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Effect of early age thermal conditioning on expression of heat shock proteins in liver tissue and biochemical stress indicators in colored broiler chicken

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ABSTRACT

The present study was aimed at finding the effects of early age thermal conditioning on liver HSP 90 alpha, 70 and 60 mRNA expression and plasma biochemical parameters in colored broiler chicken. Chicks from two breeds (Naked neck and Punjab Broiler-2) were divided in to control (C) and heat exposed (H). The chicks were reared in controlled environmental chamber (25±1 °C). H group chicks were exposed to 43±1° C for 6 hours during 5-7th day post hatch. Chicks from H group and a sample of chicks from control group (CE) were exposed to 43±1 °C for 6 hours on 42nd day. On 7th and 42nd day chicks from each group were sacrificed for sample collection and processing. On 7th day the mRNA expression of all the three genes were significantly (P<0.05) higher in H group in both the breeds. On 42nd day CE group chicks had significantly (P<0.05) higher mRNA expression for all HSP genes compared to HE birds. T_3 concentration was significantly (P<0.05) higher in H group birds in both the breeds at 7th day. The biochemical parameters significantly (P<0.05) varied between the C and H groups at 7th day. The biochemical parameters were significantly lower (P<0.05) in HE group birds compared to CE group birds after acute heat exposure on 42nd day. Based on the results of this study it can be concluded that early age thermal conditioning of chicks improves the thermo tolerance in post natal life in colored broiler chicken.

Key words: Biochemical indicators, Chicks, Heat Shock Protein, T_3, Thermal conditioning,

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INTRODUCTION

Adaptation to thermal stress during early life has advantageous effects in the later stages of life that improves the heat tolerance in broiler chicken**.** The epigenetic adaptive response has been successfully demonstrated by early age thermal manipulation of chicks by exploiting the incomplete maturation of hypothalamic thermoregulatory system (Labunsky and Meiri, 2006; Yossifoff et al., 2008; Yahav, 2009). Temperature is one of the most important factors that exerts a negative influence on the performance of poultry and causes huge losses in terms of loss of productivity, reduced growth rate, feed efficiency, egg shell quality, survivability, reduced reproductive efficiency, reduced immune competence and increased investment costs to mitigate the effects of climate change (Quinteiro-Filho et al., 2010; Rajkumar et al., 2015a). Increase in temperature beyond thermo neutral zone (18-24°C) due to environment or other factors will lead to cascading effects on thermoregulation and could be lethal to the birds as birds are more vulnerable to high temperatures.

Sudden changes in the environmental temperature are the earliest and most common phenomenon that cells have to cope with and have to preserve their structural and enzymatic integrity (Nadeau and Landry, 2006). Heat shock proteins (HSPs) are a set of proteins synthesized in response to physical, chemical or biological stresses including heat exposure (Staib et al., 2007)**.** HSPs are broadly classified into 6 distinct families based on their molecular weights ranging from 10 to 150 kDa (Benjamin and McMillan, 1998). The stimulated thermal tolerance degree is related with the expression of *HSPs* (Krebs and Bettencourt, 1999) in living organisms.

In tropical countries, summer stress is severe, especially when the environmental temperature goes up to 45 ^{o}C which leads to production of excess quantities of reactive oxygen species (ROS) causing damages to cell phospholipid membrane and other macromolecules (Wiseman and Halliwell, 1996). Early adaptation to thermal stress will reduce the effects during summer, especially in tropical countries where summer temperatures are high. The change in biochemical indicators and their trend is important in assessing the stress condition in birds and to implement the effective mitigation strategies to combat stress. Different studies are there to explore the mechanism underlying stress response and differential expression of genes in chicken (Rimoldi et al., 2015; Sun et al., 2015). Very few studies are available in relation to early age thermal conditioning/manipulation in chicken.

Having known these facts the purpose of the present study was to investigate the possible effects of thermal conditioning to high temperature during the early stages of life on *HSP 90 alpha, 70* and *60* mRNA expression in liver tissue and biochemical indicators in plasma of colored broiler chicken.

MATERIALS AND METHODS

The experiment was conducted at ICAR-Directorate of Poultry Research, Hyderabad in collaboration with Veterinary College and Research Institute (VCRI), Namakkal, Tamilnadu. The experiment was approved by the Institutional Animal Ethics Committee (IAEC).

2.1. Experimental Population

Naked neck (NN) and Punjab Broiler (PB-2) were chosen for this study to observe the effect of early age conditioning of chicks on the expression of *HSP* genes in liver tissue. The Na gene from NN was introduced in to broiler population and the base population was developed after four successive generations of backcrossing and is maintained under mild selection pressure for six weeks body weight for the last nine generations. It is an important ecotype and distributed along the hot humid coastal regions of India and is known for its heat tolerance. PB-2, a synthetic coloured broiler line was under long term selection for high 5 weeks body weight and 40 weeks part period egg production which is being utilized for the development of improved colored broiler chicken varieties.

A total of 180 day old chicks, 90 each from NN and PB-2 were collected from ICAR-Directorate of Poultry Research, Hyderabad was transported to VCRI Namakkal. The chicks were reared under normal condition for 4 days to adapt to the local condition and also to reduce the transportation stress. Then, the birds were randomly divided in to two equal halves in each breed as control (C) and heat exposed (H**).** On 5th, 6th and 7th day H group was exposed to 43±1^{o} C for 6 hours. On 7th day, 6 chicks from both breed and groups from each breed were sacrificed and used for gene expression studies. The remaining birds from C and H groups were placed in an automatically controlled environmental chamber and maintained at 25±1°C temperature, 70% relative humidity (RH) till 42nd day. The chicks were offered broiler ration (2,900 K cal: ME, 22%: CP) *ad libitum* throughout the experimental period. The chicks were vaccinated at 1st day against Marek's disease, 7th day against Newcastle disease, finally at 14th and 24th day for infectious bursal disease.

On 42nd day chicks from both C and H groups were exposed to high temperature at 43±1^{o} C for 6 hours and non exposed birds were considered as control making three groups in each breed control (C), control exposed (CE) and heat exposed (HE).

2.2. Sample collection

Six birds from each group were sacrificed on 7th and 42nd day and liver tissue was collected and stored at -70^{o} C for further use. Blood was collected in tubes containing 0.5 M EDTA from the brachial vein before slaughter. Plasma was separated by centrifuging at 2000 rpm for 15 minutes and used for biochemical and hormonal assay.

2.3. RNA Isolation and cDNA synthesis

Total RNA was extracted from liver tissue using SV total RNA isolation system (Promega, USA) according to the standard instruction. The concentration and purity of the RNA was determined by measuring absorbance in Genova nano (Jenway, UK). cDNA was synthesized from 1.5 µg of each total RNA samples using a random primer and reverse transcriptase enzyme (Applied Biosystems, USA). The reverse transcription was processed at 25º C for 10 minutes followed by 37º C for 120 minutes and the enzyme reaction was inhibited at 85º C for 5 minutes

2.4. Quantitative PCR (qPCR)

The first-strand cDNAs were used as a template to amplify gene specific primers for *HSP 90 alpha, HSP 70, HSP 60* and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*. The reactions were performed in a 25 µl volume of KAPA SYBR FAST qPCR kit (Kapa biosystems, USA) with 10 picomole of forward and reverse primer from each gene (Table 1) using Mx-3000P spectro flourometric thermocycler (Stratagene, USA). *GAPDH* was used as an endogenous control. The amplification protocol used was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of cyclic denaturation at 94°C for 20 s, annealing at 59°C for 1min and extension at 72°C for 15 s. Standard curve was generated to check the primer efficiency of the above genes by tenfold dilution of cDNA.

Table 1. Heat shock proteins (HSP) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* primer sequence information used for this study

Primers	Sequence (5'…3')	Product Size (bp)	Annealing Temperature , ºC	Accession Number
HSP 90 alpha	F- GCATTCTCAGTTCATTGGCTACC R-CTGTCTTCTCCTCCTTCTCCTCT	122	62	NM_206959.1
HSP 70	F-ATGAGCACAAGCAGAAAGAG R-TCCCTGGTACAGTTTTGTGA	95	61	J02579
HSP 60	F- AGAAGAAGGACAGAGTTACC R- GCGTCTAATGCTGGAATG	116	50	NM_001012916.1
GAPDH	F-CTGCCGTCCTCTCTGGC R-GACAGTGCCCTTGAAGTGT	105	60	NC_006088

2.5. Lipid peroxidation (LP)

RBC lysate (1:20) was mixed with Tris KCl buffer (1.3ml) and TBA reagent (1.5 ml) and boiled for 1 hour. The reagents were cooled and pyridine-butanol mixture (3 ml) and 1N NaOH (1 ml) were added. The absorbance of the test sample was measured against blank at 548 nm and the total amount of lipid peroxidation present in the haemolysate was calculated in terms of moles of malondialdehyde (MDA)/mg protein (Placer, 1966)

2.6. Reduced Glutathione (GSH)

GSH contents in plasma were determined by reduction of Ellman`s reagent by SH groups (GSH) to form 5.5-dithio-2-nitrobenzoic acid (DTNB) in phosphate buffer (Ellman 1959). The OD value of the yellow color developed was measured at 412 nm using spectrophotometer (Optima Tokyo, Japan). GSH was used as a standard solution and expressed as µmol/g protein.

2.7. Superoxide Dismutase (SOD)

SOD enzyme activity was assessed in a microtiter plate (96 well) using plasma (Madesh and Balasubramanian, 1998). Reduction of tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2yl) 2, 5-diphenyl tetrazoium bromide] to its formazan was generated by pyrogalol auto- oxidation and super oxide inhibition and the reaction was inhibited by DMSO and read at 570 nm and is expressed as U/ml.

2.8. Nitric oxide (NO)

Nitric oxide level was estimated as nitrite and nitrate by acidic Griess reaction after reduction of nitrate to nitrite by vanadium trichloride according to the method described by Miranda et al. (2001). The Griess reaction relies on a simple colorimetric reaction between nitrite, sulfonamide and N-(1-naphthyl) ethylenediamine to produce a pink azo product with maximum absorbance at 543 nm. The concentrations were determined using a standard curve of sodium nitrate and the results were expressed as µM of nitrate.

2.9. Ferric reducing ability of plasma (FRAP)

The colorimetric assay determines the antioxidant capacity of samples and was known to reduce the intense blue ferric tripyri –dyltriazine complex to its ferrous form (Benzie and strain, 1996) and is expressed as micro mole of $FESO_4$ per liter.

2.10. Alkaline Phosphatase (ALP)

ALP catalyses the hydrolysis of colourless p-Nitrophenyl phosphate to yellow coloured p-Nitrophenol phosphate at pH 10.3 and was estimated in the plasma using commercially available kit (Autospan, India). The yellow colored product was kinetically measured at 405 nm and it is proportional to ALP activity, expressed in IU/L.

2.11. Triiodothyronine (T_3)

The total T_3 level was determined using commercially available EIA kit (RFCL Ltd, India). The intra assay coefficient of variation and sensitivity level was 10% and 0.04 ng/ml respectively.

2.12. Statistical Analysis

Relative quantification of gene expression was estimated using Ct values. The Ct value is the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The level of target genes relative to endogenous control was assessed by $2^{-\Delta\Delta Ct}$ formulae and expressed in fold change. The data were analyzed on a 2-factorial ANOVA, the main effects (breed and treatment) and their interactions were evaluated using the SPSS 15 software. Treatment means that were significant were further subjected to Turkey's post-hoc test.

RESULTS

3.1. Gene expression

The results revealed that the interaction between the treatment and breed was not significant for gene expression and all stress indicators. The mRNA expression of all the three genes in liver viz., *HSP 90 alpha, 70 and 60* was significantly ($P<0.05$) higher in H group in both NN and PB-2 chickens on 7th day (Fig 1&2). CE group chicks, that were not exposed to high temperature during early age had significantly ($P<0.05$) higher m RNA expression for all the *HSP* genes, while earlier exposed birds (HE) had significantly ($P<0.05$) lower gene expression in both NN and PB-2 (Fig 3&4) on re exposure to high temperature on 42nd day, indicating the lesser stress due to pre adaptation.

3.2. Biochemical parameters

The estimates for all the stress indicators on 7th day are presented in Table 2. The body temperature was significantly ($P<0.05$) higher in H group in both NN and PB-2 chickens. The T_3 concentration was significantly ($P<0.05$) higher in C group birds in both the breeds. The stress indicators such as NO, SOD, LP and ALP concentrations significantly ($P<0.05$) varied between the C and H groups at 7 days of age (Table 2) in both the breeds. The body temperature in HE group was significantly ($P<0.05$) lower than CE and significantly ($P<0.05$) higher than C on 42nd day in both the breeds (Table 3). HE group had significantly ($P<0.05$) lower T_3 concentration in PB-2, while not varied significantly in NN, though numerically lower than the CE group. The concentrations of NO, GSH and SOD activity did not show any significant variations among the treatment groups as well as two chicken genotypes. The LP in HE group was significantly ($P<0.05$) lower and almost similar to the C group in both the breeds. The FRAP concentration was significantly ($P<0.05$) higher in HE birds in both breeds (Table 3). ALP concentration was significantly ($P<0.05$) lower in HE than the CE group in PB-2, while not varied in NN chicken.

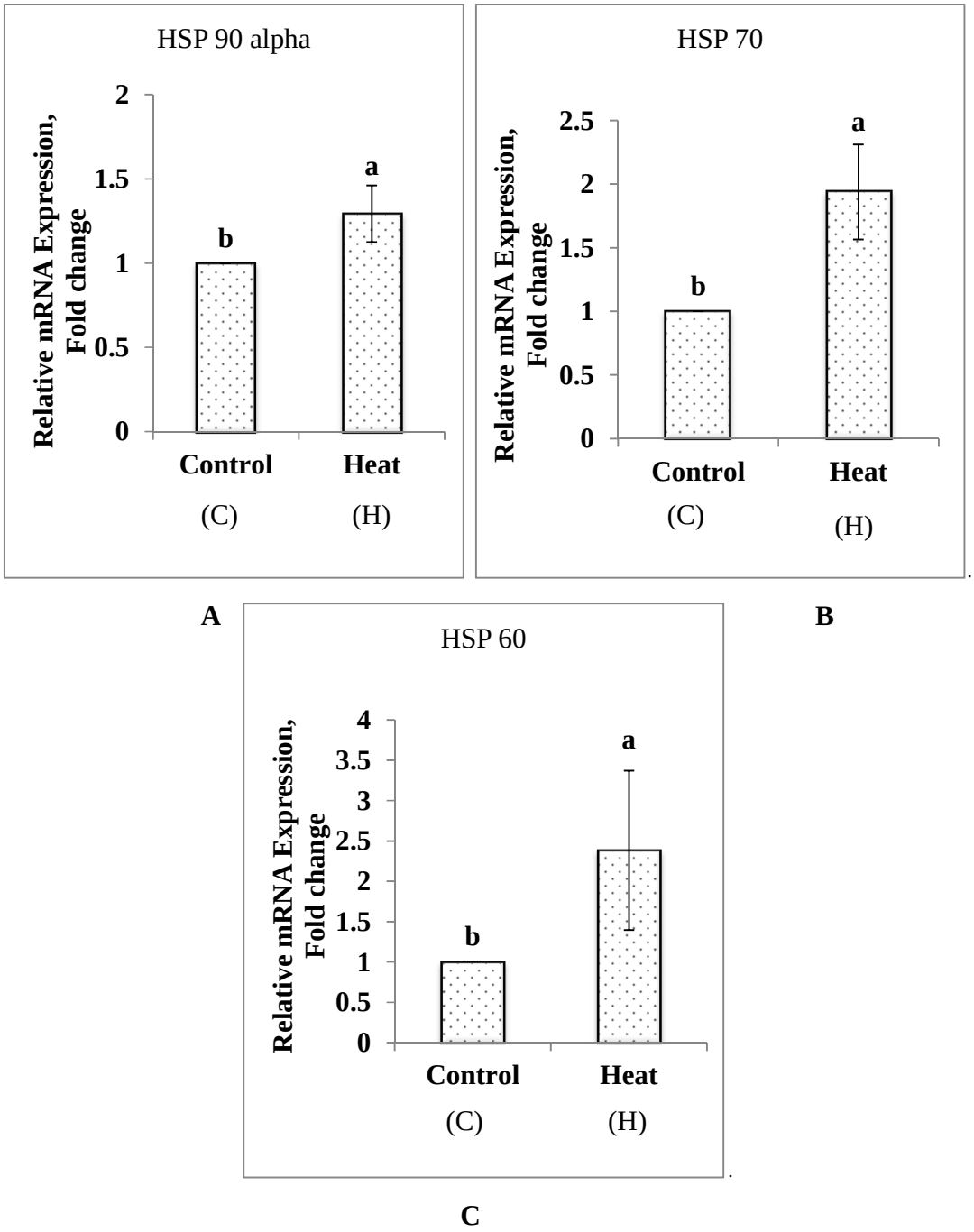

Fig. 1. Heat shock protein (*HSP*) gene expression in liver of Naked neck (NN) chicks at 7th day of age. A. HSP 90 alpha, B. HSP 70, C. HSP 60. ^{a,b} Means with different superscripts differ significantly (P<0.05)

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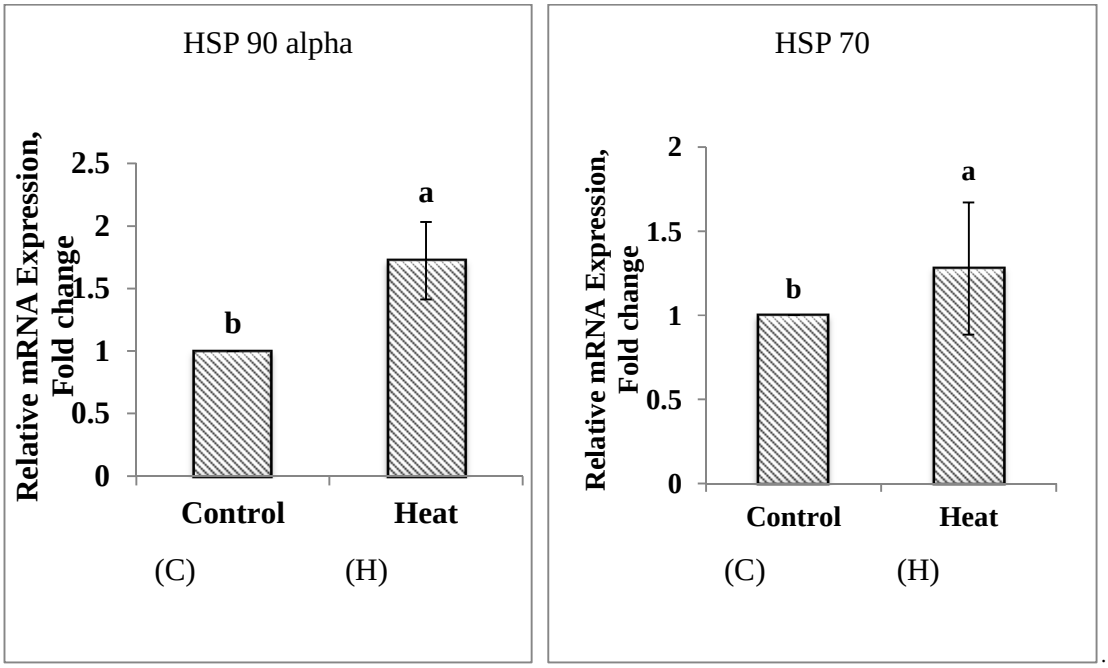
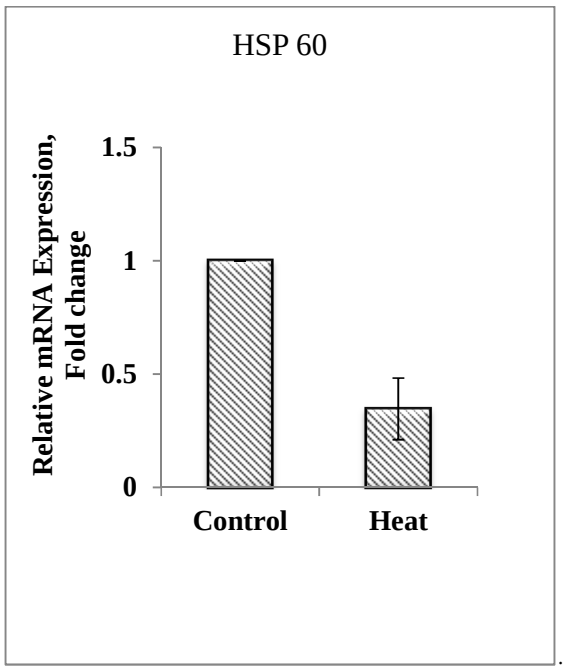


A **B**



(C)

Fig. 2. Heat shock protein (*HSP*) gene expression in liver of Punjab broiler-2 (PB-2) chicks at 7th day of age. A. HSP 90 alpha, B. HSP 70, C. HSP 60. [a, b] Means with different superscripts differ significantly (P<0.05)

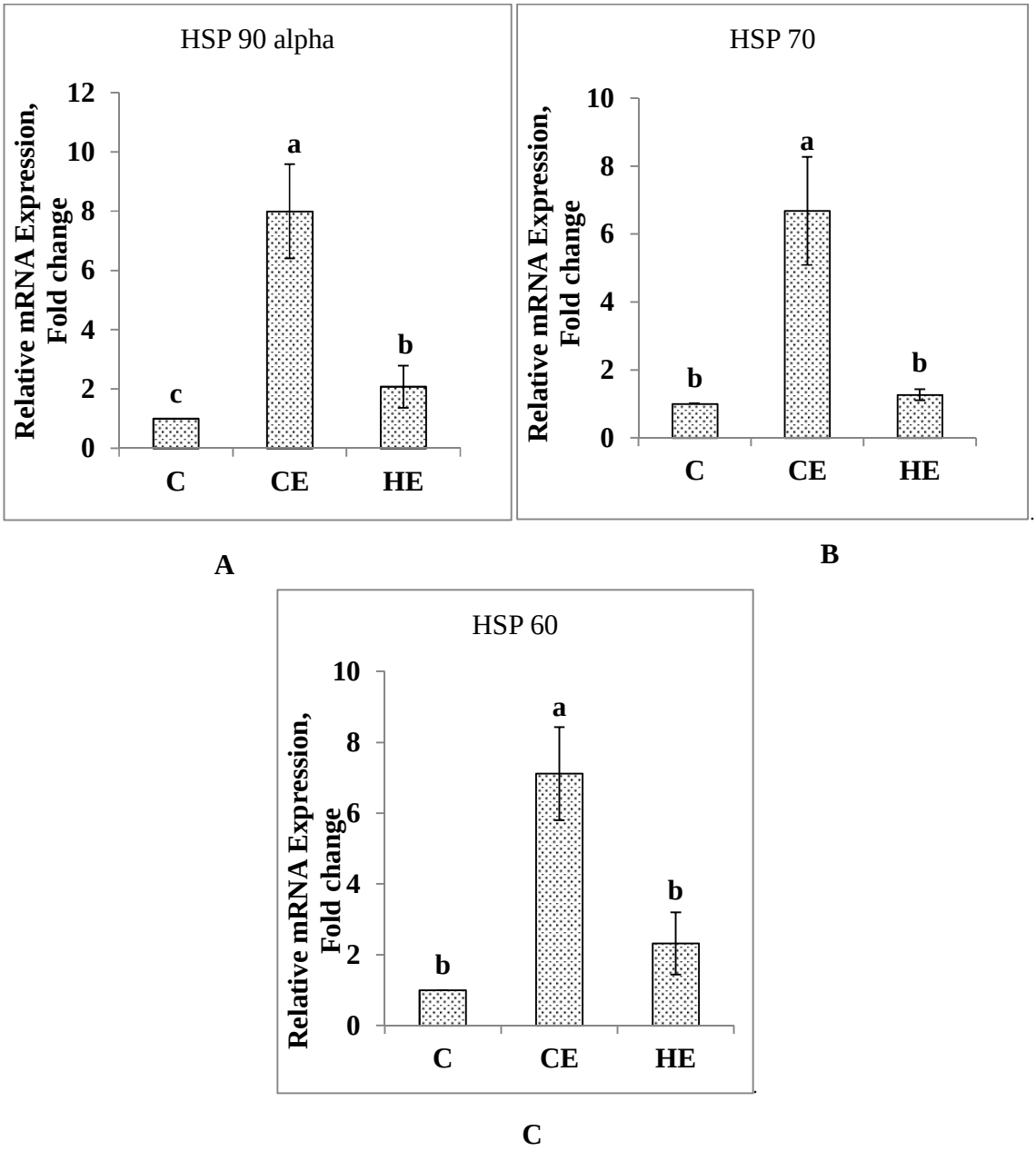


Fig. 3. Heat shock protein (*HSP*) gene expression in liver of Naked neck (NN) chicks at 42nd day of age. (C- Control; CE- Control exposed; HE- Heat exposed). A. HSP 90 alpha, B. HSP 70, C. HSP 60. [a, b] Means with different superscripts differ significantly (P<0.05)

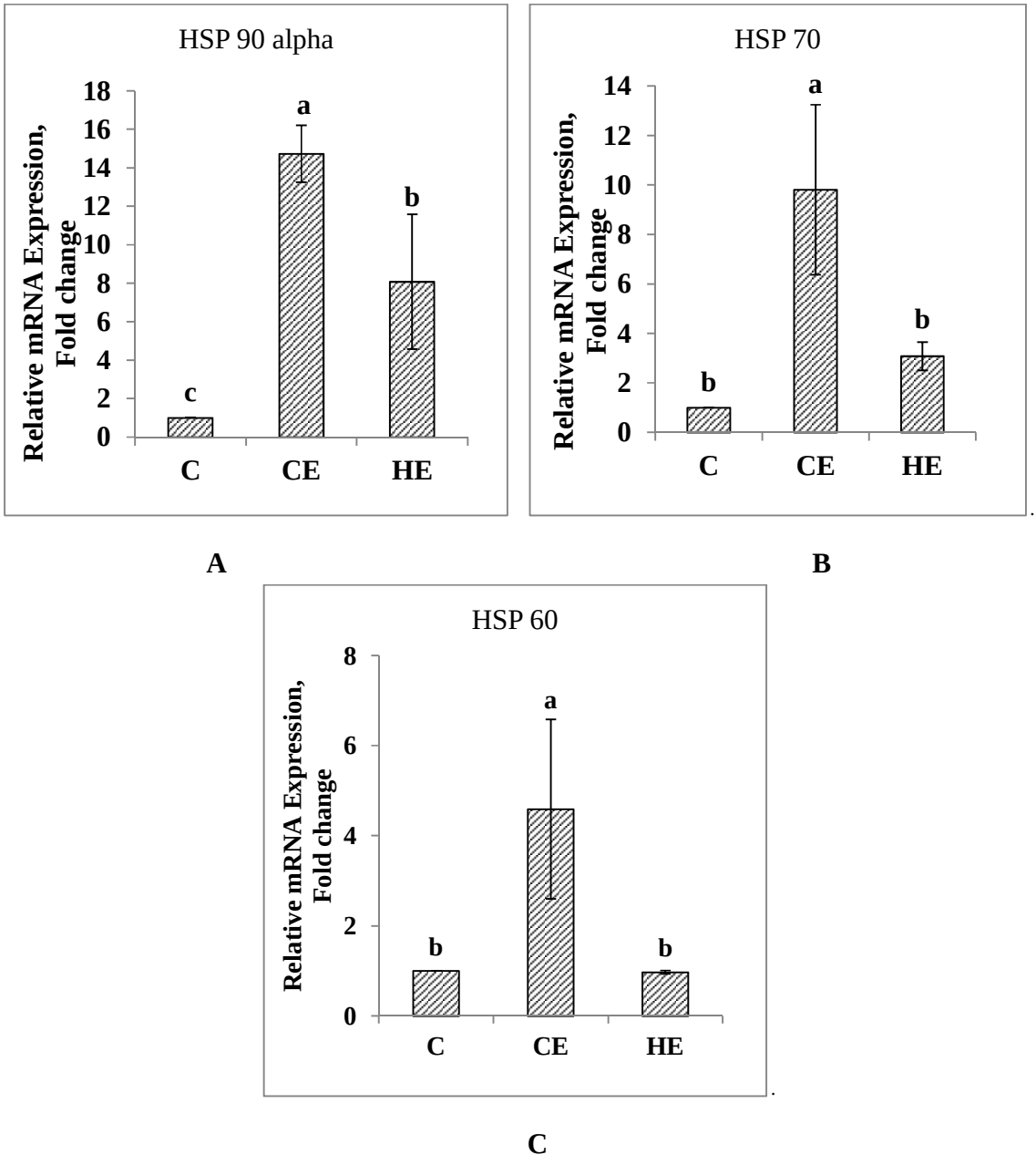


Fig. 4. Heat shock protein (*HSP*) gene expression in liver of Punjab broiler-2 (PB-2) chicks at 42nd day of age. (C- Control; CE- Control exposed; HE- Heat exposed). A. HSP 90 alpha, B. HSP 70, C. HSP 60. [a, b] Means with different superscripts differ significantly (P<0.05).

Strong positive correlation exists between body temperature and *HSP* synthesis (Yahav et al., 1997) which was similar in the present study also; however, the body temperature range was less in the present study (Table 3). Thyroid hormone regulates metabolism in birds during growth and production (Renden et al., 1994), therefore it is important in adaptation of chicks to heat. On 7th day, the T_3 concentration was significantly lower in heat exposed birds due to the stress condition immediately after the exposure to high temperature. At 42nd day of age the T_3 concentration was significantly higher in CE group indicating the stress condition of birds. The HE group and C group had similar T_3 levels indicating that those birds were adapted to higher temperature during pre-exposure on 7th day. Similar findings were reported by Rajkuamr et al., (2015a) in selected broiler chickens, pre-exposed to higher temperatures during incubation, which were attributed to the epigenetic adaptation. Heat stress markedly depress the activity of the thyrotrophic axis in birds as reflected by reduced plasma T_3 concentration (Williamson, 1985; Decuypere and Kuhn, 1988) resulting in functional hypothyroidism (Mitchell and Carlisle, 1992). The improved thermotolerance was indicated by a significantly lower metabolic rate and significant declines in T_3 concentration (Yahav et al., 2004). Significantly lower levels of T_3 and T_4 concentration and lower metabolic rate was observed in thermally conditioned chicken (Piestun et al., 2011).

Table 2. Biochemical stress indicators Nitric oxide (NO), Reduced glutathione (GSH), Super oxide dismutase (SOD), Lipid per oxidation (LP), Ferric reducing ability of plasma (FRAP), Alkaline phosphatase (ALP) and Triiodothyronine (T_3) hormone in plasma of Naked neck (NN) and Punjab broiler-2 (PB-2) chicks at 7th day of age

Parameters	NN		PB-2	
	Control (C)	Heat (H)	Control (C)	Heat (H)
Body temperature	41.13±0.09^{b}	41.55±0.16^{a}	41.12±0.11^{b}	41.19±0.18^{a}
T_3 (ng/ml)	2.17±0.05^{a}	1.89±0.05^{b}	2.16±0.08^{a}	2.14±0.07^{b}
NO (µM /L)	242.36±9.22^{b}	297.21±25.51^{a}	245.52±16.74^{b}	267.91±12.23^{a}
GSH (µM/g protein)	0.13±0.01	0.14±0.02	0.13±0.01	0.13±0.01
SOD (U/ml)	151.87±42.38^{a}	84.83±26.35^{b}	144.31±11.28^{a}	99.77±4.14^{b}
LP (moles of MDA/g protein)	0.54±0.07^{b}	0.65±0.04^{a}	0.45±0.03^{b}	0.72±0.16^{a}
FRAP (µmol/L)	437.00±96.09	372.64±62.03	465.00±132.85	429.00±68.31
ALP (IU/L)	74.78±11.16^{b}	97.38±8.30^{a}	66.46±14.51^{b}	113.23±1.32^{a}

$^{a, b}$ *Means with different superscripts in a row differ significantly within a breed (P<0.05).*

In the present study, the NO, ALP and LP levels were significantly higher and SOD activity was significantly lower in heat exposed birds on 7th day indicating the stress condition in the birds. The results were similar to that of Ramnath et al. (2008) where egg type chicken exposed to high environmental temperature had lower blood SOD activity. On 42nd day, the levels of LP, FRAP and ALP in HE group are almost similar to the C and significantly lower than the CE group indicating stress free condition in HE group which was pre exposed to thermal stress on 7th day. Similar observations for lipid peroxidation and SOD were reported by Vinoth et al. (2015) in coloured broilers. This finding is contrast with Rimoldi et. al. (2015) in fast and slow growing broiler strain where SOD was not affected by heat stress. A positive effect of thermal conditioning could be observed in the HE group birds which might be due to the epigenetic adaptation to high temperature. Thermal manipulation has been reported to decrease the intensity of liver energy metabolism and thereby lower heat production (Loyau et al., 2014). A decrease in energy metabolism leads to reduced oxidative stress due to lower production of free radicals in liver.

Table 3. Biochemical stress indicators Nitric oxide (NO), Reduced glutathione (GSH), Super oxide dismutase (SOD), Lipid per oxidation (LP), Ferric reducing ability of plasma (FRAP), Alkaline phosphatase (ALP) and Triiodothyronine (T_3) hormone in plasma of Naked neck (NN) and Punjab broiler-2 (PB-2) chicks at 42nd day of age. (C- Control; CE- Control exposed; HE-Heat exposed

$^{a, b, c}$ *Means with different superscripts in a row differ significantly within a breed (P<0.05).*

Parameters	NN			PB-2		
	C	CE	HE	C	CE	HE
Body temperature	41.63±0.07^{c}	42.18±0.17^{a}	42.03±0.10^{b}	41.60±0.11^{c}	42.25±0.09^{a}	41.83±0.18^{b}
T_3 (ng/ml)	2.11±0.10^{a}	1.55±0.19^{ab}	1.59±0.05^{ab}	1.84±0.18^{a}	1.54±0.30^{ab}	1.44±0.08^{ab}
NO (µM /L)	287.00±26.89	294.67±12.69	291.55±30.29	319.33±28.13	334.34±92.92	284.00±48.66
GSH (µM/g protein)	0.19±0.05	0.11±0.01	0.11±0.02	0.09±0.01	0.08±0.01	0.15±0.02
SOD (U/ml)	216.96±40.44	182.22±37.58	204.32±50.67	117.73±18.28	107.23±20.54	127.55±8.60
LP (moles of MDA/g protein)	0.38±0.05^{b}	0.51±0.08^{a}	0.37±0.05^{b}	0.32±0.06^{b}	0.60±0.08^{a}	0.39±0.08^{b}
FRAP (µmol/L)	516.50±40.60^{a}	304.71±24.09^{b}	519.00±44.51^{a}	484.00±49.51^{a}	339.00±48.01^{b}	357.33±54.25^{a}
ALP (IU/L)	20.26±2.74^{b}	27.62±5.13^{a}	22.43±1.28^{ab}	20.52±2.98^{b}	41.04±10.86^{a}	25.47±2.62^{ab}

DISCUSSION

Adaptation to increased temperature in early part of the life improves the thermo tolerance in later stages of life accompanied by alteration in sensible heat loss through convection and radiation (Yahav et al., 2005); reduction in corticosterone concentration and reduction in heat shock proteins (Yahav et al., 1997). The H group had significantly higher *HSP* levels immediately after exposure to high temperature due to increased stress condition of the birds in order to maintain the thermoregulatory mechanisms in the body on 7th day. Higher HSP (HSP 90 alpha, 70 and 27) protein concentrations were observed in broilers on exposure to high temperature (36ºC and 70-80 % relative humidity) on 5th day of post hatch (Yahav, 1997). The advantageous effects of thermal conditioning during incubation and early age reflected during later ages of life in broilers was well documented in the literature (Surai, 2015; Rajkumar et al., 2015a & b; Vinoth et al., 2015; Yahav, 2009). Pre-exposure to high temperature induces physiological memory due to epigenetic adaptation to high temperature resulting in improved thermo tolerance during the post-natal life (Yahav, 2009). Under the influence of stressful conditions, heat shock factors (HSFs) are responsible for the activation of the heat shock element (HSE), which is an upstream promoter sequence in the heat shock gene (Mizuno et al., 1997). The birds on re-exposure to thermal stress on 42nd day showed significantly lower *HSP* levels in HE group indicating the adaptation of birds to high temperature because of pre-conditioning and reducing the stress condition and resulting in lower levels of expression. In CE group, the *HSP* levels were significantly higher indicating the effect of thermal stress in the birds. This clearly substantiates the hypothesis of pre-thermal conditioning has advantageous effects during later stages of life due to epigenetic adaptation in chicken (Yahav, 2009; Vinoth et al., 2015).

In general, all cells respond to increased temperature by rapid gene transcription and subsequent mRNA translation to yield highly conserved HSPs to protect them from damage due to heat stress (Locke and Noble, 1995). Cellular mechanisms to the long term thermal conditioning are complex and contradicting observations were reported in earlier studies. One school of thought suggesting the high *HSP* expression is associated with better heat tolerance (Wang and Edens, 1993; 1998) while the other is low level of expression (Yahav et al., 1997). The present findings conforms to the reports of Yahav et al. (1997); Rajkumar et al. (2015a); Vinoth et al. (2015) wherein thermal conditioning during early age induced thermo tolerance by adaptation and subsequent low level of *HSP* expression. The mechanism to combat heat stress involves reducing hyperthermia, which might be partly due to the stability of tissues to hyperthermia due to earlier conditioning (Rajkumar et al., 2015a) which was true in the present study also. On re-exposure to high temperature (43°C) on 42nd day the HE birds perceived it as a lesser stress factor at cellular level due to pre-adaptation to increased temperature during early age resulting in reduced *HSP* mRNA expression in the birds.

CONCLUSION

The study concludes that thermal conditioning of chicks during early age had positive effect and improves the thermo tolerance ability of the chicks in post natal life as revealed by the reduced expression of *HSP* genes and stress indicators in colored broiler chickens.

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