Title Genetic Analysis of HSP70 and HSF3 Polymorphisms and Their Association with Egg Production Traits of Bangladeshi Hilly Chickens

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Article

Title Genetic Analysis of HSP70 and HSF3 Polymorphisms and Their Association with Egg Production Traits of Bangladeshi Hilly Chickens

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Simple Summary: Heat stress is a challenging envsironmental factor affecting poultry performance and adaptation thereto is regulated by heat shock proteins (HSPs). HSP70 expression is controlled by the heat shock factor 3 (HSF3) in chickens. It has been reported that the genetic diversity of these genes is closely associated with the reproductive performance of farm animals. The present study aimed to explore the relationship between single nucleotide polymorphisms (SNPs) in these genes of Bangladeshi Hilly chickens and egg production traits. Sequencing and allele-specific PCR identified two novel SNPs (G-399A and A-68G) in the 5′-flanking region of the HSP70 gene. In addition, already-known SNPs (A258G, C276G, and C1431A) in *HSP70* and two SNPs (A-1388G and A-1703G) in *HSF3* were also genotyped. A population of 150 breeding Hilly chickens was used to analyze the association between SNPs in these genes and egg production traits. Among all the analyzed SNPs, two novel G-399A and A-68G SNPs were significantly associated with egg number and egg weight respectively. Furthermore, all known SNPs except A-1703G were also found to be associated with egg production traits. This suggests that the identified SNPs in these genes might be useful in a selective breeding program to enhance productivity in hot environments.

Abstract: In hot environments, thermoregulation in poultry is regulated by heat shock protein 70 (*HSP70*), a member of HSPs, and its expression is controlled by the heat shock factor 3 (*HSF3*). Although the association between genetic polymorphism of these genes and thermotolerance as well as reproductive traits has been extensively studied in mammals but scarce in chickens. This study aimed to explore the relationship between single nucleotide polymorphisms (SNPs) in these genes and egg production traits of Bangladeshi Hilly chickens. To detect new SNPs and perform genotyping, sequencing and allele-specific PCR (AS-PCR) were used. We identified two novel SNPs(G-399A and A-68G) in the 5′-flanking regions of the *HSP70* that were significantly associated with higher egg numbers(EN) at 161–190 days and increased egg weight(EW) at 40 weeks of age respectively. Furthermore, SNPs A258G, C276G, and C1431A in *HSP70* and A-1388G in *HSF3* also existed and were associated with EN at different ages. Haplotype and combined genotypic effects of these two genes were found to be associated with age at sexual maturity(ASM), body weight at ASM, EN, and EW. Identified SNPs and corresponding haplotypes might be useful in selective breeding to enhance the productivity of chickens in hot environments..

Keywords: chicken; egg production; HSF3; HSP70; SNPs

1. Introduction

In Bangladesh, indigenous poultry farming plays a vital role in providing nutrition to many people, with almost 89 % of rural households engaged in this sector [1]. Commercial strains and

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indigenous chicken breeds contribute almost equal numbers of eggs (50:50) and meat (60:40) to satisfy the national demand in Bangladesh [2,3]. Among the Indigenous chicken breeds, the Hilly breed has exhibited superior disease resistance, early sexual maturity, and higher egg production when compared with nondescriptive Deshi chickens in Bangladesh's hot weather. Additionally, this breed has shown a lower mortality percentage in rearing in rural areas, further highlighting its potential as a promising indigenous chicken breed in Bangladesh [4].

However, because of global climate change, indigenous poultry farming in Bangladesh also faces the economically harmful challenge of high ambient temperatures [5]. During heat stress, birds generally thermoregulate through reduced feed intake, hormonal regulation, and panting; thus, subsequently negatively influencing reproduction [6,7]. Chicken thermoregulation also involves expressing heat shock-related genes such as heat shock proteins (HSPs) and heat shock transcription factors (HSF) [8,9].

The heat shock protein 70 (HSP70) gene is a member of the HSP family and is expressed in almost all types of cells [10]. This gene plays a protective role in various stress responses, including heat stress, and maintains homeostasis by balancing the synthesis and degradation of cellular proteins [11,12]. Previous studies have shown that genetic variations (i.e., single nucleotide polymorphisms (SNPs) in the 5'-flanking region of HSP70 affect the functions of this gene, leading to changes in cellular processes and influencing phenotypic performance [13,14]. Similarly, certain SNPs (e.g., A258G, C276G, and C1431A) in the coding region of chicken HSP70 have been linked to thermotolerance, production, and reproductive performance [15,16]. Moreover, polymorphisms in the 5′-flanking region of HSP70 have also been associated with thermotolerance and reproductive traits in mammals, but the correlation between these SNPs and egg production traits in chickens remains unclear [17–19].

Heat shock proteins, including HSP70, are transcriptionally regulated by HSF3, a member of the HSF protein family in chickens [20,21]. HSF3 mainly regulates the expression of HSP70 and acts as a primary defense against heat stress [21,22]. A previous study found that the genetic polymorphism A-1388G alters the activity of the CdxA transcription binding site, resulting in changes to the promoter activity of the HSF3 gene in chickens [23,24]. This alteration has been associated with heat-resistance parameters in Lingshan chickens, but associations between chicken reproductive traits and SNPs of HSF3 remain unexplored [23].

The poultry industry in Bangladesh, particularly the indigenous chicken breeds, plays a critical role in ensuring food security and nutrition for rural households [25]. However, the increasing impacts of global climate change, specifically rising ambient temperatures, pose significant challenges to the productivity and reproductive performance of chickens [26]. Heat stress negatively affects poultry through reduced feed intake and altered reproductive processes, significantly hindering production [27,28]. Although indigenous breeds like the Hilly chicken exhibit resilience to harsh conditions, the genetic mechanisms underlying their thermotolerance, particularly in relation to HSPs and heat HSFs, remain inadequately explored [29]. Specifically, understanding how genetic variations in key genes such as HSP70 and HSF3 affect reproductive traits under heat stress is crucial to optimizing poultry breeding programs for improved productivity in tropical climates [30,31].

Building on previous studies and the challenges identified by earlier research, the following key research questions arise: What genetic variations (SNPs) are present in the HSP70 and HSF3 genes of the Hilly chicken breed in Bangladesh? How do these SNPs relate to egg production and reproductive traits in chickens exposed to heat stress? Furthermore, can these specific SNPs in the HSP70 and HSF3 genes serve as genetic markers to enhance thermotolerance and improve reproductive performance in chickens?

This research hypothesizes that genetic variations (SNPs) in the HSP70 and HSF3 genes are associated with significant differences in reproductive traits and egg production in Hilly chickens. These variations may serve as potential genetic markers for enhancing thermotolerance and productivity in chickens raised under high ambient temperatures.

The main objective of the study was to identify and characterize genetic variations (SNPs) in the HSP70 and HSF3 genes in the Hilly chicken breed of Bangladesh. The study further aims to evaluate

 doi:10.20944/preprints202410.1037.v1

the associations between these SNPs and reproductive traits, with a particular focus on identifying potential genetic markers that could be used for selecting chickens with enhanced thermotolerance and improved egg production traits in hot environments. The findings of the study could contribute to the development of genetic selection strategies aimed at improving thermotolerance and reproductive performance in poultry, enhancing the sustainability of chicken farming in hot climates.

2. Materials and Methods

2.1. Experimental Birds and Trait Records

A total of 150 female Hilly chickens maintained at Bangladesh Livestock Research Institute (9th generation of the breeding flock) were used in the present study (Figure 1). These hens were reared under the standard management protocol of the BLRI from hatching. At 16 weeks of age, the birds were transferred to separate cages in a naturally ventilated poultry house with a 16-h photoperiod that included 12 h of daylight and 4 h of artificial light. During the laying age period (17–72 weeks), the birds were fed twice daily (morning and evening) with a diet containing 16.33 % crude protein and 2845 Kcal ME/kg DM. They were provided free access to water. From that point until reaching 310 days of age, the following parameters were recorded: the age at sexual maturity (ASM), body weight (BW) at ASM, egg weight (EW) at ASM (g), monthly egg production (number/bird), and EW at 40 weeks of age (g).

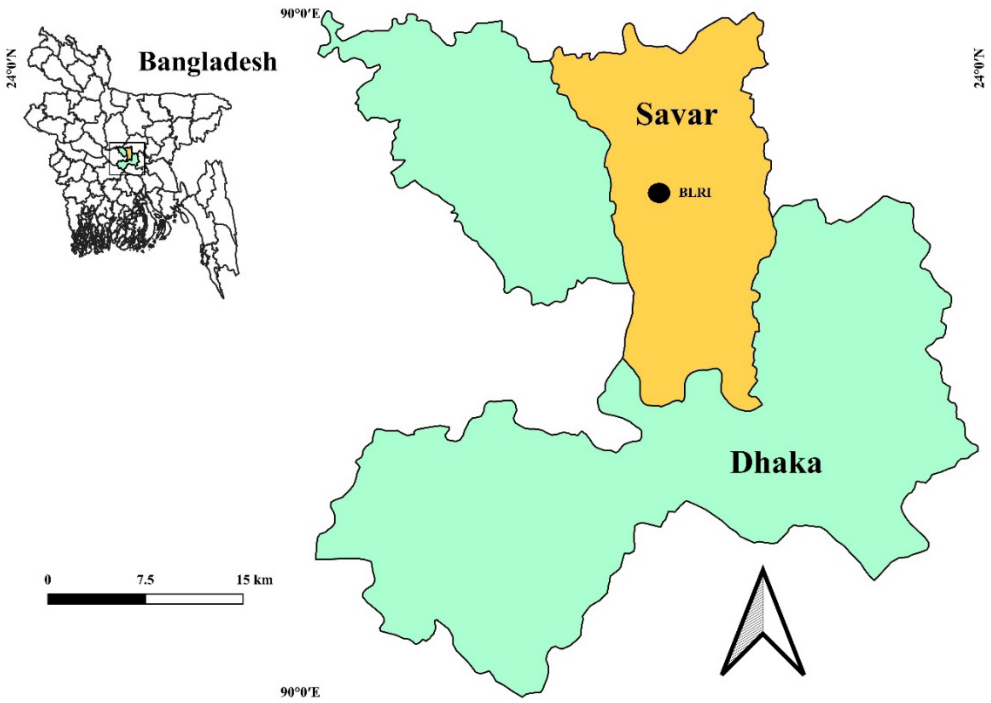

Figure 1. The study is conducted at the Bangladesh Livestock Research Institute (BLRI).

2.2. Blood Collection and Genomic DNA Extraction

Blood samples from mature Hilly hens were collected at 310 days of age and stored on FTA cards (QIAGEN GmbH, Hilden, Germany). Genomic DNA was extracted from the cards according to the manufacturer's instructions. After genomic DNA was extracted, the DNA concentrations were measured using a Bio Spec-Nano (Shimadzu Corp., Kyoto, Japan) and stored at -20 °C for further analysis.

2.3. Primer Design, PCR Amplification, and Sequencing of the HSP70 Fragment

A pair of primers listed in Table 1 were designed using Primer3 software from NCBI utilizing the complete DNA sequence of HSP70 (NC_052536.1). PCR was performed with a 20 μL final reaction

 doi:10.20944/preprints202410.1037.v1

volume containing 100 ng of genomic DNA, 10 µL of 2 × GoTaq Green Master Mix (Promega Corp., WI, USA), and 10 pmol/µL of each primer (HSP70_F_Common and HSP70_R) to amplify the 5'-flanking region of the HSP70 gene. Amplification was conducted for 30 cycles, beginning with an initial denaturation at 94°C for 5 min, then denaturation at 94 °C for 30 s, followed by 30 s of annealing at 64 °C, 30 s of extension at 72 °C, and finally 5 min of final extension at 72 °C. The PCR products were electrophoresed on a 1 % agarose gel and stained with ethidium bromide to visualize the amplicons. The amplified PCR products were purified from the agarose gel using a NucleoSpin Gel and PCR Clean-up Kit (Macherey–Nagel GmbH & Co. KG, Germany). The purified DNA provided the template for direct sequencing utilizing the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA) for each strand primer (HSP70_F/Seq and HSP70_R/Seq) (Table 1). An AB1 3130 sequencer (Applied Biosystems) was used to sequence the products according to the manufacturer's protocol. The sequence data for the 5'-flanking region of HSP70 were edited, assembled, and aligned; and polymorphism detection was conducted using GENETYX ver. 15 (GENETYX Corp., Tokyo, Japan).

Table 1. Primer sequences for sequencing and genotyping of *HSP70* gene.

No	Primer name	Sequence (5′ to 3′)	Anneal. Temp.	PCR cycle	Product length
P1	*HSP70*_R_S1_G	CCAATCACAACGCGCTCTC	64	30	300
P2	*HSP70*_R_S1_A	CCAATCACAACGCGCTCTT			
P3	*HSP70*_R_S2_A	TCGCTCGCAGTCACGTCT		30	630
P4	*HSP70*_R_S2_G	TCGCTCGCAGTCACGTCC			
P5	*HSP70*_F_Com	AGAAGTTGTGTGAGTCGCGA		30	729
P6	*HSP70*_R	AATACGTGGTGCCCAGATCG			
P7	*HSP70*_F_Seq	GTCGCGACCAAATAAGGGTA			
P8	*HSP70*_R_Seq	GTGCCCAGATCGATGCCGATG			
P9	*HSP70*_R_S1_A	GAAGGGCCAGTGCTTCATGTCT	60	30	600
P10	*HSP70*_R_S1_G	GAAGGGCCAGTGCTTCATGTCC			
P11	*HSP70*_R_S2_C	CCTCGTTCACCACACGGAAG	58	30	616
P12	*HSP70*_R_S2_G	CCTCGTTCACCACACGGAAC			
P13	*HSP70*_F_Com	CGATCTGGCTGCAATCTACG			
P14	*HSP70*_R_S3_C	CTATGTCAAAAGTGACCTCG	55	30	198
P15	*HSP70*_R_S3_A	CTATGTCAAAAGTGACCTCT			
P16	*HSP70*_S3_F_Com	AGCGTAACACCACCATTC			

F; Forward primer, R; Reverse primer, Com; Common, Primer sets P1-P6, and P9-P16 were used for the amplification of specific alleles, and primers P7-P8 were used for sequencing the 5′ flanking in *HSP70*.

2.4. Genotyping of SNPs and Reconstruction of Haplotypes in the HSP70 Gene

SNPs were genotyped separately using allele-specific PCR (AS-PCR). The AS-PCR was performed using a 20 µL reaction volume containing 10 pmol/µL of each primer (Table 1) using combinations of P1, P5 or P2, P5 for G-399A; P3, P5 or P4, P5 for A-68G; P9, P13 or P10, P13 for A258G; P11, P13 or P12, P13 for C276G; and P14, P16 or P15, P16 for C1431A SNPs. In the PCR reaction, 100 ng of DNA, 10 µL of 2 × GoTaq Green Master Mix (Promega), and the remainder of the reaction volume were made up with nuclease-free water. The PCR protocol consisted of the following steps: initial denaturation at 94 °C for 5 min, denaturing at 94 °C for 30 s, annealing temperatures, and cycle numbers as in Table 1, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. The PCR product was separated by 1.5 % (>200bp) or 2 % (<200bp) agarose gel depending on the amplicon size and was stained with ethidium bromide. Haplotypes were constructed using population genotyping data (Table 2).

 doi:10.20944/preprints202410.1037.v1

Table 2. Haplotype construction using 5 SNPs in the *HSP70* gene and their frequencies.

Haplotype	Position of SNP					Frequency
	G-399A	A-68G	A258G	C276G	C1431A	
H1	G	A	A	C	C	0.340
H2	G	A	A	C	A	0.039
H3	G	A	G	G	C	0.078
H4	G	A	G	C	A	0.029
H5	A	A	A	C	C	0.029
H6	G	A	A	G	C	0.029
H7	G	G	A	C	C	0.087
H8	G	A	G	G	A	0.068
H9	G	A	G	C	C	0.117
H10	G	G	G	C	C	0.039
H11	G	G	G	C	A	0.039
H12	G	G	G	G	A	0.019
H13	G	G	A	G	A	0.029
H14	G	G	A	C	A	0.029
H15	G	G	G	G	C	0.029

2.5. Genotyping of SNPs and Reconstruction of Haplotypes within HSF3 Gene

Genotyping of SNPs within HSF3 was performed using AS-PCR. The primer (Table 3) combinations of P17, P19 or P18, P19 for the SNP A-1388G; and P20,P22 or P21,P22, for the A-1703G SNP, were utilized. The PCR conditions and mixtures are detailed above. Haplotypes were constructed using genotyping data as mentioned above.

Table 3. Primer sequences for genotyping of *HSF3* gene.

No	Primer name	Sequence (5′ to 3′)	Anneal. Temp.	PCR cycle	Product length
P17	*HSF3*_F_S1_A	GTCCCCATAATACCTCCCCA	60	25	354
P18	*HSF3*_F_S1_G *HSF3*_S1_R_Com	GTCCCCATAATACCTCCCCG			
P19		TTTTAGCTGCCAGTTCCTTT			
P20	*HSF3*_R_S2_ A	TTTTAGCTGCCAGTTCCTTT	59	30	420
P21	*HSF3*_R_S2_G *HSF3*_S2_F_Com	TTTTAGCTGCCAGTTCCTTC			
P22		AAGAATGGCTCCTTGCCACC			

F; Forward primer, R; Reverse primer, Com; Common, Primer sets P17-P22 were used for the amplification of specific alleles in *HSF3 gene*.

2.6. Statistical Analysis

To assess the association between egg production traits and SNPs or haplotypes, a general linear model procedure (GLM) in IBM SPSS Statistics for Windows, ver. 20.0 (IBM Corp., Armonk, NY, USA), and the following equation was employed for the analysis [32].

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Yij is the phenotypic value of the specific traits (e.g., egg production, EW, ASM, and BW), μ is the population mean of the target trait, Gi is the genotype effect (where I = 3 genotypes), and eij is the random residual error associated with the Yij observation. The population in the Hardy–Weinberg Equilibrium (HWE) was fixed using a $\chi 2$ test. The parameter values are presented as the least square means ± standard error, and statistical significance (least significant difference) was evaluated at $P<0.05$. Haplotype frequencies with a minimum threshold of >3 % were considered for the association study.

 doi:10.20944/preprints202410.1037.v1

3. Results

3.1. Identification of Novel SNPs in the 5′-Flanking Region of HSP70

Based on a reference sequence of HSP70 from the NCBI database (https://www.ncbi.nlm.nih.gov/; NC_052536.1), two novel SNPs, G-399A (Table 4 and Figure 2A) and A-68G (Table 4 and Figure 2B) were identified in the 729-bp length of the 5'-flanking region of the HSP70 gene in Bangladeshi Hilly chickens.

Table 4. Genotypic and allelic frequencies with Hardy-Weinberg equilibrium test at the SNPs locus of the *HSP70* gene.

SNPs	Genotype frequency			Allele frequency		$\chi^{2\ (HWE)}$	*P*-value
G-399A	GG	AG	AA	G	A		
	0.92(137)	0.08(12)	–	0.95	0.05	7.44	*P*<0.01
A-68G	AA	AG	GG	A	G		
	0.67(72)	0.22(24)	0.10(11)	0.78	0.22	11.68	*P*<0.01
A258G	AA	AG	GG	A	G		
	0.20(28)	0.77(113)	0.03(5)	0.58	0.42	50.44	*P*<0.00
C276G	CC	CG	GG	C	G		
	0.58(86)	0.39(58)	0.03(5)	0.77	0.23	1.63	*P*>0.05
C1431A	CC	CA	AA	C	A		
	0.74(110)	0.26(39)	–	0.87	0.13	3.42	*P*>0.05

SNP; Single nucleotide polymorphism, *P*<0.05 statistically significant using Pearson's χ^2 test, *P*>0.05; Non-significant, HWE; Hardy-Weinberg Equilibrium.

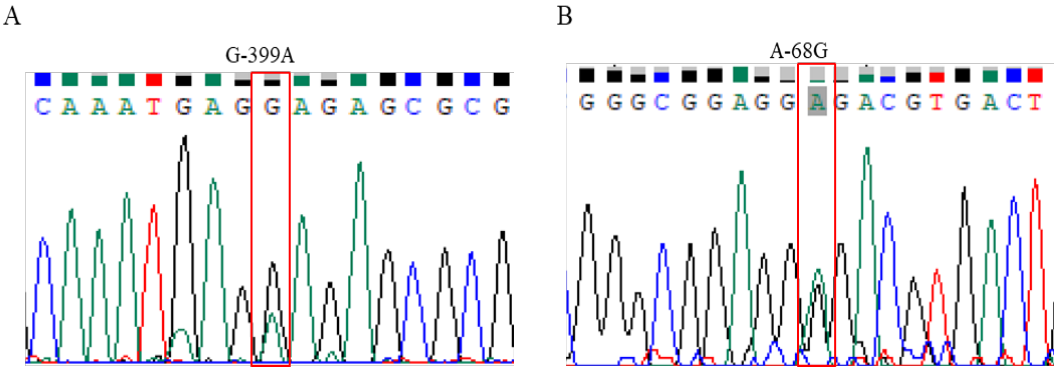

Figure 2. Determination of SNPs in the 5′-flanking region of the *HSP70* gene by sequencing. **(A)** The unique SNP G-399A was detected as multiple peaks at the same positions of 399 bp upstream from the start codon. **(B)** The unique SNP A-68G was detected as multiple peaks at the same positions of 68 bp upstream from the start codon.

3.2. Genotypic and Allelic Frequencies and Haplotype Combinations in HSP70

A total of five SNPs (Table 4) were genotyped, including two novel SNPs, G-399A (Figure 3A) and A-68G (Figure 3B) as well as three previously reported SNPs, A258G (Figure 3C), C276G (Figure 3D), and C1431A (Figure 3E) in the *HSP70* of Hilly chickens.

 doi:10.20944/preprints202410.1037.v1

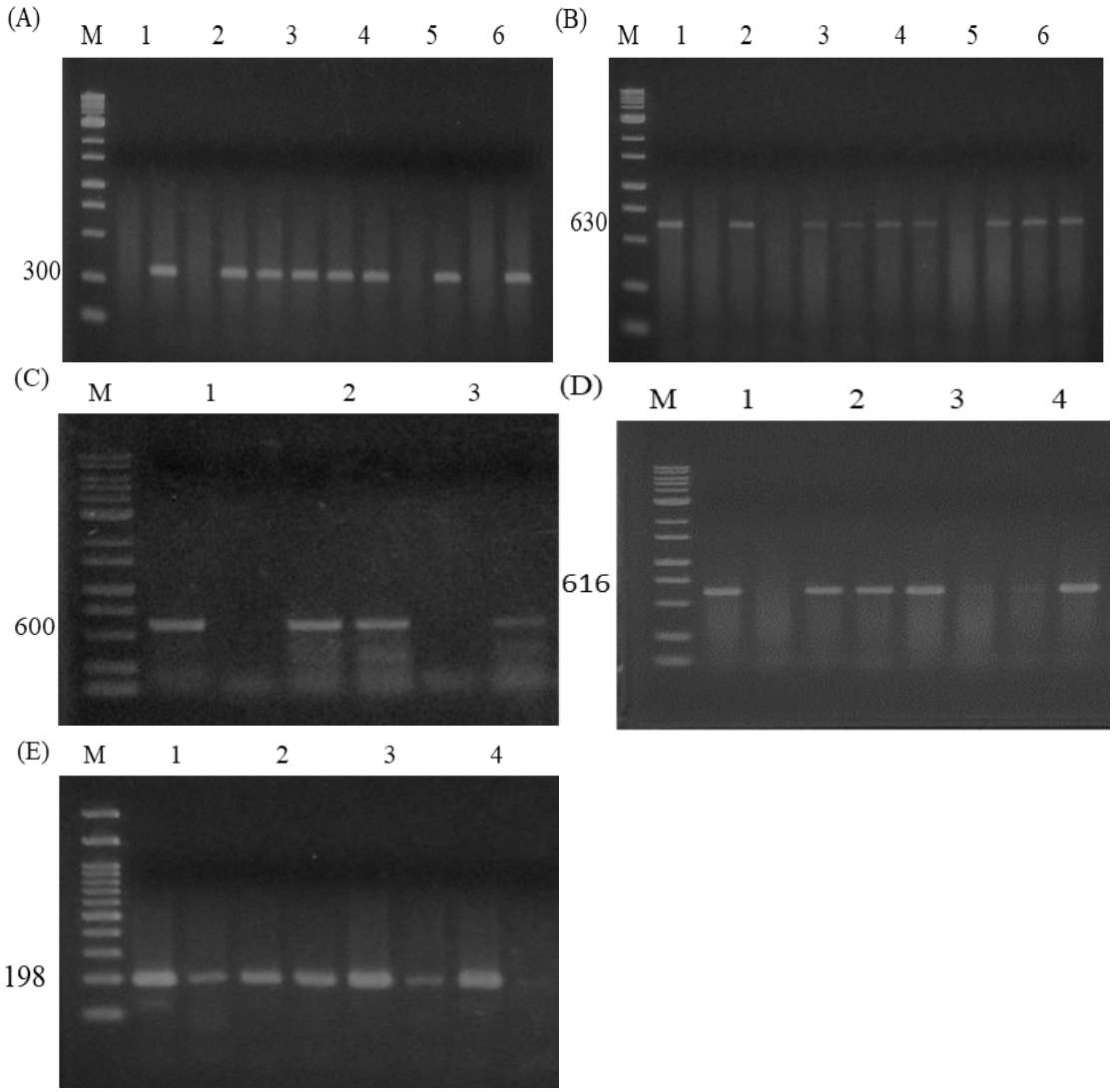

Figure 3. The electrophoresis image of AS-PCR for 5 SNPs including two novel SNPs (G-399A and A-68G) and three known in the coding region (CDs) of the *HSP70* gene. **(A)** Image of AS-PCR for G-399A SNP generated 300bp. Samples 1,2,5 represent wild GG while 3,4 for AG genotypes respectively. M shows a 1kb DNA ladder marker. **(B)** Photograph of AS-PCR for the A-68G SNP, which produced 630 bp. AA (Sample 1, 2), and AG (Sample 3, 4 & 6) while GG (Sample 5) represent mutated homozygous genotypes. M shows a 1kb DNA ladder marker. **(C)** Photograph of AS-PCR for the A258G SNP produced 600bp. AA (Sample 1) represented wild type, while AG (Sample 2) indicates heterozygous, and mutated homozygous GG represented in Sample 3 respectively. M shows a 1kb DNA ladder marker. **(D)** The image of AS-PCR for the C276G SNP yielded 616 bp. Mutated GG (Sample 4) genotypes while, CC (Sample 1&3), and CG (Sample 2) indicate wild and heterozygous genotypes respectively. M shows a 1kb DNA ladder marker. **(E)** Picture of AS-PCR for the SNP of C1431A created 198bp. Heterozygous CA (Sample 1-3) genotype while CC (Sample 4) represents a wild genotype. M shows a 100bp DNA ladder marker.

Table 4 shows the genotypic and allelic frequency for the analyzed SNPs in *HSP70*. For the G-399A SNP, the wild-type GG genotype frequency (0.92) was significantly (P<0.05) greater than those of AG (0.08) genotypes, and the frequency of the G allele (0.95) was notably greater (P<0.05) than its A allele (0.05) counterpart. Regarding the A-68G SNP, the ratio of the AA genotype (0.67) was significantly greater (P<0.05) compared with the AG (0.22) and GG (0.10) genotypes, and the frequency of the A allele (0.78) was greater than that of the G allele (0.22).

The genotypic frequency of AG (0.77) in the previously known SNP A258G was greater (P<0.05) relative to the AA (0.20) and GG (0.03) genotypes. The frequency of the A allele (0.58) was

 doi:10.20944/preprints202410.1037.v1

comparatively greater (P<0.05) than the G allele (0.42) for this SNP. Regarding, the C276G SNP, the CC genotype had a greater (P<0.05) frequency (0.58) than the CG (0.39) and GG (0.03) genotypes. Additionally, an increased frequency of the C allele (0.77) was noted compared with the G allele (0.23). In the C1431A SNP of *HSP70*, only two genotypes, CC (0.74) and CA (0.26), were observed with greater (P<0.05) frequencies for the C allele (0.87) compared with the A allele (0.13). All SNPs, except C276G and C1431A, were outside the Hardy–Weinberg Equilibrium (P<0.05). Based on the genotyping data from the five SNPs, haplotypes were constructed and 15 distinct haplotypes (H1 to H15) were identified within the study population as shown in Table 5. The most common haplotype was H1, with a frequency of 34 %, while the frequencies of the other haplotypes ranged from 0.019 to 11.70 %.

Table 5. Analyzed Single-Nucleotide Polymorphisms(SNPs) in the Chicken HSP70 gene.

SNP name	Mutation	Location	Genomic position
G-399A	G>A*	5'-flanking	52383334
A-68G	A>G*	5'-flanking	52383665
A258G	A>G	Coding	[15]
C276G	C>G	Coding	[15]
C1431A	C>A	Coding	[33]

* Novel SNP found in the HSP70 gene.

3.3. Association between Genotypes and Egg Production Traits for HSP70

Table 6 shows the association of specific polymorphisms in the HSP70 gene with egg production traits in chickens, where significant effects were observed for certain SNPs on traits like egg number and egg weight. Notably, G-399A and A258G were linked to egg production during specific intervals, while A-68G is associated with egg weight at 40 weeks (Table 6).

Table 6. Association of Polymorphisms in *HSP70* Gene with egg production traits.

	Traits (P value of significant test)									
SNPs	**ASM (days)**	**BW at ASM**	**EW at ASM**	**EW at 40 WK**	**EN 130-160d**	**EN 161-190d**	**EN 191-220d**	**EN 221-250d**	**EN 251-280d**	**EN 281-310d**
G-399A	0.890	0.148	0.129	0.660	0.578	0.020	0.421	0.218	0.484	0.225
A-68G	0.365	0.537	0.707	0.009	0.547	0.520	0.478	0.444	0.413	0.186
A258G	0.543	0.593	0.426	0.960	0.561	0.563	0.590	0.016	0.377	0.805
C276G	0.382	0.246	0.275	0.795	0.568	0.304	0.759	0.262	0.222	0.050
C1431A	0.121	0.464	0.651	0.855	0.969	0.681	0.012	0.058	0.341	0.363

Significant; P<0.05, ASM; Age at sexual maturity, EW; Egg weight, EN; Egg number, WK; Week.

The G-399A was significantly (P<0.05) correlated with egg number (EN) at 161–190 days of age. Birds with the GA genotype of this SNP had significantly (P<0.05) greater EN than birds with the GG genotype. The A-68G SNP was significantly associated with EW at 40 weeks of age, and a significantly (P<0.05) higher value was observed in birds with the mutant GG genotype compared with the AA and AG genotypes (Table 7).

Table 7. Genotypic effects of SNPs in *HSP70* gene on egg production traits.

SNPs	Traits	Genotypes (Mean ± SE)			*P* value
G-399A	EN at 161–190d	GG 16.27±0.26^{b}	AG 18.83±0.86^{a}	AA –	0.020
A-68G	EW at 40 wk	AA 46.56±0.11^{b}	AG 47.03±0.18^{a}	GG 47.30±0.27^{a}	0.009
A258G	EN at 221–250d	AA 16.00±0.19^{a}	AG 14.82±0.63^{b}	GG 14.60±1.53^{ab}	0.016
C276G	EN at 281–310d	CC	CG	GG	

 doi:10.20944/preprints202410.1037.v1

		13.01±0.24[ab]	12.67±0.29[b]	13.60±0.93[a]	0.050
C1431A	EN at 191–220d	CC	CA	AA	
		16.49±0.21[b]	17.61±0.39[a]	–	0.012

[ab]Means with uncommon superscript within the same raw differed significantly, $P<0.05$; statistically significant, EW; Egg weight, EN; Egg number.

In the case of the A258G SNP, it was related ($P<0.05$) to the EN at 221–250 days of age. Additionally, the AA genotype hens produced more eggs than the AG and GG genotype hens. The SNP C276G showed a significant ($P<0.05$) association with the EN at 281–310 days of age, and a notably higher EN ($P<0.05$) was observed in hens with the GG genotype compared with those with the CC and CG genotypes. For the SNP C1431A, it was found to be associated ($P<0.05$) with EN at 191–220 days of age, and the birds with the heterozygous CA genotype produced significantly higher EN than the CC genotype birds (Table 7).

3.4. Association of the Haplotypes for HSP70 with Egg Production Traits in the Hilly Chicken

The study considered a total of eight haplotypes (H1 to H3, and H7 to H11) with frequencies of >3 % that were used in the association analysis. These haplotypes were significantly ($P<0.05$) associated with ASM, EW at ASM, EW at 40 weeks, BW at ASM, and EN. Among all these haplotypes, H11 had significantly earlier ASM (days) and greater EN at 130–160 and 161–190 days of age compared with the other haplotypes. Haplotype 2 (H2) significantly correlated with lower BW at ASM and higher EN at 191–220 days of age compared with the other haplotypes. Furthermore, H7 showed significantly higher EW values at ASM and 40 weeks compared with the other haplotypes (Table 8).

Table 8. Association of haplotypes of the *HSP70* polymorphisms with egg production traits in Hilly chicken.

Haplotypes	**Traits (Mean ± SE)**						
	ASM	**EW at ASM**	**EW at 40 wk**	**BW at ASM**	**EN 130-160d**	**EN 161-190d**	**EN 191-220d**
H1(GAACC)	160.71±1.61[ab]	25.85±0.52[b]	46.812±0.16[ab]	1728.52±24.49[b]	2.00±0.74[b]	15.91±0.54[b]	16.03±0.39[b]
H2(GAACA)	156.75±4.68 [ab]	26.00±0.74 [ab]	45.915±0.46[b]	1543.75±77.24[a]	4.50±2.15[ab]	17.25±1.58 [ab]	18.75±1.13[a]
H3(GAGGC)	157.00±5.41 [ab]	26.62±0.52 [ab]	46.356±0.33[b]	1648.25±54.62[ab]	4.25±1.53 [ab]	16.25±1.12 [ab]	16.75±0.80 [ab]
H7(GGACC)	161.44±3.12 [ab]	27.00±0.49 [a]	47.342±0.31[a]	1839.55±51.49[c]	4.33±1.44 [ab]	14.66±1.05 [ab]	16.44±0.75 [ab]
H8(GAGGA)	165.28±3.54[b]	25.85±0.55 [ab]	46.526±0.35 [ab]	1688.85±58.39[abc]	1.71±1.63 [ab]	15.57±1.19 [ab]	17.57±0.86 [ab]
H9(GAGCC)	158.88±3.12 [ab]	26.33±0.49 [ab]	46.399±0.31[b]	1778.88±51.49[c]	2.66±1.43 [ab]	17.22±1.05 [ab]	17.11±0.75 [ab]
H10(GGGCC)	159.25±4.68 [ab]	26.75±0.74 [ab]	47.125±0.46 [ab]	1711.25±77.24 [abc]	2.75±2.15 [ab]	16.75±1.58[ab]	16.75±1.13 [ab]
H11(GGGCA)	153.25±4.68[a]	27.00±0.73 [ab]	46.853±0.46[ab]	1685.25±77.24 [abc]	7.00±2.16[a]	18.25±1.58[a]	18.25±1.13[ab]

[abc] Within a column with no common superscript differed significantly ($P<0.05$). ASM; Age at sexual maturity, EW; Egg weight (g), BW; Body weight (g), EN; Egg number/bird/month, WK; Week.

3.5. Genotypic and Allelic Frequencies and Haplotype Combinations in HSF3

Two previously known SNPs, A-1388G (Figure 4A) and A-1703G (Figure 4B), in the 5'-untranslated region (UTR) of the HSF3 gene, were genotyped in the studied flock.

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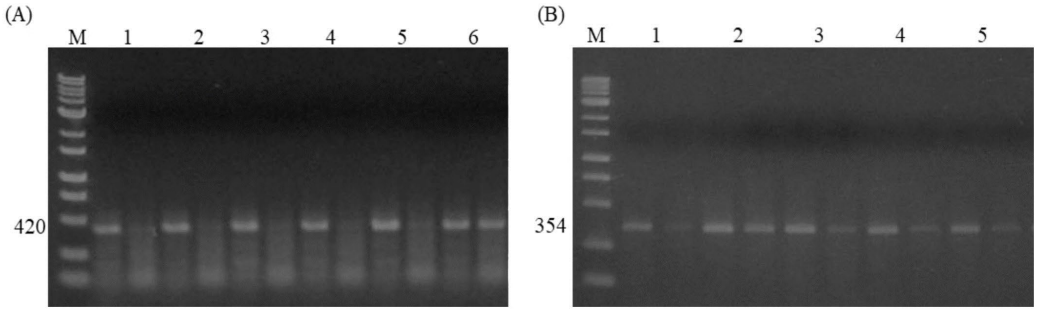

Figure 4. The electrophoresis image of AS-PCR for two SNPs in the 5′-UTR region of *HSF3* gene **(A)** Photograph of AS-PCR for the A-1388G SNP produced 420bp. Only two categorized genotypes, AA (Samples 1-5) and AG (Sample 6) were found. M shows a 1kb DNA ladder marker. **(B)** Image of AS-PCR for the SNP A-1703G produced 354bp. Samples 1,3,4,5, represent AA while sample 2 indicates AG genotype. M shows a 1kb DNA ladder marker.

Table 9 presents the genotype and allele frequencies and reveals that the A-1388G SNP showed only two categories of genotypes, with the dominant AA genotype (0.94) remarkably greater than the AG genotype (0.06). The percentage of allele A (0.97) was much higher compared with the G allele (0.03), and the allocation of genotypes in the studied flock did not conflict with the HWE (P>0.05).

Table 9. Genotypic and allelic frequencies with Hardy-Weinberg equilibrium (HWE) test at the SNPs locus of *HSF3* gene.

SNPs	Genotype frequency			Allele frequency		$\chi^{2\ (HWE)}$	*P*-value
A-1388G	AA	AG	GG	A	G		
	0.94(141)	0.06(9)	–	0.97	0.03	0.143	P>0.05
A-1703G	AA	AG	GG	A	G		
	0.91(136)	0.09(14)	–	0.96	0.04	6.88	P<0.01

SNP; Single nucleotide polymorphism, P<0.05 statistically significant using Pearson's χ^2 test, P>0.05; Non-significant, *HWE*; Hardy-Weinberg Equilibrium.

Regarding the A-1703G SNP, the frequency of the AA genotype was notably greater (0.91) than the AG genotype (0.09), with a significantly greater frequency of the A allele (0.96) compared with the G allele (0.04). Additionally, according to the χ2 test, this SNP deviated from the HWE (P<0.05). The genotype data was used to perform haplotype reconstruction, revealing the existence of only two haplotype categories: H1(AA) and H2(AG), among the 150 individual birds that were examined. Among these haplotypes, H1 was the most frequently observed with a frequency of 85 %, while H2 was observed in only 15 % of the individuals.

3.6. Association between Genotypes and Egg Production Traits and HSF3

The significant association analyses between the SNPs and egg production traits are shown in Table 10. For the SNP A-1388G, a significant (P<0.05) association was found in the EN at 130–160 days of age, with a greater value observed in the AG genotype compared with the AA genotype.

Table 10. Effects of SNPs in *HSF3* gene on egg production.

SNPs	Traits	Genotypes (mean ± SE)			*P* value
A-1388G		AA	AG	–	
	EN at 130–160d	2.85±0.36^{a}	5.55±1.22^{b}	–	0.037
	EN at 251–280d	11.04±0.25^{a}	8.82±0.84^{b}	–	0.013

^{ab}Means with uncommon superscript within the same raw differed significantly, P<0.05, EN; Egg number.

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However, a significantly reduced EN was also found at 251–280 days of age for this SNP compared with the AA genotypes. Lastly, the A-1703G SNP did not show any significant (P<0.05) correlation with egg production traits in the studied flock (Table 11).

Table 11. Association of polymorphisms in *HSF3* gene with egg production traits.

SNPs	Traits (P value of significant test)									
	ASM (days)	**BW at ASM(g)**	**EW at ASM(g)**	**EW at 40 wk(g)**	**EN 130-160d**	**EN 161-190d**	**EN 191-220d**	**EN 221-250d**	**EN 251-280d**	**EN 281-310d**
A-1388G	0.071	0.948	0.679	0.450	0.037	0.803	0.083	0.913	0.013	0.200
A-1703G	0.363	0.484	0.406	0.510	0.731	0.572	0.342	0.569	0.598	0.818

Significant; P<0.05, ASM; Age at sexual maturity, EW; Egg weight, EN; Egg number, WK; Week.

3.7. Association of HSF3 Haplotypes with Hilly Chicken Egg Production Traits

A significant association (P<0.05) was observed between haplotypes and ASM and EN at different ages. Compared with Haplotype H1, Haplotype H2 exhibited significantly earlier ASM (days) and higher EN during the 130–160 days of age period. In contrast, Haplotype H1 showed a significant correlation with higher EN during the 251–280 days of age period compared with Haplotype H2 (Table 12).

Table 12. Association of haplotypes in *HSF3* gene with egg production traits in Hilly chicken.

Haplotypes	Traits (Mean ± SE)						
	ASM	**EW at ASM**	**EW at 40 wk**	**BW at ASM**	**EN 130-160d**	**EN 161-190d**	**EN 251-280d**
H1(AA)	159.95±0.76^{b}	26.14±0.14	46.77±0.08	1746.38±14.54	2.76±0.35^{b}	16.48±0.27	11.03±.25^{a}
H2(AG)	155.44±1.78^{a}	25.96±0.33	46.85±0.18	1704.08±34.18	4.60±0.82^{a}	16.61±0.64	9.52±.59^{b}

^{ab} within a column with no common superscript differed significantly (P<0.05). ASM; Age at sexual maturity, EW; Egg weight (g), BW; Body weight (g), EN; Egg number/bird/month, WK; Week.

3.8. Evaluation of Combined Genotypic Effects of SNPs G-399A and A-68G in HSP70 with A-1388G SNP in HSF3 on Egg Production Traits

We analyzed the effects of combined genotypes of G-399A with A-1388G SNP and A-68G with A-1388G SNP on the phenotypic performance of the studied population. Birds with wild/heterozygote combinations for G-399A and A-1388G SNPs showed significantly (P<0.05) earlier ASM and higher EN at 130 – 160 days of age compared to wild/wild. BW at 40 weeks, EN at 161 – 190, and 251 – 280 days of age were also significantly (P<0.05) influenced by different combinations of these two SNPs (Table 13).

Table 13. Combined genotypic effects of two SNP(G-399A) in HSP70 and SNP(A-1388G) in HSF3 genes on productive and reproductive performances.

Parameter	Genotype (Mean±SE)				P value
	WildxWild(GGxAA) 0.84(120)	**WildxHet(GGxAG) 0.06(9)**	**HetxWild(AGxAA) 0.08(11)**	**MutxWild(AAxAA) 0.01(2)**	
ASM(d)	160.08±0.79^{b}	154.11±2.90^{a}	156.0±2.63^{ab}	157.0±6.16^{ab}	0.05
EW at ASM(g)	26.16±0.15	25.56±0.53	25.91±0.48	27.0±1.13	0.28
BW at ASM(g)	1748.71±14.11	1730.89±51.51	1672.91±46.60	1715.0±109.27	0.74
BW at 40 Wks	2047.77±24.65^{a}	1947.78±90.0^{ab}	1838.73±81.41^{b}	2260.5±190.92^{a}	0.02
EW at 40 Wks	46.78±0.08	46.39±0.30	46.95±0.28	46.72±0.65	0.23
EN at 130-160d	2.71±0.36^{b}	6.11±1.33^{a}	3.64±1.20^{ab}	3.49±2.82^{ab}	0.02
EN at 161-190d	16.26±0.28^{b}	16.89±1.02^{ab}	18.91±0.92^{a}	16.50±2.16^{ab}	0.01
EN at 191-220d	16.85±0.20	15.44±0.73	16.45±0.66	18.5±1.55	0.07
EN at 221-250d	15.13±0.19	15.0±0.71	14.91±0.64	12.50±1.51	0.86
EN at 251-280d	11.14±0.25^{a}	8.56±0.92^{b}	10.18±0.84^{ab}	12.5±1.96^{ab}	0.01

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EN at 281-310d	13.0±0.19	13.67±0.69	11.91±0.63	13.0±1.48	0.36

[abc] Means different superscripts within the same row differed significantly, Significant; *P*<0.05, Non-significant *P*>0.05, Het; Heterozygous, Mut; Mutant, ASM; age at sexual maturity, EN; egg number/month, EW; egg weight, BW; body weight, Wks; weeks, HW; hatched weight.

Regarding the combined effects of A-68G and A-1388G SNPs, birds with any mutant combinations within these SNPs significantly (*P*<0.05) influenced the EW compared to the combinations with wild/wild (Table 14).

Table 14. Combined genotypic effects of A-68G SNP in HSP70 and A-1388G SNP of HSF3 genes on productive and reproductive performance.

Parameter	Combined genotypes(Mean±SE)				*P* value
	WildxWild(AAxAA) 0.62(65)	**WildxHet(AAxAG) 0.07(7)**	**HetxWild(AGxAA) 0.22(23)**	**MutxWild(GGxAA) 0.09(10)**	
ASM(d)	160.56±1.11	156.42±3.29	160.18±1.86	164.0±2.76	0.37
EW at ASM(g)	26.27±0.18[b]	25.85±0.56[b]	26.09±0.31[b]	27.4±0.47[a]	0.03
BW at ASM(g)	1731.06±20.81	1757.85±61.92	1763.36±34.93	1834.1±51.81	0.31
BW at 40 Wks(g)	2008.54±33.73	2075.0±100.38	2027.14±56.62	2093.5±83.98	0.77
EW at 40 Wks(g)	46.56±0.12[b]	46.49±0.34[ab]	47.07±0.19[a]	47.33±0.29[a]	0.03
EN at 130 to 160 d	2.17±0.48	4.43±1.42	3.0±0.80	2.1±1.19	0.43
EN at 161 to 190 d	16.45±0.42	16.86±1.26	16.27±0.71	14.9±1.05	0.55
EN at 191 to 220 d	16.66±0.28	16.43±0.84	16.95±0.48	16.5±0.70	0.92
EN at 221 to 250 d	15.05±0.28	15.0±0.84	15.36±0.47	14.7±0.70	0.88
EN at 251 to 280 d	11.56±0.37[a]	8.57±1.09[b]	10.32±0.62[ab]	10.5±0.92[ab]	0.01
EN at 281 to 310 d	13.08±0.23[b]	14.57±0.69[a]	12.45±0.39[b]	12.9±0.58[ab]	0.04

[abc] Means different superscripts within the same row differed significantly, Significant; *P*<0.05, Non-significant *P*>0.05, Het; Heterozygous, Mut; Mutant, ASM; age at sexual maturity, EN; egg number/month, EW; egg weight, BW; body weight, Wks; weeks, HW; hatched weight.

4. Discussion

High ambient temperatures negatively affect livestock reproduction, and this impact may be exacerbated by ongoing global warming, particularly for backyard poultry farms. Using heat-resistant markers in animal breeding programs proves beneficial to efforts aimed at improving productivity in hot climates [34,35]. Heat shock protein 70 (HSP70) is one of the most common biological response markers of thermal stress and participates in numerous physiological processes including protein folding, transportation, and assembly within the cells [36,37].

It also protects the cells by preventing the apoptotic pathway which might positively impact animal health and productivity [38]. The present study investigated SNP identification in the HSP70 and HSFF3 genes which regulate the transcription of HSP70 and their association with the reproductive traits of Bangladeshi Hilly chickens.

In this study, analysis of the HSP70 gene in Hilly chickens exhibited heterogeneity in the 5'-flanking region. Genetic variations observed in the 5'-flanking region of the HSP70 gene are caused by the transitions of the nucleotides at positions -399 and -68 bp 5'-upstream from the start codon. The 5'-flanking region of HSP70 is polymorphic and several SNPs have also been reported in the broiler, Naked Neck, and Indonesian local chickens [18,39,40]. Furthermore, three previously reported synonymous SNPs in the coding region of this gene (A258G, C276G, and C1431A) were also found in the studied flock. All the SNPs in HSP70, except C276G and C1431A, were outside Hardy–Weinberg equilibrium (Table 3). This might be due to the selection and breeding strategy used to improve the studied flock.

Nevertheless, the present study revealed a significant association between the tested HSP70 SNPs and specific egg production traits among Hilly chickens (Table 6). The novel G-399A SNP was found to be significantly correlated with greater EN (*P*<0.05), and A-68G showed a significant association with increased EW (*P*<0.05) (Table 7). These associations indicate that genetic variations

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in HSP70 may influence egg production efficiency in chickens in hot environments. Similar effects associated with HSP70 SNPs in the 5'-flanking region were previously reported in other vertebrates. This includes the finding that several SNPs in the 5'-flanking region of the HSP70 gene were associated with mRNA stability, stress response, milk production, and calf weaning weight in cattle [14,17]. Variations in the 5'-flanking region of HSP70 may alter the specific binding site of the transcription factors and could modulate the binding efficiency that affects gene functions. This could lead to changes in cellular processes and ultimately alter phenotype performance [14,41]. The precise mechanism of how HSP70 affects egg production in chickens remains unknown. However, a possible explanation might be that during heat stress, HSP70 regulates thermotolerance that inhibits apoptosis in ovarian cells, which may lead to protecting granulosa cells and ultimately improving folliculogenesis and egg production [42–44]. Therefore, further physiological research is required.

Regarding the three examined synonymous SNPs of HSP70, the AA genotype of A258G had higher EN (P<0.05) at 221–250 days compared with other genotypes. The AA genotype of the same SNP was significantly associated with improved thermotolerance and BW at an early stage, but not EW or EN until 280 days of age in Taiwanese chickens [16]. This discrepancy might be due to differences in chicken breeds or environmental factors such as temperature and duration of heat stress exposure. Furthermore, the GG genotype of C276G SNP was significantly associated with greater EN (Table **7**), and in a previous study the C276G polymorphism was found to produce a novel haplotype in combination with the A258G SNP reported as a heat stress marker in Indonesian Walik chickens [15]. These silent mutations in the coding region of the HSP70 gene have been previously reported as heat-resistant markers in chickens [45]. However, this earlier study was not an association study considering reproductive performance in chickens. In the present study, the C1431A SNP was significantly correlated with increased EN as shown in Table 7. A significant association between this SNP and EW, fertility, and hatchability percentage was previously observed in Iranian Mazandaran native breeder chicken but the authors did not report the association of this SNP with EN [33].

Although important egg production traits such as ASM, EW at ASM, and BW at ASM were not correlated with any individual HSP70 SNPs (Table 6), the haplotype combinations resulting from five SNPs significantly affected these traits (Table 8). This suggests that the individual effects of these SNPs are relatively small compared with their combined effects. In chickens, combined genotypes have a greater impact on BW than individual genotypes [46].

Regarding the SNPs in the HSF3 gene, although the A-1703G SNP did not significantly affect egg production traits in the studied flock, the A-1388G SNP was found to be significantly associated with EN (Table 11). Notably, A-1388G polymorphism has been found to change the CdxA transcription factor binding site associated with thermotolerance in Chinese Lingshan chickens [23]. Haplotype analysis of HSF3 also revealed significant associations with several egg production traits, including EN and ASM for H2 (Table 12). Laying hens with the H2 haplotype exhibited earlier ASM and significantly greater EN compared with hens with the H1 haplotype. However, it is noteworthy that EN was reduced at a later stage (251–280 days) for H2, indicating that this haplotype may be less advantageous for long-term egg production.

Our findings revealed significant associations between specific SNPs within the *HSP70* and *HSF3* genes and egg production traits in the studied population. Moreover, analyzing the combined effects of two novel SNPs (G-399A and A-68G) in HSP70 and the A-1388G SNP in HSF3 genes on phenotypic performance, we found a significant interaction that suggests that these genes may play a synergistic or compensatory role in egg production in the hot environments.

5. Conclusions

In conclusion, two novel SNPs (G-399A and A-68G) were identified in the 5'-flanking region of *HSP70* in Hilly chickens, and three previously known SNPs (A258G, C276G, and C1431A) of *HSP70* were also existed in *HSP70* in the same breed. Moreover, HSF3 also possessed two reported SNPs (A-1388G and A-1703G) in this studied breed. All the SNPs in both genes, except A-1703G in HSF3, and their corresponding haplotypes, were associated with egg production traits in Hilly chickens. These significant SNPs and haplotypes might be available in the future for molecular marker-based

selection programs to enhance Hilly chickens' egg productivity in the high ambient temperature experiences in Bangladesh. Further research is needed to elucidate the precise mechanisms enabling HSPs to improve chickens' reproductive performance.

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