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The variation in the promoter region of the ovalbumin gene and its correlation with egg quality characteristics in chickens

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Abstract
An investigation was carried out on the promoter region of the ovalbumin gene to uncover genetic variations and evaluate their impact on egg quality characteristics in chickens. Single-stranded conformation polymorphism analysis, followed by sequencing, was employed to identify polymorphisms within the gene. Three distinct haplotypes were discerned across two distinct white leghorn chicken lines (IWI and IWK). These haplogroups were found to have a significant impact on egg weight by the 52nd week. Additionally, a notable correlation was observed between haplogroups and traits such as Haugh unit and albumin index, particularly within the IWK line. In summary, the partial promoter region of the ovalbumin gene demonstrated polymorphism and exerted a noteworthy influence on both the quality and quantity of eggs in layer chickens.

Keywords: Association, egg quality, ovalbumin gene, polymorphism

Introduction

Ovalbumin is the principal protein responsible for the functional characteristics of egg white in food. Acting as a storage protein, it serves as a significant source of amino acids essential for the embryo's development (Mine *et al*., 2008) [1]. The ovalbumin gene spans approximately 7.6 kb of DNA, encoding mRNA consisting of 1859 nucleotides (McReynolds *et al*., 1978) [2]. Intact ovalbumin has been detected in extracts from various embryonic organs, including the head, eye, heart, liver, intestine, spinal cord, muscle, dermis, and bone. The presence of ovalbumin in these organs, alongside the absence of ovalbumin mRNA expression and the declining presence of ovalbumin shortly after hatching in neonatal organs, suggests a potential multifaceted role for egg white ovalbumin beyond mere amino acid provision, possibly exerting a direct influence on tissue development (Sugimoto *et al*., 1999) [3]. Within the vitelline membrane, five serpins have been identified, speculated to be involved in folliculogenesis, angiogenesis, and defense mechanisms, although the exact role of ovalbumin remains ambiguous (Mann, 2008) [4]. Ovalbumin within the eggshell is believed to play a pivotal role in the formation of calcium carbonate and the stabilization of amorphous calcium carbonate, contributing to biomineralization—a process defined by the creation of hard tissue marked by a distinct minerals/organic matrix framework by living organisms (Schwahn *et al*., 2008) [5]. With these insights in mind, the study aims to pinpoint genetic polymorphisms within the partial promoter of the ovalbumin gene and evaluate their relationship with egg quality traits in chickens.

Materials and Methods

Birds, husbandry practices and collection of samples

The study involved chickens from two distinct lines within the White Leghorn breed: the IWI and IWK lines. The IWI line has been selectively bred for increased egg production up to 64 weeks of age over the last three generations at ICAR-DPR. Conversely, the IWK line has undergone selection for higher egg production up to 64 weeks of age and increased egg weight at 28 weeks of age over the past 11 generations at the institute. Chicks from these two selected lines (IWI and IWK) were bred through pedigree mating, involving 50 sires and 250 dams at a ratio of 1:5. After hatching, chicks were sexed, wing-banded, and raised in open-sided houses on deep litter until reaching 16 weeks

of age. They were provided with a layer chick starter ration containing 2,600 kcal/kg metabolizable energy (ME) and 18% crude protein (CP) up to 8 weeks of age. Subsequently, they received grower ration (2,500 kcal/kg ME and 16% CP) from 9 to 16 weeks of age, given ad libitum. From 16 weeks of age onwards, birds were individually housed in cages and fed a layer ration (2,600 kcal/kg ME and 16% CP) throughout the egg production period until the conclusion of the experiment. Lighting was provided for 16 hours per day, including natural daylight, during the laying period. The management and rearing conditions were kept consistent across all four genetic groups throughout the experiment. Upon hatching, chicks were vaccinated against Marek's disease, followed by standard vaccination protocols to protect against other important diseases such as Newcastle disease (RD), infectious bursal disease (IBD), and fowl pox. Cooling facilities were implemented during the summer season through water sprinkling on the roof, and appropriate lighting arrangements were made in the shed to provide birds with an optimal environment for reaching their full potential.

Isolation

At the age of 4 weeks, blood samples were collected from all birds using a 0.5-mL tube containing 20 µL of 0.05 M EDTA anticoagulant. Subsequently, genomic DNA was extracted from the blood cells following the established protocol outlined by Sambrook and Russell (2001) [9].

PCR

To examine polymorphism within the coding region of the ovalbumin gene, researchers focused on the partial promoter region located upstream of the transcription initiation site. Using DNASTAR software (Lasergene Inc., Madison, WI), two pairs of primers were designed based on the chicken ovalbumin gene promoter sequence available at the National Center for Biotechnology Information (accession number NM_205152.2). The primer sequences are detailed in Table 1.

For the PCR reactions, 50 µg of DNA template, 10 pM of each primer, 1.5 mM of MgCl2, 100 µM of each dNTP, 1× assay buffer, and 0.25 U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) were used. The amplification conditions for all exons included an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C, and extension at 72°C for 45 seconds, with a final extension at 72 °C for 10 minutes.

Table 1: Details of primers designed to amplify ovalbumin gene promoters for SSCP analysis

Primer	Primer Sequence (5'-3')	Product Size (bp)	Annealing Temp (°C)	NCBI Acc. No.
OA F1 (Fragment 1)	F:GCAACTGGCTTCTGGGACAG R:GGTGAACTCTGAGTTGTCTAG	286	58	NC_006089.4
OA F2 (Fragment 2)	F:TAAATTACATTCCTTATCTATTCTGC R:CTGTCCCAGAAGCCAGTTGC	323	60	NC_006089.4

Single-Stranded Conformation Polymorphism and Sequencing

Single-stranded conformation polymorphism (SSCP) analysis was conducted on a 12% native polyacrylamide gel (PAGE) containing a ratio of 50:1 acrylamide and bisacrylamide, supplemented with 5% glycerol. A mixture of 3 µL of PCR product and 15 µL of formamide dye (consisting of 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, and 0.5 M EDTA) underwent denaturation at 95 °C for 5 minutes, followed by rapid cooling on ice for 15 minutes. Subsequently, the denatured product was loaded onto the gel, and electrophoresis was carried out at 4 °C for 12 hours at 200 V. Once electrophoresis was completed, the gel was stained with silver nitrate to visualize the banding patterns of the fragments. All SSCP variants underwent sequencing using fragment-specific primers from both ends to confirm the sequence of each SSCP type. Sequencing was performed using either the ABI 3130 (48 capillary) or 3730Xl (96 capillary) electrophoresis instruments. Each SSCP type was designated as an allele for each fragment, and the alleles from two fragments in each individual were combined to construct haplotypes.

Measurement of traits for association

Age at sexual Maturity (ASM)

The age at sexual maturity was measured from day of hatch till the pullets laid their first egg.

Egg quality traits

Over the course of three consecutive days, eggs were gathered to assess various egg quality traits. These included egg weight (g), dimensions such as length and width (mm), albumen length (mm), albumen width (mm), albumen height (mm), yolk color, eggshell color, shell thickness, eggshell weight, shape index, albumen index, yolk index, and Haugh unit.

Egg Shape Index (%)

The egg's shape index was calculated by multiplying 100 with the ratio of its maximum breadth to maximum length. Measurements of the egg's length and breadth were taken using a Vernier caliper, and the shape index was determined according to the method described by Schultz (1953) [6].

Egg Shell weight (g)

The egg shell was removed, dried and weighed without the shell membrane after the left over albumen.

Egg Shell thickness (mm)

The eggshell thickness (mm) was measured using an AMES micrometer gauge at three distinct points: the narrow, middle, and broad ends of the egg.

Albumen Index (%)

Following the egg's breakage on a flat surface, the height (mm) of the thick albumen was measured at three distinct points using an AMES spherometer, and their average was determined. Additionally, the width of the thick albumen was measured at three different locations using a Vernier caliper, and the average width was computed. The albumen

index was then calculated according to the formula established by Heiman and Carver (1936) [7].

Haugh unit score HU)

The Haugh unit stands as the most commonly employed measure of albumen quality. It is determined by the relationship between egg weight in grams and the height of the thick albumen in millimeters. The albumen's height was assessed at three distinct locations using an AMES spherometer (mm), and the average value was utilized in the calculation of the Haugh Unit, following this formula:

Yolk Index (%)

The roundness of the yolk can be quantified as the yolk index upon separating it from the egg white while maintaining its integrity. Following the measurement of the albumen height, the yolk was delicately detached from the albumen using a blunt knife. Subsequently, the height of the yolk was measured using an AMES micrometer, and the width was recorded using a Vernier caliper. The yolk index was then computed according to the formula developed by Funk (1948) [8].

Yolk colour

Roche yolk colour fan with numerals was used to determine the yolk colour.

Results

The PCR products corresponding to fragments of the gene, measuring 286 bp and 323 bp in size, underwent single-strand conformation polymorphism (SSCP) analysis, followed by silver staining. This revealed a distinctive banding pattern from the second fragment (286 bp) in both the IWI and IWK lines, designated as the "H1" haplotype. In the first fragment (323 bp), four different banding patterns were observed in both populations, labeled as H1, H2, H3 haplotypes. By combining the haplotypes from the two fragments, haplogroups were established and denoted as H1H1, H2H2, H1H3, and H2H3.Representative amplified products belonging to different haplogroups were sequenced using gene-specific primers in both forward and reverse directions. Comparison with the reference sequence identified transitional mutations among the observed haplogroups. The first fragment measured 323 bp and the second 286 bp in both lines. These fragments were combined and analyzed as a single fragment. In haplotype H, a G>A transition was found at position 119 (119G>A). Similarly, in H2 haplotype, a T>C transition at position 160 (160T>C), and in H3 haplotype, a T>C transition at position 216 (216T>C) were observed. The different haplotypes were submitted to GeneBank, with accession numbers MH368655 for H1 haplotype, MH368656 for H2 haplotype, and MH368657 for H3 haplotype.

Association of haplogroups with egg quality and egg production traits

The various haplogroups were examined for their correlation with egg quality and egg production traits in both the IWI and IWK White Leghorn chicken lines. The measurements of different traits are presented in Tables 4.9 and 4.10, along with their least square means (LSM), which were calculated using the Univariate General Linear Model technique. Among all the traits assessed, in the IWI line, the haplogroups showed associations with egg weight and age of sexual maturity (ASM). The H2H2 haplogroup birds exhibited higher egg weight (53.1±0.69 g), whereas the H1H1 haplogroup birds displayed lower egg weight (50.63±0.63 g). Regarding ASM, the H2H3 haplogroup birds had a shorter ASM (139.05±1.96 days), while the H1H3 haplogroup birds had a longer duration of 144.45±1.04 days. The LSM for Haugh unit in H1H3 haplogroup birds was the highest (54.27±1.4), whereas in the H2H2 haplogroup, it was found to be the lowest (48.256±2.423). In terms of albumin index, the H2H3 haplogroup exhibited the maximum LSM among all haplogroups, i.e., 0.058±0.005, while the H2H2 haplogroup had the lowest value of 0.049±0.005.

Discussions

The examination of the single-strand conformation polymorphism (SSCP) patterns allowed for the identification of three haplotypes labelled as H1, H2, and H3, with respective frequencies of 0.47, 0.28, and 0.25 in the IWI line, and 0.47, 0.23, and 0.29 in the IWK line. These formed four haplogroups, namely H1H1, H2H2, H1H3, and H2H3, with frequencies of 0.29, 0.21, 0.36, and 0.14 in the IWI line, and 0.26, 0.14, 0.41, and 0.17 in the IWK line, respectively. Upon thorough examination of the sequence, alterations in the base pair composition of the promoter fragment were observed. The fragment size remained consistent at 589 bp in both lines. In haplotype H1, a G>A transition was detected at position 119 (119G>A). Similarly, in the H2 haplotype, a T>C transition occurred at position 160 (160T>C), and in the H3 haplotype, a T>C transition was observed at position 216 (216T>C). In the IWI line, haplogroups were associated with egg weight and age of sexual maturity (ASM). Birds in the H2H2 haplogroup exhibited a 5.9% higher egg weight than those in the H1H1 haplogroup. Regarding ASM, birds in the H2H3 haplogroup had a 3.73% advantage over those in the H1H3 haplogroup. In the IWK line, haplogroups were significantly linked to the Haugh unit and albumin index, key egg quality traits. The H1H3 haplogroup demonstrated a 12.5% higher Haugh unit compared to the H2H2 haplogroup, indicating superior albumin quality in eggs laid by birds in the H2H3 haplogroup.

Polymorphisms in the ovalbumin gene have been associated with various egg-related traits across different chicken populations. Notably, studies have revealed single-nucleotide polymorphisms (SNPs) influencing traits such as eggshell thickness, hatchability, and egg shell ultrastructure organisation. These findings underscore the importance of the ovalbumin gene as a candidate for marker-assisted selection to enhance egg-related traits in poultry breeding programmes.

Conclusion

In summary, the investigation unveiled three haplotypes (H1, H2, and H3) within the partial promoter region of the ovalbumin gene, with notable associations observed between these haplogroups and diverse egg quality traits in both the IWI and IWK chicken lines. Furthermore, prior studies have underscored the significance of genetic variations in the ovalbumin gene in impacting eggshell

quality and hatchability in poultry, accentuating the gene's importance in breeding initiatives aimed at enhancing egg production and quality. These findings underscore the potential efficacy of employing marker-assisted selection targeting the ovalbumin gene to augment desirable traits in poultry breeding endeavours.

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