System-level Insights into the Adaptation of Domestic Dairy Buffalo to Chronic Heat Stress

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Abbreviations: CATHL2 (LL-37), cathelicidin-2; CP, ceruloplasmin; GPX3, glutathione peroxidase 3; Hb, hemoglobin; HBA1, hemoglobin subunit alpha 1; HCT, hematocrit; HS, heat stress; LFQ, label-free quantification; LPL, Lipase; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PLT, platelet hematocrit; PRM, parallel reaction monitoring; RBC, red blood cell count; ROS, reactive oxygen species; RR, Respiration rates; SRM/MRM, Single or multiple reaction monitoring; THI, Temperature-humidity index; WBC, white blood cell count; TN, thermal-neutral conditions
Abstract: Chronic heat stress (HS), aggravated by global warming, reduces the production efficiency of the buffalo dairy industry. Here, we conducted a proteomic analysis to investigate the adaptation strategies used by buffalo in response to heat stress. Seventeen differentially abundant proteins with known functions were detected using label-free quantification (LFQ), and five of these differentially expressed proteins were validated with parallel reaction monitoring (PRM). These five proteins were associated with various aspects of heat stress, including decreased heat production, increased blood oxygen delivery, and enhanced natural disease resistance. Lipase (LPL), glutathione peroxidase 3 (GPX3), cathelicidin-2 (CATHL2, LL-37), ceruloplasmin (CP), and hemoglobin subunit alpha 1 (HBA1) were shown to play cooperative roles in the tolerance of chronic HS in dairy buffalo. We found that high levels of HBA1 increased blood oxygen transport capacity. Our results increase our understanding of the adaptation of dairy buffalo to chronic heat stress.

Keywords: chronic heat stress; dairy buffaloes; proteomics; adaptation mechanisms
1. Introduction

In 2016, the global population of domestic buffalo (*Bubalus bubalis*) was ~199.0 million; about 99% of these buffalo inhabit tropical and subtropical regions [1]. At present, the mean daily maximum temperature across the regions where buffalo are raised is 19.7–41.2 °C, but it is predicted that the daily maximum temperature in these regions will increase by 1–4.5 °C over the next 50 years [2]. Although buffalo are well adapted to hot climates [3,4], they show signs of thermal discomfort, such as low milk yield and poor reproductive performance, at temperatures exceeding 25°C [5]. Indeed, the body temperatures of buffalo kept at 42.1°C for 2–3 h increased from 37.5–38.5°C to 41.1°C, and the respiratory rates increased from 28.7 breaths/min to 128 breaths/min [6]. At ambient temperatures of 29.4°C and 38.9°C, nutrient intake and milk yield decreased ~30.0% and ~27.6%, respectively [7,8]. In addition, buffalo have fewer sweat glands than do cattle, increasing heat sensitivity [3,9,10]. Thus, buffalo may be increasingly prone to heat stress as global warming progresses, threatening the buffalo dairy industry [11-13].

Chronic heat stress (HS) occurs after prolonged exposure to elevated ambient temperatures and high humidity, when the animal is unable to dissipate excessive heat load to the surrounding environment [14]. HS stimulates the excessive accumulation of reactive oxygen species (ROS), which triggers the oxidative stress response [15-17]. High concentrations of ROS limit energy production and utilization in heat-stressed animals [18], negatively affecting animal growth, production, and reproductive performance [19-22]. To improve the production efficiency of dairy buffalo, it is
important to investigate the mechanisms underlying thermal tolerance using protein biomarkers.

Proteomics have been widely used to assess the ability of livestock to adapt to temperature increases [23]. Specifically, label-free quantification (LFQ) is a high-resolution method based on mass spectrometry, which determines the relative amounts of protein among two or more biological samples without the use of stable isotopes [24]. LFQ extracts peptide signals on the MS1 level, and thus uncouples the quantification process from the identification process [25]. Single and multiple reaction monitoring (SRM/MRM) methods have been widely used for protein absolute quantification due to their high sensitivity, accuracy, and repeatability. Parallel reaction monitoring (PRM) is high-resolution ion monitoring technique (5–6 orders of magnitude), which detects all product ions in parallel in a high-precision mass spectrometer [26]. The principle of this technique is similar to SRM/MRM, but PRM is more convenient and more selective than SRM/MRM [27]. PRM is thus most suitable for the absolute quantification of multiple proteins in a complex sample with an attomole-level of detection.

For livestock, blood sampling is convenient, and blood biomarkers are useful indicators of the physiological state of the animal [28]. We thus hypothesized that some serum proteins would be important for HS tolerance in buffalo. To address this hypothesis, we aimed to detect targeted serum proteins closely related to HS with LFQ, and then perform an absolute quantification of these proteins with PRM. In this way, we aimed to identify candidate protein biomarkers of chronic HS to improve the thermal tolerance of dairy buffalo.
2. Results

The average THI values were 75.76 and 54.26 under the HS and TN conditions, respectively \((p < 0.001)\). The body temperatures of the HS and TN buffalo \((38.24\, ^\circ C\) and \(38.16\, ^\circ C\), respectively) were not significantly different \((p > 0.05)\). However, the RR of the HS buffalo \((32.5\) breaths/min) was significantly greater than that of the TN buffalo \((19.0\) breaths/min; \(p < 0.001)\).

2.1. Identification of proteins related to HS adaptation

Using LFQ, 344 low-abundance proteins were identified in the serum samples. Of these, our LFQ results indicated that 17 were differentially expressed in HS buffalo as compared to the TN buffalo (Table 1). Our volcano plot indicated that four of these proteins were more abundant in HS buffalo, while 13 were less abundant (Figure 1). Our hierarchical clustering analysis indicated that the 17 differentially abundant proteins grouped well by function: proteins similar to RETN formed one cluster, and the other cluster comprised CP \((\text{L8I5R0 and D2U6Z5})\), HBA1, and LL-37. \text{L8I5R0 and D2U6Z5}, both representing CP, were obviously closely clustered (Figure 2).

Interestingly, four of the differentially abundant proteins identified by both LFQ and PRM were enzymes. Of these, CP \((\text{L8I5R0, D2U6Z5})\), HBA1 \((\text{Q9TSN7})\) and LL-37 \((\text{A0A0A7V3V9})\) were significantly more abundant in HS buffalo as compared to TN buffalo \((p < 0.05\) for all): HBA1 \((3.76\)-fold increase\), CP \((1.50\)-fold increase\) and LL-37 \((1.84\)-fold increase\) (Figure 3). The remaining enzymes, LPL and GPX3, were significantly less abundant in HS buffalo as compared to TN buffalo \((0.59\)-fold and \(0.35\)-fold decrease, respectively; \(p < 0.05\); Table 1).
2.2. GO, KEGG, and protein-protein interaction

We identified three groups of GO terms that were highly enriched in the 17 differentially expressed proteins identified by LFQ (Table 2). One set of enriched GO terms was associated with redox reactions, lipase activity, hydrolysis, and oxygen transport, while a second set was associated with correct protein folding or the degradation of mis-folded proteins (ubiquitin protein ligase binding, heat shock protein binding, and unfolded protein binding). The third set of enriched GO terms were associated with nitric-oxide synthase regulator activity (i.e., vasodilatation), ferroxidase activity, and oxygen binding.

In parallel studies, we analyzed the correlations among the 17 differentially abundant proteins identified by LFQ, and quantified the metabolites [29]. Only four proteins could be mapped to KEGG metabolic pathways: GPX3, CP, CD14, and LL-37. GPX3 and CP produce glutamic acid; similar amounts of glutamic acid were produced by the HS buffalo (34.2 µM) and the TN buffalo (26.8 µM; $p > 0.05$). CD14 and LL-37 produce acetylcholine; significantly more acetylcholine was produced by the HS buffalo (0.19 nM) than the TN buffalo (0.16 nM; $p < 0.001$). GPX3 was associated with the glutathione metabolism pathway; CP was associated with the porphyrin metabolism pathway; CD14 was associated with the regulation of the actin cytoskeleton; and LL-37 was associated with salivary secretions.

We analyzed the PPI networks of 16 of the 17 differentially abundant proteins identified by LFQ. CD14, CPN1, HBA1, HAVCR1, CHST4, THBS4, SPARCL1 and PLA1A were not included in the network (Figure 4). This network had eight edges,
with an average node degree of 1, and an average local clustering coefficient of 0.479.

The expected number of edges was 2, giving a network enrichment p-value of 0.000221.

In particular, two of the PPIs (LPL and RETN; CP and GPX3) function synergistically to increase thermal tolerance in HS buffalo.

2.3. Validation of LFQ results with PRM

We used PRM to validate the differential abundance of five proteins: CP (L8I5R0 and D2U6Z5), HBA1, LPL, LL-37, and GPX3 (Table 1). Three of these proteins (HBA1, CP, and LL-37) were identified as significantly more abundant in HS buffalo as compared to TN buffalo by both LFQ and PRM (Figure 3). However, although LFQ analysis identified GPX3 as significantly more abundant in HS buffalo, PRM did not identify a significant difference in GPX3 abundance between HS and TN buffalo ($p=0.363$).

2.4. Biochemical blood analysis

HS buffalo had significantly lower levels of MCHC ($p < 0.05$) and MCH ($p < 0.01$) than did TN buffalo, and significantly higher levels of HCT ($p < 0.01$) and MCV ($p < 0.01$; Figure 5). Although HS buffalo had slightly higher levels of RBC, Hb, and PLT, as well as slightly lower levels of WBC, these differences were not significant (Figure 5).

3. Discussion

No significant differences in body temperature were detected between the HS and TN buffalo, indicating that buffalo can maintain normal body temperatures under HS conditions. However, HS buffalo breathed significantly faster than TN buffalo ($p <
improving respiratory evaporative heat loss. In addition to physiological regulation, animals cope with high heat and high humidity primarily through biochemical strategies [30]. In particular, we here identified 17 proteins that were differentially abundant in HS buffalo as compared to TN buffalo. Several of these proteins were associated with functions related to HS. For example, HBA1, which was significantly more abundant in HS buffalo, is involved in heme and iron binding, and transports oxygen from the lungs to various inner organs and peripheral tissues [31]. CP was significantly more abundant in HS buffalo, which plays an important role in iron transport and protects cellular lipids from iron-dependent lipid peroxidation [32-34]. Heat-stressed animals have poor appetites and low nutrient intake, requiring efficient energy usage [35]. Under HS conditions, triglycerides are hydrolyzed by LPL to free fatty acids for supplemental energy [36]. However, triglyceride degradation decreases fatty acid oxidation, resulting in reduced ATP production [37]. This was consistent with our results, as the HS buffalo had significantly higher LPL levels than the TN buffalo.

Under normal condition, ROS (e.g., H$_2$O$_2$ and O$_{2^-}$) play important roles as signal molecules [38]. However, HS triggers excessive ROS accumulation, causing damage to biological macromolecules [39]. As a well-known redox protein and antioxidant enzyme, GPX3 protects cells from oxidative stress by decomposing H$_2$O$_2$ [40,41]. LFQ abundance analysis indicated that HS buffalo had significantly lower levels of serum GPX3 than did TN buffalo, suggesting that the HS buffalo were experiencing HS [42]. Consistent with this result, previous studies of livestock (e.g., finishing broilers, cattle,
reported that HS decreased GPX3 activity. [43-46]. It is worth noting that, although PRM analysis indicated that mean GPX3 levels in HS buffalo were lower than those in TN buffalo, these differences were not significant. This suggested that HS buffalo maintain normal GPX3 levels to protect cell membranes from oxidative damage. In addition, H$_2$O$_2$ inhibits protein refolding and leads to protein denaturation, which is detrimental to cellular structure and function. [47]. Indeed, several GO terms significantly enriched in the differentially abundant proteins are known to promote correct protein folding and degrade mis-folded proteins (i.e., ubiquitin protein ligase binding, heat shock protein binding, and unfolded protein binding). This might imply that buffalo use posttranslational protein processing to handle HS.

Chronic HS impairs immune function in animals, and also increases susceptibility to infection [48]. LL-37, a small cationic antimicrobial peptide produced in the epithelial cells, disrupts the activity of gram-negative bacteria by damaging and destroying bacterial membranes [49,50]. LL-37 plays key roles in inflammatory response regulation and immune mediator induction [51,52]. In heat-stressed mice, LL-37 production was suppressed by the nicotinic acetylcholine system, increasing the susceptibility of the mice Staphylococcus aureus [53]. In HS buffalo, LL-37 was significantly more abundant than in TN buffalo (based on both LFQ and PRM), suggesting that the HS buffalo might also be more susceptible to infection. As LL-37 is important for the immune and inflammatory response [54,55]. it is possible that the higher LL-37 serum levels seen here in HS buffalo might compensate for the relatively low GPX3 levels.
The differential abundant proteins in HS buffalo as compared to the TN buffalo were also significantly enriched in the GO term nitric-oxide synthase regulator activity, which is associated with peripheral vasodilatation [56]. HS buffalo deliver more oxygen per unit volume of blood than cattle [57]. In vasodilation, blood flow to the body surface is increased to enhance heat dissipation, and oxygen transport to internal organs is improved. This was consistent with the high levels of HBA1 and RBC detected in the HS buffalo.

We were unable to identify metabolic (KEGG) pathways enriched in most of the 17 differentially abundant proteins identified by LFQ. However, we successfully mapped four proteins (GPX3, CP, CD14 and LL-37) and their corresponding metabolites to metabolic pathways. As a major neurotransmitter in animals, acetylcholine participates in homoeothermic thermoregulation [58]. Acetylcholine is also known to regulate glutamic acid release, and glutamic acid generates glutamate via pyroglutamic acid [59]. Glutamate and GPX produce glutathione via the glutathione metabolism pathway [60], and GPX3 and glutathione protect the cell from heat injury [61]. Glutamic acid, along with CP, is also involved in the porphyrin metabolism pathway, which increases HBA1 levels to improve oxygen transport in HS animals. LL-37 and acetylcholine were mapped to the salivary secretion pathway. As buffalo, which have few sweat glands [3], dissipate only 12% of their excess heat through skin evaporation [9], salivary secretions have been shown to help decrease body temperature [62,63].

Under HS conditions, high levels of hemoglobin lead to increased oxygen delivery
Based on these pathways and our results, we propose a model of HS adaptation in dairy buffalo (Figure 6): an increase in HBA1 abundance to eliminate ROS and maintain normal oxygen supply, and an increase in LL-37 abundance to enhance host disease resistance. PPI networks provide collaborative working modes for physiological and biological processes at the system-level [65]. We found that most of the 17 differentially abundant proteins in HS buffalo as compared to TN buffalo were linked in the *H. sapiens* database (Fig. 4). CD14, CHST4, HBA1, SPARCL1, CPN1, THBS4, SPARCL1, and PLA1A were not linked to each other, possible because these proteins have dissimilar functions.

It is well known that Hb and CP play a collaborative role in oxygen delivery, as CP oxidizes Fe2+ to Fe3+ without releasing ROS [66]. Indeed, we observed a concomitant increase in Hb and CP in HS buffalo, consistent with a previous study [54]. However, to date, no biological interaction networks have shown a direct relationship between these proteins, although CP was shown to have an inverse linear relationship with soluble transferrin receptor levels [67]. Therefore, we speculated that Hb might have an indirect relationship with CP. That is, these proteins were not linked in the PPI network, but Hb and CP are functionally similar, and their serum levels increased simultaneously. Previous studies have reported that RETN and LPL are important for glucose and lipoprotein metabolism [68], and the PPI between RETN and LPL indicated that the metabolic profiles of glucose and lipoprotein were altered by HS. Moreover, the enzymatic oxidant GPX3 and the nonenzymatic antioxidant CP protect the cell from damage due to oxidative stress [69].
YWHAZ (P63103), YWHAE (P62261; 14-3-3E), and YWHAG (A7Z057) are isoforms of 14-3-3 proteins, which act as molecular chaperones [70]. YWHAZ negatively regulates cell death, and YWHAE facilitates the movement of phosphorylated HSF1 from the nucleus to the cytoplasm, increasing cell thermotolerance. The three 14-3-3 proteins were downregulated in the HS buffalo, suggesting that other proteins may perform these biological functions under HS.

Under HS, homoeothermic animals facilitate heat dissipation by increasing body temperature, respiratory rate, and blood flow to the peripheral tissues [71,72]. The demands on the cardiovascular system also increase with HS, as does arterial blood pressure, causing internal tissues and organs to compete with peripheral areas for blood supply [73,74]. Eventually, low blood flow volume may result in the hypoxia of the internal organs [75]. Indeed, body temperature and blood flow may be closely associated [76]: it was shown that blood flow to internal organs, such as the reproductive organs, decreased 20–40% in heat-stressed rabbits [77].

During HS, RBC decrease [78]. Indeed, HS cattle decreased ~12–20% as compared to TN cattle [79]. Here, the RBC of the HS buffalo was not significantly different from that of the TN buffalo. This might indicate HS buffalo protect RBCs from heat injury. Hb is associated with the delivery of oxygen from the lungs to various other tissues [80]. An increase in serum Hb may compensate for a decrease in RBC and low MCV, enabling the maintenance of normal oxygen supply under chronic HS. It may be that high levels of serum Hb protect the red blood cells from oxidative stress [81,82]. Hb increases have been reported in several other animal species subjected to HS,
including sheep, goats, piglets, ruminants (Holsteins, Jerseys, and Zebus), and tilapia [83-88].

Hb increases may be due to low oxygen pressure and saturation, or to an improved resistance to ROS [89]. In contrast, cooling increased blood Hb concentrations in HS Murrah buffalo [90]. Thus, high Hb levels may alleviate the deleterious effect of HS and hypoxia. However, one study showed that HS decreased blood Hb concentration as compared to normal temperatures [91]. which may be because the acute HS animals in that study were exposed to extremely high temperatures (40 ± 2°C). Here, the chronic HS buffalo had higher serum Hb as compared to TN buffalo. The HS buffalo also had significantly higher concentrations of HBA1 than the TN buffalo, indicating that high HBA1 concentrations may alleviate the tissue and organ hypoxia caused by an increased respiratory rate. Indeed, it has been shown that HBA1 plays an important role in increasing blood flow to peripheral tissues. HS buffalo also had significantly lower levels of MCHC and MCH, as well as significantly higher levels of HCT and MCV. This might indicate that dairy buffalo under chronic HS maintain a normal oxygen supply by modulating crucial blood parameters.

HS animals decrease nutrient intake, leading to a negative energy balance7. Because fatty acid oxidation produces more heat (146 ATP) than does carbohydrate (38 ATP), heat-stressed animals decrease fatty acid oxidation [7,92]. LPL is synthesized at the surface of the vascular endothelium and released into the blood. LPL hydrolyzes circulating triacylglycerides to free fatty acids, promoting fat synthesis while decreasing the production of ATP and heat [93]. In addition to decreasing heat
production, fat deposition is beneficial under HS as fat insulates the body to decrease solar radiant heat absorption [94]. Indeed, low levels of glycerol in the plasma of ruminants were found in summer [95]. However, few studies have focused on serum profiles of LPL abundance. LPL levels in pig adipose tissue increased in response to mild HS [96], as did adipogenesis in porcine adipocytes [97]. LPL activity increased in the plasma and muscle of HS rats, but not in the white adipose tissue [98]. We found that serum LPL abundance decreased in response to chronic HS. We thus speculated that dairy buffalo, unlike monogastric animals, adapt to chronic HS by decreasing fatty acid oxidation to reduce heat production. Based on the HS-related functions of LL-37 (physiological defense response), ceruloplasmin (copper ion binding and ferroxidase activity), and HBA1 (oxygen delivery), as well as the associated metabolites, we proposed a model of the anti-HS adaptive mechanisms used by dairy buffalo to cope with chronic HS (Figure 6).

4. Materials and Methods

4.1. Animals

Animals were treated according to the strictures of the Animal Welfare Act, as issued by Animal Ethics Committee of Yunnan Province, China in 2007. All animals were loose housed, and provided with fresh drinking water ad libitum. Animals were fed mixed rations (80% whole-plant corn silage ad libitum, 12.5% feed concentrate, and 7.5% corn protein powder).

The temperature-humidity index (THI) was calculated based on air temperature and relative humidity. As THI is closely related to the body temperatures of dairy
animals under HS, THI is widely used to assess the degree of HS [99]. THI values ≥72 have been shown to affect dairy cattle [100]. In Dehong, Yunnan province, China (24.43°N, 98.57°E; altitude: 870 m), average THI has been ≥72 between June and August 2005–2015, based on meteorological data. Therefore, six multiparous Nili-Ravi × Murrah × local crossbred female buffaloes (group HS; daily average milk yield 4.5 kg) with similar lactation were used for the heat stress experiment at this site between July 20 and August 4, 2016 (average THI 75.76). We tested another six dairy buffaloes (daily average milk yield 6.1 kg) under thermal-neutral conditions (group TN) between January 3 and January 18, 2017 (average THI 54.26).

4.2. Environmental and physiological parameters

Temperature and humidity loggers (±0.2°C; Testo 175H1), placed 2.0 m above the floor of the bedding area, recorded ambient temperature and relative humidity at 30 min intervals. We used these figures to calculate THI: $\text{THI} = (1.8 \times T + 32) - (0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26)$ [89], where $T$ was the ambient temperature (°C) and RH was the relative humidity (%).

Respiration rates (RR; breaths/min) were measured daily at 08:00, 13:00, and 18:00 h using a stopwatch. A waterproof micro-temperature sensor (±0.5°C; DS1922L, Wdsen Electronic Technology Co., Ltd), fixed in the slot of a T-shaped controlled internal drug release device (without progesterone; DEC International NZ Ltd), was placed in the vagina of each buffalo. This device recorded body temperature at 30 min intervals.

4.3. Depletion of high-abundance proteins from the serum
Blood samples were collected with vacutainer tubes via jugular venipuncture. Each sample was centrifuged at 1400 g for 10 min to separate the serum. Serum samples were stored at -80°C. High-abundance proteins in the serum samples were depleted using affinity chromatographic columns (Agilent Multiple Affinity Removal LC Columns). The low-abundance proteins in the serum samples were isolated by first performing an ultrafiltration concentration, then adding one double volume of SDT buffer (4% SDS, 100 mM Tris-HCl, and 1 mM DTT; pH 7.6) to the concentrated sample. The resultant mixture was then incubated in a boiling water bath for 15 min. The mixture was then centrifuged at 14000 g for 20 min, and the supernatant was removed. We then added 20 µg of the supernatant to 5×loading buffer (10% SDS, 0.5% bromophenol blue, 50% glycerol, 500 mM DTT, and 250 mM TrisHCl; pH 6.8). This mixture was incubated in a 90°C water bath for 5 min, and then analyzed using 12.5% SDS-PAGE (constant 14 mA current for 90 min) with Coomassie blue staining.

4.4. Filter-added sample preparation protein digestion

Trypsin protein digestion was performed using the filter-aided sample preparation (FASP) procedure described previously [101]. In brief, serum samples were lysed, and proteins were extracted using SDT buffer. Proteins were quantified with BCA Protein Assay Kits (Bio-Rad). We then analyzed 20 µg of each extracted protein sample with SDS-PAGE. Next, 100 µg of extracted protein was dissolved in DTT (to generate a final concentration of 10 mM) in a 90°C water bath for 15 min, and then cooled to room temperature. Small molecules and detergent were extracted using 10 kD ultrafiltration with 200 µL UA buffer (8 M Urea and 150 mM Tris-HCl; pH 8.0). The mixture was
centrifuged four times at 14,000 g for 30 min, and the filtrate was discarded. We then added 100 IAA buffer (50 mM IAA in UA buffer) to the concentrate. Prior to centrifugation, this mixture was shaken at 600 rpm for 1 min, and then incubated for 30 min in the dark. After incubation, the mixture was centrifuged at 14000 g for 20 min. The filter was washed three times with 100 µL of UA buffer and centrifuged at 14000 g for 20 min. Then, the filter was washed twice with 100 µL of 50 mM NH4HCO3 buffer. Finally, the protein suspension was added to 40 µL of NH4HCO3 buffer (2 µg Lys-C) and incubated in a 37°C water bath for 4 h. We added 2 µg trypsin to the protein mixture, and then incubated the mixture in a 37°C water bath for 16 h. After incubation, the protein mixture was transferred to a fresh reaction tube, and centrifuged at 14000 g for 15 min. Finally, the filter was washed with 40 µL of 50 mM NH4HCO3 buffer, and centrifuged at 14000 g for 30 min. The filtrate was collected and desalinated with a C18 Cartridge (Empore SPE Cartridges C18 [standard density], Sigma; bed inner diameter 7 mm; volume 3 ml). Desalted samples were freeze-dried, and peptide levels were determined at OD280. Peptide samples were then stored at −80°C.

4.5. LFQ with LC-MS/MS analysis

We performed relative LFQ with LC-MS/MS. LC-MS/MS was performed using a Q-Ex active mass spectrometer (Thermo Scientific) coupled to an Easy nLC 1200 (Proxeon Biosystems, now Thermo Fisher Scientific) with an analysis time of 60 min. First, peptides were loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100; 100 µm × 2 cm; nanoViper C18) connected to a C18-reversed phase analytical column (Thermo Scientific Easy Column; 10 cm long, 75 µm inner
diameter, 3 µm resin) in buffer A (0.1% formic acid). Peptides were separated using a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nl/min, controlled by an IntelliFlow technology. The linear gradient in the trap column was as follows: 0–35% solution B from 0–50min; 35–100% solution B from 50–55min; 40–100% solution B from 55–60min; and hold at 100% solution B.

MS raw data for all samples were combined and searched using MaxQuant v1.3.0.5 [102], which uses the Andromeda search engine against the Bovinae Uniprot database [103]. The precursor mass tolerance was set to 20 ppm for the first Andromeda search, and to 6 ppm for the main search. Carbamidomethylation (C) was considered a fixed modification, while oxidation (M) and acetylation (of the protein N-terminus) were considered variable modifications. We eliminated all peptide and protein identifications where the false discovery rate (FDR) was >0.01 against the reversed-sequence database.

We used the MaxLFQ approach in MaxQuant for label-free quantification [104], enabling ‘match between runs’ with a retention time window of 2 min, and setting the LFQ minimum ratio count to 1. Using the relative abundance of proteins in TN buffalo as a reference, we identified HS-buffalo proteins as significantly differentially expressed if the HS protein was identified in at least two of three replicates with a fold change >1.5 and a $p < 0.05$. We used volcano plots to visualize the differentially expressed proteins. We then performed a hierarchical clustering analysis of the proteins differentially expressed between the HS and TN buffalo using the log2-transformed expression values and a cutoff of $p < 0.05$ in Treeview (Java Runtime Environment).
4.6. Absolute quantification of targeted proteins with PRM

The Easy nLC 1200 (Thermo Scientific) was used for chromatographic separation. First, peptides were separated with a binary solvent system consisting of 0.1% formic acid (solvent A) and 0.1% formic acid acetonitrile (84% acetonitrile) (solvent B). We used 95% solvent A as the equilibrium liquid for the analytical column (ThermoScientific EASY column). We added 20 fmol of a standard peptide to 2 μg of the separated peptides. The peptide mixture was then separated at a flow rate of 250 nl/min, with a linear gradient in the trap column (5–23% solution B from 0–42 min; 23–40% solution B from 42–50 min; 40–100% solution B from 50–60 min; and hold at 100% solution B).

We only selected proteins for PRM validation that were significantly more abundant and closely associated with heat stress adaptation. We examined the peptides eluted by the nano-high-performance LC using PRM positive ion mass spectrometry on a Q-Ex active HF mass spectrometer system (Thermo Scientific). MS spectra were acquired in the m/z range 300–1800, with an R of 60000 at m/z 200. Automatic gain control (AGC) was set to 3e6, with a max injection time of 200 ms. We then selected 20 PRM/MS2 scans for higher-energy collisional dissociation experiments that conformed to the following parameters: 1.6 Th isolation window; R of 30,000 at m/z 200; 3e6 AGC; 120 ms maximum injection time; MS2 higher-energy collisionally activated dissociation (HCD); and a normalized collision energy of 27.

We analyzed the PRM raw data from the 12 HS and TN samples with Skyline.
v3.5.0 [106]. The peptide settings used for the Skyline import were consistent with the MaxQuant search parameters (i.e., enzyme set to trypsin; max missed cleavages set to 2). For each set of Skyline analyses, we selected two or three consecutive high-intensity peptides for import into Skyline. Skyline was used to determine the number of targeted proteins, the sequences of the targeted peptides, the charges of the parent ions, and the peak areas. Total peak areas were calibrated against internal standard peptides, heavy isotopes (absolute quantification) [94]. Based on these total peak areas, we quantified each target peptide.

4.7. Bioinformatics analysis

We identified protein sequences homologous to those of the selected differentially expressed proteins with the NCBI BLAST+ client (ncbi-blast-2.2.28+-win32.exe) and InterProScan [107,108]. We then annotated protein sequences using gene ontology (GO) terms with Blast2GO [109,110]. The GO annotation results were plotted with R scripts. Following annotation, the differentially expressed protein sequences were blasted against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) to identify KEGG orthology. Differentially expressed proteins were subsequently mapped to KEGG pathways [111].

Finally, we constructed a functional protein interaction network for our differentially expressed proteins using STRING (https://string-db.org/), searching by multiple protein names. As few protein–protein interactions (PPI) are yet available in the Bos taurus database of STRING, we searched for these interactions in the Homo sapiens database instead.
4.8. Biochemical parameters

We measured hemoglobin level (Hb), red blood cell count (RBC), white blood cell count (WBC), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), platelet hematocrit (PLT), and mean corpuscular volume (MCV) in the whole blood samples using a fully automated biochemical analyzer (Sysmex XT-1800i) within 24 h of blood collection.

5. Conclusions

Our results indicated that, in buffalo, pathways associated with oxidation-reduction, hydrolysis, protein-mis-folding repair, and oxygen transport were activated by chronic HS, and that LPL, GPX3, LL-37, CP, and HBA1 played cooperative roles to mitigate the effects of chronic HS. Our results, along with our newly developed model, increase our understanding of the adaptation to heat stress in dairy buffalo. Our results will thus help to support the vigorous development of the buffalo dairy industry in tropical and subtropical regions.

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Figure 1. Quantitative proteomic comparison of serum samples of heat-stressed and normal-temperature buffalo. (a) Principal components analysis (PCA). (b) Volcano plot representation of moderated t-test analysis. Each point represents one protein. Fold change calculated based on the average of six samples. The horizontal bar represents a q-value cutoff of 0.01. Significantly more abundant proteins in the heat-stress buffalo are shown in red; significantly less abundant proteins are shown in green. The significantly more abundant proteins were verified with parallel reaction monitoring analysis.

Figure 2. Hierarchical clustering of the proteins identified as differentially abundant in the heat-stressed buffalo as compared to the normal-temperature buffalo with label-free quantification analysis. Proteins are in rows; protein details are given in Table 1. Colors indicate level of abundance. HS1–HS6: heat-stress buffalo 1–6; TN1–6: normal-temperature buffalo 1–6. Asterisks indicate proteins that are significantly differently abundant between groups: *p < 0.05, **p < 0.01.

Figure 3. Parallel reaction monitoring verification of five of the proteins indicated to be significantly differentially abundant by label-free quantification. HS: heat-stressed buffalo; TN: normal-temperature buffalo. Bars represent means (n = 6) ± standard error. Asterisks indicate significant differences in abundance between the two groups: *p < 0.05, **p < 0.01. L8I5R0 and D2U6Z5 represent CP. For full protein names, please refer to Table 1.

Figure 4. Protein–protein interaction network including the proteins differentially abundant in heat-stressed buffalo as compared to normal-temperature buffalo. Blue
edges indicate co-occurrence evidence; purple edges indicate experimental evidence;
yellow edges indicate text-mining evidence; light blue edges indicate database evidence;
and black edges indicate co-expression evidence. For full protein names, please refer
to Table 1.

**Figure 5.** Blood parameters of heat-stressed (black bars) and normal-temperature (gray
bars) buffalo. WBC: white blood cells \((\times 10^9)\); RBC: red blood cells \((\times 10^{12})\); MCHC:
mean corpuscular hemoglobin concentration (g/L); MCV: mean corpuscular volume
(fL); Hb: hemoglobin (g/L); MCH: mean corpuscular volume (pg); PLT: platelet
hematocrit \((\times 10^9)\). Bars represent means \((n = 6) \pm \text{standard error. Asterisks indicate}
significant differences in abundance between the two groups: \(*p < 0.05\), \(***p < 0.001\).

**Figure 6.** A proposed model of heat stress adaptation in dairy buffalo. Proteins and
metabolites in red or blue are significantly more or less abundant in heat-stressed
buffalo as compared to normal-temperature buffalo.
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**Table 1. Proteins Differentially Abundant in Heat-stressed Buffalo as Compared**
to Normal-temperature Buffalo, as Indicated by Label-free Quantitation

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein symbol</th>
<th>Protein name</th>
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<th>P</th>
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<tbody>
<tr>
<td>Q9TSN7</td>
<td>HBA1</td>
<td>Hemoglobin subunit alpha1</td>
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<tr>
<td>L8I5R0</td>
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<td>A0A0A7V3V9</td>
<td>CAMP (LL-37)</td>
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<tr>
<td>D2U6Z5</td>
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<td>YWHAZ</td>
<td>YWHAZ</td>
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<tr>
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Table 2. Gene Ontology (GO) Analysis of the Proteins Differentially Abundant in Heat-stressed Buffalo as Compared to Normal-temperature Buffalo

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<th>Proteins associated</th>
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<tbody>
<tr>
<td>GO:0004602</td>
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</tr>
<tr>
<td>GO:0016787</td>
<td>Hydrolase activity</td>
<td>E1ACW2, G5E5V0, L8HYL8</td>
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<tr>
<td>GO:0019825</td>
<td>Oxygen binding</td>
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<td>GO:0016491</td>
<td>Oxidoreductase activity</td>
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<td></td>
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<tr>
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<td>L8IAK0</td>
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<tr>
<td>GO:0051082</td>
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</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.