

**Gene study of Oval and OCX-32 in laying and non-laying hens****Fadhil Aamer Fadhil¹ and Ali Saeed Alchalabi^{*2}**

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ABSTRACT

Specific genes tightly control the laying hen's oviduct's egg development process, and each section of the oviduct adds a distinct element to the final product. The aim of the study was to study the oval, and oxc32 genes in both laying and non-laying hens. A total of 38 adult hens were split into two groups; laying and non-laying hens were involved in this study. From the magnum, isthmus and uterus tissues RNA was isolated and transcribed to cDNA for gene expression study. In comparison to other places, the data indicated that oval was significantly increased in laying hens at the Magnum region as opposed to non-laying chickens. Compared to non-laying hens, laying hens' uterine and isthmus regions showed significantly higher levels of oxc32. Gene ontology study revealed that oval gene plays a key role in matrix protein regulation while oxc32 play a key role in eggshell quality via calcium and lipid metabolism. Moreover, gene analysis showed negative regulation of endopeptidase activity, regulation of proteolysis, and regulates the mineralization process and contributes to the structural integrity and antimicrobial properties of the eggshell. In conclusion, a common method for understanding high throughput molecular data and generating hypotheses about the biological mechanisms underlying investigations is Gene Ontology enrichment analysis. by providing a shared vocabulary for describing how genes function.

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P- ISSN: 2077-9798**E- ISSN:** 2959-8478**KEYWORD:** oval, oxc32, gene ontology, laying hens, gene expression**INTRODUCTION**

The egg formation process in the laying hen's oviduct is strictly regulated, and each oviduct segment contributes a distinct component to the final product. A healthy egg is produced by a combination of genes and proteins. This suggests that the different oviductal segments exhibit variable gene and protein expression with time and tissue [1]. The oviduct is a long, tubular structure containing five functionally and histomorphologically distinct segments: the infundibulum, where fertilization takes place;

the magnum, which produces components of the egg white; the isthmus, which forms the membranes of the egg shell; the shell gland or uterus, which forms the calcified eggshell; and the vagina, where oviposition or egg laying takes place [2,3]. A variety of genes are expressed in different locations and times in the uterus for eggshell biomineralization, the isthmus for shell membrane synthesis, and the magnum for albumen synthesis and deposition around the yolk [4]. This research will focus on the genes *oval* and *ocx32*, which are essential for albumin synthesis and eggshell mineralization respectively. Ovalbumin is one of the key proteins in egg whites. With 386 residues in its whole amino acid sequence, it is a monomeric phosphor glycoprotein [5]. Despite lacking any inhibitory qualities, it is a member of the serpin superfamily due to its structural similarity to serine protease inhibitors (Ser PIns). Poultry's ovalbumin gene is approximately 7.6 kb long, with 7 exons and 6 introns. It codes for 1859 mRNA nucleotides [6]. *OVAL* mRNA expression peaks in the magnum of laying chickens 4–23 hours after ovulation. The process that releases the stored proteins is the mechanical distention of the magnum wall brought on by the egg's entrance [7]. One of these substances, relaxin (*RLN3*), triggers the release of proteins from the epithelial cells. In laying hens, the *RLN3* is expressed in the magnum, a part of the oviduct that aids in albumen secretion and synthesis. According to research, *RLN3* expression in the magnum is increased throughout egg development and contributes to regulating how long an egg remains in the magnum for efficient protein deposition. Furthermore, *RLN3* contributes to the egg's oviduct transit [8]. The magnum of laying chickens expresses more *RLN3* mRNA when there is an egg present [9]. This pathway involves the *oval* gene signaling system and protein secretion.

During eggshell calcification, calcite crystals are known to be structured by matrix

proteins like osteopontin, ovocleidins, and ovocalyxins [10]. Ovocalyxins are three major proteins that play a role in the mineralization of eggshells (*OCX*) [11]. In the calcification termination phase, the *OCX-32* controls the structure of the calcite crystals and has a somewhat anti-mineralization function. While *OCX-36* protects the egg from microbial invasion, it has not been demonstrated that it directly contributes to eggshell calcification [12]. The quality of eggshell formation is ensured by the favorable environment provided by *OCX-21*, another ovocalyxin [10]. Because it affects the composition of the shell and other characteristics of egg quality, the *OCX-32* gene has become a prominent target. The *OCX-32* gene, which is mostly expressed in the eggshell gland, gives this structure its shape and integrity [13]. Ovocalyxin-32 is a protein that is present in the cuticle and outer calcified layers of laying hens' eggshells [6]. This protein, which is 32 kDa in size, is involved in the structure and defense mechanisms of the eggshell. In the uterine portion of the oviduct, *OCX-32* is produced, and as the eggshell forms, its expression increases [5]. During the active calcification phase of eggshell production, the protein is produced in the isthmus and the distal oviduct, more precisely in the uterus [14]. *OCX32* is released into the uterine fluid and is believed to be involved in both the overall process of eggshell formation and the cessation of mineral deposition. Because of its correlation with a number of eggshell quality attributes, *OCX32* is regarded as a candidate gene for selection in commercial chicken breeding programs [15]. *OCX-32*, may play a part in the mineralization and general structure of the shell. It's possible that *OCX-32* has a role in stopping mineral deposition during eggshell production. The expression of the ovalbumin gene may provide amino acids to the developing embryo or facilitate biomineralization, but its primary function is in the production of egg whites. In this study,

the oval and ocx32 genes in the oviducts of laying and non-laying chickens will be examined.

MATERIAL AND METHODS

Ethics Statement

All research involving animals was approved by the University of Mosul's Veterinary Medicine College's Animal Care and Use Committee (UM.VET.2024.013). The Laboratory Animal House and Scientific Unit/Veterinary Medicine College/University of Mosul's animal ethics guidelines were followed at every stage.

Animals and Housing

The white laying chickens utilized in this study were of the Meso breed from the College of Agriculture at the University of Mosul. A total of 38 laying chickens were used in the study strategy, and they were 24 weeks old when they laid their eggs. In this study, every chicken was fed the same food and housed in the same way until it was killed. To help them get used to their new surroundings, the hens were kept in ground cages for two weeks while their health was regularly checked. After being divided into two groups, the laying chickens (group A) (n = 28) and the non-laying hens (group B) (n = 10), were discovered to have dormant ovaries upon hen dissection following slaughter.

Sample Collection

Samples of healthy laying eggs have been obtained from the uterus, the white isthmus, and the magnum, three different locations inside the oviducts. Tissue samples were collected from laying hens for the whole egg-producing season. Prior to the extraction of RNA, tissue samples were rapidly frozen at -85 °C.

RNA Isolation and CDNA Synthesis

Total RNA was isolated from all oviduct areas using the Addbio total RNA extraction kit (Korea). After extracting the RNA according to the manufacturer's recommendations, the RNA integrity was tested using the thermal scientific Nano drop ONE system (ThermoFisher, USA), which served as the first step in the PCR procedure. The isolated RNA was transcribed to cDNA using reverse transcriptase (RT), and the first and second strands of cDNA were produced using AddScript Rt Master (Addbio, Korea) in a thermal cycler involved priming at 25°C for 10 minutes, reverse transcription at 50°C for 60 minutes, RT inactivation at 80°C for 5 minutes, and holding at 12°C.

RT-PCR analysis

The RT-PCR stages, which were carried out with the Step-One Applied Biosystems tool system, USA, included further evaluation of the cDNA samples for the oval and ocx32 genes (Table 1). Each reaction required 25 µl, including 12.5 µl of Add SYBER Master Addbio, Korea, 5.5 µl of PCR-grade water, 0.5 µmol of sense and antisense specific primers, and 5 µl of cDNA. To denaturize the template cDNA, it was pre-incubated at 95°C for three minutes. The amplification procedure was performed 40 times, with denaturation (15 seconds at 95 °C), annealing (15 seconds at 60 °C), and extension (30 seconds at 72 °C). Fluorescence was recorded after each cycle at 72 °C. Every experiment included a negative control run where no cDNA template was used. Standard curves were generated by graphing the Ct values (cycle threshold) against the log cDNA dilution using the typical techniques for each target and monitoring gene. This enabled relative quantitation after PCR. To express the values, the relative mRNA level of control implants was set to 1. After retrieving the data as Ct values, we used the $2^{-(\Delta\Delta Ct)}$ technique to compute the fold

change.

RT-PCR ANALYSIS

Table 1. Gene sequencing of studied genes

Gene name	Forward sequence	Reverse sequence	Gene ID
β -actin*	TATGTGCAAGGCCGGTTTC	TGTCTTTCTGGCCCATACCAA	396526
OVAL	TTCCTGGGTAGAAAGTCAGACAAAT	GACAATGGCATTAAACCAGAACCA	396058
OCX-32	GGACAGCACTGCACTACATCAA	GGAATTTCTGTGGAGCAAGACAA	395209

* β -actin was used as a house-keeping gene

Gene analysis

The clean data's GC content and sequence duplication level were assessed after removing low-quality raw readings or utilizing an adapter and ploy-N. Q30. The Hisat2 software was used to align these clean reads to the reference genome (*Gallus gallus* 5.0; <http://asia.ensembl.org/index.html>). The datasets used for function annotation were GO (Gene Ontology; <http://geneontology.org>), COG (Clusters of Orthologous Groups; <http://www.ncbi.nlm.nih.gov/COG>), and Nt (NCBI non-redundant nucleotide sequences; <https://www.ncbi.nlm.nih.gov/>). [16]. The Wallenius non-central hyper-geometric distribution based on the Goseq R packages was used to do GO analysis of expressed genes ($FC > 1.5$, $FDR < 0.05$) [17-19].

Statistical Analysis

All data were displayed as mean $\pm$ SE using IBM's SPSS program, version 26, USA. Gene expression was analyzed using a p-value of less than 0.05. An independent student t-test was used to compare laying and non-laying chickens.

RESULTS

The oval gene's fold change within the magnum region (3.7) (1.49) varied more in the laying and non-laying hen groups versus the white isthmus and uterus (p values 0.0031 and 0.047, respectively), based to fold change analysis (Fig. 1)

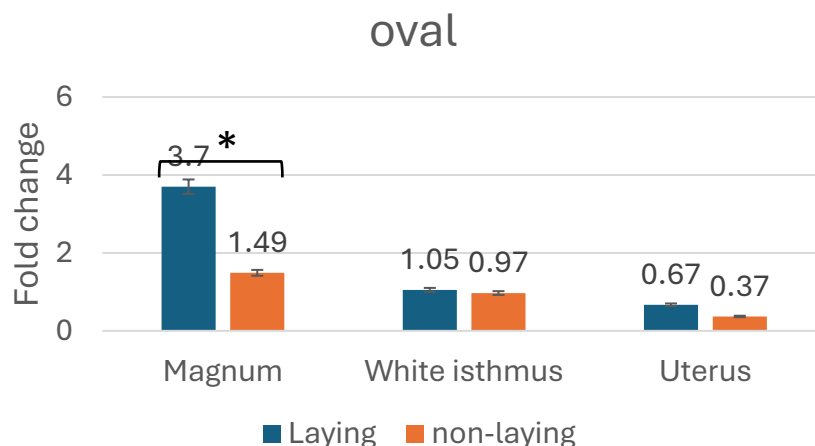


Fig. 1. Oval fold change in laying and non-laying hens

Interestingly, *ocx32* mRNA analysis in non-laying hens' oviduct regions revealed no variation in terms of gene expression and fold change analysis, while transcriptomic analysis of the uterus region revealed that *ocx32* was

highly significant (2.94) in laying hens compared to non-laying in terms of gene expression and fold change analysis with comparison to white isthmus and magnum regions 1.76, 0.58, respectively (Fig. 2).

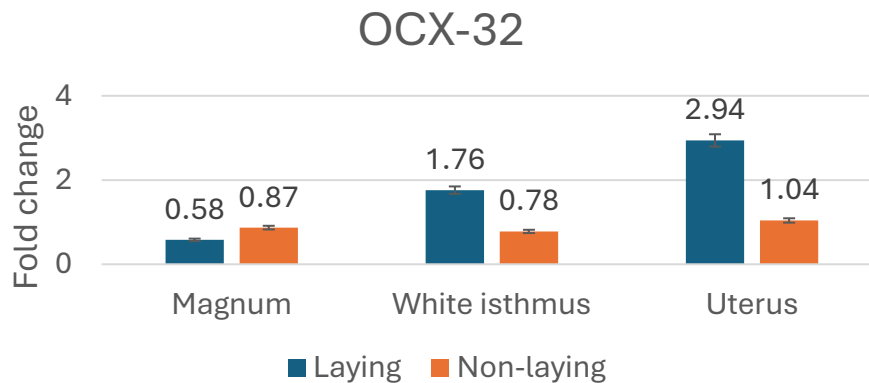


Fig. 2. OCX32 fold change in laying and non-laying hens

A total of 760828153 clean reads were generated from the 16 libraries that were split into two groups from the sample that was being studied. The *Gallus gallus* genome was uniquely mapped to roughly 355–405 × 106 reads of laying and non-laying hens (88.21–92.62%, 78.07–92.72%) all of the entire raw

data. A reliable quality of RNA sequencing data was demonstrated by the libraries' GC contents, which varied between 48.38–50.44%, 48.62–50.15%, and almost 50% of both laying and non-laying hens. Furthermore, 91.70% of the bases outperformed 93.09% with a quality score of > Q30.

Table 2. Sequence quality and alignment information of uterus in laying and non-laying hens

Laying hen group					Non-laying hen group				
Sample	Total reads	Mapped reads	GC content	Q30	Sample	Total reads	Mapped reads	GC content	Q30
1	43507350	91.01%	48.38%	94.00%	1	53519126	78.07%	50.15%	91.59%
2	45291354	88.81%	50.28%	93.93%	2	53019942	91.83%	48.64%	92.79%
3	42874072	90.94%	49.94%	93.17%	3	49974265	91.95%	48.95%	90.86%
4	43068518	90.30%	49.02%	92.53%	4	51507914	90.63%	48.90%	90.59%
5	45015057	88.21%	50.44%	91.44%	5	46935009	90.63%	48.94%	90.41%
6	43163504	91.51%	48.93%	93.36%	6	56402094	92.18%	48.93%	92.67%
7	51639809	89.59%	50.09%	92.25%	7	43750975	91.89%	48.62%	92.36%
8	40940558	92.62%	49.81%	94.00%	8	50218606	92.72%	48.69%	92.35%

GC, guanine-cytosine; Q30, the proportion of bases with a Phred quality score greater than 30. Data are from 4 replicates with 2 birds each of laying and non-laying hens.

Gene Ontology (GO) Of Studied Genes

Through functional annotation and pathway enrichment analysis of expressed genes (EGs) across two groups, the gene ontology (GO) of genes enrichment study was conducted to collect crucial information for functional prediction of EGs. The EGs were functionally classified across groups using the

eight COG (Clusters of Orthologous Groups) categories: transport and metabolism of amino acids, transport and catabolism of secondary metabolites, posttranslational modification, protein turnover, cytoskeleton, and inorganic ion transport and metabolism. To annotate the EGs, three primary functional categories were employed: biological process, cellular component, and molecular function. The

biological process category had the most common terms: "cellular process," "single-organism process," and "biological regulation.", and two EGs encoding matrix

proteins and calcium transport proteins were suspected to play a role in eggshell formation, table 3 and table 4.

Table 3. Gene ontology (GO) analysis of expressed genes (fold change > 1.5 at a false discovery rate < 0.05) in association with matrix protein, calcium transport

GO Terms	GO.ID	Potential function	Fold change	Annotation
Biological Process				
cellular protein modification process	GO:0006464	Matrix protein	3.7	Ovalbumin, regulate shell structure and present in eggshell
protein modification by small protein conjugation or removal	GO:0070647	Calcium transport	2.94	calcium and lipid metabolism in chicken uterine cells
Cellular Component				
endosome	GO:0005768			
extracellular matrix	GO:0031012			
Molecular Function				
serine-type endopeptidase inhibitor activity	GO:0042605			
endopeptidase inhibitor activity	GO:0005509			

Table 4. Gene analysis by AmiGO2

Gene name	Transcript ID (Stable ID)	Molecular function	Biological process	Cellular Component	protein class	Biotype
Ovalbumin (Oval) SERPINB14	ENSGALT00010027940	serine-type endopeptidase inhibitor activity calcium ion binding	negative regulation of endopeptidase activity regulation of proteolysis	extracellular space cytosol early endosome lumen phagolysosome	protease inhibitor	Protein coding
Ovocalyxin-32 OCX-32	ENSGALT00010002737	endopeptidase inhibitor activity	regulates the mineralization process and contributes to the structural integrity and antimicrobial properties of the eggshell	extracellular space	protease inhibitor	Protein coding

Oval: Exons: 8, Coding exons: 7, Transcript length: 1,875 bps, Translation length: 386 residues

OCX32: Exons: 6, Coding exons: 6, Transcript length: 1,676 bps, Translation length: 275 residues

DISCUSSION

The ovary's ovulated yolk develops into an egg in the poultry oviduct, a very dynamic and intricate organ. However, oviduct

tissues experience neovascularization, extracellular matrix remodeling, and massive proliferation of luminal and glandular epithelial cells during egg production. Understanding the oviductal proteins and

genes involved in egg production is therefore crucial [20]. A mature hen's oviduct receives the ovum from the ovary and provides the biological conditions required for the egg's development and potential fertilization [11]. During egg formation, albumin from the magnum is deposited around the yolk, and the egg is then wrapped by the eggshell membranes from the isthmus. As the yolk travels through the oviduct, the uterus deposits calcium on it, hardening the eggshell. The functional roles of the different portions of the oviduct have been found to influence the expression of several genes related to albumin deposition, eggshell membrane formation and shell calcification, and the protein secreted by the oviduct [21,22]. Certain sections of the oviduct, such as the magnum (where albumen is added) and the shell gland (where the shell is formed), express distinct gene sets. Within a segment, several cell types (e.g., gland cells and epithelial cells) will have unique gene expression profiles linked to their specific functions [23]. While the magnum is primarily responsible for creating the proteins contained in egg whites, the shell gland (uterus) is necessary for the formation of eggshells [24]. While genes involved in the synthesis of egg white protein are significantly expressed in the magnum shortly after ovulation, genes linked to the formation of the eggshell are boosted as the egg travels through the shell gland. In current study, the oval mRNA expression was highly upregulated in the magnum region followed by isthmus and down regulated in the uterus which is mainly controls the albumin synthesis. Increased oval synthesis is triggered by the ovum's presence in the magnum through hormonal signaling and mechanical stimulation [25]. The magnum is the main location for oval synthesis, but other oviduct components, such as the uterus and isthmus, also affect its expression, which is connected to the development of eggshells [4]. The oviduct's magnum region is where oval, a key component of egg white, is generated. The

oviduct wall's mechanical distension and signaling molecules like relaxin cause its expression to be elevated during ovulation and to increase when the egg is in the magnum. Oval expression in laying hens is upregulated rather than downregulated in non-laying hens due to low estrogen concentrations, which further explains why oval expression was low in non-laying hens. It may also be because the cells in the uterus and isthmus lacked estrogen receptors, which are crucial to this process. Hormonal control, such as estrogen, plays a significant role in albumin regulating gene expression. In order to control gene transcription, estrogen interacts to estrogen receptors (ERs), which then attach to DNA. There is a correlation between the amount of ovalbumin mRNA synthesis and the quantity of ER molecules attached to nuclei. This opinion is strongly agreed with [25], [26], [27], [28] that mention through transcriptional control, estrogen significantly affects the expression of the ovalbumin gene in the chicken oviduct. Ovalbumin mRNA, the template used to create the ovalbumin protein found in egg whites, is produced and concentrated more readily when estrogen is present. This regulation is evident when ovalbumin mRNA levels decrease with estrogen withdrawal and quickly increase with readministration, indicating a direct correlation between estrogen and gene transcription. Furthermore, our results indicating ERs play a crucial role in this process is supported by [29]. Following their attachment to ERs, estrogen causes the ERs to bind to DNA and regulate gene transcription. There is an association between the number of ER molecules affixed to nuclei and the amount of ovalbumin mRNA production.

The OCX-32 gene, is the other gene that regulates eggshell proteins. It is crucial for the eggshell's quality. The cuticle and outer layers of the eggshell include a matrix protein that it encodes. Changes in the OCX-32 gene

have been connected to traits like eggshell thickness, strength, and color, according to research [30]. In current study we found that *ocx32* expression was highly expressed in uterus of laying hens rather than non-laying hens also was downregulated in other parts of oviduct in laying hens. OCX32 contributes significantly to the organic matrix of the eggshell. During the last stages of eggshell formation, it is thought to play a role in the mineral deposition and eggshell completion processes. It encodes a matrix protein that is present in the cuticle and outer layers of the eggshell and is mainly expressed in the uterus, specifically in the eggshell gland. Given that it is secreted into uterine fluid, it may play a part in eggshell structure and mineral deposition. This opinion is agreement with [31-33] the OCX-32 gene has gained attention because to its role in shell composition and other aspects of egg quality and only the tissue of the uterus and isthmus exhibited high levels of *ocx32* expression. Therefore, *ocx32* is identified as a novel protein generated in the distal oviduct, where hen eggshell formation occurs.

Gene ontology study revealed that the structural molecule in the eggshell matrix is OCX-32's as main molecular function. Being a part of the organic matrix, it helps to maintain the shell's structural integrity. Numerous biological functions linked to the development of eggshells are mediated by OCX-32, including. The process of calcification contributes to the formation and arrangement of calcium carbonate crystals. Shell produced in the uterus and released to aid in the development of the eggshell. Assembly of the Proteinaceous Extracellular Matrix (ECM), being a matrix protein, OCX-32 affects how the eggshell's extracellular matrix is assembled and arranged. As cellular component, OCX-32 is found in the extracellular space, more precisely in the cuticle and eggshell. The shell gland, where it is generated, also contains it. And according to its important function in

eggshell quality, OCX-32 is regarded as a top candidate gene for poultry selective breeding [34,35]. Supporting our results, the molecular mechanisms and pathways involved in egg production can be discovered by using Gene Ontology (GO) analysis of differentially expressed genes (DEGs) in laying hens. DEGs are genes whose expression levels vary significantly between groups (e.g., high vs. poor egg-producing hens). Researchers can find enhanced biological processes, molecular activities, and cellular components linked to egg production by employing GO to analyze these DEGs. As well as, GO Enrichment Analysis offers a regulated, organized terminology to explain the roles of genes and gene products. GO enrichment analysis determines which GO terms, in comparison to a background gene set, are over-represented (enriched) in a list of DEGs [37], [38]. The biological processes, molecular processes, and cellular sites that are most pertinent to the observed variations in gene expression can thus be identified [39].

CONCLUSION

The current research found that both *ocx32* and *ocx31* are expressed gene in different parts of hen's oviduct in both laying and non-laying hens. *Ocxl* gene was highly upregulated in magnum region while *ocx32* was highly upregulated in uterus and white isthmus tissues and mainly controlling albumin synthesis and biomineralization of eggshell. In transcriptomics research, gene ontology is a potent tool for examining gene function and regulation. GO gives researchers a common language to describe gene activities, which makes it easier to spot connections and patterns that might otherwise be hard to spot. GO is a crucial part of contemporary transcriptomics research, whether it is utilized for enrichment analysis, clustering and visualization, or integration with other omics data.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

1. Sah N, Mishra B. Regulation of Egg Formation in the Oviduct of Laying Hen. *Worlds Poult Sci J.* 2018;74(3):509-522. <https://doi.org/10.1017/S0043933918000442>
2. Poultry Hub Australia. Reproductive System. Available from: <https://www.poultryhub.org/anatomy-and-physiology/body-systems/reproductive-system>
3. Pennsylvania State University Extension. Hen Reproduction. Available from: <https://extension.psu.edu/hen-reproduction>
4. Mishra B, Sah N, Wasti S. Genetic and Hormonal Regulation of Egg Formation in the Oviduct of Laying Hens. *IntechOpen.* 2019. <https://doi.org/10.5772/intechopen.85011>
5. Wilson PB. Recent Advances in Avian Egg Science: A Review. *Poult Sci.* 2017;96(10):3747-3754. <https://doi.org/10.3382/ps/pex187>
6. Gautron J, Stapane L, Le Roy N, Nys Y, Rodriguez-Navarro AB, Hincke MT. Avian Eggshell Biomineralization: An Update on Its Structure, Mineralogy and Protein Toolkit. *BMC Mol Cell Biol.* 2021;22(1):11. <https://doi.org/10.1186/S12860-021-00350-0>
7. Zhao JP, Zhang Q, Jiao HC, Wang XJ, Jiang MJ, Luo H, et al. Ovalbumin Expression in the Oviduct Magnum of Hens Is Related to the Rate of Egg Laying and Shows Distinct Stress-Type-Specific Responses. *J Anim Physiol Anim Nutr.* 2016;100(5):876-883. <https://doi.org/10.1111/jpn.12475>
8. Sah N, Kuehu DL, Khadka VS, Deng Y, Jha R, Wasti S, et al. RNA Sequencing-Based Analysis of the Magnum Tissues Revealed Novel Genes and Biological Pathways Involved in the Egg-White Formation in the Laying Hen. *BMC Genomics.* 2021;22(1):1-16. <https://doi.org/10.1186/S12864-021-07634-X>
9. Lv C, Zheng H, Jiang B, Ren Q, Zhang J, Zhang X, et al. Characterization of Relaxin 3 and Its Receptors in Chicken: Evidence for Relaxin 3 Acting as a Novel Pituitary Hormone. *Front Physiol.* 2022; 13:1010851. <https://doi.org/10.3389/fphys.2022.1010851>
10. Hernández-Hernández A, Gómez-Morales J, Rodríguez-Navarro AB, Gautron J, Nys Y, García-Ruiz JM. Identification of Some Active Proteins in the Process of Hen Eggshell Formation. *Cryst Growth Des.* 2008;8(12):4330-4339. <https://doi.org/10.1021/cg800786s>
11. Hincke MT, Nys Y, Gautron J, Mann K, Rodriguez-Navarro AB, McKee MD. The Eggshell: Structure, Composition and Mineralization. *Front Biosci.* 2012;17(4):1266-1280. <https://doi.org/10.2741/3985>
12. Cordeiro CMM, Esmaili H, Ansah G, Hincke MT. Ovocalyxin-36 Is a Pattern Recognition Protein in Chicken Eggshell Membranes. *PLoS One.* 2013;8(12):e84112. <https://doi.org/10.1371/journal.pone.0084112>

13. Moreau T, Gautron J, Hincke MT, Monget P, Réhault-Godbert S, Guyot N. Antimicrobial Proteins and Peptides in Avian Eggshell: Structural Diversity and Potential Roles in Biomineralization. *Front Immunol.* 2022;13:946428. <https://doi.org/10.3389/fimmu.2022.946428>
14. Duan G, Liu W, Han H, Li D, Lei Q, Zhou Y, et al. Transcriptome and Histological Analyses on the Uterus of Freckle Egg-Laying Hens. *BMC Genomics.* 2023;24(1):746. <https://doi.org/10.1186/S12864-023-09828-X>
15. Song X, Li S, He S, Zheng H, Li R, Liu L, et al. Integration of Whole-Genome Resequencing and Transcriptome Sequencing Reveals Candidate Genes in High Glossiness of Eggshell. *Animals.* 2024;14(8):1141. <https://doi.org/10.3390/ani1408114>
16. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene Ontology Analysis for RNA-Seq: Accounting for Selection Bias. *Genome Biol.* 2010;11(2):R14. <https://doi.org/10.1186/GB-2010-11-2-R14>
17. Mao X, Cai T, Olyarchuk JG, Wei L. Automated Genome Annotation and Pathway Identification Using the KEGG Orthology (KO) as a Controlled Vocabulary. *Bioinformatics.* 2005;21(19):3787-3793. <https://doi.org/10.1093/bioinformatics/bti430>
18. Lomax J. Get Ready to GO! A Biologist's Guide to the Gene Ontology. *Brief Bioinform.* 2005;6(3):298-304. <https://doi.org/10.1093/bib/6.3.298>
19. Thomas PD, Mi H, Lewis S. Ontology Annotation: Mapping Genomic Regions to Biological Function. *Curr Opin Chem Biol.* 2007;11(1):4-11. <https://doi.org/10.1016/j.cbpa.2006.11.039>
20. Jeong W, Lim W, Kim J, Ahn SE, Lee HC, Jeong JW, et al. Cell-Specific and Temporal Aspects of Gene Expression in the Chicken Oviduct at Different Stages of the Laying Cycle. *Biol Reprod.* 2012;86(6):172. <https://doi.org/10.1095/biolreprod.111.098186>
21. Du J, Hincke MT, Rose-Martel M, Hennequet-Antier C, Brionne A, Cogburn LA, et al. Identifying Specific Proteins Involved in Eggshell Membrane Formation Using Gene Expression Analysis and Bioinformatics. *BMC Genomics.* 2015;16(1):792. <https://doi.org/10.1186/s12864-015-2013-3>
22. Willems E, Decuyper E, Buyse J, Everaert N. Importance of Albumen During Embryonic Development in Avian Species, with Emphasis on Domestic Chicken. *Worlds Poult Sci J.* 2014;70(3):503-518. <https://doi.org/10.1017/S0043933914000567>
23. Chang X, Wang BB, Zhang H, Qiu K, Wu S. The Change of Albumen Quality During the Laying Cycle and Its Potential Physiological and Molecular Basis in Laying Hens. *Poult Sci.* 2024;103(10):104004. <https://doi.org/10.1016/j.psj.2024.104004>
24. Zhang XY, Uchechukwu EO, Wang J, Zhang HJ, Zhou JM, Qiu K, et al. Histological and Molecular Differences in Albumen Quality Between Post-Adolescent Hens and Aged Hens. *Poult Sci.* 2024;103(6):103618. <https://doi.org/10.1016/j.psj.2024.103618>
25. Swaneck GE, Nordstrom JL, Kreuzaler F, Tsai MJ, O'Malley BW. Effect of Estrogen on Gene Expression in Chicken Oviduct: Evidence for Transcriptional Control of Ovalbumin Gene. *Proc Natl Acad Sci. USA.* 1979;76(3):1049-1053. <https://doi.org/10.1073/pnas.76.3.1049>

26. Tsai SY, Tsai MJ, Schwartz R, Kalimi M, Clark JH, O'Malley BW. Effects of Estrogen on Gene Expression in Chick Oviduct: Nuclear Receptor Levels and Initiation of Transcription. *Proc Natl Acad Sci. USA.* 1975;72(11):4228-4232. <https://doi.org/10.1073/pnas.72.11.4228>
27. McNaught RW, Smith RG. Characterization of a Second Estrogen Receptor Species in Chick Oviduct. *Biochemistry.* 1986;25(8):2073-2081. <https://doi.org/10.1021/bi00356a036>
28. Smith RG, Taylor RN. Estrogen Receptors as Mediators of Gene Transcription. *J Steroid Biochem.* 1981;15(C):321-328. [https://doi.org/10.1016/0022-4731\(81\)90292-2](https://doi.org/10.1016/0022-4731(81)90292-2)
29. Seneca Learning. Regulating Transcription – Biology: AQA A Level. Accessed Jul. 16, 2025. Available from: <https://senecalearning.com/en-GB/revision-notes/a-level/biology/aqa/8-2-5-regulating-transcription>
30. Zhi Y, Wang D, Zhang K, Wang Y, Geng W, Chen B, et al. Genome-Wide Genetic Structure of Henan Indigenous Chicken Breeds. *Animals.* 2023;13(4):753. <https://doi.org/10.3390/ani13040753>
31. Gautron J, Hincke MT, Mann K, Panhéleux M, Bain M, McKee MD, et al. Ovocalyxin-32, a Novel Chicken Eggshell Matrix Protein: Isolation, Amino Acid Sequencing, Cloning, and Immunocytochemical Localization. *J Biol Chem.* 2001;276(42):39243-39252. <https://doi.org/10.1074/jbc.M104543200>
32. Zhao Y, Liao C, Ding Y, Fu H, Guo X, Zhang Y. Overexpression of the OCX-32 Gene Regulates Ca^{2+} and Lipid Concentration in Chicken Uterine Epithelial Cells. *Gene.* 2025;964:149653. <https://doi.org/10.1016/j.gene.2025.149653>
33. Yacoub HA, Fathi MM, Al-Homidan IH, Badawy MI, Abdelfattah MH, Elzaref MF, et al. Association of Ovocalyxin-32 Gene Variants with Egg Quality Traits in Indigenous Chicken Breeds. *Animals.* 2024;14(20):3010. <https://doi.org/10.3390/ani14203010>
34. Wolc A, Drobik-Czwaro W, Jankowski T, Arango J, Settar P, Fulton JE, et al. Accuracy of Genomic Prediction of Shell Quality in a White Leghorn Line. *Poult Sci.* 2020;99(6):2833-2840. <https://doi.org/10.1016/j.psj.2020.01.019>
35. Fulton JE, Soller M, Lund AR, Arango J, Lipkin E. Variation in the Ovocalyxin-32 Gene in Commercial Egg-Laying Chickens and Its Relationship with Egg Production and Egg Quality Traits *Anim Genet* 2012;43(Suppl.1):102-113. <https://doi.org/10.1111/j.1365-2052.2012.02384.x>
36. Haynes WA, Tomczak A, Khatri P. Gene Annotation Bias Impedes Biomedical Research. *Sci Rep.* 2018;8(1):1362. <https://doi.org/10.1038/s41598-018-19333-x>
37. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: Tool for the Unification of Biology. *Nat Genet.* 2000;25(1):25-29. <https://doi.org/10.1038/75556>
38. Tomczak A, Mortensen JM, Winnenburg R, Liu C, Alessi DT, Swamy V, et al. Interpretation of Biological Experiments Changes with Evolution of the Gene Ontology and Its Annotations. *Sci Rep.* 2018;8(1):5115. <https://doi.org/10.1038/s41598-018-23395-2>