.

Romanov, M.N., Talbot, R.T., Wilson, P.W., Sharp, P.J., 1999. Inheritance of broodiness in the domestic fowl. Br. Poult. Sci. 40, S20–S21. https://doi.org/10.1080/00071669986611.

Shen, X., Zeng, H., Xie, L., He, J., Li, J., Xie, X.J., Luo, C.L., Xu, H.P., Zhou, M., Nie, Q.H., Zhang, X.Q., 2012. The GTPase activating Rap/RanGAP domain-like 1 gene is associated with chicken reproductive traits. PLoS One 7, e33851. https://doi.org/10.1371/journal.pone.0033851.

Sharp, P., Macnamee, M.C., Sterling, R.J., Lea, R.W., Pedersen, H.C., 1988. Relationships between prolactin, LH and broody be-haviour in bantam hens. J. Endocrinol. 118, 279–286. https://doi.org/10.1677/joe.0.1180279.

Taraborrelli, S., 2015. Physiology, production and action of progesterone. Acta Obstet. Gynecol. Scand. 161, 8–16. https://doi.org/10.1111/aogs.12771.

Tao, Q.H., Sun, N.N., Wang, M.S., Zhao, W.X., Liu, W., Zhang, X., Li, A., 2024. Effect of the LIF gene on the cell cycle and apoptosis of ovarian granulosa cells in white Muscovy ducks. Theriogenology 230, 91–100. https://doi.org/10.1016/j.theriogenology.2024.09.008.

Wang, J., Liu, Z.S., Cao, D.G., Liu, J., Li, F.W., Han, H.G., Han, H.X., Lei, Q.X., Liu, W., Li, D.P., Wang, J.X., Zhou, Y., 2023. Elucidation of the genetic determination of

clutch traits in Chinese local chickens of the Laiwu black breed. BMC Genomics 24, 686. https://doi.org/10.1186/s12864-023-09798-0.
Wang, H., Yang, C., Wang, J., Xi, Y., Qi, J., Hu, J., Bai, L., Li, L., Mustafa, A., Liu, H., 2023. Genome-wide association analysis of neck ring traits in NongHua ma male ducks. Br. Poult. Sci. 64, 670–677. https://doi.org/10.1080/00071668.2023.2249840.
Wu, X., Yan, M.J., Lian, S.Y., Liu, X.T., Li, A., 2014. GH gene polymorphisms and expression associated with egg laying in muscovy ducks (Cairina moschata). Hereditas 151, 14–19. https://doi.org/10.1111/j.1601-5223.2013.00016.x.
Wu, X., Wan, X.P., Lan, J.J., Yan, M.J., Lian, S.Y., Rijal, M., Huang, Z.B., Li, A., 2015. Cloning, expression, and polymorphism at the 5′-flanking region of the GnRH gene and their association with laying traits in Muscovy duck (Cairina moschata). Br. Poult. Sci. 56, 531–542. https://doi.org/10.1080/00071668.2015.1077502.
Wu, X., Jiang, L., Xu, F.Q., Cao, S.M., Chen, Y., Zhang, Y.Y., He, W.F., Yan, M.J., Lian, S. Y., Li, A., 2021. Long noncoding RNAs profiling in ovary during laying and nesting in Muscovy ducks (Cairina moschata). Anim. Reprod. Sci. 230, 106762. https://doi.org/10.1016/j.anireprosci.2021.106762.
Xiao, Y.Q., Ni, Y.D., Huang, Y.B., Wu, J., Grossmann, R., Zhao, R.Q., 2011. Effects of kisspeptin-10 on progesterone secretion in cultured chicken ovarian granulosa cells from preovulatory (F1-F3) follicles. Peptides 32, 2091–2097. https://doi.org/10.1016/j.peptides.2011.09.001.
Xie, C., Mao, X.Z., Huang, J.J., Ding, Y., Wu, J.M., Dong, S., Kong, L., Gao, G., Li, C.Y., Wei, L.P., 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic. Acids. Res. 39, W316–W322. https://doi.org/10.1093/nar/gkr483.
Xu, H.P., Shen, X., Zhou, M., Fang, M.X., Zeng, H., Nie, Q.H., Zhang, X.Q., 2010. The genetic effects of the dopamine D1 receptor gene on chicken egg production and broodiness traits. BMC Genet. 11, 17. https://doi.org/10.1186/1471-2156-11-17. Mar 3.
Yang, W.L., Yu, S.Q., Peng, J.Z., Chang, P.H., Chen, X.Y., 2023. FGF12 regulates cell cycle gene expression and promotes follicular granulosa cell proliferation through ERK phosphorylation in geese. Poult. Sci. 102, 102937. https://doi.org/10.1016/j.psj.2023.102937.
Yao, Y., Yang, Y.Z., Gu, T.T., Cao, Z.F., Zhao, W.M., Qin, H.R., Xu, Q., Chen, G.H., 2019. Comparison of the broody behavior characteristics of different breeds of geese. Poult. Sci. 98, 5226–5233. https://doi.org/10.3382/ps/pez366.
Ye, P.F., Ge, K., Li, M., Yang, L., Jin, S.H., Zhang, C., Chen, X.Y., Geng, Z.Y., 2019. Egg-laying and brooding stage-specific hormonal response and transcriptional regulation in pituitary of Muscovy duck (Cairina moschata). Poult. Sci. 98, 5287–5296. https://doi.org/10.3382/ps/pez433.
Yuan, J.W., Wang, K.H., Yi, G.Q., Ma, M., Dou, T.C., Sun, C.J., Qu, L.J., Shen, M.M., Qu, L., Yang, N., 2015. Genome-wide association studies for feed intake and efficiency in two laying periods of chickens. Genet. Sel. Evol. 47, 82. https://doi.org/10.1186/s12711-015-0161-1.
Yu, J., Lou, Y.P., He, K., Yang, S.B., Yu, W.S., Han, L., Zhao, A.Y., 2016. Goose broodiness is involved in granulosa cell autophagy and homeostatic imbalance of follicular hormones. Poult. Sci. 95, 1156–1164. https://doi.org/10.3382/ps/pew006.
Zhang, X.Y., Yang, F.X., Zhu, T., Zhao, X.R., Zhang, J.X., Wen, J.H., Zhang, Y.L., Wang, G., Ren, X.F., Chen, A.Q., Wang, X., Wang, L., Lv, X.Z., Yang, W.F., Qu, C.Q., Wang, H., Ning, Z.H., Qu, L.J., 2024. Whole genome resequencing reveals genomic regions related to red plumage in ducks. Poult. Sci. 103, 103694. https://doi.org/10.1016/j.psj.2024.103694.
Zhou, X., Stephens, M., 2012. Genome wide efficient mixed model analysis for association studies. Nat. Genet. 44, 821–824. https://doi.org/10.1038/ng.2310.
Zhou, M., Du, Y., Nie, Q., Liang, Y., Luo, C., Zeng, H., Zhang, X., 2010. Associations between polymorphisms in the chicken VIP gene, egg production and broody traits. Br. Poult. Sci. 51, 195–203. https://doi.org/10.1080/00071661003745786.