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Genome-wide association study on the body temperature changes of a broiler-type strain Taiwan country chickens under acute heat stress

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ABSTRACT

Body temperature is the simplest parameter for evaluating the physiological conditions of animals under thermal stress. Genome-wide association studies (GWAS) have identified candidate genes related to economic traits in domestic animals. The present study conducted a GWAS on body temperature changes in a broiler-type strain Taiwan country chickens (TCCs) under acute heat stress. A total of two hundred 30-week-old roosters of a broiler-type strain TCCs were used. The roosters were subjected to acute heat stress at 38 °C for 4 h, and their body temperature was recorded before and during heat stress. The change in body temperature (ΔT) of each rooster was calculated according to the difference between the initial temperature and the highest body temperature during heat stress. The roosters were categorized according to survival and ΔT at the end into dead, susceptible, resistant, and intermediate groups. Collected red blood cells were genotyped using a 600 K chicken single-nucleotide polymorphism (SNP) array. A GWAS for ΔT was conducted using the Cochran–Armitage trend test. Significant SNPs were annotated as candidate genes according to the nearest genes. Results indicated that the ΔT of the heat-resistant group was significantly lower than that of the heat-susceptible group. A total of 17 SNPs belonging to 8 candidate genes, 352 SNPs for 78 candidate genes, and 174 significant SNPs for 63 candidate genes were identified in the association analyses in the dead *vs.* survival, resistant *vs.* susceptible, and intermediate *vs.* susceptible groups, respectively. The annotated candidate genes are associated with apoptosis, cellular response to external stimuli, and signal transduction pathways. In conclusion, the significant SNPs located in and proximal to genes in the GWAS analysis were related to apoptosis or responses to external stimuli which serve as potential candidates underlying physiological adaptation to heat stress or thermotolerance in chickens.

1. Introduction

Rapid climate change is a major threat to the sustainability of livestock production worldwide (Silanikove and Koluman, 2015; Nawab et al., 2018). High ambient temperature accompanied by high humidity causes the major economic loss of livestock in subtropical countries such as Taiwan (Liang et al., 2016). The necessity of reducing the detrimental effects of environmental challenges applies to all species. However, chickens apparently are more sensitive to high environmental temperatures than other domestic animals (Geraert et al., 1993). The degree of thermotolerance differs among species and breeds as well as within breeds (Hoffmann, 2010; Renaudeau et al., 2012; Boettcher et al., 2015) due to genetic variations in their thermoregulatory mechanisms and energy exchange (Salah et al., 1995). Moreover, domestic animals with high productive performance are more susceptible to stress (Washburn et al., 1980; Cahaner et al., 1995). Heat stress is a reflex reaction in overbearing environmental conditions and causes poor growth performance (Bottje and Harrison, 1985), immunosuppression (Young, 1990), and high mortality (Yahav et al., 1995) in domestic animals. The broiler-type B strain Taiwan country chickens (TCCs) comprise a closed population that have been selected for their high growth rate as the sire line since 1982 (Lee, 1992). In contrast to exotic breeds, male TCCs demonstrate favorable thermotolerance, but their semen quality and fertility also decrease under hot

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conditions (Lee, 1992); moreover, a functional genomics analysis revealed altered testicular responses to acute heat stress (Wang et al., 2013, 2014).

Under high temperature environments, chickens change their behavioral responses and physiological homeostasis to maintain normal core body temperature (Lara and Rostagno, 2013). Body temperature is considered the simplest parameter for evaluating the physiological conditions of animals under thermal stress (Bianca and Kunz, 1978). Chen et al. (2013) suggested that change in body temperature (ΔT) was the most credible phenotypic indicator for assessing the heat stress of chickens. Breeding with genetically heat-resistant chickens is a necessary strategy for commercial poultry production in tropical areas (Liang et al., 2016). Efforts have been made to identify specific genes associated with tolerance or sensitivity to heat stress in chickens (Varasteh et al., 2015; Lan et al., 2016). Identification of candidate genes and mutations in quantitative trait loci (QTL) regions for body temperature is a potential strategy for mitigating the effects of heat stress on livestock (Dikmen et al., 2013). Few studies have been attempted to elucidate the genetic component underlying body temperature change during heat stress. Microsatellites mapping of QTL for body temperature under heat stress have been mapped in Japanese quail (Minvielle et al., 2005). Many genome-wide association studies (GWASs) have been conducted to identify candidate genes related to economic traits (Hayes et al., 2009; Snelling et al., 2010; Van Goor et al., 2015; Lien et al., 2017). GWASs have been conducted to identify QTL for body temperature and blood chemistry components under shorter periods of chronic heat stress in an advanced intercross line of broiler and Fayoumi chickens (Van Goor et al., 2015, 2016). The purpose of this study was to perform a GWAS for body temperature change in a broiler-type strain TCCs under acute heat stress.

2. Materials and methods

2.1. Animals managements, acute heat-stress treatment, body temperature measurements, and rooster categorization

All procedures involving the handling and treatment of chickens in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University, Taiwan (IACUC Permit NO. 104-112) prior to the initiation of this study. Broiler-type chickens are more susceptible to thermal stress and may causes sudden death syndrome. Our IACUC committee had awareness of mortality aspects and approved with sudden death in this study. The day-old chicks were wing-banded brooded in floor pens (2.35 m wide x 2.35 m deep) of a tunnel house with 24 h/day heating for two weeks. At eight weeks of age, female chickens were removed and the males were retained in the pens until 16 weeks of age. The roosters were then transferred to individual wire-floored cages. The average air temperature in the house was between 25 °C and 30 °C during the 3th to 28th weeks of age. The chicks were fed a starter diet (22.3% crude protein (CP) and 3270 kcal/kg metabolizable energy (ME)) until 6 weeks of age, followed by a grower diet (15.0% CP and 2930 kcal/kg ME) until 16 weeks of age. The roosters were given pellet breeder diet (16.9% CP, 2930 kcal/kg ME, and 3.24% Ca) until the end of the experiment. Feed and water were provided *ad libitum*. Before treatment, roosters were transferred to individual wire floored cages of climate chamber and allowed to adapt to the chamber for 2 weeks under the following conditions: a 14:10-h light:dark photoperiod, 25 °C, and 55% relative humidity (RH). Blood samples were collected through the jugular vein during the adjusting period and stored at −80 °C until further DNA isolation. Optimal environments for experimental chickens were maintained by careful monitoring twice per day to ensure the health at adaptation period. In the present study, the acute stress models of heat exposure were created to simulate the sudden heat waves that occur in nature. All roosters were used for heat-stress experiments involving the same procedure. At 30 weeks of age, the roosters were treated with acute heat stress at 38 °C and 55% RH for 4 h according to the procedures of Wang et al. (2013). To reduce uncomfortability and distress of the bird during treatment, birds were provided with improved air flow system for a stable normal humidity in the chamber and more cool water supply to prevent dehydration due to over-ventilation. Individual body temperature were measured at 0.5, 1, 2, 3, and 4 h to monitor the status of the chickens during heat treatment. The body temperature at each time point or lethal body temperature of roosters with sudden death was recorded during the adaptation and treatment periods by inserting an alcohol thermometer approximately 2.5 cm into the cloaca (Chen et al., 2013). The precision of the thermometer was 0.1 °C. The ΔT of each individual was calculated according to the difference between the highest body temperature during heat stress and body temperature before heat stress. The ΔT distribution in the population was assessed through the SAS® univariate procedure (SAS, 2010). The surviving roosters ($n = 175$) were categorized according to ΔT into the susceptible group ($\Delta T > 4.2$ °C, $n = 18$), resistant group ($\Delta T < 2$ °C, $n = 37$), and intermediate group ($\Delta T = 3$ °C, $n = 45$). Roosters that died during heat treatment were designated as the dead group ($n = 25$). The 12.5% (25 died in the total of 200 chickens) of mortality (sudden death) in this study. Differences among groups were examined using the Kruskal–Wallis test (Zar, 2013), and ΔT was considered to be statistically significant if $P < 0.05$. Blood samples collected from the wing vein were used for DNA isolation and genotyping analysis.

2.2. DNA isolation, genotyping, and quality control

Genomic DNA from the blood samples of 92 roosters (25 in the dead group, 18 in the susceptible group, 37 in the resistant group, and 12 in the intermediate group, Table 1) were extracted using a standard Gentra Puregene Blood Kit (Qiagen, Valencia, CA). The extracted DNA was diluted to 15–20 ng/μL for SNP genotyping. Individuals were genotyped using the Affymetrix 600 K chicken SNP Axiom Array containing 580,954 SNPs (Affymetrix, Clara, CA) according to manufacturer protocol at the National Center for Genome Medicine at Academia Sinica, Taiwan. Quality control was performed using Axiom Analysis Suite (Affymetrix). Individuals and SNPs were excluded if they met any of the following criteria: (1) individual call rate < 97%; (2) no chromosomal or physical location; (3) minor allele frequency < 0.05; (4) Fisher's exact test P value for Hardy–Weinberg equilibrium $\leq 1 \times 10^{-6}$. After filtering, all samples were deemed eligible for subsequent GWAS analysis.

2.3. Population stratification and GWAS analysis

Detection of possible population stratification that could influence association analysis was performed using EIGENSTRAT 2.0 (Patterson et al., 2006). We estimated the variance inflation factor for genomic controls. A GWAS and genomic control correction were conducted to compare allelic and genotypic frequencies between groups (dead *vs.* survival, intermediate *vs.* resistant, intermediate *vs.* susceptible, resistant *vs.* susceptible) using the Cochran–Armitage trend test implemented in PLINK (Purcell et al., 2007). A quantile–quantile (Q–Q) plot was used to determine P value distribution. The Manhattan plot and Q–Q plot were produced using the R language (R Development Core Team, 2007).

2.4. Identification of positional candidate genes, gene-enrichment analysis, and overlap with known quantitative trait loci

Gene annotation of significant SNPs was based on the Ensembl Genes 92 database of *Gallus gallus* genomes (Gallus_gallus-5.0). The official gene symbols were downloaded from the Ensembl database (https://www.ensembl.org/index.html) and further analyzed. Significant SNPs were annotated as candidate genes according to the nearest genes. Functional classification of the candidate genes were

Table 1
The body temperature and body temperature change (ΔT) of broiler-type B strain Taiwan country chickens before and after heat stress.

Time	Traits	Survival group			Dead group
		Resistant	Intermediate	Susceptible	
Pre-heat stress	No. of roosters	37	45	18	25
	Body temperature (Mean ± SEM)	40.2 ± 0.1^{a}	39.7 ± 0.1^{ab}	39.4 ± 0.1^{b}	40.1 ± 0.1^{a}
30 min	No. of roosters	37	45	18	25
	Body temperature (Mean ± SEM)	42.1 ± 0.1^{b}	42.2 ± 0.1^{b}	42.7 ± 0.1^{a}	42.6 ± 0.1^{a}
60 min	No. of roosters	37	45	18	25
	Body temperature (Mean ± SEM)	42.2 ± 0.1^{c}	42.3 ± 0.1^{c}	42.8 ± 0.1^{b}	43.4 ± 0.1^{a}
120 min	No. of roosters	37	45	18	24
	Body temperature (Mean ± SEM)	42.0 ± 0.1^{c}	42.2 ± 0.1^{c}	42.9 ± 0.2^{b}	44.4 ± 0.1^{a}
180 min	No. of roosters	37	45	18	15
	Body temperature (Mean ± SEM)	41.9 ± 0.1^{c}	42.1 ± 0.1^{c}	42.9 ± 0.2^{b}	44.6 ± 0.2^{a}
240 min	No. of roosters	37	45	18	0
	Body temperature (Mean ± SEM)	41.7 ± 0.1^{c}	42.3 ± 0.1^{b}	44.6 ± 0.2^{a}	N/A
	No. of roosters	37	45	18	25
	Average of ΔT (Mean ± SEM)	1.8 ± 0.1^{c}	3.0 ± 0.1^{b}	4.6 ± 0.1^{a}	5.1 ± 0.1^{a}
	Range of ΔT (min.-max.)	1.0–2.0 °C	3.0 °C	4.2–5.0 °C	4.5–7.0 °C

^{a,b,c} Means with different superscripts differ significantly among groups by Kruskal-Wallis test ($P < 0.05$).

annotated for their biological processes using the gene ontology (GO) database (http://www.geneontology.org/). GO enrichment analysis was performed with DAVID tools (https://david.ncifcrf.gov/). Additionally, the Ensembl database and Reactome pathway database (https://reactome.org/) were used to identify the pathways and or sub-pathways involving these candidate genes. Moreover, regions within 5 Mb from a candidate gene were searched for previously reported QTL regions with physiology traits in chicken QTL database (https://www.animalgenome.org/cgi-bin/QTLdb/GG/index).

3. Results

3.1. Populations and phenotype statistics

The body temperature of chickens during heat stress (0.5–4 h) was higher than that before heat treatment (Fig. 1). The body temperature before heat stress of dead and resistant groups was higher than that of intermediate group (Table 1). The susceptible and dead groups had higher body temperatures than those in the resistant and intermediate groups during heat stress treatment (Table 1). The ΔT differed significantly ($P < 0.05$) in the comparison of dead *vs.* survival, intermediate *vs.* resistant, intermediate *vs.* susceptible, and resistant *vs.* susceptible groups (Table 1).

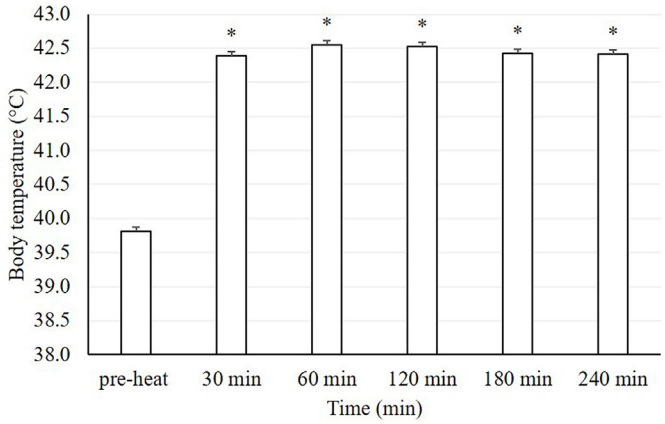

Fig. 1. The body temperature of broiler-type B strain Taiwan country chickens before and during heat stress. * Means differ before and during heat stress ($P < 0.05$).

3.2. Population stratification

A GWAS analysis was performed to identify genes associated with ΔT between the comparison groups (dead *vs.* survival, intermediate *vs.* resistant, intermediate *vs.* susceptible, and resistant *vs.* susceptible). In the single association analysis on ΔT, we discovered that the *P* values from the analysis reflect an overestimation with an inflation factor markedly higher than 1. Thus, these *P* values should not be used to identify significant genetic variants. The *P* value of the association test were adjusted using the genomic control (GC) method to correct the residual inflation. The distributions in GC *P* values and lambda values (λ) between groups were 1.52, 1.07, 1.23, and 1.00, respectively (Fig. 2). The λ values for ΔT were close to 1 except for the dead *vs.* survival and intermediate *vs.* resistant groups (λ = 1.52 and 1.23). However, the GC correction method failed to effectively correct the *P* value in intermediate *vs.* resistant group. The Q–Q plots and λ values indicated that no strong stratifications between the intermediate vs. susceptible or resistant *vs.* susceptible groups (Fig. 2).

3.3. GWAS results for significant SNPs for ΔT, gene annotation, pathways, and overlap with QTL regions

In total, 17 significant SNPs were identified through the GWAS in the dead *vs.* survival group. A global view of Manhattan plots for ΔT is presented in Fig. 3. One SNP belonging to sciellin (*SCEL*) was located on chromosome 1. A total of 15 significant SNPs belonging to *KCNS2*, *STK3*, *OSR2*, *NPAL2*, *VPS13B*, and *POP1* were located on chromosome 2. The other SNP belonging to armadillo repeat containing (*THADA*) was located on chromosome 3. Significant SNPs of chromosome 2 were located within the QTL regions associated with body temperature (#30853) QTL (Table S1). A GO analysis indicated that the candidate genes were associated with the biological processes of cellular process, biological regulation, and metabolic process (Fig. 4). These genes were linked to pathways involved in signal transduction, RNA metabolism, programmed cell death, and transport of small molecules (Table 2). A total of 352 significant SNPs were identified through the GWAS in resistant *vs.* susceptible groups (Table S2). A global view of Manhattan plots for ΔT is presented in Fig. 5. These SNPs were identified on chromosome 1, 4, 5, 6, 8, 9, 10, 11, 13, 14 17, 19, 21, and 26. A high genetic correlation revealed that these SNPs were located on chromosome 5, 9, and 11. The QTL regions were identified on chromosome 1, 5, 6, 9, 10, 14, and 17. Details of significant SNPs and QTLs are provided in Table S2. The GO analysis suggested that the candidate genes were associated with cellular processes, metabolic processes, and biological regulation (Fig. 6). The cellular processes, metabolic processes,

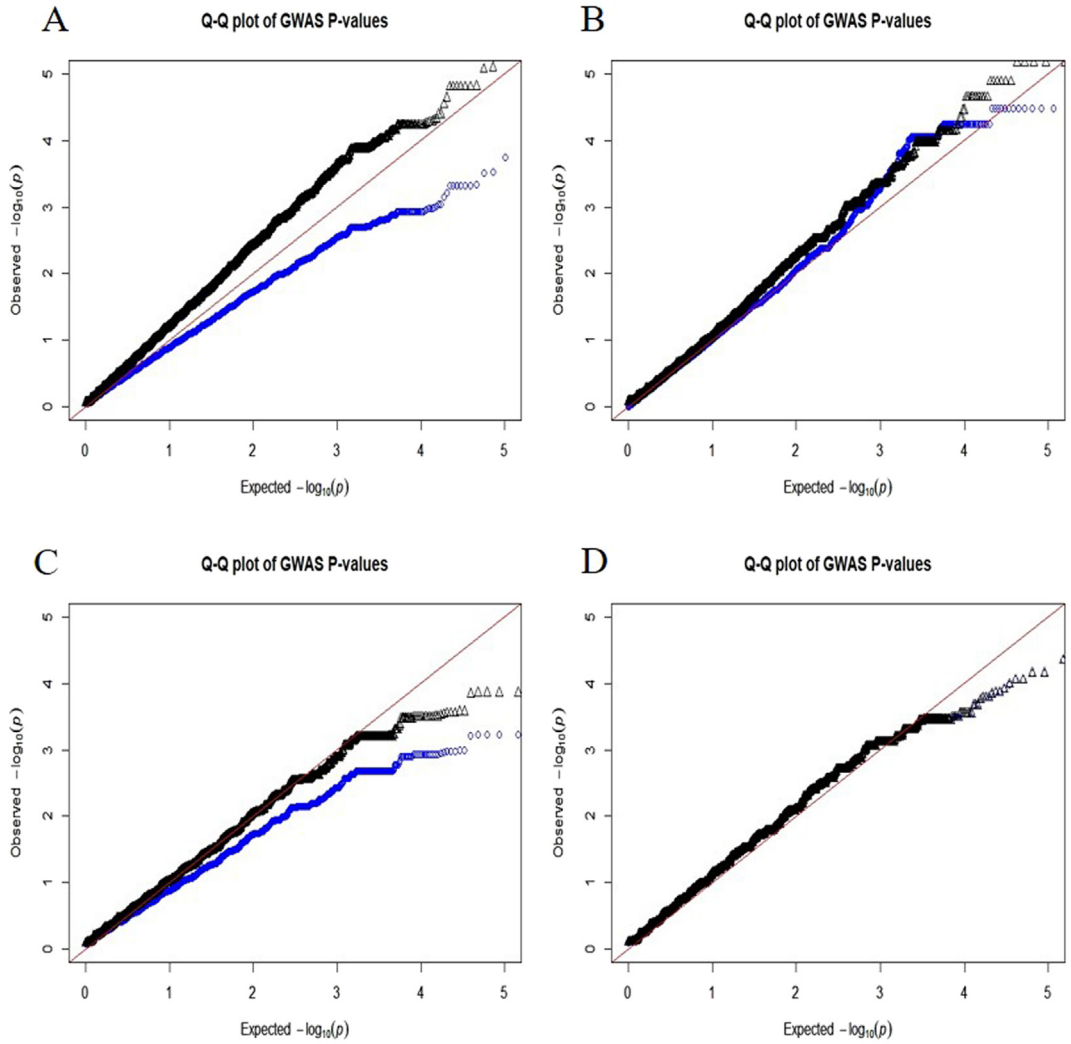

Fig. 2. The quantile–quantile (Q–Q) plots of body temperature change between groups of broiler-type B strain Taiwan country chickens. Panel A: dead *vs.* survival, Panel B: resistant *vs.* susceptible, Panel C: intermediate *vs.* resistant, and Panel D: intermediate vs. susceptible. Black triangles indicate the $-\log10\,P$ values, blue circles represent the genome-wide $-\log10\,P$ values after genomic control correction. The lambda values of dead *vs.* survival, resistant *vs.* susceptible, intermediate *vs.* resistant, and intermediate *vs.* susceptible were 1.52, 1.07, 1.23, and 1.00, respectively.

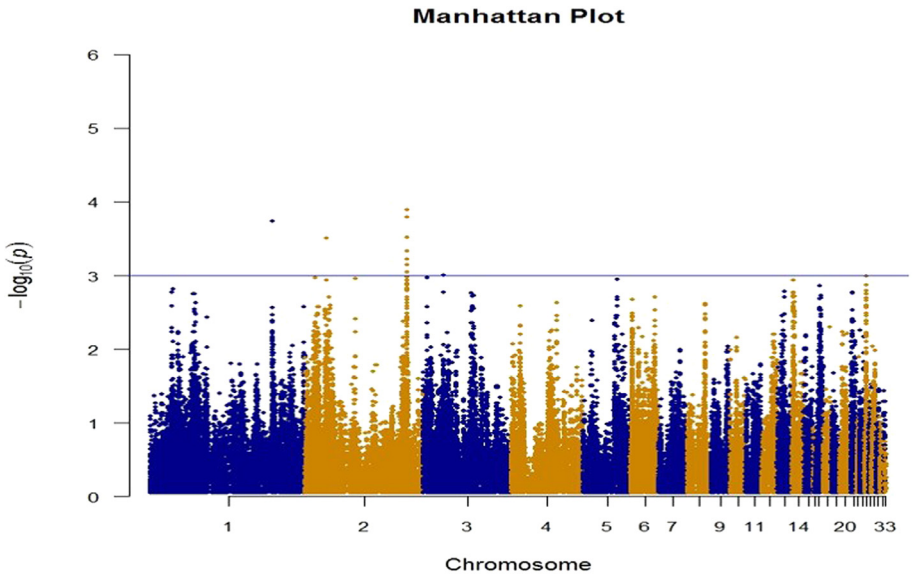

Fig. 3. Manhattan plot of the observed *P* values for body temperature change in the genome-wide association study between dead *vs.* survival groups of broiler-type B strain Taiwan country chickens. The Manhattan plot presents *P* values for the association of single nucleotide polymorphisms (y-axis) plotted against their chromosomal positions (x-axis). The blue line indicates the genome-wide significant threshold ($P \leq 0.001$ of Fisher's exact test with genomic control method correction).

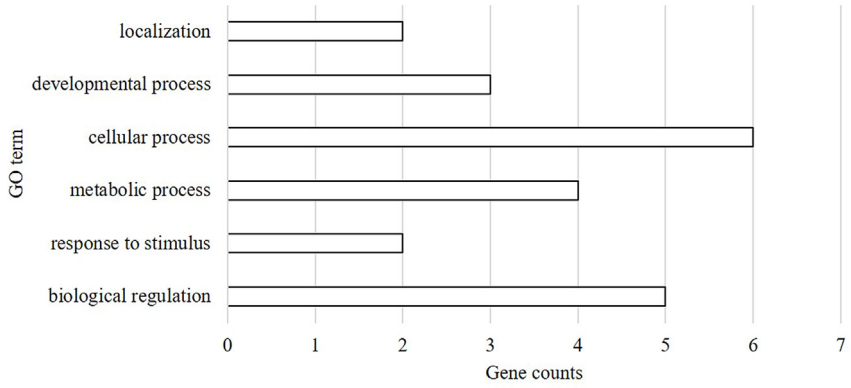

Fig. 4. Gene ontology (GO) analysis of the significant genes between dead *vs.* survival groups of broiler-type B strain Taiwan country chickens. This bar chart presents the classification of GO biological processes as determined with the Ensembl web tool. Bars reflect the number of genes in the specified category.

biological regulation, localization, and cellular component organization or biogenesis were significantly enriched GO terms (Fig. 6). There genes were linked to pathways involved in signal transduction, metabolism, the immune system, and the transport of small molecules (Table 2). Manhattan plots for ΔT in intermediate *vs.* susceptible groups are presented in Fig. 7. The GWAS results indicated that 174 SNPs were different in the loci of intermediate and susceptible groups (Table S3). These SNPs were identified on chromosome 1, 2, 3, 4, 5, 6, 7, 10, 15, 18, 19, and 28. A strong genetic correlation indicated that these SNPs were located on chromosome 3, 5, and 7. The QTL regions were identified on chromosome 1, 2, 3, 4, 5, 6, 7, 10, and 18. Details of significant SNPs and QTLs are provided in Table S3. The results of the GO analysis indicated that these candidate genes were primarily involved in the biological processes of cellular processes, response to stimuli, and biological regulation (Fig. 8). The cellular process, response to stimulus, and biological regulation, which were significantly enriched GO terms (Fig. 8). These genes were linked to pathways involved in hemostasis, the immune system, signal transduction, and metabolism (Table 2). Sub-pathways are listed in Table S4.

Table 2
Functional pathway of genes with significant single nucleotide polymorphisms between different groups of Taiwan country chickens.

Group	Pathway	Significant genes after heat stress
Dead *vs.* Survival	Metabolism of RNA	*POP1, THADA*
	Neuronal System	*KCNS2*
	Programmed Cell Death	*STK3*
	Signal Transduction	*STK3*
	Transport of small molecules	*NPAL2*
Resistant *vs.* Susceptible	Developmental Biology	*TRPC7, DLG1, SNW1, TRPC1*
	Disease	*SNW1, TNKS2, SLC9A9, DAB2IP*
	Extracellular matrix organization	*PCOLCE2, ELN, PLOD2*
	Gene expression (Transcription)	*SNW1, CPSF3L*
	Hemostasis	*TRPC7, ATP1B3*
	Immune System	*TRPC7, CUL3, TRPC1, BLNK, STOM, TRIP12*
	Metabolism	*NDUFA8, ETNK2, GPT2, ST7, UQCRFS1, PLEKHA6*
	Metabolism of proteins	*MRPL20, TTLL5, TNKS2, CUL3, MXRA8, GALNTL4, EXOC6, AURKAIP1*
	Metabolism of RNA	*TBL3, SNW1, XRN1, U2SURP*
	Muscle contraction	*TRPC1, ATP1B3*
	Neuronal System	*DLG1, ARHGEF9, NRXN3*
	Organelle biogenesis and maintenance	*EXOC6*
	Signal Transduction	*TRPC7, DLG1, DEPDC7, ARHGEF9, SNW1, TNKS2, CUL3, DVL1, DNER, ST7, DAB2IP*
	Transport of small molecules	*TRPC7, TRPC1, SLC9A9, STOM, ATP1B3*
	Vesicle-mediated transport	*PLDN, EXOC6*
Intermediate *vs.* Susceptible	Cell Cycle	*DYNC1I2*
	Cell-Cell communication	*ITGA6, MPP5*
	Cellular responses to stress	*DYNC1I2*
	Developmental Biology	*EFNA2, TRPC3, SCN2A*
	Disease	*THSD7A, LRRFIP1*
	DNA Repair	*RAD51B*
	Extracellular matrix organization	*TGFB2, ITGA6*
	Gene expression (Transcription)	*TRPC3, MOV10L1, ESRRG, IL2*
	Hemostasis	*RAD51B, TGFB2, TRPC3, ANXA5, PDE5A, ITGA6, RAPGEF4*
	Immune System	*IL21, DYNC1I2, CCL4, TRIM58, LRRFIP1, MPO, IL2, RAPGEF4*
	Metabolism	*FABP2, BZRAP1, ARG2, GPHN, RAPGEF4*
	Metabolism of proteins	*DYNC1I2, TNIP3, THSD7A, PIGH*
	Muscle contraction	*SCN2A*
	Neuronal System	*BZRAP1*
	Organelle biogenesis and maintenance	*DYNC1I2, TTC21B*
	Signal Transduction	*DYNC1I2, TRPC3, TTC21B, CCL4, IL2, RAPGEF4*
	Transport of small molecules	*SCARB1, TRPC3, CYBRD1*
	Vesicle-mediated transport	*SCARB1, DYNC1I2*

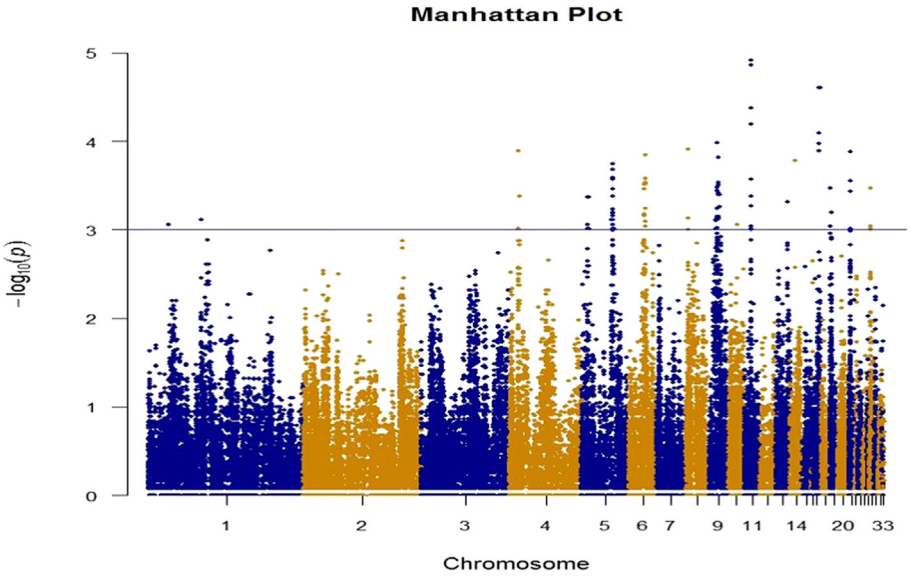

Fig. 5. Manhattan plot of the observed *P* values for body temperature change in the genome-wide association study between resistant *vs.* susceptible groups of broiler-type B strain Taiwan country chickens. The Manhattan plot presents *P* values for the association of single nucleotide polymorphisms (y-axis) plotted against their chromosomal positions (x-axis). The blue line depicts the genome-wide significant threshold (*P* values ≤ 0.001 of Fisher's exact test with genomic control method correction).

4. Discussion

GWASs constitute a promising method for identifying common genetic variants with respect to the economic traits of domestic animals. This study identified genetic variants and positional candidate genes associated with ΔT during heat stress in a broiler-type strain of TCCs. The identified genes related to thermotolerance provide potential information to improve our understanding how genetic influence operates at the physiological response to acute heat stress.

Several physiological parameters, such as body temperature (Chen et al., 2013), initial glucose levels (Xie et al., 2015), and feather follicle density (Jiang et al., 2010), have been proposed as indicators for determining the ability of chickens to respond to stressful situations under heat stress. However, initial glucose levels and feather follicle density are difficult to measure when selecting heat-tolerant chickens. Body temperature increased dramatically after the initiation of acute heat stress (Fig. 1) is in agreement with the results of our previous studies (Wang et al., 2013, 2015). In this study, we discovered that chickens in the dead group exhibited a greater ΔT (Table 1) and those in the resistant group, who had smaller ΔT during heat stress and similar body temperature as that before heat stress. Sykes and Salih (1986) suggested that ΔT can reflect the regulation ability of core body temperature in hens and a significant correlation between ΔT and survival in chickens subjected to heat stress was reported later (Chen et al., 2013). In contrast to other groups, however, slow change in body temperature during acute heat stress was observed in the resistant group, suggesting superior heat tolerance (Table 1). Differences in heat tolerance were also observed through the changes in body temperature of the chickens when subjected to heat stress (Cheng et al., 2018). Therefore, the results suggest a considerable variation in the heat tolerance of chickens, thereby providing a foundation for the selection of chickens with high heat resistance.

In the analysis of a homogeneous population, the correction of potential population stratifications is crucial to ensure the accuracy of the statistical analysis. Because purebred native chickens were used in this study, the results revealed inflation factors higher than 1 among the groups. Ma et al. (2012) suggested that artificial insemination and genetic selection are major factors contributing to population stratifications. This phenomenon is common in GWASs on domestic animals because domestic animals contain a large population composed of a minority group with a high linkage disequilibrium, such as Korean Holstein chickens (Shin et al., 2017) and Rhode Island Red chickens in China (Liu et al., 2018). Broiler-type B strain TCCs constitute a closed population that has been artificially selected for early maturity and body confirmation since 1982 (Lee, 1992). These selection strategies may reduce genetic variability and increase the linkage disequilibrium of the population. We believed that the inflation of *P* values in animal GWASs is a common phenomenon, and thus we applied GC methods to correct for residual inflation and further detect significant genetic variants (Fig. 2). GC *P* values did not exhibit an inflation problem in the intermediate *vs.* susceptible or resistant *vs.* susceptible groups in the present study (Fig. 2). Furthermore, the GC correction method effectively corrected the *P* values between the dead and survival groups (Fig. 2).

In chickens, response to heat stress has been reported to associate with several physiological and gene pathways involved in a range of

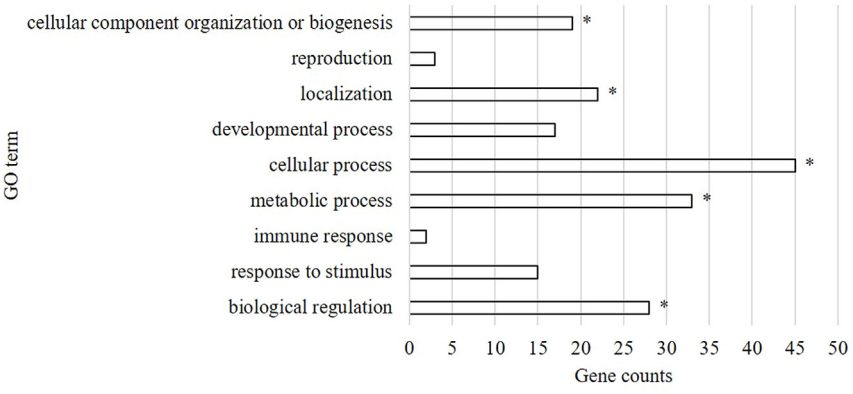

Fig. 6. Gene ontology (GO) analysis of the significant genes between resistant *vs.* susceptible groups of broiler-type B strain Taiwan country chickens. This bar chart presents the classification of GO biological processes as determined using the Ensembl web tool. Bars reflect the number of genes in the specified category. The GO enrichment analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) tools (The enrichment *P* values were corrected using Benjamini's methods). *Significantly enriched GO terms ($P < 0.05$).

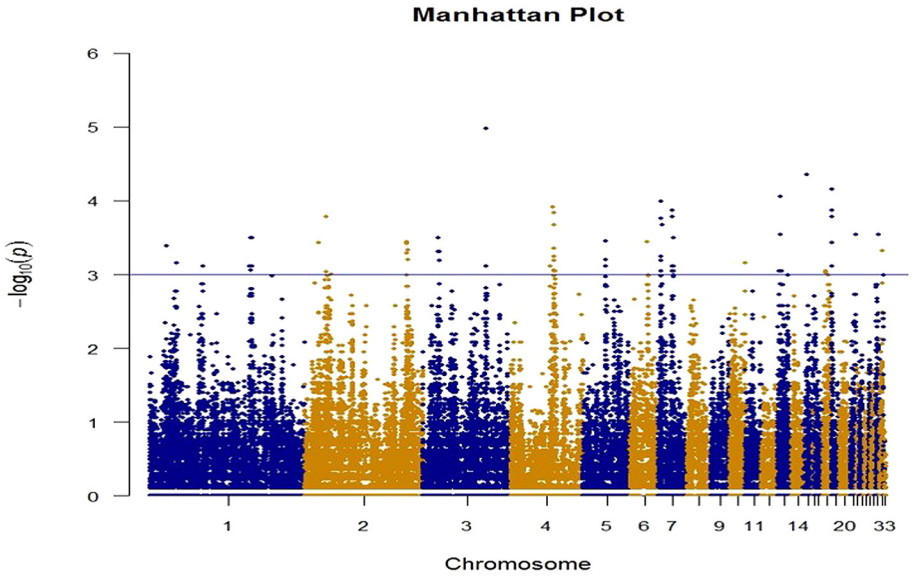

Fig. 7. Manhattan plot of the observed *P* values for body temperature change in the genome-wide association study between intermediate *vs.* susceptible groups of broiler-type B strain Taiwan country chickens. The Manhattan plot presents *P* values for the association of single nucleotide polymorphisms (y-axis) plotted against their chromosomal positions (x-axis). The black line depicts the genome-wide significant threshold (*P* values ≤ 0.001 of Fisher's exact test with genomic control method correction).

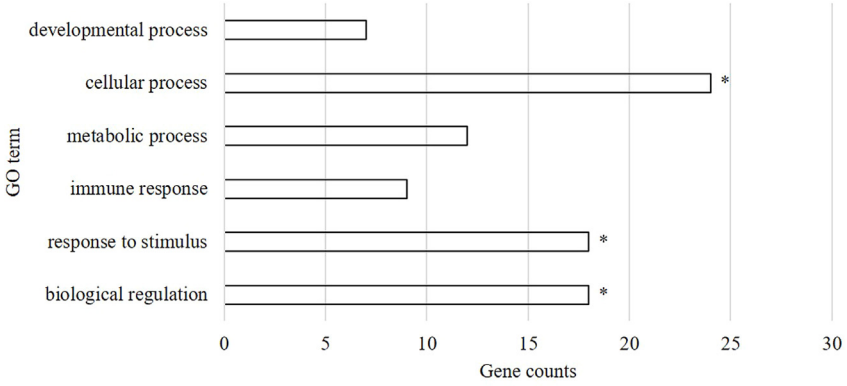

Fig. 8. Gene ontology (GO) analysis of the significant genes between intermediate *vs.* susceptible groups of broiler-type B strain Taiwan country chickens. This bar chart presents the classification of GO biological processes as determined using the Ensembl web tool. Bars represent the number of genes in the specified category. The GO enrichment analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) tools (enrichment *P* values were corrected using Benjamini's methods). *Significantly enriched GO terms ($P < 0.05$).

biological mechanisms. For example, these genes are involved in the biological processes of stimuli response, signal transduction, cellular processes, apoptosis, metabolic processes, DNA synthesis, transcription, translation, protein folding and degradation, developmental processes, and immune system processes (Matsuki et al., 2003; Wang et al., 2013, 2015; Cheng et al., 2015, 2018; Lan et al., 2016; Tu et al., 2016; Zahoor et al., 2017).

Heat shock and heat stress tend to result in DNA damage, lipid peroxidation, protein inactivation, and aberrantly folded proteins (Lord-Fontaine and Averill-Bates, 2002; Mujahid et al., 2007). The responses reflect cellular resistance to heat stress known as thermotolerance (Calderwood et al., 2010). Studies have reported that heat stress causes severe injury to the intestine and heart in chickens, as well as the induction of cell apoptosis (Gu et al., 2012; Islam et al., 2013; Zhang et al., 2017b). In this study, the 15 SNPs associated with integrated death phenotypes under heat stress and loci identified were located on chromosome 2 (Table S1). The serine/threonine kinase family is involved in the regulation of cellular responses, including cell proliferation, differentiation, and survival (Su and Karin, 1996). The functional pathway analysis revealed that *STK3* is involved in signal transduction and programmed cell death pathway (Table 2). STK3 is a pro-apoptotic kinase that is activated by stress; it enters the nucleus and induces chromatin condensation followed by internucleosomal DNA fragmentation (Pruitt et al., 2011). Our other studies have demonstrated that STK-related gene and protein expressions were upregulated after acute heat stress in the small yellow follicles of broiler-type B strain TCCs (Cheng et al., 2015, 2018). Because *STK3* is also located in body temperature (#30853) QTL regions, these results suggest that SNPs are suitable markers for the genetic selection of thermotolerance in TCCs (Table S1).

The ability to respond to external stimuli and regulate internal temperatures is required for animal survival, maintenance of homeostasis, and avoidance of tissue-damaging noxious temperatures (Dhaka et al., 2006). The transient receptor potential (TRP) channel is the first link between TRP channel and sensory transduction (Kaneko and Szallasi, 2014). TRPs form cationic channels that detect sensory stimuli such as temperature, pH, or oxidative stress and transduce the change with either electrical or chemical signals (Dhaka et al., 2006). The TRPC3 channel is crucial to the regulation of vascular smooth muscle tone and the autoregulation of cerebral blood flow because of its role in regulating cerebral vascular contractility (Brayden et al., 2008). Thilo et al. (2009) proposed that TRPC3 expression is associated with hypertension and hypoxic conditions in human cerebral vascular tissue. Thus, the association of SNPs with TRPC7 and TRPC1 in resistant *vs.* susceptible groups (Table S2) and TRPC3 in intermediate *vs.* susceptible groups (Table S3) suggests that thermotolerance by TCCs involves heat disputation from the vascular blood flow underlying autonomous nerve regulation (Table S4). TRPC3 is located in body temperature (#9426) QTL regions, suggesting that SNPs may contribute to ΔT under heat stress (Table S3). Moreover, the association of SNPs with *DYNC1I2* in intermediate *vs.* susceptible groups is involved in cellular responses to stress pathway (Table 2). *DYNC1I2* encodes a member of the dynein intermediate chain family (Crackower et al., 1999). Cytoplasmic dynein plays a crucial role in intracellular transport and nuclear migration

(Cianfrocco et al., 2015). Our previous studies have shown that dynein family related genes and proteins are differentially expressed in the hypothalamus of birds after heat stress (Tu et al., 2016, 2018) and seasonal change (Mukai et al., 2009). The SNPs of TRP and motor-related genes in our findings provide a foundation for further research to discern the functions of these genes with respect to thermotolerance in chickens.

ROS production was significantly increased in the mitochondria of heat-stressed chickens (Mujahid et al., 2010). Respiratory electron transport is considered to be the primary source of cellular ROS, and inhibition of respiratory chain complex activity has been proven to compromise adenosine triphosphate synthesis (Letellier et al., 1994; Davey and Clark, 1996; Rossignol et al., 1999). *NDUFA8* and *UQCRFS1* are related to respiratory electron transport, the citric acid cycle and respiratory electron transport, ATP synthesis through the chemiosmotic coupling, and heat production by uncoupling proteins (Pennacchio et al., 1995; Mimaki et al., 2012). Tsolakidou et al. (2010) discovered that NDUFA8 was decreased in the hypothalamic paraventricular nuclei of mice after 4 h of forced exposure to swimming stress. Mitogen-activated protein kinase (MAPK) signaling pathways are involved in heat stress–induced organ injury (Yu et al., 2013). *DLG1*, *CUL3*, and *DAB2IP* in resistant *vs.* susceptible groups and *IL2* in intermediate *vs.* susceptible groups are involved in the MAPK family signaling cascades, MAPK1/MAPK3 signaling, and the RAF/MAP kinase cascade pathway (Table S4). Additionally, heat stress also causes profound modulation in the signaling pathway that leads to protein kinase activation and phosphorylation of HSF1 at numerous regulatory serine residues, which ultimately results in cellular response (Calderwood et al., 2010). *IL2* is located in body temperature (#9426) QTL regions; therefore, SNPs may contribute to ΔT under heat stress (Table S3). Furthermore, *NDUFA8* and *DAB2IP* are located in troponin T concentration (#2358) QTL regions (Table S2). When chickens undergo significant temperature fluctuations, the blood system is a component involved in thermoregulatory response (Yahav et al., 1997). Zhang et al. (2017a) suggested that the heart has a higher pumping load and pressure to dissipate heat through increased blood circulation under high temperatures. Furthermore, one mechanism for stabilizing body temperature during heat stress may act on the regulation of blood flow to the skin (Wolfenson et al., 1981); this results in higher cardiac output and may increase strain on the heart from pumping blood, leading to cardiovascular injury. Troponin is considered a highly sensitive and specific biomarker of cardiac injury (O'Brien et al., 2006). Another study suggested that serum cardiac troponin T and effective blood flow is stable in humans (Grzegorzewska et al., 2016). The mechanism for regulating troponin T concentrations may redirect blood flow and enable the cardiovascular system to acclimate to thermal environments.

In conclusion, the GWAS analysis identified significant SNPs, genes, and QTL regions associated with programmed cell death pathways, signal transduction, and cellular responses to stress may be potential candidates underlying the physiological response to heat stress or thermotolerance in chickens.

Declarations of interest

None.

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Appendix A. Supplementary data

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