Original Article

Effect of daily environment and season in a tropical environment on the expression of heat shock protein, and steroidogenic and apoptotic genes in bovine cumulus-oocyte complex

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Abstract

The objectives of this study were to determine the differences in the expression of heat shock protein, and steroidogenic and apoptosis genes by cumulus-oocyte complex (COC) of beef cattle in a tropical environment as affected by seasonal and day effect of temperature and relative humidity. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to measure gene expression in COC in different seasons including cool (Jan to Feb), summer (Mar to Apr) and rainy (May to Jul) seasons. Ambient temperature and relative humidity recorded on the day before oocyte collection were used to classify environment groups as: low temperature and low humidity (LT-LH), high temperature and low humidity (HT-LH) and high temperature and high humidity (HT-HH). Expression of HSP70 was higher in the cool than rainy season, and for LT-LH than HT-HH ($p < 0.05$). For the steroidogenic gene, STAR genes were expressed less in HT-LH than either LT-LH or HT-HH ($p < 0.05$), but no effect of season was observed. Expression of MCL-1 was lower ($p < 0.05$) for HT-LH than LT-LH or HT-HH and BAX was higher ($p < 0.05$) for HT-LH than LT-LH or HT-HH. Except for HSP70, expression of heat shock proteins and steroidogenic and apoptotic genes in COC was not affected by season. However, expression of these genes in COC was altered when environmental conditions were characterized by HT-LH. Higher humidity appears to mediate expression of these genes when climates are hot.

Keywords: Tropical environment, Gene expression, Bovine, Cumulus-oocyte complex

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Introduction

Successful early follicular development is crucial for female reproductive function. Cumulus and granulosa cells originate from a common progenitor in preantral follicles and differentiate as cell compartments that are physically separated by antrum formation, resulting in the cumulus-oocyte complex (COC), surrounded by follicular fluid with granulosa layers lining the follicle wall. Communication between cumulus cells and the oocyte allows a bidirectional supply of nutrients and signaling molecules that regulate the process of oocyte development and maturation. This phenomenon requires appropriate gene specific expression of the different types of ovarian cells at different developmental stages (Choi and Rajkovic, 2006). For example, the spatial and temporal patterns of ovarian steroidogenic acute regulator protein (STAR) expression appear to coincide with the follicle's steroidogenic potential during folliculogenesis (Ronen-Fuhrmann *et al*., 1998; Thompson *et al*., 1999). As expressed in granulosa cells of ovaries, BCL-2 (transcript name BCL2L1) as anti-apoptotic gene or pro-survival is found mainly in the developing follicles. In contrast, BAX (pro-apoptotic) is observed mainly in the atretic follicles suggesting a possible role in atresia (Hussein, 2005).

The loss of livestock reproductive productivity due to heat stress has been documented in beef and dairy cattle (Bernabucci *et al*., 2010). Heat stress is defined as the period when the combination of ambient temperature and relative humidity result in a temperature–humidity index (THI) >72 (Armstrong, 1994). In response to heat stress, cells typically produce heat shock protein (HSP) which is critical for cell survival and blocks apoptosis (Kregel, 2002). When the THI exceeds 75, and especially when THI is 80, around the time of artificial insemination, the fertility rate is dramatically reduced (García-Ispierto *et al*., 2007). Oocyte developmental competence is extremely sensitive to high temperatures which hasten oocyte maturation (Schrock *et al*., 2007) and disrupt oocyte maturation (Roth and Hansen, 2005) by impaired gene expression in granulosa cells (Wolfenson *et al*., 2000; Li *et al*., 2016). In a tropical climate, the THI does not differ greatly between the hot-dry summer and the hot-humid rainy season (Suriyasathaporn *et al*., 2006). However, the reproductive performance of dairy cattle is lower in the hot-humid environment (Pongpiachan *et al*., 2003). Raymond *et al*. (2017) reported that the variation in humidity was more important than increasing temperature in creating extreme heat stress. However, the effect of heat stress on gene expression of COC has not been evaluated to determine whether it is a seasonal effect or an effect of conditions on the day before oocyte collection. Therefore, the objectives of this study were to determine the differences between seasonal and current day effects of ambient temperature and relative humidity on the expression of heat shock protein, steroidogenic genes and apoptosis genes expressing from COC of beef cattle reared in a tropical heat stress environment.

Materials and Methods

Chemicals: Unless otherwise mentioned, all chemicals were purchased from Sigma – Aldrich (St. Louis, MO, USA).

Environmental data collection: The study was conducted in Chiang Mai, Thailand from January to July 2018 and included collections during the cool (Jan to Feb), summer (Mar to Apr) and rainy (May to Jul) seasons. Ambient temperature (temp) and relative humidity (RH) 1 day prior to oocyte collection were obtained from the Chiang Mai local Meteorological Observing Station, Meteorological Department, Thailand. Temp (°C) and RH (%) were used to calculate the temperature humidity index (THI) according to Dikmen and Hansen (2009):

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26.8)]$$

Bovine ovaries and immature oocyte collection: A total of 53 ovaries were collected from Zebu cattle (*Bos indicus*) at a slaughter house on 15 days during the study, 5 collections within each season. Fourteen ovaries that were cystic or inactive were excluded from the study. The remaining 39 active ovaries were immediately transferred into phosphate buffer saline (PBS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate at 37 °C and transported within 3 h to the laboratory. Cumulus oocyte complex (COC) were aspirated from follicles measuring 2 to 8 mm diameter by a syringe fitted with an 18G needle. Class I COC, defined as oocytes with more than three complete layers of cumulus cells and homogenous ooplasm (Mayes and Sirard, 2001), were selected. The total number of COC class I were 228, 206, and 222 for cool, summer, and rainy seasons, respectively. Class I COC were washed three times with PBS, preserved in RNAlater solution (Ambion, USA) for RNA stabilization, and storage at -20 °C until extraction.

RNA isolation and cDNA synthesis: Total RNA was extracted from COC using RNAzol RT reagent according to the manufacturer's recommendations. The quality and quantity of RNA samples were determined by A_{260}/A_{280} using a DU 730 nanoVette UV/VIS (Beckman Coulter, CA, USA) . First-strand cDNA was synthesized from an initial amount of 3 µg of total RNA in a 20 µl total reaction volume containing 4 µl 5X RT Buffer, 1 µl 10 mM dNTP mix (10 mM each), 1µl oligo$(dT)_{20}$ primer (50 µM), 1 µl 0.1 M DTT, 1 µl RNase Inhibitor (40 U/ µl), 1 µl Superscript III Reverse Transcriptase (200 U/ µl) (Invitrogen, Life Technologies, New York), 1 µl RNase H, 3 µg of total RNA, and DEPC-treated H_2O. The cDNA synthesis was performed in A200 Thermal cycler (Denville Scientific Inc., Metuchen, NJ, USA) by incubating at 50 °C for 50 min. The cDNA was stored at -20 °C until analysis.

Real – time reverse transcription – polymerase chain reaction (Real– time RT – PCR) : Real– time PCR analysis was performed using an Applied Biosystems 7300 Real– time PCR system equipped with SDS Software v1.4 (Applied Biosystems) using the SensiFast Sybr-Green Hi-ROX Kit (Bioline, USA). The PCR reaction solution was prepared in a final volume of 20

µl, consisting of 5 µl of 2X SensiFAST SYBR Hi - ROX Mix, 0.4 µl of each forward and reverse primer, 1 µl of cDNA template (100 ng), and UltraPure Distilled water (Invitrogen, NY, USA). The reaction mixers were incubated in a 96-well plate at 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 sec, 60 °C for 30 sec, and followed by dissociation curve analysis to confirm specificity (T_m). Each sample was performed in duplicate with 8 genes including a reference gene (Table 1). Three category of target genes were 1) heat shock related gene: heat shock protein family A (HSP70) and heat shock protein 90 Beta family member 1 (HSP90B1), 2) steroidogenic gene: steroidogenic regulatory protein (STAR) and Chromosome P450 Family 19 Subfamily A member 1 (CYP19A1), and 3) apoptotic related genes including, BCL2 like 1 (BCL2L1), Myeloid cell leukemia 1 (MCL-1), and BCL2 associated X (BAX). All genes were normalized the expression level by the reference gene as β – actin (ACTB). The levels of gene expression were analyzed in duplicate with $2^{-\Delta CT}$ method.

Table 1 Details of Genes, primer sequences, and GeneBank accession number and product length of genes used for RT – PCR.

Full name		Primer sequence (5' – 3')	Accession number	Product length
Heat shock protein family A	HSP70	F: CAAGATCACCATCACCAACG	NM_203322.3	239 bp
		R: AAATCACCTCCTGGCACTTG		
Heat shock protein 90 Beta family member 1	HSP90B1	F: TTGCCTTCCAAGCTGAAGTT	NM_174700.2	216 bp
		R: TGACATGCAGCAGGTTCTTC		
Steroidogenic regulatory protein	STAR	F: CCCATGGAGAGGCTTTATGA	NM_174189.3	115 bp
		R: TGATGACCGTGTCTTTTCCA		
Chromosome P450 Family 19 Subfamily A member 1	CYP19A1	F: GGTTAGCTCCAGGAGTGCAG	NM_174305.1	219 bp
		R: CTTGGCTTAGGGTCATGGAA		
BCL2 associated X	BAX	F: TTTGCTTCAGGGTTTCATCCA	NM_173894.1	126 bp
		R: CCGATGCGCTTCAGACACT		
BCL2 like 1	BCL2L1	F: GAGTCGGATCGCAACTTGGA	NM_001077486.2	120 bp
		R: CTCTCGGCTGCTGCATTGT		
Myeloid cell leukemia 1	MCL-1	F: AGAAATGTGCTGCTGGCTTT	NM_001099206.1	219 bp
		R: AGCCACAATCCTCCTGTCAC		
β - actin	ACTB	F: TCCAGCCTTCCTTCCTGGGCAT	NM_173979.3	116 bp
		R: GGACAGCACCGTGTTGGCGTAGA		

F, Forward primer; R, Reverse primer; bp, base pairs.

Statistical analysis: Logarithm transformations were used when data of gene expression were not normally distributed. Mean and standard deviation of temperature, humidity, and THI between the difference seasons was analyzed using ANOVA and pairwise comparison by Turkey's analysis. For reaching at THI ≥75, the value causing drastic decreases in production performance (De Rensis *et al*., 2015), the calculated relative humidity must be 65% for the ambient temperature at 26°C, the upper limit of ambient temperatures at which Holstein cattle can maintain stable body temperature (Berman *et al*., 1985). Averages of temperatures, humidity, and THI of a day before oocyte collection were used to determine the environment with high (HT) or low temperature (LT) when they were higher than or up to 26°C, respectively, while the high (HH) or low humidity (LH) were defined with the cut-off point at 65%. The combination of HT or LT and HH or LH were used to define environment groups. Due to the non-distributed data, gene expression values from all genes were logarithm transformed. Factors used for statistical analysis including seasons (cool, summer and rainy season) and environment group (LT-LH, HT-LH and HT-HH). The mean differences in each specified target genes' expression values within each factors were determined by analysis of variance (ANOVA) (Proc ANOVA, SAS University edition). The pairwise comparison was performed using the Tukey procedure. Significance was declared when $p < 0.05$ and trends when $0.05 > p < 0.1$.

Results

The average daily mean and range of ambient temperature and relative humidity within each season and collection date during the study period are presented in Figure 1. In our study period, cool-season temperatures were mostly lower than 26°C, but not for summer and rainy season. Within season, the distribution of the environmental groups was: LT-LH in cool (n=4), HT-LH in cool (n=1) and summer (n=3), and HT-HH in summer (n=2) and rainy (n=5). Means and standard deviation of temperature and humidity for cool, summer, and rainy seasons and corresponding values for LT-LH, HT-LH, and HT-HH were showed in Table 2. In general, the cool, summer, and rainy seasons in Thailand would be expected to

match with the environment groups of LT-LH, HT-LH, and HT-HH, respectively. THI were gradually increased between difference season ($p < 0.05$), whereas environment groups, LT-LH was lowest THI when compared to the other groups. This finding supports to the study of Suriyasathaporn *et al*. (2006). In this study, differences between season and environment group, defined by the ambient temperature and relative humidity the day before sample collection, were reflective of the duration of heat stress duration. Therefore, a season effect would indicate the long-term heat stress effect, while the environment group as defined for this study is more reflective of short term heat stress.

Means and standard deviations of expression values of seven target genes in COCs separated by seasons or environment groups were shown in Table 2. Expression of HSP70 and BCL2L1 expression were lower ($p < 0.05$) in rainy season compared to the cool season; however, no differences were observed in gene expression due to season. The expression of HSP70, HSP90B1, STAR, MCL-1, and BAX was related to the environment group. Both HSP70 had lower expression ($p < 0.05$) and BCL2L1 tended to have lower expression ($p < 0.1$) in HT-HH environment compared with HT-LH.

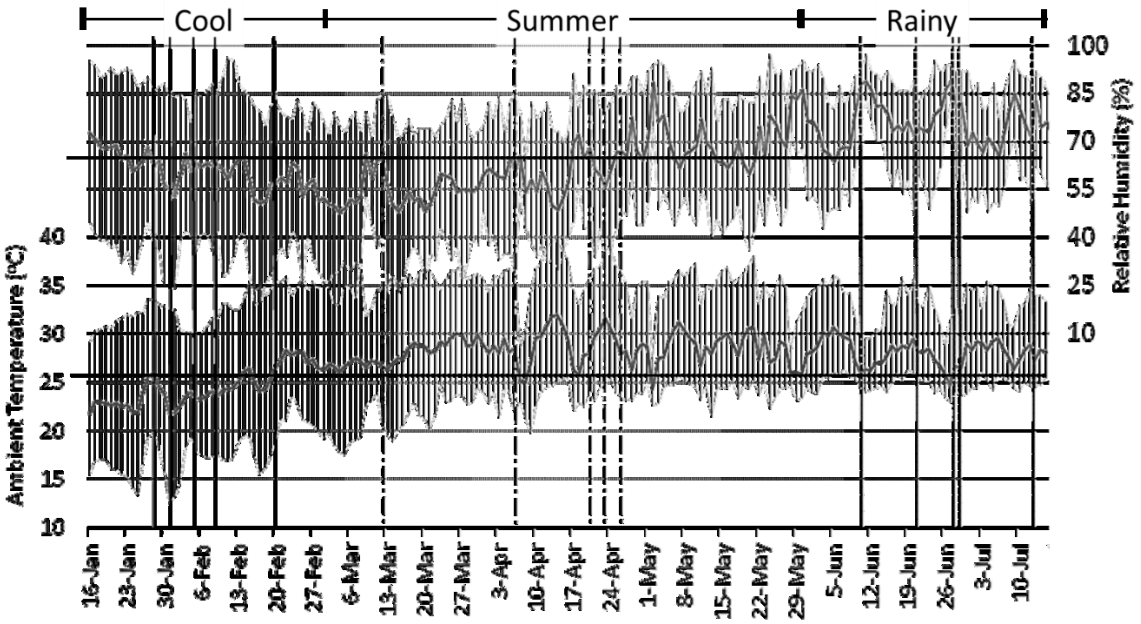

Figure 1 Averages and ranges of daily ambient temperature and relative humidity during the study period. ' | ¡ ¦ ' vertical lines indicate dates at oocyte collection for cool, summer, and rainy seasons, respectively, and long horizontal lines indicates the cut-off point determining either high temperature or high humidity.

Table 2 Means* and standard deviations of environmental data and seven genes in COC by season or environment groups. Environment groups were defined base on average daily ambient temperature and relative humidity 1 day before ovaries collection. Low (LT, < 26°C) and high temperature (HT. > 26°C) and low (LH, < 65%) and high humidity (HH, > 65%).

	Season			Environment group		
	Cool	Summer	Rainy	LT-LH	HT-LH	HT-HH
Environmental data						
Ambient temperature (°C)	24.3±1.8^{a}	28.5±1.5^{b}	27.0±0.8^{b}	23.7±1.5^{a}	28.3±2.2^{b}	27.3±0.8^{b}
Relative humidity (%)	60.2±3.7^{a}	61.5±5.5^{a}	81.6±4.6^{b}	61.0±3.7^{a}	57.4±2.1^{b}	77.6±7.6^{c}
THI	72.4±3.0^{a}	77.4±2.2^{b}	79.3±1.4^{c}	72.9±1.2^{a}	78.1±2.0^{b}	79.5±1.3^{b}
Gene expression in COC						
Heat shock protein family						
HSP70	40.4±37.5^{a}	29.9±40.7^{ab}	10.7±8.6^{b}	40.9±40.8^{ab}	47.5±42.0^{a}	9.6±7.2^{b}
HSP90B1	1.8±1.0	1.2±1.1	1.7±1.2	2.0±1.0^{a}	1.0±0.9^{b}	1.6±1.2^{ab}
Steroidogenic genes						
STAR	86.8±100.4	41.4±75.9	52.9±38.3	106.4±106.9^{a}	12.9±10.5^{b}	65.8±71.9^{a}
CYP19B1	44.2±52.0	63.6±64.4	22.9±30.0	48.8±58.8	16.1±12.1	53.9±55.8
Apoptotic genes						
MCL-1	51.1±51.3	17.4±20.4	31.5±36.2	61.8+52.2^{a}	7.0±6.8^{b}	30.3±30.4^{a}
BAX	2002±2156	1439±2315	119.0±24.0	369.0±177^{a}	4058±1471^{b}	219.0±177^{a}
BCL2L1	3342±2092^{a}	2129±2582^{ab}	467±695^{b}	2325±603^{xy}	6393±0.0^{x}	1021±1536^{y}

*Logarithm transformation was applied to all gene expression values before statistical analyses due their non-normal distributed data.

$^{a, b}$different letter indicating the significant different at $p < 0.05$.

$^{x, y}$different letter indicating the trend of different at $p < 0.1$.

Discussion

In this study, THI at 75 (De Rensis *et al*., 2015) and cut-off values of relative humidity at 65% and ambient temperature at 26°C for Holstein cattle (Berman *et al*., 1985) were used. Cattle from zebu breeds are better able to regulate body temperature in response to heat stress than B. taurus breeds (Hammond *et al*., 1996; Gaughan *et al*., 1999; Hansen, 2004). Because of thermotolerant ability of zebu cows, the consequences of exposure to heat stress are less severe in reductions in reproductive function (Rocha *et al*., 1988). However, both *Bos taurus* and *Bos indicus* cattle experience physiological change to high ambient temperatures starting at 26 °C (Beatty *et al*., 2006). Therefore, zebu cows in HT-HH and HT-LH might have higher heat stress problem than LT-LH, but the actual heat stress problem might not be occurred in these zebu cows. In addition, the rainy season was represented by May to Jul, which in fact is just the beginning of rainy season. Therefore, the field application of the result of this study must be carefully performed.

Biologically, HSP family expression is associated with cell survival as these genes work to limit cell damage or induced cell apoptosis (Arya *et al*., 2007). Li *et al*. (2016) reported that HSP70, and HSP90B1 were up-regulated in the bovine ovary's granulosa cells under heat stress conditions. In our study, HSP70 exhibited the same higher expression in the cool season and during LT-LH as in the rainy season and HT-HH, respectively (Table 1) possibly because heat stress occurs most of the year in Thailand (Suriyasathaporn *et al*., 2006). The decrease in HSP70 and HSP90B1 during the higher heat stress environment observed in the summer and rainy seasons or HT-LH and HT-HH might be related to the decrease of this heat shock protein after long term exposure in heat stress environment (Kim *et al*., 2020). In a study of long-term chronic environmental stress, plasma HSP70 concentration was highest on d 30 and decreased at day 60, 90, and 110 of heat stress (Gaughan *et al*., 2013). However, the lowest expression of HSP70 in our study was observed during the rainy season and for HT-HH. Chen *et al*. (2015) reported that HSP concentrations decreased *in vivo* during later stages of heat exposure in rats and the decreased HSP concentrations were associated with a reduced protective effect on cells. Thus, cumulus cells might be damaged and alter developmental competence. Heat stress was associated to reduce cytoplasmic events of oocyte maturation, thereby decreasing the oocyte competence (Ahmed *et al*., 2017). Nevertheless, the mechanism of this lower expression needs further investigation.

For the steroidogenic genes, expression level of the STAR gene was lower for HT-LH compared with LT-LH and HT-HH, but no effect of season was observed (Table 2). This finding indicated that STAR gene expression was negatively affected by recent exposure to high temperature. In support of an *in vitro* study, the STAR gene expression was down regulated when cultured in heat condition (Li *et al*., 2016; Khan *et al*., 2020). In contrast, the STAR gene expression increased in COC of cows housed in high temperature and high humidity conditions. The anti-apoptotic gene, MCL-1, was expressed higher ($p < 0.05$) for LT-LH and HT-HH compared with HT-LH suggesting that the recent high temperature suppressed expression. In contrast, expression of BAX, a pro-apoptotic gene, was lower ($p < 0.05$) for LT-LH and HT-HH compared with HT-LH indicating that only the recent high temperature increased expression. In contrast to Paes *et al*. (2016), higher expression of MCL-1 in matured oocytes exposed to short term heat stress condition during *in vitro* maturation induced the BCL-2/BAX pathway (Li *et al*., 2016; Khan *et al*., 2020), and MCL-1 expression (Omari *et al*., 2015). However, HT-HH expressed increased both steroidogenic gene and apoptotic gene indicating that that recent increases in humidity might compensate for high ambient temperature.

In conclusion, heat shock protein expression in COC, especially HSP70, was more suppressed in the long-term and recent environmental effects due to high temperature and high humidity. The steroidogenic gene, STAR, and apoptotic related gene expression were more suppressed in the current hot environment. However, the suppression was relieved when the COC was from the hot-humid condition. Therefore, the lower fertility of cows in the hot-humid environment compared with that observed in hot, low humidity environment in tropical countries could not be explained by related genes expression in COC.

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