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Effects of heat stress on the hepatic transcriptome of yellow-feathered chickens

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Abstract

Heat stress is one of the main environmental factors that hamper the efficiency of poultry production. Heat stress is known to be less harmful to yellow-feathered chickens (YFC) than to commercial broilers, especially in its effect on the relative weight of organs. The transcriptome was used to examine the gene expression profile of the livers of YFC that were exposed to heat stress. Mean liver weight (HW) and the hepatic weight index (HWI) of birds in the heat stress group did not differ significantly from those of the control group. A total of 596 genes were differentially expressed in the liver in response to heat stress. The enrichment results of differentially expressed genes (DEG) showed that many of these genes were related to the Fanconi anaemia pathway and that the cell cycle pathway was modulated. Thus, it can be concluded that these genes might be involved in improving the stability of the YFC liver genome and controlling the physiological response to heat stress.

Keywords:, heat stress, liver, transcriptome, yellow-feathered chickens
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Introduction

Yellow-feathered chickens are less affected by heat stress than commercial broilers, especially for its effect on weight gain (Azad *et al.*, 2010; Zhang *et al.*, 2019). Transcriptome studies are an effective method of analysing tissue gene expression and can be used to study the effect of heat stress on poultry. At a phenotypic level, the decreased growth performance that is observed in poultry exposed to heat stress affects the relative weights of various tissues (Quinteirofilho *et al.*, 2010; Zhang *et al.*, 2017; Zhang *et al.*, 2019). This might be interpreted to indicate that heat stress can suppress tissue cell proliferation at various rates depending on the organ.

Liver tissue has been shown to be more susceptible to heat stress than that of other organs (Lin *et al.*, 2006). Differences in gene expression related to the cell cycle in liver of white Leghorn chickens indicated their down-regulation under heat stress (Sun *et al.*, 2015). Coble *et al.* (2014) examined the liver transcriptome of broilers that had been exposed to heat stress and found that 40 differentially expressed genes (DEG) were involved in cell signalling and the development and function of the endocrine system. A parallel study by the same group analysed the transcriptome of YFC liver tissue exposed to acute heat stress, and found 834 DEG, almost half of which were involved in lipid and energy metabolism (Zhang *et al.*, 2020). In YFC that were exposed to chronic heat stress, an RNA-seq analysis of heart tissue revealed 37 DEG that were involved in oxidative phosphorylation and cardiac muscle contraction pathways (Zhang *et al.*, 2019). However, to the authors' knowledge, a transcriptomic study has not been undertaken on livers of YFC exposed to chronic heat stress. Therefore, the goal of this study was to compare the transcriptomes of YFC subjected to heat stress (38 ± 1 °C for eight hours/day over seven days) with the transcriptome of similar birds in a non-stressful environment.

Materials and Methods

Twelve 17-day-old male YFC were selected, divided into two equal groups (control and heat stress), and placed in two environmentally controlled rooms for a five-day acclimatization period. The period of heat stress consisted of seven days. It started after the acclimatization, when the YFC were 22 days old and ended when they were 28 days old. During acclimatization and the experimental period, the YFC were

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provided with ad libitum access to food and water. The heat stress group was exposed to the temperature of 38 ± 1 °C for 8 hours each day (9h00 - 17h00) and was kept at 25 ± 1 °C for the rest of the time. The control group was kept at 25 ± 1 °C, 24 hours a day. Bodyweight (BW) and cloacal temperature (CT) were measured according to (Zhang *et al.*, 2020; Zhang *et al.*, 2019). All 28-day-old YFC from the treatment and control groups were euthanized at the end of the final day of the heat stress treatment, and their livers were removed. Their hepatic weight (HW) was determined, and the hepatic weight index (HWI) was calculated (HWI = HW/BW × 100%). Twelve liver tissue samples (six control and six heat stress group) were collected and stored at -80 °C for further study. All YFC were cared for and treated in accordance with the Guangdong Ocean University Animal Care and Use Committee guidelines (permit number SYXK 2014-0053).

The total RNA of each liver sample was extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The RNA-seq libraries of each sample were prepared with the RNA Library Prep Kit for Illumina, according to the manufacturer's recommendations. Illumina HiSeq 4000 platform was used to sequence the 12 individual liver RNA libraries. The software HISAT2 (v2.0.5) was used to map the sequences to the Gallus gallus genome assembly (Kim *et al.*, 2015), and gene expression levels were quantified as fragments per kilobase million (Trapnell *et al.*, 2010). The DEG analysis was conducted using DESeq (1.16.1) (Anders & Huber, 2010) (P <0.05) (false discovery rate (FDR) < 0.01). The functional groups of DEG were determined using DAVID 6.8. All RNA-seq raw data have been submitted to the sequence read archive (SRA) under SRA number PRJNA627128.

The 16 DEG were determined with qRT-PCR, and the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to analyse the gene expression levels. The primer pairs are shown in Table 1. The values of BW, HW, HWI, CT and qRT-PCR were analysed with one-way analysis of variance (ANOVA) with SPSS software (v19.0).

Table 1 Forward and reverse primers used for qPCR validation of RNA-seq

Gene name	Forward primer	Reverse primer	Product length
FANCA1.64	TGCGATCGTGTTCCTTCAGA	TACCAACAGCATCGAGGACC	131 bp
FANCG1.66	TCAAGGCATGGAGAACACCT	GCAAAGGGTAGTGGAGAGGT	146 bp
FANCI0.94	TCCTGCCTCCACTGAAACTT	TACCAGGCTAGACAGTGCTG	112 bp
PMS2	CTCTCAGTTCGTTGTGTGCA	TGCTCACAGTTGTTCCTTGC	147 bp
APITD1	AGACCTCGAGATGTTTGCCA	GCAAGCTCGTCACTCTTCTG	123 bp
BRCA1	ACAACAGGGTCAAAGGGTCT	TAACCCAGTACTGCTTGCCA	128 bp
BRCA2	GGAGCAGAAGTGGATCCAGA	TTCATCCTGCTGCTTTGCTC	123 bp
BUB1	AGCCACTCAGTACCTATGCC	TCCTTCTCCAACCAAGCTGT	122 bp
E2F1	TTCTGCCCAGGATGTGAACA	AAATCGTCCTTGCCATGCTC	141 bp
MAD2L1	GAGTGTGACAAGAACGCCAG	CAGTTTCCAGGAGAGGCAGA	130 bp
WEE1	AGAGCTGAAGGACCTACTGC	CACCGACGTCCTGGATATGA	115 bp
CDC20	TGCACCAGAGATTCGCAATG	GCAGCTGAATGATTTCCCCA	128 bp
CCNA2	TGTCAGCGATATCCACACGT	CCAGCCAGTCCACCAGAATA	122 bp
CDK1	AGGGGTGATTAAACTGGCAGA	CCTCTGGAGACCTGTACCAC	104 bp
MCM6	ACACAGCCAAACATCTGCAG	GCCTGAGTCTCTTGAATGCG	116 bp
PLK1	TGTCCCTCTGCCTTGAACAA	CACGTGCAGAGGTAGGGTAA	140 bp
GAPDH	TAGTGAAGGCTGCTGCTGAT	AAGGTGGAGGAATGGCTGTC	103 bp

Results and Discussion

The mean BW at the age of 28 days (BW28) of the heat stress group was lower (P =0.047) than that of the control (Figure 1A). The HW and HWI of the heat stress group did not differ from those of the control (P =0.100 and P =0.911), indicating normal physiological development of the liver (Figure 1B, 1D). The current measurements of the CT showed that the heat stress treatment (38 ± 1 °C) caused a significantly elevated CT in YFC (Figure 1C).

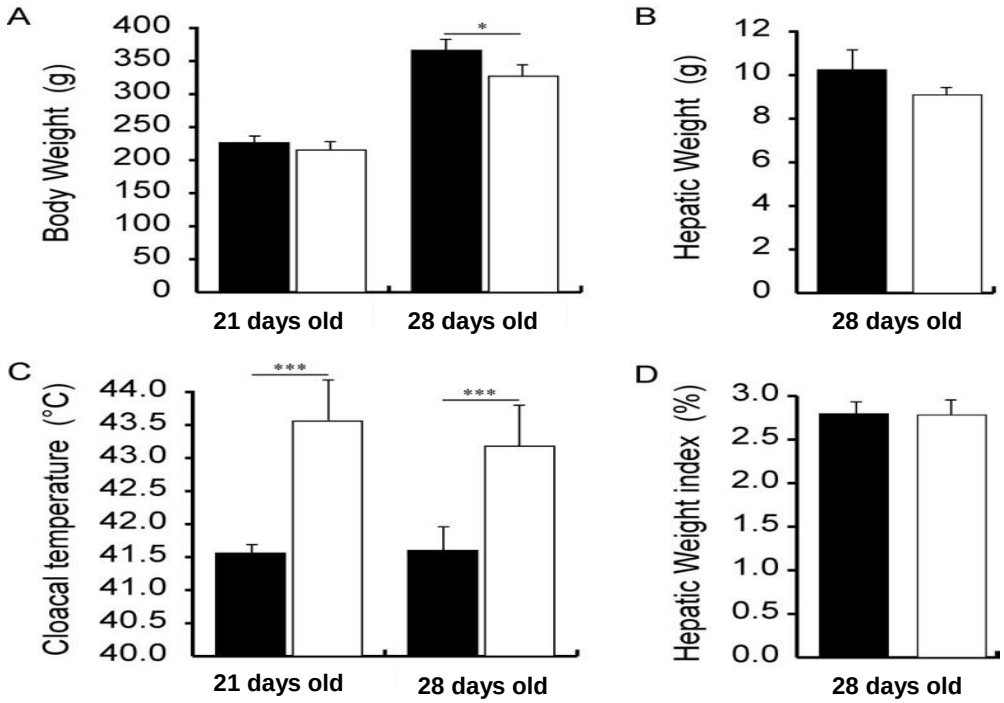

Figure 1 Mean (± SE) bodyweight (A), hepatic weight (B), cloacal temperature (C) and hepatic weight index (D) of yellow-feathered chickens subjected to heat stress (open bars) and contemporary control birds (solid bars) *(P <0.05) and ***(P <0.001)

The total clean reads of these DEG ranged from 43 to 52 million, and approximately 93% of the clean reads were mapped to the reference genome of the chicken, of which 78% were mapped to exons (Table 2). A total of 596 DEG (P <0.05, FDR < 0.01) were found in response to heat stress in their livers (Figure 2). The 368 and 228 were up-regulated and down-regulated expressions. The log2 (fold change) induced by heat stress ranged from -6.33 to 8.54.

Table 2 Number of raw, clean, mapped and uniquely reads for hepatic transcriptome of yellow-feathered chickens subjected to heat stress and contemporary control birds

cDNA library	Raw reads	Clean reads	Mapped reads	Uniquely mapped	Exon mapped (%)
Control 1	46,408,340	44,705,112	42,214,283	41,202,210	83.34
Control 2	51,286,396	49,489,496	46,795,734	45,676,045	82.77
Control 3	46,890,308	45,026,922	42,655,688	41,640,537	83.66
Control 4	46,837,116	44,498,392	40,560,906	39,809,319	73.05
Control 5	45,924,804	43,796,568	40,135,752	39,426,354	71.36
Control 6	45,741,030	43,586,872	39,330,746	38,572,107	74.10
HS group 1	47,229,822	45,163,122	42,687,367	41,657,734	82.57
HS group 2	47,381,304	45,276,764	42,951,268	41,773,468	83.71
HS group 3	45,788,802	43,814,718	41,424,514	40,302,827	83.65
HS group 4	47,321,782	45,177,940	41,519,533	40,800,706	73.30
HS group 5	53,435,846	51,197,804	47,057,005	46,270,076	71.42
HS group 6	46,292,372	44,238,778	40,599,199	39,911,081	72.44
Average	47,544,826	45,497,707	42,327,666	41,420,205	77.95

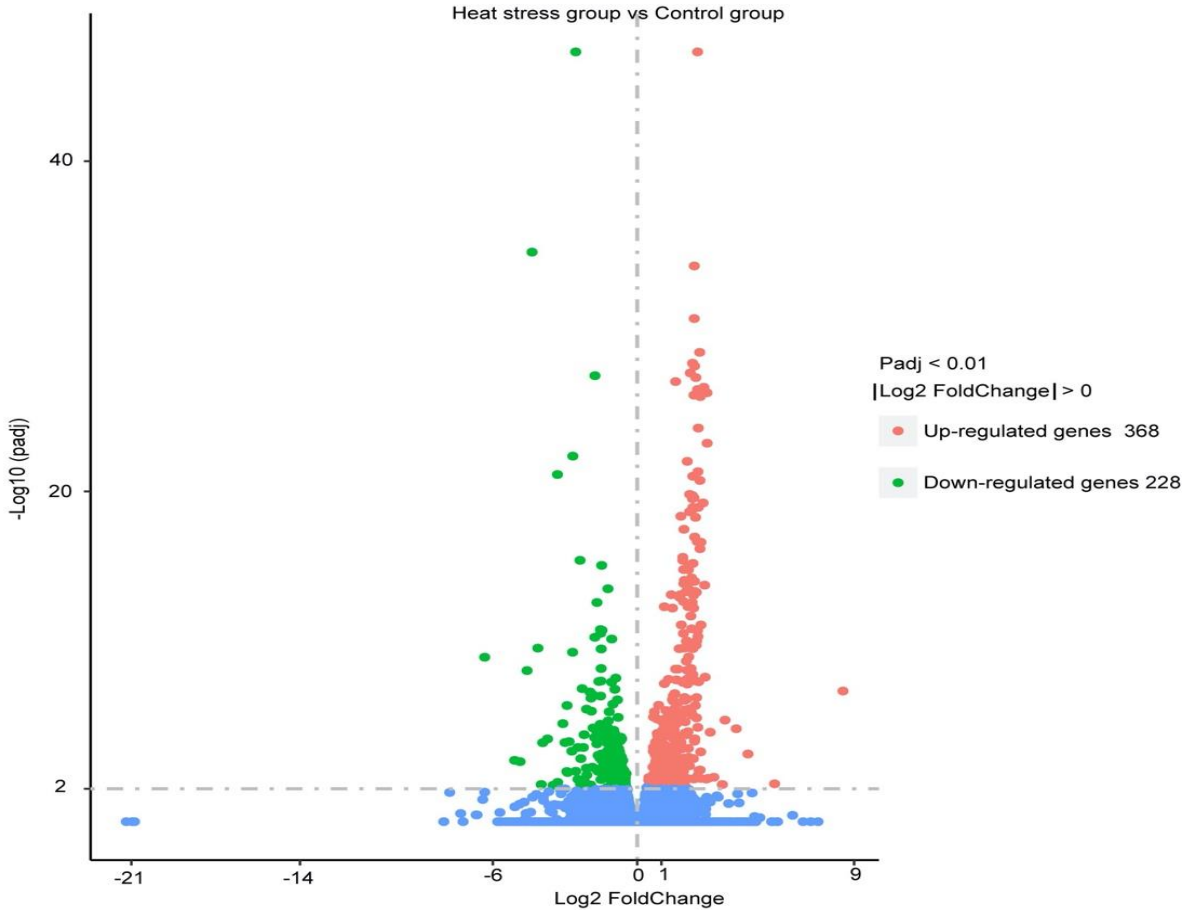

Figure 2 Volcano plot of differentially expressed genes in heat stress yellow-feathered chickens compared with the control birds

DAVID (Huang *et al.*, 2009) was used to analyse the gene ontology terms for DEG to investigate the biological processes related to heat stress. The enriched biological process terms (FDR <0.05) of the DEG expressed in the liver were related to chromosome segregation and cell division (Figure 3). The Kyoto encyclopaedia of genes and genomes (KEGG) (Kanehisa & Goto, 1999) pathway analysis of these DEG revealed the enrichment of two pathways (P <0.05, FDR < 0.01), namely the Fanconi anaemia pathway and the cell cycle pathway (Table 3). Ten DEG (*FANCA, FANCG, FANCI, PMS2, APITD1, BRCA1, BRCA2, EME1, POLN,* and *UBE2T*) were associated with the Fanconi anaemia pathway, whereas fourteen (*BUB1, E2F1, MAD2L1, TTK, WEE1, CDC20, CDC45, CCNA2, CCNB2, CDK1, CDKN2C, MCM6, PTTG1,* and *PLK1*) were associated with the cell cycle pathway. Interestingly, these DEG were up-regulated in the current experiment and verified by qRT-PCR (Figure 4). The current results suggest that the liver of YFC might provide a molecular mechanism defending against heat stress.

Table 3 KEGG pathway enriched analysis for differentially expressed genes

KEGG pathway	*P*-value	FDR	Gene names	Gene description
Fanconi anaemia pathway	1.54E-05	0.017	*FANCA*	Fanconi anaemia complementation group A
			FANCG	Fanconi anaemia complementation group G
			FANCI	Fanconi anaemia complementation group I
			PMS2	PMS1 homolog 2
			APITD1	Centromere protein S
			BRCA1	Breast cancer 1
			BRCA2	Breast cancer 2
			EME1	Essential meiotic structure-specific endonuclease 1
			POLN	Polymerase (DNA) Nu
			UBE2T	Ubiquitin conjugating enzyme E2 T
Cell cycle	3.78E-05	0.042	*BUB1*	BUB1 mitotic checkpoint serine/threonine kinase
			E2F1	E2F transcription factor 1
			MAD2L1	MAD2 mitotic arrest deficient-like 1
			TTK	TTK protein kinase
			WEE1	WEE1 G2 checkpoint kinase
			CDC20	Cell division cycle 20
			CDC45	Cell division cycle 45
			CCNA2	Cyclin A2
			CCNB1	Cyclin B1
			CDK1	Cyclin-dependent kinase 1
			CDKN2C	Cyclin-dependent kinase inhibitor 2C
			MCM6	Minichromosome maintenance complex component 6
			PTTG2	Pituitary tumour-transforming 2
			PLK1	Polo like kinase 1

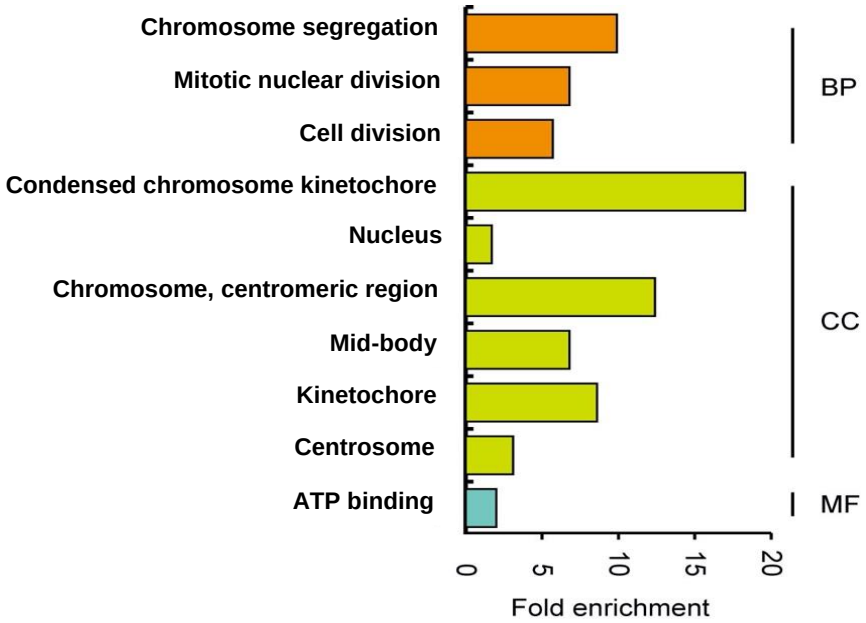

Figure 3 Histogram displaying gene ontology terms of differentially expressed genes enrichment in group subjected to heat stress

BP: biological processes, CC: cellular components, MF: molecular functions

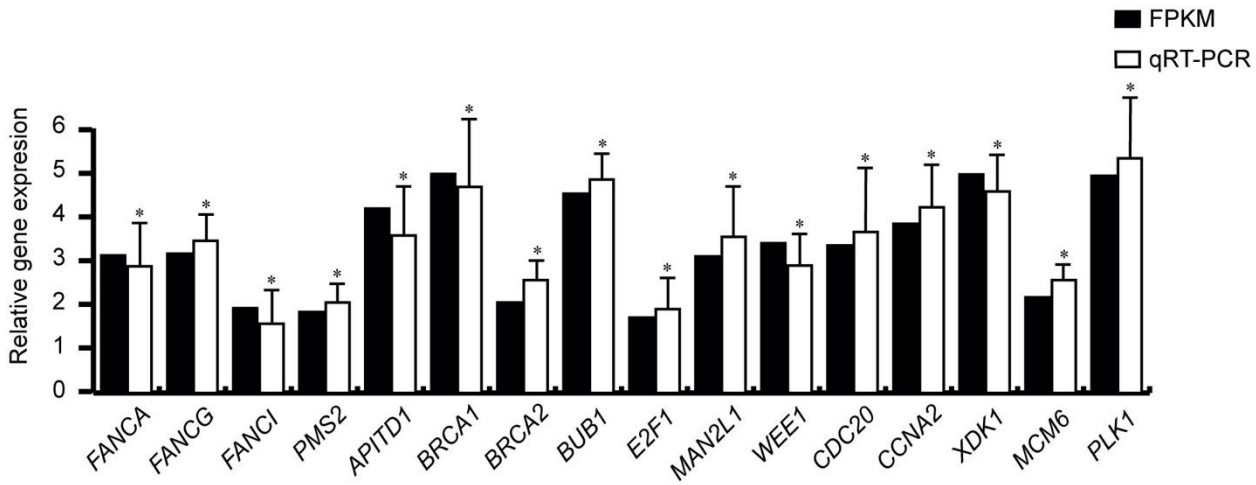

Figure 4 Validation of gene expression of differentially expressed genes in KEGG enrichment pathway
*Represents genes with significant differential expression (P <0.05)

After exposure to heat stress, the BW gain in YFC decreased by 9.9% and the BW gain ratio was 55.7%. In a study of chickens under heat stress conditions, the BW gain between 21 and 28 days of age in broilers decreased by 18% (Azad *et al.*, 2010). In Advanced Intercross Line chickens bred for thermo-tolerance (Deeb & Lamont, 2002), exposure to heat stress resulted in a reduction of BW gain by 58.9% (Angelica *et al.*, 2015). The similarity of these rates suggests that YFC can adapt to high ambient temperatures (Deeb & Lamont, 2002; Van Goor *et al.*, 2015). In poultry, relative weights of tissue are usually assessed as indicators of physiological development (Van *et al.*, 2013). Previous studies have shown that the heat stress-driven decline in the growth performance of fast-growing broilers has varying effects on organs, including the relative weight reduction of the spleen, thymus, bursa, and heart (Quinteirofilho *et al.*, 2010; Zhang *et al.*, 2017), but no significant difference in relative heart weight was found in slow-growing chickens and YFC under chronic heat stress (Zhang *et al.*, 2017; Zhang *et al.*, 2019). In the present study, the authors found that HWI was not affected significantly by chronic heat stress, and they observed normal physiological development of the liver in the treatment group YFC (Van *et al.*, 2013). In birds, body temperature is commonly recorded in the rectum (Poole & Stephenson, 1977) and the current result is in line with previous studies, which showed that chicken body temperature increased significantly under heat stress (Cooper & Washburn, 1998; Feng, 2010; Zhang *et al.*, 2020).

In contrast, only 37 DEG (FDR < 0.1) were identified in the heart transcriptome of YFC subjected to heat stress in a previous study (Zhang *et al.*, 2019). The current findings (596 DEG) suggest that the liver of YFC is more susceptible to heat stress than the heart. In terms of gene ontology, the biological processes affected by the DEG in the liver were related to chromosome segregation and cell division. A study on a hepatocellular cell line of male white Leghorns found that heat stress affected the expression of genes related to DNA replication, DNA repair, and chromosome segregation (Sun *et al.*, 2015). The KEGG pathway analysis of these DEG revealed the enrichment of the Fanconi anaemia pathway and the cell cycle pathway. Interestingly, while these DEG were up-regulated in the current experiment, the expression of DEG related to the cell cycle had previously been down-regulated in liver cell line of white Leghorn chickens under heat stress (Sun *et al.*, 2015). These results suggest that the response of tissues to heat stress is different from that of cell lines. This might be related to the genetic composition of the species, genomic variations, and the nature of heat stress (Sonna *et al.*, 2002).

The role of the Fanconi anaemia pathway and its proteins is to maintain genomic stability through responses to replication, and oxidative and mitotic stress (Palovcak *et al.*, 2017). For instance, *PMS2* is a mismatch repair gene, coding for a mismatch repair endonuclease, which maintains the stability of the genome by correcting the base mismatches and insertion-deletion mismatches during DNA replication and recombination (Sameer *et al.*, 2014). Replication stress occurs when the DNA secondary structure, DNA damage, or another factor, blocks the DNA replication mechanism and leads to stagnation (Cortez & David, 2015). Inter-strand crosslinks (ILCs) are one of the factors which can cause replication stress by completely blocking the progress of the replication fork and inhibiting DNA replication and RNA transcription (Clauson *et al.*, 2013). Fanconi anaemia proteins assist in the recovery of replication forks that were stalled because of ICLs. The product of the *FANCA* gene, which was up-regulated in the current study, has DNA-binding activity and regulates the activity of the crossover junction endonuclease *EME1* (encoded by the *EME1*

gene, which was also up-regulated in the current results) in an ICL damage-dependent manner (Benitez *et al.*, 2013), which could help to verify ICL damage. The function of the *EME1* product is in DNA repair and maintaining genome integrity. An *EME1* deficiency has been shown to cause spontaneous genomic instability (Abraham, 2003). Furthermore, the *POLN* gene codes for a DNA polymerase (Marini *et al.*, 2003), which participates in ICL repair (Bernard *et al.*, 2017; Zietlow *et al.*, 2009).

Fanconi anaemia pathway proteins can also maintain genome stability by preventing oxidative damage. Reactive oxygen species (ROS) are a known source of DNA damage that can drive genomic instability. Exogenous ROS can arise from heat stress. Under the conditions of oxidation, the proteins coded for by *FANCA* and *FANCG* were induced to be monoubiquitinated (Park *et al.*, 2004). Fanconi anaemia proteins and UBE2T monoubiquitination have been shown to be necessary to limit DNA damage (Alpi *et al.*, 2007). The FANCG protein interacts with cytochrome P4502E1 to support the roles of Fanconi anaemia proteins in redox metabolism (Makoto *et al.*, 2002). Fanconi anaemia proteins, specifically FANCA, could protect the promoters of antioxidant genes from oxidative stress (Wei *et al.*, 2012).

The Fanconi anaemia pathway is also involved in the regulation of chromosomal abnormalities in mitosis to maintain chromosomal stability (Naim & Rosselli, 2009). The current results indicate that one function of the Fanconi anaemia pathway may be to respond to a harmful cellular environment that is caused by oxidative damage. *FANCI* has been shown to be involved in maintaining chromosome stability (Dorsman *et al.*, 2014). The *FANCA* gene was demonstrated to be related to genome stability in the G2/S phase of mitosis, and DNA replication in the S phase (Howlett *et al.*, 2005). The product of *BRCA1* is involved in the G2/M checkpoint activation and repair of DNA damage (Zhu & Dutta, 2006). The gene APITD1 promotes the Fanconi anaemia proteins to chromatin. In the light of this research, the current results indicate that the up-regulation of the genes related to the Fanconi anaemia pathway in the liver of YFC may be responsible for regulating gene stability in response to heat stress.

The current analysis of the up-regulated DEG related to the cell cycle pathway indicated that they may also be involved in regulating DNA replication, repair, and mitosis in response to heat stress. The process of eukaryotic cell proliferation includes G1, S, G2, and M phases (Bell & Dutta, 2002). CDC45 codes for a DNA replication factor that participates in the activity of DNA helicase in *MCM6* ensures the correct loading of DNA polymerase, and is involved in the unwinding of DNA for initial DNA replication (Depamphilis *et al.*, 2006; Jeannine *et al.*, 2015). *CCNA2* is a highly conserved family of cyclins (Ko *et al.*, 2013), which regulate the initiation and progress of the S phase (Yam *et al.*, 2002) and interact with *CDK1* to regulate the cell cycle control of the G2/M transition (Arsic *et al.*, 2012; Pagano *et al.*, 1992). The product of the *PTTG1* gene is involved in the repair of DNA damage, and is up-regulated during cell replication (Vlotides *et al.*, 2007), and overexpression of the PTTG1 protein was shown to promote cell-cycle progression and cell proliferation (Zhang *et al.*, 1999) and it is considered a cell cycle regulator in the G1/S and G2/M transitions (Vlotides *et al.*, 2007). WEE1 has been shown to be a kinase involved in the cell cycle, regulating the G2 checkpoint block caused by DNA damage (Wang *et al.*, 2004) for pre-mitotic DNA repair (Raleigh & O'Connell, 2000). The *MAD2L1* (Zhang & Lees, 2001), *CDC20* (Shi *et al.*, 2017), *BUB1* (Kim *et al.*, 2015), and *TTK* (Yu *et al.*, 2015) code for the components of the spindle physical examination protein. These genes regulate the transition from the metaphase to the anaphase of the M phase by monitoring the attachment of sister chromatids to the spindle to ensure the fidelity of chromosome separation and genome stability (May & Hardwick, 2006). The function of the product of *PLK1* is mainly to regulate the M phase (Takaki *et al.*, 2008). Depletion of *PLK1* has been shown to cause mitotic arrest (Lei & Erikson, 2008), while the knockout of *PLK1* caused embryo death in mice (Lu *et al.*, 2008). In light of previous studies, the authors concluded that the up-regulation of the cell cycle genes observed in the liver of YFC is probably adaptive, and is responsible for the maintenance of normal DNA replication, repair and mitosis in liver under heat stress conditions.

Conclusion

The current findings provide evidence for the transcriptomic regulation of heat stress responses in the liver of YFC. A total of 596 DEG were detected to be involved in the heat stress response of the YFC liver. The DEG associated with the Fanconi anaemia pathway may be involved in heat stress-related genome stability. Meanwhile, the DEG associated with the cell cycle pathway may be involved in DNA replication, repair, and mitosis during cell proliferation. In the present study, these DEG were up-regulated in the heat stress group. The authors concluded that these genes may be involved in improving the stability of the YFC liver genome and may control the physiological response to heat stress. The current results provide insight into the potential mechanisms of YFC to protect against this important environmental stressor. In addition, this study should provide important insight into future efforts to improve the ability of species to withstand heat stress through genome-wide association studies and breeding.

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Authors' Contributions

YZY and QZ designed the study, QZ, YQL and ZWC performed the experiment. QZ and YQL analysed the data. QZ contributed to the writing of the manuscript. All the authors agreed with the final version to be submitted.

Conflict of Interest Declaration

The authors certify they have no conflicting interests regarding the material discussed in the manuscript.

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