Proteomic Study of Hypothalamus in Pigs Exposed to Heat Stress

CURRENT STATUS: UNDER REVIEW

Tianyue Yu
Guangdong Ocean University

ORCiD: https://orcid.org/0000-0001-6834-3761

Yan-Hong Yong
Guangdong Ocean University

Jun-yu Li
Guangdong Ocean University

Biao Fang
Guangdong Ocean University

Can-ying Hu
Guangdong Ocean University

Lian-yun Wu
Guangdong Ocean University

Ravi Gooneratne
Lincoln University Faculty of Agriculture and life science

Yadnyavalkya Patil
Lincoln University Faculty of Agriculture Horticulture Viticulture

Xianghong Ju
Guangdong Ocean University

Corresponding Author:
juxh77@163.com

DOI:
10.21203/rs.2.17191/v1

SUBJECT AREAS
Large Animal Medicine

KEYWORDS
Pigs, Heat stress, Hypothalamus, Quantitative proteomics
Abstract

Background: With evidence of warming climates, it is important to understand the effects of heat stress in farm animals in order to minimize production losses. Study of changes in the brain proteome induced by heat stress may aid in understanding how heat stress impacts brain function. The hypothalamus is a key region in the brain that controls the pituitary gland, which is responsible for the secretion of several important hormones.

Result: In this study, we examined the hypothalamic protein profile of ten pigs (30–40 kg body weight), 5 of which were subjected to heat stress (35 ± 1°C; relative humidity = 90%) and 5 acting as controls (28 ± 3°C; RH = 90%). The isobaric tags for relative and absolute quantification (iTRAQ) analysis of the hypothalamus identified 1710 peptides corresponding to 360 proteins, and 295 differentially expressed proteins (DEPs), 148 of which were up-regulated and 147 down-regulated in heat-stressed animals. Ingenuity Pathway Analysis software predicted 30 canonical pathways, 4 functional groups, and 4 regulatory networks of interest and the DEPs mainly concentrated in the cytoskeleton of the pig hypothalamus during heat stress.

Conclusions: The upstream regulators of these 295 DEPs in the hypothalamus of the pig under HS are mainly transcriptional regulators, chemical drugs, and sRNA. This study provides reference data for further study of the mechanism of HS on hypothalamic physiology and metabolism (Illustration 1).

Background

Pigs are homeotherm with under-developed sweat glands. Therefore, at high temperature, pigs experience stress more than other species [1]. Heat stress (HS) affects pigs markedly including changing the metabolism of several organ systems [2], deposition of fat [3], disruption to energy balance [4], and lowering of meat quality [5]. Bell [1993] reported that during HS, the barrier function of the intestinal mucosa may be compromised due to increased hypoxia and formation of free radicals in pig visceral organs[6]. Ju et al. [2009] reported that HS alters immune and biochemical indexes leading to metabolic and endocrine disorders in Bama miniature pigs[7]. It is increasingly important to explain the role of immune system function in the mechanism of stress regulation and the susceptibility of livestock to disease, due to the emerging animal diseases are mostly zoonotic and
they also threaten public health.

The hypothalamic–pituitary–adrenal axis is the most important pathway in the physiological control of stress [8]. Its activation begins with the secretion of corticotropin-releasing hormone in the hypothalamic paraventricular nucleus, which promotes the release of pituitary adrenocorticotropic hormone, which in turn releases glucocorticoids from the adrenal cortex. [9]. Soria et al. [2012] reported that sodium tungstate plays an important role in controlling energy metabolism in adult mice orally treated with this sodium salt and that it could modulate hypothalamic plasticity and networks [10]. Sutherland et al. [2006] observed a decrease in levels of plasma cortisol (a major glucocorticoid) in pigs exposed to HS for 21 days [11]. Kataria and Kataria [2013] detected significantly higher levels of serum prolactin and cortisol in pigs exposed to a higher temperature (45°-46°C)[12]. In cattle, cortisol increases in the early stages of HS but subsequently returns to normal levels [13]. So, the objective of our study was to examine the function and interaction of differentially expressed proteins (DEPs) in the hypothalamus of pigs subjected to HS. This would provide the basis for further study of the mechanism of HS effects on the metabolism and physiological function of the hypothalamus. iTRAQ technology is a comparative proteomic science developed in recent years [14]. It has been used to describe proteomic analysis of the hypothalamic response during sleep regulation [15], feeding patterns [16], microgravity conditions [17], anesthesia [18], exposure to environmental toxicants [19], and during reproductive cycles [20]. In previous studies, when the ambient temperature was maintained at 35 ± 1 °C and relative humidity 90%, the body temperature of the pig was increased 0.5 °C to 1 °C and the heart and respiratory rates were increased ~1.2-fold and 2.3-fold[21]. In this study, we used iTRAQ technology to study proteomic changes in the hypothalamus of pigs exposed to HS for 7 days to determine the mechanism of immune regulation and to provide a theoretical basis for the control and prevention of HS.

Results
Identification and Quantitative Analysis of DEPs
Using iTRAQ technology, 7072 total, 4095 spectra and 2882 unique spectra were identified, 1710 peptides and 1451 unique peptides were matched, and 360 total proteins were identified (Fig. 1A).
Among all the proteins, according to the level of protein abundance, when the difference multiple reached 1.5 or more, and the statistical test showed that P < 0.05, it was regarded as a differential protein. Thus, there were 295 DEPs identified in the hypothalamus, of which 148 were up-regulated and 147 were down-regulated (Table 1).

Most DEPs have molecular weights in the range of 20–60 kD (157 DEP) (Figure 1B). In addition, the identified DEPs had a higher polypeptide coverage, of which 80% and 54% showed more than 10% and 20% sequence coverage, respectively (Fig. 1C). About 74% of the identified DEPs had 3 or more peptides (Fig. 1D).

Subcellular Localization, Functional Characteristics and Bioinformatics Analysis of DEPs

To elucidate the functional characteristics of DEPs in the hypothalamus under HS, the DEPs of hypothalamic tissues were analyzed based on the basic biological functions of proteins in the UniProtKB / Swiss-Prot, TrEMBL protein database and Gene Ontology database, clustering of molecular functions, and cell location in which the proteins are located. A total of 295 DEPs were identified in the hypothalamus of pigs in HS. The location proteins are shown in Fig. 2A. And to better understand these 295 DEPs, further analysis was undertaken using the Ingenuity Pathways Analysis (IPA) tool. Canonical pathways were examined first, and the top 30 pathways are shown in Fig. 2B. Compared with the human genome database, annotation in the pig genome database is relatively scarce, and many protein features are not identified or classified. The differential proteins identified in our study were converted to the human protein gene bank identification (gi) number. The gi numbers and regulatory levels of these proteins were entered into the IPA software, and based on the database, protein–protein interaction signal pathways were constructed. The proteins identified by iTRAQ in the hypothalamus were clustered according to different functions and it found 4 statistically significant functional groups, namely, diseases and disorders, molecular and cellular functions, physiological and phylogenetic functions, and toxicological functions (Fig. 3).

The 295 DEPs identified in the hypothalamus of pigs under HS were consistent, which corresponded to 23 diseases and disorders including neurological disease, psychological disorders, skeletal and
muscular disorders, hereditary disorder, metabolic disease, dermatological disease and conditions, hematological disease, immunological disease, and inflammatory diseases, among others (Fig. 3A). These DEPs can also be assigned to 27 molecular and cellular functions groups, including cell death and survival, cell assembly and organization, cell function and maintenance, molecular transport, free radical scavenging, cell morphology, small molecule biochemistry, nucleic acid metabolism, lipid metabolism, cellular development (Fig. 3B); 16 physiological system development and functions groups, including nervous system development and function, tissue morphology, organ morphology, organismal development, tissue development, behavior, organismal survival, embryonic development, organismal function, hematological system development and function, immune cell trafficking, organ development (Fig. 3C); and 12 toxicity functions groups, namely, liver hyperplasia/hyper-proliferation, renal damage, renal tubule injury, cardiac necrosis/cell death, kidney failure, cardiac arrhythmia, cardiac damage, cardiac inflammation, renal dilation, cardiac dysfunction, liver fibrosis (Fig. 3D).

Among the DEPs identified in the hypothalamus, 13 functional networks were constructed (Fig. 4). The 4 networks of interest correspond to (1) Cell assembly and organization, neural development and function, intercellular signal and interaction (Fig. 4A); (2) Nucleic acid metabolism, small molecular biochemistry and cell morphology (Fig. 4B); (3) Cell assembly and organization, cell function and maintenance and neuropathic disease (Fig. 4C); (4) Free radical scavenging, small molecule biochemistry and cancer (Fig. 4D). In our analysis, proteins with DEP present in the pathway and identified as up-regulated were shown in red shades, while proteins identified as down-regulated were shown in green. Proteins in the network that were not recognized as DEP are marked in white. Predictors of upstream regulators of hypothalamic DEPs identified as transcriptional regulators, chemicals, chemical poisons, kinases, cytokines, endogenous mammalian compounds and mature small RNA were activated. Growth hormones, kinases, G-protein coupled receptors, transcriptional regulators and compounds were inhibited.

Validation of Protein Identification and Quantification
Histone H2A is a type of innate immune molecule discovered in recent years that plays a key role in
the phagocytosis of neutrophils and in the clearance of pathogenic microorganisms. To verify the reliability of DEPs identified by iTRAQ, the hypothalamus of pigs on day 7 of HS was used to detect the expression of Histone H2A by Western blot. As shown in fig. 5, the ratios of HS to control group Histone H2A hypothalami were consistent with those obtained using the iTRAQ technique.

Discussion
The body’s stress system consists of the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system and adrenal medulla. The hypothalamus is a vital subcortical center and plays a crucial role in maintaining homeostasis in the body. The hypothalamus is the body’s temperature regulation center, but is also involved in the balance of water and salt, blood pressure stability, reproductive function integration and regulation of a variety of stress responses. Since the hypothalamus plays such a major role in temperature regulation, in HS pigs it is important to study the hypothalamus and particularly its regulation of DEPs. Because when stressors stimulate, endocrine factors such as glucocorticoids and catecholamines can cause immune cell apoptosis, lymphocyte proliferation damage and dendritic cell dysfunction [22, 23], thereby inducing immunosuppression in animals. In this study, iTRAQ technology enabled the identification of 295 DEPs from the hypothalami of pigs under HS and IPA analysis software analyzed cell location, molecular function, regulatory pathways, regulatory networks and upstream regulators, which laid a foundation for systematically revealing the regulatory mechanism of HS and the mechanism of stress-induced immunosuppression.

Tubulin is related to the structure of neurons and participates in the process of synapse formation. It is a molecular marker of synaptic plasticity. Sodium tungstate, an anti-obesity drug, causes abnormal changes in the hypothalamus tubulin, internexin alpha and pyruvate dehydrogenase. The functions of these proteins involve axonal growth, neuronal plasticity, cytoskeleton and neuronal structure. Mouse studies indicate that sodium tungstate treatment regulates the structure and function of hypothalamic circuits and thus regulates changes in ingestion centers while suppressing small mouse appetite [10]. In addition to the down-regulation of tubulin-α, tubulin-β and tubulin polymerization promoting family members P25, dihydrolipoyl lysine-residue acetyltransferase and neuronal
intermediate silk protein were also down-regulated in HS. Activation of actin-associated proteins is thought to be necessary for cytoskeletal plasticity and cell repair, whereas actin itself regulates highly dynamic postsynaptic site structures, especially the dendritic spine [24]. Hypothalamic actin is also significantly up-regulated in HS. This shows that adaptive changes in hypothalamic neural circuits and synaptic plasticity are caused by HS.

The heat shock protein (HSP) response is a highly conserved cellular response to outward stress in all species. HSPs include heat shock protein 27 (HSP27) and take part in the processing such as antigen presentation, intracellular transport and apoptosis, it acts as a molecular chaperone by assisting new peptides to assume their proper conformation [25]. HSP27 is a multidimensional protein that acts as a protein chaperone and antioxidant and it plays a role in the inhibition of apoptosis and actin cytoskeletal remodeling [26]. Fujita et al. (2004) found that HSP27 phosphorylation could maintain actin stability, thereby stabilizing the cytoskeleton and inhibiting apoptosis to some extent[27]. In this study, HSP27 was up-regulated in porcine hypothalamus tissue under HS (Table 1), further indicating the role of HSP27 in hypothalamic injury and highlighting its association with inflammatory responses. The 17β-hydroxysteroid dehydrogenases (17β-HSD) family play an important role in lipid metabolism. The 17β-HSD are a group of alcohol oxido-reductases that catalyze the reduction of 17-ketosteroids and the dehydrogenation of 17β-hydroxysteroids in steroidogenesis and steroid metabolism [28, 29]. Su et al. [2014] reported that adenovirus-mediated over-expression of human 17β-HSD13 induced fatty liver phenotype in C57BL/6 mice, with significant increase levels of mature sterol regulatory element-binding protein 1 and fatty acid synthase[30]. And increased hepatic 17β-HSD13 levels were confirmed in dB/db (diabetic) and high-fat-diet-fed mice. 17-βHSD can catalyze the dehydrogenation of 17-hydroxysteroid to participate in the synthesis and secretion of steroid hormones. The expression of 17-βHSD in the hypothalamus of pigs under HS is up-regulated significantly (Table 1), suggesting abnormal metabolism of steroid hormones in the hypothalamus of pigs in HS.

In the proteomics study of cattle, histone 2A1 was thought to be a new innate immune molecule that was down-regulated in immunosuppressed neutrophils in cattle [13]. When neutrophils exert a killing effect, they release a series of DNA, histone and antibacterial peptides and form the “traps” for
The expression of histone 2A1 in the hypothalamus of pigs in HS was up-regulated (Table 1). IPA analysis indicates that it plays a role in certain disease processes such as cancer, infectious diseases, and diseases of the reproductive system, liver and immune system. Histone H2A plays an important role in DNA folding, and is also a conserved cationic protein of eukaryotes, which is involved in the antibacterial activity of cells. Either in eukaryotes or prokaryotes, its variants take the form of antimicrobial peptides involved in the body’s immune response. The molecule composed of the alpha helix has both hydrophilic and hydrophobic ends, which enhances its antibacterial activity [33]. So, in HS, whether the change of H2A is related to immunosuppression requires further study.

Annexins (ANXA) are calcium-dependent phospholipid binding proteins and they are widespread in eukaryotes. Shortly after apoptosis induction, phosphatidylserine (PS) can be transferred from the inside of the cell membrane to the outside. In the presence of calcium ions, ANXA5 has a high affinity for PS and can specifically bind to exposed PS. Therefore, ANXA5 can specifically recognize apoptotic cells and serve as a new molecular probe for detecting apoptosis. ANXA5 is one of the most widely distributed and largest members of the family and is involved in many important physiological processes, such as anti-coagulation, calcium channel activity and protein kinase inhibition. It is also closely related to inflammation and cellular stress [34, 35]. In HS, ANXA2, 4, 5, 6 and 11 were significantly up-regulated in the hypothalamus of pigs (Table 1). At the same time, they are involved in function and maintenance of cell, cell-cell communication and interaction, cell survival and death, cell migration and neurological diseases.

Thirteen proteins, including Ulip2, STX1, synapse-associated proteins and glial fibrillary acidic protein, show obvious changes in the hypothalamus of heat-stroke rats [36], and most of these proteins were also identified in the hypothalamus of pigs in HS. In our study, we hypothesize that HS and heat stroke may have similar regulatory processes in the central nervous system. Other studies have shown that sleep interruptions can also cause stress responses in animals. These proteins are mainly related to metabolism: they catalyze the transfer of carboxylesterase and aldehyde dehydrogenase, and are involved in aminobutyric acid dehydrogenation, serine conversion and ATP (adenosine...
triphosphate) and NADH (nicotinamide adenine dinucleotide) dehydrogenase synthesis. The formation of this ATP may be beneficial to the metabolic process of these pathways. In the hypothalamus of pigs subjected to HS, carboxylesterase and acetaldehyde dehydrogenase families 1, 2, 4, 6 and 9 were significantly increased, and ATP synthesis was also induced. These molecules play an important role in biological processes such as cell survival, molecular transfer, cell migration, tissue damage and abnormalities, organ morphology, tissue development, free radical scavenging, and nervous system development and function. Different stressors may induce similar adaptive physiological processes to maintain homeostasis.

Conclusion
In conclusion, using iTRAQ technology, 295 DEPs were identified in the hypothalamus of the pigs under HS, 148 of which were up-regulated and 147 down-regulated. These DEPs were involved in 23 diseases and disorders, and 13 functional networks could be plotted. Moreover, the upstream regulators of these 295 DEPs are mainly transcriptional regulators, chemical drugs, and small RNA.

Methods
Pigs feeding and researching were followed in accordance with the National Institutes of Health (NIH) guidelines for the arrange and study of laboratory animals, and all projects have been examined and approved by the Animal Care and Use Committee of Guangdong Ocean University, China.

Experimental Animals and Groups
Ten Bama miniature pigs, weighing about 30-40 kg, were purchased from Bama miniature pig breeding farm in Guangxi Zhuang Autonomous Region of China, and randomly divided into HS group (n = 5) and control group (n = 5). The air temperature of the control pigs was 28 ± 3 °C, while the temperature of the HS group was maintained at 35 ± 1 °C. The relative humidity for both groups was 90%. All pigs had free access to water. Their diet was based on the recommended nutrients for this breed and pig age.

Hypothalamus Collection
After 7 days of heat stress treatment, pigs were euthanized by only using corona pliers on the head and by artificial bloodletting. Immediately after slaughter, the hypothalamus tissue was removed and
weighed. Subsequently, the tissues were washed with PBS\(\text{PH}=7.4\) to remove any blood and contaminants from the tissue surface. The hypothalamic tissue was placed in sterile tubes and quickly frozen in liquid nitrogen. Three pigs were used as control group and five pigs in the heat-stress group. After entering the laboratory, the frozen specimens were stored at -80 °C prior to biochemical and molecular analysis.

**Protein Extraction**

Frozen hypothalamus tissue from control and heat stress were immersed into liquid nitrogen and ground into fine powder by use a mortar. The powder (~ 100 mg per pig) was homogenized with RIPA buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 1mM PMSF, 1mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 2% SDS). Then the tissue was homogenized with an Sonic Cell Disruptor (VCX130, USA) at 20% power output for 10 min with 2-s-on and 4-s-off cycles. The homogenates were held on ice for 30 min and centrifuged at 25,000 \(\times\) g for 30 min at 4°C, and the supernatant was stored at −80°C for further analysis. The protein concentration was measured by Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, MA, USA).

**iTRAQ Labeling and Strong Cation Exchange (SCX) Fractionation**

According to Wisniewski et al. [2009] [37], protein digestion was performed based on previous protocols described and according to the manufacturer’s instructions, the resulting peptide mixture was labeled using the iTRAQ Reagent-4plex Multiplex Kit (AB SCIEX, Framingham, MA, USA). According to the manufacturer’s instructions, 100 \(\mu\)g of control sample and heat stress samples were labeled with Traq Reagent-4plex multiplex kit (AB SCIEX), respectively. The control samples were labeled with reagent 116; The experimental samples (high temperature) were labeled with reagent 117. Other two separate biological experiments were labeled with the same tab. Three independent biological experiments were performed. The labeling solution reaction was incubated for 1 hour at room temperature before further analysis. Then, labeled peptides were combined and separated by cation exchange (SCX) chromatography [38] and desalted on a C18 Column (66872-U; Sigma, St. Louis, MO, USA). The dried peptide mixture was reconstituted and acidified with 2 mL of buffer A (pH 3.0, 10 mM KH 2 PO 4 in 25% of CAN) and loaded onto a 4.6 \(\times\) 100 mm polysulphoethyl column (5
The peptides were eluted with a gradient of 0%-5% buffer B (pH 2.7, 2 m KCl, 10 mm KH2PO4 in 25% of acetonitrile) at a flow rate of 1 mL/min for 5 min, 5-10% buffer B for 10-15 min, 10%-30% buffer B for 25-35 min, and 30%-50% buffer B for 35-50 min. The elution was monitored by absorbance at 214 nm and fractions were collected each minute. Finally, the collected fractions (about 30 fractions) were finally combined into 10 wells and desalted on a C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), inner diameter 7 mm bed I.D., 3 ml volume, Sigma). Using vacuum centrifugation to concentrate each fraction and reconstituted in 40 µl of 0.1% (v/v) trifluoroacetic acid. Before nanoLC-MS/MS analysis, all samples were stored at −80 °C.

**LC–MS/MS Analysis**

The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 µm × 2 cm, nanoViper C18) connected to the C18-reverse phase analytical column(Thermo Scientific Easy Column, 10 cm long, 75 µm inner diameter, 3 µm resin) in buffer A(0.1% formic acid) and control the flow velocity of 300 nL/min by IntelliFlow technology separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid). The peptides eluted by high performance liquid chromatography was directly poured into the Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA). The Data was acquired in a positive ion mode and the selected mass range is 300-1800 mass/charge (m/z). The Q-Exactive survey scans achieved resolutions of 70,000 (m/z 200) and 17,500 (m/z 200), respectively, and the resolution and maximum ion implantation time of the high-energy collision dissociation spectra were fixed at 20 and 60 MS, respectively. Dynamic exclusion (40.0 s duration) was used. MS/MS data were collected using the top 10 most abundant precursor ions. The normalized collision energy was 30 EV and the bottom filling rate was defined as 0.1%. The instrument was run with the peptide recognition mode enabled.

**iTRAQ data analysis**

The protein identifications were performed using the MASCOT engine (version 2.3.02; Matrix Science, London, UK) embedded in Proteome Discoverer 1.4 (Thermo Fisher Scientific). The search parameters as listed: (1) database, Uniprot; (2) taxonomy, Homo sapiens; (3) enzyme, trypsin; (4) fixed modifications, carbamidomethyl of C, iTRAQ 4plex(N-term), iTRAQ 4plex(K); (5) variable modifications,
oxidation of M; (6) max missed cleavages, 2; (7) peptide charges state, +2, +3, and + 4; (8) peptide mass tolerance, 20 ppm; (9) mass/mass tolerance, ±0.05 Da. The differentially expressed proteins were defined as those that changed twice between the two groups. The data analysis and graphics creation was supported by Wayen Biotechnologies Co., Ltd. (Shanghai, China).

**Bioinformatics Analysis**

According to Gene Ontology, published reports and databases such as Uniprot and TrEMBL, the Go ID of tightly regulated proteins and some unaltered proteins was introduced into Ingenuity Pathway Analysis software (IPA, www.ingenuity.com) for bioinformatics Learning analysis. The typical pathways and protein interaction networks of DEPs were analyzed.

**Western Blot Analysis**

Pig hypothalamus samples from both control and HS groups were homogenized in Radio-Immunoprecipitation Analysis buffer (Thermo Fisher Scientific, MA, USA) and then sonicated on ice. The homogenate was incubated on ice for 30 min and centrifuged at 12,000×g for 15 min at 4°C. The supernatant was collected and the total protein concentration was measured by the BCA assay. Next, protein denaturation was carried out 100°C for 10 min. After the proteins were separated on SDS-PAGE gels, transferred it to PVDF membranes and then blocked in 5% non-fat milk/TBST solution at room temperature for 2 h. The membrane was incubated overnight at 4°C in Western blotting diluent containing primary antibodies: histone H2A (1:1000, Cell Signaling Technology) and GAPDH (Cell Signaling Technology) (1:1000). After washing them 4 times with TBST, the membrane was incubated for 1 h at room temperature in a blocking solution containing anti-rabbit secondary antibody (1:1000). Blots were visualized using ECL detection system and proteins were quantified using ChemiDoc XRS + image analyzer (Bio-Rad, Hercules, CA, USA). The expression level of the target protein was evaluated using densitometry. The ratio of the target protein of the control group and the HS group to the optical density measurement of GAPDH was compared. The tests were repeated three times and the statistical significance of the results data was evaluated by Student's t-test.

**Statistical Analysis**

Statistical analysis used by SPSS Statistics 23.0. Protein expression differences analysis between the
HA group and the CA group were performed using the T-test, and p < 0.05 was taken to indicate statistical significance.

**Abbreviations**

17β-HSD: 17β-Hydroxysteroid dehydrogenases

ANXA: Annexins

DEPs: Differentially expressed proteins

HS: Heat stress

HSP: Heat shock protein

IPA: Ingenuity Pathway Analysis

iTRAQ: Isobaric tags for relative and absolute quantification

LC-MS/MS: Mass spectrometry

**Declarations**

**Acknowledgement**

We acknowledge Prof. Dr. Ju’s team at the Stress student laboratory, Department of Veterinary Medicine, Guangdong Ocean University for all of their assistance with this work.

**Authors’ contributions**

TY conceived the project, designed the tests, prepared samples for the mass spectrometry and analyzed the data, carried out the experimental work, created the graphics and wrote the manuscript.

YY participated in a streamlined analysis, created the pathway figure to assist in reviewing and modifying the manuscript. JL, BF, LW, CH and YP were responsible for animal care and participated in the writing and revision of the manuscript. RG aided in conceptual framework of the study and revised the manuscript. XJ provided financial support, designed the experiments and modified the manuscript. All the authors confirmed the final version of the manuscript.

**Funding**

This work was supported by the National Natural Science Foundation (NSFC) of China [grant nos. 31101862, 31472243] and Shenzhen Projects for Basic Research [JCYJ20170306162414058]. The funding bodies had no role in research design, data collection, analysis and interpretation, and
manuscript writing.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

The protocol was approved by the Committee on the Ethics of Animal Experiments of the Guangdong Ocean University (Permit No.: 201-1231). All surgeries were performed under sodium pentobarbital anaesthesia, and every effort was made to minimize suffering.

**Consent for publication**

Not applicable

**Competing interests**

The authors have no conflicts of interest to declare.

**Author details**

1. Department of Veterinary Medicine, Guangdong Ocean University, Zhanjiang 524088, China

2. Department of Animal Science, Guangdong Ocean University, Zhanjiang 524088, China

3. Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, New Zealand

4. Shenzhen Institute of Guangdong Ocean University, Shenzhen 518018, China

**References**

1. Xiong Y, Xianyong MA, Zheng C, Tian Z, Zhang J, Chen W, Youjun HU, Wang L: *Effects of Heat Stress on Intestinal Health, Immune System and Meat Quality in Pigs and Its Mechanisms*. *Chinese Journal of Animal Nutrition* 2017.

2. Pearce SC, Lonergan SM, Hufflonergan E, Baumgard LH, Gabler NK: *Acute Heat Stress and Reduced Nutrient Intake Alter Intestinal Proteomic Profile and Gene Expression in Pigs*. *Plos One* 2015, 10(11):e0143099.

3. Cui Y, Hao Y, Li J, Bao W, Li G, Gao Y, Gu X: *Chronic Heat Stress Induces Immune Response, Oxidative Stress Response, and Apoptosis of Finishing Pig Liver: A Proteomic Approach*. *International
Journal of Molecular Sciences 2016, 17(5):393.

4. Gregory NG, Sant’Ana ADS: *How climatic changes could affect meat quality*. Food Research International 2010, 43(7):1866–1873.

5. Feng YJ, Xian-Hong GU: *Research Progress on the Effect of Heat Stress on Meat Quality and its Mechanism in Pigs*. China Animal Husbandry & Veterinary Medicine 2013, 40(2):96–99.

6. Bell JA: *Selective blockade of spinal reflexes by ω-conotoxin in the isolated spinal cord of the neonatal rat*. Neuroscience 1993, 52(3):711.

7. Xiang-Hong JU, Yong YH, Jian-Chang HE, Deng FC: *Effect of Heat Stress on Baseline Immune and Blood Biochemical Index in Ba-Ma Miniature Pig*. Chinese Journal of Animal Science 2009.

8. Zhao Z, Liu J, Hui L, Li D, Rong C, Qi W, Gang Z, Rao Z: *Expression of GFAP in rat hypothalamus after different temperature heat stresses*. Acta Anatomica Sinica 2007, 38(5):525–527.

9. Odeon MM, Yamauchi L, Grosman M, Acosta GB: *Long-term effects of repeated maternal separation and ethanol intake on HPA axis responsiveness in adult rats*. Brain Research 2017, 1657:193–201.

10. Soria G, Krezymon A, Benani A, Planas AM, Carmona MDC, Gomis R: *Anti-obesity sodium tungstate treatment triggers axonal and glial plasticity in hypothalamic feeding centers*. Plos One 2012, 7(7):e39087.

11. Sutherland MA, Niekamp SR, Rodriguez-Zas SL, Salak-Johnson JL: *Impacts of chronic stress and social status on various physiological and performance measures in pigs of different breeds*. Journal of Animal Science 2006, 84(3):588–596.

12. Kataria N, Kataria AK: *Alterations in prolactin and cortisol levels in heat stressed pigs from arid tracts in India*. Porcine Research 2013:4–8.

13. Duff GC, Galyean ML: *Board-invited review: recent advances in management of highly stressed, newly received feedlot cattle*. Journal of Animal Science 2007, 85(3):823–840.

14. Zhi XX, Xin W, Hua LL, Lei DS, Xiong E PI, Wei L: *ITRAQ Technology and Its Application in Proteomics*. Chinese Journal of Biochemistry & Molecular Biology 2011.

15. Poirrier JE, Guillonneau F, Renault J, Sergeant K, Luxen A, Maquet P, Leprince P: *Proteomic changes in rat hippocampus and adrenals following short-term sleep deprivation*. Proteome Science 2008,
16. Ribeiro EB: Studying the central control of food intake and obesity in rats. Revista De Nutrição 2009, 22(1):163-171.

17. Sarkar P, Sarkar S, Ramesh V, Kim H, Barnes S, Kulkarni A, Hall JC, Wilson BL, Thomas RL, Pellis NR: Proteomic Analysis of Mouse Hypothalamus under Simulated Microgravity. Neurochemical Research 2008, 33(11):2335-2341.

18. Fouillen L, Petruzziello F, Veit J, Bhattacharyya A, Kretz R, Rainer G, Zhang X: Neuropeptide alterations in the tree shrew hypothalamus during volatile anesthesia. Journal of Proteomics 2013, 80(6):311-319.

19. Martyniuk CJ, Kroll KJ, Doperalski NJ, Barber DS, Denslow ND: Genomic and Proteomic Responses to Environmentally Relevant Exposures to Dieldrin: Indicators of Neurodegeneration? Toxicological Sciences 2010, 117(1):190.

20. GarciaSegura LM, Lorenz B, Doncarlos LL: The role of glia in the hypothalamus: implications for gonadal steroid feedback and reproductive neuroendocrine output. Reproduction 2008, 135(4):419.

21. Ju XH, Xu HJ, Yong YH, An LL, Jiao PR, Liao M: Heat stress upregulation of Toll-like receptors 2/4 and acute inflammatory cytokines in peripheral blood mononuclear cell (PBMC) of Bama miniature pigs: an in vivo and in vitro study. Animal 2014, 8(9):1462-1468.

22. Fontana JM, Bankamp B, Rota PA: Inhibition of interferon induction and signaling by paramyxoviruses. Immunological Reviews 2010, 225(1):46-67.

23. Schneider-Schaulies J, Schneider-Schaulies S: Receptor interactions, tropism, and mechanisms involved in morbillivirus-induced immunomodulation. Advances in Virus Research 2008, 71:173.

24. Bradke F, Fawcett JW, Spira ME: Assembly of a new growth cone after axotomy: the precursor to axon regeneration. Nature Reviews Neuroscience 2012, 13(3):183.

25. Khar A, Ali AM, Pardhasaradhi BV, Varalakshmi CH, Anjum R, Kumari AL: Induction of stress response renders human tumor cell lines resistant to curcumin-mediated apoptosis: role of reactive oxygen intermediates. Cell Stress & Chaperones 2001, 6(4):368-376.

26. Vidyasagar A, Wilson NA, Djamali A: Heat shock protein 27 (HSP27): biomarker of disease and
therapeutic target. *Fibrogenesis & Tissue Repair*, 5(1) (2012-05-07) 2012, 5(1):7.

27. Fujita Y, Ohto E, Katayama E, Atomi Y: *alphaB*-Crystallin-coated MAP microtubule resists nocodazole and calcium-induced disassembly. *Journal of Cell Science* 2004, 117(9):1719-1726.

28. Breuer H, Dahm K: *Anreicherung und charakterisierung einer 17β-hydroxysteroid: NAD(P)-oxydoreductase der rattenniere. BBA - Enzymological Subjects* 1964, 85(1):29–37.

29. Schultz RM, Groman EV, Engel LL: *3(17)beta-Hydroxysteroid Dehydrogenase of Pseudomonas testosteroni. Ligand binding properties. Journal of Biological Chemistry* 1977, 252(11):3784–3790.

30. Su W, Wang Y, Jia X, Wu W, Li L, Tian X, Li S, Wang C, Xu H, Cao J: *Comparative proteomic study reveals 17β-HSD13 as a pathogenic protein in nonalcoholic fatty liver disease. Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(31):11437.

31. Lippolis JD, Reinhardt TA: *Centennial paper: Proteomics in animal science. Journal of Animal Science* 2008, 86(9):2430–2441.

32. Kimura K, Goff JP, Canning P, Wang C, Roth JA: *Effect of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil number and function in periparturient dairy cows. Journal of Dairy Science* 2014, 97(8):4842–4851.

33. Arockiaraj J, Gnanam AJ, Kumaresan V, Palanisamy R, Bhatt P, Thirumalai MK, Roy A, Pasupuleti M, Kasi M: *An unconventional antimicrobial protein histone from freshwater prawn Macrobrachium rosenbergii: analysis of immune properties. Fish & Shellfish Immunology* 2013, 35(5):1511–1522.

34. Lokman NA, Ween MP, Oehler MK, Ricciardelli C: *The role of annexin A2 in tumorigenesis and cancer progression. Cancer Microenvironment* 2011, 4(2):199–208.

35. Gauer J, Knutson K, Jaworski S, Rice A, Rannikko A, Lentz B, Hinderliter A: *Membrane Modulates Affinity for Calcium Ion to Create an Apparent Cooperative Binding Response by Annexin a5. Biophysical Journal* 2013, 104(11):2437–2447.

36. Campos-Martorell M, Salvador N, Monge M, Canals F, García-A-Bonilla L, Hernández-GM, Ayuso MI, Chacón N P, Rosell A, Alcazar A: *Brain proteomics identifies potential simvastatin targets in acute phase of stroke in a rat embolic model. Journal of Neurochemistry* 2014, 130(2):301–312.

37. JR W, Zougman A, N N, M M: *Universal sample preparation method for proteome analysis. Nature*
Methods 2009, 6(5):359–362.

38. Han X, Shao W, Liu Z, Fan S, Yu J, Chen J, Qiao R, Zhou J, Xie P: *iTRAQ-based quantitative analysis of hippocampal postsynaptic density-associated proteins in a rat chronic mild stress model of depression. Neuroscience* 2015, 298:220–292.

Tables

Table 1 The significantly changed protein in hypothalamus of pigs under HS

| Protein name                                           | Accession number | Ratio (Heat stress/Control) | Peptides | Functions                                      |
|--------------------------------------------------------|------------------|------------------------------|----------|-----------------------------------------------|
| **Down-regulated in Hypothalamus**                     |                  |                              |          |                                               |
| Similar to V-type proton ATPase subunit F-like         | gi|311275455         | 0.643                        | 4        | hydro transport synthase                      |
| Similar to microtubule-associated protein tau-like     | gi|350590233         | 0.152                        | 11       | microtub                                       |
| Similar to neurofilament heavy polypeptide            | gi|311270880         | 0.493                        | 9        |                                               |
| Similar to dynamin-1 isoform 1                         | gi|194033645         | 0.333                        | 8        | phospholip                                    |
| Similar to cytoplasmic dynein 1 heavy chain 1          | gi|350587231         | 0.425                        | 12       | protein binding and microtubule activity     |
| Similar to microtubule-associated protein 6            | gi|350588280         | 0.27                         | 11       |                                               |
| Similar to annexin A6-like                            | gi|335304211         | 0.611                        | 3        | calcium ion                                   |
| Similar to clathrin coat assembly protein AP180        | gi|350578374|ref|XP_00 3121434.3 | 0.21     | 5        | binc                                          |
| Similar to ankyrin-2                                   | gi|350587861         | 0.543                        | 15       | spectrin                                       |
| Similar to dihydropyrimidinase-related protein 1       | gi|350587318         | 0.135                        | 9        | hydrolase acting on nitrogen peptide         |
| Protein Description                                      | gi|     |  Activity                  |
|----------------------------------------------------------|-----|---------------------------|
| Similar to alpha-centractin                             | gi194041937 | 0.52 | 5 | ATP binding                |
| Similar to beta-adducin isoform 1                       | gi335285320 | 0.381 | 8 | calmodulin binding         |
| Muscle glycogen phosphorylase                           | gi106073338 | 0.329 | 4 | glycogen phosphorylase     |
| RAB10, member RAS oncogene family                       | gi340007402 | 0.455 | 3 | GTP binding                |
| Similar to tubulin beta-2C chain-like                   | gi335281298 | 0.331 | 18 | GTP binding class I protein|
| AP-2 complex subunit beta                               | gi342187276 | 0.444 | 9 | protein transactin binding |
| Similar to prefoldin subunit 5-like isoform 1           | gi335287837 | 0.492 | 2 | transactivation            |
| Similar to septin-7-like isoform 1                      | gi311275636 | 0.368 | 5 | protein binding structural |
| Glia maturation factor beta                             | gi346986485 | 0.401 | 2 | protein kinase activity    |
| Similar to platelet-activating factor acetylhydrolase IB subunit beta-like | gi311263970 | 0.509 | 2 | 1-alk-2-acetylglycerol ester |
| Similar to creatine kinase U-type, mitochondrial-like isoform 1 paralemmin-1 | gi311244870 | 0.284 | 9 | creatine kinase            |
| Similar to dihydropromidinase-related protein 3-like    | gi335283727 | 0.442 | 1 | D3 dopamine binding        |
| Extracellular signal-regulated kinase-2                 | gi310789265 | 0.291 | 3 | chondroitin binding        |
| Proteolipid protein                                     | gi5679718  | 0.085 | 6 | RNA polymerase domain kinase |
| Acylphosphatase                                          | gi353524  | 0.398 | 2 | structural protein myelin  |
| RTN4-Aw                                                  | gi38488990 | 0.266 | 4 | acylphosphatase activity   |
| Heat shock 70 kDa protein 12A                           | gi350593095 | 0.439 | 7 | ATP binding                |
| Peptidyl-prolyl cis-trans isomerase D                   | gi346986322 | 0.559 | 3 | peptide and heat shock     |
| Unnamed protein product                                 | gi1900   | 0.428 | 5 | sodium:potassium exchanging ATP binding |
| Similar to guanine                                       | gi311245496 | 0.293 | 2 | signal transduction binding |
nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2-like isoform 3
Glyceraldehyde-3-phosphate dehydrogenase; gi|2506441 0.664 10 NAD binding and microtubule binding
Alpha-soluble NSF attachment protein
Similar to uncharacterized membrane protein C1orf95-like
Similar to rab GDP dissociation inhibitor alpha-like isoform 1
Similar to septin-6-like isoform 2
Parvalbumin
Similar to microtubule-associated protein RP/EB family member 3-like
Similar to complexin-1-like
Similar to neuroplastin, partial

Lipotropin gamma gi|229326 0.322 4 -
Metallothionein-III gi|2073002 0.195 2 -
Similar to V-type proton ATPase subunit E1 isoform 2
Rab-3A gi|115394766 0.116 2 -
Similar to annexin A6-like
Similar to V-type proton ATPase 116 kDa subunit a isoform 1-like isoform 1
Unnamed protein product gi|1921 0.464 4 -
Similar to gamma-enolase isoform 1
Similar to protein S100-B-like
Similar to secernin-1-like
Similar to synaptic vesicle membrane protein VAT-1 homolog
Similar to microtubule-associated protein 1A
Similar to endophilin-A2-like
Similar to ADP/ATP translocase 1-like isoform 2
fascin gi|225382133 0.24 6 -
Similar to glycogen phosphorylase, brain form-like, partial gi|335310649 7 0.665 glycc phosphoryl and pyrphosphat actin b
Thymosin beta-4 gi|85700161 1 0.572 GTP binc prot heterodir acti chaperon-
Similar to tubulin alpha-1D chain gi|194043861 17 0.121 protein bi GTP bi omega p acti phosphat decarboxyl acti
Prefoldin subunit 6 gi|297747282 3 0.516 protein-L-i protein-i deiminasi cholesterol l transporte
Similar to tubulin alpha-4A chain OTUB1 gi|335303414 18 0.161 protein bi GTP bi omega p acti phosphat decarboxyl acti
Similar to protein-arginine deiminase type-2-like gi|335290579 9 0.563 protein-L-i protein-i deiminasi
Myelin P2 protein gi|297307127 4 0.647 tubulin bir calcium io
Similar to tubulin polymerization-promoting protein family member 3-like isoform 1 gi|311257146 4 0.349 translation activity, n binc
Similar to transcriptional activator protein Pur-alpha isoform 1 gi|335283572 5 0.629 metal ion b NAD b
NADP-dependent malic enzyme, mitochondrial gi|346716344 6 0.579 Signal tra mechar actin b
Similar to bolA-like protein 2-like, Cofilin 2 gi|335307557 1 0.653 metal ion b sodium:pt exchangir acti
Na+/K+ transporting alpha 3 polypeptide gi|283443672 25 0.282 calcium ion spectrin
Similar to spectrin alpha chain, brain gi|311246557 51 0.402 high-affinity transme transpo
Excitatory amino acid transporter 1 gi|346986408 1 0.32 hist methyltra activity (H4-
Similar to protein arginine N-methyltransferase 1 isoform 1 gi|335290012 1 0.621 Posttran: modificat turnover, cf
Similar to 26S proteasome non-ATPase regulatory subunit 12-like gi|350590194 2 0.56 hydrolase acting on nitrogen peptide l
Similar to dihydropyrimidinase-related protein 5 gi|311252980 4 0.371
| Protein Name                                      | GI Number       | Score | Activity                                      |
|--------------------------------------------------|-----------------|-------|-----------------------------------------------|
| Similar to syntaxin-1A-like                      | gi|335284236     | 3     | myosin head binding activ                      |
| Similar to syntaxin-1B-like                      | gi|350581587     | 5     | extracellular glutamate channel               |
| Mitochondrial NAD+isocitrate dehydrogenase 3 beta variant 1 | gi|98283612      | 2     | NAD binc isocit dehydrogen actin              |
| Similar to cysteine and glycine-rich protein 1-like isoform 1 | gi|335296245     | 4     | protein binn calcium ion binn                 |
| Beta-synuclein                                   | gi|144227406      | 6     | calcium ion alpha-tubu                        |
| Synapsin Ib                                     | gi|212525788      | 13    | ATP binding activ calcium ion                 |
| HPCA                                            | gi|115394790      | 7     | transport activ                               |
| Similar to mitochondrial 2-oxoglutarate/malate carrier protein | gi|335298430     | 6     | Cytoske                                       |
| Similar to transgelin-3-like                    | gi|350591990      | 5     | hydrogend ATPase activ mech                   |
| Putative V-ATPase G subunit                     | gi|6624727        | 2     | protein activ serine/threonine phosphatase activi |
| Similar to protein phosphatase inhibitor 2-like | gi|335300158      | 2     | signal tr activity activ         |
| Galectin-1                                      | gi|47716872       | 7     | GTP, CTP b activ                               |
| Heat shock 90kD protein 1, beta                 | gi|346986428      | 15    | UDP b activ                                  |
| Similar to 14-3-3 protein eta                   | gi|194043292      | 5     | sodium b activ                               |
| Similar to protein kinase C and casein kinase substrate in neurons protein 1-like | gi|335292069      | 7     | cytoskeleton activ                           |
| Protein, myelin basic                           | gi|224358         | 4     | structural cc myelin                          |
| Similar to alpha-internexin                     | gi|350593043      | 11    | structural cc cytoskeleton                    |
| Protein phosphatase 1 regulatory subunit 1B     | gi|2499741        | 4     | receptor bi activ protein kinase activ         |
| Similar to synaptic vesicle glycoprotein 2A     | gi|194036298      | 6     | Recept transme transport activ hexokinas       |
| hexokinase 1                                    | gi|342187282      | 15    | Hsp90 prote                                  |
| CDC37 cell division cycle 37 protein            | gi|51870491       | 5     | glycoprotein and carbi activ                   |
| Similar to contactin-1-like isoform 2           | gi|350584500      | 12    |                                              |
| HN1                                             | gi|54151071       | 1     |                                              |
| Protein Name                                      | gi | Homology | Function                                                                 |
|--------------------------------------------------|----|----------|--------------------------------------------------------------------------|
| Prostaglandin D synthase                         | 1064940 | 1        | 0.443; retinoid binding and prostaglandin synthase activity              |
| Endorphin gamma                                  | 229600  | 2        | 0.202; protein C binc prot homodimer activity                            |
| RTN4-C                                          | 38327590 | 1        | 0.31; protein homodimer activity                                         |
| Similar to cell adhesion molecule 3             | 350583264 | 2        | 0.212; protein homodimer activity                                         |
| somatostatin                                    | 46850198 | 2        | 0.44; protein homodimer activity                                          |
| Rho GDP dissociation inhibitor alpha            | 315321426 | 4        | 0.625; Rho GDP-d inhibitor activity                                       |
| Similar to LanC lantibiotic synthetase component C-like 1 | 335307003 | 2        | 0.444; G-protein receptor activity                                       |
| Similar to synaptotagmin-1                      | 350584732 | 4        | 0.235; 1-phosphatidyl ethanol and metal ion binding                      |
| Similar to myosin-10-like                       | 350590878 | 26       | 0.503; nucleotide binding                                                |
| Similar to 14-3-3 protein beta/alpha isoform 1  | 194044626 | 6        | 0.352; transci corepressor histone de binc motor                        |
| Similar to dynactin subunit 2-like isoform 3    | 335310032 | 8        | 0.579; Posttranslational modification, protein turnover, chaperones       |
| Similar to gamma-soluble NSF attachment protein-like | 350596353 | 3        | 0.416; Posttranslational modification, protein turnover, chaperones       |
| peptidyl-Pro cis trans isomerase                 | 226256  | 6        | 0.629; dihydrolipoamide acetyltransferase                               |
| Similar to endophilin-A1-like                   | 311245746 | 2        | 0.209; dihydrolipoamide acetyltransferase                               |
| Dihydrolipoamide acetyltransferase              | 14587786 | 4        | 0.62; dihydrolipoamide acetyltransferase                                |
| Similar to ubiquitin-conjugating enzyme E2 N-like | 335288894 | 4        | 0.649; ubiquitin binding and ubiquitin-protein ligase activity           |
| Similar to neurofilament medium polypeptide-like isoform 1 | 350592300 | 24       | 0.497; structural activity                                               |
| Tubulin polymerization promoting protein p25 alpha | 170178280 | 7        | 0.225; tunnel and calcium                                                |
| Similar to rap1 GTPase-GDP dissociation stimulator 1 | 350587954 | 4        | 0.281; GTPase GDP dissociation stimulator 1                               |
| Tubulin beta-2B chain                            | 343478189 | 16       | 0.154; structural activity                                               |
| Clathrin heavy chain                             | 24492556 | 38       | 0.362; ankyrin bii structural activity                                   |
| Gamma-synuclein                                 | 132269870 | 7        | 0.548; type I trar                                                        |
| FKBP1A-like                                     | 61098747 | 3        | 0.628; type I trar                                                        |
| Similar to astrocytic phosphoprotein PEA-15 | gi|194035847 | 5 | 0.262 | growth factor receptor protein kin binding
| Stathmin-1 | gi|49615355 | 5 | 0.523 | signal transducer
| Glutaminase | gi|1583522 | 3 | 0.438 | glutaminase: protein kinase binding
| Adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1 | gi|197130945 | 2 | 0.54 | phospholipid and protein binding
| Similar to myristoylated alanine-rich C-kinase substrate | gi|335279372 | 3 | 0.362 | protein binding a filament actin binding structural actin binding GTP binding ubiquitin protein binding
| Similar to erythrocyte membrane protein band 4.1-like 1 isoform 1 | gi|335304751 | 9 | 0.601 | -
| Similar to dynamin 1 | gi|350584292 | 3 | 0.5 | pyruvate kinase
| Similar to neural cell adhesion molecule 2 | gi|350592100 | 2 | 0.307 | GPI anchor integrin binding
| Similar to pyruvate kinase isozymes M1/M2 isoform 1 | gi|194038728 | 17 | 0.4 | -
| CD90 protein | gi|224697007 | 3 | 0.376 | GPI anchor integrin binding
| Neuromodulin | gi|346716243|ref|NP_0 01231264.1| 4 | 0.1 | protein binding
| Nucleoside diphosphate kinase A | gi|325652098 | 4 | 0.394 | nucleoside diphosphate kinase binding
| Similar to dihydropyrimidinase-related protein 3 | gi|350581250 | 5 | 0.234 | chondroitin sulfate binding phosphorylase oxidoreductase activity acting on NAD
| Similar to NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5-like | gi|311275515 | 2 | 0.598 | -
| Similar to MARCKS-related protein-like isoform 2 | gi|311258852 | 2 | 0.382 | sodium:potassium-exchanging ATPase activity
| Similar to sodium/potassium-transporting ATPase subunit beta-2-like like | gi|311268313 | 2 | 0.172 | -
| Similar to neural cell adhesion molecule 1-like | gi|311263926 | 10 | 0.256 | -
| Similar to guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2-like isoform 2 | gi|311251041 | 5 | 0.474 | signal transducer activity
| Protein Name                                                                 | gi/Uniprot ID   | P-value | Function/Activity                                                                 |
|-----------------------------------------------------------------------------|-----------------|---------|----------------------------------------------------------------------------------|
| Similar to mu-crystallin homolog                                           | gi|335284508      | 4       | Thyroid hormone binding and thiomorpholine-carboxylate dehydrogenase             |
| Hexokinase II                                                              | gi|90820093        | 2       | Carbohydrate and metabolism                                                      |
| Similar to NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10-like | gi|335310935       | 2       | Hexokinase activity and glucose binding                                          |
| Similar to AP-2 complex subunit alpha-1 isoform 1                          | gi|311258000       | 5       | Protein C-terminus binding                                                       |
| calbindin 2                                                                 | gi|300827489       | 5       | Calcium ion binding                                                              |

*Up-regulated protein in hypothalamus*

| Protein Name                                                                 | gi/Uniprot ID   | P-value | Function/Activity                                                                 |
|-----------------------------------------------------------------------------|-----------------|---------|----------------------------------------------------------------------------------|
| Annexin A2                                                                  | gi|52631987       | 14      | Phosphatidylinositol-4,5-bisphosphate binding                                    |
| Small acidic protein                                                         | gi|349585075       | 1       | Low-density particle binding                                                     |
| Apolipoprotein B-100                                                         | gi|350597081       | 3       | Copper-dependent protein binding and copper-chaperone activity                   |
| Antioxidant protein 1 homolog                                                | gi|262036932       | 3       | Copper-dependent protein binding and copper-chaperone activity                   |
| Hsp27                                                                       | gi|55666280       | 6       | Copper-dependent protein binding and copper-chaperone activity                   |
| Nuclease-sensitive element-binding protein 1-like                           | gi|350586335       | 4       | Copper-dependent protein binding and copper-chaperone activity                   |
| 78 kDa glucose-regulated protein                                             | gi|350579657       | 22      | Copper-dependent protein binding and copper-chaperone activity                   |
| L-3-hydroxyacyl-CoA dehydrogenase                                            | gi|4454537         | 6       | Copper-dependent protein binding and copper-chaperone activity                   |
| Isocitrate dehydrogenase [NADP] cytoplasmic isoform 1                       | gi|350593816       | 4       | Copper-dependent protein binding and copper-chaperone activity                   |
| Histone H2B type 1-like                                                     | gi|194039814       | 2       | Chaperone binding and unfolded protein binding                                   |
| Flavin reductase-like                                                        | gi|335289705       | 5       | ATP binding and unfolded binding, tetra-17-beta-dehydrogenase                   |
| Immunoglobulin lambda-chain                                                  | gi|164511          | 5       | ATP binding and unfolded binding, tetra-17-beta-dehydrogenase                   |
| Stress-70 protein, mitochondrial                                            | gi|311250237       | 16      | Electron carrier activity                                                        |
| 17-beta-hydroxysteroid Dehydrogenase 14-like                                | gi|311257850       | 3       | Stress-70 protein, mitochondrial                                                |
| Synaptosomal-associated protein                                             | gi|347800627       | 2       | Syntaxin                                                                        |
| Ppk 98; a protein kinase                                                     | gi|431944          | 20      | Virion and calcium ion binding                                                   |
| Aldehyde reductase                                                          | gi|1185557         | 3       | Electron carrier activity                                                        |
| **Gene Name**                                   | **gi** | **E-value** | **Score** |
|-----------------------------------------------|--------|-------------|-----------|
| Argininosuccinate synthase                    | gi|335281104 | 19.913    |
| Immunoglobulin heavy chain variable region    | gi|119663061 | 1.985     |
| Ribosomal protein L6                          | gi|56384243  | 1.956     |
| Soluble epoxide hydrolase                     | gi|45551399  | 3.051     |
| Alpha-1B-glycoprotein-like                    | gi|311259609 | 3.927     |
| V-type proton ATPase subunit G 1              | gi|298104120 | 3.062     |
| Electron transfer flavoprotein beta subunit precursor | gi|35384838  | 2.808     |
| Cathepsin D protein                           | gi|56417363  | 4.76      |
| Similar to protein disulfide-isomerase A4-like | gi|311264773 | 1.889     |
| Serpin H1 precursor                           | gi|346421378 | 3.195     |
| IgG heavy chain precursor                     | gi|5052050   | 5.141     |
| Prolyl 4-hydroxylase beta polypeptide         | gi|358009193 | 1.926     |
| Solute carrier family 25 member 3             | gi|255964672 | 1.595     |
| Similar to ezrin                              | gi|350578005 | 4.869     |
| Similar to calcium-regulated heat stable protein 1-like isoform 2 | gi|350581733 | 3.533     |
| Similar to ribonuclease UK114-like isoform 1  | gi|194037005 | 5.011     |
| Similar to prohibitin                         | gi|3a50590415| 1.519     |
| Alternative Pig Liver Esterase                | gi|164414678 | 5.891     |
| Similar to proenkephalin-A                    | gi|311253816 | 1.63      |
| Sorbitol dehydrogenase                        | gi|346421435 | 2.822     |
| Similar to branched-chain - amino-acid aminotransferase, mitochondrial isoform 2 | gi|335289923 | 7.182     |
| Hemoglobin alpha                              | gi|229176    | 3.057     |
| Gene Name                          | gi/Accession   | Rank | q Value |
|-----------------------------------|----------------|------|---------|
| Lumican precursor                 | gi|343183420     | 7    | 2.116   |
| Thioredoxin                       | gi|14326453      | 3    | 1.604   |
| Transaldolase                     | gi|349732238     | 5    | 1.532   |
| Lysosome membrane protein 2 precursor | gi|346421419    | 4    | 2.332   |
| Bcl-2-like protein 13             | gi|350584479     | 4    | 2.387   |
| Glycerol-3-phosphate dehydrogenase 1 | gi|283765396    | 3    | 2.003   |
| Similar to transgelin-2-like isoform 1 protein SET | gi|335286672    | 6    | 5.511   |
| Similar to annexin A5             | gi|335293906     | 12   | 2.117   |
| tumor protein D52                 | gi|346986324     | 4    | 1.576   |
| Dihydropyrimidinase               | gi|311253507     | 3    | 3.762   |
| Isovaleryl Coenzyme A dehydrogenase | gi|262204892    | 5    | 2.146   |
| Sterol carrier protein 2          | gi|262263195     | 4    | 1.851   |
| Formiminotransferase-cyclodeaminase | gi|433003      | 2    | 1.723   |
| Enoyl-CoA hydratase, mitochondrial | gi|298104076    | 8    | 2.514   |
| Long-chain acyl-CoA dehydrogenase | gi|1695729      | 2    | 2.921   |
| Similar to glyoxalase domain-containing protein 4-like APEX nuclease 1 | gi|335298275    | 7    | 1.616   |
| CArG-binding factor A             | gi|160858224     | 5    | 1.718   |
| Similar to 40S ribosomal protein S24 | gi|194042179   | 2    | 1.72    |

- **binc:** extracellular structural protein oxidoreductase
- **sedohept:** phosphoglycerald phosphatase receptor activity enzyme
- **cystein:** endopeptidase activator involved in proc cystein endopeptidase activator involved in proc
- **NAD:** binc glycerol-3-phosphate dehydrogenase
- **protein:** histone binding, phosphatase activity
- **calcium ion binding:** histone binding, phosphatase activity
- **propanoyl acyltransferase:** folic acid, intermediol binc fatty-acyl-CoA and palm oxidase Amino acid and metabolism transci corepressi sequence-specific binding transcription factor translation, structure
| Protein Name | gi/Accession | Fold Change | Function |
|--------------|--------------|-------------|----------|
| isoform 4 | gi|311254836 | 2 | 2.85 |
| Similar to kynurenine-oxoglutarate transaminase 3 isoform 2 | | | | bioger kynurenine transamina cysteine-S-beta-lyase kynure oxoglutarate transamina prot homodim acti |
| prohibitin 2 | gi|343780941 | 8 | 1.594 |
| Similar to 3-hydroxybutyrate dehydrogenase type 2-like isoform 1 | | | | estrogen binc 2,3-dihy dihydroxy dehydrogen |
| Annexin A4 | gi|4033507 | 4 | 3.162 |
| Similar to vimentin | gi|335296459 | 22 | 1.906 |
| Cytochrome b5 fragment | gi|229384 | 2 | 6.781 |
| Succinyl-CoA synthetase beta-subunit, partial | gi|164669 | 6 | 4.231 |
| Histidine-rich glycoprotein precursor | gi|347582595 | 5 | 1.891 |
| Eukaryotic translation elongation factor 1 alpha | gi|110287842 | 9 | 1.53 |
| Similar to alpha-actinin-4-like isoform 2 | gi|335289608|ref|XP_003355931.1| | 13 | 2.541 |
| unconventional myosin | gi|516155 | 8 | 2.048 |
| aminoacylase I | gi|1845|emb|CAA48565.1 | 6 | 9.061 |
| Similar to putative aminopeptidase C13A | gi|350587379 | 3 | 2.756 |
| Glutathione S-transferase | gi|1185280 | 2 | 18.659 |
| Histidine triad nucleotide-binding protein 2, mitochondrial isoform 1 precursor | gi|346716222 | 4 | 2.219 |
| Apolipoprotein A-I | gi|164359 | 14 | 4.284 |
| Epididymal secretory protein E4 | gi|22535477 | 2 | 1.859 |
| Antithrombin protein | gi|106647532 | 5 | 3.182 |
| Description                              | gi    | Value | Activity                      |
|------------------------------------------|-------|-------|-------------------------------|
| Similar to annexin A1                    | gi194042189 | 6     | 2.757            |
| Aldose 1-epimerase                       | gi11611545 | 3     | 5.151 |
| Similar to aldehyde dehydrogenase        | gi350597032 | 1     | 5.491 |

**family 1 member A3**

| Description                              | gi    | Value | Activity                      |
|------------------------------------------|-------|-------|-------------------------------|
| Similar to alpha-2-macroglobulin         | gi311256211 | 7     | 1.728 |
| Apolipoprotein A-II precursor            | gi297747304 | 2     | 5.64  |
| 2,4-dienoyl-CoA reductase 1              | gi295442674 | 3     | 3.733 |
| Similar to selenium-binding protein 1    | gi194036227 | 20    | 2.677 |
| Monoamine oxidase A                      | gi45551418 | 4     | 1.979 |
| Similar to ganglioside GM2 activator-like isoform 1 | gi311274101 | 1     | 14.478 |
| Similar to hypothetical protein LOC100038023 | gi311245732 | 2     | 1.724 |

| Description                              | gi    | Value | Activity                      |
|------------------------------------------|-------|-------|-------------------------------|
| Similar to methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial SMP-30 | gi115371745 | 2 | 5.202 |
| Complement component 4A                  | gi147780441 | 2     | 1.581 |
| Similar to hepatoma-derived growth factor | gi335286747 | 7     | 2.187 |
| Gastrin-binding protein                  | gi433066 | 9     | 2.614 |
| Protein canopy homolog 2 precursor       | gi297307133 | 6     | 1.89  |
| Alpha-1 acid glycoprotein, partial       | gi164302 | 8     | 6.08  |
| Similar to glutaredoxin-related protein 5, mitochondrial | gi194038359 | 2 | 2.363 |
| G-beta like protein                      | gi495144 | 3     | 2.11  |
| Apolipoprotein C-III                     | gi164361 | 4     | 4.813 |
| Catechol-O-methyltransferase             | gi285818436 | 6     | 2.321 |

**turnover, ct**

**serine endopeptidase acti**

**carbohydrate and aldose J acti**

**aldel dehydro [NAD(P)+**

**endopeptidase acti**

**prot homododim acti**

**oxidoreductacti, acting on NAE**

**selenium a binc**

**primary am acti**

**phospholipa acti**

**protein bindingand constituent**

**thiolester acti**

**zinc binding, regul**

**endopeptidacti, growth facl**

**acetyl-1 acetyltyra acti**

**protein**

**metal ion b electron car**

**protein b binc**

**phospholipid and lipase acti**

**catecl methyltra acti**
| Protein Name | Accession | Log2 Fold Change | Activity/Function |
|--------------|-----------|-----------------|------------------|
| Phosphotriesterase-related protein | gi|3466448441177.1| 4 | 4.447 | hydrolase acting on e |
| Chromogranin B | gi|10121853| 25 | 3.564 | - |
| Adipocyte fatty acid-binding protein | gi|4160392| 5 | 324.583 | transporter |
| Similar to TP53-regulated inhibitor of apoptosis 1 | gi|350592546| 2 | 3.42 | cysteine endopeptidase activity in apoptotic |
| Similar to peroxiredoxin-1 isoform | gi|311259408| 7 | 1.631 | thioredoxin acti |
| Similar to alpha-aminoadipic semialdehyde dehydrogenase-like 3-hydroxyanthranilate 3,4-dioxy genase | gi|349732262| 2 | 3.249 | 3-hydroxya 3,4-dioxy acti |
| Transgelin | gi|346421409| 9 | 2.033 | electron car |
| Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial | gi|356582295| 7 | 3.568 | | |
| Similar to carbonic anhydrase 2 | gi|194037097| 6 | 1.943 | carbonate d acti |
| Leukotriene A4 hydrolase | gi|262204898| 3 | 3.469 | aminopeptid zinc ion |
| Similar to histone H2A type 1 | gi|194039812| 2 | 1.797 | DNA binc enzyme |
| Myosin light chain isoform LC17b | gi|253578| 7 | 2.162 | structural cc mus |
| uncoupling protein 3 | gi|4165892| 1 | 1481.875 | oxidphosho uncouple |
| Heat shock 10kD protein | gi|30525868| 7 | 10.249 | chaperon |
| Mitochondrial aldehyde dehydrogenase 2 | gi|81295909| 6 | 7.509 | aldel dehydro |[NAD(P)+]acti |
| Similar to cytosol aminopeptidase-like | gi|350587377| 2 | 2.194 | metalloexc acti |
| Similar to adipocyte plasma membrane-associated protein | gi|335308355| 2 | 2.602 | Arylester strictosidin acti |
| Similar to hemoglobin subunit beta-like | gi|311263245| 5 | 2.37 | oxygen tr acti |
| Ribophorin I | gi|9857227| 4 | 1.592 | dolic diphospho de-pr glycotrai acti |
| superoxide dismutase [Mn], mitochondrial | gi|312283580| 3 | 1.959 | manganese |
| Similar to hypothetical protein LOC100516841 | gi|350595403| 3 | 2.421 | 3-hydroxyi dehydro |
| Proteasome activator 28 beta subunit | gi|30315381| 4 | 1.865 | - |
| Gene Name                                                                 | gi/|Length| Activity                                      |
|--------------------------------------------------------------------------|------|-------|-----------------------------------------------|
| Lon peptidase 1, mitochondrial                                           | 342349346 | 10    | 3.016 mitochondrial strand promoter sense    |
| Ig heavy chain variable VDJ region, partial                              | 558859 | 1     | 2.341 glutathione acti                      |
| Glutathione peroxidase 3 precursor                                       | 169646366 | 3     | 7.594 glutathione acti                      |
| Signal sequence receptor, alpha S-adenosylhomocysteine hydrolase        | 297632426 | 1     | 2.434                                          |
| Similar to 4-trimethylaminobutyl dehyde dehydrogenase                    | 40644231 | 4     | 2.286                                          |
| Hexosaminidase A alpha polypeptide                                        | 169117926 | 3     | 2.462                                          |
| Similar to Ig kappa chain V-II region RPMI 6410                          | 350582129 | 3     | 2.706                                          |
| Similar to fumarylacetoacetate hydrolase domain-containing protein 2     | 311252000 | 5     | 2.18                                           |
| Similar to acyl-coenzyme A thioesterase 6                                | 347300323 | 3     | 3.05                                           |
| Thioredoxin-dependent peroxide reductase, mitochondrial                  | 309795  | 1     | 1.862                                          |
| Rho GDP dissociation inhibitor (GDI) beta                                | 346716314 | 2     | 2.344                                          |
| Glucosidase 2 subunit beta precursor                                     | 347446687 | 6     | 1.733 phosphatidic acti                      |
| Similar to caldesmon                                                     | 311275365 | 5     | 2.353 Calmodulin, myosin                     |
| Similar to purine nucleoside phosphorylase                               | 194038973 | 5     | 2.615                                          |
| Similar to fumarylacetoacetase                                           | 335292272 | 5     | 6.573 fumarylacetase activity and acti       |
| Similar to eukaryotic translation initiation factor 3 subunit A          | 194042126 | 2     | 1.961 translation factor acti                |
| Similar to apoptosis-inducing factor 1, mitochondrial isoform 1          | 311276941 | 2     | 3.164 flavin a dinucleotic                   |
| Similar to ES1 protein homolog, mitochondrial-like                       | 335300836 | 4     | 1.726 Secondary r biosynthesis: and cati     |
| Non-selenium glutathione phospholipid hydroperoxide peroxidase (PHGPx)  | 6689393 | 7     | 2.148 glutathione acti                      |
| Cytochrome b5 type B                                                     | 227430316 | 2     | 5.38                                           |
| Similar to chromobox protein homolog 3-like                             | 350595422 | 2     | 1.834                                          |
### isoform 1

| Enzyme Description                                      | gi|   | Activity                      |
|----------------------------------------------------------|----|----|-------------------------------|
| long-chain 3-ketoacyl-CoA thiolase gij6165556            | 7  | 3.162 | NAD binding and long-chain-	|
|                                                          |    |      | enoyl-CoA hydratase activity  |
| Similar to serpin A3-8 gi|350587171 | 10 | 4.428 | serine endopeptidase activity |
| Similar to hydroxyacid-oxoacid transhydrogenase, mitochondrial-like gi|311253769 | 2 | 5.101 | hydroxyacid transhydrogenase activity |
| Similar to adenylate kinase 2, mitochondrial-like isoform 1 gi|335309396 | 4 | 9.89 | adenylate kinase activity and ATP binding |

**Figures**
Figure 1
Protein identification in the hypothalami of heat-stressed (HS) and Control pigs. (A) Numbers of proteins identified, (B) distribution of the DEPs in molecular weight (kD), (C) the coverage of the identified peptides for DEP, and (D) the distribution of DEPs containing different numbers of identified peptides.
Figure 2

Bioinformatics analysis of the DEPs identified in the hypothalamus of pigs. (A) Subcellular locations of the proteins with differential expression ($B \leq 0.05$) in heat-stressed (HS) and Control pigs. (B) Thirty most related canonical pathways from Ingenuity Pathways Analysis.
Figure 3

Functional characterization of DEPs in the hypothalamus of pigs under heat stress. (A) diseases and disorders, (B) molecular and cellular functions, (C) physiological system development and functions, and (D) toxicological functions.
Figure 4

Ingenuity pathway analysis of differentially expressed proteins in the hypothalamus of pigs under heat stress. Red is up-regulated, green is down-regulated, and white is a protein that is involved in pathway regulation but not identified in this study. A darker color indicates a greater change in the expression level of the protein. Different shapes represent different molecular types (e.g., protein families), the lines connecting the molecules represent intermolecular relationships, the dashed lines are indirect effects, the solid lines are direct effects, and the arrows represent specific molecular relationships and directions of action.
Western blot identification of DEPs: (A) Expression of Histone H2A in the hypothalamus of pigs at day 7 of heat stress. Con = control pigs; HS = heat-stresssed pigs. (B) Expression of Histone H2A was significantly increased in the HS group compared with the control group.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

supplemental instrument.docx
illustration.tif