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Hsp70 Genotypes and Heat Tolerance of Commercial and Native Chickens Reared in Hot and Humid Conditions

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ABSTRACT

Heat tolerance in poultry production was obtained attention due to the need for genetic lines that can withstand climate changes. This study aimed at investigating heat tolerance in commercial and native broiler genetics, as well as the physiological and growth performance responses of HSP70 genotypes submitted to heat stress. In Experiment I, heterophil:lymphocyte (H:L) ratio, as an indicator of heat tolerance, was compared between commercial broilers (n = 100) and Thai native chickens (n = 100). Growing chickens (with similar initial weight) of each genetic strain were randomly divided into two groups: 1) thermoneutral environment (26 °C ± 2 °C) and 2) heat stress (36 °C ± 2 °C). The results showed that native chickens originating from a tropical environment presented lower H:L ratio and mortality rate compared with commercial broilers. In Experiment II, HSP70 genotypes were compared. PCR-RFLP was applied to identify the genotypes (C1C1, n = 38; C1C2, n = 38; and C2C2, n = 28). Ten-week-old chickens of each genotype were evaluated in the same environments described in Experiment I. Heat-stress indicators – respiratory rate (RR), cloacal temperature (CT), packed cell volume (PCV), and average daily gain (ADG) – were measured for three weeks. The significant difference in PCV indicated that C2C2 chickens were less tolerant to heat stress compared to other genotypes. The RR, CT, and ADG were not significantly different among all genotypes. Since the C2C2 genotype was shown to be sensitive to heat stress, C1C1 and C1C2 could be used as markers for heat-tolerant genetic strains of Thai indigenous chickens and hybrid commercial lines.

INTRODUCTION

Heat stress has negative effects on the productivity and welfare of poultry. In the poultry industry, heat stress is responsible for the morbidity and mortality of one million broilers per month (Guerreiro *et al.,* 2004). Several recent reports show that heat stress adversely affects feed intake (Cooper & Washburn, 1998), water intake (Bruno *et al.*, 2011), growth performance (Altan *et al.*, 2000; Abu-Dieyeh, 2006), blood variables (Altan *et al.*, 2000; Aengwanich, 2007), electrolytes (Borges *et al.*, 2004), and the immune system (Zulkifli *et al.,* 2000b; Mashaly *et al.,* 2004; Tirawattanawanich *et al.,* 2011), increasing mortality (Al-Fataftah *et al.,* 2007).The optimal temperature range for commercial broilers is about 21-26 °C (Ewing *et al.,* 1999), and a temperature of 32 °C causes heat stress (Daghir, 1995).

The genetic selection for heat-tolerant broilers is a challenge in global poultry production. In tropical and sub-tropical countries, the evaporative systems applied in broilers houses are not fully functional due to high environmental humidity. In Southeast Asia, crossbreeding between indigenous and exotic breeds is the most popular breeding method of obtaining a commercial hybrid resistant to tropical conditions,

and capable of producing a reasonable amount of eggs and meat (Mekki *et al.,* 2005; Aengwanich, 2007; Bekele *et al.*, 2010; Kingori *et al.*, 2010). The genetic control of heat tolerance is complex and has low heritability (El-Gendy & Washburn, 1995). Not all indigenous chickens, and very few exotic breeds, have adequate heat tolerance (Duangduen *et al.,* 2007). The introgression of genes from heat-tolerant lines into grand parental stock is an effective strategy applied to accelerate the genetic progress of commercial lines that can tolerate heat stress. The genes associated with heat tolerance have been investigated in chickens, including the naked-neck (Patra *et al.,* 2002), frizzle (Sharifi *et al.,* 2010), dwarfism (Sharifi *et al.,* 2010), and slow/rapid feathering (Fotsa *et al.,* 2001) genes. In all instances, the genes affected the appearance and some performance parameters of the chickens.

The heat shock protein 70 (HSP70) is a chaperone protein that effectively protects several proteins and cell organelles from stressors (Kiang & Tsokos, 1998). Changes in HSP70 expression were detected during heat stress in vital organs, such as the heart, liver, and kidneys. The relationship of HSP70 with the survival ability of cells affected by heat stress was extensively investigated by Yu & Bao (2008). Mazzi *et al.* (2003); recently reported a nucleotide polymorphism in the coding region of HSP70 in chickens. Duangduen *et al.* (2007) found that this mutation, although silent, may possibly be used as a heat tolerance marker in indigenous and commercial broiler strains. However, due to the unbalanced number of chickens in each genotype, a lower number of chickens was submitted to heat stress. Hence, this study aimed at comparing the heat tolerance of commercial and indigenous broiler strains, as well as the effects of HSP70 genotypes on the physiological responses and growth performance of those strains under heat stress conditions.

MATERIALS AND METHODS

Birds were handled and managed according to the Guidelines of Experimental Animal Care of the National Research Council of Thailand (National Research Council of Thailand, 1999). The project was reviewed and approved by KKU – TRF contract number RDG5320010.

Experiment I: Effects of heat stress on commercial and Thai Native chickens

Bird management and heat stress conditions

Commercial broilers were compared with native Thai chickens (*Gallus domesticus*) both under normal and heat stress conditions. A total of 200 chickens with equal numbers of males and females were used in the study. Broiler chickens (n = 100) and Thai native chicken (n = 100) were obtained from commercial company and fromthe Research and Development Network Center, Khon Kaen University, Khon Kaen, Thailand, respectively. Initial body weight and agewere 613.3 ± 12.5 g and 3 weeks in commercial broilers, and 601.2 ± 15.8 g and 8 weeks in native chickens, respectively. Body weight was controlled to allow comparing the physiological responses of both groups. Birds were housed in individual cages in two separate rooms (thermoneutral and heat-stress rooms), fed twice daily with a conventional commercial broiler diet (20% CP; 3,250 kcal ME/kg), and given water *ad libitum*.

The heat stress conditions applied in the present study attempted to mimic the natural environment conditions of tropical regions, where temperatures increase with the intensity of daylight. Relative humidity was not artificially controlled. Heat stress was induced by increasing house temperature from 26 °C to 36 °C for six hours daily (10 am to 4 pm), using thermostat-controlled equipment with 20 light bulbs (200 watts). The other group was housed at room temperature, and the house temperature was set at 26 °C from 10 am to 4 pm, using an air conditioner.House temperature and relative humidity were recorded hourly using an automatic data logger. A 7-day adaptation period was applied before the collection of physiological data. Birds were closely monitored until the end of the experimental period of three weeks, when commercial broilers and native chickens were 7 and 10 weeks old, respectively.

Data Collection

The experiment was carried out for three weeks. Respiratory rate (RR; breaths/min), cloacal temperature (CT; °C), heterophil/lymphocyte ratio (H/L), and body weight (BW; g) were determined on days 1, 7, 14, and 21.Physiological variables and blood samples were measured three hours after heat-stress induction. In addition, the body weight (BW), and feed intake (FI) were measured at 08.00 am. Average daily gain (ADG) and feed conversion ratio (FCR) were calculated considering the entire experimental period. Mortality rate was recorded during the experimental period.

In order to determine H/L ratio, blood was individually collected from all experimental birds in Eppendorf tubes with EDTA on day 7, 14 and 21. Whole blood was smeared on a glass slide, and air dried for one minute. Each slide was fixed with absolute methyl alcohol,

stained with Gimsa, heterophil and lymphocyte counts were performed under a microscope to calculate the H/L ratio.

House temperature and humidity were hourly recorded by an automatic data logger. The temperature-humidity index (THI) was calculated according to the formula THI = 0.8 * T_{db} + Rh * (T_{db} – 14.4) + 46.4

where T_{db} is the temperature and *Rh* is the percentage of relative humidity (Hahn *et al.,* 2009).

HSP70 Genotyping

Approximately 1 mL of blood was collected from the wing vein of each chicken, with 0.1 mLof 0.5 M EDTA added as an anticoagulant in the first experimental week. Genomic DNA was isolated using the guanidine method. Briefly, whole blood samples were washed twice with 0.9% NaCl and centrifuged for five minutes at 2,500 rpm (Hermle Z323K rotor, Labnet International, Edison, NJ, USA). Thirty μL of blood cell pellets were transferred to a 1.5 mL tube, followed by the addition of 70 μL of 20% SDS, 25 μL of 1% proteinase K (1 mg/mL), 50 μL of 7.5 m sodium acetate, and 740 μL of cell lysis buffer (5 MGuHCl, 20 mMTris-HCl, and 0.5 M EDTA). After vortexing, incubation at 60 °C for 45–60 minutes, and centrifugation at 1,000 rpm for five minutes, the supernatant was transferred to a new microtube, to which absolute isopropanol was added. The DNA was precipitated at 14,000 rpm for 15 minutes. The supernatant was discarded, and the DNA pellet was washed 2-3 times with 75% ethanol. The DNA pellet was air-dried at room temperature and dissolved in a DNA hydration buffer. DNA quality and concentration were determined by UV spectroscopy (NanoVue, GE Healthcare, UK).The DNA was diluted to 50 mg/μL for use as a working solution and stored at –20 °C before analyses.

The HSP70 gene was amplified by PCR, as explained in Mazzi *et al.* (2003). Primers HSP70-F (5'- AAC CGC ACC ACA CCC AGC TAT G - 3') and HSP70-R (5'- CTG GGA GTC GTT GAA GTA AGC G - 3') were used. The reaction was performed in a total volume of 20 μL containing 100 mg of genomic DNA (2 μL of 50 mg/μL genomic DNA), 0.5 M of each primer (2 μL of 5 M of each primer), 10 MdNTP (2 μL of 100 mMdNTP), 5 M $MgCl_2$ (2 μL of 50 m $MgCl_2$), 1x PCR buffer (2 μL of 10x PCR buffer), and 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI). Reactions were performed in a 96-well thermal cycler (GeneAmp® PCR System 9600, Perkin-Elmer/Applied Biosystems, Carlsbad, CA) according to the following cycling profile: initial denaturation at 94 °C for five minutes; 35 cycles at 94 °C for 30 seconds, 64 °C for 45 seconds, and 72 °C for 45 seconds; and a final extension at 72 °C for five minutes.

PCR products (360 bp) from individual chickens were genotyped for the loci*C* and *M* by digestion with *Cfr*I and *Mme*I (New England Biolabs, Ipswich, MA), respectively. The digestion reaction contained 4 μL of PCR products, 2 μL 10x buffer, and 5 U enzymes, in a total volume of 20 μL (incubated at 37 °C overnight). DNA fragments were subjected to 2% agarose gel electrophoresis with 1 m TBE buffer (0.089 m Tris base, 0.089 m boric acid, and 0.002 m EDTA, pH 8.0). After electrophoresis at constant 100 mA and 100 Volt for 25 minutes, gels were stained with GelStar® (GelStar, Patchogue NY) for 10 minutes. DNA fragments were visualized by UV transillumination and photographed with a gel documentation system (Syngene, Cambridge, UK).

Genotype frequencies were determined separately for loci *C* and *M* according to the formula: $G_i = \sum n_i / N$ where G_i is the *i* genotype frequency, n_i is the number of animals with genotype *i*, and *N* is the total number of samples.

Statistical Analysis

A split-plot in time experimental design was applied. For the analysis of RR, CT, ADG, FI, and FCR, the environmental temperature was set as the main plot, and chicken genetics was set as the subplot.Data were analysed according to the following model:

$$y_{ijkl} = \mu + \rho_i + \alpha_j + \delta_{ij} + \beta_k + \alpha\tau_{jk} + \varepsilon_{ijkl}$$

where y_{ijkl} = observation, μ = overall mean, ρ_i = effect of sex, α_j = effect of environmental temperature (thermoneutral or heat stress), δ_{ij} = main plot error, β_k = effect of genetics (commercial broilers or native chickens), $\alpha\tau_{jk}$ = effect of the environmental temperature × genetic interaction, and ε_{ijkl} = subplot error. Means were compared by Duncan's new multiple range test. For the analysis of the H/L ratio, the subplot measures repeated on days 1, 7, 14, and 21 were included.

Experiment II: Effect of the HSP70 genotype on the heat tolerance of native chickens

The objective of experiment II was to study the effect of the HSP70 genotype on the heat tolerance in native chickens. Based on the results of experiment I, allele C1 may be a putative allele for heat stress tolerance. However, the HSP70 genotypes found in the samples were not equally distributed, and no C1C1 was detected in this experiment. Therefore,

the three genotypes (C1C1, C1C2, and C2C2) were simultaneously evaluated. In Experiment II, native chicken mating was designed to obtain these three genotypes in the offspring. The three genotypes (C1C1, C1C2, and C2C2) were produced by mating C1C1 x C1C1, C1C1 x C2C2, and C2C2 x C2C2 parents (30 sires and 150 dams per mating group). In order prevent confusion with locus M, parents with the M1M1 and M1M2 genotypes were discarded from the study, as <5% of both genotypes were found in native chickens. Therefore, only M2M2 parents were used in Experiment II.

All chickens were reared according to identical management procedures and fed the same feed. The experiment was conducted from August to December, 2010,on the experimental farm of Khon Kaen University, Khon Kaen, Thailand. DNA isolation and genotyping were conducted in the Laboratory of Animal Biotechnology, Department of Animal Science, Khon Kaen University.

Management and Induction of Heat Stress

Before the beginning of the experiment, all birds were reared in an environmentally-controlled house. Birds were submitted to heat stress four weeks before market age. Because Thai indigenous chickens typically achieve market weight at 14-16 weeks of age, 10-wk-old chickens were used in this experiment. Birds of the genotypes C1C1 (n = 38), C1C2 (n = 38), and C2C2 (n = 28) were randomly divided in two groups with equal numbers of males and females and submitted to heat stress or not. Each group was randomly assigned into separate closed compartments (3.5 × 8 m each) in the same room equipped with ventilation. Chickens were kept in individual cages in each compartment and received feed twice daily and water *ad libitum*. Birds were submitted to a 7-day adaptation period before the treatments were applied. The temperature heat-stress room was increased to 36 °C for six hours daily (10 am to 4 pm) using thermostat-controlled equipment with 9 bulbs (250 watts). In the thermoneutral room, temperature was set at 26 °C and maintained air conditioner equipment. Birds were closely monitored during until the end of the 3-week experimental period. House temperature and relative humidity were recorded hourly using an automatic data logger.

Data Collection and HSP70 Genotyping

Respiratory rate (RR; breaths/min), cloacal temperature (CT; ° C), packed cell volume (PCV; %), and heterophil/lymphocyte ratio (H/L) were determined weekly three hours after heat-stress induction for four weeks. Body weight (BW; g) was measured at 08.00 am on the following day, and was used to calculate average daily gain (ADG; g/d). Thereafter, these parameters were collected weekly from 10 to 14 weeks of age.

For PCV measurement, a hematocrit capillary was used to collect blood from the wing vein of individual birds, followed by centrifugation at 12,000 rpm for five minutes. The PCV was calculated as volume of red blood cells relative to whole blood volume, using the hematocrit scale, and expressed as percentage. The H/L ratios were determined as described in Experiment I.

The HSP70 genotypes in the offspring were examined using primers, as described in Experiment I. PCR products were digested with C*fr*I (New England Biolabs, Ipswich, MA, USA) to confirm the mating genotypes. The digestive reaction is detailed in the previous experiment.

Statistical Analysis

A split-plot in time experimental design was applied. The HSP70 genotype was set as the main plot, and time (weeks) was set as the subplot. Physiological data were analysed separately for both thermoneutral and heat stress conditions, using the following model:

$$y_{ijklm} = \mu + \beta_i + \rho_j + \alpha_k + \delta_{kl} + \tau_m + \alpha\tau_{km} + \varepsilon_{ijklm}$$

Where y_{ijklm} = observation, μ = overall mean, β_i = effect of sex, ρ_j = effect of hatch number, α_k = effect of HSP70 genotype (C1C1/M2M2, C1C2/M2M2, and C2C2/M2M2), τ_m = effect of time, $\alpha\tau_{km}$ = main plot error, ε_{ijklm} = effect of genotype × time interaction, and ε_{ijklm} = subplot error. Means were compared by Duncan's new multiple range test. Trends of the physiological and daily gain responses during the experimental period were analyzed by orthogonal polynomials.

RESULTS AND DISCUSSION

Experiment I: Effects of Heat Stress on Commercial broilers and Thai Native Chickens

Daytime THI values observed during the three weeks of the experiment are shown in Figure 1. There was a clear difference in THI patterns between the control and heat stress environments, and the maximum THI difference (approximately 10 degrees of THI) was recorded between 12:00 to 14:00 hours.

Effects of Heat Stress on Growth Performance and Mortality

The results clearly show the growth performance differences between of two genetic strains reared

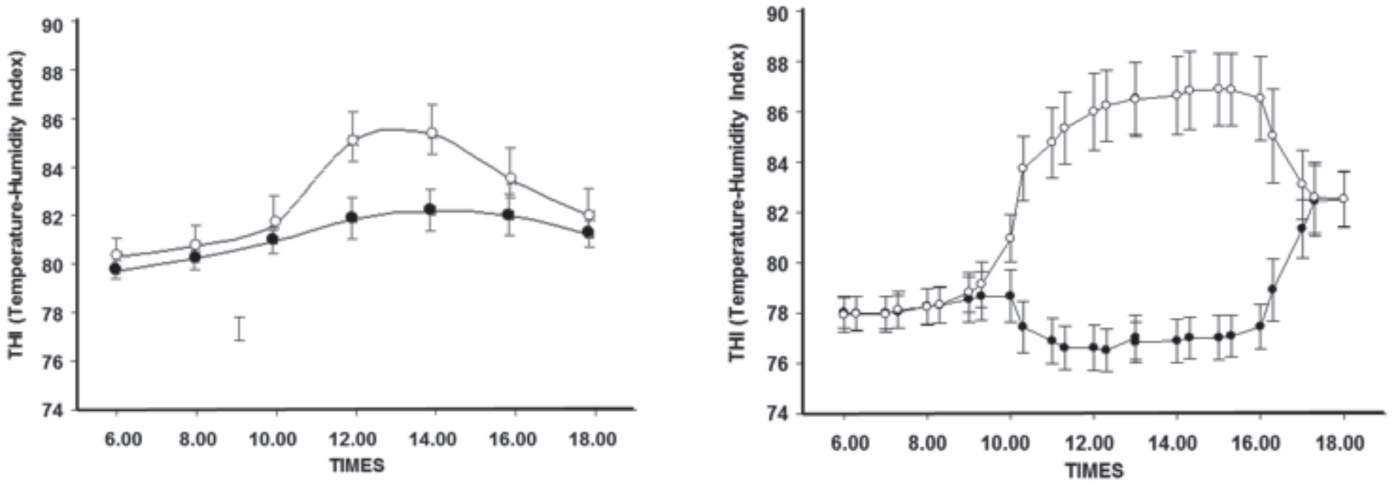

Figure 1 – Temperature-humidity index from 6 a.m. to 6 p.m. in the heat-stress environment at 36 ± 2 °C (O), and thermoneutral environment 26 ± 2 °C (●) recorded in Experiments I (a) and II (b).

under different temperatures (Table 1). The average daily gain (ADG) and feed intake (FI) of commercial broilers were significantly ($p<0.05$) reduced under heat stress conditions. On the other hand, the ADG of native chickens was not affected by heat stress, indicating the heat tolerance of this native breed. No feed conversion ratio (FCR) differences were detected ($p>0.05$) between treatments within the same genetics. However, genetic x environmental interactions (GxE) were observed. The rate of ADG reduction in commercial broilers (20.4%) was twice as high as in native chickens (9.6%) when temperature was increased from 26 (±2) °C to 36 (±2) °C. In contrast, the rate of FI reduction in commercial broilers (7.9%) was nearly twice as low as in native chickens (14.0%). Due to their lower tolerance to heat, the mortality rate of commercial broilers (46.0%) was extremely high, whereas it was only 2% in native chickens (2%). Reduced feed intake and growth performance due to heat stress has been documented in several reports (Mehta & Singari, 1999; Yuming *et al.,* 1999; Altan *et al.,* 2000; Oliveira *et al.,* 2000; Temim *et al.,* 2000; Deeb & Cahaner, 2001; Xin *et al.,* 2002).

Effects of heat stress on physiological responses

Under thermoneutral environments, body temperature of chickens should range between 41-42 °C (Donkoh, 1989; Reddy, 2000). When submitted to high environmental temperatures (36 °C), broiler body temperature increases, resulting in panting and high water consumption (Pathom, 1997; Cooper & Washburn, 1998; Zhou *et al.,* 1999; Puvadolpirod & Thaxton, 2000). Therefore, body temperature is an indicator of heat stress tolerance. In the present study, the cloacal temperature of commercial broilers was higher ($p<0.05$) than that of native chickens, in both under thermoneutral and heat stress conditions. Increased cloacal temperatures in birds of both evaluated genotypes were observed from days 1 to 21 of the experiment in the heat-stress environment. Panting is the main route for heat dissipation in chickens, according to Aengwanich (2007), who also reported higher heat tolerance in Thai native chickens

Table 1 – Effect of heat stress on the respiratory rate, cloacal temperature, average daily gain, feed intake, feed conversion ratio and mortality of commercial broilers and Thai native chickens maintained at thermoneutral (26 ± 2 °C) or heat stress (38 ± 2 °C) temperatures.

	Commercial Broilers		Thai Native chicken		SEM
	26 ± 2 °C	38 ± 2 °C	26 ± 2 °C	38 ± 2 °C	
Respiratory rate, RR (breaths/min)	39.18 [b]	162.57 [c]	33.26 [a]	158.87 [c]	1.54
Cloacal temperature, CT (°C)	41.58 [b]	43.14 [d]	41.42 [a]	42.24 [c]	0.04
Average daily gain, ADG (g/d)	42.26 [a]	33.63 [b]	17.47 [c]	15.78 [c]	1.70
Feed intake, FI (g/d)	87.31 [a]	80.33 [b]	72.21 [c]	62.09 [d]	2.12
Feed conversion ratio, FCR	2.08 [a]	2.39 [a]	4.13 [b]	4.09 [b]	0.23
Mortality (%)	0.00	46.00	0.00	2.00	-

[a,b] Means with different superscripts indicate significant differences between genetic strains ($p< 0.05$).

than in commercial broiler strains when reared in hot temperatures (38 ± 1 °C).

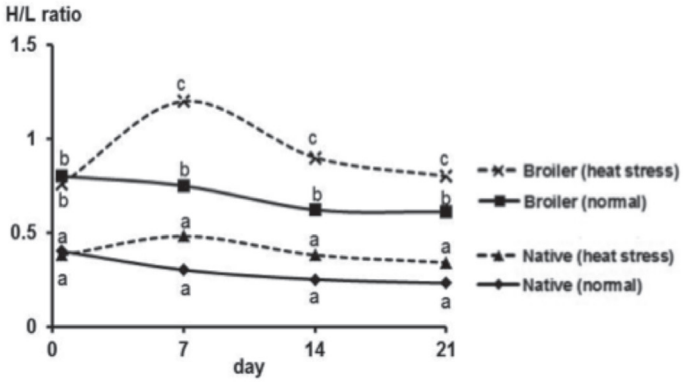

Figure 2 – Heterophil:lymphocyte (H/L) ratio measured on days 0, 7, 14 and 21 of the experiment of commercial broilers and Thai native chickens maintained at thermoneutral (26 ± 2 oC) or heat stress (38 ± 2 oC) temperatures. Different letters within the same day indicate significant differences among groups (p<0.05).

Experiment II: Effect of the HSP70 genotype on the heat tolerance of native chickens

HSP70 Genotypes

The polymerase chain reaction with the given primer gave a PCR product size of 360 bp. The genotype at locus C was defined after cutting with *Crf*I; a fragment size of 229 bp was indicated for the C1 allele, and of 240 bp for the C2 allele. The genotype at locus M was defined after cutting with *Mme*I; a fragment size of 229 bp was indicated for the M1 allele, and of 360 bp (non-cutting) for the M2 allele. The expected C1C1, C2C2, and C1C2 genotypes from C1C1 x C1C1, C2C2 x C2C2, and C1C1 x C2C2 parents, respectively, were confirmed by PCR-RFLP. All chickens were also confirmed for the M2M2 genotype. A total of 102 chicken genotypes at locus C and locus M were also confirmed. The fragments from gel electrophoresis indicating genotypes C1C1, C1C2, and C2C2 are shown in Figure 3.

Effect of HSP70 genotypes on physiological responses

The effects of genotypes on the physiological responses, as measured by respiratory rate (RR) and cloacal temperature (CT) under both control and heat-stress conditions, are shown in Table 2.The RR of native chickens submitted to heat stress was significantly higher than that of the controls. No RR and CT differences (p>0.05) among genotypes were detected in none of the experimental weeks. The considerable RR increase in chickens under heat stress was in agreement with several previous reports (Abu-Dieyeh, 2006; Aengwanich & Suttajit, 2010). Body heat dissipation increased with increasing RR, but CT remained unchanged. The absence of effects of genotypes on RR and CT is in agreement with Duangduen (2007) and Puvadolpirod & Thaxton (2000). The evaluated parameters were different between environments. Under heat stress, during the three-week period, RR and CT presented cubic (p<0.01) and tentatively quadratic (p<0.10) polynomial responses, respectively (Figure 4). The rate of RR increase was high during the first week after exposure to heat, and then gradually slowed until the third

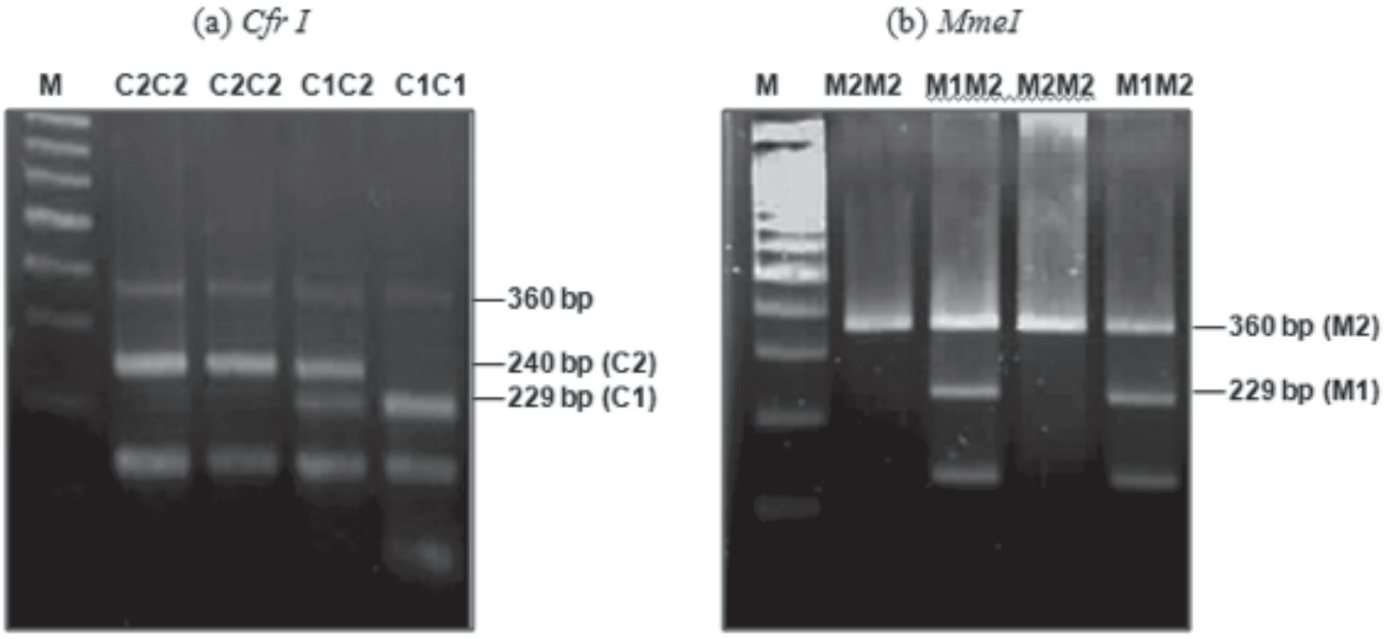

Figure 3 – DNA bands from PCR and polymorphism of HSP70 by PCR-RFLP, showing PCR product size of 360 bp. Genotype at locus C: fragment size of 229 bp for the C1 allele and of 240 bp for the C2 allele. Genotype at locus M: fragment size of 229 bp for the M1 allele and of 360 bp for the M2 allele.

week. This pattern indicates that the birds acclimated to the hot temperature (Moberg & Menh, 2000). CT peaked in the second week and then remained stable. In the heat-stressed chickens, RR was more than four times higher than that of chickens maintained in the thermoneutral environment.

Table 2 – Genotypic frequency distribution for the loci C and M in commercial broilers and Thai native chickens.

	Locus C			Locus M		
	C1C1	C1C2	C2C2	M1M1	M1M2	M2M2
Commercial broilers	-	0.08	0.92	-	0.15	0.85
Native chicken	0.18	0.54	0.28	-	0.07	0.93

Effect of HSP70 Genotypes on Hematological Response

The effects of genotypes on hematological response, as measured by packed cell volume (PCV) and heterophil/lymphocyte ratio (H/L) under thermoneutral and heat stress conditions are shown in Table 3. Under heat stress, PCV presented different patterns among genotypes. The PCV of Thai native chickens is typically 28-37% (Simaraks *et al.*, 2004). In this study, the PCV of control chickens was ranged between 29.79 and 30.48%. Under heat stress conditions, PCV linearly ($p<0.01$) decreased in C1C1 and C1C2 genotypes, whereas C2C2 presented a quadratic ($p<0.01$)

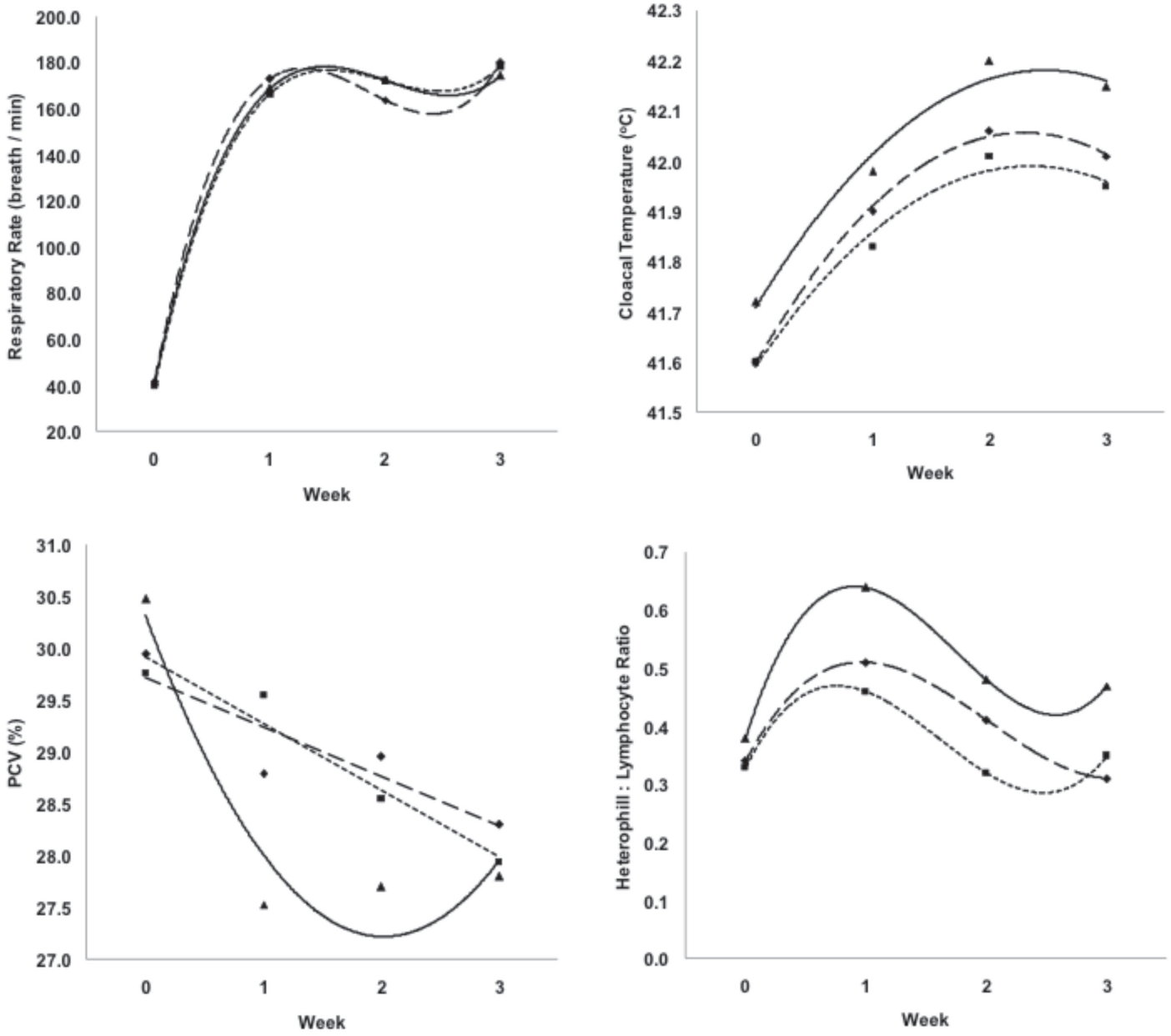

Figure 4 – Responses and trends of: a) respiratory rate, b) cloacal temperature, c) packed cell volume, and d) heterophil/lymphocyte ratio before heat stress (week 0) and during heat stress (weeks 1–3) among three genotypes (C1C1 = ●, – –; C1C2 = ●, ------; C2C2 = ▲, ———).

Table 3 – Effects of *HSP70* genotypes on the respiratory rate, cloacal temperature, packed cell volume, H/L (heterophil/lymphocyte ratio) and average daily gain in Thai Native chickens maintained at thermoneutral (26 ± 2 °C) or heat stress (38 ± 2 °C) temperatures.

Trait	Genotype	Control (26 ± 2 °C)								Heat stress (36 ± 2 °C)							
		Week				SEM	Contrast			Week				SEM	Contrast		
		0	1	2	3		L	Q	C	0	1	2	3		L	Q	C
Respiratory rate (breath/min)	C1C1	33.00	27.98	23.40	20.07	1.54	NS	NS	NS	41.48	173.18	163.84	179.94	4.04	< 0.01	< 0.01	< 0.01
	C1C2	30.89	26.05	20.92	16.70	1.78	NS	NS	NS	39.89	166.59	172.28	178.44	4.26	< 0.01	< 0.01	< 0.01
	C2C2	39.63	26.44	25.46	22.83	1.86	NS	NS	NS	41.52	168.98	172.65	174.64	3.27	< 0.01	< 0.01	< 0.01
	SEM	3.20	4.37	2.59	1.74					2.69	8.23	6.01	7.78				
Cloacal temperature (°C)	C1C1	41.44	41.64	41.53	41.60	0.04	NS	NS	NS	41.60	41.90	42.06	42.01	0.05	< 0.01	< 0.05	NS
	C1C2	41.44	41.56	41.43	41.44	0.04	NS	NS	NS	41.60	41.83	42.01	41.95	0.05	< 0.01	< 0.05	NS
	C2C2	41.47	41.66	41.67	41.59	0.04	NS	NS	NS	41.72	41.98	42.20	42.15	0.04	< 0.01	< 0.10	NS
	SEM	0.07	0.06	0.09	0.07					0.09	0.06	0.07	0.09				
Pack cell volume (%)	C1C1	30.25	29.73	29.52	29.77	0.33	NS	NS	NS	29.95	28.79[a]	28.96	28.30	0.31	< 0.01	NS	NS
	C1C2	30.94	30.83	30.41	30.13	0.43	NS	NS	NS	29.76	29.55[a]	28.55	27.94	0.32	< 0.01	NS	NS
	C2C2	30.58	30.14	30.18	29.77	0.43	NS	NS	NS	30.48	27.53[b]	27.70	27.80	0.25	< 0.01	< 0.01	NS
	SEM	0.85	0.42	0.63	0.87					0.52	0.35	0.41	0.61				
H/L[1/]	C1C1	0.31	0.38	0.35	0.35	0.02	NS	0.02	NS	0.34	0.51	0.41	0.31[a]	0.03	NS	< 0.01	0.11
	C1C2	0.29	0.31	0.29	0.25	0.02	NS	NS	NS	0.33	0.46	0.32	0.35[a]	0.04	NS	NS	0.04
	C2C2	0334	0.37	0.38	0.36	0.02	NS	NS	NS	0.38	0.64	0.48	0.47[b]	0.03	NS	0.11	0.04
	SEM	0.04	0.03	0.04	0.04					0.03	0.07	0.06	0.05	0.03			
Average daily gain (g/day)[2/]	C1C1	-	27.42	13.34	8.87	0.80	< 0.01	< 0.01	-	-	27.18	9.78	8.39	0.91	< 0.01	< 0.01	-
	C1C2	-	25.71	14.43	10.20	0.94	< 0.01	< 0.01	-	-	26.64	11.59	7.30	0.96	< 0.01	< 0.01	-
	C2C2	-	27.67	12.86	11.19	0.97	< 0.01	< 0.01	-	-	27.14	9.98	8.16	0.76	< 0.01	< 0.01	-
	SEM		1.48	1.70	1.88						1.42	2.06	1.62				

[1/ a,b] Means with different superscripts within the same trait indicate significant differences among genotypes ($p<0.10$)

NS = not significant; SEM = standard error of mean; L = linear; Q = quadratic; C = cubic response.

decreasing response. The decrease of PCV in chickens under heat stress was in agreement with the report by Altan *et al.* (2003). PCV decreases because heat is released from the cells by losing water to the interstitial fluid and blood circulation. The increase of water in the plasma, therefore, reduces the PCV (Abdel-Azeemet *et al.,* 2007). Hemodilution is an important mechanism related to evaporative heat loss (El-Nouty *et al.,* 1990) in Thai indigenous chickens. Figure 4c shows that the PCV of C2C2 chickens under heat stress decreased quadratically ($p<0.01$), while C1C1 and C1C2 chickens presented a linear ($p<0.01$) reduction. The PCV difference between the C2C2 (27.53%) and the C1C1 (28.79%) and C1C2 (29.55%) genotypes tended to be different ($p<0.10$). The rapid PCV decrease observed in the first week of heat stress shows that the C2C2 chickens were more sensitive to heat stress. However, all genotypes presented similar PCV values by the third week. This indicates that the PCV recovery time from heat stress effect is of at least two weeks.

The H/L ratio is an acceptable indicator of heat stress (Gross & Siegel, 1983). It has also been found to be related to blood glucocorticoids levels (Davis *et al.,* 2008). Therefore, H/L has been used to evaluate the welfare of chickens in stressful environments (Altan *et al.,* 2000). In the present study, the H/L of C1C1 chickens presented a quadratic response ($p<0.05$) and C1C2 and C2C2 a cubic response ($p<0.05$) under heat stress (Figure 4d). The H/L sharply increased after the first week of heat exposure, but tended to return to normal level by the third week due to homeostasis. This increase of H/L in response to heat stress response is consistent with Zulkifli *et al.* (2000a), who observed a significant H/L increase in broilers around 4-7 days after exposure to heat stress. Similar findings were reported by Aengwanich (2007) and Duangduen *et al.* (2007). The increase of H/L under chronic heat stress is explained by the simultaneous increase in heterophils and decrease in lymphocytes in response to glucocorticoid hormones (stress hormones), which promote the release of heterophils from the bone marrow into the circulation (Bishop *et al.,* 1968). This may further cause an increase in the attachment of lymphocytes to the vascular epithelial lining (Fauci, 1975; Dhabhar, 2002). H/L ratios of 0.2, 0.5, and 0.8 have been suggested as indicators of no stress, moderate, and severe stress in chickens, respectively (Gross & Siegel, 1983). According to this criterion, Thai native chickens in our study were not stressed under thermoneutral conditions and were moderately stressed under heat stress. Previous reports (Aengwanich, 2007; Duangduen *et al.,* 2007) showed that broilers may present moderate stress (H/L in a range of 0.4–0.5) even under thermoneutral conditions, and severe stress when submitted to heat stress (H/L in a range of 0.6–0.9). Our results show that C2C2 chickens presented a wide oscillation of H/L ratio, especially in the first week, indicating that this genotype was sensitive to heat stress. The significantly higher ($p<0.01$) H/L ratios of C2C2 compared with other genotypes in the third week indicates the unsuccessful recovery of H/L in C2C2 chickens.

Effect of HSP70 Genotypes on Average Daily Gain

The effects of genotype on average daily gain (ADG) under control or heat stress conditions are shown in Table 3. Chickens in the heat stressed group presented slightly lower ADG than the control group. However, no significant differences in ADG among genotypes ($p>0.05$) maintained under either condition were detected. The quadratic reduction ($p<0.01$) of ADG over the three-week period can be attributed to the increasing age of the chickens. The lack of ADG differences between heat-stressed and control chickens is also in agreement with the findings of Aengwanich (2007) and Duangduen (2007). On the other hand, ADG reduction due to heat stress is commonly observed in commercial broiler chickens (Cooper & Washburn, 1998; Altan *et al.,* 2000).

The results of this study indicate that RR, PCV and H/L are effective indicators for heat stress in chickens. Significant responses to heat stress – increasing RR to remove heat from the body, increasing hemodilution (decreasing PCV) to remove heat from the cells, and increasing H/L ratios in response to glucocorticoid hormones – were demonstrated in our study. The HSP70 genotypes presented different tolerances to heat stress. The C2C2 genotype presented the highest susceptibility to heat stress. Therefore, the selection of C1C1 or C1C2 genotypes may be applied in commercial poultry breeding programs to enhance heat tolerance.

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