Heat challenge influences serum metabolites concentrations and liver lipid metabolism in Japanese quail (Coturnix japonica)

Running head: HIGH TEMPERATURE AND LIPID METABOLISM

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ABSTRACT

High temperature induces various metabolic disturbances in animals. However, no comprehensive information is currently available on the metabolic pathway affected by high environmental temperature. The present study examined metabolite content in the serum of heat challenged quails using metabolomic analysis. In the present study, female quails with normal laying rate at 20 weeks kept in standard condition (control group) or exposed to 34°C 4hr per day (12:00hr to 16:00hr)(heat group) for 10 consecutive days. The metabolomic analysis identified 165 metabolites in the serum, and significant differences were observed in the serum for 7 metabolites between two groups. An analysis by MetaboAnalyst, a web-based metabolome data tool, indicate that high temperature affect ketone body metabolism, butyrate metabolism, arginine and proline metabolism. Furthermore, histological examination of liver indicates a heat challenge induced abnormal lipid metabolism. Triglyceride and cholesterol level in the liver increased, however cholesterol level decreased in the serum. Genes related to lipid metabolism significantly increased in the liver after heat challenge. The present study demonstrated that high temperature cause liver damage, thus lipid metabolic was affected. Protect liver under high temperature could be one solution for coping with high temperatures in summer.

KEY WORDS: heat challenge, Japanese quail, lipid metabolism, liver, serum metabolite
High temperature in summer is one of the major factors restrict poultry industry. Heat stress is a prime consideration in poultry production systems and affects animal health and productivity [6], which causes huge economic loss. In poultry industry, heat stress can reduce egg laying number [8], decrease ovarian weight [19] and hierarchical follicles number [23].

The change of physiological and biochemical characteristics during heat stress have been reported, for example, heat stress increased apical glucose transport in the chicken jejunum [3], acute heat stress induced superoxide radical production in chicken skeletal muscle [16]. Increased the vasoconstrictor responses to 5-HT by a mechanism that involved extracellular Ca\(^{2+}\) influx through calcium channels [22]. Heat stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens [26]. And affect gene expression of brain-gut neuropeptides in broiler chickens (Gallus gallus domesticus)[12].

Few researches reported on metabolites change under high temperature, and then they found multiple pathways affected by heat stress including glucose, amino acid, and lipid metabolism [7]. Environmental high temperature may affects multiple tissues, the organism alter its physiology to maintain homeostasis. As metabolite content should reflect the net results of physiological changes, it may provide an insight into the metabolic disturbance under high temperature.

Liver plays a central role in metabolism. Liver regulates glycogen storage and decomposition of red blood cells. Liver also plays as an accessory digestive gland that produces bile, an alkaline compound that helps the breakdown of fat. In sexually matured birds, the elevated level of estrogen indirectly regulates the rapid growth of vitellogenic oocytes by potentiating the synthesis of yolk precursor proteins in the liver [21]. It is reasonable to hypothesize that the liver will be responsive to high temperature due to its central role in maintaining the overall metabolism of the organism.
The objective of the present study was to investigate metabolic change in heat challenged quails. Gas chromatography mass spectrometry (GC/MS) was used to analyze low molecular weight metabolites in heat-challenged quails. Their livers were investigated for understanding the reason of metabolic disturbance.

MATERIALS AND METHODS

Animals and experimental design

Female Japanese quails were hatched and raised in a breeding colony at Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology. Quails were provided with commercial quail food (Quail Cosmos, Tahara, Japan) and water, and were allowed to feed ad libitum. Quails were housed in metal cages in a controlled environment (lights on, 05:00hr to 19:00hr; temperature, 23±2°C; humidity, 50±10%; air exchanged 20 times hourly).

At the age of 20 weeks, 18 healthy female quails with normal laying rate were selected and divided into control and heat group. For the heat group females were exposed to 34°C for 4hr per day (12:00hr to 16:00hr) during 10 consecutive days in Biomulti incubator (LP-80CCFL-6CTAR, NK system, Tokyo, Japan). At day 10th of treatment, quails were killed by decapitation after heat treatment. Blood was obtained from carotid artery and separated by centrifugation at 3,000 ×g for 30 min at 4°C, serum was separated and stored at -20°C for metabolic analysis and triglyceride and cholesterol measurement.

Liver was removed and measured weight. Middle lobe of fresh liver samples were fixed in 4% paraformaldehyde for 24 hr. The fixed samples were transferred through a graded series of ethanol and xylene and then embedded in paraffin wax. Other part of livers were collected and stored at -80°C for RT-PCR, triglyceride and cholesterol measurement.
The ethical committee of animal experimentation of Tokyo University of Agriculture and Technology permitted all experimental protocols.

**Non-targeted analysis of low molecular weight metabolites in serum**

A serum metabolomics analysis was performed using GC/MS as described previously [24] with some modifications. In brief, a sample of 50 μl of serum was mixed with 5 μl of 1 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) in distilled water as an internal standard, and 250 μl of methanol–chloroform–water (2.5:1:1) mixture. Then samples were lyophilized, and added with 40 μl of 20mg/ml methoxyamine hydrochloride (Sigma-Aldrich), dissolved in pyridine for oximation. After mixing, the samples were shaken for 90 min at 30°C. Next 20 μl of N-methyl N-trimethylsilyl-trifluoroacetamide (GL Science, Tokyo, Japan) was added for trimethylsilylation, and the mixture was incubated at 37°C for 45 min. The sample was subjected to GC/MS (GCMS QP2010-Ultra; Shimadzu, Kyoto, Japan). The Shimadzu Smart Metabolites Database (Shimadzu) was used to identify metabolites. Samples were normalized by a pooled sample from control group. A metabolic pathway analysis was performed using MetaboAnalyst [25]. Metabolites that significantly differed between two groups were subjected to an enrichment analysis (http://www.metaboanalyst.ca/faces/upload/EnrichUploadView.xhtml).

**Histological analysis**

Paraffin-embedded samples were serially sectioned (5 μm thick) onto 3-aminopropyl-triethoxysilane (APES)-coated slides and stained with hematoxylin–eosin (HE). The stained sections were observed and photographed under a microscope (Keyence BX-51, Osaka, Japan)

**Triglyceride and cholesterol in serum and liver**

One hundred milligram of liver from each quail was put into centrifuge tube containing 0.2 ml saline, homogenized on ice, added with 0.8 ml chloroform-methanol
(1:1), then mixed well and stayed at 4°C for 18 hr, followed by centrifugation with 3,500 ×g for 10 min at 4 °C. Three layers were clearly separated, and the bottom layer of lipid phase was gently collected and stored at 4°C for triglyceride and cholesterol measurement.

Triglyceride of serum and liver was detected by LabAssay™ Triglyceride kit (Wako, Osaka, Japan). The cholesterol was detected by LabAssay™ Choleserol kit (Wako).

**RNA extraction, reverse transcription (RT) and quantitative PCR**

Total RNA was extracted from liver using Isogen reagent kit (Nippon Gene, Tokyo, Japan). The concentration and purity of the isolated total RNA was determined spectrophotometrically at 260 and 280 nm wave length with a Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). The total RNA (1 μg) was reversely transcribed to cDNA with an Omniscript® Reverse Transcription kit (Takara, Tokyo, Japan) with Oligo-dT primers (Takara) according to the manufacturer’s protocol.

Target genes and the housekeeping gene (GAPDH) were quantified by real-time PCR using an ABI 7500 Fast and a commercial kit (SYBR Premix Ex Taq™ II, Takara). The specificity of the PCR product was verified by a melting curve and by agarose gel electrophoresis. The relative concentration of mRNA was calculated using the 2^ΔΔCt method [21]. Ct values from control samples were used as calibrators. The gene-specific primers were designed on the basis of chicken mRNA sequences using Primer Version 5.0 (Table 1).

**Statistical analysis**

All data are presented as mean ± standard error of the mean (SEM) and were analyzed by t-test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the P < 0.05..
RESULTS

Metabolites and metabolic pathway affected by heat challenge

A total of 165 metabolites in the serum were identified using the non-target analysis, and heat challenge significantly affected the concentration of 7 metabolites (Fig.1). A total 6 metabolites were significantly higher in the heat-challenged group than in the control group (Beta-Alanine, L-Serine, Acetoacetic acid, 3-Aminoisobutanoic acid, Succinic acid and Urea), whereas one metabolite was lower in the heat-challenged group (1-Butylamine).

The MetaboAnalyst analysis indicated that ketone body metabolism, butyrate metabolism, arginine and proline metabolism were affected in the serum of heat-challenged quails (Fig. 2).

Histopathology analysis of liver

To investigate the effect of heat challenge on liver structure, morphological change in quail livers was examined. As shown by microscope (Fig.3), no obvious change was observed in the control liver (Fig.3 A and a). On the other hand, high ambient temperature induced lipid deposition in hepatocytes. The typical morphological changes of lipid steatosis were detected in the heat livers (Fig.3 B and b). This is the histologic appearance of hepatic macrovesicular steatosis (fatty change). The lipid accumulates in the hepatocytes as vacuoles. These vacuoles have a clear appearance with HE staining.

Serum and liver triglyceride and cholesterol level

Serum and liver triglyceride and cholesterol were detected. Both serum triglyceride and cholesterol lower in heat group (Fig.4 A, B), and cholesterol significantly lower in heat challenged quails (P < 0.05). However, triglyceride and cholesterol in the liver showed significantly higher in heat group (Fig 4 C, D)(P < 0.05).
**Lipid metabolic gene expression in liver**

The mRNA transcript levels of *PPARα* and *AGPAT5* significantly higher in heat challenged liver comparing with those in control group (P < 0.05) (Fig.5 A, D), *SREBF* and *AGPAT2* also higher in heat challenged liver, however did not have significant difference (P > 0.05).

**DISCUSSION**

The present study explored metabolite contents in the serum of quail under 10 days heat challenge. The results analyzed by MetaboAnalyst analysis revealed that (1) serum beta-alanine, l-serine, acetoacetic acid, 3-aminoisobutanoic acid, succinic acid and urea increased by heat challenge, (2) ketone body metabolism, butyrate metabolism, arginine and proline metabolism were affected by heat challenge. Increase of triglyceride and cholesterol level and lipid metabolic gene expression in liver indicates that heat challenge may cause disturbance in some metabolic pathway such as lipid metabolism in liver.

In the present study, acetoacetic acid significantly increased in heat-challenged quail, which suggest the rate of synthesis exceeds the rate of utilization. Ketone bodies, namely, acetone, acetoacetic acid, and β-hydroxybutyrate, are small lipid-derived molecules that serve as a circulating energy source for tissues in times of fasting or prolonged exercise [1]. During fasting, muscle and liver stores of glycogen are depleted first. Then, fatty acids are mobilized from adipocytes and transported to the liver for conversion to ketone bodies. Ketone bodies are transported from the liver to the extrahepatic tissues and converted into acetyl-CoA, which then enters the citric acid cycle and is utilized for energy production. The relative proportion of ketone bodies present in blood may vary from 78% (β-hydroxybutyric acid) to 20% (acetoacetic acid) and 2% (acetone)[10]. In normal conditions, the ketone bodies are produced constantly by the liver and utilized by peripheral tissues.

The increase of acetoacetic acid is commonly detected in the serum of diabetic
humans and rodents, which are more susceptible to heat-related illnesses and 
exogenous insulin rescues this phenotype [17, 22]. Furthermore, thermal therapy 
improves insulin sensitivity in diabetic and obese rodents [4, 9].

PPARα gene expression increased in heat challenged quail liver, since PPARα is 
a key transcriptional regulator of lipid metabolism and activated by a lipid surge to 
induce lipid catabolism. It plays an important role in the formation of fatty liver [15]. 
PPARα can also reduce inflammation, improve liver oxidative stress, and prevent 
liver tissue damage [13]. Present results indicate serum triglyceride and cholesterol 
level decreased after heat challenge, which agreed by Fujita et al, showed PPARα 
agonist can significantly reduce plasma triglyceride content, improve liver steatosis, 
and increase insulin sensitivity [2, 14].

Another gene, AGAPT5 increased in the liver after heat. Acylglycerophosphate 
acyltransferases/lyso phosphatidic acid acyltransferases (AGPAT) are a homologous 
family of enzymes that catalyze the formation of phosphatidic acid (PtdOH). Parks et 
al.[18] conducted antisense oligonucleotides (ASOs) to silence Agpat5 expression in 
liver and adipose tissue, and identify AGPAT5 as a gene associated with plasma 
insulin levels and insulin resistance, inhabitation of AGPAT5 could significantly 
improve glucose tolerance after high-fat or high-fructose feeding. PPARα can 
increase insulin sensitivity, however AGPAT5 cause insulin resistance, The 
triglyceride and cholesterol level increased in live, but cholesterol level decreased in 
serum may due to lipid metabolic disturbance in liver after 10 days heat challenge.

The liver of mammals and birds are different [11]. In avian species, lipids and 
especially triglycerides stores in adipocytes, hepatocytes and growing follicles [5]. 
When hepatic lipogenesis exceed the capacity of very low density lipoprotein (VLDL) 
secretion, triglycerides accumulated in the liver, and cause a steatosis [5]. One of the 
reasons contribute to the decreased ovarian and follicular weight might be liver 
damage and lipid metabolic dysfunction under high environmental temperature, the
synthesis of yolk precursor proteins in the liver were decreased.

High temperature may influence many aspects of animal. It takes further researches to clarify the mechanism under this situation. The present study demonstrated that high temperature influence nutrition absorption. Ketone body metabolism affect may be due to the change of insulin regulation. Consequently hepatic steatosis, hepatic metabolism dysfunction, especially lipid (triglyceride and cholesterol) may occur. The present results may suggest prevent in damage of liver under high temperature, increasing nutrition intake could be one solution for against problems caused by summer high temperature in poultry farm.

ACKNOWLEDGMENTS

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REFERENCES


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FIGURE LEGENDS

Fig.1 Seven serum metabolites in control group (Con) and heat group (Treat). A: Beta-Alanine, B: L-Serine, C: Acetoacetic acid, D: 1-Butylamine, E: 3-Aminoisobutanoic acid, F: Succinic acid, G: Urea. H: t. stat and P value of each metabolites (P < 0.05, t-test).

Fig.2 Metabolic pathway affected in the serum of quails under heat challenge.

Fig.3 HE staining of the sections derived from Japanese quail liver tissues, control group (A and a), heat group (B and b), bar = 50μm in A and B, bar = 10μm in a and b. Arrow indicate lipid deposition in hepatocytes in heat group.

Fig.4 Serum and liver triglyceride and cholesterol level. Serum triglyceride (A), serum cholesterol (B), liver triglyceride (C) and liver cholesterol (D) in control (Control) and heat treated (Heat) groups. Each bar represents the mean ± SEM. *Significantly different from the values at control (P < 0.05, t-test), n = 9/group.

Fig.5 Expression of lipid metabolic gene expression in liver in control (Control) and heat treated (Heat) groups. Each mRNA was normalized to the GAPDH mRNA expression level in the same preparation, and the mean of each experimental control was assigned a value of 1.0. A: liver peroxisome proliferator activated receptor alpha (PPARα) mRNA; B: liver sterol regulatory element binding factor (SREBF)
mRNA; C: liver 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2) mRNA; D: liver 1-acylglycerol-3-phosphate O-acyltransferase 5 (AGPAT5) mRNA. The values shown are the mean ± SEM. *Significantly different from the values at control (P < 0.05, t-test), n = 9/group.
### Table 1 Primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Size of PCR product (bp)</th>
<th>Annealing Temperature (°C)</th>
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<tbody>
<tr>
<td>PPARa</td>
<td>F: 5'-TTGTCGCTGCCCATATTGC-3'</td>
<td>189</td>
<td>60</td>
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<td></td>
<td>R: 5'-CTGGCAGTCGTGTTGAGAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBF</td>
<td>F: 5'-GCCCTTTGTCTTCCTGCT-3'</td>
<td>152</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATGCTCTTTCAGGACCACGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGPAT2</td>
<td>F: 5'-GTCATCACACATACCTGGG-3'</td>
<td>194</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGGACAGCGGATGAAAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGPAT5</td>
<td>F: 5'-TGCTGTATTCCTAGGTTGGCCTG-3'</td>
<td>184</td>
<td>59.8</td>
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<tr>
<td></td>
<td>R: 5'-ACCCACAGCCAGCAATTAA-3'</td>
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<td>GAPDH</td>
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<td>60</td>
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<tr>
<td></td>
<td>R: 5'-TAAGACCCCTCCAGATGCA-3'</td>
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Figure 1

![Box plots showing concentration changes for various metabolites](image)

### H. Summary of Significant Changes

<table>
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<tr>
<th>Name</th>
<th>t.stat</th>
<th>p.value</th>
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<td>Beta-Alanine</td>
<td>-10.391</td>
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<td>L-Serine</td>
<td>-4.1361</td>
<td>0.014422</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>-3.7704</td>
<td>0.019598</td>
</tr>
<tr>
<td>1-Butylamine</td>
<td>3.4003</td>
<td>0.02727</td>
</tr>
<tr>
<td>3-Aminoisobutanoic acid</td>
<td>-3.1316</td>
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</tr>
<tr>
<td>Succinic acid</td>
<td>-3.1211</td>
<td>0.03549</td>
</tr>
<tr>
<td>Urea</td>
<td>-3.1147</td>
<td>0.035709</td>
</tr>
</tbody>
</table>
Figure 2

Fold enrichment

Arginine and Proline Metabolism
Butyrate Metabolism
Ketone Body Metabolism
Figure 3

Control

Heat

A

B

a

b

a

b