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Seasonal heat stress alters the milk yield, antioxidative levels and serum metabolites in primiparous and multiparous dairy cows

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Abstract

This study aimed to explore the seasonal heat stress affects the milk yield, antioxidative levels and serum metabolites in primiparous and multiparous dairy cows during early lactation. A total of two hundred dairy cows were selected according to their calving months (June, temperature humidity index (THI) =66.72; July, THI=70.30; August, THI=69.32; September, THI=67.20; October, THI=59.45). Blood samples were collected on day 0, 21, 50, 80, 100 after calving for serum oxidative status analysis and milk yield was recorded every day. The lower average daily milk yield in cows that calved in June and July ($P<0.05$), and average daily milk yield of multiparous cows was higher than that of primiparous cows that calved in the same month ($P<0.05$), suggesting that seasonal (June) heat stress negatively affected milk yield in both primiparous and multiparous cows at early lactation. Besides, 15 and 11 serum metabolites were changed in heat stress (average THI = 70.30) group compared with non heat stress (average THI = 59.45) group in primiparous cows and multiparous cows, respectively. These metabolites were mainly involved in the pathways of aminoacyl-tRNA biosynthesis, glyoxylate and dicarboxylate metabolism, and the metabolism of glycine, serine and threonine. These data suggested that heat stress negatively affected the milk yield and thus caused the the elevation of

the serum oxidative and antioxidative index Metabolic biomarkers associated with the heat stress in serum were found which providing the basement of evaluating indicator between heat stress and non heat stress groups in primiparous and multiparous cows.

Key words Early lactation, Milk yield, Oxidative status, Serum metabolites, Heat stress

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Declarations

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Conflict of interest

The Authors declare that they have no conflict of interest.

Ethics approval

The cows were managed according to the guidelines for the care and use of experimental animals of Jilin Agricultural University (JLAUACUC-2018-019).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

All the data are included in the manuscript.

Code availability

51 Not applicable.

52 **Authors' contributions**

53 ZS and YZ contributed to conception and design of the study. XC, ND, YR, XQ and FZ organized the database.
54 XC, ZS performed the statistical analysis. XC and SZ wrote the first draft of the manuscript. XC, TW, JX, WZ and ND
55 wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted
56 version.

57 Introduction

58 The effects of heat stress on dairy cows are multifactorial and not only have great impact on the productivity of
59 cows, present hazards to animal health and the oxidative-antioxidative balance, and even negatively affect an animal's
60 offspring when they mature to adulthood (Dahl et al. 2017). It has been reported that THI is a major factor that can
61 decrease the milk production of dairy cows when it is higher than 72, and which usually considered the threshold of
62 heat stress (Du et al. 1996(a); Du et al. 1996(b)). Milk yield is closely related to heat stress especially for the
63 high-producing cows (Gantner et al. 2017). Ravagnolo et al. (2000) founded that milk yield declines by about 0.2 kg
64 when THI (> 72) increased one unit. According to Kadzere et al. (2002) and Igono and Johnson (1990), cows in early
65 lactation are more sensitive to heat stress than those in late lactation. THI has been used as an indicator of thermal
66 comfort for dairy cows (Savaliya et al. 2019).

67 Many studies have assessed oxidative status by monitoring malondialdehyde (MDA) levels since increased MDA
68 concentration is a biomarker of free radical-mediated damage and oxidative stress (Bouwstra, et al. 2008; Castillo et al.
69 2006) and antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) that represent
70 major antioxidant responses by protecting the cells against increased reactive oxygen species (Konvicna et al. 2015).
71 Metabolomics is a powerful analytical technology for understanding biological systems from hundreds of thousands of
72 low-molecular weight metabolites in animals, and gas chromatography mass spectrometry (GC-MS) has also been used
73 in metabolomics studies to provide a global understanding of the pathophysiological changes brought about by the
74 environment (Zhao et al. 2015). For example, Tian et al. (2016) used metabolomics to study the heat stress candidate
75 biomarkers in milk serum. We hypothesized that the damage degree via THI before and after calving and serum
76 metabolites associated with the heat stress on primiparous and multiparous cows would be different. Therefore, this
77 study investigated the effect of THI on the milk yield, oxidative status, and serum metabolites in primiparous and

78 multiparous cows during early lactation. Our findings may provide guidance for the management of heat stress in dairy
79 farms and draw people's more attention to the health of dairy cows.

80 **Materials and Methods**

81 **Animals and experimental design**

82 This study was performed from June to the following February (next year) at a commercial dairy farm in Shandong
83 Province, P.R. China. Primiparous Holstein cows (n=100) and multiparous (parity: 2.5 ± 0.5) Holstein cows (n=100)
84 were selected according to their calving days in June, July, August, September and October, respectively. All the cows
85 suffered different THI before and after calving. All experimental cows were fed the total mixed ration (TMR) ad libitum
86 as described previously (Zhao et al. 2019). Air temperature and humidity at a height of 1.5 m above the ground at both
87 ends and in the middle of the cowshed were recorded three times per day. Average daily temperature humidity index
88 (THI) was calculated every day (NRC, 1971). The cows were managed according to the guidelines for the care and use
89 of experimental animals of Jilin Agricultural University (JLAUACUC-2018-019).

90 **Sample's collection**

91 Blood was individually sampled from coccygeal veins on the calving day and 3–4 h after the morning feed on d 0,
92 21, 50, 80, and 100. Serum was collected after centrifuging at 3000 rpm at 4°C for 10 min and stored at -40°C for the
93 latter analysis.

94 **Oxidative and antioxidative index analysis**

95 MDA, SOD, GSH-PX, and total antioxidant capacity (T-AOC) in serum were analyzed using a commercial bovine
96 enzyme-linked immunosorbent assay kit (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China).

97 **GC-MS analysis**

98 The serum of dairy cows with the highest THI (July-calved, average THI = 70.30) and lowest THI (October-calved,

average THI = 59.45) were selected for GC-MS analysis (sampling on calving day from cows calved in July and October, respectively). Thus, the cows that calved in July and October were in the heat stress (HS) group and in non-heat stress (NHS) group, respectively. The serum samples (100 μ L) and methoxyamine hydrochloride (20 μ L, 0.3 mg/mL) were mixed with 300 μ l methyl alcohol:chloroform (3:1) for 30 s. After approximately 10 min latter, the mixed liquor was centrifuged at $12,000 \times g$ at 4°C for 10 min. The supernatant (300 μ l) was transferred to a centrifugal tube and dried using nitrogen gas. Subsequently, a 60 μ L solution (methoxyamine hydrochloride and pyridine) was added to the serum samples and incubated in a water bath at 37°C for 120 min. After that, 60 μ l BSTFA (bis(trimethylsilyl)-trifluoroacet-amide) with 1% TMCS (trimethylchlorosilane) was added and incubated at 37°C for 60 min. The samples were centrifuged at $13,000 \times g$ at 4°C for 5 min, and then 50 μ l supernatant of each sample was transferred to a GC vial. Each sample (1 μ l) was injected into an Agilent 7890/5975C system equipped with a fused silica capillary column (30.0 m $\times$ 0.25 mm i.d.) with a 0.25- μ m HP-5MS stationary phase (Agilent, Shanghai, China) (Chen et al. 2019).

Statistical analyses

The THI, milk yield, serum oxidative and antioxidative index, and Pearson correlation were analyzed using SPSS computer software (SPSS Statistics for Windows, Version 17.0; Chicago: SPSS Inc.), and P-values < 0.05 were statistically considered to be significant. Metabolic data was preprocessed including noise reduction, retention time correction, peak alignment, and other data standardization using AMDIS in Agilent, and then analyzed by SIMCA 14.0 software (Umetrics AB, Umeå, Sweden). Principal component analysis (PCA) was performed on the data to visualize the sample distribution and separation trends. An orthogonal partial least squares-discriminate analysis (OPLS-DA) model was used to identify differences in metabolites. Both the variable importance in the projection (VIP > 1) values and Student's t-test ($P < 0.05$) were compared to find potential metabolites.

Results

Variation of temperature humidity index (THI) during experimental period

The maximum THI, minimum THI, and average THI by month during the experiment are shown in Fig. 1. Briefly, the average THI decreased from July to January. Average THI values in July and August were higher than that in other months ($P < 0.05$). During the calving time (from June to October), the THI in October was lower than in other months ($P < 0.05$). The maximum THI values in June, July, and August were 74.78, 75.65, and 75.17, respectively. All of these maximum THI values were higher than 72, which are in the range of heat stress for dairy cows (Armstrong 1994) and recent data indicates that a THI of approximately 68 affected the milk yield of high-yielding dairy cows (Zimbelman et al. 2009), consistently, we found that cows were affected by heat stress in June, July, and August months.

Fig. 1 Temperature humidity index (THI) during experimental period

a-f: A different letter means significant difference ($P < 0.05$) and same letter means no significant difference ($P > 0.05$) among different months

Milk yield

The effect of different calculated THI before and after calving on the cows as shown in Table 1. The cows that calved in June and July suffered high calculated THI after calving. The average daily milk yield (from 1- 100 days) and average daily milk yield after peak milk to 100 days of the primiparous cows that calved in June and July were lower

than those calved in September and October ($P < 0.05$). No significant difference was found among multiparous cows that calved in different months ($P > 0.05$). And average daily milk yield after peak milk to 100 days of multiparous cows that calved in June were lower than those calved in the other months ($P < 0.05$). In the same calving month, the average daily milk yield of multiparous cows over 100 days and average daily milk yield after peak milk to 100 days were higher than that of primiparous cows ($P < 0.05$). When compared with the cows that calved in June, the average daily milk yield of primiparous cows that calved in September and October increased by 19.15% and 13.83%, respectively. However, the average daily milk yield of multiparous cows increased by 2.86% and 7.64%, respectively. Milk yield negatively correlated with THI in both primiparous cows and multiparous cows ($P < 0.05$).

Table 1 Effect of temperature humidity indexes (THI) on milk yield (kg/d)

Calving month			June	July	August	September	October
Average THI (100 days)			69.08±0.48 ^c	66.00±0.80 ^d	60.70±1.18 ^c	53.04±1.07 ^b	49.16±1.04 ^a
Calculated	THI	(before calving, >72)	1549.13	2361.60	2947.86	3240.90	3240.90
Calculated	THI	(after calving, >72)	1691.77	879.29	293.03	0.00	0.00
Average daily milk yield (ADMY, 100 days, primiparous cows)			35.87±1.26 ^{Aa}	35.67±1.93 ^{Aa}	39.06±1.26 ^{Aa} b	42.74±1.11 ^{Ab}	40.83±0.76 ^A b
Average daily milk yield (ADMY, 100 days, multiparous cows)			47.63±1.26 ^B	46.69±1.54 ^B	48.88±2.04 ^B	48.99±0.76 ^B	51.27±2.07 ^B
Average daily milk yield after peak milk to 100 days			37.22±0.88 ^{Aa}	38.20±0.81 ^{Ab}	42.26±1.31 ^{Ac}	44.55±1.18 ^{Ad}	44.04±1.24 ^A d

(primiparous cows)

Average daily milk yield after

peak milk to 100 days(multiparous cows) 46.73±2.49^{Ba} 49.66±2.31^{Bb} 51.12±1.90^{Bc} 51.51±1.48^{Bc} 54.30±1.68^B_d

Correlation analysis of average

THI and ADMY (primiparous cows) -0.65**

Correlation analysis of average

THI and ADMY(multiparous cows) -0.45**

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Calculated THI before calving: THI above 72 from April 1st to calving day were calculated.

146

Calculated THI after calving: THI above 72 during early lactation days (100 days) were calculated.

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Different lowercase letters (a,b) means significant difference ($P < 0.05$) in the same row.

148

Different capital letters (A, B) means average daily milk yield and average daily milk yield after peak milk to 100 days is significantly different ($P < 0.05$) in the same column.

149

** Means correlation analysis between average THI and average daily milk yield is significantly different ($P < 0.01$).

150

The lactation curve of primiparous and multiparous cows from days 1 to 100 is shown in Fig. 2. The milk yield of

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primiparous cows reached a peak from about the 46th to 50th days of lactation and the peak milk yield was 38.86, 38.77,

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42.35, 44.84 and 44.03kg/d for the calves that calved in June, July, August, September and October. And multiparous

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cows reached a peak milk yield was from about the 37th to 43rd day of lactation and the peak milk yield was 52.52,

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54.66, 54.05, 54.07 and 58.07kg/d for the calves that calved in June, July, August, September and October. The time at

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which the milk yield of primiparous and multiparous cows that calved in June reached a peak was later than that of

other groups, and the peak milk yield and persistency of milk yield after peak milk to 100 days were lower than that of other groups. The downward trend of milk curve for multiparous cows was much more obvious than primiparous cows.

Fig. 2 Lactation curves of primiparous and multiparous cows from days1 to 100.

a: primiparous cows, b: multiparous cows.

Oxidative and antioxidative index

Our results showed that all of the serum oxidative indexes showed different variation trends during the early lactation period, however, the overall concentration of every index in primiparous cows was higher than that in multiparous cows in the same month (Fig. 3). On days 50 and 100, all the indexes were relatively low compared to other times. Although no significant difference in MDA, SOD, and T-AOC in serum was found among the groups in primiparous dairy cows on the calving day ($P > 0.05$), but GSH-Px of primiparous cows that calved in August, September, and October was significantly higher than that in June and July ($P < 0.05$). No significant differences were found in the above serum oxidative indexes were found in multiparous dairy cows on calving day. On day 21 after calving, SOD and GSH-PX values in cows that calved in September and October were significantly higher than in those that calved in June and July ($P < 0.05$). On day 50 after calving, SOD in primiparous cows that calved in October was

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Fig. 3 Effect of temperature humidity indexes (THI) on serum oxidative indexes.

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a: primiparous cows, b: multiparous cows.

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Fig. 4 Correlation among serum oxidative indexes and temperature humidity index (THI)

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Metabolic candidate and pathway analysis

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The original data obtained from GC/MS were classified into a two-dimensional matrix, and then analyzed by

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SIMCA-P+14.0 software for PCA pattern recognition. Serum PCA plots had an obvious separation among different

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groups, and all the samples were within the 95% confidence interval (Fig. 5-A). On the basis of the PCA model,

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OPLS-DA was used to further analyze the different metabolites in serum between HS and NHS groups of primiparous

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cows and multiparous cows (Fig. 5-B, C).

195

196

A

Fig. 5 PCA score plots of four groups and OPLS-DA plots of of primiparous cows and multiparous cows

A: PCA score plots of different groups and each dot stands for a serum sample of four groups; B: OPLS-DA plots of primiparous cows; C: OPLS-DA plots of multiparous cows.

GC-MS identified the different metabolites between heat stress (average THI in calving month = 70.30) and non heat stress (average THI in calving month = 59.45) groups of primiparous and multiparous cows. According to the metabolic candidates, a heat map was constructed as shown in Fig. 6. A total of 15 and 11 metabolic candidates were obtained in primiparous and multiparous cows, respectively. The concentrations of L-threonine and D-fructose in the HS group of primiparous cows were higher than in the NHS group of primiparous cows ($P < 0.01$). Urea and dodecane in the HS group were higher than in the NHS group of multiparous cows ($P < 0.01$). Four different metabolites were found in both primiparous cows and multiparous cows: L-alanine, glycine, serine, and DL-ornithine, and the levels were lower in the HS group compared to the NHS group of primiparous cows. However, the opposite result was found in multiparous cows, in which the abundance was higher in the HS group.

Fig. 6 Heat map representation of metabolite concentration of the HS and NHS samples.

The pathway enrichment analysis and pathway topology analysis of metabolic candidates were performed by metaboanalyst 4.0 (<http://www.metaboanalyst.ca>). The Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) was used to reveal metabolic pathways. A total of 30 and 15 pathways were related to primiparous and multiparous cows, respectively (Fig. 7), and the pathways with P values < 0.05 and impact value > 0 are listed in Table 2. The metabolic candidates in the primiparous cows were mainly involved in 8 pathways that are related to translation, carbohydrate metabolism, amino acid metabolism, metabolism of other amino acids, metabolism of cofactors and vitamins, and lipid metabolism. The metabolic candidates in the multiparous dairy cows were mainly involved in the pathways of aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; glycine, serine, and threonine metabolism; valine, leucine, and isoleucine degradation; and glyoxylate and dicarboxylate metabolism, which are related to translation, amino acid metabolism, and carbohydrate metabolism. The common metabolic candidates in both primiparous and multiparous cows are involved in aminoacyl-tRNA biosynthesis; glyoxylate and dicarboxylate metabolism; and glycine, serine, and threonine metabolism.

226 **Table 2.** Affected metabolic pathways based on KEGG classification

Class	Pathway Name	Metabolites	P-value	Impact value
Primiparous cows				
Translation	Aminoacyl-tRNA biosynthesis	Glycine, Serine, L-Alanine, L-Threonine, L-Tyrosine, Glutamic acid	<0.01	0.24
Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	Serine, Glycine, Glutamic acid	<0.01	0.19
	Glycine, serine and threonine metabolism	Serine, Glycine, L-Threonine	<0.01	0.38
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	L-Alanine, Glutamic acid	<0.05	0.16
	Phenylalanine, tyrosine and tryptophan biosynthesis	L-Tyrosine	<0.05	0.25
Metabolism of other amino acids	Glutathione metabolism	Glycine, Glutamic acid	<0.05	0.05
Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism	Glycine, Glutamic acid	<0.05	0.08

Lipid metabolism	Linoleic acid			
	metabolism	9,12-Octadecadienoic acid	=0.05	0.75
Multiparous cows				
Translation	Aminoacyl-tRNA	Glycine, Serine, L-Alanine,		
	biosynthesis	L-Isoleucine, L-Leucine,	<0.01	0.24
		L-Proline		
	Valine, leucine and			
	isoleucine biosynthesis	L-Leucine, L-Isoleucine	<0.01	0.25
Amino acid metabolism	Glycine, serine and			
	threonine metabolism	Glycine, Serine	<0.05	0.36
	Valine, leucine and			
	isoleucine degradation	L-Isoleucine, L-Leucine	<0.05	0.04
Carbohydrate metabolism	Glyoxylate and			
	dicarboxylate metabolism	Serine, Glycine	<0.05	0.15
Pathway of common metabolic candidates				
Translation	Aminoacyl-tRNA			
	biosynthesis	Glycine, L-Serine, L-Alanine	<0.01	0.14
Carbohydrate metabolism	Glyoxylate and			
	dicarboxylate metabolism	L-Serine, Glycine	<0.01	0.15

Amino acid metabolism	Glycine, serine and threonine metabolism	L-Serine, Glycine	<0.01	0.36
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Fig. 7 Pathway topology analysis related to primiparous cows and multiparous cows.

Discussions

Studies have demonstrated a negative effect of heat stress on daily milk yield of dairy cows. Bouraoui et al. (2002) reported that a negative correlation existed between THI and milk yield and daily milk yield reduced by 21% when the THI value increased from 68 to 78. Collier et al. (2012) found milk yield decreased 2.2 kg/day when daily THI was 68. Dunn et al. (2014) studied the effect of heat stress on milk yield in the UK, and milk yield decreased during the heatwaves of 2003 and 2006. A study by Zimbelman et al. (2009) indicated that milk yield decreases by an average of 2.2 kg when animals experience a minimum THI of 65 or greater. The results of previous studies above were similar with the results of current study. In the present study, when compared with the cows that calved in June, the average daily milk yield of primiparous cows that calved in September and October increased by 19.15% and 13.83%, respectively. However, the average daily milk yield of multiparous cows increased by 2.86% and 7.64%, respectively.

239 These results indicate that the effect of THI on milk yield may be cumulative and delayed, and the multiparous cows are
240 more susceptible to higher THI, which supported the hypothesis that the damage degree of calculated THI on dairy
241 cows was more severe after calving than that before calving, and the effect of THI on multiparous cows was more
242 severe than multiparous cows. In agreement with the present study by Bernabucci et al. (2014), milk yield started to
243 decline when THI ranged from 73 to 76, and multiparous cows were more susceptible to heat stress than primiparous
244 cows. The negative effects of heat stress on milk yield can be explained by decreased dry matter intake and decreased
245 rumination and nutrient absorption (Liu et al. 2008; Baumgard and Rhoads 2012). Furthermore, there is an increase in
246 peripheral blood flow for cooling, and mammary blood flow is reduced, which contributes to lower milk yield (West,
247 2003).

248 MDA is a product of lipid peroxidation, and previous studies have used increased levels of MDA to represent the
249 imbalance between oxidants and antioxidants (Sharma, et al. 2011). Also, SOD, GSH-PX, and T-AOC are biomarkers
250 for antioxidant status. The present study found that MDA was usually higher in cows that calved in June and July
251 during the experimental period, especially in primiparous dairy cows compared to those that calved in September and
252 October, which indicated that the cows calved in June and July were affected by heat stress. The concomitant increase
253 in MDA, SOD, GSH-PX, and T-AOC may be a reaction of oxidative -antioxidative balance and a compensatory
254 mechanism of homeostatic control in order to decrease the risk of oxidative damage (De et al. 1992). Consequently,
255 heat stress affected the oxidative status, and the antioxidant mechanism would be activated by increasing the levels of
256 SOD, GSH-PX, and T-AOC. Bernabucci et al. 2002 found that SOD was higher in cows that gave birth during the
257 summer when THI ranged from about 71.2 to 75.2, which is similar with the results of the current research. Safa et al.
258 (2019) found that the MDA and T-AOC were higher in heat-stressed cows, heat-stressed cows exhibited higher rectal
259 temperatures, and milk yield decreased by 1442 kg over the whole lactation period when compared with cooled cows,

260 which is similar to the findings on milk yield and serum index in the current study.

261 Potential metabolic pathways that heat stress affected included aminoacyl-tRNA biosynthesis, amino acid
262 metabolism, and carbohydrate metabolism of multiparous cows, and in addition to these, metabolism of cofactors and
263 vitamins and lipid metabolism were influenced in primiparous cows. Amino acids are important substrates for the
264 synthesis of proteins, and most of them in the present study are involved in the pathway of aminoacyl-tRNA
265 biosynthesis. Aminoacyl-tRNAs are essential substrates for translation, providing precursors for the synthesis of
266 proteins (Sheppard et al. 2008). Glycine is synthesized from serine, threonine, choline, glyoxylate, and hydroxyproline.
267 Serine is derived from the diet or synthesized from glucose and glutamic acid, and alanine provides the amino group for
268 glyoxylate (Wang et al. 2013; Thompson and Richardson 2014). Also, serine, glycine, and glutamic acid are involved in
269 glyoxylate and dicarboxylate metabolism. Furthermore, glycine plays a critical role in anti-oxidative reactions by
270 stimulating the synthesis of glutathione and reducing oxidative stress (Wang et al. 2014), and Senthilkumar et al. (2004)
271 found that glycine inhibits the oxidative stress by increasing the GSH-Px and SOD in livers and brains of rats. The
272 levels of amino acids in the HS group of primiparous cows were lower except for threonine, when compared with the
273 NHS group. This could be due to the effect of heat stress, as animals will consume amino acids to synthesize
274 antioxidant enzymes to resist heat stress and maintain an oxidative-antioxidative balance. However, the opposite results
275 were found in multiparous cows, this could be due to the multiparous cows were more sensitive to heat stress than
276 primiparous cows and the synthesis of antioxidant enzymes was restrained, which could explain that SOD, T-AOC and
277 GSH-Px was higher in primiparous cows than that in multiparous cows. 9,12-Octadecadienoic acid is one isomer of
278 conjugated linoleic acid (CLA) have antioxidant properties (Yurawecz et al. 1995). And the present study showed
279 9,12-Octadecadienoic acid in the serum of NHS group was higher when compared with HS group in primiparous cows.

280

281 **Conclusions**

282 This study demonstrated that heat stress negatively affected the milk yield and thus caused the elevation of the
283 serum oxidative and antioxidative index. Metabolic biomarkers associated with the heat stress in serum were found
284 which providing the basis of evaluating indicator between heat stress and non heat stress groups in primiparous and
285 multiparous cows. Moreover, we found that the cows were more susceptible to the heat stress after calving than that
286 before calving, and the multiparous cows were more sensitive to heat stress than the primiparous cows, and discover the
287 candidate biomarkers in serum.

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293 **Conflict of interest**

294 The Authors declare that they have no conflict of interest.

295

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