104 (2025) 105069

Contents lists available at ScienceDirect

Poultry Science

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

Genetic parameter estimation and molecular foundation of Double-yolk eggs trait in white leghorn

Anqi Chen [a], Xiaoyu Zhao [d], Haiyan Wang [e], Xiurong Zhao [a], Gang Wang [a], Xinye Zhang [a], Xufang Ren [a], Yalan Zhang [a], Xue Cheng [a], Xiaofan Yu [a], Xiaohan Mei [a], Huie Wang [b], Menghan Guo [a], Xiaoyu Jiang [a], Fuping Zhang [c], Zhonghua Ning [a], Lujiang Qu [a,b,*]

[a] National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, PR China
[b] Xinjiang Production and Construction Corps, Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin, Tarim University, Alar 843300, PR China
[c] Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, College of Animal Sciences, Guizhou University, Guiyang 550025, Guizhou Province, PR China
[d] Xingrui Agricultural Stock Breeding, Baoding 072550, Hebei Province, PR China
[e] Hohhot Customs District, Hohhot 010010, Inner Mongolia Autonomous Region, PR China

ARTICLE INFO

Keywords:
White Leghorn
Double-yolk eggs
Heritability
Genome-wide association study

ABSTRACT

Double-yolk (**DY**) eggs refer to the presence of two yolks in an egg, and they are often present in fowl flocks. As abnormal eggs, DY eggs occur frequently in the early stages of egg-laying in hens, as well as in hens with early sexual maturity. In order to understand the inheritant pattern of DY eggs and explore candidate genes associated with DY eggs, we selected over 10,000 white Leghorn (**WL**) chickens from 4 generations and recorded the data during the egg laying period, including total egg number and the rate of DY egg number during the first 2 months and the period of 18-58 weeks of age (**EN2month, EN38, EN58, DY2month, DY38, and DY58**), age at first egg (**AFE**), first egg weight (**FEW**), and body weight at first egg (**BWA**). The results of genetic parameter analysis showed that the DY egg rate was a trait with low to medium heritabilities with the values from 0.15 to 0.29. And there were strong positive phenotypic and genetic correlations between DY egg rate and egg production at different age stages, and they were all strongly negatively correlated with AFE. However, the DY egg rate and egg production at different stages had strong positive and negative genetic correlations with BWA and FEW, respectively. We also found that significant differences in these trait values between different generations and cage layers, indicating that generations and cage layers had a certain influence on these traits. Furtherly, we used whole genome-wide association (**GWA**) analysis to identify genes underlying DY, and 5 candidate genes (EZH2, CNTNAP2, TMEM163, GPC1, and ACMSD) associated with DY2month in WL. Our study improved the understanding of DY eggs in hens, and the genetic parameters of DY eggs, and also provided insights into reducing the production of DY eggs by various selection strategies.

Introduction

As abnormal eggs, double-yolk (**DY**) eggs mostly occur in birds with early sexual maturity, mainly in poultry (Jaap and Muir, 1968; Benoff, 1980; Gebhardt-Henrich and Marks, 1995; Van Middelkoop, 1978; Salamon and Kent, 2014; Wolc et al., 2012; Zelenka et al., 1986) and also wild birds (Deeming, 2011). DY eggs are mainly found in young birds just entering the reproductive maturity stage (Benoff, 1980; Curtis, 1914; Johnston and Gous, 2007; Navara and Wrobel, 2019). The mechanism for regulating follicle development and excretion during this period is still to be improved, and the occurrence of abnormal ovulation might lead to two or more ova with similar developmental levels being retained in one egg (Benoff, 1980; Conrad and Warren, 1940; Curtis, 1914; Hocking et al., 1987; Lowry et al., 1979; Navara and Wrobel, 2019). Therefore, the number of DY eggs produced during this stage accounted for the vast majority of the total number of DY eggs in the entire egg laying cycle (Benoff, 1980; Jaap and Muir, 1968). Mature birds could also produce DY eggs, but most of these birds already had the

* Corresponding author. Full postal address: 2 Yuanmingyuan West Road, Haidian District, Beijing, PR China.
E-mail address: quluj@163.com (L. Qu).

https://doi.org/10.1016/j.psj.2025.105069
Received 3 January 2025; Accepted 18 March 2025
Available online 18 March 2025

phenomenon when they were young (Curtis, 1914). Multiple yolk eggs were longer than the normal eggs of same individual (Curtis, 1914), and there was no significant difference between the two yolks in DY eggs of pheasant (Deeming, 2011) and duck (Salamon and Kent, 2013). Compared to normal duck eggs, DY eggs in ducks had low fertility, and the yolk (yolk1) closer to the airspace inside had higher fertility (Salamon and Kent, 2016). With the progress of egg production, the regulatory mechanisms for controlling ova development and excretion were also improving, so the number of DY eggs would gradually decrease and disappear (Benoff, 1980; Christmas and Harms, 1982; Jaap and Muir, 1968).

The production of DY eggs was influenced by various factors, mainly including the combined effects of genetic and environmental factors. Previous studies showed that the heritabilities of DY eggs ranged from 0.25 to 0.5 (Lowry, 1967; Van Middelkoop, 1978; Dunn et al., 2004; Wolc et al., 2012), and some genes were associated with this trait, including naked neck gene (NA) (Chen and Tixier-Boichard, 2003), GNRHR (Dunn et al., 2004), IGF1 (Liu et al., 2022). The effect of nutritional levels on the production of defective eggs was considered significant, and high body weight was associated with an increase in DY egg rate (Benoff, 1980; Wolc et al., 2012). The defect egg rate of high weight chickens was higher than that of low weight chickens, and relaxing the selection of hen weight could reduce the number of DY eggs and defect eggs (Reddy and Siegel, 1977). Light stimulation was also one of the main environmental factors affecting the production of DY eggs (Johnston and Gous, 2007), and early exposure of hens to light stimulation could significantly increase the number of DY eggs (Benson et al., 2022; Renema et al., 2008). In addition, high concentrations of like growth factor 1 (**IGF1**) in the blood could stimulate the development and selection of goose follicles, which also contributed to the production of DY eggs (Liu et al., 2022). In order to further reveal the genetic mechanism of DY eggs, we recorded the egg production data of 11,309 white Leghorn (**WL**) chickens from 4 generations. Then we used this data to estimate the heritabilities of DY egg rate at different weeks of age and their correlations with other traits. Overall, our results enriched the researches on the genetic basis of DY eggs in chickens and provided insights for subsequent systematic breeding work on DY eggs.

Materials and methods

Ethics statement

This study was conducted following the guidelines for the experimental animals established by the Animal Care and Use Committee of China Agricultural University.

Animals and data collection

To further reveal the genetic patterns of DY eggs across multiple generations, we used the egg production data from 4 generation (13th, 14th, 15th and 16th) of hens. A total of 11309 WL hens with complete pedigrees from 4 generations were selected for analysis. At around 10 weeks of age, the chickens were transferred to 3-layer single cages and were fed in the same conditions. We recorded complete production data during the egg laying period, mainly including the total egg number during the first 2 month (**EN2month**), total egg number in the period of 18-38 weeks of age (**EN38**), total egg number in the period of 18-58 weeks of age (**EN58**), the rate of DY egg number during the first 2 month (**DY2month**), the rate of DY egg number in the period of 18-38 weeks of age (**DY38**), the rate of DY egg number in the period of 18-58 weeks of age (**DY58**), age at first egg (**AFE**), first egg weight (**FEW**), body weight at first egg (**BWA**). Because DY eggs were generally larger than normal eggs, the identification of DY eggs was determined by the breeder by visual observation during egg collection. To investigate the effect of cage layers and generation on DY eggs and other traits, we compared the phenotype values between different generations and between different cage layers in 15th and 16th. We first used Kolmogorov-Smirnov test for data, and found that only FEW in 15th followed normal distribution (P value > 0.05). Then we used the bartlett test to perform homogeneity of variance for FEW in 15th, and the results showed the homogeneity of variance between different groups (P value > 0.05). For FEW in 15th, we compared the values of different cage layers using least significant difference (**LSD**) method and displayed the differences between groups using the letter marking method, the p-value was corrected using Bonferroni method. For other traits, we used Kruskal Wallis test and Wilcoxon rank sum test to compare the values by different generations and cage layers and displayed the differences between groups using the letter marking method, the P-value was corrected by Bonferroni method (Tables 1 and 2).

Genetic parameter estimation

Considering the generation effect, we used univariate and multivariate model in DMU software (Madsen and Jensen, 2013) to estimate the genetic parameters of DY eggs and other traits, as shown below.

$$y = X\mu + Za + e$$

In this model, y is the phenotypic observations of known traits (DY2month, DY38, DY58, EN2month, EN38, EN58, AFE, BWA, and FEW), X is relation matrix of fixed effects, μis vector composed of fixed effects, Z is correlation matrix of random effects, a is vector composed of random effects, e is random residuals.

GWAS for DY2month in WL chickens

Most of DY eggs were produced at the first two months of egg laying. Based on DY2month in 16th WL chickens, we selected 45 hens with the highest rate of DY eggs during the first 2 months and 45 hens with the least rate of DY eggs during the first 2 months as case (0) and control (1) groups for GWAS, respectively (Additional file 1 Table S1). We collected 2ml of blood from the wing vein of each chicken and stored it in the collection tube. DNA was extracted from the blood using FlaPure Animal Tissue/Cell/Blood DNA Extraction Kit (Genesand Biotech Co.,Ltd, Beijing, China). We used next-generation sequencing by DNBSEQ-T7 with 150-bp paired-end (Biomarker Technologies Corporation, Beljing, China). And then we performed quality control by cutting adapter and removing low quality reads for the raw data. The processed data was mapped to the chicken reference genome (GRCg6a) by Burrows-Wheeler Aligner (BWA, version 0.7.17) (Li and Durbin, 2010) for BAM files. Then SAMtools (v.1.16.1) (Danecek et al., 2021) and Picard tools (v2.25.2) (https://broadinstitute.github.io/picard/) were used respectively for sorting BAM files and handling duplicated reads. Local realignment was performed by "RealignerTargetCreator","IndelRealigner"parameter of Genome Analysis Toolkit (GATK, version3.8) (Mckenna et al., 2010), and the adjustment of base quality scores was also performed by "BaseRecalibrator"and "PrintReads" parameter of Genome Analysis Toolkit. Finally, we used "Haplotypecaller", "GenotypeGVCFs","SelectVariants"and "VariantFiltration" for variation detection and filtering. Hard filtration standards were "QUAL < 30.0 || QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < 8.0. Finally, we obtained a total of 9231619 single nucleotide polymorphisms (**SNPs**) and 1212907 insertion-deletions (**Indels**) remained. In addition, we used delly (v1.1.5) (Rausch et al., 2012) to detect structural variation (**SV**) in the sorted and marked duplicate BAM files and performed genotyping on them. Finally, we used BCFtools (Danecek et al., 2021) to merge genotype files and converted them into VCF files. And we obtained 14290 deletions for subsequent GWAS.

In order to obtained high quality SNPs, we used PLINK software (Purcell et al., 2007) to further filter low quality of SNPs with SNP call rate < 90 %, minor allele frequencies < 0.01, and Hardy-Weinberg equilibrium P $<$ 1E-8. After filtering, there were 7455792 SNPs and 793212 Indels remaining. Then we used "blocks" and "indep-pairwise

Table 1
Phenotypic description of DY2month, DY38, DY58, EN2month, EN38, EN58, AFE, BWA, FEW in 4 generations.

Generation	DY2month (%)	DY38 (%)	DY58 (%)	EN2month (eggs)	EN38 (eggs)	EN58 (eggs)	AFE (days)	BWA (g)	FEW (g)
13	$3.53^{a}\pm4.97$	$1.55^{a}\pm2.70$	$1.00^{a}\pm2.26$	$29.6^{d}\pm8.2$	$112.5^{d}\pm33.9$	$219.8^{b}\pm76.1$	$129.9^{b}\pm10.2$	$1475.4^{c}\pm156.5$	$39.5^{b}\pm5.0$
14	$0.03^{c}\pm0.36$	$0.01^{c}\pm0.26$	$0.01^{c}\pm0.25$	$32.6^{c}\pm9.5$	$120.3^{a}\pm25.8$	$211.6^{d}\pm48.6$	$126.9^{c}\pm9.2$	$1426.7^{d}\pm140.8$	$38.2^{c}\pm4.4$
15	$1.97^{b}\pm3.50$	$0.93^{b}\pm1.69$	$0.54^{b}\pm1.03$	$37.7^{b}\pm9.4$	$118.8^{b}\pm16.2$	$219.1^{c}\pm40.1$	$138.7^{a}\pm9.1$	$1486.4^{b}\pm149.5$	$40.9^{a}\pm4.1$
16	$1.67^{b}\pm2.72$	$0.87^{b}\pm1.43$	$0.48^{b}\pm0.94$	$52.1^{a}\pm7.5$	$117.3^{c}\pm11.5$	$246.7^{a}\pm27.8$	$139.3^{a}\pm8.9$	$1537.3^{a}\pm149.3$	$41.2^{a}\pm5.0$

The data was expressed as mean±sd. The letters a, b in the superscript of the table represent levels of significance for differences between values ($P < 0.05$). Abbreviations: DY2month, the rate of DY eggs during the first 2 month, DY38, the rate of DY eggs in the period of 18-38 weeks of age, DY58, the rate of DY eggs in the period of 18-58 weeks of age, EN2month, total egg numbers during the first 2 month, EN38, total egg numbers in the period of 18-38 weeks of age, EN58, total egg numbers in the period of 18-58 weeks of age, AFE, age at first egg, BWA, body weight at first egg, FEW, first egg weight.

Table 2
Phenotypic data of different cage layers in 15 and 16th chickens.

Generation	Layer	DY2month (%)	DY38 (%)	DY58 (%)	EN2month (eggs)	EN38 (eggs)	EN58 (eggs)	AFE (days)	BWA (g)	FEW (g)
15	First	$2.22^{a}\pm3.7$	$1.08^{a}\pm1.9$	$0.63^{a}\pm1.2$	$36.3^{b}\pm9.2$	$116.8^{b}\pm17.4$	$219.2^{a}\pm43.0$	$139.8^{a}\pm9.4$	$1499.5^{a}\pm148.0$	$41.1^{a}\pm4.1$
	Second	$2.40^{a}\pm4.0$	$1.09^{a}\pm1.8$	$0.62^{a}\pm1.1$	$37.8^{a}\pm9.4$	$119.0^{a}\pm16.2$	$219.6^{a}\pm38.7$	$138.6^{ab}\pm9.3$	$1473.3^{b}\pm148.7$	$40.8^{a}\pm4.2$
	Third	$1.42^{b}\pm2.8$	$0.70^{b}\pm1.5$	$0.41^{b}\pm0.9$	$38.3^{a}\pm9.4$	$120.0^{a}\pm15.6$	$218.6^{a}\pm39.9$	$138.3^{b}\pm8.8$	$1493.4^{a}\pm150.3$	$40.8^{a}\pm4.0$
16	First	$2.18^{a}\pm3.2$	$1.18^{a}\pm1.8$	$0.56^{a}\pm1.0$	$51.7^{a}\pm6.5$	$117.1^{a}\pm10.4$	$251.4^{a}\pm18.8$	$139.8^{a}\pm8.0$	$1560.4^{a}\pm145.5$	$41.4^{a}\pm5.0$
	Second	$1.61^{b}\pm2.6$	$0.83^{b}\pm1.4$	$0.43^{a}\pm0.7$	$52.0^{a}\pm7.7$	$117.2^{a}\pm12.1$	$250.0^{a}\pm18.2$	$139.4^{a}\pm9.2$	$1537.3^{b}\pm147.7$	$41.2^{a}\pm5.1$
	Third	$1.60^{b}\pm2.7$	$0.81^{b}\pm1.3$	$0.44^{a}\pm0.7$	$52.3^{a}\pm7.5$	$117.6^{a}\pm11.3$	$250.0^{a}\pm18.4$	$139.0^{a}\pm8.7$	$1530.7^{b}\pm151.4$	$41.1^{a}\pm5.0$

The data was expressed as mean±sd. The letters a, b, c in the superscript of the table represent levels of significance for differences between values ($P < 0.05$). The same letter indicates no significant difference, while different letters indicate a significant difference. Abbreviations: DY2month, the rate of DY eggs during the first 2 month, DY38, the rate of DY eggs in the period of 18-38 weeks of age, DY58, the rate of DY eggs in the period of 18-58 weeks of age, EN2month, total egg numbers during the first 2 month, EN38, total egg numbers in the period of 18-38 weeks of age, EN58, total egg numbers in the period of 18-58 weeks of age, AFE, age at first egg, BWA, body weight at first egg, FEW, first egg weight.

50 10 0.1″ commands in PLINK for linkage disequilibrium (**LD**) analysis for SNPs and datasets, and obtained 1009710 blocks and 300809 independent SNPs. We applied the same LD analysis to Indels datasets and obtained 66356 independent Indels and 66832 blocks. We used the general linear model (**GLM**) in TASSEL software (Bradbury et al., 2007) for DY2month GWAS, and the formula was as follows.

$$Y = Wa + Zb + e$$

Y is the vector of phenotypic values of DY2month, W is a matrix of covariates (fixed effects, this is the population structure), a is the corresponding coefficients of the fixed effects (the weight vector of each group), Z is a vector of markers genotype (markers effect), b is the effect size of the markers (the weight vector of each marker), e is random error. Considering the over-conservatism of the Bonferroni correction method (Gao et al., 2010), we adjusted the threshold line to 7.63E-7 (1/1310519) and 3.82E-8 (0.05/1310519) for SNPs GWAS. And we also adjusted the threshold line to 7.50E-6 (1/133188) and 3.75E-7 (0.05/133188) for Indels GWAS. Then we used the CMplot package (https://cran.r-project.org/web/pack ages/CMplot/index.html) in R software to generate Manhattan and quantile-quantile (Q-Q) plots for GWAS results. And we also used BioMart in Ensembl and selected the reference genome of chicken (GRCg6a) to annotate SNPs, Indels, and deletions (http://www.ensembl.org/biomart/martview). We also conducted gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using KOBAS (http://bioinfo.org/kobas/) for gene function analysis.

Post GWAS statistical power calculations

We calculated the effect values (b) of 10 loci that reached the suggestive level in our research using the following formula (Sham and Purcell, 2014).

$$r^2 = 2b^2 f(1-f)$$

In this formula, r^2 is the proportion of variance explained by the markers, f is the frequency of minor alleles (MAF), b is the effect size of allele. We calculated the power analysis of 10 loci for sampling size at suggestive level (7.63E-7) by qchisq and pchisq in R software. The power values of three loci with significance level (7.63E-7) were also calculated by the pwc packages in R software.

Results

Phenotypic data analysis

We showed phenotypic data of DY2month, DY38, DY58, EN2month, EN38, EN58, AFE, BWA, FEW for 4 generations in Table 1. The results showed that there were certain fluctuations in phenotypic data between different generations, but the difference in data between 15th and 16th was not significant. In terms of egg production data, the DY egg rate of current generation significantly decreased and the egg production performance of this generations significantly improved. The result was mainly due to the continuous breeding of egg production traits from 13th to 16th. After analyzing the data of 15th and 16th generations, we found that the cage layer had a certain impact on the rate of DY eggs, etc. The rate of DY eggs in bottom layer chickens at different stages was significantly higher than that of high layer chickens (Table 2). The main reason for this phenomenon might be the significant difference in light intensity between different cage layers, as we found in previous studies (Chen et al., 2024). Different light intensities might affect the development and discharge of follicles, thereby affecting egg production, which was consistent with the research results on the effect of light on DY eggs mentioned earlier.

Genetic parameter estimates

Based on complete pedigree and phenotype data, we estimated the parameters of DY2month, DY38, DY58, EN2month, EN38, EN58, AFE, BWA, and FEW (Table 3).

The heritability of DY egg rate at different stages ranged from 0.15 to 0.3, indicating a medium level of heritabilities for DY egg rates. The heritability of EN2month was 0.4, but heritabilities of EN38 and EN58 were all less than 0.1.

And there were positive genetic and phenotypic correlations between the rate of DY eggs at different stages, and they were all positively genetic and phenotypic correlated with BWA and FEW. DY2month was

Table 3
Genetic parameter estimates of DY2month, DY38, DY58, EN2month, EN38, EN58, AFE, BWA, FEW.

	DY2month	DY38	DY58	EN2month	EN38	EN58	AFE	BWA	FEW
DY2month	**0.29 (0.02)**	0.93	0.92	0.20	0.27	0.29	-0.26	0.18	0.21
DY38	0.78	**0.24 (0.02)**	0.98	0.16	0.23	0.24	-0.24	0.20	0.18
DY58	0.64	0.93	**0.15 (0.01)**	0.21	0.21	0.18	-0.29	0.17	0.16
EN2month	0.10	0.03	0.01	**0.40 (0.02)**	0.73	0.40	-0.94	-0.27	-0.57
EN38	0.04	-0.15	-0.25	0.57	**0.10 (0.01)**	0.88	-0.62	-0.12	-0.33
EN58	0.02	-0.17	-0.29	0.36	0.92	**0.08 (0.01)**	-0.28	-0.02	-0.12
AFE	-0.13	-0.09	-0.07	-0.81	-0.33	-0.17	**0.52 (0.02)**	0.25	0.59
BWA	0.13	0.14	0.10	-0.24	-0.09	-0.05	0.29	**0.53 (0.02)**	0.33
FEW	0.13	0.13	0.11	-0.40	-0.15	-0.08	0.49	0.29	**0.37 (0.02)**

The value on the diagonal in this table is the heritability of each trait and the values in upper and lower triangles are genetic and phenotypic correlations, respectively. SE values are present in parenthese. Abbreviations: DY2month, the rate of DY eggs during the first 2 month, DY38, the rate of DY eggs in the period of 18-38 weeks of age, DY58, the rate of DY eggs in the period of 18-58 weeks of age, EN2month, total egg numbers during the first 2 month, EN38, total egg numbers in the period of 18-38 weeks of age, EN58, total egg numbers in the period of 18-58 weeks of age, AFE, age at first egg, BWA, body weight at first egg, FEW, first egg weight.

also positively correlated with EN2month. In addition, there were positive genetic and phenotypic correlations between egg number at different stages, and they were all negative genetic and phenotypic correlated with AFE, BWA, and FEW.

GWAS

We used 90 WL chickens as samples for GWAS to identify SNPs associated with DY2month. The Q20 and Q30 of the filtered sample data were both greater than 95 %, and we obtained data with more than 620 Gp and 2,080,580,183 reads. After comparing with the reference genome and merging samples, we obtained a total of 9,231,619 SNPs for further analysis. 7 regions including 1 SNP on chromosome 1, 2, 4, 9, and 12 and 2 SNPs on chromosome 23 and 3 SNPs on chromosome 7 reached suggestive level (P value < 7.63E-7), which belonged to intergenic variant and intron variant (Fig. 1, Additional file 2 Table S2). And we found 4 genes associated with DY2month, including contactin associated protein 2 (CNTNAP2), transmembrane protein 163 (TMEM163), aminocarboxymuconate semialdehyde decarboxylase (ACMSD), glypican 1 (GPC1) (Fig. 1, Additional file 2 Table S2), which were all related to protein coding and were involved in pathways such as Tryptophan metabolism, Cell adhesion molecules (CAMs), and Metabolic pathways. In the GWAS of Indels and deletions, we obtained 1 Indel on chromosome 7, 2 Indels and 1 deletion chromosome 2, 2 Indels on chromosome 2, 3 Indels on chromosome 4, and also annotated enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), TMEM163, and CNTNAP2 genes (Fig. 2 and 3, Additional file 3 Table S3, Additional file 4 Table S4). In post GWAS power analysis, the MAF of 10 loci reached suggestive significance level were 0.17, 0.27, 0.41, 0.31, 0.30, 0.32, 0.42, 0.49, 0.34, and 0.28. The r^2 of 10 loci were 0.28, 0.31, 0.29, 0.30, 0.32, 0.34, 0.29, 0.30, 0.28, and 0.28. We set the power size to 0.85, and calculated the theoretical sample size for the 9 loci to be 95, 80, 90, 85, 80, 70, 90, 85, 95, and 95 (Additional file 5 Table S5). It was obvious that the sample size we used was greater than or close to these theoretical values.

Discussion

Genetic parameter estimation and the impact of environmental factors on DY eggs

We used phenotypic and pedigree data from 4 generation to estimate the genetic parameters of DY egg rate and other traits. And the use of multiple generations of data could make the estimation results more accurate and applicable to numerous other studies (Benoff, 1980; Lowry, 1967; Wolc et al., 2012). In our study, we found significant differences in DY egg rate and egg production between different generations in our study, as well as significant differences in DY egg rate between different

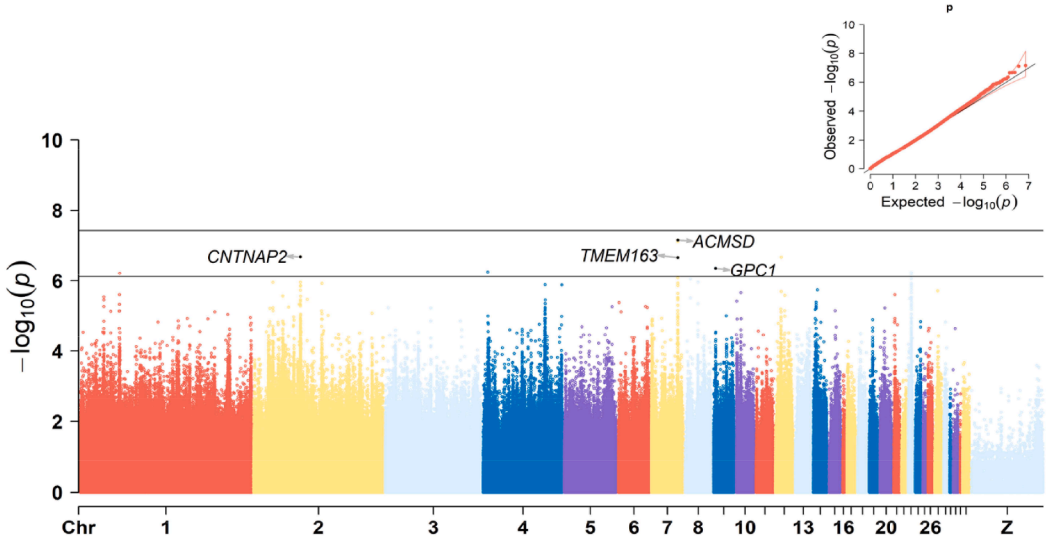

Fig. 1. Manhattan plot and Q-Q plot of genome-wide association study using SNPs for DY2month in WL. Each point in the graph corresponds to the SNP in the datasets, and the two lines represent genome-wide significance thresholds (3.82E-8) and suggestive significance thresholds (7.63E-7). The vertical axis (y-axis) of the Manhattan plot represents - log10 observed P-values of SNPs, and the horizontal axis (x-axis) represents the position of these SNPs on the chromosome. The Q-Q plot contains the observed - log10-transformed p-value plotted against expected - log10-transformed p-value.

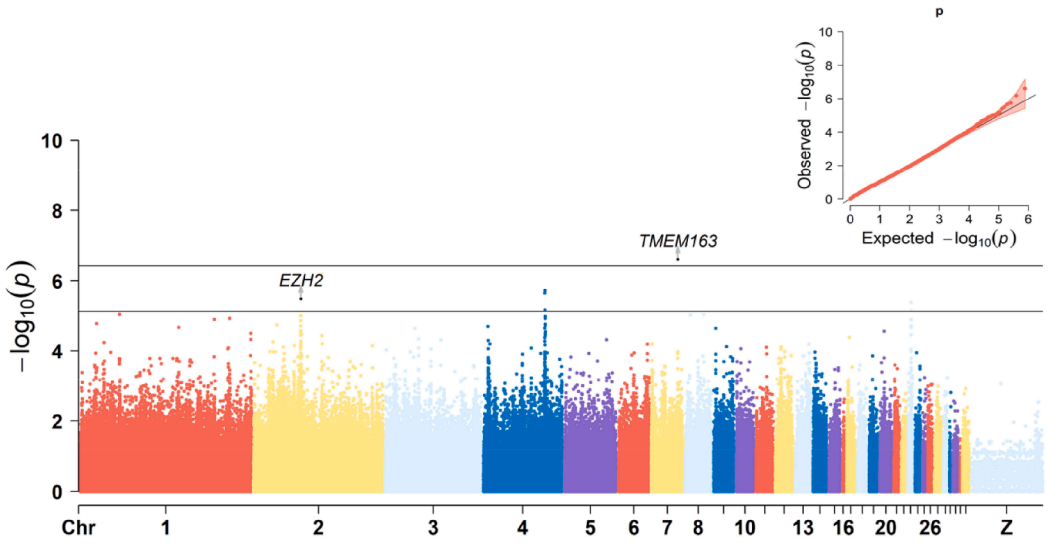

Fig. 2. Manhattan plot and Q-Q plot of genome-wide association study using Indels for DY2month in WL. Each point in the graph corresponds to the Indels in the datasets, and the two lines represent genome-wide significance thresholds (3.75E-7) and suggestive significance thresholds (7.50E-6). The vertical axis (y-axis) of the Manhattan plot represents - log10 observed P-values of Indels, and the horizontal axis (x-axis) represents the position of these Indels on the chromosome. The Q-Q plot contains the observed - log10-transformed p-value plotted against expected - log10-transformed p-value.

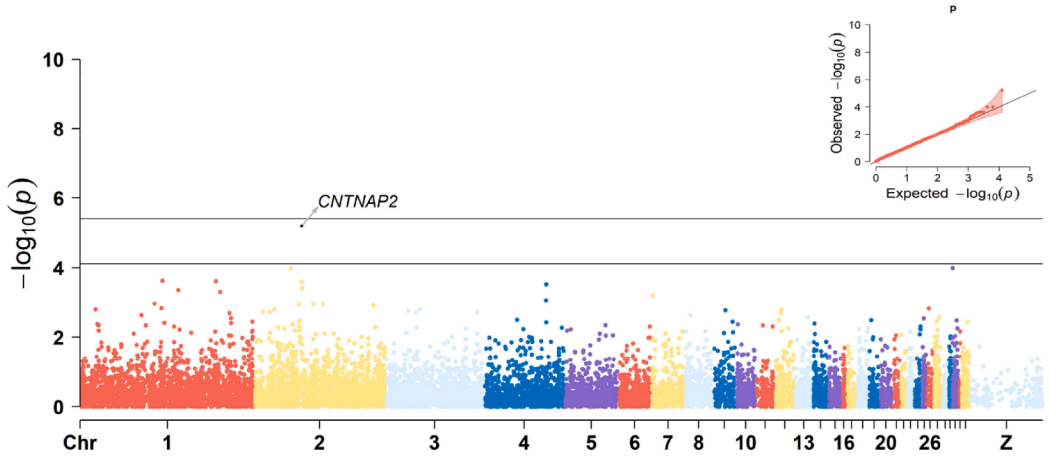

Fig. 3. Manhattan plot and Q-Q plot of genome-wide association study using deletions for DY2month in WL. Each point in the graph corresponds to the deletions in the datasets, and the two lines represent genome-wide significance thresholds (3.88E-6) and suggestive significance thresholds (7.77E-5). The vertical axis (y-axis) of the Manhattan plot represents - log10 observed P-values of deletions, and the horizontal axis (x-axis) represents the position of these Deletions on the chromosome. The Q-Q plot contains the observed - log10-transformed p-value plotted against expected - log10-transformed p-value.

cage layers. This analysis also supported our previous results. Overall, the DY egg rate significantly decreased and the egg production significantly increased in 15th and 16th generations, which was closely related to long-term targeted breeding. But in Lowery's study, the selection of chicken flocks was a long-term plan to increase egg production, which increased the heritability of DY eggs (Lowry, 1967). However, in Middelkoop's study, only the selection of DY eggs in chicken flocks was conducted, resulting in an increase in the number of DY eggs (Van Middelkoop, 1978). The above results also supported our findings across different generations, proving that long-term breeding was effective in improving the production performance of chickens. In our previous research, we found significant differences in the light intensity received by chickens in different cage layers, which specifically meant that light intensity accepted by the upper layer chickens was greater than that accepted by the bottom layer chickens (Chen et al., 2024). In this study, it was found that the DY egg rate of bottom layer chickens were significantly higher than that of upper layer chickens, which indicated that the light intensity might be negatively correlated with the DY egg rate. Based on relevant research, we concluded that light was crucial in the sexual maturation and egg production processes of birds. Appropriate light intensity was an important factor in ensuring normal sexual maturation of birds. The mature ovulation mechanism would greatly reduce the occurrence of DY eggs during early egg production of birds.

The heritability of DY2month was 0.29, while the heritability of DY58 was 0.15, indicating that DY eggs belonged to intermediate level of heritable trait. And it was also worth noting that the heritability of EN2month was as high as 0.4, while the heritability of EN58 was only 0.08. Related studies had also found that the heritability of DY eggs was generally between 0.3 and 0.5 (Lowry, 1967; Mikami and Yamada, 1975; Van Middelkoop, 1978; Wolc et al., 2012), which was consistent with the results of our study. In the early of egg-laying, hens were just placed in individual cages and were exposed to regular light sources and other uncontrollable environmental factors. As egg-laying activity progressed, the influence of environmental factors on different traits also increased. Therefore, we assumed that increasing environmental factors and other uncertain factors would increase their influence in production,

which might be the reasons for the decline of heritability of DY eggs and egg production. The correlation result showed that there were strong positive genetic and phenotypic correlations between DY egg rate or egg production at different stages, both of which were strongly negatively correlated with AFE. And there were strong positively genetic and phenotypic correlations between DY egg rate at different stages and BWA and FEW, but there were strong negative correlations between egg production at different stages and BWA and FEW. DY eggs mainly appeared in the early stage of egg-laying, so chickens with higher DY2month also had higher DY38 and DY58, which could be used to explain the correlations of DY eggs rate at different stages. Related studies found that DY eggs were produced by heavier and earlier-maturing pullets (Benoff, 1980), and high body weight and egg weight were associated with an increase in DY egg rate (Wolc et al., 2012). The various defective egg rates of high weight chickens were significantly higher than those of low weight chickens, and relaxed selection of high weight chickens could reduce the number of DY eggs (Reddy and Siegel, 1977). And there was significant positive genetic and phenotypic correlation between DY2month and EN2month. A study found that a decrease in excessive yolk formation in the ovary could lead to a decrease in abnormal eggs while also causing an increase in normal eggs (Van Middelkoop, 1978). Wolc et al. (2012) found that high-producing hens had lower frequency of egg defects, and there were strong negative correlations between early DY egg rate and both early egg production and early egg production rate (Wolc et al., 2012). But Benoff et al. (1980) found that DY egg production produced by WL chickens during the entire laying period was significantly positively correlated with normal egg production (Benoff, 1980). In Machal et al 's study, it was also found that the number of eggs produced by brown-egg laying hybrids during the early stages of egg production was significantly positively correlated with DY egg rate (Machal et al., 2004). The above research conclusions all supported the correlations between DY eggs rate and body weight or egg number in this study, but there were still some differences in results due to lines.

GWAS and Gene function analysis

After analyzing the heritability of DY2month, we believed that genetic factors accounted for a large proportion and wanted to explore candidate genes significantly associated with this trait through genomic methods. We used DY2month data from the 16th generation for subsequent analysis and selected 2 groups of individuals with extreme phenotypic as samples for SNPs GWAS. In our study, we found 4 genes associated with DY2month, including CNTNAP2, TMEM163, ACMSD, and GPC1. And in the GWAS of Indels and large fragment deletions, we also identified the EZH2, TMEM163, and CNTNAP2 genes. The GPC1 gene was involved in the chicken Wnt signaling pathway, and its expression played a regulatory role in Wnt signaling pathway (Shiau et al., 2010; Liu et al., 2016). The Wnt signaling pathway could affect cell proliferation (Guo et al., 2021) and had been found to play a role in regulating chicken ovarian function and egg production (Mishra et al., 2020; Zhang et al., 2019), as well as in chicken follicle development and selection (Isa et al., 2022; Ma et al., 2024; Nie et al., 2022, 2024; Tai et al., 2022; Wang et al., 2017, 2018; Xu et al., 2024). Xiong et al. (2019) found that GPC1 was also a maker gene associated with the development ability of yak oocytes (Xiong et al., 2019). In general, DY eggs were produced due to the immature development of the ovary, which lead to the discharge of multiple follicles. Therefore, we believed that GPC1 gene might regulate chicken follicle development selection and ovarian function by participating in the Wnt signaling pathway, thereby affecting the production of DY eggs. The above results indicated that GPC1 was the most likely candidate gene to affect the production of DY eggs.

Conclusion

In our research, we found that the heritabilities of the DY eggs at different stages ranged from 0.15 to 0.29, the heritabilities of the egg number at different stages ranged from 0.08 to 0.4. And the heritabilities of AFE, BWA, and FEW were 0.52, 0.53, and 0.37. There were positive genetic and phenotypic correlations between the rates of DY eggs at different stages, as well as a similar correlation between egg production at different stages. And there were positive genetic and phenotypic correlations between the 3 stages of DY egg rate and BWA and FEW, and negative correlations between the 3 stages of total egg number and these two traits, the same positive correlation existed between the DY2month and EN2month. There were significant differences in DY egg rate between different cages and generations, as well as significant differences in egg number between different generations. Compared to previous generations, the egg production performance of 16th generation had significantly improved. And 5 genes were found to be associated with DY2month, including EZH2, CNTNAP2, TMEM163, ACMSD, and GPC1. The above results comprehensively analyzed the genetic mechanism of DY eggs in chickens and provided the possibility of using molecular methods to reduce the production of DY eggs.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

Funding: This work was supported by the Construction and demonstration application of breeding system for high yield green shell layer (NY2502130025), Beijing Innovation Team of the Modern Agro-industry Technology Research System for Poultry (BAIC06-2024-G01), China Agriculture Research System (CARS-40),and Investigation and Demonstration of Key Technologies for Safe and Efficient Breeding of Laying Hens in Sanming City (2023-N-14). Additionally, we also appreciate the support of High-performance Computing Platform of China Agricultural University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105069.

References

Benoff, F.H., 1980. Defective egg production in a population of dwarf white leghorns 1. Br. Poult. Sci. 21, 233–240. https://doi.org/10.1080/00071668008416661.

Benson, A.P., Blocher, R.H., Jarrell, Z.R., Meeks, C.K., Habersang, M.B., Wilson, J.L., Davis, A.J., 2022. Effect of early photostimulation at 15-weeks of age and everyday spin feeding on broiler breeder performance. Poult. Sci. 101, 101872. https://doi.org/10.1016/j.psj.2022.101872.

Bradbury, P.J., Zhang, Z., Kroon, D.E., Casstevens, T.M., Ramdoss, Y., Buckler, E.S., 2007. Tassel: software for association mapping of complex traits in diverse samples. Bioinformatics 23, 2633–2635. https://doi.org/10.1093/bioinformatics/btm308.

Chen, A., Zhao, X., Wen, J., Zhao, X., Wang, G., Zhang, X., Ren, X., Zhang, Y., Cheng, X., Yu, X., Mei, X., Wang, H., Guo, M., Jiang, X., Wei, G., Wang, X., Jiang, R., Guo, X., Ning, Z., Qu, L., 2024. Genetic parameter estimation and molecular foundation of chicken beak shape. Poult. Sci. 103, 103666. https://doi.org/10.1016/j.psj.2024.103666.

Chen, C.F., Tixier-Boichard, M., 2003. Correlated responses to long-term selection for clutch length in dwarf brown-egg layers carrying or not carrying the naked neck gene. Poult. Sci. 82, 709–720. https://doi.org/10.1093/ps/82.5.709.

Christmas, R.B., Harms, R.H., 1982. Incidence of double yolked eggs in the initial-stages of lay as affected by strain and season of the year. Poult. Sci. 61, 1290–1292. https://doi.org/10.3382/ps.0611290.

Conrad, R.M., Warren, D.C., 1940. The production of double yolked eggs in the fowl1. Poult. Sci. 19, 9–17. https://doi.org/10.3382/ps.0190009.

Curtis, M.R., 1914. Studies on the physiology of reproduction in the domestic fowl vi double- and triple-yolked eggs. Biol. Bull. 26, 55–83. https://doi.org/10.2307/1536071.

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, 4. https://doi.org/10.1093/gigascience/giab008.
Deeming, D.C., 2011. Double-yolked pheasant eggs provide an insight into the control of albumen secretion in bird eggs. Br. Poult. Sci. 52, 40–47. https://doi.org/10.1080/00071668.2010.538372.
Dunn, I.C., Miao, Y.W., Morris, A., Romanov, M.N., Wilson, P.W., Waddington, D., 2004. A study of association between genetic markers in candidate genes and reproductive traits in one generation of a commercial broiler breeder hen population. Heredity 92, 128–134. https://doi.org/10.1038/sj.hdy.6800396.
Gao, X.Y., Becker, L.C., Becker, D.M., Starmer, J.D., Province, M.A., 2010. Avoiding the high bonferroni penalty in genome-wide association studies. Genet. Epidemiol. 34, 100–105. https://doi.org/10.1002/gepi.20430.
Gebhardt-Henrich, S.G., Marks, H.L., 1995. Effects of feed restriction on growth and reproduction in randombred and selected lines of japanese quail. Poult. Sci. 74, 402–406. https://doi.org/10.3382/ps.0740402.
Guo, C., Dong, J., Ma, Y., Zhou, S., Zeng, W., Liu, G., Zhang, C., 2021. Lif and bfgf enhanced chicken primordial follicle activation by wnt/β-catenin pathway. Theriogenology 176, 1–11. https://doi.org/10.1016/j.theriogenology.2021.09.008.
Hocking, P.M., Gilbert, A.B., Walker, M., Waddington, D., 1987. Ovarian follicular structure of white leghorns fed ad libitum and dwarf and normal broiler breeders fed ad libitum or restricted until point of lay. Br. Poult. Sci. 28, 493–506. https://doi.org/10.1080/00071668708416983.
Isa, A.M., Sun, Y., Li, Y., Wang, Y., Ni, A., Yuan, J., Ma, H., Shi, L., Tesfay, H.H., Fan, J., Wang, P., Chen, J., 2022. Micrornas with non-additive expression in the ovary of hybrid hens target genes enriched in key reproductive pathways that may influence heterosis for egg laying traits. Front Genet 13, 974619. https://doi.org/10.3389/fgene.2022.974619.
Jaap, R.G., Muir, F.V., 1968. Erratic oviposition and egg defects in broiler-type pullets. Poult. Sci. 47, 417. https://doi.org/10.3382/ps.0470417. -&.
Johnston, S.A., Gous, R.M., 2007. Extent of variation within a laying flock: attainment of sexual maturity, double-yolked and soft-shelled eggs, sequence lengths and consistency of lay. Br. Poult. Sci. 48, 609–616. https://doi.org/10.1080/00071660701573037.
Li, H., Durbin, R., 2010. Fast and accurate long-read alignment with burrows-wheeler transform. Bioinformatics 26, 589–595. https://doi.org/10.1093/bioinformatics/btp698.
Liu, J., Zhao, X.F., Dai, Z.C., Yang, P.X., Chen, R., Guo, B.B., Lei, M.M., Shi, Z.D., 2022. A possible mechanism for double-yolked eggs in the early stage of egg-laying in zhedong white goose-function of igf1 and lhr signaling. Animals 12, 10. https://doi.org/10.3390/ani12212964.
Liu, Z., Sun, C., Qu, L., Wang, K., Yang, N., 2016. Genome-wide detection of selective signatures in chicken through high density snps. PLoS One 11, e0166146. https://doi.org/10.1371/journal.pone.0166146.
Lowry, D.C., 1967. The incidence of double-yolked eggs in relation to improvement in egg production. Der Züchter 37, 82–85. https://doi.org/10.1007/BF00329571.
Lowry, D.C., Dobbs, J., Abplanalp, H.A., 1979. Yolk deposition in eggs of a line selected for simultaneous multiple ovulations. Poult. Sci. 58, 498–501.
Ma, X., Han, X., Wang, W., Zhang, Q., Tang, H., 2024. B-catenin regulates ovarian granulosa cell cycle and proliferation in laying hens by interacting with tcf4. Poult. Sci. 103, 103377. https://doi.org/10.1016/j.psj.2023.103377.
Machal, L., Jerabek, S., Zatloukal, M., Strakova, E., 2004. Defective eggs and their relationship to egg yield, egg and body weight in hens of five original laying lines. Czech J. Anim. Sci. 49, 51–57. https://doi.org/10.17221/4279-cjas.
Madsen, P., Jensen, J., 2013. A user's guide to dmu. Pack. Analys. Multivar. Mixed Models Vers. 6, 1–33.
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.
Mikami, H., Yamada, Y., 1975. The heritability of liability to laying anormal eggs. J. Poultry Sci. 12, 253–258.
Mishra, S.K., Chen, B., Zhu, Q., Xu, Z., Ning, C., Yin, H., Wang, Y., Zhao, X., Fan, X., Yang, M., Yang, D., Ni, Q., Li, Y., Zhang, M., Li, D., 2020. Transcriptome analysis reveals differentially expressed genes associated with high rates of egg production in chicken hypothalamic-pituitary-ovarian axis. Sci. Rep. 10, 5976. https://doi.org/10.1038/s41598-020-62886-z.
Navara, K.J., Wrobel, E.R., 2019. Frequent double ovipositions in two flocks of laying hens. Poult. Sci. 98, 1903–1910. https://doi.org/10.3382/ps/pey518.
Nie, R., Zheng, X., Zhang, W., Zhang, B., Ling, Y., Zhang, H., Wu, C., 2022. Morphological characteristics and transcriptome landscapes of chicken follicles during selective development. Animals (Basel) 12 (6). https://doi.org/10.3390/ani12060713.
Nie, R., Zhang, W., Tian, H., Li, J., Ling, Y., Zhang, B., Zhang, H., Wu, C., 2024. Regulation of follicular development in chickens: Wif1 modulates granulosa cell proliferation and progesterone synthesis via wnt/β-catenin signaling pathway. Int. J. Mol. Sci. 25 (3). https://doi.org/10.3390/ijms25031788.
Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J., Sham, P.C., 2007. Plink: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575. https://doi.org/10.1086/519795.
Rausch, T., Zichner, T., Schlattl, A., Stütz, A.M., Benes, V., Korbel, J.O., 2012. Delly: structural variant discovery by integrated paired-end and split-read analysis. Bioinformatics 28, i333–i339. https://doi.org/10.1093/bioinformatics/bts378.
Reddy, P.R.K., Siegel, P.B., 1977. Selection for body weight at eight weeks of age: 12. Egg production in selected and relaxed lines. Poult. Sci. 56, 673–686. https://doi.org/10.3382/ps.0560673.
Renema, R.A., Sikur, V.R., Robinson, F.E., Korver, D.R., Zuidhof, M.J., 2008. Effects of nutrient density and age at photostimulation on carcass traits and reproductive efficiency in fast- and slow-feathering turkey hens. Poult. Sci. 87, 1897–1908. https://doi.org/10.3382/ps.2007-00431.
Salamon, A., Kent, J., 2013. Double and single yolked duck eggs: their contents and dimensions compared and the mechanical stimulation hypothesis for albumen secretion is supported. Int. J. Poultry Sci. 12, 254–260. https://doi.org/10.3923/ijps.2013.254.260.
Salamon, A., Kent, J., 2014. Changes during incubation within double-yolked duck (anas platyrhynchos domesticus) eggs: yolk position, mortality, hatchability and the importance of an optimal egg size. Int. J. Poultry Sci. 13, 695–702. https://doi.org/10.3923/ijps.2014.695.702.
Salamon, A., Kent, J.P., 2016. Yolk size and ovulation order determine fertility within double-yolked duck (anas platyrhynchos domesticus) eggs. Reprod. Fertil. Dev. 28, 440–445. https://doi.org/10.1071/rd14059.
Sham, P.C., Purcell, S.M., 2014. Statistical power and significance testing in large-scale genetic studies. Nat. Rev. Genetics 15, 335–346. https://doi.org/10.1038/nrg3706.
Shiau, C.E., Hu, N., Bronner-Fraser, M., 2010. Altering glypican-1 levels modulates canonical wnt signaling during trigeminal placode development. Dev. Biol. 348, 107–118. https://doi.org/10.1016/j.ydbio.2010.09.017.
Tai, Y., Yang, X., Han, D., Xu, Z., Cai, G., Hao, J., Zhang, B., Deng, X., 2022. Transcriptomic diversification of granulosa cells during follicular development between white leghorn and silky fowl hens. Front Genet. 13, 965414. https://doi.org/10.3389/fgene.2022.965414.
van Middelkoop, J.H., 1978. Types of egg produced in white plymouth rock hens. World's Poultry Sci. J. 34, 69–80. https://doi.org/10.1079/WPS19960029.
Wang, W., Wu, K., Jia, M., Sun, S., Kang, L., Zhang, Q., Tang, H., 2018. Dynamic changes in the global micrornaome and transcriptome identify key nodes associated with ovarian development in chickens. Front Genet. 9, 491. https://doi.org/10.3389/fgene.2018.00491.
Wang, Y., Chen, Q., Liu, Z., Guo, X., Du, Y., Yuan, Z., Guo, M., Kang, L., Sun, Y., Jiang, Y., 2017. Transcriptome analysis on single small yellow follicles reveals that wnt4 is involved in chicken follicle selection. Front. Endocrinol. (Lausanne) 8, 317. https://doi.org/10.3389/fendo.2017.00317.
Wolc, A., Arango, J., Settar, P., O'Sullivan, N.P., Olori, V.E., White, M.S., Hill, W.G., Dekkers, J.C.M., 2012. Genetic parameters of egg defects and egg quality in layer chickens. Poult. Sci. 91, 1292–1298. https://doi.org/10.3382/ps.2011-02130.
Xiong, X.R., Lan, D.L., Li, J., Yin, S., Xiong, Y., Zi, X.D., 2019. Identification of differential abundances of mrna transcript in cumulus cells and ccnd1 associated with yak oocyte developmental competence. Anim. Reprod. Sci. 208, 106135. https://doi.org/10.1016/j.anireprosci.2019.106135.
Xu, Z., Liu, Q., Ning, C., Yang, M., Zhu, Q., Li, D., Wang, T., Li, F., 2024. Mirna profiling of chicken follicles during follicular development. Sci. Rep. 14, 2212. https://doi.org/10.1038/s41598-024-52716-x.
Zelenka, D.J., Siegel, P.B., van Krey, H.P., 1986. Ovum formation and multiple ovulation in lines of white plymouth rocks and their crosses. Br. Poult. Sci. 27, 409–414. https://doi.org/10.1080/00071668608416897.
Zhang, T., Chen, L., Han, K., Zhang, X., Zhang, G., Dai, G., Wang, J., Xie, K., 2019. Transcriptome analysis of ovary in relatively greater and lesser egg producing jinghai yellow chicken. Anim. Reprod. Sci. 208, 106114. https://doi.org/10.1016/j.anireprosci.2019.106114.