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THREE CANDIDATE GENES AND ITS ASSOCIATION WITH QUANTITATIVE VARIATION OF EGG PRODUCTION TRAITES OF LOCAL QUAIL BY USING PCR-RFLP

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ABSTRACT

The aim of study was to investigate the potential candidate gene *SEMA3E, TLX* and *GH* and their association with the economic traits, which is becoming increasingly important in poultry breeding program. A total of 72 genomic DNA samples from three female local lines of quail including (desert, brown and white) were collected to determine the association of genes with reproduction trait using PCR-RFLP Technique. According to growth performance the Best Linear Unbiased Prediction (BLUP) value in selected females for high (H) and low (L) production traits was ranged from -9.2173 to 0.3827. This value is used to estimate body weight at first egg (BWFE), age at first egg (AFE), weight at first egg (WFE), Egg number per Bird (ENPH), Average egg weight (EWTA), and hen day (HD) in three lines. The results indicate that there was a wide intra specific SEMA3E, TLX and GH variability among these local quails which identifying twelve differences genotypes. The identified genotypes for all genes had a significantly (P<0.05) affected on the reproduction trait during first 150 days. The quail's genotype AAABAA always exhibited the largest body weight at first egg in desert H line, while L line of white was greater in Egg number per bird and hen day of the genotype ACCCAC. No significant associations were observed between all loci and age at first egg trait among local quails. The results indicate that there are agreements between BLUP values with PCR-RFLP results to achieve a favorable selection response in reproductive performance of local quail in Kurdistan region, Iraqi.

Key words: Local quail, RFLP, Polymorphism, egg production traits.

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تعدد المظاهر لثلاث جينات المرشحة وارتباطها بالتباين الكمي لصفات إنتاج البيض من السمان المحلي باستخدام تقنية PCR–RFLP

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المستخلص

اجرى البحث لتحديد التراكيب الوراثية لجينات الثلاثة *SEMA3E* و *TLX* و *GH*, وارتباطها بالصفات الاقتصادية التي لها أهمية كبيرة في تحسين برامج تربية الدواجن.تم استخلاص DNA من 72 عينات الدم من ثلاث خطوط محلية للإناث السمان وهي (الصحراء والبني والأبيض) إعتمادا على النتائج أفضل تنبؤ خطي غير متحيز (BLUP) لتحديد ارتباط الجينات بصفات إنتاج البيض لهذه الخطوط باستخدام تقنية PCR –RFLP. تراوحت قيمة أفضل التنبؤ الخطي غير المتحيز (BLUP) بين 9.2173– إلى 0.3827 توضح هذه القيم وجود اختلافات وراثية كبيرة بين افراد طيور السمان المحلي المستخدم في التقدير وزن الجسم عند وضع اول البيضة (BWFE), العمر عند وضع اول البيضة (AFE), الوزن عند وضع اول البيضة (WFE), عدد البيض الكلي لطائر (ENPH), متوسط وزن البيضة (EWTA), وانتاج يومي (HD) في ثلاثة خطوط ضمن هذه الدراسة. تشير النتائج إلى وجود تباين واسع داخل SEMA3E و TLX و GH بين هذه السمانات المحلية التي تعطيها اثني عشر تراكيب وراثية. وإن تركيب وراثي لجميع الجينات له تاثير المعنوي على مستوى (P<0.05) على الصفات الانتاجية خلال 150 يومًا. أظهر النتائج بان التركيب الوراثي AAABAA لها أعلى وزن الجسم عند وضع اول بيضة في خط الصحراء عالي إنتاج (H) , بينما خط الأبيض منخفض إنتاج (L) لها أكبر عدد البيض لكل طائر من التركيب الوراثي ACCCAC. لم يلاحظ أي ارتباط بين جميع المواقع في العمر عند وضع اول بيضة بين السمان المحلية. وعلى ضوء هذه النتائج استخلص بأن هناك التوافق بين قيم BLUP مع نتائج RFLP–PCR للحصول على عائد وراثي المثالي في الأداء الإنتاجي للسمان المحلي في إقليم كردستان العراق.

الكلمات المفتاحية: السمان, RFLP , التباين الوراثي انتاج البيض.

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INTRODUCTION

Quail is one of the poultry species, which have been used in various biological and genetic experiments including meat and egg production. Quail is popular bird model in numerous fields of research because of its small body size, short generation interval (3-4 generation per year), resistance to many common avian disease and high egg production it has been considered as an excellent laboratory experimental bird, less feed and easy maintenance Vali, (29) and Akpa *et al*., (2). Early sexual maturity quails t the age of six weeks, females start to lay eggs, but their full production usually begin at the age of 50 days (about 7 weeks). Females are averagely lay 300 eggs during their entire reproductive period which generally lasts 10-12 months Kayang *et al*., (13), Chełmońska *et al*., (7) and Alkan *et al*., (3). The production of egg is regarded as one of the most performance parameters of laying birds. Despite effective roles of additive genetics on egg production, other factors including age at sexual maturity, bird weight, its nutrition, management and environmental systems might also affect egg production of Japanese quail Daikwo *et al*., (9). The creation of high-performance lines is the main goal of modern breeding in the poultry industry Kulibaba *et al*., (16). Such poultry lines have moderately been of importance to both breeders and farmers. Genetic roles have experimentally been confirmed to affect traits that are associated with both production and reproduction King'ori, (14) and Miazi *et al*., (20). Best Linear Unbiased Prediction (BLUP) has been widely used in genetic evaluation of poultry species Konig *et al*., (15) and Rozempolska-Rucinska *et al*., (23). Traditionally, selection of animals for breeding is based on two types of data pedigree and phenotypes. BLUP combines these data to generate estimated of breeding values Baumung *et al*., (4). DNA based molecular markers for candidate gene studies have been developed and identified for economic traits in several species including poultry. The identification of genetic/ DNA markers and the development of marker assisted selection (MAS) provides an effective approach for genetic improvement programs and enhance-animals ability to adapt to the environment of animals Liu, (18) and Chethan *et al*., (8) .Generally, growth and reproduction of organisms is naturally regulated by growth hormone (*GH*). This polypeptide component is effectively involved in a wide range of physiological activities such as aging, production of egg, reproduction and also body composition Shaw *et al.*, (27). The *GH* gene is located on the tip of the long arm of the chromosome 1. Vasilatos-Younken et al., (30)In addition, egg numbers and laying rates are associated with SacI locus which locates in intron 4 of *cGH* gene Markhous *et al*,, (19). Moreover, a secreted class 3 semaphorinis encoded by *SEMA3E* gene are specifically involved in the repulsion of endothelial cells of vascular beds. This gene product is also contributed in regulating axonal growth and synaptic connectivity leading to gain an adequate of the central nervous system CNS Cariboni *et al*., (6). Moreover, Song *et al*., (28) demonstrated that T-cell leukemia translocation, also known as Hox11 (*TLX*) is considered as an important target in neural development through the progressive regulation of cell cycle in Neural Stem Cells (NSCs). Modern poultry production is generally aimed at elevation of egg production and inhibition of incubation behavior Xu *et al.*, (31). Data from this study can be used as basic input for innovative breeding program to select for reproductive traits associated with three candidate genes (*SEMA3E*, *TLX* and *GH*) which plays an essential role in reproduction, metabolism, and regulation of the immune system. It has been reported that the candidate genes such as Growth Hormone affect on reproductive characteristics of quails Nie *et al*., (22), This study was aimed to investigate the variability of three candidate genes and economically importance traits of local quail in Kurdistan region, Iraq using Restriction Fragment Length Polymorphism (RFLP-PCR).

MATERIALS AND METHODS

Location and morphological measurements

The experiments were carried out at Grdarasha research center, animal resources department, College of Agriculture, University of Salahaddin. For this purpose 363 newly hatched female chicks of three local lines, desert (111), brown (123) and white (125) each line were randomly distributed into ten families; the mating system was in a ratio of one male to three females. The estimated BLUP of %10 (top and bottom) of three female lines was according to their differences in feather color and body weight. The birds were raised in cages and had free access food and water. The egg production performance of quails were recorded as body weights at first egg (BWFE) in g; Age at first egg (AFE) in days; Mean weight of eggs (MEW) in g 150 days, hen days (HD), weights at first egg (WFE) in g and Egg number per Bird (ENPH) at 150 days of age.

DNA extraction

Blood samples were collected from each bird after slaughtering and 1 mL of blood sample was placed in a 3ml of anti-coagulant Tris-ethylene di amine tetra acetic acid (EDTA) tube and stored at -20°C until DNA extraction. Genome DNA was extracted from the blood using a blood DNA extraction kit (GeNet Bio, korea). Quality and quantity of DNA were examined by Nanodrop (1000 UK) spectrophotometer and gel electrophoresis.

RFLP-PCR

The final volume of PCR mixture was 25 µL composed of 10 µL of Green Master Mix (200 µM dNTPs, 25 units/mL Taq polymerase and also1.5 mM MgCl2), 1 µLfor each forward and reverse primer,1 µL of extracted DNA and the final volume was performed by the addition of 13 µL of DNAse free water. Two respective genes (CJA1 and CJA3) were amplified using a particular setting consisted of initial denaturation step at 94ºC for 5 min (one cycle), then it is followed by [94 °C for1 min, annealing step at 60 °C for 1 min, and finally 72 °C for 1 min (32 cycles)]. These steps are followed by a final extension step 72 °C for 5 min. The digestion of 10 µL of PCR product was made using the restriction enzyme Sasazaki *et al.,* (24) with some minor modifications and also based on the instructions of the manufacturer (Thermo Scientific). This process was investigated by gel electrophoresis preparing 2.5% of agarose that was stained with 3 µL of safe dye (Cat. No. B-2010, GeNet Bio, Korea). The agarose gel was run at a constant voltage of 100 V/cm for 45 min. The bands were subsequently visualized by UV transilluminator and the gel photographed (Proxima 2500 Isogene Life science, Netherland).

Statistical analysis

Genotypes of polymorphic loci were determined by direct counting of the bands. The gene frequencies for each locus in each sample were calculated using the following equations:

$$\mathbf{p} = \frac{2(\mathrm{AA})+\mathrm{AB}}{2\mathrm{N}} \qquad \mathbf{q} = \frac{2(\mathrm{BB})+\mathrm{AB}}{2\mathrm{N}}$$

where p = the gene frequency of allele A, q = the gene frequency of allele B and N = the total number of birds tested and tested to Hardy-Weinberg ratios using was calculated using GENPOP software version 4.13 Xu *et al*., (32). The association between genotypes with reproductive traits was investigated using the GLM procedure of SAS software (25) and the genetic effects on average egg weight and total number of eggs laid during first 150 days after flocks maturity (when 5% of the flock are in egg production) were analyzed by following model:

$$Y_{ijk} = \mu + G_i + H_j + E_{ijk}$$

Where: Yijk= observed trait values at 150 day, µ= overall means, Gi= Genotype with a variation for the candidate gene (i = 1-3), Hj=fixed effect of reproductive trait, Eijk=random residual effect. The genotypes effects on reproductive traits were fitted to following equations:

$$Y_{ijklo} = \mu + A_i + S_j + C_k + P_l + \varepsilon_{ijklo}$$

Where: Y ijklo = reproductive traits of o^{th} bird, of i^{th} GH (Ai, i=1, AC, i=2, AB and i=3, CC), of j^{th} SEMA3E (Sj, j= 1, AB, j=2, BC and j=3, CC), of k^{th} TLX (Ck, k =1, AA, k=2, AB, and k=3, AC), of l^{th} all genes combinations (Pl, l=1 ,2,3,4,5,6,7,8,9,10,11,and 12), µ = Population mean, ε_{ijklo} = random error. It was assumed to be normally and independently distributed with mean zero and variance $\delta^2 e$.

For genetics evaluation of bird for various performance traits, Best Linear Unbiased Prediction (BLUP) procedure described by SAS, (25) was applied. The model used for this purpose was the Mixed Model (fixed + random effects) of SAS, (25) software.

Table 1. primer sequences, restriction enzyme used in this study

Gene	Primer Sequence (5’-3’)	Ta(°C)	Enzyme	PCR-RFLP size (bp)	References
SEMA3E	*F:ATACTCCAGCTGAGTGGGGA* *R:CAGAAGTATGAGGGAGATCAG*	60	*Hae III*	412/ 362+50/ 335+77	Sasazaki *et al.*, 2006
TLX	*ACACTAGGAACATAATGGGCT* *RTCACTGTGGCGTTTCAGATT*	60	*PstI*	546/ 404+142	Sasazaki *et al.*, 2006
GH	*F:ATCCCCAGGCAAACATCCTCG* *:CCTCGACATCCAGCTCACAT*	56	*Msp 1*	776/ 529+241	Setiati *et al.*, 2014

F: Forward primer; R: Reverse primer; Ta: Annealing temperature

RESULTS AND DISCUSSION

The estimated Best Linear Unbiased Prediction (BLUP) value of local quail according to high (H) and low (L) body weight was ranged from (-8.6613 to -1.6933g, -9.2173 to -2.4293g and -4.3113 to 0.3827) females of desert, brown and white, respectively at six month. These results indicated that there are big genetic variations among quails for reproductive trait. It means that selection can play a big role in improving egg production trait.

Gene Polymorphism Detection

The fragment sequences amplified with three primers of (SEMA3E, TLX, and GH) genes were expressed differently and the restriction enzymes cut the gene in different locations in three different lines of local quail are shown in Fig. 2. The RFLP pattern of SEMA3E locus observed three different alleles (A, B and C) and three genotypes (AB, BC and CC), while three different alleles (A, B and C) and three genotypes (AA, AB and AC) were found for the TLX locus. Also, GH locus produces two kinds of alleles (A and C) with three genotypes (AA, AC and CC). Polymorphisms SEMA3E/ Hae III gene is indicated two bands: 412 bp and 362 bp for AB genotype, two bands: 362 bp and 50 bp for BC genotype, and two bands: 335 bp and 77 bp for CC genotype. For the TLX/ PstI gene were obtained a single bands 546 bp for the AA genotype, 518bp, 404bp and 142 bp for AC genotype, While the obtained one fragment from GH/MspI was 776bp for the AA genotype, 776bp, 529bp and 241bp for AC genotype ,and 529bp and 241bp for the AC genotype. In agreement with Bozkaya *et al*. (5) who describe the possibility of using *SEMA3E* and *TLX* loci for studying recombination frequencies in the populations of Japanese quails. Out of the eight loci (*SEMA3E*, *IFR1*, *HAL*, *LOC396025*, *UGP2*, *LOC396192*, *TLX and BMP5*), polymorphism was detected in the *SEMA3E* and *TLX* loci; five loci were found to be monomorphic and one locus (HAL) could not be amplified by PCR -RFLP. Similar results were previously reported by Deef, (11) which was performed PCR-RFLP in terms of revealing the genetic characterization and also genetic relationship of the five species of quails. The Common quails are found to be Coturnix coturnix, bobwhite quail Colinus virginianus, and three quails belong to Coturnix japonica including panda quail, japanese quail, dotted white quail. Highly polymorphic restriction profiles were recorded from the analysis of fragments that were generated by digestion of PCR products with the restriction enzyme *NlaIII*. A wide variability in intra specific *COI*, *SEMA3E* and *TLX* genes was obtained among the respective quails. Also, another study discovered that the genetic characterization and relationships between divergence levels of chicken lines as Red Junglefowl (Gallus gallus gallus) and commercial chicken lines (Gallus gallus domesticus) by using PCR-RFLP technique for five genes (*COL1*, *SEMA3E*, *TLX*, *COL2 and COL3*). The results showed that there is a wide intraspecific *COI* (Cytochrom Oxidas I), *SEMA3E* (Semaphorin-3E) and NR2E1 (*TLX*) (Nuclear receptor subfamily 2 group E member 1) defrentional among these chicken lines with restriction enzymes *NlaIII* where this enzyme produced polymorphic intra specific and intere specific restriction fragments in white, Red and Sasso broilers and without any fragments in Red Jungle fowl except *COL2* and *COL3* primers Deef and El-Nabi, (10).

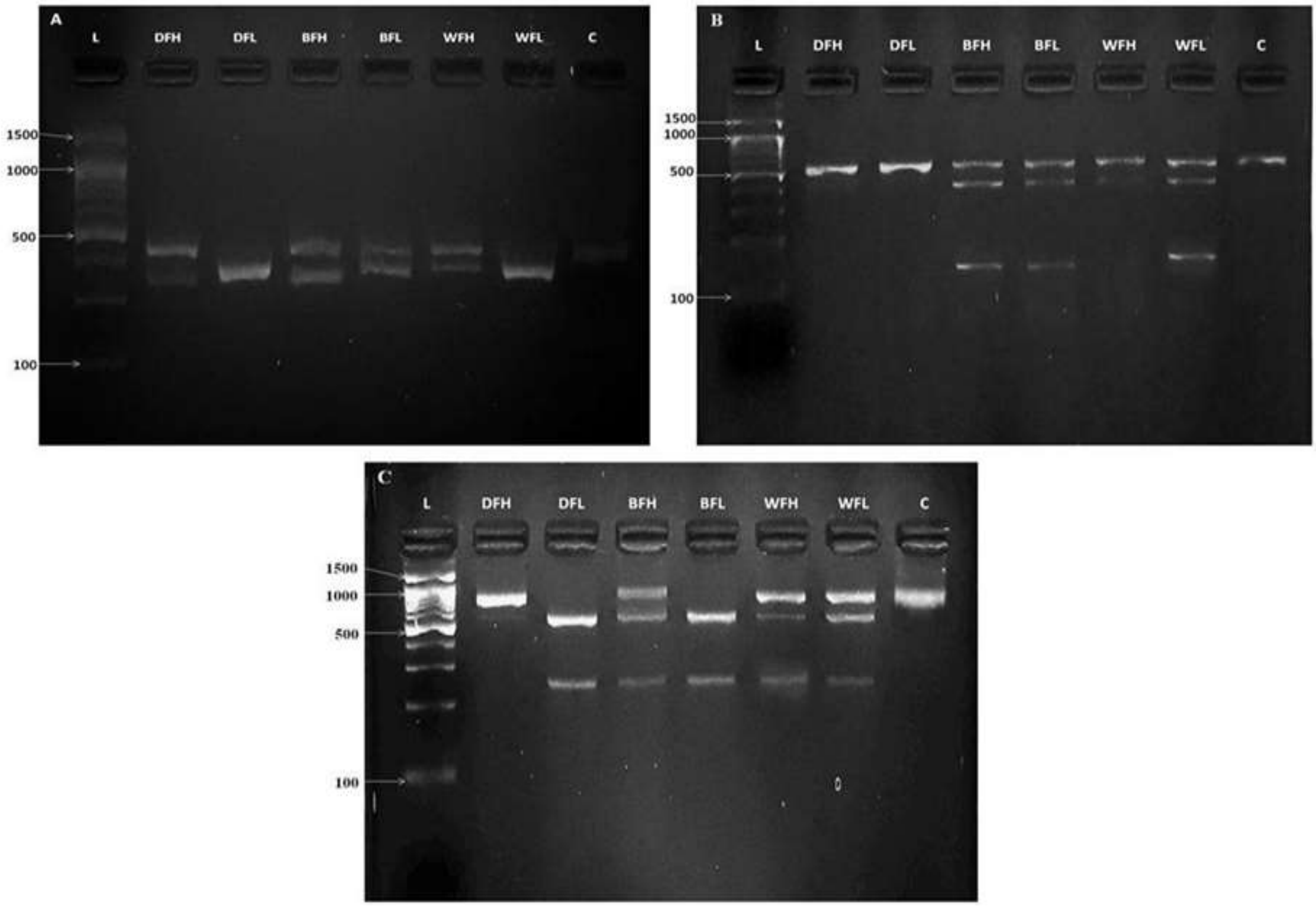

Fig 1. Digestion of PCR products of A) SEMA3E; B) TLX and C) GH of pulled samples in three local lines. L: DNA marker, DFH: desert female high production, DFL: desert female low production, BFH: brown female high production, BFL: brown female low production, WFH: white female high production and WFL: white female low production.

Allele and genotype frequency in local quail populations

The electrophoresis results of PCR-RFLP analysis products that were digested with restriction enzymes are shown in table 2. For SEMA3E and GH loci, allele C was the most frequent allele and ranged from 0.583 to 0.542, while allele A was identified as a dominant allele in TLX locus due to the highest frequency (0.708). Whereas, the frequency of allele B was higher in SEMA3E (0.333) than TLX (0.083) loci. The frequency of AB heterozygous genotype was the lowest (0.113) in SEMA3E and (0.143) TLX loci but the highest frequency of BC genotype was (0.523). The probability of random mating in the population was estimated by Chi-square ($\chi2$) test to examine Hardy-Weinberg equilibrium (HWE) at each locus. The analysis of chi-square test showed that GH loci in (HWE) Hardy-Weinberg equilibrium, while both TLX and SEMA3E loci were not in Hardy-Weinberg equilibrium (Table 2). This is in agreement with the report by Nasirifar *et al*., (21) showed that the genetic variability in growth hormone association with quantitative variation of live weight, carcass traits in Japanese quail , the population was under the Hardy-Weinberg equilibrium ($P<0.005$).

Table 2. Genotypic and allelic frequencies of SEMA3E, TLX and GH genes

	Gene	n	Allele frequency			Genotype frequency			X^2	HWE
Local quail	***SEMA3E***	72	**A**	**B**	**C**	**AB**	**BC**	**CC**		
			0.083	**0.333**	**0.583**	**0.113**	**0.523**	**0.364**	**2.09**	**NS**
	TLX	72	**A**	**B**	**C**	**AA**	**AB**	**AC**		
			0.750	**0. 000**	**0.250**	**0.562**	**0.000**	**0.438**	**1.12**	**NS**
	GH	72	**A**		**C**	**AA**	**AC**	**CC**		
			0.417		**0.583**	**0.174**	**0.486**	**0.340**	**8.89**	*****

***p<0.05, NS: Non-significant**

Genotypes association with egg production traits in local quails

The analysis results of association between the three (SEMA3E, TLX and GH) genes polymorphisms and egg production traits of three local quail lines are shown in Table 3. In accordance to the genotype for all genes in present study showed a significant association with the egg production traits of local quails in 150 days of lying (P<0.05). The highest BWFE was (221.74±3.187) reported in desert H line in AAABAA genotype but lower in WFE (8.33±0.577) and EWTA (13.19±0.853) than H line of white population with genotype ACBCAA, while L line was greater in ENPB (121.94±1.453) and HD (83.77±1.714) of the genotype ACCCAC (P<0.05). Likewise, for the H line in the brown population of the genotype CCBCAC was (38.33±1.528) higher in AFE than desert and white population. Obviously, the genotype for all genes had positive effects on egg production traits in the H and L lines among local quails in our experiments. The association of these genes with egg reproduction traits in local quail was also observed by Setiati *et al*., (26) .who found the effect of divergent selection of high and low weights of the egg production of quail egg traits (*Coturnix coturnix japonica*) to identify gene polymorphisms *GH* for six generations which showed variance between high and low due to selection in earlier generations (P < 0.05), higher weight groups where egg production is lower compared with the low weight group. Lan *et al*., (17) was studied the effect of growth hormone (*GH*) polymorphisms in egg production of Japanese quails detected that the polymorphic sites were no significant effect on egg production and egg numbers. Doan et al., (12) showed that the average egg weights were 11.2 and 11.7 g of Japanese quails, respectively. Makhsousi *et al*., (17) studied growth hormone polymorphism in a native chicken population and the results showed molecular marker association with laying performance has a significant effective on breeding programmers. It can conclude that the *SEMA3E, TLX* and *GH* loci has been shown to be effective marker associations with reproductive performance of local quail lines, in which, AAABAA genotype for all genes are significantly associated with body weight at first egg in desert lines and higher value of egg number per bird. Also the average egg weights at 150 days of age and hen day were higher in genotype ACCCAC in white lines. Evidently, there were no significant differences among the different genotype and lines of age at first egg. And the brown line means tended to be closer to the desert line than the white line for all traits but different from age at first egg and weight at first egg of two lines. This study opens interesting prospects for selection programs in future, particularly marker assisted selection process (MAS).

Table 3. Relationship between genotype of all genes and egg production trait in local quails.

Population	line	Genotype all genes	Association of genes with egg production traits					
			BWFE	AFE	WFE	ENPB	EWTA	HD
Desert	H	AAABAA	221.74 ± 3.187^{a}	39.67 ± 0.577^{a}	8.00 ± 0.000^{ab}	114.44 ± 1.638^{d}	12.76 ± 0.524^{b}	78.34 ± 2.694^{c}
	L	CCCCAA	192.16 ± 2.702^{c}	39.67 ± 0.557^{a}	7.33 ± 0.577^{abc}	116.61 ± 1.317^{bc}	$12.37\pm0.380c^{d}$	80.79 ± 1.248^{b}
Brown	H	ACBCAC	206.00 ± 2.745^{b}	38.33 ± 1.528^{a}	7.67 ± 0.577^{ab}	111.64 ± 1.861^{e}	12.62 ± 0.333^{bc}	76.37 ± 1.239^{d}
	L	CCBCAC	180.02 ± 2.331^{d}	38.67 ± 0.577^{a}	6.33 ± 0.577^{c}	115.85 ± 2.112^{cd}	11.92 ± 0.415^{e}	80.57 ± 1.679^{b}
White	H	ACBCAA	199.91 ± 2.621^{b}	39.00 ± 1.732^{a}	8.33 ± 0.577^{a}	117.85 ± 2.031^{b}	13.19 ± 0.853^{a}	79.82 ± 1.180^{bc}
	L	ACCCAC	177.62 ± 2.447^{d}	39.33 ± 2.082^{a}	7.00 ± 1.000^{bc}	121.94 ± 1.453^{a}	12.18 ± 0.322^{de}	83.77 ± 1.714^{a}

Data are expressed as means ± SE. BWFE = body weight at first egg; AFE = age at first egg; WFE = weight at first egg; ENPH = Egg number per Bird at 150 days of age; EWTA =Average egg weight at 150 days of age; HD= hen day. Different letters in each column represent significant difference according to Duncan multiple range test, (P < 0.05), n=72.

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