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# Dynamic Expression of *HSP90B1* mRNA in the Hypothalamus of Two Chinese Chicken Breeds under Heat Stress and Association Analysis with a SNP in Huainan Chickens

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**ABSTRACT**

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The effects of heat stress on *HSP90B1* messenger RNA (mRNA) expression in the hypothalamus of chicken were investigated and *HSP90B1* variations were detected. Females of two Chinese chicken breeds (Huainan and Wenchang) were used for the experiments. At 64 days of age, the ambient temperature (24 ± 1°C) was increased to 35 ± 1°C for 24 h (heat stress), then decreased to 24 ± 1°C for 24 h (recovery). Hypothalamus samples were collected at 0, 12, and 24 h during heat stress, as well as 12 and 24 h during recovery. The *HSP90B1* mRNA expression increased significantly during heat stress and significantly decreased during recovery being higher in Huainan chickens. Fifteen primer pairs were designed to amplify the exons of *HSP90B1* by a polymerase chain reaction, and single nucleotide polymorphisms (SNPs) were detected by Sanger sequencing. In Huainan chickens, we identified a SNP (NC_006088.3:g.6798G>A) in exon 14 of *HSP90B1* which did not cause amino acid variation but caused a codon for glutamic acid change from GAG to GAA. The frequencies for genotypes *AA*, *GA*, and *GG* were 0.49, 0.27, and 0.24, respectively. Individuals with the *GG* genotype survived heat stress at 42°C for a longer time (248.2 ± 39.3 min) than individuals with *GA* and *AA* genotypes, which survived for 227.2 ± 44.5 min and 179.3 ± 36.5 min, respectively. The results suggested that the increased heat tolerance was associated with the higher expression of *HSP90B1*, and genotype *GG* could be used as a potential marker for heat resistance in chickens.

**Keywords**: heat shock protein; mRNA level; single nucleotide polymorphism; thermal stress

Heat stress is one of the main limiting factors in poultry production because of its adverse effects on feed intake, growth rate, egg production, hatchability, and mortality of birds (Bartlett and Smith 2003; Ryder et al. 2004). This can be a tremendous economic burden in the livestock industry (St-Pierre et al. 2003). Many researchers have investigated the effect of high environmental temperature on a variety of physiological and biochemical indices in different poultry breeds, including the Red Jungle and Village Fowl (Soleimani and Zulkifli 2010), the Ross Broiler (Cooper and Washburn 1998), and

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the Beijing-You chicken and the commercial AA broiler (Lu et al. 2007), and have confirmed the deleterious effects of heat stress.

When the ambient environment changes dramatically, stress responses are initiated by activating corticotropin releasing factor (CRF). Then a group of highly conserved proteins known as heat shock proteins (HSPs) are rapidly synthesized by regulation of the hypothalamic pituitary adrenal (HPA) axis (Benjamin and McMillan 1998; Figueiredo et al. 2007). HSPs are a subset of molecular chaperones first discovered in Drosophila larvae by inducing expression during heat stress (Ritossa 1962). HSPs are categorized into families on the basis of their molecular weights, e.g. HSP27, HSP60, HSP70, HSP90, and HSP110/104 (Kregel 2002). Proposed functions of HSPs include microfilament stabilization (HSP27), protein refolding and preventing aggregation of denatured proteins (HSP60), acting as molecular chaperones (HSP70), regulating steroid hormone receptors and participating in protein translocation (HSP90) and protein folding (HSP110/104) (Kregel 2002).

HSP90 has been shown to act as a ubiquitous molecular chaperone. Its most important function is to protect organisms from being affected by heat (Bhat et al. 2015). Hao and Gu (2014) compared *HSP90* messenger RNA (mRNA) expression in the breast muscle of broilers at 22°C and 40°C, and found that it was significantly upregulated under acute heat stress ($P < 0.01$). Chen et al. (2013) examined *HSP90β* mRNA expression in the brain and small intestine of female White Recessive Rock chickens, and found it was increased sharply after 2 h of heat stress ($P < 0.05$) and reached its peak after 3 h ($P < 0.01$), then decreased significantly from 3 h to 6 h ($P < 0.01$). Lei et al. (2009) found *HSP90* expression increased in the liver of heat-treated broilers after 2 h of acute heat stress, then showed a continual drop with extended duration of heat stress. There are many previous studies on the various changing profiles of *HSP90* mRNA expression during heat stress. However, *HSP90* mRNA expression during the recovery period after heat stress has not been studied extensively.

The genetic effects of *HSP90* on heat stress tolerance have been investigated in several studies. Charoensook et al. (2012) detected polymorphisms in the *HSP90AB1* gene, and found that the *T* allele at single nucleotide polymorphism (SNP) g.4338T>C occurring in intron 3 improved heat tolerance in cattle. A SNP of c.-141G>A in the 5' promoter region of the *HSP90β* gene was found in chickens which had some effects on thermal tolerance traits (Chen et al. 2013). However, Simona et al. (2015) found that there was no significant interaction effect between temperature and genotype on the expression of *HSP90* in the liver of chronically heat-stressed broilers.

Huainan chicken originated in central China, while Wenchang chicken originated in the south of China; both are used as dual-purpose breeds. In the present study, the dynamic mRNA expression profile of *HSP90B1* in the hypothalamus during heat stress and recovery periods was investigated in these two breeds, and the association of polymorphisms of *HSP90B1* with heat tolerance was analyzed to help identify potential genetic markers for heat tolerance in chickens.

## MATERIAL AND METHODS

***Birds and sample collection***. All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals at Anhui Agricultural University. Huainan and Wenchang chickens were used as experimental stocks to investigate *HSP90B1* mRNA expression during and after heat stress. Female Huainan chickens ($n = 200$) and female Wenchang chickens ($n = 200$) with similar body weights at 60 days of age were selected and transferred to cages in the same housing facility. The ambient temperature was maintained at 24 ± 1°C for 3 days before the beginning of heat stress (60–63 days of age). At 64 days of age, the ambient temperature was increased to 35 ± 1°C and maintained there for the next 24 h (heat stress). After 24 h, the temperature was quickly decreased to 24 ± 1°C for the next 24 h (recovery period). Birds had free access to food and water throughout the experiment. For each breed, 90 hens were randomly selected for sampling, as 18 hypothalamus samples were collected at 0, 12, and 24 h during heat stress and 12 and 24 h during the recovery period. The hypothalamus collection procedure was carried out as previously described (Sun et al. 2015). Samples were stored in RNAfixer (Transgen Biotech Co., China) at –80°C and then subjected to mRNA expression analysis.

The association of SNPs in *HSP90B1* with heat resistance was studied in another group of Huainan

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chickens, using 113 females at 65 days of age. Before the initiation of heat stress, birds were kept in cages at a temperature of 24 ± 1°C. The temperature was quickly increased and maintained at 42 ± 2°C. The lethal time for each individual was defined as the period from the start of the heat stress until they could no longer stand erect and were short of breath. All birds were removed at 300 min. A blood sample from each chicken was collected for genotyping.

***RNA extraction and complementary DNA synthesis***. Total RNA was extracted from the hypothalamus using Trizol reagent (Tiangen Biotech Co., China), and then used for first strand synthesis with a Reverse Transcription Kit (Transgen Biotech Co.). Sequences of *HSP90B1* and *GAPDH* were obtained from GenBank (GenBank Accession Nos. NC_006088.3 and K01458, respectively). Polymerase chain reaction (PCR) primers (Table 1) were designed using Primer 5.0 software (PREMIER Biosoft, USA). The amplified PCR fragments were purified and sequenced directly using a DNA sequencer ABI3730XL (Applied Biosystems, USA) by a commercial sequencing company to verify the specificity (Sangon Biotech Co., China).

***Real-time qPCR analysis***. Quantitative real-time polymerase chain reaction (RT-q PCR) was used to elucidate the differential expression of *HSP90B1* between Huainan and Wenchang chickens. The PCR reaction was performed in a final reaction volume of 15 µl using the SYBR® Green PCR Master Mix Kit (Applied Biosystems), according to the manufacturer's instructions. All reactions and determinations were performed in triplicate, and the mean mRNA value was calculated. Negative controls were also run in each set of PCR assays, including without complementary DNA (cDNA) (no template controls, NTCs) and without reverse transcriptase. The fold expression or repression of the target gene relative to the internal control gene *GAPDH* (Tu et al. 2015) in each sample was then calculated by the $2^{-\Delta\Delta Ct}$ method (Deo et al. 2011).

***Sequencing and genotyping***. DNA was extracted from blood samples using a DNA isolation Kit (Tiangen), and quantified using a Nucleic Acid Protein Analyzer Beckman DU730 (Beckman Coulter, Inc., USA). In total, 50 genomic DNA samples were mixed equivalently in a pool for PCR. Fifteen primer pairs (Supplementary Table S1) were designed to amplify the exons of *HSP90B1* using Primer Premier 5.0 according to the DNA sequences (GenBank Accession No. NC_006088.3). The PCR reaction was performed with a reaction mix containing 12 µl 2×*Taq* PCR StarMix (0.1 U/µl; Transgen Biotech Co.), 1 µl of each forward and reverse primer (10 pmol/µl), 1 µl DNA template (50 ng/µl), and 10 µl diethylpyrocarbonate-treated (DEPC) water, according to the manufacturer's instructions. The PCR conditions were as follows: 95°C for 2 min; 35 cycles of 94°C for 30 s, a primer specific annealing temperature (Supplementary Table S1) for 30 s, 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were purified with DNA Purification Kit (Tiangen) and were sent to Sangon Biotech Co. for sequencing on ABI3730XL DNA sequencer (Applied Biosystems).

A SNP NC_006088.3:g.6798G>A, located in exon 14 of *HSP90B1*, was identified (coordinates of the gene: 54869423–54879269, reverse complement; in SNP database: rs15273262). Genotyping was realized by sequencing of the PCR fragments of all chickens obtained with the use of the primer pair HSP90B1-12 (Supplementary Table S1). The analysis of sequences was conducted using the software DNASTAR (http://www.biologysoft.com/; Steve Shear Down, 1998–2001 version reserved by DNASTAR Inc., USA).

***Statistical analysis***. Comparisons of the mRNA expression level in the two breeds and the lethal time between genotypes were conducted by one-way ANOVA using the GLM procedure of the SAS software (Statistical Analysis System, Version 8.2, 2001). The Hardy-Weinberg equilibrium of distribution of genotypes was analyzed by chi-squared

Table 1. Primer sequences for quantitative real-time polymerase chain reaction (RT-q PCR)

| Gene | Primer sequence | Amplicon length (bp) |
|---|---|---|
| *HSP90B1* | F: 5'-AGTCGTGAAGCCTTGGAGAA-3'<br>R: 5'-ACGAGTGCACATGGAGACTG-3' | 125 |
| *GAPDH* | F: 5'-CATCACACGGACACTTCAAG-3'<br>R: 5'-ACAAACATGGGGGCATCAG-3' | 152 |

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test using the FREQ procedure of the SAS software. All data are expressed as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

***mRNA expression profile of HSP90B1 in Huainan and Wenchang chickens***. The *HSP90B1* mRNA expression in the hypothalamus of Huainan and Wenchang chickens during heat stress and recovery is shown in Figure 1. In both breeds it exhibited a dynamic trend during heat stress and recovery, similar to that in a previous report from Yu et al. (2008). The level of *HSP90B1* mRNA sharply increased during heat stress ($P < 0.05$), and then decreased in recovery ($P < 0.05$). Comparing the two breeds revealed that the mRNA expression of *HSP90B1* was higher in Huainan than in Wenchang chickens during the heat stress and recovery periods ($P < 0.05$), although the baseline expression was the same in both breeds. During heat stress the *HSP90B1* expression increased by 220% in Huainan chickens; Wenchang chickens showed a 145% increase. During the recovery period, the *HSP90B1* expression decreased by 59% in Wenchang chickens, which was greater than the decrease of 39% observed in Huainan chickens.

When animals suffer from heat stress, a heat shock response will occur, which decreases cellular injury and upregulates *HSP90* in response, so as to recover damaged proteins or other cellular structures (Lee and Tsai 2005). During the initial period of heat stress, the expression of *HSP90* is raised due to increasing damage to cells that promotes the secretion of *HSP90* to adapt to the environment (Hao and Gu 2014). In the present study, *HSP90B1* mRNA increased in the hypothalamus during 24 h of heat stress and decreased as the temperature returned to ambient. However, Lei et al. (2009) found that *HSP90* expression increased in the liver of heat-treated broilers after 2 h at 34°C, but then showed a continuous drop after 3, 5, and 10 h of heat stress. It was hypothesized that *HSP90* expression enhanced the resistance of cells during the initial period of heat stress, and that long-term heat stress may result in a decline (Lei et al. 2009). We found that the increase in *HSP90B1* mRNA during heat stress in Huainan chickens was higher than that in Wenchang chickens, indicating that Huainan chickens are more sensitive to heat stress than Wenchang chickens. The difference in heat tolerance may be associated with the geographical origin of the breeds: Huainan chickens originated in central China (Chen 2004) where there is a temperate, monsoon climate; Wenchang chickens originated in south China (Chen 2004) where the climate is hot and humid throughout the year.

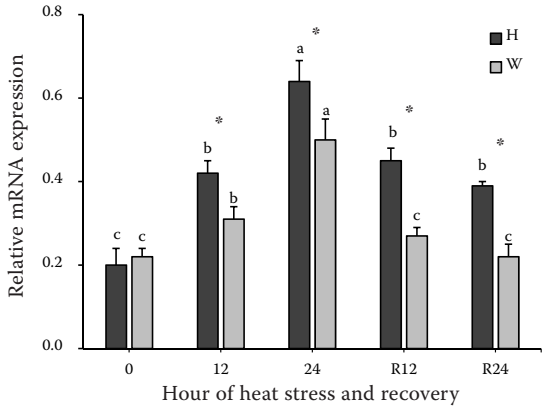


Figure 1. Dynamic expression of *HSP90B1* mRNA during heat stress and recovery periods in two Chinese chicken breeds

H = Huainan chickens, W = Wenchang chickens

In the same breed, values at different time points with different letters differ at $P < 0.05$; at the same time point, values marked with asterisk differ at $P < 0.05$

***SNPs in HSP90B1 and association with heat tolerance in Huainan chicken***. A SNP NC_006088.3:g.6798G>A, located in exon 14 of *HSP90B1*, was identified (coordinates of the gene: 54869423–54879269, reverse complement; in dbSNP: rs15273262); this polymorphism did not cause an amino acid sequence change, but caused a codon for glutamic acid to change from GAG to GAA. The genotypic frequencies of *AA*, *GA*, and *GG* were 0.49, 0.27, and 0.24, respectively, and allelic frequencies were 0.62 for *A* and 0.38 for *G* (Table 2). The genotypic

Table 2. Genotypic frequency of *HSP90B1* g.6798G>A and association of genotypes with lethal time for Huainan female chickens

| Genotype | Genotypic frequency | Lethal time (min) |
|---|---|---|
| *AA* | 0.49 ($n$ = 55) | 179.3 ± 36.5$^{b}$ |
| *GA* | 0.27 ($n$ = 31) | 227.2 ± 44.5$^{ab}$ |
| *GG* | 0.24 ($n$ = 27) | 248.2 ± 39.3$^{a}$ |

$^{a,b}$values of lethal time with different superscripts differ at $P < 0.05$

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distribution in the population was in the Hardy-Weinberg equilibrium ($P > 0.05$). The lethal time for each genotype is presented in Table 2. Individuals with genotype *GG* of *HSP90B1* g.6798G>A survived for a longer time at 42 ± 2°C (248.2 ± 39.3 min), suggesting higher heat tolerance compared to genotypes *GA* and *AA*, which survived for 227.2 ± 44.5 min and 179.3 ± 36.5 min ($P < 0.05$), respectively.

Heat tolerance is a complex trait (Li et al. 2011). Chen et al. (2013) identified a mutation at c.141G>A in the 5' region of *HSP90β* which was associated with heat tolerance in Lingshan and White Recessive Rock chickens, identifying *GG* as the genotype with high heat tolerance. However, Simona et al. (2015) found there was no significant association between temperature, genotype, and liver *HSP90* expression in two broiler breeds. In the present study, the SNP (NC_006088.3:g.6798G>A) we identified in exon 14 of the *HSP90B1* gene produced three genotypes – *AA*, *GA*, and *GG*. Genotype *GG* displayed a longer survival time, indicating that allele *G* was favourable for heat tolerance. Although this was a silent SNP, which did not change the amino acid sequence or the function of the resulting protein, such SNPs may be associated with target traits by affecting the stability of mRNA and translation efficiency (Laws et al. 2003; Capon et al. 2004). Therefore, the mutation g.6798G>A could be linked with other important SNPs which influences the stability of *HSP90B1* mRNA expression.

In conclusion, the SNP NC_006088.3:g.6798G>A has the potential to be used in breeding for improved heat tolerance after further study of various chicken populations and proof of the effect.

## REFERENCES

Bartlett J.R., Smith M.O. (2003): Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poultry Science, 82, 1580–1588.

Benjamin I.J., McMillan D.R. (1998): Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circulation Research, 83, 117–132.

Bhat R., Tummalapalli S.R., Rotella D.P. (2015): Progress in the discovery and development of heat shock protein 90 (Hsp90) inhibitors. Journal of Medicinal Chemistry, 57, 8718–8728.

Capon F., Allen M.H., Ameen M. (2004): A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. Human Molecular Genetics, 13, 2361–2368.

Charoensook R., Gatphayak K., Sharifi A.R., Chaisongkram C., Brenig B., Knorr C. (2012): Polymorphisms in the bovine HSP90AB1 gene are associated with heat tolerance in Thai indigenous cattle. Tropical Animal Health and Production, 44, 921–928.

Chen G.H. (2004): Genetic resources of Chinese poultry. In: Yang N.: Indigenous Avian Breeds. Shanghai Scientific and Technical Publishing, Shanghai, China, 18–21. (in Chinese)

Chen Z.Y., Gan J.K., Xiao X., Jiang L.Y., Zhang X.Q., Luo Q.B. (2013): The association of SNPs in Hsp90β gene 5' flanking region with thermo tolerance traits and tissue mRNA expression in two chicken breeds. Molecular Biology Reports, 40, 5295–5306.

Cooper M.A., Washburn K.W. (1998): The relationships of body temperature to weight gain, feed consumption, and feed utilization in broilers under heat stress. Poultry Science, 77, 237–242.

Deo A., Carlsson J., Lindlof A. (2011): How to choose a normalization strategy for miRNA quantitative real-time (qPCR) arrays. Journal of Bioinformatics and Computational Biology, 9, 795–812.

Figueiredo D., Gertler A., Cabello G., Decuypere E., Buyse J., Dridi S. (2007): Leptin downregulates heat shock protein-70 (HSP-70) gene expression in chicken liver and hypothalamus. Cell and Tissue Research, 329, 91–101.

Hao Y., Gu X.H. (2014): Effects of heat shock protein 90 expression on pectoralis major oxidation in broilers exposed to acute heat stress. Poultry Science, 93, 2709–2717.

Kregel K.C. (2002): Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology, 92, 2177–2186.

Laws S.M., Hone E., Gandy S., Martins R.N. (2003): Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription. Journal of Neurochemistry, 84, 1215–1236.

Lee S., Tsai F.T. (2005): Molecular chaperones in protein quality control. Journal of Biochemistry and Molecular Biology, 38, 259–265.

Lei L., Yu J., Bao E. (2009): Expression of heat shock protein 90 (Hsp90) and transcription of its corresponding mRNA in broilers exposed to high temperature. British Poultry Science, 50, 504–511.

Li Q., Han J., Du F.L., Ju Z.H., Huang J.M., Wang J., Li R.L., Wang C.F., Zhong J.F. (2011): Novel SNPs in HSP70A1A gene and the association of polymorphisms with thermo tolerance traits and tissue specific expression in Chi-

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nese Holstein cattle. Molecular Biology Reports, 38, 2657–2663.

Lu Q., Wen J., Zhang H. (2007): Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. Poultry Science, 86, 1059–1064.

Ritossa F. (1962): A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia, 18, 571–573.

Ryder A.A., Feddes J.J.R., Zuidhof M.J. (2004): Field study to relate heat stress index to broiler performance. Journal of Applied Poultry Research, 13, 493–499.

Simona R., Emiliano L., Francesca M.S., Stefano P.M., Maria C.C., Giovanni B., Genciana T. (2015): Expression profile of six stress-related genes and productive performances of fast and slow growing broiler strains reared under heat stress conditions. Meta Gene, 6, 17–25.

Soleimani A.F., Zulkifli I. (2010): Effects of high ambient temperature on blood parameters in red jungle fowl, village fowl and broiler chickens. Journal of Animal and Veterinary Advances, 9, 1201–1207.

St-Pierre N.R., Cobanov B., Schnitkey G. (2003): Economic losses from heat stress by US livestock industries. Journal of Dairy Science, 86, 52–77.

Sun H., Jiang R.S., Xu S.Y., Zhang Z.B., Xu G.Y., Zheng J.X., Qu L.J. (2015): Transcriptome responses to heat stress in hypothalamus of a meat-type chicken. Journal of Animal Science and Biotechnology, 6: 6.

Tu W.L., Cheng C.Y., Wang S.H., Tang P.C., Chen C.F., Chen H.H., Lee Y.P., Chen S.E., Huang S.Y. (2015): Profiling of differential gene expression in the hypothalamus of broiler-type Taiwan country chickens in response to acute heat stress. Theriogenology, 85, 483–494.

Yu J.M., Bao E.D., Yan J.Y., Lei L. (2008): Expression and localization of Hsps in the heart and blood vessel of heat-stressed broilers. Cell Stress and Chaperones, 13, 327–335.

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