INTRODUCTION

Egg productivity is the most important of economic traits in laying poultry (Kim et al., 2004). It includes egg production, egg weight (EW), sexual maturity and the number of recruited dominant follicles that are regulated by various endocrine, paracrine and autocrine factors (Su et al., 1996; Qin et al., 2015a). Many of the modern chicken breeds have been generated by conventional breeding methods (such as self-selection and family selection); with enhanced egg production traits a key focus (Qin et al., 2015b). However, egg productivity is inherited as a polygenic trait, with low to moderate heritability, making the level of genetic improvements difficult to estimate (Luo et al., 2007; Biscarini et al., 2010; Venturini et al., 2013). It has been proposed that a candidate gene approach is a cost-effective means of investigating associations of gene polymorphisms and quantitative trait loci responsible for variations in traits of interest (Rothschild and Soller, 1997; Linville et al., 2001). The identification and utilization of single nucleotide polymorphisms (SNP) in candidate genes associated with genotypes that have significant effects on economically important traits has become increasingly important in chicken breeding programs (Liu et al., 2010; Zhang et al., 2012; Qin et al., 2015b). This technique has...
attracted a growing number of animal breeding researchers in recent years. Nevertheless, more candidate genes and SNPs are required for marker-assisted selection in chicken breeding. Previous studies have demonstrated adrenergic, alpha-1B-, receptor (ADRA1B) and peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PPARGC1B) genes are involved in the regulation of ovarian follicular development, selection and maturation in broilers and rats (Aguado and Ojeda, 1986; Tcherepanova et al., 2000; McDerment et al., 2012), with a potential effect on egg production in laying hens.

Gene ADRA1B encodes alpha-1B adrenergic receptor (α1B ADR) that is a member of the G-protein-coupled family of transmembrane receptors (Allen et al., 1991), located at chromosome 13 in chickens (Gene ID: 373890). Southern blot analysis and nucleotide sequencing indicate that this gene comprises two exons and a single large intron of at least 16 kb in rats or 20 kb in humans that interrupts the coding region at the end of the putative sixth transmembrane domain (Ramarao et al., 1992; Gao and Kunos, 1993). In chickens, due to alternative splicing of pre-mRNA following transcription of primary transcript mRNA, there are at least three spliced variants of ADRA1B transcript (Ping and Faber, 1993; Port and Bristow, 2001). Not only has the α1B ADR been shown to activate mitogenic response and regulate growth and proliferation of many cells (Waldrop et al., 2002; Kodama and Togari, 2013), but also it was suggested that the receptor is implicated for follicle development, selection and maturation in hen ovaries (Aguado and Ojeda, 1986; McDerment et al., 2012).

The PPARGC1B gene encodes peroxisome proliferator-activated receptor gamma (PPARγ), coactivator 1 beta (PPARGC1B, or PGC-1β), it belongs to a small family of nuclear receptor coregulators that coordinate responses to metabolic stimuli and stressors (Handschin and Spiegelman, 2006; Lin, 2009). This gene is located at chromosome 13 and consists of 11 exons that encode 997 amino acids in chicken (Accession No.XP_414479), with its synonymous name as PPARγ coactivator 1 beta (PGC1B) gene, or PPARγ coactivator-1 (PGC-1) related estrogen receptor alpha coactivator (PERC) gene in human and mouse (Kessler et al., 2002; St-Pierre et al., 2003). It was shown that PGC-1, including PPARγ coactivator 1 alpha (PGC-1α) and PGC-1β, is a coactivator of estrogen receptor-alpha (ERα)-dependent transcriptional activity, which may serve as a convergence point between PPARγ and ERα signaling (Tcherepanova et al., 2000). The role of estrogens and ovarian estrogen receptors (ERα, β) in hen folliculogenesis, follicle maturation, ovulation and reproduction has been well established (Drummond et al., 1999; 2012; Hrabia et al., 2004; 2008; Brunström et al., 2009). Therefore, it was inferred that variations of PPARGC1B and ADRA1B gene sequences may greatly influence chicken ovarian development and egg laying performance, although the molecular mechanisms of both of them in regulating follicle development and growth remain unknown. While the Chinese Dagu chicken is an important animal resource, little characterization of ADRA1B and PPARGC1B genetic polymorphisms and possible correlations with egg performance in indigenous Chinese breeds has been performed (Park et al., 2006; Akhmetov et al., 2009; Lee et al., 2011).

In this study, ADRA1B and PPARGC1B fragments were examined for novel sequence polymorphism using a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) approach and sequencing analysis. Associations between the newly identified genotypes and the egg production traits were explored in local Chinese Dagu hens. The aim of this work is to provide a potential genetic molecular marker able to facilitate selection for improved egg production traits in chicken breeding.

MATERIALS AND METHODS

Birds and trait measurements

The Chinese Dagu chickens were provided by College of Animal Science and Technology of Jilin Agricultural University. As reported by Qin et al. (2015b), the eggs used for hatching were randomly selected from the Dagu chicken population, with 360 hens hatched and raised in layered batteries under the same rearing conditions, to include free access to water and feed in accordance to the nutrient requirements of local Chinese Dagu hens (NY/T 33-2004, Beijing, China). Approaching 16 wks of age, hens were reared in individual cages under constantly maintained conditions. All of the birds were exposed to a 16 L:8 D photoperiod, with lights on at 5:00 am. After the start of laying, eggs were collected and recorded daily, with EWs determined on one day in a week. Body weight was recorded following feed and water restrictions at 30 and 43 wks of age, with the individual laying performance calculated. Egg production traits examined in this study included hen-housed egg production (egg laying number) at 30, 43, 57, and 66 wks of age, EW, and body weight at 30 and 43 wks of age. All animal experiments were performed in accordance with laws of the People’s Republic of China regarding animal protection.

DNA samples and PCR amplification

A total of 360 individuals from Chinese Dagu chickens were sampled in this work. For each bird, about 1 mL of blood was collected from the wing vein at 300 days of age and DNA was extracted using a standard phenol-chloroform method. To detect genomic DNA purity, 1% agarose gel electrophoresis and ultraviolet-spectrophotometer assay
were performed, with final concentrations between 2 and 10 ng/µL detected. Primers were designed based the ADRA1B (GenBank accession No. XM_414483.3) and PPARC1B (Accession No. XM_414479.4) gene sequences in chicken. The primer pairs used to amplify the fragments for ADRA1B (forward 5’-TTAACAGAGCGCAACGGACA-3’; reverse 5’-ACAAATAGCTGAAATATGGTCTT-3’) and PPARC1B gene (forward 5’TAGGAAAGGAGGCC GATGT-3’; reverse 5’-AACGCCAGAAGGAAAGAT-3’) were screened after examination in advance.

PCR reactions were performed in a total volume of 50 µL, including: 20 µL of 2X Taq Master Mix, 100 nM of each primer, 25 to 50 ng of template DNA, and 22 µL RNase-free Water. The PCR conditions included: 94°C for 2 min, followed by 35 cycles at 94°C for 30 s for denaturing, 59°C (55°C) for 30 s for annealing of ADRA1B gene (PPARC1B gene), 72°C for 30 s for extension, and a final extension at 72°C for 2 min.

Cloning of PCR products, sequencing and alignment

PCR products were purified with the Wizard prep PCR purification system (Promega, Madison, WI, USA), cloned into the Promega pGEM-T easy vector according to the methods published by Sambrook and Russell (2001), and the obtained fragments commercially sequenced. Two independent PCR amplifications were performed for each sampled bird, with sequences analyzed using BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) to confirm the expected chicken ADRA1B and PPARC1B gene sequences. The obtained sequences were then aligned using the DNAMAN software version 6.0 to identify nucleotide substitutions.

Genotyping by PCR-SSCP and reconstruction of haplotypes

To screen for ADRA1B and PPARC1B gene polymorphism, the confirmed PCR products were further analyzed using the SSCP assay as previously described (Qin et al., 2015b). Briefly, Every 10 µL PCR product was mixed with 6 µL of loading dye (0.025% bromophenol blue, 0.025% xylene cyanol, 98% deionized formamide, 2% glycerin and 10 mM ethylenediaminetetraacetic acid [EDTA]). Denatured at 99°C for 10 min beforehand, the samples were cooled rapidly on ice, and then loaded on 10% polymerized gels (acrylamide:bisacrylamide, 39:1) of size 16 cm×18 cm. Electrophoresis was carried out at 130 V for 7 to 10 h at room temperature in 1×tris-borate-EDTA (TBE) buffer. After silver stain, the gels were examined under upper white by gel photography system (GeneSnap from SynGene). To avoid false positive/negative results due to artificial manipulation in the experiment, each sample was confirmed by repeated amplifications and detections. Haplotypes were reconstructed according to the genotyping data obtained from all 360 individuals with the PHASE program (Stephens et al., 2001).

Polymorphism evaluation

Genotype and allelic frequencies at each SNP site were calculated, with each polymorphism evaluated for Hardy-Weinberg equilibrium using a Pearson's goodness-of-fit chi-square test (degree of freedom = 1). Gene homozygosity (Ho), heterozygosity (He), effective number of alleles (Ne) and polymorphism information content (PIC) were statistically analyzed using the POPGENE v. 1.32 software (Yeh et al., 1997).

Marker-trait association analysis

Associations of single polymorphisms or haplotypes with laying performance traits were analyzed using general linear model procedure in SPSS 18.0. The model was as below:

\[ Y_{ijk} = \mu + L_i + G_j + F_k + e_{ijk} \]

where \( Y_{ijk} \) is the phenotypic value of the target trait, \( \mu \) is the population mean, \( L_i \) is the fixed effect of the line, \( G_j \) is the fixed effect of the SNP genotype or haplotype, \( F_k \) is the random effect of the family and \( e_{ijk} \) are the residuals. Type III sum of squares was used in each test. Values were considered significant at p<0.05 and presented as least square means±standard errors.

RESULTS

Analysis of the nucleotide sequence amplified

A 389 bp PCR amplicon obtained for ADRA1B gene and a 276 bp fragment for PPARC1B were cloned using specifically designed primers, with the obtained fragments sequenced. Comparisons of the predicted sequences with the corresponding GenBank sequences (XM_414483.3 for ADRA1B and XM_414479.4 for PPARC1B gene) were performed using the BLAST software provided by the NCBI server. Primer specificity was confirmed, with PCR amplicon sizes corresponding to the expected sequences of the candidate genes (Figure 1), and further confirmed by comparing the cloned PCR product sequences with the direct genomic PCR products from the same individual. For all of the examined birds, no more than two allelic sequences were observed, suggesting that the primer pair specifically amplified a single gene.

Genotyping by PCR-SSCP and reconstruction of haplotypes

In the study, genotyping of the amplified ADRA1B and
PPARGC1B target fragments was conducted by PCR-SSCP analysis. The typing results showed that there were two genotypes (AA and AG) in the coding region of ADRA1B gene (Figure 2) and two genotypes (TT and TC) in the 3′-untranslated region (UTR) of PPARGC1B gene within the Dagu hen population (Figure 3). Haplotype reconstruction was performed based on these genotype data and four haplotypes (AATT, AATC, AGTT, and AGTC) were identified among the 360 individual hens examined. The haplotype present at the highest frequency was the AATT haplotype (0.55), with the AGTT haplotype the next most frequent (0.18), followed by AATC (0.17), and AGTC (0.10).

Polymorphism of the target sequences

Based on the PCR-SSCP banding patterns of the ADRA1B and PPARGC1B fragments, polymorphism of the target sequences was revealed following sequencing and aligning of the PCR products. For the ADRA1B fragment, an A/G transition at base position 1915 in the exon 2 of ADRA1B gene (Accession No. XM_414483.3) was found by sequencing and alignment. The bird with homotype A1915A was classified as genotype AA, and the heterotype of A1915G named genotype AG basing on the band patterns of the PCR-SSCP and the sequencing information. PCR-SSCP: polymerase chain reaction-single strand conformation polymorphism; ADRA1B: adrenergic, alpha-1B-, receptor; SNP, single nucleotide polymorphism.

For this SNP, the birds sampled were typed as either AA or AG genotypes by PCR-SSCP analysis. In PPARGC1B gene, a T/C transition at base position 6146 in the 3′-UTR of PPARGC1B gene (Accession No. XM_414479.4) was detected by sequencing and alignment. The bird with homotype T6146T was termed genotype TT, and the heterotype of T6146C named genotype TC. PCR-SSCP: polymerase chain reaction-single strand conformation polymorphism; PPARGC1B: peroxisome proliferator-activated receptor gamma, coactivator 1 beta; SNP, single nucleotide polymorphism.

Polymorphism of the target sequences

Based on the PCR-SSCP banding patterns of the ADRA1B and PPARGC1B fragments, polymorphism of the target sequences was revealed following sequencing and aligning of the PCR products. For the ADRA1B fragment, an A/G transition at base position 1915 in the exon 2 of the coding region was detected (Figure 2), and named SNP A1915G. Furthermore, this noted A/G transition leads to a non-synonymous substitution, aspartic acid 489-to-glycine. For this SNP, the birds sampled were typed as either AA or AG genotypes by PCR-SSCP analysis. In PPARGC1B gene, a T/C transition at base position 6146 in the 3′-UTR was identified and named SNP T6146C (Figure 3), with birds typed as either TT or TC following PCR-SSCP analysis.
Allele and genotype frequencies

Genotypic and allelic frequencies for the ADRA1B and PPARGC1B genes are given in Table 1. For the SNP A1915G of ADRA1B gene, the frequency of allele A was notably higher than allele G, with the frequency of genotype AA higher than genotype AG in this population. For the SNP T6146C of PPARGC1B gene, the frequency of allele T was higher than allele C, with the frequency of genotype TT higher than genotype TC. The polymorphism at both sites, A1915G of ADRA1B and T6146C of PPARGC1B, were evaluated and found to exhibit significant genetic disequilibrium between the A and G alleles of ADRA1B and the T and C alleles of PPARGC1B (p<0.05).

As shown in Table 2, gene homozygosity (Ho) was higher than gene heterozygosity (He) for both the SNP A1915G (P1) of ADRA1B or for the SNP T6146C (P2) of PPARGC1B, with effective allele numbers of 1.314 (P1) and 1.288 (P2). The value of PIC for He in P1 (ADRA1B gene) was higher than that of P2 (PPARGC1B gene), but the polymorphism was not higher and only varied from moderate (0.210) to low (0.198).

Association of genotypes with laying performance in Dagu hens breed

The SNP A1915G (ADRA1B) genotype AG was significantly associated with higher hen-housed egg production (HHEP) at 30, 43, 57, and 66 wks of age and with EW at 30 and 43 wks (Table 3; p<0.05). For the SNP T6146C (PPARGC1B), genotype TC was markedly correlated with higher HHEP at 57 and 66 wks of age and with EW at 30 and 43 wks (Table 3; p<0.05). However, no significant difference was observed between AA and AG or between TT and TC regarding body weight at 30 and 43 wks (p>0.05).

Among the four haplotypes, haplotype AGTC was found to be correlated with the highest HHEP at 30 to 66

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**Table 1. Genotypic and allelic frequency at the SNP locus of ADRA1B and PPARGC1B genes in the Dagu chicken population**

| SNP          | Genotype | No. of chickens | Genotype frequency | Allele | Allele frequency | $x^2$ |
|--------------|----------|-----------------|--------------------|--------|-----------------|------|
| A1915G (ADRA1B) | AA       | 260             | 0.722              | A      | 0.861           | 12.69* |
|              | AG       | 100             | 0.278              | G      | 0.139           |      |
| T6146C (PPARGC1B) | TT    | 268             | 0.744              | T      | 0.872           | 7.70* |
|              | TC       | 92              | 0.256              | C      | 0.128           |      |

SNP, single nucleotide polymorphism; ADRA1B: adrenergic, alpha-1B-, receptor; PPARGC1B: peroxisome proliferator-activated receptor gamma, coactivator 1 beta.

* p<0.05 was accepted to be statistically significant when the data were analyzed using a Pearson's goodness-of-fit chi-square test (degree of freedom=1).

**Table 2. Polymorphism information analysis of chicken ADRA1B and PPARGC1B genes in the Chinese local Dagu chicken population**

| SNP          | Gene homozygosity (Ho) | Gene heterozygosity (He) | Effective allele number (Ne) | Polymorphism information content (PIC) |
|--------------|------------------------|--------------------------|-------------------------------|--------------------------------------|
| A1915G (ADRA1B) | 0.761                  | 0.239                    | 1.314                         | 0.210                                |
| T6146C (PPARGC1B) | 0.777                  | 0.223                    | 1.288                         | 0.198                                |

ADRA1B: adrenergic, alpha-1B-, receptor; PPARGC1B: peroxisome proliferator-activated receptor gamma, coactivator 1 beta; SNP, single nucleotide polymorphism.

**Table 3. Association of the polymorphism in chicken ADRA1B and PPARGC1B genes with egg production trait in the local Dagu hen population**

| Egg production trait | Genotypes |
|----------------------|-----------|
|                      | ADRA1B    | PPARGC1B  |
|                      | AA (260)  | AG (100)  |
| (X ± SE)             | (X ±SE)   | (X ±SE)   |
| Body weight at 30 wks (kg) | 2.79±0.43 | 2.77±0.55 |
| Body weight at 43 wks (kg) | 2.94±0.70 | 3.04±0.10 |
| Hen-housed egg production at 30 wks (No.) | 19.00±2.30 | 24.69±2.10 |
| Hen-housed egg production at 43 wks (No.) | 88.97±4.41 | 96.09±5.81 |
| Hen-housed egg production at 57 wks (No.) | 125.73±5.40 | 137.74±5.82 |
| Hen-housed egg production at 66 wks (No.) | 137.45±7.48 | 151.73±6.49 |
| Egg weight at 30 wks (g) | 55.25±0.54 | 56.97±0.59 |
| Egg weight at 43 wks (g) | 57.45±0.56 | 60.17±0.64 |

ADRA1B: adrenergic, alpha-1B-, receptor; PPARGC1B: peroxisome proliferator-activated receptor gamma, coactivator 1 beta; SE, standard error.

The No. represents the total number of eggs at the corresponding age. Numbers in the parenthesis indicate the number of hen individuals in each group. Sample size, n = 360.

* Means within a row for each gene lacking a common superscript differ (p<0.05).
wk of age and highest EW at 30 and 43 wks (Table 4; p<0.05), followed by haplotype AGTT and AATC associated with the higher HHEP at 43 to 66 wks of age, while with highest EW at 43 wks (p<0.05); and with the lowest levels associated with haplotype AATT (p<0.05). Concerning body weight at 30 and 43 wks, there were no significant differences between the four haplotypes (p>0.05).

**DISCUSSION**

China has a wide variety of indigenous chicken resources. Innovatively improving and utilizing the egg production trait is becoming one of the important tasks in the Chinese local chicken industry. However, egg production is inherited as a polygenic trait, with low to moderate heritability (Luo et al., 2007; Biscarini et al., 2010; Venturini et al., 2013), making genetic improvement of this trait more costly based on estimated breeding values, but poorly effective by using traditional breeding method (Qin et al., 2015b). Therefore, genetic factors are destined to play a pivotal role in promoting egg production traits to further this economically important resource. Involved in the process of egg production are not only members of the glycoprotein hormone family of gonadotropins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), but also a wide variety of cellular and tissue-level signal transductions that play critical roles in regulating normal follicular development and oocyte maturation (Qin et al., 2015a). These processes are also controlled by many local intraovarian factors in an autocrine or/and paracrine manner, such as the α1B ADR and PGC-1β proteins (Aguado and Ojeda, 1986; Tcherepanova et al., 2000; McDerment et al., 2012). Polymorphisms associated with egg production related hormones, growth factors, and sex hormones such as FSH beta subunit (FSHb), LH, prolactin (PRL), growth hormone (GH), transcription factor forkhead box L2 (FOXL2) and growth differentiation factor-9 (GDF9) have been intensively studied in chickens (Feng et al., 1997; Cui et al., 2006; Onagbesan et al., 2006; Qin et al., 2015b). However, little is known regarding polymorphisms in chicken *ADRA1B* and *PPARGC1B* genes (Park et al., 2006; Akhmetov et al., 2009; Lee et al., 2011). In an attempt to identify novel DNA markers associated with egg production traits in chickens, we examined polymorphisms in *ADRA1B* and *PPARGC1B* and evaluated their associations with egg production traits in Chinese local hens.

The present work, was the first to provide information about genetic polymorphisms in *ADRA1B* and *PPARGC1B* genes in chickens. The A/G transition in exon 2 of *ADRA1B* gene was identified to bring about a non-synonymous substitution, aspartic acid 489-to-glycine, in the deduced amino acid sequence of ADRA1B receptor. The substitution of amino acid residue was located in intracellular region of the carboxy terminus tail of the receptor (Piirainen et al., 2011). Previous studies in mammals and humans have elucidated the structural feature of the region that is implicated to the intracellular signal transduction of this receptor (Eason and Liggert, 1995; Jewell-Motz et al., 1997; Olah and Stiles, 1998; Shim et al., 2002). It is possible that sequence substitution may influence overall receptor architecture and subsequently disrupt the conformation of additional domains (Jewell-Motz et al., 1997; Olah and Stiles, 2000; Scheer et al., 2000). However, the possible functional alterations of ADRA1B receptor caused by the A/G mutation was unclear, and the potential varied effects on the ovarian follicle development and egg production by the change remain to be further explained in chicken. As for the transition of a T/C at base position 6146 in the 3′-UTR of *PPARGC1B* gene, it is reasonable to assume that a possible MicroRNA (miRNA) regulatory change might occur with the presence of this mutation in the 3′-UTR (Hossain et al., 2009; Yao et al., 2009; Yang et al., 2013), as

| Table 4. Association of different haplotype polymorphism in chicken *ADRA1B* and *PPARGC1B* genes with egg production trait in the local Dagu hen population |
|---------------------------------------------------------------|---------------------------------------------------------------|
| **Haplotype**                      | **Egg production trait** |
|                                  | Body weight at 30 wks (kg) | Hen-housed egg production at 30 wks (No.) | Hen-housed egg production at 43 wks (No.) | Hen-housed egg production at 57 wks (No.) | Hen-housed egg production at 66 wks (No.) | Egg weight at 30 wks (g) | Egg weight at 43 wks (g) |
|                                  | (±SE)                      | (±SE)                                     | (±SE)                                      | (±SE)                                      | (±SE)                                      | (±SE)                      | (±SE)                      |
| **AATT** (198)               | 2.79±0.06                  | 20.64±2.50*                              | 86.62±4.95*                               | 126.27±7.28*                              | 138.18±8.49*                              | 55.87±0.62*                | 57.94±0.64*               |
| **AATC** (61)               | 2.70±0.06                  | 22.00±2.48*                              | 94.50±6.96*                               | 130.86±8.59*                              | 147.63±9.88*                              | 56.27±0.89*                | 59.92±0.73*               |
| **AGTT** (66)               | 2.82±0.07                  | 19.93±3.27*                              | 93.40±5.78*                               | 133.93±8.59*                              | 145.50±8.78*                              | 57.57±1.10*                | 60.89±0.97*               |
| **AGTC** (35)               | 2.81±0.09                  | 25.00±3.35*                              | 102.13±7.25*                              | 148.80±8.44*                              | 160.63±9.74*                              | 58.55±0.97*                | 60.39±1.06*               |

*ADRA1B*: adrenergic, alpha-1B-, receptor; *PPARGC1B*: peroxisome proliferator-activated receptor gamma, coactivator 1 beta; SE, standard error.
The No. represents the total number of eggs at the corresponding age. Numbers in bracket indicate the number of hen individuals in the group.

*ab* Means within a row for each gene lacking a common superscript differ (p<0.05).
any mutations present in the binding sites may reverse the biological effect of the target gene on hen ovary development and growth. In recent years, several miRNAs localized in the first intron of the PPARGC1B gene and in the regulatory region flanking the gene's transcription start site in mouse and rat, such as mir-182/96/183, mir-205, miR-378 and miR-378*, were identified to attenuate the function of PGC-1β protein that is a coactivator of the ERα signaling (Tcherepanova et al., 2000; John et al., 2012; Mallat et al., 2014). Nevertheless, the investigation for a candidate regulator of endogenous miRNA targeting the site might be due to a tight linkage with either an advantageous allele or with an artificially selected economically favorable trait, such as higher egg production. Collectively, the results of the present study strongly suggest that the two novel ADRA1B and PPARGC1B polymorphisms are associated with egg production and EW, thus are potential molecular markers for egg productivity in Chinese Dagu chicken breeding.

**CONFLICT OF INTEREST**

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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