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Chronic heat stress facilitates lipoprotein lipase expression and triglyceride biosynthesis in the adipose tissue of growing broiler chickens

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Exposure to chronic heat stress not only reduces body weight gain in growing broilers, but also enhances fat deposition. To elucidate this effect, numerous studies have investigated the changes in the liver, the primary site of *de novo* fat synthesis in avian species, and a marked increase in fat accumulation has been observed under chronic heat stress. However, the impact of heat stress on adipose tissue remains largely unclear. To investigate alterations in adipose tissue following heat-exposure, we reared growing broilers in a high-temperature environment (32 °C) for 14 d. Despite the decreased feed intake, the ratio of abdominal fat to body weight in the heat-exposed group remained comparable to that in the control group. Furthermore, the heat-exposed group exhibited a marked decrease in plasma free fatty acid concentrations. Transcriptome analysis of abdominal fat from heat-exposed and control groups indicated 459 differentially expressed genes. In the heat-exposed group, genes associated with lipid synthesis showed increased expression in the adipose tissue. Heat stimulation induced the expression of the lipoprotein lipase gene in cultured primary adipocytes. These results suggest that chronic heat exposure promotes lipoprotein lipase expression and triglyceride biosynthesis in the adipose tissue of growing broilers.

Keywords Broiler, Chronic heat stress, Adipose tissue, Lipoprotein lipase

Meat-type chickens are a primary protein source for humans in most parts of the world[1,2]. The incidence of heat stress has increased across nearly all poultry species with the rise in global temperatures. Heat stress results in annual economic losses of US$ 128–165 million in the poultry industry[3]. Modern commercial poultry produce more metabolic heat due to their rapid metabolism. This makes broilers more sensitive to environmental temperatures[4,5]. Therefore, efforts to maintain broiler chicken homeostasis are necessary to enhance bird survival and optimize performance in these environments[6].

Chronic heat stress induces anabolic alterations in lipid metabolism and increases fat deposition in broiler chickens[7–10]. Broilers subjected to chronic heat stress show a marked reduction in feed intake, leading to diminished body weight gain. However, the ratio of fat deposition to body weight increases, especially in the abdominal, subcutaneous, and intramuscular regions, compared to chickens reared at optimal temperatures[7–10]. Chronic heat stress exerts an anabolic effect on the metabolic pathways of broiler chickens in vivo, particularly those involved in lipid synthesis. Lipogenesis, the metabolic process that synthesizes fatty acids and triglycerides from glucose and other substrates, plays a crucial role in fat storage as a long-term energy source during periods of feed scarcity. Lipogenesis in avian species has unique characteristics compared to mammals, especially in terms of synthesis sites and endocrine regulation[11]. In birds, over 90% of *de novo* fat synthesis occurs in the liver, whereas *de novo* fat synthesis in adipose tissue plays a less important role than in mammals[12,13]. A substantial proportion of fatty acids in avian adipose tissue are triglycerides synthesized in the liver, which are packaged as

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plasma lipoproteins (e.g., very-low-density lipoprotein, VLDL; low-density lipoprotein, LDL), transported to the adipose tissue, and subsequently hydrolyzed into fatty acids for fat storage[14].

Due to its specific lipid metabolism, the liver is the primary site of *de novo* fat synthesis in broilers, and a marked increase in fat accumulation has been observed under chronic heat stress conditions. In addition to elevated triglyceride and cholesterol levels in the liver, the expression of genes related to fatty acid synthesis is upregulated[15]. An increase in plasma VLDL levels has also been observed, which facilitates the distribution of liver-synthesized fat to various tissues[15]. Transcriptomic and metabolomic analyses of the liver revealed that numerous genes encoding enzymes involved in various stages of fatty acid synthesis were upregulated by heat stress[16,17]. Consequently, increased fat synthesis in the liver of broilers is considered the primary driver of elevated fat accumulation throughout the body.

However, it is unclear how elevated lipid synthesis in the liver leads to increased systemic fat deposition, i.e., increased adipose tissue development. Although there is the possibility that several changes may occur in adipose tissue under chronic heat stress, most studies on fat deposition under chronic heat stress have concentrated on the liver and less attention has been given to change in adipose tissue. Although adipose tissue is the primary site of fat deposition, this gap in knowledge is partly due to the distinct lipid synthesis process in birds compared to that in mammals, and the relatively limited *de novo* fat synthesis in avian adipose tissue. Therefore, this study aimed to clarify the changes in lipid metabolism in chickens exposed to chronic heat stress in vivo, with a particular focus on adipose tissue alterations.

Results

Chronic heat exposure did not reduce fat accumulation in chickens despite a reduction in feed intake

Twenty-one-day-old chickens were divided into three groups: control, heat-exposed, and pair-fed groups. The control and heat-exposed group chickens were raised under optimal conditions at 24 °C and under chronic heat stress at 32 °C, respectively, for 14 d (Fig. 1a). The pair-fed group received restricted feed that matched the intake of the heat-exposed group on the previous day (Fig. 1a). The rectal temperature of the heat-exposed group was consistently higher by 0.4–1.4 °C higher than that of the control and pair-fed groups throughout the experimental period (Fig. 1b).

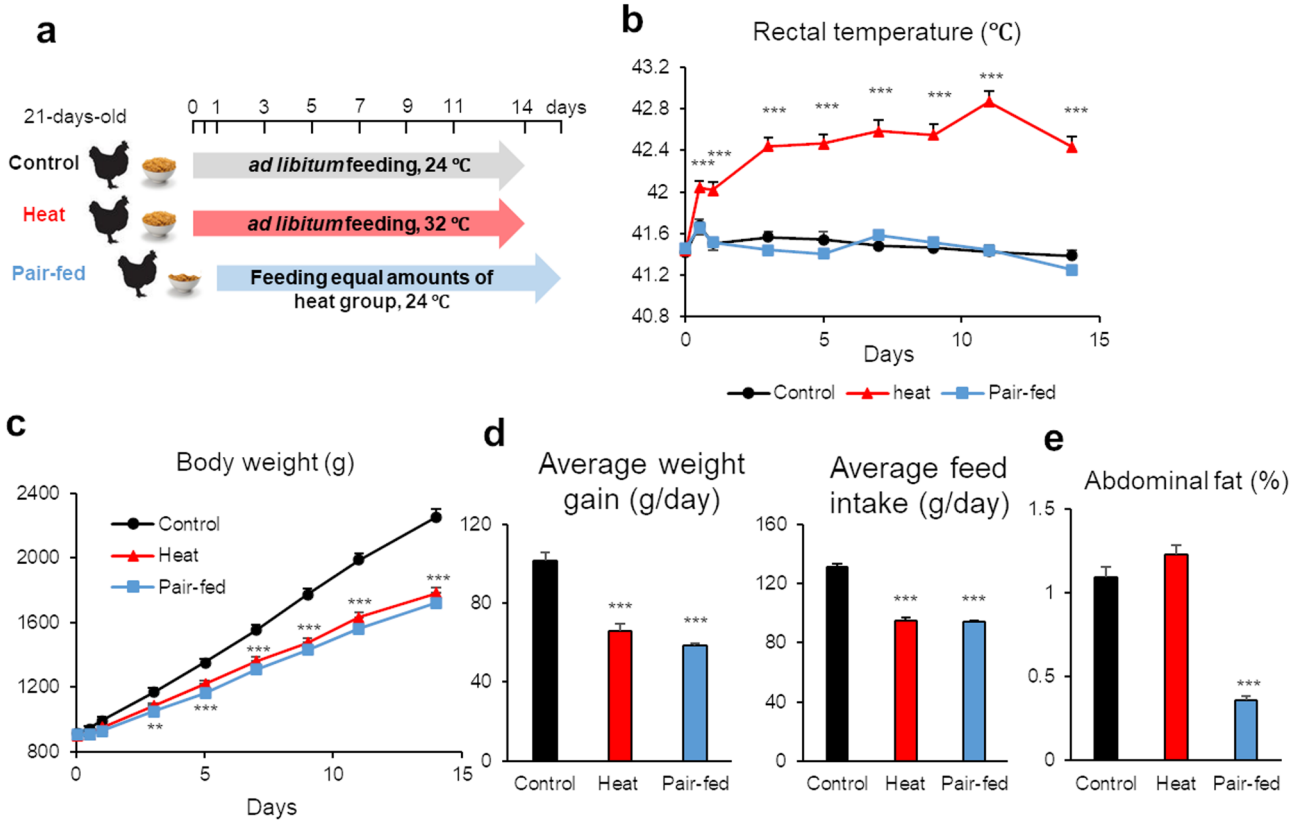

Fig. 1. Heat stress maintains adipose tissue development despite decreasing feed intake. (**a**) Schematic diagram of the experimental group. (**b**) Rectal temperature of 21–35-day-old broiler chickens. (**c**) Body weight change of 21–35-day-old broiler chickens. (**d**) Average body weight gain and feed intake of 21–35-day-old broiler chickens. (**e**) The ratio of abdominal fat to final body weight. All data were shown means ± standard error of mean (SEM), $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $n=12$.

Both the heat-exposed and pair-fed groups exhibited lower body weight gain than the control group, with final body weight reduced to approximately 80% of that of the control group (Fig. 1c). There was no marked difference in the body weight gain between the pair-fed and heat-exposed groups (Fig. 1d left). Average feed intake was approximately 70% lower in the heat-exposed and pair-fed groups compared to the control group (Fig. 1d right). The abdominal fat-to-body weight ratio was higher ($P < 0.001$) in the heat-exposed group, despite identical feed intake to the pair-fed group (Fig. 1e). Although the increase in adipose tissue percentage between the control and heat-exposed groups was modest ($P = 0.15$), these findings suggest that heat stress promotes fat accumulation at levels comparable to those in the control group, despite a marked reduction in feed intake.

Chickens exposed to chronic heat had similar lipid parameters as the control and lower plasma non-esterified fatty acid (NEFA) concentrations.

To elucidate the changes in lipid metabolic dynamics in vivo during heat exposure, plasma energy substrates and lipid parameters were measured. Blood glucose concentrations remained unchanged ($P > 0.05$) in all three groups (Fig. 2a). The pair-fed group showed a typical low-energy state characterized by increased NEFAs and markedly decreased triglyceride contents; these changes were not observed in the heat-exposed groups. The plasma triglyceride and total cholesterol concentrations in the heat-exposed group were comparable to those in the control group (Fig. 2c, d). Plasma NEFA contents were substantially lower in the heat-exposed group than in the other groups (Fig. 2b), with a decrease in NEFA concentrations beginning within 12 h of heat exposure.

Cholesterol present in the liver and other tissues is released into the plasma in the form of lipoproteins (high-density lipoprotein, HDL; LDL; VLDL). Lipoprotein-derived cholesterol concentration was determined. Plasma HDL, which acts as a carrier of cholesterol back to the liver for cholesterol reuse, was substantially higher in the pair-fed than in the control group. There were no statistical differences in the breakdown of LDL and VLDL, which are important for transport from the liver to the tissues (Fig. 2e). These results suggest that the lipid parameters in the pair-fed group reflected a starvation response and hypercholesterolemia due to feed restriction, whereas the lipid dynamics in the heat-exposed group were similar to those in the control group. Plasma NEFA concentrations were markedly lower throughout the experimental period in the heat-exposed group.

Chronic heat stress upregulated lipid biosynthesis-related genes in adipose tissue

Adipose tissue is crucial for fat metabolism, including the conversion of triglycerides to free fatty acids and glycerol. Because heat exposure reduces plasma NEFA concentrations, we hypothesized that adipose tissue (the primary source of NEFA) would be affected by heat stress. To investigate the effects of chronic heat stress on adipose tissue, transcriptome analysis was conducted to identify differentially expressed genes (DEGs) in the control and heat-exposed groups. Adipose tissue samples from three individuals in each group were subjected to RNA-Sequencing (RNA-Seq) (Fig. 3a, b). Raw data stats are listed in supplementary Table 1. A total of 459 DEGs with expression levels that differed by more than two-fold ($p < 0.05$, false discovery rate (FDR) < 0.1) from the control group were identified, with 244 upregulated and 215 downregulated genes in the heat-exposed group (Fig. 3c).

The upregulated DEGs included several genes crucial for fat accumulation such as lipoprotein lipase (*LPL*), which hydrolyzes lipoproteins to enhance fatty acid uptake by tissues, and perilipin 4 (*PLIN4*) and diacylglycerol O-acyltransferase 2 (*DGAT2*), which are both essential for triglyceride synthesis and maintain[18]. The expression of adipose triglyceride lipase (*PNPLA2*), a key enzyme in lipolysis, decreased in the heat-exposed group.

To elucidate the biological functions and processes associated with the DEGs, we conducted a Gene Ontology (GO) enrichment analysis. The GO enrichment analysis of all DEGs (Fig. 4a), as well as separate analyses of upregulated and downregulated DEGs, indicated that the upregulated DEGs were associated with terms related to lipid biosynthesis, such as lipid and glycerolipid biosynthesis (Fig. 4b). In contrast, downregulated DEGs were linked to cell division processes, including nuclear division and the mitotic cell cycle (Fig. 4c). These findings suggest that chronic heat exposure enhanced the function of genes related to lipid uptake and triglyceride biosynthesis in the adipose tissue.

The RNA-seq data indicated changes in the expression of genes involved in triglyceride and HDL synthesis, including *LPL*, *PLIN4*, *DGAT2*, and *APOA1*. Consequently, gene expression analysis of these genes was conducted using quantitative polymerase chain reaction (qPCR). *LPL* expression increased 1.5-fold in the adipose tissue of heat-exposed chickens compared to that in control and pair-fed chickens (Fig. 5a). The enzymatic activity of LPL was higher in the heat-exposed group (Fig. 5b). The expression of *PPARG*, a transcription factor that promotes *LPL* gene expression and regulates adipogenesis, was substantially higher in the heat-treated group. This suggests that the induction of *PPARG* enhanced *LPL* expression and activity in the adipose tissue under heat stress. Although *APOA1* gene expression was higher in the pair-fed group than that in the other groups, no statistical differences were observed between the control and heat-exposed groups. Substantial differences in the expression of *DGAT2* and *PLIN4* were found between the pair-fed and the other groups, but not between the control and heat-exposed groups. These results indicate that the *PPARG–LPL* pathway may be activated in the adipose tissue of heat-exposed chickens.

Heat stress enhanced LPL expression in cultured chicken adipocytes

Although heat-exposed chickens exhibited increased expression of *LPL* and *PPARG* genes in adipose tissue, it was unclear whether this upregulation in vivo was due to endocrine alterations or the direct effects of heat stress. To clarify this point, heat stimulation was applied to isolated chicken adipocytes to investigate the changes in lipid accumulation and gene expression. The extent of lipid accumulation in heat-stimulated adipocytes was similar to that in control cells (Fig. 6a). Quantification of lipid droplet formation was similar in terms of the number, area, and diameter of lipid droplets between the groups ($P > 0.05$) (Fig. 6b). However, there was a slight

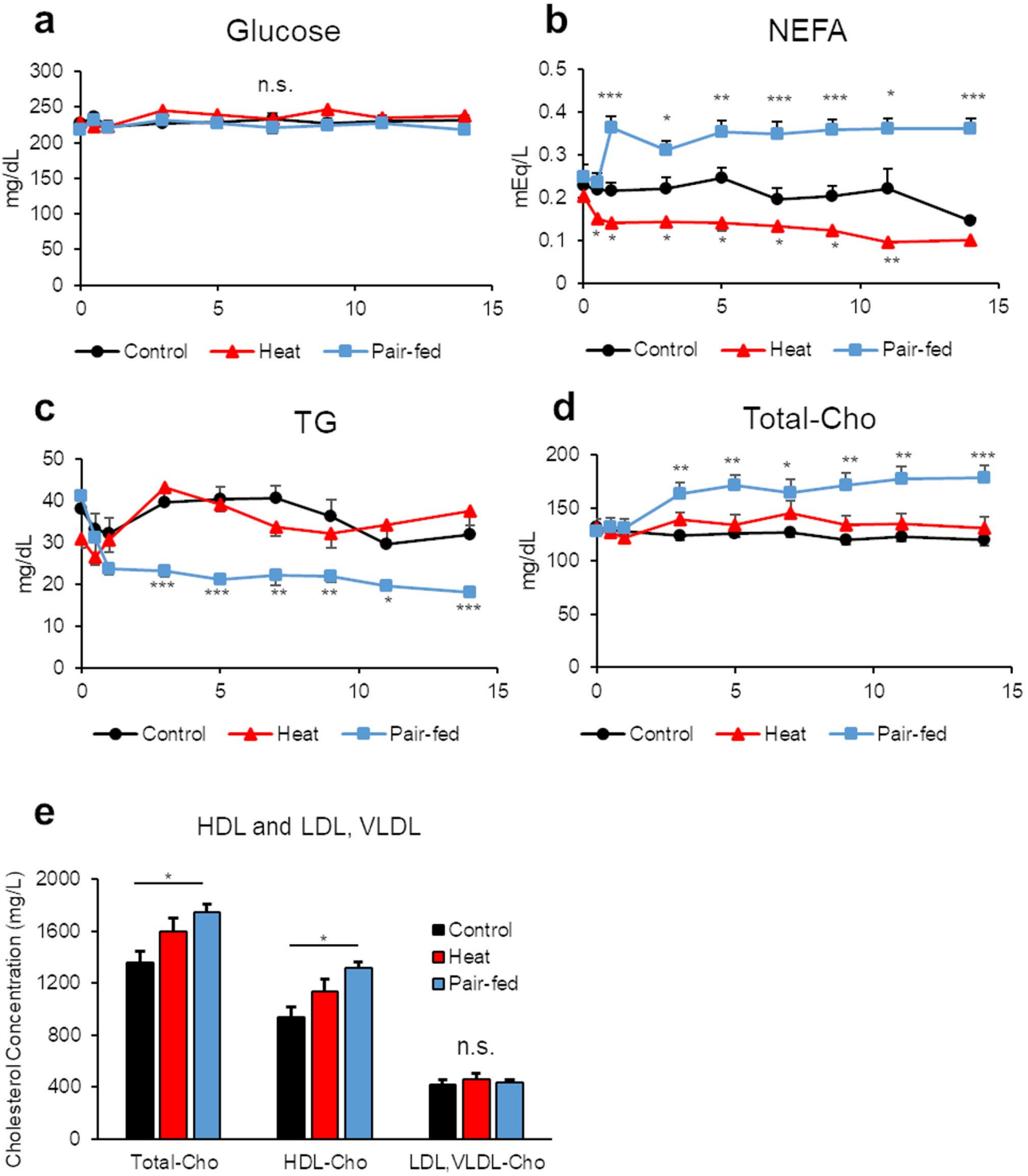

Fig. 2. Effects of heat exposure on plasma energy metabolite levels in broiler chickens. Plasma concentrations of glucose (**a**), non-esterified fatty acids (NEFA) (**b**), triglycerides (TG) (**c**), and total cholesterol (Cho) (**d**). All data were shown as means ± standard error of mean (SEM), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control at each time point, $n = 12$. (**e**) Plasma concentrations of total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol/very low-density lipoprotein cholesterol. Data are shown as means ± standard error of mean (SEM), $^{*}P < 0.05$; $n = 12$; ns = not significant.

increase ($P = 0.07$) in the percentage of lipid droplets with diameters greater than 10 μm in heat-stimulated cells (Fig. 6b).

Gene expression analysis indicated a marked increase (1.3-fold) in *LPL* expression in heat-stimulated adipocytes (Fig. 6c). Although the gene expression of *DGAT2*, *PLIN4*, and *PPARG* was increased in the adipose

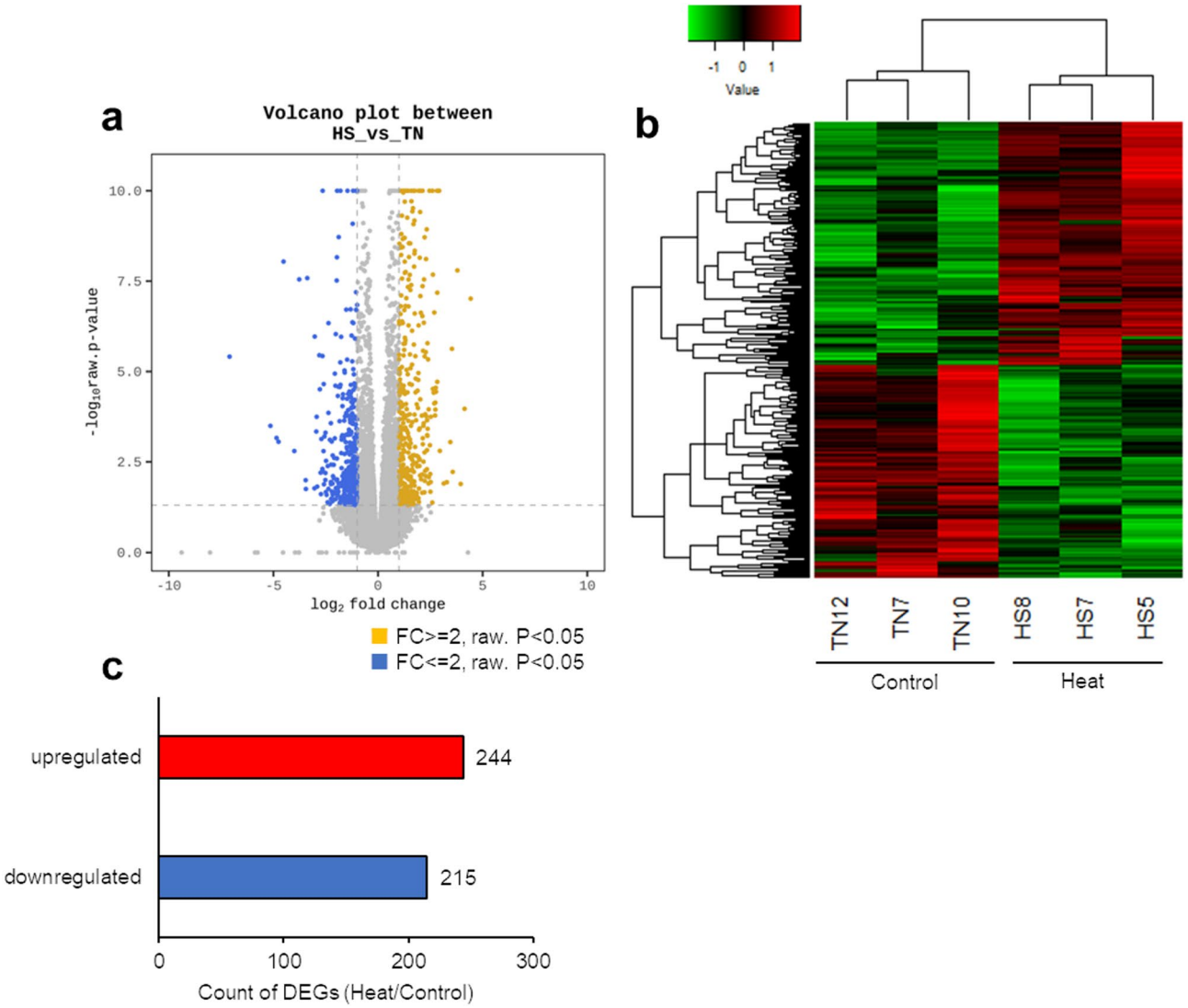

Fig. 3. Differentially expressed genes in the control versus heat stress-induced broiler chicken adipose tissue. (**a**) Volcano plot showing the fold changes ≥|2| (equivalent to an absolute log $_2$ FC ≥ 1) and P-values (< 0.05) of differentially expressed genes (DEGs) between control and heat stress-induced broiler chickens. (**b**) Heatmap showing the DEG profiles. (**c**) The number of DEGs. ($n = 3$ each)

tissue of heat-exposed chickens in vivo (Fig. 5c), no differences ($P > 0.05$) were observed in heat-stimulated adipocytes (Fig. 6c). These results suggest that heat stress alone can stimulate the expression of *LPL* in differentiated chicken adipocytes and increase lipid uptake.

Discussion

Since the initial report on increased fat deposition in broiler chickens due to heat exposure, numerous studies have focused on blood components, endocrine changes, and the liver. However, the precise mechanisms underlying these alterations in adipose tissue under heat stress remain unclear. In the liver, chronic heat stress facilitated excess lipid deposition and was involved in increasing the levels of fatty acid synthesis-related factors, such as ACC and FASN[15]. The results of transcriptome and metabolome analyses together provide convincing evidence of increased fat deposition under heat stress[16,17]. However, there is no evidence that chronic heat stress has an effect on adipose tissue, which merely receives lipids from the liver. We proposed that the transcript expression profiles in the adipose tissue were susceptible to change due to chronic heat stress.

This study is the first to investigate the effects of heat exposure on growth rate and blood components in chickens. Heat-exposed chickens exhibited body weight changes comparable to those of the pair-fed group, indicating that the reduction in body weight gain was primarily attributable to decreased feed intake. However, the fat accumulation ratio in the heat-exposed group remained the same as that of the control group. In contrast, fat accumulation was substantially reduced in the pair-fed group (Fig. 1). This finding is consistent with the blood lipid metabolic parameters of heat-exposed chickens, such as triglyceride and cholesterol, which were maintained at levels similar to those of the control group (Fig. 2). Based on the results of plasma NEFA levels,

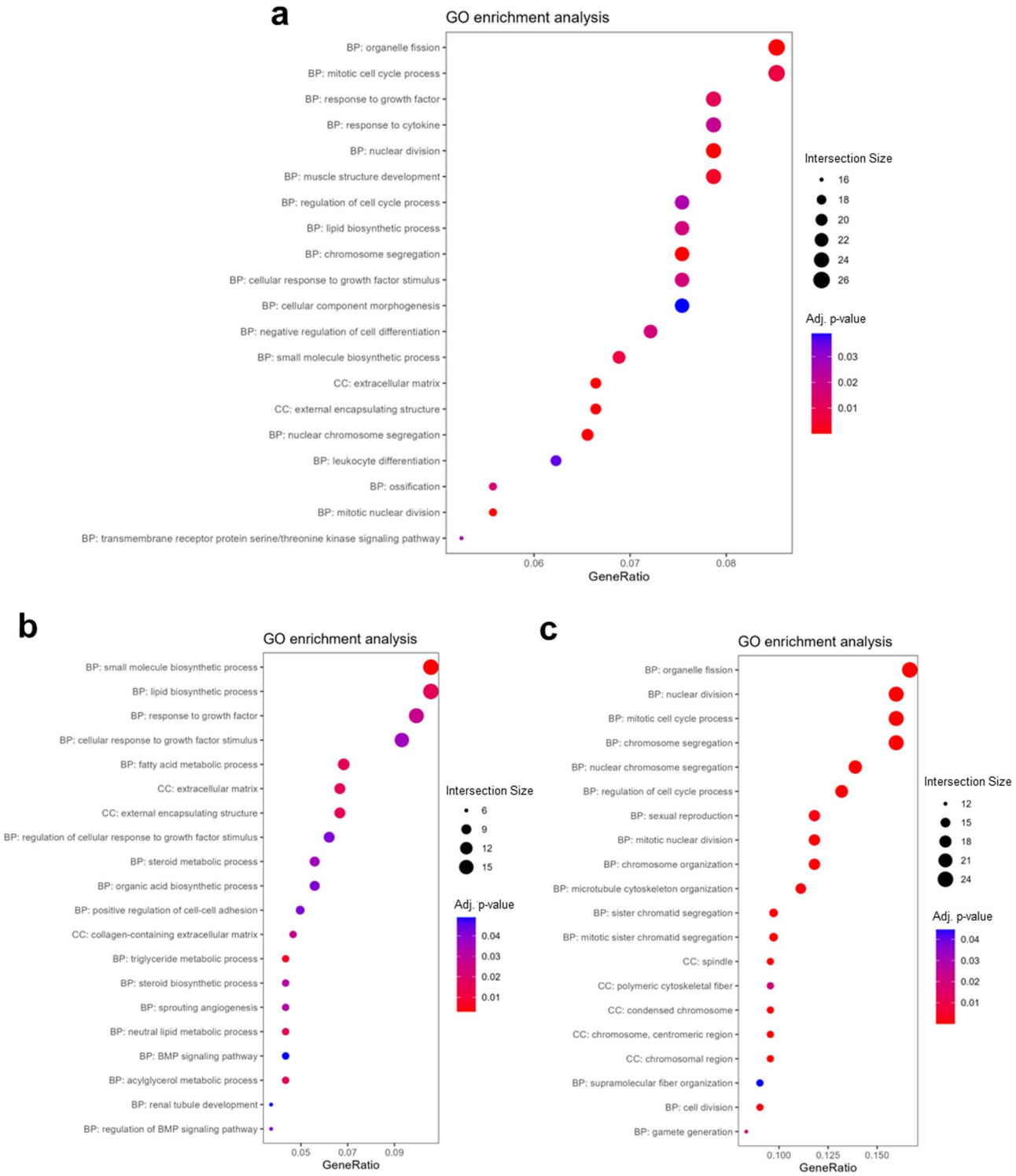

Fig. 4. Gene ontology (GO) enrichment analysis of adipose tissue of control and heat stress-induced broiler chickens. GO enrichment analysis of all identified (**a**), upregulated (**b**), and downregulated DEGs (**c**).

it can be speculated that the Pair-fed group was in a nutrient deficient state in vivo as a reason of the restricted feeding. An increase in blood NEFA levels indicates a significant acceleration in fat utilization due to energy deprivation. On the other hand, NEFA levels in the heat-exposed group were lower than those in the control group as well as in the pair-fed group, despite the same amount of feed intake. The plasma NEFA concentrations decreased substantially half a day after heat exposure (Fig. 2). Reductions in NEFA concentrations following chronic heat exposure have been reported[17,19,20]. Wang et al. suggested that the decrease in NEFA concentrations associated with chronic heat exposure was due to increased levels of the antilipolytic hormone, insulin[19].

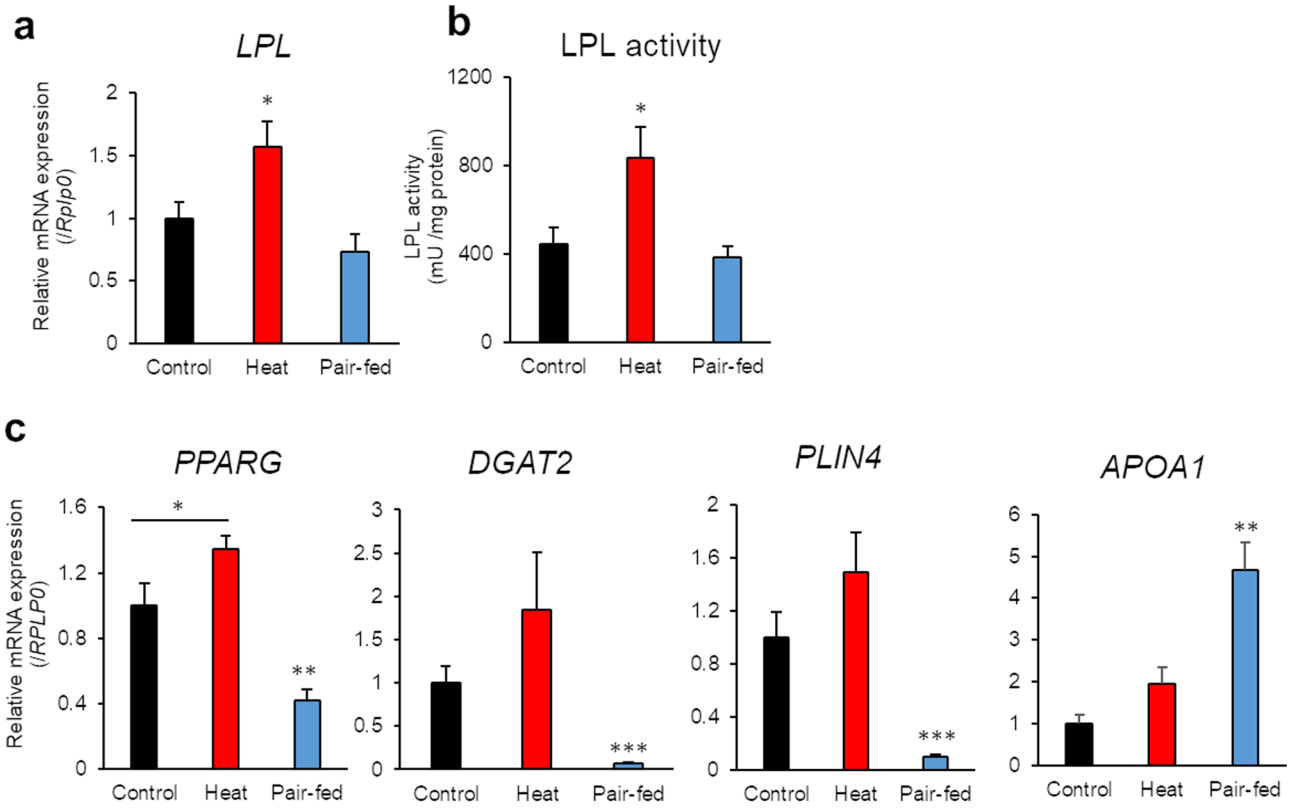

Fig. 5. Heat stress facilitates gene expression and activity of lipid assimilation-related factors in abdominal fat. (**a**) Gene expression analyses of *LPL* in 35-d-old broiler chickens. (**b**) LPL activity in abdominal fat of 35-d-old broiler chickens. (**c**) Expression of *PPARG*, *DGAT2*, *APOA1*, and *PLIN4* in the abdominal fat of 35-d-old broiler chickens. *RPLP0*: internal control gene. Data are shown as means ± standard error of mean (SEM), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 12$.

The transcriptome data in the current study suggest that heat stress repressed lipolysis in the adipose tissue because the gene expression of *PNPLA2*/ATGL (an enzyme involved in triglyceride hydrolysis) decreased in the abdominal fat of heat-exposed chickens[21]. The observed reduction in NEFA concentrations suggests that heat exposure may alter endocrine mechanisms and inhibit lipolysis in the adipose tissue.

Transcriptome analysis of adipose tissue indicated that chronic heat exposure altered the expression of lipid synthesis-related genes in adipose tissue. Specifically, the gene expressions of *LPL* and *PPARG* were elevated (Fig. 5a, b), suggesting that heat exposure promoted lipid synthesis in the adipose tissue. In broiler chickens, heat exposure enhances fat accumulation and increases the expression of genes involved in fat synthesis in the liver[15,22]. Based on the findings of the current study, the entire body appears to be oriented towards fat assimilation during chronic heat exposure.

Lipoprotein lipase (LPL) is a key enzyme involved in the hydrolysis of triglycerides and facilitates the uptake of fatty acids by tissues[23]. LPL plays a central role in avian adipose tissue. As *de novo* fat synthesis in birds predominantly occurs in the liver, fat stored in the adipose tissue is largely derived from the liver. Nascent fat synthesized in the liver takes the form of lipoproteins, which are hydrolyzed into fatty acids by LPL in various tissues, including adipose tissue, and are subsequently incorporated into the tissue for fat storage[11]. In addition, peroxisome proliferators activated receptor γ (PPARγ), a well-established upstream regulator, activates *LPL* transcription. *PPARγ* gene expression was elevated in adipose tissue exposed to chronic heat (Fig. 5c). PPARγ is also a master regulator of adipogenesis[24]. Heat stress was also reported to increase the transcription levels of fat synthesis genes, including *PPARγ*, and facilitated the formation of a large number of lipid droplets in 3T3–L1 adipocytes[25]. Thus, chronic heat exposure could partially affect the expression of lipid synthesis-related genes in adipose tissue. Besides, sterol regulatory element-binding protein (*SREBP*) expression was increased 2-fold, that acts in downstream of Liver X receptors (LXRα and LXRβ). LXR-SREBP have a pivotal role in the metabolic conversion carbohydrate to lipid[26]. Because LXRs are highly expressed in adipose tissue and involved in lipid accumulation in mature adipocyte[26,27], heat stress may also facilitate this pathway and contribute to increasing the fat deposition.

The DEGs included several genes related to the cell cycle and cell division. Downregulation of the cell cycle- and mitosis-related genes suggests that heat exposure may suppress cell proliferation in the adipocytes. Similar suppression of the cell cycle has been observed in the livers of broilers under chronic heat stress, potentially allowing time for DNA damage repair caused by heat stress[16]. However, GO terms related to DNA repair were

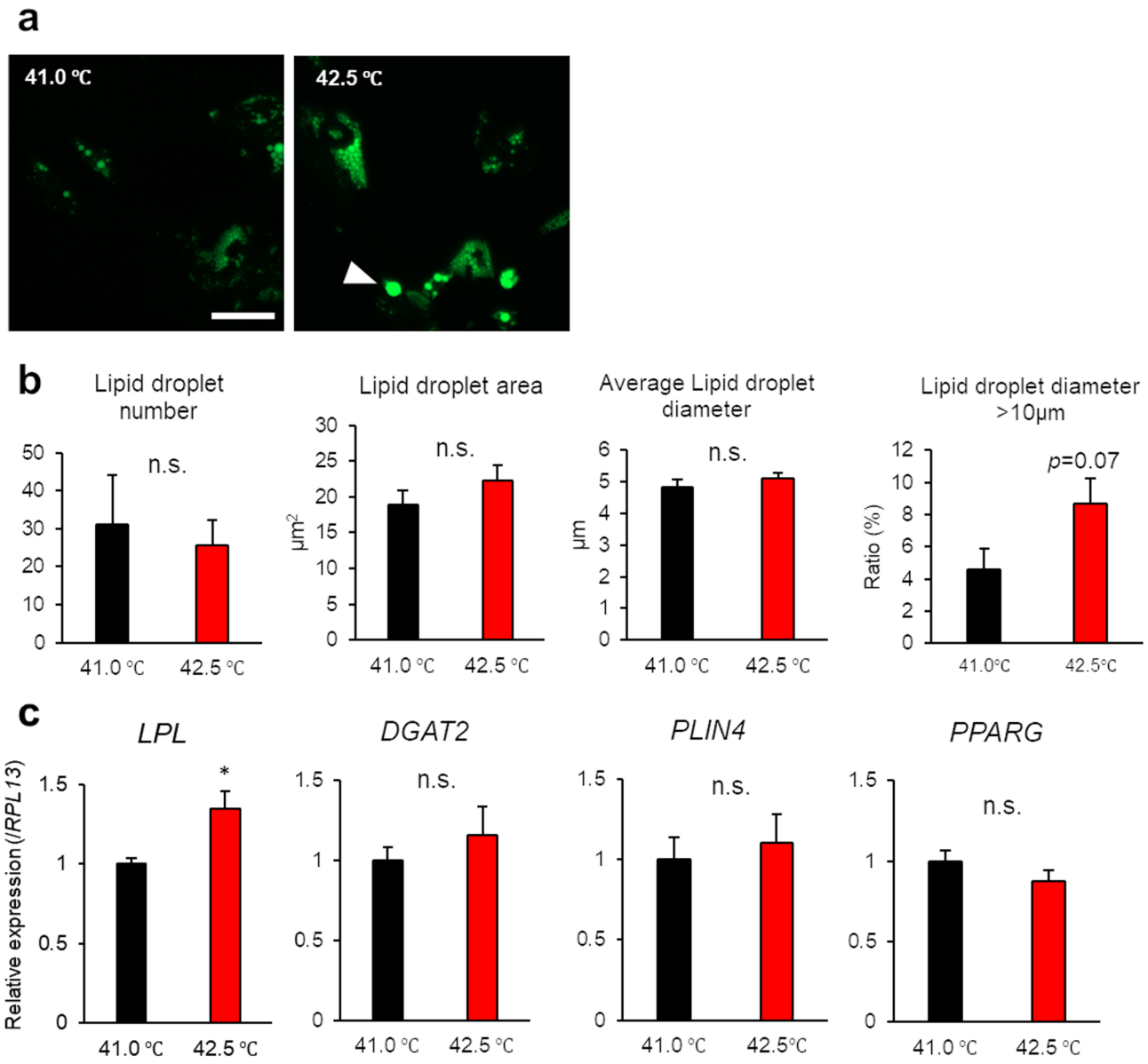

Fig. 6. Heat stress enhances lipid accumulation in cultured chicken adipocytes. (**a**) Differentiation was induced in cultured adipocytes and cells were incubated at 41.0 ℃ or 42.5 ℃. After 48 h, the cells were stained with BODIPY 493/503. Green fluorescence indicates lipid droplets. White arrowhead shows the lipid droplet with diameter > 10 μm. Scale bar = 50 μm. (**b**)The ratios of lipid droplet number, area, diameter, and diameter > 10 μm. (**c**) Gene expression analysis of *LPL*, *DGAT2*, *PLIN4*, and *PPARG*. *RPL13*: internal control gene. Data are shown as means ± standard error of mean (SEM), $^{*}P < 0.05$, $n = 6$.

not identified in the enrichment analysis of adipose tissue in the current study. Further investigation is required to clarify these findings.

Gene expression analyses in in vivo and in vitro models of the effects of heat alone indicated increased gene expression and LPL activity. It is interesting that thermal stimulation increases *LPL* mRNA expression, despite it has been shown that *LPL* mRNA expression is less responsive to aging and nutritional manipulation in growing chickens[28]. Similar results have been observed in pigs, where triglyceride uptake from the plasma and accumulation in adipose tissue were thought to be enhanced, leading to increased fat deposition[29]. Although in vitro experiments showed that thermal stimulation directly upregulated *LPL* expression, there was no change in *PPARG* expression, which was increased in vivo. This discrepancy suggests that heat alone may not influence *PPARG* expression, or that *PPARG* might already be upregulated 48 h after differentiation.

The key question here is the physiological significance of the increased fat accumulation induced by chronic heat exposure. Heat exposure has been shown to enhance fat accumulation in pigs and 5-w-old rats[29–31], whose growth potential is comparable to that of broilers; however, this effect was not confirmed in 48-w-old egg-laying hens[32], suggesting that increased fat deposition is a response specific to growing animals. One possible factor

is underactivity of the thyroid gland, which may slow metabolism and promote fat storage. The thyroid gland synthesizes two major hormones, T4 (3,5,3′,5′-tetraiodo-l-thyronine) and T3 (3,5,3′-tri-iodo-l-thyronine), which are essential for normal growth and development[33]. T4 is classically viewed as a prohormone that must be converted to T3 via tissue-level deiodinases for biological activity. Decreasing serum T4 and T3 contents lead to a marked decline in the basal metabolic rate, along with a decrease in protein and fat catabolism[34,35]. In chickens, both T3 and T4 play important roles in the regulation of metabolism and thermogenesis. To support this, Beckford et al. reported that heat stress significantly reduced the T3 concentration by 62% in broilers[36]. However, this alone may not fully explain the observed fat accumulation, as thermal stimulation was shown to directly increase LPL expression in the current study. The current study provides novel insights into how chronic heat exposure affects energy assimilation at both the tissue and cellular levels, emphasizing the need for further research on the effects of heat from alternative perspectives.

In summary, heat exposure affects not only the liver, which is traditionally considered the primary driver of increased fat accumulation, but also adipose tissue, which serves as the fat storage organ. Specifically, heat exposure enhances lipid synthesis in the adipose tissue, with LPL, a key enzyme involved in the uptake of lipoproteins from the liver, upregulated under heat stress. As chicken fat is not typically an edible component, increased fat accumulation in broilers represents an inefficient use of energy[37]. Therefore, reducing heat-induced fat accumulation is essential for improving the energy efficiency and productivity of broilers. To achieve this, it is important to address the changes that occur in both the adipose tissue and the liver. This study is the first to demonstrate that enhanced LPL expression, which is involved in lipid uptake and triglyceride synthesis in adipose tissue, contributes to the increased fat accumulation observed in broilers. These findings provide new insights into the effects of chronic heat stress on broilers and highlight the importance of managing fat deposition to improve production outcomes.

Methods

Animal experiment

All animal experiments were performed in accordance to the principles of the Basel Declaration and were approved by the Tohoku University Institutional Animal Care and Use Committee (no. 2021-020-01), and performed under humane endpoints to minimize pain in the broiler chickens. All animal experiments were performed in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University (https://www.clag.med.tohoku.ac.jp/wordpress/wp-content/uploads/2024/04/3-kitei-E-17th.pdf) and relevant guidelines. The study is reported in accordance with ARRIVE guidelines. Thirty newly-hatched male broiler chicks (Ross strain, *Gallus domesticus*) were purchased from a commercial hatchery (Miyagi, Japan) for use in the control and heat-stress groups. The following day, 15 newborn broiler chicks were purchased from a same hatchery as a pair-fed group. All the chicks were housed in electrically-heated battery cages. At 14 d of age, nine chicks with markedly different weights were excluded, and the chicks were allocated to three treatments with 12 chickens in a completely randomized design. All chickens were transferred into individual wire cages in environmentally-controlled chambers and housed at an optimum temperature (22.0 ± 0.5 °C, humidity 50 ± 0.5%). The chickens were housed under 24-h light conditions throughout the experimental period. All chicks were fed the same experimental diet until the end of the experiment (Table 1). The nutrient composition of the diets in present study was based on the Japanese Feeding Standard for Poultry[38].

Chronic heat exposure

An overview of the experiments is presented in Fig. 1a. At 21 d of age, control and heat-stressed group chickens were exposed to control conditions (22.0 ± 0.5 °C, humidity 50 ± 0.5%) or heat stress (32.0 ± 0.5 °C, humidity 50 ± 0.5%) until 35 d of age (Fig. 1a). The feed intake and body weight were recorded daily. Pair-fed chickens started the experiment one day later in thermoneutral conditions (22.0 ± 0.5 °C, humidity 50 ± 0.5%) and were fed twice daily with and amount of feed equal to the average feed intake of the heat-stressed group on the previous day. Blood samples were collected 0.5, 1, 3, 5, 7, 9, 11, and 14 d after the start of heat exposure. At 35 d of age, the chickens in each group were euthanized by cervical dislocation ($n = 12$). Isolated abdominal fat tissues were weighed and frozen in liquid nitrogen, powdered, and stored at -80 °C until use.

Blood analysis

To separate plasma from blood cells, collected blood samples were immediately centrifuged at 1500 × *g* for 15 min at 4 °C. The plasma was collected as small aliquots and stored at -80 °C until analysis. Several blood analyses were performed using the following kits: glucose CII-Test to measure glucose concentration, cholesterol E-test to measure total cholesterol concentration, triglyceride E-test to measure triglyceride concentration, and NEFA-C-Test to measure the NEFA concentration (all from Wako Pure Chemical Industries, Osaka, Japan), according to the manufacturer's instructions. The serum lipoprotein cholesterol content was determined using HDL and LDL/VLDL cholesterol assay kits (Cell Biolabs, Inc. San Diego, CA, USA) according to manufacturer's instructions.

RNA-seq

Total RNA was extracted from adipose tissues using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The extracted RNA was purified using the RNeasy Mini Kit (QIAGEN). The RNA samples were submitted to Macrogen Japan Co., Ltd. (Tokyo, Japan) for RNA-seq analysis. Double-stranded cDNA was synthesized using the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA), and next-generation sequencing analysis was conducted using a NovaSeq 6000 (100-bp paired-end reads). The sequence reads were trimmed using Trimmomatic version 0.38 and then mapped to the reference genome (*Gallus gallus* genome: ARS-UCD1.2) using HISAT2 version 2.1.0, followed by transcript assembly

Ingredient	(%)
Corn	46.61
Sorghum grain	10.00
Soya bean meal	20.00
Corn gluten meal	9.00
Rice bran, de-oiled	4.00
Fishmeal (CP 65%)	1.50
Fat	4.00
Salt	0.32
Limestone	0.90
Dicalcium phosphate	1.80
Choline chloride	0.10
DL-methionine	0.25
L-lysine hydrochloride	0.55
L-arginine	0.32
L-threonine	0.20
L-tryptophan	0.10
L-valine	0.10
Vitamin-mineral premix *1,2	0.25
Total	100.00
Calculated nutritional values	
ME (Mcal/ kg) *3	3.10
CP (%) *4	21.20

Table 1. Composition of the diet. *1 Provided per kilogram of diet: retinol acetate, 1 mg; cholecalciferol, 5 µg; α-tocopherol acetate, 10 mg; thiamin hydrochloride, 1.8 mg; riboflavin, 3.6 mg; pyridoxine hydrochloride, 3.5 mg; calcium pantothenate, 10 mg; 2-methyl-1,4-naphthoquinone, 0.5 mg; folic acid, 0.55 mg; cyanocobalamin, 0.01 mg; biotin, 0.15 mg. *2 Provided per kilogram of diet: $MnSO_4 \cdot 5H_2O$, 316.4 mg; $ZnSO_4$, 129.5 mg; $FeSO_4 \cdot 7H_2O$, 522 mg; $CuSO_4$, 26.34 mg, KI, 0.6 mg; Na_2SeO_3, 3.92 mg; $CoCl_2 \cdot 6H_2O$, 3.92 mg; MoO_3, 0.6 mg. *3 ME = metabolizable energy. *4 CP = crude protein.

and quantification using StringTie version 1.3.4d. Finally, differential expression analysis was carried out using edgeR[39]. P-value < 0.05 and FDR < 0.1 was set as the threshold to declare significant associations. All data analyses were performed by Microgen Japan Co., Ltd. The data have been disclosed in the DDBJ database as Submission No. GSE292022 (https://www.ncbi.xyz/geo/query/acc.cgi?acc=GSE292022).

Gene ontology enrichment analysis

GO analyses of the DEGs were performed using the g: Profiler online tool (https://biit.cs.ut.ee/gprofiler), with $P < 0.05$ used as the threshold for significance[40,41]. GO was divided into three gene categories: biological processes (BP), cellular components (CC), and molecular functions (MF). Only the top 20 BP, CC, and MF terms were used. The figures were generated using R version 4.0.3.

Lipoprotein activity

Approximately 200 mg of frozen abdominal fat tissue was weighed and homogenized using a Micro Smash MS-100 (Tomy Seiko Co., Tokyo, Japan) at 3,000 rpm for 30 s in 1 mL of cold buffer (20 mM Tris, pH 7.5, 150 mM NaCl). The homogenized samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. LPL activity was determined using a Lipoprotein Lipase Activity Assay Kit (fluorometric; Cell Biolabs. Inc.) according to the manufacturer's instructions. The fluorescence intensity was detected at 510 nm using a microplate reader (POWERSCAN HT; DS Pharma Biomedical, Osaka, Japan). Protein concentration quantification was carried out using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture

Chicken stromal–vascular cells were isolated according to the method of Matsubara et al. (2005)[42]. Briefly, the abdominal adipose tissue was collected from 10-d-old male broiler chicks (Ross strain) by sterile dissection following rapid decapitation of the birds. The abdominal tissue was minced into fine sections with scissors and incubated in 10 mL of digestion buffer (PBS (−), 0.1% collagenase, 2.8 mM glucose, 4% bovine serum albumin (all from Wako Pure Chemical Industries)) for 45 min at 37 °C in a water bath. After the incubation, Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich, St Louis, MO, USA) containing 10% FBS (Hyclone; Cytiva, Marlborough, MA, USA), 100 U/mL penicillin and streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) was added. Flask contents were mixed and filtered through nylon screens with 100-µm mesh openings to remove undigested tissue. The filtered cells were centrifuged at 500 × *g* for 5 min to separate the floating adipocytes from the stromal–vascular cell pellets. The stromal–vascular cells were then seeded in 75-cm^2 collagen type I-coated dishes at a

Gene name	Accession no.	Sequence (5'-3')	Product length (bp)
LPL	NM_205282.2	Fwd: ATG TTC ATT GAT TGG ATG GAG GAG	159
		Rev: AAA GGT GGG ACC AGC AGG AT	
PPARG	NM_001001460.2	Fwd: CAC TGC AGG AAC AGA ACA AAG AA	67
		Rev: TCC ACA GAG CGA AAC TGA CAT C	
DGAT2	XM_419374.8	Fwd: ATC ATC ATC GTG GTG GGA GG	249
		Rev: GCC ATG GAA GAT GCA TGG AG	
PLIN4	NM_001317088.2	Fwd: TAG CTT CCC CCA GTG CCT AT	147
		Rev: CAC CAG GCA CCA TCA CTG G	
APOA1	NM_205525.5	Fwd: GTG ACC CTC GCT GTG CTC TT	217
		Rev: CAC TCA GCG TGT CCA GGT TGT	
RPLP0	NM_204987.2	Fwd: TTG GGC ATC ACC ACA AAG ATT	83
		Rev: CCC ACT TTG TCT CCG GTC TTA A	
RPL13	NM_204999.2	Fwd: GGC CCG TGT TAT CTC AGA GG	89
		Rev: GGA TCC CAA AGA GAC GAG CG	

Table 2. Primer sequences.

density of 1×10^4 cells/cm^2 and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. At 90% confluence, cells were detached using trypsin-EDTA (Nacalai Tesque, Inc.), and collected cells were centrifuged at 500 × *g* for 5 min. The resuspended cells were seeded at a density of 1×10^4 cells/cm^2 in collagen-coated culture dishes containing the respective media. The next day, the medium was changed in DMEM containing 10% FBS, 500 nM dexamethasone (Wako), 0.5 mM 3-isobutyl-1-methylxanthine (Wako), 20 µg/ mL bovine insulin (Sigma) and 300 µM oleate (Nacalai Tesque, Inc.) for differentiation induction. Considering that the body temperature of heat-exposed chickens was approximately 0.4–1.4 °C higher than the normal temperature of 41.0 °C in the control group, control cells were cultured at 41.0 °C and heat-stressed cells were incubated at 42.5 °C. After 48 h, cells were used for RNA extraction or lipid droplet staining.

RNA extraction and qPCR

Total RNA was extracted from the isolated adipose tissues or cultured cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Frozen abdominal fat tissues were homogenized using a Micro Smash MS-100 (Tomy Seiko) at 3,000 rpm for 30 s in 1 mL of TRIzol reagent. Total RNA purity and concentration were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). One microgram of total RNA was reverse transcribed with mixed primers consisting of oligo (dT) and random hexamers into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Gene expression levels were determined using a TB Green Premix Ex Taq II Kit (Takara Bio Inc., Shiga, Japan). PCR was performed using a CFX Connect system (Bio-Rad Laboratories, Hercules, CA, USA) and quantified based on the Pfaffl method[43]. The results were normalized to *RPLP0* or *RPL13* gene expression levels and are shown as fold changes relative to the control. The primer sequences are listed in Table 2.

Lipid droplet staining and quantification

Differentiated stromal–vascular cells were washed twice with PBS and stained for 1 h at room temperature with 1 µg/mL suspension of Bodipy493/503 (Thermo Fisher Scientific) in PBS-T (PBS and 0.1% Triton-X), a permeabilization buffer. The stained cells were washed three times with PBS and observed using a KEYENCE BZ-X800 all-in-one microscope (Keyence, Osaka, Japan). The lipid droplet number, area, and diameter were quantified using a BZ-X800 Analyzer software.

Statistical analysis

All statistical analyses were performed using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean ± standard error of the mean. All data were analyzed using a completely randomized design. Statistical significance was determined using a one-way analysis of variance, followed by a post-hoc Tukey–Kramer test or Steel–Dwass test. In the cell culture experiment, Student's *t*-test was used to compare the control and heated groups; $P < 0.05$ was considered to be significant.

Data availability

All data supporting the findings of this study are available within the article and its supplementary information file or are available from the corresponding author upon request. Sequence data that support the findings of this study have been deposited in the Gene Expression Omnibus of NCBI with the primary accession code GSE292022. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE292022.

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Conceptualization, Y.T. and K.S.; methodology, Y.T. and K.S.; validation, Y.T. and T.M.; formal analysis, Y.T.; investigation, Y.T. (prepared Figs. 1, 2, 3, 4, 5 and 6), T.M. (prepared Figs. 5 and 6), and C.A. (prepared figures 6); data curation, Y.T.; writing—original draft preparation, Y.T., M.R.H., and S.Y.; writing—review and editing, Y.T., S.Y. and K.S.; supervision, K.S.; project administration, Y.T. and K.S.; funding acquisition, Y.T. and K.S. All authors have read and reviewed the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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