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Alteration of *HSF3* and *HSP70* mRNA expression in the tissues of two chicken breeds during acute heat stress

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ABSTRACT. This study aimed to estimate changes in *HSF3* and *HSP70* mRNA expression in stress-sensitive tissues of 2 chicken breeds during acute heat stress. Lingshan chickens (LSC) and White Recessive Rock (WRR) (24 chickens of each breed) were randomly divided into 4 groups (0, 2, 3, and 6 h of heat treatment). With increasing heat treatment time, both *HSF3* and *HSP70* expression first declined and then showed a significant increase in both breeds. However, *HSP70* expression decreased in the heart following 6 h of heat treatment, whereas *HSF3* expression continued to increase. After 2 h of heat treatment, *HSF3* expression was significantly higher in the brain and leg muscle of LSC compared to WRR ($P < 0.05$, $P < 0.01$). In comparison, *HSP70* expression was significantly higher in the liver and leg muscle of WRR

compared to LSC ($P < 0.01$, $P < 0.05$). After 3 h of heat treatment, *HSF3* expression was significantly higher in the brain and leg muscle of LSC compared to WRR ($P < 0.01$). In comparison, *HSP70* expression was significantly higher in the liver and heart of LSC compared to WRR ($P < 0.01$). These results indicate that the expression of *HSF3* and *HSP70* mRNA in LSC and WRR exhibit species-specific and tissue-specific differences during heat treatment.

Key words: Heat shock factor-3; Heat shock protein 70; Chicken; Heat treatment

INTRODUCTION

High temperatures have detrimental effects on chicken feed intake and reproductive performance, causing major economic damage to the food industry (Meehl and Tebaldi, 2004; Abidin and Khatoon, 2013; Al-Aqil et al., 2013). Heat shock factor (HSF) protein expression is induced during the signaling response to heat shock. This response, combined with the effect on heat stress genes through HSEs (heat shock element), results in the expression of heat shock proteins (HSP; Yenari et al., 1999). Fujimoto and Nakai (2010) documented that *HSF1* is the major HSP gene in mammals, while *HSF3* is the major *HSP* gene in poultry. The *HSP* gene plays an important role in protecting cellular homeostatic processes from heat stress by preserving the structure of normal proteins and repairing damaged ones (Tytell and Hooper, 2001). HSPs are divided into 6 families (HSP110, HSP90, HSP70, HSP60, small molecule HSPs, and ubiquitin). The HSP70 family is one of the most thoroughly studied groups. The lack of *HSP70* expression in *Drosophila* results in reduced heat resistance and developmental delays, indicating that the *HSP70* gene plays an important role in heat shock (Gong and Golic, 2006).

Shabtay and Arad (2006) observed that differential combinations of HSFs and DNA have varying effects on *HSP* transcription and translation. *HSF1* mRNA expression of pig hearts is significantly altered following heat treatment, with changes in *HSP70* mRNA expression occurring in other tissues (Zhang et al., 2012). In another study, *HSP70* and *HSF1* mRNA expression in coral was the highest on the first day of heat treatment and declined with further exposure to heat stress (Nakamura et al., 2012). Islam et al. (2013) noted that the *HSF1* gene is specifically expressed in the liver of mice upon heat treatment, while expression in the heart is not detected. However, *HSF3* mRNA expression in different chicken breeds has received limited investigation. This study investigated the expression of *HSF3* and *HSP70* mRNA in various tissues of 2 chicken breeds, Lingshan chickens (LSC) and White Recessive Rock (WRR), to determine species-specific differences in both basal and heat treatment-regulated expression.

MATERIAL AND METHODS

Animals and experimental design

In total, 48 pure lines of LSC (N = 24) and WWR (N = 24) breeds were purchased

from the Guangdong South Winchester Food Group poultry breeding company (China), and raised according to standard procedures. The heat shock experiments were initiated 8 days after purchasing the chickens. The control chickens (N = 12, 6 LSC and 6 WWR, which were randomly selected from the population) were fed at room temperature (25° ± 1°C), while the remaining 3 groups (experimental heat stress group) were fed in an artificial climate chamber. The artificial climate chamber was heated from 32°C to 40 ± 1°C, with a maintenance temperature of 40° ± 1°C. During the experiment, the humidity of the artificial climate chamber was maintained above 70%. The experimental group was raised at 40° ± 1°C and included 12 chickens (6 LSC and 6 WRR) that were sacrificed after 2, 3, and 6 h of heat treatment. Blood and heart, liver, brain, and leg muscle tissues were immediately collected. The control group was prepared in a parallel fashion. Blood and tissue samples were stored at -80°C until further assay. All procedures were conducted under protocols approved by the Committee for the Care and Use of Experimental Animals at South China Agricultural University.

Isolation of total RNA and preparation of first-strand cDNA

After washing in ice-cold surface RNase Erasol, 0.5 g of the heart, liver, brain, and leg muscle tissues from the heat shock and control animals stored at -70°C were placed in 2-mL centrifuge tubes with 1 mL surface RNase Erasol using small centrifugal mills. Total RNA was isolated from the tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer protocols. RNA concentrations were determined with a spectrophotometer (ND2000C Thermo NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 260 nm. Five-fold serial dilutions of RNA were prepared in ribonuclease-free water, and 0.5 μg of each sample was transcribed into cDNA using the PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China), following the manufacturer protocol. The resulting cDNA was stored at -20°C prior to use.

Primer design for *HSP70* and *HSF3* and gga-β-actin mRNA detection

Complementary polymerase chain reaction (PCR) primers were designed based on the mRNA sequences for *HSP70*, *HSF3*, and β-actin obtained from the GenBank database of the National Center for Biotechnology Information (NCBI, Rockville Pike, Bethesda MD, USA). Primers were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). β-actin was chosen as the internal control. Primer specificity was evaluated against the NCBI database using the NCBI BLAST software. Highly purified, salt-free primers specific for *HSF3* (forward primer, 5'-TGGCTTACCTATTTGTCA-3'; reverse primer, 5'-GGGTCTTCTGGAGCATTA-3'; 153-bp product), *HSP70* (forward primer, 5'-GCGCCAG GCCACCAAAGATG-3'; reverse primer, 5'-GCCCCCTCCCAAGTCAAAGATG-3'; 135-bp product), and β-actin (forward primer, 5'-CTCCCCCATGCCATCCTCCGTCTG-3'; reverse primer, 5'-GCTGTGGCCATCTCCTGCTC-3'; 165-bp product) were synthesized by Sangon Biotech (China). *HSF3* and *HSP70* had optimized annealing temperatures of 59° and 60°C, respectively.

Measurement of *HSF3* and *HSP70* mRNA levels by fluorescent quantitative real-time PCR (qRT-PCR)

Each cDNA sample (1 µL, 20X dilution in ribonuclease-free water) was added to a reaction mixture containing 10 µL SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), the indicated primers (0.2 µL each), and double-distilled water in a total volume of 20 µL. The qRT-PCR was performed using a CFX96 real-time PCR thermocycler (Bio-Rad, USA), following manufacturer protocols. In brief, the reactions were incubated at 94°C for 30 s for 1 cycle to activate the enzyme, followed by 45 cycles of denaturation at 94°C for 30 s, and annealing and elongation at 59°C for *HSF3* and 60°C for *HSP70* for 30 s. For each run, a negative control tube lacking cDNA was processed along with the experimental samples. Amplification efficiencies of the target (*HSP70* and *HSF3*) and reference (β-actin) mRNA sequences were approximately equal. *HSP70* and *HSF3* mRNA levels in all samples were normalized using the following formula: relative quantity of *HSP70* or *HSF3* mRNA = $2^{-\Delta\Delta Ct}$, where ΔΔCt corresponds to the difference between the ΔCt measured for the mRNA level of each tissue. Here, $\Delta Ct = Ct_{HSP70 \text{ or } HSF3} - Ct_{\beta\text{-actin}}$.

Statistical analysis

Statistical differences between each group were assessed by a one-way analysis of variance (ANOVA) using the SAS 8.0 software (SAS Institute Inc., Cary, NC, USA). Comparisons between the mean values of the control group and those of each experimental group were performed using the Duncan test for multiple comparisons. P values of less than 0.05 were regarded as statistically significant.

RESULTS

HSF3 and *HSP70* mRNA expression in different LSC tissues

The qRT-PCR results showed that both *HSF3* and *HSP70* mRNA expression first declined and then significantly increased with increasing heat treatment time. After 2 h of heat treatment, *HSF3* mRNA expression reached the highest level in the brain, liver, and leg muscle, whereas the highest expression level of the heart was reached after 6 h. After 3 h of heat treatment, the highest *HSP70* mRNA expression level was reached. *HSF3* mRNA expression peaked faster compared to *HSP70* mRNA expression. After 2 h of heat treatment, *HSF3* mRNA expression was the highest in the leg muscle, followed by the heart, liver, and brain. In comparison, *HSP70* mRNA expression was the highest in the heart, followed by the leg muscle, brain, and liver. *HSP70* and *HSF3* mRNA tissue expression levels of the heat treatment group (HTG) were significantly higher compared to the control group (CG) ($P < 0.01$). After 3 h of heat treatment, *HSF3* and *HSP70* mRNA expression was the highest in the leg muscle, followed by the heart, liver, and brain. The *HSP70* and *HSF3* mRNA tissue expression levels were significantly higher in the HTG compared to the CG ($P < 0.01$). After 6 h of heat treatment, *HSF3* mRNA expression levels were the highest in the heart, followed by the leg muscle, liver, and brain. *HSP70* mRNA expression was the highest in

the heart, followed by the brain, leg muscle, and liver. *HSF3* mRNA expression levels in the leg muscle and heart were significantly higher in the HTG compared to the CG ($P < 0.01$). *HSP70* mRNA expression in brain and heart were significantly higher in the HTG compared to the CG ($P < 0.01$; Table 1).

Table 1. *HSP70* and *HSF3* mRNA expression in LSC tissues following heat treatment.

	Control group (N = 6)	Heat treatment group (N = 18)		
	0 h	2 h	3 h	6 h
Liver				
HSF3	0.58 ± 0.20	1.54 ± 0.49**	1.17 ± 0.20**	0.69 ± 0.25
HSP70	0.04 ± 0.02	0.51 ± 0.28**	2.11 ± 0.9**	0.08 ± 0.03
Brain				
HSF3	0.29 ± 0.09	0.56 ± 0.20**	0.56 ± 0.06**	0.30 ± 0.14
HSP70	0.12 ± 0.04	0.87 ± 0.39**	1.31 ± 0.71**	0.53 ± 0.36**
Leg muscle				
HSF3	1.07 ± 0.25	3.07 ± 0.35**	3.05 ± 0.61**	2.22 ± 0.65**
HSP70	0.38 ± 0.19	1.28 ± 0.37**	3.24 ± 1.00**	0.44 ± 0.17
Heart				
HSF3	0.99 ± 0.23	1.58 ± 0.40**	1.80 ± 0.54**	3.18 ± 0.94**
HSP70	0.14 ± 0.12	2.44 ± 1.08**	2.84 ± 0.65**	0.80 ± 0.62**

**$P < 0.01$ compared to the 0 h. Values are reported as means ± SD.

HSF3 and *HSP70* mRNA expression in WRR tissues

The qRT-PCR results showed that both *HSF3* and *HSP70* mRNA expression initially declined followed by a significant increase with increasing heat treatment time. After 2 h of heat treatment, *HSF3* mRNA expression reached the highest level in the brain, liver, and leg muscle, whereas the highest expression of the heart was reached after 6 h. After 3 h of heat treatment, *HSP70* mRNA expression reached the highest level in the brain, heart, and leg muscle, whereas the highest expression level was reached after 2 h in the liver. *HSF3* mRNA expression peaked faster than *HSP70* mRNA expression. After 2 h of heat treatment, *HSF3* mRNA expression was the highest in the leg muscle, followed by the liver, heart, and brain. *HSP70* mRNA expression was the highest in the leg muscle, followed by the heart, brain, and liver. *HSP70* and *HSF3* mRNA expression was significantly higher in the HTG compared to the CG ($P < 0.01$). After 3 h of heat treatment, *HSF3* mRNA expression was the highest in the leg muscle, followed by the heart, liver, and brain. *HSP70* mRNA expression was the highest in the leg muscle, followed by the heart, brain, and liver. *HSP70* and *HSF3* mRNA tissue expression levels were significantly higher in the HTG compared to the CG ($P < 0.01$). After 6 h of heat treatment, *HSF3* mRNA expression was the highest in the heart, followed by the leg muscle, liver, and brain. *HSP70* mRNA expression was the highest in the heart, followed by the leg muscle, brain, and liver. *HSF3* mRNA expression levels in the leg muscle, liver, and heart were significantly higher in the HTG compared to the CG ($P < 0.01$, $P < 0.05$, $P < 0.01$). *HSP70* mRNA expression levels in the brain, leg muscle, and heart were significantly higher in the HTG compared to the CG ($P < 0.01$, $P < 0.05$, $P < 0.05$) (Table 2).

Table 2. *HSP70* and *HSF3* mRNA expression in WRR tissues following heat treatment.

	Control group (N = 6)	Heat treatment group (N = 18)		
	0 h	2 h	3 h	6 h
Liver				
HSF3	0.59 ± 0.15	1.76 ± 0.63**	1.52 ± 0.58**	0.89 ± 0.28*
HSP70	0.06 ± 0.06	0.93 ± 0.19**	0.33 ± 0.16**	0.04 ± 0.01
Brain				
HSF3	0.18 ± 0.06	0.35 ± 0.12**	0.35 ± 0.11**	0.21 ± 0.09
HSP70	0.08 ± 0.05	0.95 ± 0.30**	1.13 ± 0.51**	0.28 ± 0.17**
Leg muscle				
HSF3	0.97 ± 0.23	2.51 ± 0.60**	2.25 ± 0.24**	1.44 ± 0.39**
HSP70	0.24 ± 0.14	1.91 ± 0.51**	2.53 ± 1.42**	0.41 ± 0.16*
Heart				
HSF3	0.68 ± 0.23	1.50 ± 0.32**	1.65 ± 0.26**	2.11 ± 0.58**
HSP70	0.23 ± 0.16	1.31 ± 0.45**	1.85 ± 0.68**	0.48 ± 0.27*

*P < 0.05 and **P < 0.01 compared to the 0 h. Values are reported as means ± SD.

Higher *HSF3* mRNA expression in multiple tissues of LSC compared to WRR after heat treatment

At 0 h of heat treatment, *HSF3* mRNA expression in the brain and heart was significantly higher in LSC compared to WRR (P < 0.01). After 2 h of heat treatment, *HSF3* mRNA expression in the brain and leg muscle was significantly higher in LSC compared to WRR (P < 0.05 and P < 0.01, respectively). After 3 h of heat treatment, *HSF3* mRNA expression in the brain and leg muscle was significantly higher in LSC compared to WRR (P < 0.01). After 6 h of heat treatment, *HSF3* mRNA expression in the brain and leg muscle was significantly higher in LSC compared to WRR (P < 0.05 and P < 0.01, respectively; Table 3).

Table 3. Expression of *HSF3* mRNA in different tissues of LSC and WRR following heat treatment.

Tissues	Control group (N = 6)		Heat treatment group (N = 18)					
	0 h		2 h		3 h		6 h	
	LSC	WRR	LSC	WRR	LSC	WRR	LSC	WRR
Liver	0.58 ± 0.20	0.59 ± 0.15	1.54 ± 0.49	1.76 ± 0.63	1.17 ± 0.20	1.52 ± 0.58	0.69 ± 0.25	0.89 ± 0.28
Brain	0.29 ± 0.09**	0.18 ± 0.06	0.56 ± 0.20*	0.35 ± 0.12	0.56 ± 0.06**	0.35 ± 0.11	0.30 ± 0.14	0.21 ± 0.09
Leg muscle	1.07 ± 0.25	0.97 ± 0.23	3.07 ± 0.35**	2.51 ± 0.60	3.05 ± 0.61**	2.25 ± 0.24	2.22 ± 0.65**	1.44 ± 0.39
Heart	0.99 ± 0.23**	0.68 ± 0.23	1.58 ± 0.40	1.50 ± 0.32	1.80 ± 0.54	1.65 ± 0.26	3.18 ± 0.94*	2.11 ± 0.58

*P < 0.05 and **P < 0.01 between LSC and WRR in four times. Values are reported as means ± SD.

Higher *HSP70* mRNA expression in multiple tissues of LSC compared to WRR after heat treatment

At 0 h of heat treatment, *HSP70* mRNA expression was not significantly higher in LSC compared to WRR. After 2 h of heat treatment, *HSP70* mRNA expression in the liver and leg muscle was significantly higher in WRR compared to LSC (P < 0.01 and P < 0.05, respectively). After 3 h of heat treatment, *HSP70* mRNA expression in the liver and heart was significantly higher in LSC compared to WRR (P < 0.01). After 6 h of heat treatment, *HSP70* mRNA expression in the liver, brain, and heart was significantly higher in LSC compared to WRR (P < 0.01, P < 0.01, and P < 0.05, respectively; Table 4).

Table 4. Expression of *HSP70* mRNA in different tissues of LSC and WRR following heat treatment.

Tissues	Control group (N = 6)		Heat treatment group (N = 18)					
	0 h		2 h		3 h		6 h	
	LSC	WRR	LSC	WRR	LSC	WRR	LSC	WRR
Liver	0.04 ± 0.02	0.06 ± 0.06	0.51 ± 0.28**	0.93 ± 0.19	2.11 ± 0.9**	0.33 ± 0.16	0.08 ± 0.03**	0.04 ± 0.01
Brain	0.12 ± 0.04	0.08 ± 0.05	0.87 ± 0.39	0.95 ± 0.30	1.31 ± 0.71	1.13 ± 0.51	0.53 ± 0.36**	0.28 ± 0.17
Leg muscle	0.38 ± 0.19	0.24 ± 0.14	1.28 ± 0.37*	1.91 ± 0.51	3.24 ± 1.00	2.53 ± 1.42	0.44 ± 0.17	0.41 ± 0.16
Heart	0.14 ± 0.12	0.23 ± 0.16	2.44 ± 1.08**	1.31 ± 0.45	2.84 ± 0.65**	1.85 ± 0.68	0.80 ± 0.62*	0.48 ± 0.27

*P < 0.05 and **P < 0.01 between LSC and WRR in four times. Values are reported as means ± SD.

DISCUSSION

HSF1 is able to bind with HSE in the upstream region of *HSP70* in the mouse, which induces *HSP70* transcription (Kroeger et al., 1993). Sakurai and Takemori (2007) noted that targets of *HSF* included cis-HSE sequences, which are the reverse repetitive sequences of multiple 5'-nGAAn-3'. *HSF* binding to HSEs is very important in *HSP* gene transcription, indicating that *HSP* gene transcription is regulated by the *HSF* gene. The qRT-PCR results of the current study showed that both *HSF3* and *HSP70* mRNA expression first declined and then significantly increased with increasing heat treatment time in both chicken breeds (LSC and WRR). These results demonstrate that the *HSF3* gene is correlated with the *HSP70* gene.

HSF1 binding to the heat shock promoter *HSP70* gene is important in *HSP70* transcription (Westerheide et al., 2009). The upregulation of HSPs is induced by *HSF1* during stress response, which might play an important role in the regrowth of skeletal muscle (Yasuhara et al., 2011). In the chicken, under severely high temperature conditions, *HSF1* induces HSP expression in the brain. In comparison, *HSF3* is expressed in the brain and blood under mild, moderate, and severely high temperature conditions (Shabtay and Arad, 2006). Consequently, the tissue specificity of *HSF3* mRNA expression is associated to the different demands of the tissues. In this paper, *HSF3* mRNA levels in the heart continued to rise following 3 h of heat treatment, indicating that the demand for *HSF3* in the chicken heart after heat treatment differs compared to other tissues.

Upon cell stress, the rapid increase of intracellular *HSP70* transcription leads to large quantities of HSP70 protein synthesis (Song et al., 1995). In poultry cells, if the *HSF3* gene is destroyed, *HSP* gene expression declines, with a subsequent loss in heat resistance. This phenomenon demonstrates that HSPs may be induced by *HSF3* during heat treatment (Tanabe et al., 1998). Hence, at the onset of heat treatment, *HSP70* and *HSF3* mRNA expression levels were consistently altered in the current study. However, at 6 h after the onset of heat treatment, *HSF3* mRNA expression continued to increase in the heart, whereas *HSP70* mRNA expression decreased. *HSP70* and *HSF3* mRNA expression levels differed in the LSC tissue compared to the WRR tissue following heat treatment, indicating species-specificity.

Upon heat stress in pigs, *HSP70* mRNA expression varied between the heart, liver, and stomach, with variable amounts of HSF1 protein being expressed (Zhang et al., 2012). In the current study, we found distinct *HSP70* and *HSF3* mRNA expression in the 4 tissues tested from the 2 chicken breeds after heat treatment, indicating tissue-specificity. In chicken DT40 cells, *HSF3* mRNA expression increased with heat treatment time, but decreased after 4 h, while *HSP70* mRNA expression also increased with heat treatment time (Tanabe et al., 1997).

WRR individuals were vulnerable to heat stress whereas LSC individuals were well adapted to heat stress, supporting the results of previous studies (Chen et al., 2013). In the liver, brain, and leg muscle of LSC and WRR *HSF3* mRNA expression initially increased followed by a decrease with increasing heat treatment; however, *HSF3* mRNA expression in the heart continued to increase. In conclusion, multiple tissue analyses and different heat treatment times in the current study showed that *HSF3* and *HSP70* mRNA expression was significantly higher in LSC compared to WRR, indicating that LSC is better adjusted to heat stress compared to WRR.

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