RESEARCH ARTICLE

Thermal Stress Markers of Ongole Cattle in Transitional Phase: THI and HSP70

Priyanka Dhan[1], Vasantha Seshu Kumari I[1]*, Chaitanya Gollu[2], Naveen Swaroop Murikipudi[3], Raghavendra Srikanth Nistala[4]

Abstract

The effect of environment on the health and productivity of the animals is well evidenced in different species of animals. Indigenous cattle were thought to be tolerant and well adapted to environmental changes, but still the effects of varying THI during the transition period has not been explored so far in connection with the expression of stress biomarkers like heat shock proteins particularly in indigenous breeds like Ongole. The present study investigated the gene expression of heat shock proteins (HSP70) in transitional Ongole cows, a dual-purpose indigenous breed from Andhra Pradesh, India. The study measured the relative mRNA expression of HSP70 in peripheral blood mononuclear cells (PBMCs) using the $2^{-\Delta\Delta Cq}$ method. The results revealed significant changes in the parameters studied, providing insights into the stress adaptation mechanisms of Ongole cows during the transition period. It was noticed that, the day zero of parturition was found to be more stressful to the Ongole cows compared to antepartum and postpartum periods. Adoption of nutritional and summer management strategies might ameliorate the negative impacts of higher THI during transition period for optimum production and well-being of Ongole cattle.

Keywords: Gene expression, HSP70, Ongole cattle, Thermal stress, Transitional stress.

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Introduction

Livestock in India contribute 4.11% to the total GDP and 25.6% to the agricultural GDP and India holds the largest livestock population globally (20th Livestock Census). Ongole cattle, a dual purpose indigenous breed, native to the coastal districts of Andhra Pradesh are known for their utility in draft power and moderate milk production (Gaur *et al.,* 2002). Despite the fact that India holds largest livestock sector and are the leading producers of milk and meat, the productivity per animal still remains low owing to adverse effects of several biotic and abiotic stressors. Livestock species usually experience environmental stress of varying degree, while being effective in thermoregulation they initially try to cope up with stress *via* numerous behavioral and circulatory adjustments which are aimed at maintenance of homeostasis. Exposure to high ambient temperature is one of the major constraints on productivity in hot climatic areas as it reduces feed intake, induces numerous haemato-biochemical and neuroendocrine responses aimed at maintenance of thermal balance while compromising production behind.

Transition period is defined as the period from three weeks pre-partum to three weeks post-partum and it is considered as the most stressful period in the life cycle of dairy animal as it induces rapid physiological and metabolic changes due to excessive nutritive demands in late gestation, calving and early lactation (Vasantha *et al*., 2024). When the thermal stress is accompanied with transition period (Abdelrazek *et al*., 2018), the effect on the animal's health and productivity is more pronounced and the homeostasis is disturbed (Grewal *et al*., 2019). The transition period from late pregnancy to early lactation is particularly stressful for dairy cattle, marked by negative energy balance and oxidative stress (Abdelrazek *et al.,* 2018). Additionally, the synthesis of heat shock proteins (HSPs) like HSP 70 is critical indicator of thermotolerance (Hassan *et al.,* 2019). The primary factors that cause heat stress in dairy animals are high ambient temperature and high relative humidity (RH) (West, 2003). Heat shock proteins (HSPs) are highly conserved cellular proteins with chaperone activity which get activated by several physical and physiological stressors and enable the cell to survive injury. Stressful condition in animals elicits HSP

[1]Department of Veterinary Physiology, NTR College of Veterinary Science, Gannavaram-521101, Andhra Pradesh, India.

[2]School of Veterinary and Animal Sciences, Centurion University of Technology and Management, Paralakhemundi, Odisha-761211.

[3]Department of Veterinary Biochemistry, NTR College of Veterinary Science, Gannavaram-521101, Andhra Pradesh, India.

[4]Livestock Research Station, Lam, Guntur, Andhra Pradesh, India.

***Corresponding Author:** Vasantha Seshu Kumari I, Department of Veterinary Physiology, NTR College of Veterinary Science, Gannavaram-521101, Andhra Pradesh, India. E-mail: vasuinjarapu@gmail.com

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synthesis especially the HSP 70; therefore intracellular levels of HSP70 were suggested as indicators of stress.

Despite the significant roles of these biomarkers, studies on HSPs in Ongole cows are limited, however few studies revealed the impact of heat stress in transitional cows, the micro environment surrounding the animal varies with geographical location resulting in differential expression of HSPs. On this background, we evaluated the effect of combined stress on periparturient cattle which was aimed to understand the magnitude and quantum of stress in the animals to adopt suitable strategies. Therefore, this study was aimed to identify biomarkers of stress during the transition period in Ongole cattle by examining expression of HSP 70 during different weeks of transitional period in relation to THI.

Materials and Methods

Location and Experimental Design

This study was conducted on Ongole cattle at the Livestock Research Station (LRS), Lam, Guntur, Andhra Pradesh (India), which is located at 16.3067° N latitude and 80.4365° E longitude, at an elevation of 33 meters above mean sea level. Twelve adult Ongole cattle, comprising six pregnant (treatment group) and six non-pregnant (control group), were selected for the study. These 2 to 3-years-old cattle were housed in a loose housing system, with *ad libitum* access to feed and water, and were maintained under regular deworming and vaccination schedules.

The experiment took place from June to August 2023. THI was recorded daily from 1:00 PM to 2:00 PM throughout the experimental period. Ambient temperature (AT) and relative humidity (RH) were recorded using digital thermo hygrometer daily. Temperature humidity index (THI) was calculated using Johnson (1963) formula: THI = 0.72 (Tdb + Twb) + 40.6 where, T_{db} = dry bulb temperature (°C); T_{wb} = wet bulb temperature (°C).

Blood Collection and Lab Analysis

Blood samples were collected weekly from the jugular vein, with 10 mL of whole blood collected in EDTA-coated vacutainers for PBMC separation and RNA isolation. PBMCs were isolated using Histopaque-1077 (Sigma, USA) and RNA was extracted using TRIZOL reagent (Manjari *et al.,* 2015). RNA quality and quantity were assessed using a Nanodrop spectrophotometer (Nanodrop, ND-1000, Thermo-Scientific, USA) and cDNA synthesis was performed using a BIO-RAD iScript Select cDNA Synthesis kit. Primers for target genes were designed using Primer3 Plus software with the nucleotide sequence of the gene available in the Gene bank database (www.ncbi.nim.nih.gov) (Table 1), and the synthesized cDNA was used for quantitative PCR analysis to study gene expression. The end product obtained from the qPCR assay was subjected to agarose gel electrophoresis and was validated by using DNA markers.

Statistical Analysis

The results were represented as Mean ±SE and analyzed using Graph Pad PRISM 9.0, following Snedecor and Cochran (1994). Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method, with significant differences between groups determined by one-way ANOVA and Duncan's multiple range test, and comparisons between control and transition periods conducted via independent sample 't' test. Statistical significance was set at $p<0.05$.

Results and Discussion

Meteorological Data

The mean ±SE values of THI recorded during the experimental period are shown in Table 2. The overall mean for the entire experimental period was 78.46.

High environmental temperature has adverse effects on the physiological functions of dairy animals and affects their productivity (Polsky and von Keyserlingk, 2017). This can further be aggravated when it is accompanied by high humidity (Collier *et al*., 2008). To measure the intensity of environmental stress, THI has been developed (Alhussien and Dang, 2018). It is one of the best methods to evaluate heat stress in animals (Marai and Haeeb, 2010). The THI is a result of the combined effect of temperature and relative humidity (Dikmen and Hansen, 2009) which determine the microclimatic conditions of the animal. When the THI reaches 72, cows change their behaviour (Kamal *et al*., 2018; Herbut and Angrecka, 2018), yield and composition of milk gets altered (Liu *et al*., 2017). Based on the THI index (Helal *et al*., 2010), a THI of 74 or less is considered as normal, 75-78 alert, 79-83 danger and 84 and above an emergency. THI values higher than 80 units have been classified as danger zone to the well-being and productivity of cattle (Segnalini *et al*., 2013). In the present study, since the THI was in between 75-78; this indicated that the animals were under mild heat stress.

Relative mRNA Expression of HSP 70 in PBMC of Transitional Ongole Cows

The mRNA expression levels of HSP70 were examined in PBMCs from transitional Ongole cows. Mean ±SE values of ΔCq, ΔΔCq, and fold change for HSP70 across different stages are detailed in Table 3. The qPCR product amplicon size of GAPDH (reference gene) and HSP70 (gene of interest) was observed on a 2% agarose gel (Fig. 1, 4), with amplification plots and melt curves presented in Figures 2,3 and 5,6 for GAPDH & HSP70, respectively.

The cumulative effect of environment and parturition as reported by Vasantha *et al*. (2024) was evident in the present study. As the experiment was carried out in June – August, a period where the mean THI crossed the comfort zone, the peripheral warm receptors in the skin become activated and send neural signals to the warm receptors located in the anterior hypothalamus to stimulate the respiratory activity in

Table 1: Primer sequence of the genes

Genes	Primer sequence from 5' to 3'	Size (bp)	Accession No
HSP 70	F: CTCGTCGATGGTGCTGACCAAG R: TCCTGTCCAGGCCGTAGGCGAT	206	NM_203322.3
	Reference gene		
GAPDH	F: AAAGTGGACATCGTCGCCAT R: CCGTTCTCTGCCTTGACTGT	144	XM-006065800.4

Table 2: Minimum and maximum values of temperature (T), relative humidity (RH) &THI recorded during the study period (n=90) (June-August, 2023)

Month	T max	T min	RH max	RH min	THI max	THI min	Mean THI
June	43.6	25.6	79	27	96.79	62.51	79.51
July	39.7	23.6	98	39	102.67	62.70	78.30
August	36.3	23.8	93	54	94.80	66.50	77.48
Overall mean	39.9	24.3	90	40	98.09	63.90	78.46

Table 3: ΔCQ, ΔΔCQ and fold change of HSP70 during the transitional period and control in Ongole cows (Mean ±SE, n=6)

HSP	Stage of transition period						
	-3 week	-2 week	-1 week	Day 0	+1 Week	+2 Week	+3 week
CQ	23.31±0.42	24.91±0.51	23.68±0.34	21.53±0.40	23.23±0.48	21.01±0.46	21.73±0.49
ΔCQ	2.51±0.53	5.16±0.44	1.87±0.32	0.74±0.50	1.80±0.33	2.64±0.25	2.27±0.52
IΔΔCQ	0.49±0.66	0.54±0.32	0.08±0.35	-0.95±0.20	-0.02±0.26	0.05±0.21	0.14±0.46
Fold change	0.774b ±0.140	0.983b ±0.089	0.971b ±0.094	1.946a ±0.110	1.028b ±0.074	0.975b ±0.059	0.943b ±0.112

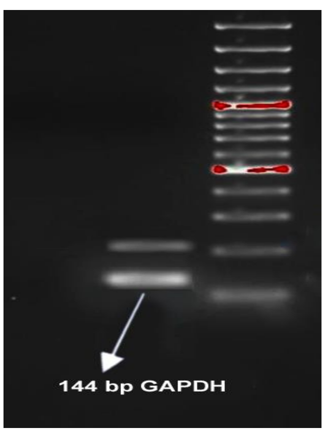

Fig. 1: Amplicon size of GAPDH (Reference gene), ladder (100bp),144b

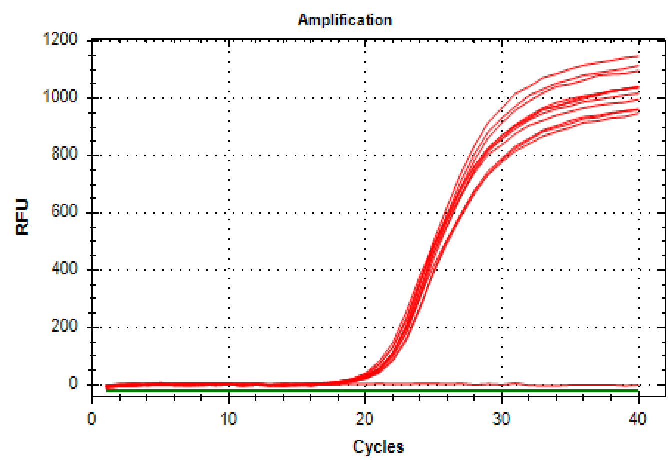

Fig. 2: Amplification plot of GAPDH

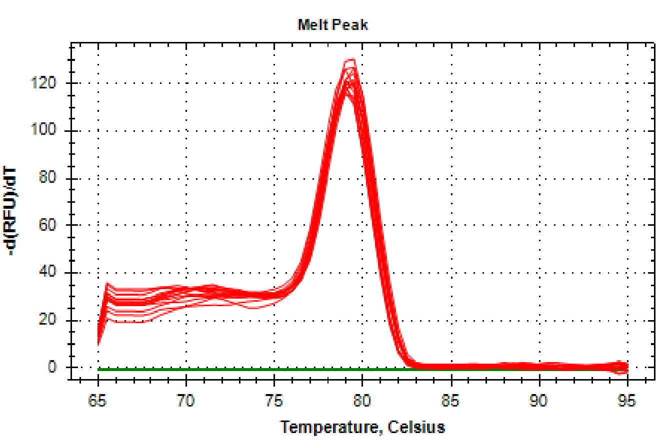

Fig. 3: Melt curve of GAPDH

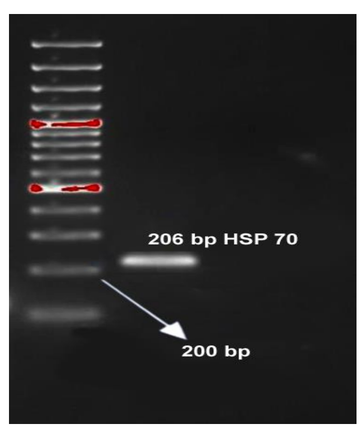

Fig. 4: Amplicon size of HSP70 (206bp)

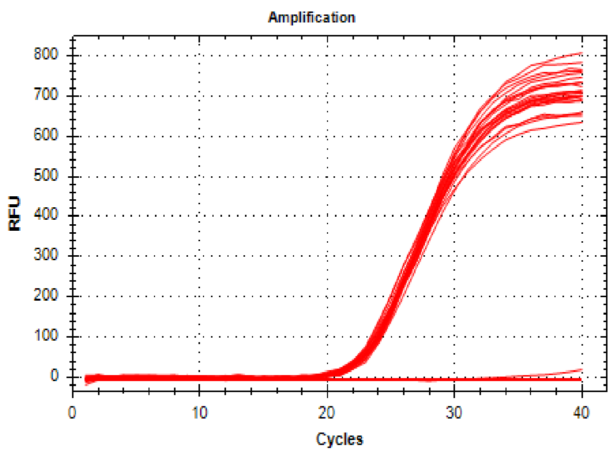

Fig. 5: Amplification plot of HSP70

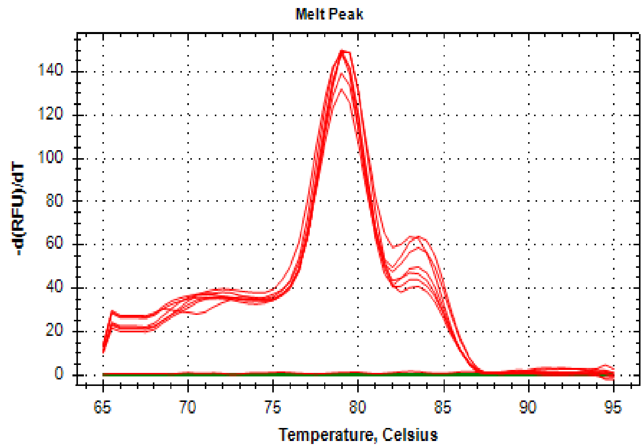

Fig. 6: Melt curve of HSP70

order to increase the rate of heat loss from the body (Krishnan *et al*., 2023).

HSPs are proven biomarkers for determining stress response in cattle and buffalo, in addition, their expression is species as well breed-specific (Kumar *et al.,* 2015). Increased ROS is suggested to be a crucial factor in triggering HSPs production during stress of any origin (Ananthan *et al*., 1986). Antioxidant enzymes and antioxidants were proved to regulate HSPs production in several studies (Lallawmkimi, 2009) indicating that oxidative stress is the fundamental mechanism behind upregulation of the same. Heat shocks as well as other stress impulses like oxidative stress, ischemia, inflammation, or aging (Favatier *et al*., 1997) are indicative of stress (Sonna *et al*., 2002).

The results of the present study indicated that the HSP70 expression was upregulated on the day 0 of parturition and declined postpartum. Sheikh *et al*. (2016) also reported highest expression of HSP70 on the day of calving in both Karan Fries (KF) and Sahiwal cows. It was opined that higher degree of stress on the day of calving, as a consequence of cellular events occurring extensively might be the reason behind. Our findings are in line with Aggarwal *et al*. (2012), who reported higher plasma levels of HSP70 in KF cows. In humans, serum HSP70 concentrations were positively correlated with the age of gestation, during the second half of pregnancy (Molvarec *et al*., 2007). In a study made by Aggarwal *et al*. (2012) significantly lower levels of HSP70 in plasma of α-tocopherol acetate treated cows was reported during transition period.

The transitional stress of the animals under our study resulted in gradual increase in HSP70 towards the day zero of parturition and a gradual decrease in the same after parturition of the transition period studied. As postulated by Xiao *et al*. (2002), the adverse effects of heat or chemical or abnormal stresses could be overcome by the protection conferred by the increased expression of HSP70. The Ongole cattle are known to be an adaptive breed for tropical heat stress particularly in its native tract. Since the study was conducted close to the native tract of the breed, the cows in the study showed their adaptive behaviour towards the climatic stress and so the heat stressor, HSP70 elevated during the transition period, that too, only towards the calving displaying the responsiveness towards calving stress.

It is a proven fact that HSP70 could be used as a sensitive biomarker for heat stress management in large farm animals and it could be used as a sensitive biomarker for heat stress management in large farm animals (Mishra *et al*., 2011). Since Ongole breed is much adaptive to tropical hot and humid climate the HSP70 levels responded only to the transitional stress in the present study. However, an enhanced cumulative stress from environmental stress and transitional stress together cannot be ignored. It may be postulated that when heat stress is combined with transitional stress the HSP 70 expression would be further enhanced. It can be concluded that the HSP 70 is not solely a marker for thermal heat stress, but can very well be presented as a marker for transitional stress in large animals.

Conclusion

The mean THI in the present study indicated that the animals were subjected to mild heat stress. This necessitates the need for microclimatic alterations to mitigate effects of the higher THI for optimum production and reproduction. The elevated levels of HSP70 on day of calving might be an indication of the intensity of combined stress (parturition and environment). As an outcome of the study on the expression of HSP70 during transition period in Ongole cattle, it is proposed that HSP70 could be used as stress biomarkers in Ongole cows to identify/determine onset of any post-parturient metabolic diseases or complications. This piece of work may help in future studies to determine and detect the alteration in the levels of these biomarkers during transition period with precision. Also, this work will help as a base line reference of biomarker genes of Ongole cattle during various stages of transition period. Hence to mitigate the combined effect of parturition stress along with heat stress necessary nutritional and management strategies need to be adapted for optimum production and reproduction.

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