Association Analysis of Polymorphisms in the 5′ Flanking Region of the HSP70 Gene with Blood Biochemical Parameters of Lactating Holstein Cows under Heat and Cold Stress

Zaheer Abbas 1,†, Lirong Hu 2,†, Hao Fang 1, Abdul Sammad 2,†, Ling Kang 1, Luiz F. Brito 3,*, Qing Xu 1,* and Yachun Wang 2,*

1 Institute of Life Science and Bioengineering, Beijing Jiaotong University, Beijing 100044, China; zaheerabbas@bjtu.edu.cn (Z.A.); 18121612@bjtu.edu.cn (H.F.); lingkang3187@163.com (L.K.)
2 School of Animal Science and Technology, China Agricultural University, Beijing 100193, China; B20193040324@cau.edu.cn (L.H.); drabulsammad1742@yahoo.com (A.S.)
3 Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA; britol@purdue.edu
* Correspondence: qingxu@bjtu.edu.cn (Q.X.); wangyachun@cau.edu.cn (Y.W.)
† These authors contributed equally to this work.

Received: 30 September 2020; Accepted: 30 October 2020; Published: 2 November 2020

Simple Summary: Thermal stress causes detrimental effects on the health, welfare, and production of dairy cows, resulting in huge economic losses to the worldwide dairy cattle industry. Understanding the genomic background of thermal stress can lead to more accurate genetic selection strategies. In this study fourteen blood biochemical parameters were evaluated as potential biomarkers for heat or cold stress. Moreover, twelve single nucleotide polymorphisms (SNPs) were detected in the 5′ flanking region of the HSP70, a gene known to be associated with thermal stress response in many livestock species. Furthermore, four SNPs were significantly associated with lactate, and lipid peroxide under heat stress, and with dopamine and superoxide dismutase under cold stress. In summary, these molecular markers and bio-markers further our knowledge of thermotolerance in Holstein cattle and can be used for improving breeding strategies for climate resilience.

Abstract: Thermal stress (heat and cold) has large economic and welfare implications for the worldwide dairy industry. Therefore, it is paramount to understand the genetic background of coping mechanism related to thermal stress for the implementation of effective genetic selection schemes in dairy cattle. We performed an association study between 11 single nucleotide polymorphisms having minor allelic frequency (MAF > 0.05) in the HSP70 gene with blood biochemical parameters. The concentrations of growth hormone (GH), lactate (LA), prolactin (PRL), and superoxide dismutase (SOD) in blood were significantly higher (p < 0.05), while the concentrations of blood urea nitrogen (BUN), c-reactive protein (CRP), potassium (K+), lactate dehydrogenase (LDH), lipid peroxide (LPO), and norepinephrine (NE) were significantly lower (p < 0.05) in heat-stressed animals as compared to the control group. A significant (p < 0.05) increase in the concentrations of cortisol (COR), corticosterone (CORT), and potassium (K+) was observed (p < 0.05), while the concentrations of adrenocorticotrophic hormone (ACTH), dopamine (DA), GH, LDH, NE, PRL, and SOD were significantly lower in cold-stressed animals as compared to the control group (p < 0.05). Furthermore, SNP A-12G and C181T were significantly associated (p < 0.05) with LA and LPO, while A72G was linked with LPO (p < 0.05) in heat-stressed animals. Moreover, the SNPs A-12G and SNP C131G were significantly associated (p < 0.05) with DA and SOD under cold stress condition, respectively. These SNPs markers significantly associated with fluctuations in blood biochemical parameters under thermal stress provide a better insight into the genetic mechanisms underlying climatic resilience in Holstein cattle.
Keywords: heat stress; cold stress; blood biochemical parameters; Chinese Holstein cattle; HSP70 gene

1. Introduction

Livestock experiences different stressors throughout their lives including physical, nutritional, chemical, psychological, and thermal stress [1,2]. Among them, thermal stress is the most intriguing factor influencing production, reproductive efficiency, health, and the well-being of the high-producing animals [3,4]. In tropical and sub-tropical regions, increased temperature and humidity are the major constraints in livestock production [5], whereas the extremely low temperature in temperate areas also negatively impacts welfare and productivity [1]. The dairy cattle thermo-neutral zone (TNZ) lies in the range of 4 °C to 24 °C, within which the animal maintains a body temperature of 38.4–39.1 °C for normal physiological functions [6]. Temperatures above or below the TNZ threshold can result in heat or cold stress, respectively. The temperature and humidity index (THI) index has been widely used to evaluate thermal stress load in dairy cattle populations [7,8]. The THI threshold for heat stress has been documented to be around 67 [9] and 72 [7,10], and cold stress response is usually activated under THI 38 [11].

Among other effects, thermal stress directly influences feed intake, milk yield, reproductive performance, growth rate and even can cause premature death in harsh conditions [12,13]. Dairy cattle are usually more vulnerable to heat stress because high-producing animals generate more metabolic heat during lactation [5,14,15]. Heat stress is a complex trait as it triggers various physiological and hormonal responses through multiple molecular mechanisms [16]. Moreover, heat stress disturbs the normal regulation of oxidant/anti-oxidants, and thus, causing severe cellular damage through enzymatic and non-enzymatic activities [17,18]. It is also well-known that ambient temperature lower than the TNZ can result in increased feed intake and decreased feed conversion [19]. Temperature-stressed cows also have altered lipids, carbohydrates, and amino acids metabolism [20,21]. Therefore, plasma hormones related to stressor effects are key indicators of the cows’ physiological state and reflect the physiological compensations underlying stress exposure [22]. Various studies have reported that the biological processes involved in complex responses to thermal stress are related to hormonal changes, including insulin, adrenocorticotropic hormone, dopamine, cortisol, nor-epinephrine, and prolactin concentration [15,21,23]; antioxidants activities, such as superoxide dismutase, glutathione peroxidase; metabolites levels (e.g., blood urea nitrogen, lactate, lipid peroxide) [16,24,25].

Within-breed genetic variation exists for thermo-tolerance and disease resistance [26,27], and thus, genetic and genomic selection for improved heat or cold resistance may increase the resilience and well-being of dairy cattle [2]. Moreover, it will have important implications in the productivity, and long-term sustainability of the dairy industry as the incidence of extreme temperatures is becoming inevitable in the majority of geographic regions around the world [28,29]. HSP70 is one of the key genes from the heat shock protein (HSP) family, which are associated with thermal stress response [30–33]. The heat shock protein 70 (HSP70) is involved in many biological activities, including the proper folding of new and abnormal proteins, apoptosis, and cellular immunity [27,33]. Furthermore, HSP70 accomplishes a key role in the normal physiological activities of cells. HSP70 has both inducive and constitutive forms that reduce stress by increasing the chaperone activity [34]. Besides, HSP70 has been shown to regulate protein transport across the cell membrane using energy and hydrolysis, and consequently, maintaining the integrity of the cellular protein [33]. Both in vivo and in vitro studies have shown that HSP70 is also involved in neuro-protection [35].

The 5′ flanking region of the HSP70 gene is rich in binding sites of various transcription factors, and the mutations in this region can affect the expression of the HSP70 mRNA, stability, and translation of the HSP70 protein [36,37]. Abundant genetic variation has been reported in the DNA sequence of the HSP70 gene to discover their link with thermal tolerance capabilities of the animal, the example of which is the investigated variance in the HSP70 gene associated with heat resistance in Tharparkar
cattle [10]. The variation in the promoter region of the HSP70 gene has been reported to significantly affect the response of fibroblasts to heat stress. Moreover, the mutation in the AP2 locus in the cattle HSP70 gene results in a significant decrease in the HSP70 transcription level [38]. Basirico et al. found that two mutation sites (g895 C/- and g1128 G/T) in the 5′ UTR region of HSP70 in Holstein cattle significantly affected the viability of PBMCs and the expression levels of the HSP70 gene after exposure to heat-stressed condition, the expression level of the HSP70 gene in mutant individual cells showed significant increase in comparison to the wild type [39]. Polymorphisms in the HSP70 gene in different sheep breeds raised under thermal stress have shown significant influence (p < 0.05) on the blood metabolites such as glucose, serum glutamic-oxaloacetic transaminase (SGOT), phosphorous, triglycerides, and cholesterol [40]. Furthermore, a study conducted by Hu et al. reported significant alteration in four blood metabolites in cattle under cold stress; ACTH (adreno-corticotropic hormone), T3 (tri-iodothyronine), T4 (thyroxine), and K+ (potassium) [27].

Genetic variation in the 5′ flanking region of the HSP70 gene in Chinese Holstein cattle population and their association with heat or cold stress-related blood biochemical parameters are still unknown. Therefore, this study investigates the naturally occurring fluctuations in blood biochemical parameters during heat and cold stress and its association with the genetic polymorphism in the 5′ flanking region of the HSP70 gene in Chinese Holstein cattle. The ultimate goal is to identify significant genetic markers and biomarkers that can be used to genetically improve thermo-tolerance in dairy cattle.

2. Material and Methods

2.1. Animals’ Selection and Sampling

The experiment was performed in agreement with the Committee on Ethics of Animal Experimentation from the Beijing Jiaotong University, Beijing, China. A total of 196 healthy lactating Holstein cows were selected to evaluate the impact of heat or cold stress on blood biochemical parameters. Out of the total 196 animals, 178 animals were sampled in August 2014 (heat stress period) when THI was higher than 78, 120 in November 2014 (TNZ, THI = 55.43), and 126 in January 2015 (cold stress period) after THI dropped to below 38. Furthermore, rectal temperature was measured three times daily during the three sampling months, and average monthly rectal temperature was calculated then. Most of the animals were repeatedly measured across sampling periods. These animals were raised at the Sanyuan dairy farm in Beijing, China. All the animals were maintained under general management practices, fed in a scattered hurdle with a full mixed ration three times a day and unrestricted access to fresh drinking water. Experimental cows were kept in a semi-open barn, with sprinklers above the feeding line and fans over the stall space. In the summer, cooling sessions were performed during feeding periods. Free-stall fans and sprinklers were operational during the entire month of August (hottest period in the area). During night time, cows were allowed to rest in the outer-barn free yard, according to the weather conditions. Milking was carried out three times a day (morning, afternoon, and evening). In August cows were subjected to a rigorous cooling session of 30 min prior to milking.

Blood samples were collected from the coccygeal vein into anticoagulant-mixed and anticoagulant-free tubes. The anticoagulant-free blood samples were centrifuged at 3000 rpm/min for 10 min. The upper serum layer was parted and frozen at −80 °C for the determination of blood biochemical parameters. Furthermore, the anticoagulant-mixed blood was used to extract genomic DNA according to the kit instruction (DP318-02, TIANGEN Biotech—Beijing, Co. Ltd., Beijing, China).

2.2. THI Calculation

The temperature and relative humidity in percentage (RH) were measured using an automatic weather station (Model: P4581, Comet System S.R.O., Bebruzova, Czech Republic) located within the lactating-cows’ pen. THI was recorded from 1 August (summer), where the THI was higher than
78 continuously for 7 days, while THI for the TNZ condition was recorded after 1 November and for cold stress after 1 January. Furthermore, THI was calculated as [41]:

\[
\text{THI} = 0.8 \times \text{AT} + (\text{RH} \, \% / 100) \times [(\text{AT} - 14.4) + 46.4]
\]

(1)

where AT is the air temperature in Celsius degree (°C) and RH denotes the percentage (%) relative humidity.

2.3. Detection of Blood Biochemical Parameters

Fourteen blood metabolites were evaluated as potential indicators of heat or cold stress response, including ACTH (adrenocorticotrophic hormone), COR (cortisol), CORT (cortisone), CRP (c-reactive protein), DA (dopamine), GH (growth hormone), LPO (lipid peroxide), NE (nor-epinephrine), PRL (prolactin), and SOD (superoxide dismutase), which were measured using radioimmuno-assay [42]. Moreover, BUN (blood urea nitrogen), LA (lactate), and LDH (lactate de-hydrogenase) were detected by the colorimetry technique [43], while K+ (potassium) was measured through an electrolyte analyzer. The laboratorial work was done at the Beijing Huaying Biotechnology Research Institute, Beijing, China.

2.4. PCR Amplification and Sequencing

The isolation of genomic DNA from the blood samples was performed according to the manufacturer instruction (DP318-02, TIANGEN Biotech Co., Ltd., Beijing, China). The primer for the targeted region (5’ flanking area) of the HSP70 gene was constructed based on DNA sequence AY149618.1 (GenBank accession number) using the Primer version 3.0 (PREMIER Biosoft, San Diego, CA, USA). The forward primer was 5’GTCTGTAGGCCCTAATCTGA3’, and the reverse primer was 5’ACGCAGGAGTAGTGGTG3’. The amplification system of PCR was 50 µL, including 2 µL bovine genomic DNA (50 ng/µL), 5 µL 10× PCR Buffer, 4 µL dNTP Mixture (2.5 mM), 2 µL forward primer (10 µM), 2 µL reverse primer (10 µM), and 0.25 µL rTaq enzyme (5 U/µL). The reaction conditions were 94 °C 5 min; 94 °C 45 s; 58 °C 30 s; 72 °C 60 s; 30 cycles; and 72 °C for 5 min. The purification of the PCR product was done using the Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). Furthermore, the amplified fragments were sequenced both in forward and reverse directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, South San Francisco, CA, USA).

2.5. Detection of SNPs in the 5’ Flanking Region of the HSP70 Gene

The PCR amplification products were sent to the Bioengineering Co. Ltd. laboratory (Shanghai, China) for sequencing. Single nucleotide polymorphisms (SNPs) located in the 5’ flanking region of the HSP70 gene were detected by analyzing the polymorphic loci of the target sequence in the DNA samples of 196 Holstein cows using the DNAMAN version 6.0 (Lynnon Biosoft, San Ramon, CA, USA) and Chromas2.0 (Technelysium, South Brisbane, Australia) software.

2.6. Association Analyses

The MIXED procedure implemented in SAS9.2 software (SAS Institute Inc., Cary, NC, USA) was applied to estimate the effect of heat or cold stress on blood metabolites of Holstein cows. The statistical model for the effect of heat or cold stress on blood metabolites is described as:

\[
y = \mu + \text{SEA} + \text{PAR} + \text{Cow} + b_1 \times \text{DIM} + b_2 \times \text{DIM}^2 + \epsilon
\]

(2)

where “y” is the individual blood biochemical content measured in January, August, or November; “µ” is the mean of each blood biochemical content; “SEA” is the fixed effect of season; “PAR” is the fixed effect of parity; “Cow” is the individual effect of the animal; “DIM” is the effect of days in milk; “b1” and “b2” are the regression coefficients and “ε” is the residual term.
Subsequently, association analyses between the *HSP70* gene polymorphisms and blood metabolites affected by heat or cold stress were performed using the GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The model is presented as:

\[ y = \mu + SNP + PAR + b1 \times DIM + b2 \times DIM^2 + e \]  

where “y” is the individual blood biochemical content measured in August or January; “\( \mu \)” is the average of each blood biochemical content; “SNP” is the fixed effect of different genotypes of each SNP; “PAR” is the fixed effect of parity; “DIM” is the effect of days in milk; “b1” and “b2” are the regression coefficients, and “e” is the residual term. The significance threshold used was \( p < 0.05 \).

3. Results

3.1. Air Temperature, THI, and Rectal Temperature during Thermal Stress and TNZ Period

The average temperature and THI in the experimental cowshed were monitored for three months as shown in Table 1. The threshold categories set for thermal stress are mild, moderate, and extreme [44]. The currently reported categories set for THI are: \( 68 \leq THI < 72 \) is considered to cause mild heat stress, \( 73 \leq THI < 79 \) represents moderate heat stress, and \( 80 \leq THI < 89 \) indicates while THI \( \geq 90 \) is considered as fatal/emergency state [45]. In Beijing, the average temperature and THI of the cows-shed in August/2014 was \( 31.80 \pm 2.80 \, ^\circ\text{C} \) and \( 81.57 \pm 3.20 \), respectively, and the daily average THI greater than 78 was observed for 21 days in August/2014, which lasted for 8 h. This indicates that the experimental animals were under severe heat stress (\( 72.40 < THI < 89.68 \)). The average temperature and THI in November/2014 were \( 12.76 \pm 3.92 \, ^\circ\text{C} \) and \( 55.43 \pm 5.43 \), respectively, showing that the cows were under TNZ conditions. The average temperature in January/2015 was \( -6.70 \pm 2.35 \, ^\circ\text{C} \) and the average THI was \( 25.63 \pm 4.67 \) revealing that the cows were under mild cold stress. The animals’ rectal temperatures were recorded three times daily during the three months of sampling, and the average monthly rectal temperature was calculated. The rectal temperature in August (summer) showed a significant increase as shown in Table 1, indicating that an external increase in temperature and THI has a positive effect on the internal body temperature.

3.2. Heat or Cold Stress Effect on Blood Biochemical Parameters of Holstein Cows

Analysis of variance shows that season, parity, and individual cows had a significant effect on a variety of blood biochemical parameters \( (p < 0.05) \). The parity and individual effects were adjusted through the least square method and season comparison was performed as shown in Table 2. The blood concentration of GH, LA, PRL, and SOD under heat stress condition increased significantly \( (p < 0.05) \) as compared to the TNZ condition, while there was a significant decrease \( (p < 0.05) \) in BUN, CRP, K\(^+\), LDH, LPO, and NE. Furthermore, the concentration of COR, CORT, and K\(^+\) was higher under cold stress conditions compared to TNZ \( (p < 0.05) \), while the concentrations of ACTH, DA, GH, LDH, NE, PRL, and SOD were significantly lower \( (p < 0.05) \) under cold stress. The significant changes in blood metabolites in response to thermal stress indicate that they are potential biomarkers for thermo-tolerance.
A22T, G105T, and C131G) were not found in the dbSNP database (www.ncbi.nlm.nih.gov). Out of these 12 SNPs, six out of the 12 SNPs (A-261T, A-221G, C-135-, CRP (mg/L) 2.99 ± 0.08 b 4.40 ± 0.09 a 4.18 ± 0.09 a <0.0001
DA (ng/mL) 73.28 ± 1.89 ab 78.12 ± 2.28 a 67.22 ± 2.32 b 0.0027
GH (ng/mL) 5.26 ± 0.05 a 3.95 ± 0.07 b 3.41 ± 0.07 c <0.0001
K+ (mmol/L) 14.49 ± 0.13 c 16.44 ± 0.16 b 17.43 ± 0.16 a <0.0001
LA (mmol/L) 2.22 ± 0.02 b 2.05 ± 0.03 a 2.06 ± 0.03 a <0.0001
LDH (U/L) 901.82 ± 17.47 b 1008.61 ± 21.03 a 951.36 ± 21.40 b <0.0004
LPO (nmol/L) 5.49 ± 0.04 b 5.80 ± 0.05 a 5.74 ± 0.05 a <0.0001
NE (pg/mL) 355.34 ± 5.53 b 425.06 ± 6.72 a 408.18 ± 6.78 b <0.0001
PRL (iu/L) 292.64 ± 2.69 a 221.04 ± 3.27 b 209.26 ± 3.30 c <0.0001
SOD (U/mL) 120.28 ± 0.92 a 111.64 ± 1.12 b 93.54 ± 1.13 c <0.0001

1 Multiple comparisons were performed by the Bonferroni t-test after adjusted for the significant fixed effects. LSM, least-square means; SE, standard error. ab Different letters in the same row indicate significant differences between the two months (p < 0.05). b p < 0.01 indicates highly significant differences among seasons; p < 0.05) indicates a significant difference among seasons.

3.3. SNPs in the 5’ Flanking Region of the HSP70 Gene

The 5’ flanking region of the HSP70 gene was amplified and sequenced in 196 Holstein cows. Based on the reference sequence of Holstein Friesian (AY149618.1), the amplified product was 624 bp including 399 bp of the 5’ flanking region, 208 bp of the untranslated region (5’ UTR), 17 bp of the coding region, and 225 bp of the first exon. The target region was then sequenced and analyzed further for SNP detection. The SNPs observed in the 5’ flanking region of the HSP70 gene in Holstein cows are shown in Figure 1. Total of 12 SNPs were identified, in which all these SNPs were reported in our previous study except SNP A-221G, and A-12G [27]. Six out of the 12 SNPs (A-261T, A-221G, C-135-, A22T, G105T, and C131G) were not found in the dbSNP database (www.ncbi.nlm.nih.gov). Out of these six SNPs, three SNPs (A-261T, A-221G, and C131G) did not have the information on chromosomal position. The gene and chromosomal location, accession number, and allelic frequency information of the 12 SNPs are presented in Table 3.

Table 3. SNPs detected in the 5’ flanking region of the HSP70 gene in Chinese Holstein cows.

| SNP | Gene Position 1 | Chromosome Position | Accession Number | Allele Frequency 2 |
|-----|-----------------|---------------------|-----------------|-------------------|
| 1   | −261            | NA                  | NA              | A:0.8053 T:0.1947 |
| 2   | −221            | NA                  | NA              | A:0.0053 G:0.9947 |
| 3   | −135            | 23:27:33:006        | NA              | C:0.7193 −0.2807 |
| 4   | −12             | 23:27:33:887        | rs445536803     | A:0.1754 G:0.8246 |
| 5   | +22             | 23:27:33:854        | NA              | A:0.9474 T:0.0526 |
| 6   | +45             | 23:27:33:831        | rs211506802     | A:0.7965 T:0.2035 |
| 7   | +72             | 23:27:33:804        | rs47160461      | A:0.9263 G:0.0737 |
| 8   | +94             | 23:27:33:782        | rs43864103      | A:0.1860 G:0.8140 |
| 9   | +102            | 23:27:33:774        | rs478612967     | A:0.4158 C:0.5842 |
| 10  | +105            | 23:27:33:771        | NA              | G:0.6456 T:0.3544 |
| 11  | +131            | NA                  | NA              | C:0.9421 G:0.0579 |
| 12  | +181            | 23:27:33:726        | rs473916108     | C:0.5895 T:0.4105 |

Note: 1 The transcription initiation site is +1; 2 allele frequency of each SNP in the 196 individuals used in this study.
which AA genotype individuals had significantly higher DA concentration compared to AG and GG genotype, while the individuals with TT genotype of SNP C181T had a higher LA concentration.

For instance, SNP 4 (A-12G) was significantly correlated with DA content. The Bonferroni t-test indicates that the LA content in blood was significantly associated with the SNP A-12G compared to the GG genotype. Moreover, the polymorphism located within the 5’ flanking region of the HSP70 gene was also significantly associated with the blood biochemical parameters under cold stress (Table 4). For instance, SNP 4 (A-12G) was significantly correlated with DA content (p < 0.05). The LA content in individuals with the GG genotype of the SNP A-12G was significantly higher compared to the AG genotype, while the individuals with TT genotype of SNP C181T had a higher LA concentration in blood (p < 0.05). The Bonferroni t-test indicates that the LA content in individuals with the GG genotype of the SNP A-12G was significantly higher compared to the AG genotype, while the individuals with TT genotype of SNP C181T had a higher LA concentration in blood (p < 0.05).

Table 4. Significantly associated SNPs in the 5’ flanking region of the HSP70 gene of Holstein cows with the blood metabolites under thermal stress.

| SNPs | Stress  | Blood Biochemical Parameters | p-Value | Genotype | Number of Animals | Least Squares Mean ± Standard Error |
|------|---------|------------------------------|---------|----------|------------------|-----------------------------------|
| 4 (A-12G) | Heat stress | LA (mmol/L) | 0.03 | AA | 13 | 2.16 ± 0.12 ab |
| | | | | AG | 33 | 2.06 ± 0.08 b |
| | | | | GG | 124 | 2.27 ± 0.05 a |
| | Cold stress | DA (ng/mL) | 0.03 | AA | 9 | 84.66 ± 7.44 a |
| | | | | AG | 26 | 62.08 ± 4.68 b |
| | | | | GG | 86 | 65.74 ± 3.02 b |
Table 4. Cont.

| SNPs       | Stress | Blood Biochemical Parameters | p-Value | Genotype | Number of Animals | Least Squares Mean ± Standard Error |
|------------|--------|-------------------------------|---------|----------|-------------------|------------------------------------|
| 7 (A72G)   | Heat stress | LPO (nmol/mL) | 0.02 | AA 152 | 5.51 ± 0.06 a |
|            |        |                               |         | AG 14   | 5.80 ± 0.15 a    |
|            |        |                               |         | GG 4    | 4.72 ± 0.36 b    |
| 11 (C131G) | Cold stress | SOD (U/mL) | 0.04 | CC 110 | 96.47 ± 1.21 b   |
|            |        |                               |         | CG 10   | 94.60 ± 3.11 ab  |
|            |        |                               |         | GG 1    | 113.12 ± 6.42 a  |
| 12 (C181T) | Heat stress | LA (mmol/L) | 0.05 | CC 81   | 2.16 ± 0.15 b    |
|            |        |                               |         | CT 28   | 2.19 ± 0.08 ab   |
|            |        |                               |         | TT 61   | 2.34 ± 0.06 a    |

Note: 1. p < 0.05 indicates significantly associated with the blood biochemical parameters. 2. a,b Different letters in the same column indicate a significant difference between genotypes (p < 0.05) based on the Bonferroni t-test. The association between all the 11 SNPs and blood biochemical parameters was done, but only 4 SNPs were found significantly associated with the above blood biochemical parameters.

4. Discussion

THI is widely used to quantify thermal stress in dairy animals [29,46]. When THI exceeds 72, the animals experience heat stress [12,29], and cold stress for THI lower than 38 [11]. In the present study, the average THI exceeded 72, showing that animals were under heat stress in August/2014 and cold stress in January/2015 when the average THI was lower than 38. The rectal temperature recorded during the summer significantly increased, while no significant changes in the rectal temperature was observed during the winter period, as shown in the Table 1. Thermal stress causes physiological, hormonal, biochemical, and molecular responses for the sake of cell survivability in harsh and stressful environments [15,16,21]. Our findings show that there were significant changes in blood metabolites of Holstein cattle under heat or cold stress as shown in Figure 2, in agreement with other studies in Holstein and Jersey cattle [47,48].

In our study, the concentration of PRL, GH, LA, and SOD significantly increased while BUN, CRP, K+, LDH, LPO, and NE decreased in heat-stressed cows compared to the TNZ. The animal body increases heat dissipation under heat stress, which manifests as shortness of breath, rapid heartbeat, and disorder of metabolic pathways [5]. The change in PRL has a direct relationship with increased rectal temperature [49], which is consistent with our study. PRL is not only correlated with milk production but also involved in thermoregulation by maintaining water and electrolytes balance [50]. The increase in PRL is to meet up with the increased demand for water and electrolytes during heat stress [51]. GH establishes a prominent position along with PRL in milk synthesis, as well as partitioning of energy toward the mammary gland for milk synthesis during heat stress [52]. The animal experiences negative energy balance during heat stress because of reduced feed intake [21]. The possible reason for increased GH is to enable maintenance of the energetic status of the animal during heat stress condition [52]. LA increased during heat stress in the leg and pectoral muscles of chickens [53], as well as in the blood of Holstein cattle [54], which corroborates with our findings. Heat-stressed cows showed increased gluconeogenesis as compared to TNZ conditions [55], and presumably increased utilization of LA and alanine for glucose synthesis [21,55,56]. Besides the animals dissipating heat through panting and sweating during heat stress that results in dehydration [57], the increase in LA may also be due to decreased blood volume as a result of dehydration and lower blood flow toward the muscle during heat stress [58]. Heat stress induces oxidative stress that stimulates certain body defense mechanisms and increase oxidative stress-related biomarkers such as SOD in order to scavenge free
Various studies claimed that heat stress increases the blood SOD in cows [60], goats [61], and mice [62], which is similar to our findings.

BUN is normally originated from rumen and through deamination of the amino acids by the liver [63]. Srikandakumar et al. reported that the BUN content in dairy cattle decreased during heat stress by 1.48 mmol/L and 0.65 mmol/L, respectively, which is consistent with the results of this study [64]. The decrease in BUN concentration suggests an alteration in protein catabolism, and the nitrogenous re-partition during heat stress [15]. Moreover, heat stress elevates the marginal vasodilation to dissipate more heat resulting in decreased blood drift to the organs [15], besides it also changes the reduced thyroid activity during heat stress [65]. CRP is known as a sensitive inflammation marker, the decrease in CRP may be due to liver dysfunction due to heat stress, as CRP is liver originated during diseases or under severe stress. Another study claimed that CRP is correlated with milk production and is at peak during high lactation [66], thus the decrease of CRP in heat-stressed cows may also be as a result of decreased milk production due to reduced feed intake during heat stress. LPO decreased during heat stress conditions, likely as defensive capability of HSP70 [67], and the increased activity of the antioxidant defense system against per-oxides, which includes various enzymes such as SOD, catalase (CAT), and glutathione per-oxidase during heat stress [68–70]. Previous studies have shown that LDH concentration is lower in the summer in comparison to the TNZ, as also noticed in the current study, and is thought to be the consequence of the reduced thyroid activity during heat stress [71,72].

The main physiological response of animals under cold stress is to up-surge heat production and decrease heat dissipation in order to up-hold a constant body temperature, besides it also changes the endocrine hormone regulation [73]. Similar to our study, the DA level in chickens decreased after exposure to severe cold stress [74], which may be due to the damage of neural spikes of DA neuron releasing DA. In our study, the COR and CORT plasma levels were higher under cold stress, while different studies showed a similar trend in the increase of blood COR in pigs and dairy cattle.
Animals 2020, 10, 2016

under cold stress [19,75]. Moreover, a recent study showed that blood COR increased in dairy cattle after the exposure to heat stress for a long duration [76], likely to reduce metabolic heat production [11]. Similar to heat stress, in order to maintain body temperature and elevate energy production, cows under cold stress can reduce production performance and immunity [77]. Based on the above evidences, the increase in COR may be an adaptive strategy and reflect a shielding action favoring an increase in metabolic heat production under cold stress. Furthermore, cold stress was also found to increase corticosterone and thyroxin levels. In summary, heat or cold stress caused significant changes in blood metabolites in Holstein cows, which had a great impact on the physiological state of the cows and reflect the physiological compensations that cows undergo at various lactation intensities and/or stress exposure. Therefore, the aforementioned metabolites can be used as bio-markers for evaluating tolerance to thermal stress.

The HSP70 protein family not only acts as a molecular chaperone to assist the folding/unfolding, congregation, and transportation of new and abnormal proteins, inhibit cell apoptosis, and protect cells from stress damage, but also stimulate the rapid response of the immune system and regulate the activation of immune cells and the production of cytokines [78,79]. SNPs provide tools to carry out association analyses and the identification of genetic markers for the selection of important traits [27,80]. It has been reported that SNPs in the 5′ flanking region of the HSP70 gene in Duroc boars show association with semen quality under heat stress, and suggested that those SNPs can contribute as genetic markers for breeding for improved heat tolerance in pigs [81]. Polymorphisms in the 5′ flanking regions of the HSP70 gene validate its association with the blood metabolites such as T3 and T4 in Sanhe cattle under severe cold stress [27]. Deb et al. reported that the variation of the AP2 binding element in the promoter region of the HSP70 gene could affect heat stress response of Frieswal hybrid cattle [82]. The rectal temperature and respiratory frequency of homozygous genotype individuals were significantly lower ($p < 0.05$) than those of heterozygous individuals. We identified a total of twelve SNPs in the 5′ flanking region of the HSP70 gene (Table 4). All these SNPs were previously reported under cold stress by our group except SNP A-221G, and A-12G [27]. However, the inclusion of three distinct weather conditions with respective representation of important biochemical parameters in high producing cows, constitute salient features of this association study. We found that four SNPs were significantly associated with the various blood biochemical parameters (Table 3) related to heat or cold stress in Chinese Holstein cows. Under heat stress three SNPs (A-12G, C181T, and A72G) were significantly associated with the blood metabolites LA, and LPO. Under cold stress SNPs, A-12G and C131G were significantly associated with DA and SOD, respectively. The validation of these blood biomarkers under heat stress and their association with the polymorphisms in the 5′ flanking region suggest them as indicators of thermal stress in Holstein cattle. Furthermore, the polymorphisms in the 5′ flanking region associated with these biochemical indicators reveal that three SNPs (A-12G, A72G, C181T) may be used as molecular marker for the selection of heat resilient animals, while A-12G and C131G may be used as biomarkers for cold tolerance. Future studies should perform associations with whole-genome markers to identify other SNPs associated with the biomarkers defined here.

5. Conclusions

This study has taken a novel approach for the characterization of blood biochemical profile of lactating Holstein cows under three thermal conditions. The association analysis performed hereafter identified SNPs in the 5′ flanking region of the HSP70 gene, which are significantly associated with the indicators of thermal tolerance. An antagonistic trend of gluconeogenesis-related biochemical parameters between cold and heat stress depicts the cow’s physiological alterations to thermal challenges. SNP A-12G and C181T were significantly associated with lactate; SNP A72G with lipid per-oxide under heat stress; while SNP A-12G was significantly associated with dopamine; and SNP C131G was linked with superoxide dismutase under cold stress. Moreover, these SNPs may be used as candidate molecular markers for thermo-tolerance in Chinese Holstein cattle.
Author Contributions: Z.A., conceptualization, methodology, visualization, validation, writing—original draft, writing—review and editing. L.H., methodology, validation, formal analysis, software, data curation. H.F. and L.K., software, formal analysis. A.S. and L.F.B., writing—review and editing. Q.X. and Y.W., supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: The funding aid for this study was provided by the Research Fund for International Young Scientists by the National Natural Science Foundation of China (Grant Number: 31750110459), China Agriculture Research System (CARS-36), and the Program for Changjiang Scholar and Innovation Research Team in University (IRT_15R62).

Conflicts of Interest: The authors have no conflict of interest and all the authors agreed to the publication of this work. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References
1. Jyotiranjan, T.; Mohapatra, S.; Mishra, C.; Dalai, N.; Kundu, A.K. Heat tolerance in goat-A genetic update. Pharma Innov. J. 2017, 6, 237–245.
2. Brito, L.F.; Oliveira, H.R.; McConn, B.R.; Schinckel, A.P.; Arrazola, A.; Marchant-Forde, J.N.; Johnson, J.S. Large-Scale Phenotyping of Livestock Welfare in Commercial Production Systems: A New Frontier in Animal Breeding. Front. Genet. 2020, 11, 793. [CrossRef] [PubMed]
3. Chaidanya, K.; Niyas, P.A.A.; Sejian, V.; Shaji, S. Adaptation of Livestock to Environmental Challenges. J. Vet. Sci. Med. Diagn. 2015, 4. [CrossRef]
4. Sammad, A.; Umer, S.; Shi, R.; Zhu, H.; Zhao, X.; Wang, Y. Dairy cow reproduction under the influence of heat stress. J. Anim. Physiol. Anim. Nutr. 2019. [CrossRef] [PubMed]
5. Das, R.; Sailo, L.; Verma, N.; Bharti, P.; Saikia, J.; Intiwati; Kumar, R. Impact of heat stress on health and performance of dairy animals: A review. Vet. World 2016, 9, 260–268. [CrossRef]
6. Hahn, G.L. Housing and management to reduce climatic impacts on livestock. J. Anim. Sci. 1981, 52, 175–186. [CrossRef]
7. Ravagnolo, O.; Misztal, I.; Hoogenboom, G. Genetic component of heat stress in dairy cattle, development of heat index function. J. Dairy Sci. 2000, 83, 2120–2125. [CrossRef]
8. Habeeb, A.A.; Gad, A.E.; Atta, M.A. Temperature-Humidity Indices as Indicators to Heat Stress of Climatic Conditions with Relation to Production and Reproduction of Farm Animals. Int. J. Adv. Biotechnol. Res. 2018, 1, 35–50. [CrossRef]
9. Heinicke, J.; Hoffmann, G.; Ammon, C.; Amon, B.; Amon, T. Effects of the daily heat load duration exceeding determined heat load thresholds on activity traits of lactating dairy cows. J. Therm. Biol. 2018, 77, 67–74. [CrossRef]
10. Bhat, S.; Kumar, P.; Kashyap, N.; Deshmukh, B.; Dige, M.S.; Bhushan, B.; Chauhan, A.; Kumar, A.; Singh, G. Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. Vet. World 2016, 9, 113–117. [CrossRef]
11. Xu, Q.; Wang, Y.C.; Hu, L.R.; Kang, L. The effect of temperature stress on milk production traits and blood biochemical parameters of Holstein cows. In Proceedings of the World Congress on Genetics Applied to Livestock Production, Auckland, New Zealand, 16 January 2018; Volume 11, p. 95.
12. Nabenishi, H.; Yamazaki, A. Effects of temperature–humidity index on health and growth performance in Japanese black calves. Trop. Anim. Health Prod. 2017, 49, 397–402. [CrossRef]
13. Dash, S.; Chakravarty, A.K.; Singh, A.; Upadhyay, A.; Singh, M.; Yousuf, S. Effect of heat stress on reproductive performances of dairy cattle and buffaloes: A review. Vet. World 2016, 9, 235–244. [CrossRef] [PubMed]
14. Yano, M.; Shimadzu, H.; Endo, T. Modelling temperature effects on milk production: A study on Holstein cows at a Japanese farm. SpringerPlus 2014, 3, 1–11. [CrossRef]
15. Sammad, A.; Wang, Y.J.; Umer, S.; Lirong, H.; Khan, I.; Khan, A.; Ahmad, B.; Wang, Y. Nutritional physiology and biochemistry of dairy cattle under the influence of heat stress: Consequences and opportunities. Animals 2020, 10, 793. [CrossRef] [PubMed]
16. Gupta, M.; Kumar, S.; Dangi, S.; Jangir, B. Physiological, Biochemical and Molecular Responses to Thermal Stress in Goats. Int. J. Livest. Res. 2015, 3, 27. [CrossRef]
17. Zhao, F.Q.; Zhang, Z.W.; Qu, J.P.; Yao, H.D.; Li, M.; Li, S.; Xu, S.W. Cold stress induces antioxidants and Hsps in chicken immune organs. Cell Stress Chaperones 2014, 19, 635–648. [CrossRef]
18. Şahin, E.; Gümüşlü, S. Cold-stress-induced modulation of antioxidant defence: Role of stressed conditions in tissue injury followed by protein oxidation and lipid peroxidation. *Int. J. Biometeorol.* 2004, 48, 165–171. [CrossRef] [PubMed]

19. Frank, J.W.; Carroll, J.A.; Allee, G.L.; Zannelli, M.E. The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide. *J. Anim. Sci.* 2003, 81, 1166–1176. [CrossRef]

20. Dahl, G.E.; Do Amaral, B.C.; Levin, Y.; Zachut, M.; Skibiel, A.L. Liver proteomic analysis of postpartum Holstein cows exposed to heat stress or cooling conditions during the dry period. *J. Dairy Sci.* 2017, 101, 705–716. [CrossRef]

21. Abbas, Z.; Sammad, A.; Hu, L.; Fang, H.; Xu, Q.; Wang, Y. Glucose Metabolism and Dynamics of Facilitative Glucose Transporters (GLUTs) under the Influence of Heat Stress in Dairy Cattle. *Metabolites* 2020, 10, 312. [CrossRef]

22. Johnson, H.D.; Vanjonack, W.J. Effects of Environmental and Other Stressors on Blood Hormone Patterns in Lactating Animals. *J. Dairy Sci.* 2010, 59, 1603–1617. [CrossRef]

23. Bova, T.L.; Chiavaccini, L.; Cline, G.F.; Hart, C.G.; Matheny, K.; Muth, A.M.; Voelz, B.E.; Kesler, D.; Memili, E. Environmental stressors influencing hormones and systems physiology in cattle. *Reprod. Biol. Endocrinol.* 2014, 12, 1–5. [CrossRef] [PubMed]

24. Koubková, M.; Knížková, I.; Kunc, P.; Hátťrová, H.; Flusser, J.; Doležal, O. Influence of high environmental temperatures and evaporative cooling on some physiological, hematological and biochemical parameters in high-yielding dairy cows. *Czech. J. Anim. Sci.* 2002, 47, 309–318.

25. Guo, J.; Gao, S.; Quan, S.; Zhang, Y.; Bu, D.; Wang, J. Blood amino acids profile responding to heat stress in dairy cows. *Asian-Australs. J. Anim. Sci.* 2018, 31, 47–53. [CrossRef]

26. Ansari-Mahyari, S.; Ojali, M.R.; Forutan, M.; Riasi, A.; Brito, L.F. Investigating the genetic architecture of HSP70 in livestock—At cellular level. *J. Anim. Physiol. Anim. Nutr.* 2010, 94, 301–310. [CrossRef] [PubMed]

27. Garner, J.B.; Douglas, M.L.; Williams, S.R.O.; Wales, W.J.; Marett, L.C.; Nguyen, T.T.T.; Reich, C.M.; Hayes, B.J. Genomic selection improves heat tolerance in dairy cattle. *Sci. Rep.* 2016, 6, 34114. [CrossRef] [PubMed]

28. Ravagnolo, O.; Misztal, I. Genetic Component of Heat Stress in Dairy Cattle, Parameter Estimation. *J. Dairy Sci.* 2000, 83, 2126–2130. [CrossRef]

29. Kiang, J.G.; Tsokos, G.C. Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* 1998, 80, 183–201. [CrossRef]

30. Liu, Y.; Li, D.; Li, H.; Zhou, X.; Wang, G. A novel SNP of the ATP1A1 gene is associated with heat tolerance traits in dairy cows. *Mol. Biol. Rep.* 2011, 38, 83–88. [CrossRef]

31. Belhadj Slimen, I.; Najar, T.; Ghram, A.; Abdrrabba, M. Heat stress effects on livestock: Molecular, cellular and metabolic aspects, a review. *J. Anim. Physiol. Anim. Nutr.* 2016, 100, 401–412. [CrossRef]

32. Hassan, F.; Nawaz, A.; Rehman, M.S.; Ali, M.A.; Dilshad, S.M.R.; Yang, C. Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. *Anim. Nutr.* 2019, 5, 340–350. [CrossRef]

33. Lindquist, P.J.G.; Svensson, L.T.; Alexson, S.E.H. Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver—Recombinant expression in *Escherichia coli*, tissue expression, and nutritional regulation. *Eur. J. Biochem.* 1998, 251, 631–640. [CrossRef] [PubMed]

34. Mishra, S.; Palai, T. Importance of HSP70 in Livestock—At cellular level. *J. Mol. Pathophysiol.* 2014, 3, 30. [CrossRef]

35. Simcox, A.A.; Cheney, C.M.; Hoffman, E.P.; Shearn, A. A deletion of the 3′ end of the *Drosophila melanogaster* hsp70 gene increases stability of mutant mRNA during recovery from heat shock. *Mol. Cell. Biol.* 2015, 5, 3397–3402. [CrossRef] [PubMed]

36. Theodorakis, N.G.; Morimoto, R.I. Posttranscriptional regulation of hsp70 expression in human cells: Effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. *Mol. Cell. Biol.* 2015, 7, 4357–4368. [CrossRef]
38. Schwerin, M.; Maak, S.; Kalbe, C.; Fuerbass, R. Functional promoter variants of highly conserved inducible hsp70 genes significantly affect stress response. *Biochim. Et Biophys. Acta—Gene Struct. Expr.* 2001, 1522, 108–111. [CrossRef]

39. Basiricò, L.; Morera, P.; Primi, V.; Lacetera, N.; Nardone, A.; Bernabucci, U. Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. *Cell Stress Chaperones* 2011, 16, 441–448. [CrossRef]

40. Singh, K.M.; Singh, S.; Ganguly, I.; Nachiappan, R.K.; Ganguly, A.; Venkataramanan, R.; Chopra, A.; Narula, H.K. Association of heat stress protein 90 and 70 gene polymorphism with adaptability traits in Indian sheep (*Ovis aries*). *Cell Stress Chaperones* 2017, 22, 675–684. [CrossRef] [PubMed]

41. Mader, T.L.; Davis, M.S.; Brown-Brandl, T. Environmental factors influencing heat stress in feedlot cattle. *J. Anim. Sci.* 2006, 84, 712–719. [CrossRef]

42. Maurer, H.H.; Fritz, C.F. Toxicological detection of pholcodine and its metabolites in urine and hair using radio immunoassay, fluorescence polarisation immunoassay, enzyme immunoassay and gas chromatography-mass spectrometry. *Int. J. Leg. Med.* 1990, 104, 43–46. [CrossRef]

43. Gill, P.; Moghadam, T.T.; Ranbar, B. Differential scanning calorimetry techniques: Applications in biology and bioscience. *J. Biomat. Tech.* 2010, 21, 167–193. [PubMed]

44. Wang, X.; Gao, H.; Gebremedhin, K.G.; Bjerg, B.S.; Van Os, J.; Tucker, C.B.; Zhang, G. A predictive model of equivalent temperature index for dairy cattle (ETIC). *J. Therm. Biol.* 2018, 76, 165–170. [CrossRef] [PubMed]

45. Collier, R.J.; Hall, L.W.; Rungruang, S.; Zimbleman, R.B. Quantifying heat stress and its impact on metabolism and performance. In Proceedings of the MidSouth Ruminant Nutrition Conference, Florida, FL, USA, 1 February 2012; pp. 74–84. [CrossRef]

46. Arieli, A.; Adin, G.; Bruckental, I. The effect of protein intake on performance of cows in hot environmental temperatures. *J. Dairy Sci.* 2004, 87, 620–629. [CrossRef]

47. Abeni, F.; Calamari, L.; Stefanini, L. Metabolic conditions of lactating Friesian cows during the hot season in the Po valley. 1. Blood indicators of heat stress. *Int. J. Biometeorol.* 2007, 52, 87–96. [CrossRef]

48. Smith, D.L.; Smith, T.; Rude, B.J.; Ward, S.H. Short communication: Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. *J. Dairy Sci.* 2013, 96, 3028–3033. [CrossRef]

49. Alam, M. The role of prolactin in thermoregulation and water balance during heat stress in domestic ruminants. *Asian J. Anim. Vet. Adv.* 2011, 6, 1153–1169. [CrossRef]

50. Gao, S.T.; Guo, J.; Quan, S.Y.; Nan, X.M.; Fernandez, M.V.S.; Baumgard, L.H.; Bu, D.P. The effects of heat stress on protein metabolism in lactating Holstein cows. *J. Dairy Sci.* 2017, 100, 5040–5049. [CrossRef]

51. Collier, R.J.; Doelger, S.G.; Head, H.H.; Thatcher, W.W.; Wilcox, C.J. Effects of Heat Stress during Pregnancy on Maternal Hormone Concentrations, Calf Birth Weight and Postpartum Milk Yield of Holstein Cows. *J. Anim. Sci.* 1982, 54, 309–319. [CrossRef]

52. Bauman, D.E.; Bruce Currie, W. Partitioning of Nutrients During Pregnancy and lactation: A Review of Mechanisms Involving Homeostasis and Homeorhesis. *J. Dairy Sci.* 1980, 63, 1514–1529. [CrossRef]

53. Zhang, Z.Y.; Jia, G.Q.; Zuo, J.J.; Zhang, Y.; Lei, J.; Ren, L.; Feng, D.Y. Effects of constant and cyclic heat stress on muscle metabolism and meat quality of broiler breast fillet and thigh meat. *Poult. Sci.* 2012, 91, 2931–2937. [CrossRef]

54. Koch, F.; Lamp, O.; Islamizad, M.; Weizel, J.; Kuhl, B. Metabolic Response to heat stress in late-pregnant and early lactation dairy cows: Implications to liver-muscle crosstalk. *PLoS ONE* 2016, 11, e0160912. [CrossRef] [PubMed]

55. Shahzad, K.; Akbar, H.; Vailati-Riboni, M.; Basiricò, L.; Morera, P.; Rodriguez-Zas, S.L.; Nardone, A.; Bernabucci, U.; Loor, J.J. The effect of calving in the summer on the hepatic transcriptome of Holstein cows during the peripartal period. *J. Dairy Sci.* 2015, 98, 5401–5413. [CrossRef] [PubMed]

56. Rhoads, R.P.; La Noce, A.J.; Wheelock, J.B.; Baumgard, L.H. Short communication: Alterations in expression of gluconeogenic genes during heat stress and exogenous bovine somatotropin administration. *J. Dairy Sci.* 2011, 94, 1917–1921. [CrossRef]

57. Gu, Z.; Li, L.; Tang, S.; Liu, C.; Fu, X.; Shi, Z.; Mao, H. Metabolomics Reveals that Crossbred Dairy Buffaloes Are More Thermotolerant than Holstein Cows under Chronic Heat Stress. *J. Agric. Food Chem.* 2018, 66, 12889–12897. [CrossRef] [PubMed]
Animals 2020, 10, 16

58. Bergh, U.; Danielsson, U.; Wennberg, L.; Sjöberg, B. Blood lactate and perceived exertion during heat stress. Acta Physiol. Scand. 1986, 126, 617–618. [CrossRef] [PubMed]

59. Takahashi, M. Heat stress on reproductive function and fertility in mammals. Reprod. Med. Biol. 2012, 11, 37–47. [CrossRef]

60. Bernabucci, U.; Ronchi, B.; Lacetera, N.; Nardone, A. Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. J. Dairy Sci. 2002, 85, 2173–2179. [CrossRef]

61. Wang, L.; Xue, B.; Wang, K.; Li, S.; Li, Z. Effect of heat stress on endotoxin flux across mesenteric-drained and portal-drained visera of dairy goat. J. Anim. Physiol. Anim. Nutr. 2011, 95, 468–477. [CrossRef]

62. Matsuzuka, T.; Ozawa, M.; Nakamura, A.; Ushitani, A.; Hirabayashi, M.; Kanai, Y. Effects of heat stress on the redox status in the oviduct and early embryonic development in mice. J. Reprod. Dev. 2005, 51, 281–287. [CrossRef]

63. Rhoads, R.P.; Nardone, A.; Ronchi, B.; Bernabucci, U.; Lacetera, N.; Baumgard, L.H. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal 2010, 4, 1167–1183. [CrossRef]

64. Srikandakumar, A.; Johnson, E.H. Effect of heat stress on milk production, rectal temperature, respiratory rate and blood chemistry in Holstein, Jersey and Australian milking Zebu cows. Trop. Anim. Health Prod. 2004, 36, 685–692. [CrossRef] [PubMed]

65. Srikandakumar, A.; Johnson, E.H.; Mahgoub, O. Effect of heat stress on respiratory rate, rectal temperature and blood chemistry in Omari and Australian Merino sheep. Small Rumin. Res. 2003, 49, 193–198. [CrossRef]

66. Lee, W.C.; Hsiao, H.C.; Wu, Y.L.; Lin, J.H.; Lee, Y.P.; Fung, H.P.; Chen, H.H.; Chen, Y.H.; Chu, R.M. Serum C-reactive protein in dairy herds. Can. J. Vet. Res. 2003, 67, 102–107.

67. Dieterich, A.; Troschinski, S.; Schwarz, S.; Di Lellis, M.A.; Henneberg, A.; Fischbach, U.; Ludwig, M.; Gärtner, U.; Triebskorn, R.; Köhler, H.R. Hsp70 and lipid peroxide levels following heat stress in Xeropicta derbentina (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs. Cell Stress Chaperones 2015, 20, 159–168. [CrossRef]

68. Aebi, H. Catalase in Vitro. In Methods in Enzymology; Academic Press: New York, NY, USA, 1984; Volume 105, pp. 121–126. [CrossRef]

69. Gutteridge, J.M.C. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin. Chem. 1995, 41, 1819–1828. [CrossRef]

70. Halliwell, B.; Gutteridge, J.M.C. Understanding Insulin Action: Principles and Molecular Mechanisms Free Radicals. In Biology and Medicine, 2nd ed.; J. Espinal Ellis Horwood: London, UK, 1990; p. 7458.

71. Goddard, P.J.; Keay, G.; Grigor, P.N. Lactate dehydrogenase quantification and isoenzyme distribution in physiological response to stress in red deer (Cervus elaphus). Res. Vet. Sci. 1997, 63, 119–122. [CrossRef]

72. Helal, A.; Youssef, K.M.; El-Shaer, H.M.; Gipson, T.A.; Goetsch, A.L.; Askar, A.R. Effects of acclimatization on energy expenditure by different goat genotypes. Livest. Sci. 2010, 127, 67–75. [CrossRef]

73. Aarif, O.; Mahapatra, P.S.; Yatoo, M.A.; Dar, S.A.; Aarif, O.; Mahapatra, P.S.; Yatoo, M.A.; Dar, S.A. Impact of Cold Stress on Physiological, Hormonal and Immune Status in Male and Female Broad Breasted White Turkeys. J. Stress Physiol. Biochem. 2013, 9, 54–60.

74. Moore, H.; Rose, H.J.; Grace, A.A. Chronic cold stress reduces the spontaneous activity of ventral tegmental dopamine neurons. Neuropsychopharmacology 2001, 24, 410–419. [CrossRef]

75. Kang, H.J.; Piao, M.Y.; Lee, I.K.; Kim, H.I.; Gu, M.J.; Yun, C.H.; Seo, J.; Baik, M. Effects of ambient temperature and dietary glycerol addition on growth performance, blood parameters and immune cell populations of Korean cattle steers. Asian-Australas. J. Anim. Sci. 2017, 30, 505–513. [CrossRef]

76. Christison Gl, J.H. Cortisol turnover in heat-stressed cow. J. Anim. Sci. 1972, 35, 1005–1010. [CrossRef]

77. Brouček, J.; Letkovičová, M.; Kovalčík, K. Estimation of cold stress effect on dairy cows. Int. J. Biometeorol. 1991, 35, 29–32. [CrossRef]

78. Šima, P.; Cervinková, M.; Funda, D.P.; Holub, M. Enhancement by Mild Cold Stress of the Antibody Forming Capacity in Euthymic and Athymic Hairless Mice. Folia Microbiol. 1998, 43, 521–523. [CrossRef]

79. Moseley, P. Stress proteins and the immune response. Immunopharmacology 2000, 48, 299–302. [CrossRef]

80. Isilk, R.; Bilgen, G. Associations between genetic variants of the POU1F1 gene and production traits in Saanen goats. Arch. Anim. Breed. 2019, 62, 249–255. [CrossRef] [PubMed]
81. Huang, S.Y.; Chen, M.Y.; Lin, E.C.; Tsou, H.L.; Kuo, Y.H.; Ju, C.C.; Lee, W.C. Effects of single nucleotide polymorphisms in the 5′-flanking region of heat shock protein 70.2 gene on semen quality in boars. *Anim. Reprod. Sci.* **2002**, *70*, 99–109. [CrossRef]

82. Deb, R.; Sajjanar, B.; Singh, U.; Kumar, S.; Brahmane, M.P.; Singh, R.; Sengar, G.; Sharma, A. Promoter variants at AP2 box region of Hsp70.1 affect thermal stress response and milk production traits in Frieswal cross bred cattle. *Gene* **2013**, *532*, 230–235. [CrossRef] [PubMed]

**Publisher’s Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.