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Negative effect of seasonal heat stress on testis morphology and transcriptomes in Angora rabbit

Haisheng Ding[1], Yuanlang Wang[1], Huiling Zhao[1], Jinzi Wang[1] and Dongwei Huang[1*]

Abstract

Background The temperature of testes exposed to hyperthermic conditions can affect livestock reproductive performance. This study aimed to explore the difference in semen quality, testicular morphological structure, and gene expression profiles of testes of Angora rabbits in spring (no heat stress), summer (intense heat stress), and autumn (no heat stress) seasons.

Results Heat stress during summer significantly reduced semen quality and damaged testicular morphology and spermatogenesis, which recovered to normal levels in autumn, although semen quality recovery was notably slow. RNA-Seq analysis showed that the expression levels of 8703 genes changed significantly in summer, but their expression levels in autumn returned to those in spring, which was consistent with the testicular morphology analysis results in different seasons. Enrichment analysis revealed that the DEGs were primarily associated with spermatogenesis, sperm motility, spermatid development, cell death, regulation of apoptotic processes, and responses to external stimuli. The MAPK, Rap1, TNF, Ras, Apoptosis, and Wnt signaling pathways regulated reproduction under heat stress. In addition, minimal variations in testicular morphology and gene expression profiles were observed between autumn and spring. Gene expression pattern analysis showed that genes with high expression in summer mainly participated in the regulation of cell apoptosis, immunity, and response to heat stress, whereas genes with low expression in summer mainly participated in the regulation of spermatogenesis.

Conclusions This study investigated the influence of different seasons on the reproductive performance of male Angora rabbits and provided initial insights into the molecular regulatory mechanisms underlying the testicular response to heat stress during summer.

Keywords Angora rabbits, Semen quality, Testicular morphology, Heat stress, RNA-Seq

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Background

High ambient temperature is an important subfertility factor and negatively affects animal and human fertility [1–4]. Heat stress is an environmental factor that affects animal reproductive performance by hindering testicular development, arresting spermatogenesis, degrading semen quality, and causing infertility in males [5, 6]. Almost all animals experience stress at high temperatures [7]. Compared to other agricultural animals, rabbits, particularly Angora rabbits, are more sensitive to heat stress because of their fewer sweat glands and a thicker coat of fur [8]. Seasonal declines in the reproductive efficiency of male rabbits occur during July, August, and September, and excessive heat leads to a reduction in ejaculate volume and sperm production, affecting semen quality by injuring testicular tissue and increasing abnormal sperm in the ejaculate [8, 9]. Thus, heat stress may decrease the reproductive performance of rabbits. Therefore, understanding the molecular mechanisms underlying heat stress-induced alterations in the testicular function is necessary.

Testes are the male reproductive organs where spermatogenesis occurs in male mammals, and ambient temperature considerably affects testicular function [10, 11]. Spermatogenesis occurs in the seminiferous tubules and is divided into spermatocytogenesis, meiosis, and spermiogenesis [12]. The mammalian testicular temperature is maintained at 2–8 °C below the core body temperature, which is essential for optimal reproductive function because a slight increase in testicular temperature can impair spermatogenesis and potentially result in infertility [13, 14]. For example, incubation of sperm samples from New Zealand White rabbits at 42 °C compared with 32.5 and 37 °C decreased total motility, mean velocity, curvilinearity, and metabolic activity [15]. Heat stimulation in the testes can induce excessive generation of reactive oxygen species (ROS), reduce oxidation resistance, cause apoptosis and autophagy of germ cells, and cause DNA damage in mature sperm [16–18]. Germ cells are susceptible to heat stress because of their high mitotic activity, particularly spermatocytes and early round sperm cells [19]. Heat stress can also reduce the number of Sertoli cells and damage their structure and function, resulting in a decline in sperm quality [20]. Testicular heat stress lowered the testosterone level of mice exposed to 40 °C [21]. Spermatogenesis depends on the testosterone produced by the Leydig cells of the testes. Exposure to heat stress at 41 °C impaired the process of spermatogenesis in mice subjected, leading to an increase in apoptosis [22], resulting in reducing fertilization capacity both in vivo and in vitro.

Tumor suppressor p53 is a potential inducer of germ cell apoptosis in response to heat [23]. Testicular p53 level is associated with germ cell loss after heat stress [24, 25]. ALOX15B is a critical enzyme in heat treatment-induced apoptosis in Sertoli cells, and the p38–p53 pathway participates in ALOX15B-induced apoptosis under heat treatment in Sertoli cells [26]. Heat stress can elevate the expression of Bcl-2 and induce redistribution of Bax from the cytoplasm to perinuclear or nuclear localization in germ cells [27]. Heat shock proteins (HSPs) are activated by heat stress to protect cells and tissues from environmental stress [28, 29]. Conversely, caspases are key mediators of apoptosis, and the activation of executioner caspase-3 and initiator caspase-9 is responsible for the death of mouse testicular cells after acute hyperthermia [30].

Mailin et al. [31]. explored the dynamic expression of genes in the recovery of multiple seminiferous epithelial cycles and spermatogenic cycles in mice testicular tissue after heat stress using RNA-seq. Multi-tissue transcriptome sequencing analysis in sheep was performed to investigate gene expression and understand the molecular mechanisms underlying heat stress [32]. Heat stress also affected negatively ovarian tissue of female rabbits and changed the expression of miRNAs using small RNA-seq [1].

The regulatory mechanisms underlying seasonal effects on male rabbit reproduction are poorly understood. Angora rabbits are highly susceptible to heat stress, as they have few functional sweat glands and long and thick hair coats and cannot effectively eliminate excess body heat when environmental temperatures are high. Therefore, this study aimed to investigate the differences in sperm production, testicular morphology, and transcriptome profiles in spring, summer, and autumn.

Materials and methods

Animals

Sixty-eight 1-year-old male Angora rabbits with healthy body conditions and normal reproductive functions were chosen from the Animal Husbandry Institute of the Anhui Academy of Agriculture Sciences. The rabbits were housed individually in cages, provided with *ad libitum* access to water, and fed a fixed amount of diet on a scheduled basis during the experiment. All animal experiment procedures were in strict accordance with protocols approved by the Animal Care Advisory Committee of the Anhui Academy of Agricultural Sciences (protocol code AAAS2021-29).

The ambient temperature and relative humidity (RH) was continuously measured using a digital thermometer (Xiaomi Technology Co., Ltd.) from May to November 2022. The temperature and humidity index (THI) were calculated using the following equations:

$$THI = db\ °C - [(0.31 - 0.31RH)(db\ °C - 14.4)]$$

db °C = dry bulb temperature, RH = relative humidity percentage/100. The obtained values were classified as

follows: <27.8, no heat stress; 27.8–28.9, moderate heat stress; 28.9–30.0, severe heat stress; >30.0, very severe heat stress.

Measurement of semen volume and sperm density

After brief exposure to female rabbits, semen was collected using an artificial vagina with a doe teaser in spring, summer, and autumn. Semen was collected in graduated test tubes, the volume of ejaculate was recorded, and the color of the semen was observed. The sperm density was analyzed using a CeroSmall Semen Quality Analyzer (Hamilton Thorne, Hamilton, OH, USA).

Testis sample collection and histological examination

Testis samples were obtained from four Angora rabbits sacrificed by injecting 20–30 ml of air into the ear vein during spring (May 9), summer (August 14), and autumn (November 10). All testicular samples were divided into two halves. A partial testis sample was fixed in 4% paraformaldehyde for histological analysis, as described in our previous study [33], whereas another testis sample was snap-frozen in liquid nitrogen until further transcriptome analysis.

cDNA library construction, sequencing, and mapping

Total RNA was extracted from twelve testis samples using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the integrity of the total RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RIN values were in the range of 7.4–8.9 of the twelve total RNA samples (Supplementary Table 1), the electropherogram of RNA samples were showed in Supplementary Fig. 1. The mRNA was enriched, fragmented, and converted into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). Adapters were ligated to the end of double-stranded cDNA, and the library was created by PCR using the Illumina Truseq RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer's protocols. Twelve libraries of the testes of Angora rabbits from spring, summer, and autumn were sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

To obtain high-quality reads, low-quality reads, adapters, and N reads were filtered from raw reads using *fastp* (version 0.18.0). Paired-end cleaned reads were mapped to the *Oryctolagus cuniculus* genome using HISAT2.2.4 [34]. The mapped reads for each testis sample were assembled using StringTie v1.3.1 [35]. To quantify gene expression abundance in the testis, RSEM was used to measure the Transcripts Per Kilobase of the exon model per million mapped reads (TPM). Differential expression analysis between the two groups was performed using DESeq2 software. Differentially expressed genes (DEGs) were selected with the parameter of false discovery rate (FDR) below 0.05 and|fold change| ≥ 2.

GO enrichment and KEGG pathway analyses

To understand the biological functions of DEGs, Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses were conducted through mapping DEGs to the GO (http://www.geneontology.org/) and KEGG (www.genome.jp/kegg/) database. A *P.* adjust value ≤ 0.05 was considered significant for GO analysis, and *Q* value ≤ 0.05 was considered significant for KEGG analysis.

Gene expression patterns analysis of genes

Gene expression patterns were analyzed to cluster genes with similar expression patterns in multiple samples. To examine the expression patterns of DEGs, the expression data from each sample were normalized to 0, log2 (v1/v0), and log2 (v2/v0) and then clustered by STEM [36]. Clustered profiles with $P \leq 0.05$ were considered significant. Then the biological functions of DEGs from clustered profiles were analyzed using the GO and KEGG database.

qRT-PCR validation of sequencing data

Total RNAs were extracted from the testes using the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). qRT-PCR was performed on an ABI 7500 (Applied Biosystems, Waltham, MA, USA) using TransStart Green qRT-PCR SuperMix (TransGen, Beijing, China). The reference gene was *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene. Eight candidate genes were examined using the $2^{-\Delta\Delta CT}$ method. The primer sequences used for qRT-PCR are listed in Supplementary Table 2. The amplification and melting curves for DEGs were showed in Supplementary Figs. 2 and 3, respectively.

Statistical analyses

All data were reported as the mean ± standard deviation from four biological replicates. A student's *t*-test was used to analyze differences between different groups. The threshold of $P < 0.05$ was considered significant.

Results

Changes in temperature, humidity, and THI of the rabbit farm in different seasons

To understand the environmental heat stress in the rabbit house, the temperature and humidity of the Angora

Table 1 Temperature, humidity, and THI changes in the rabbit house in different seasons

Time	Temperature (°C)	Humidity (%)	THI
Spring (April 21–May 20)	18.79 ± 3.32	78.70 ± 10.31	18.34 ± 3.08
Summer (August 1–August 31)	30.29 ± 1.51	74.48 ± 5.08	28.91 ± 1.18
Autumn (October 11–November 10)	15.67 ± 2.40	70.16 ± 8.17	15.39 ± 2.13

rabbit house were monitored for one month during the spring, summer, and autumn seasons. The THI was then calculated separately. The average temperature, humidity, and THI of the Angora rabbit house in spring (April 21–May 20) were 18.79 ± 3.32 °C, 78.70 ± 10.31%, and 18.34 ± 3.08, respectively (Table 1). In August, the average temperature, humidity, and THI of the rabbit house were 30.29 ± 1.51 °C, 74.48 ± 5.08%, and 28.91 ± 1.18, respectively. In autumn (October 11-November 10), the average temperature, humidity, and THI of the rabbit house were 15.67 ± 2.40 °C, 70.16 ± 8.17%, and 15.39 ± 2.13, respectively. The results indicated no heat stress in spring and autumn, but severe heat stress was observed in summer in the rabbit house.

Comparison of Angora rabbit sperm quality in different seasons

Semen volume was the highest in spring, decreased significantly in summer ($P < 0.05$), and then slightly increased in autumn ($P > 0.05$) (Fig. 1A). Sperm density in spring was significantly higher than that in summer and autumn ($P < 0.05$). Compared to summer, sperm density increased in autumn ($P > 0.05$) (Fig. 1B). The results showed that heat stress in summer had an apparent negative effect on sperm quality, which increased slightly in autumn.

Morphological change of testis of the Angora rabbit in different seasons

To better understand the morphological differences in the testes during different seasons, HE staining was conducted to compare testis and seminiferous tubule development (Fig. 2). The morphology of the testes and seminiferous tubules were intact, and all types of germ cells, including spermatogonia, spermatocytes, round spermatids, and elongated spermatids, were observed in spring and autumn (Fig. 2A, B, E, F). However, apparent atrophy of the seminiferous tubules and an increase in the gap between seminiferous tubules were observed in the testes in summer (Fig. 2C). The seminiferous tubules exhibited abnormal spaces, few spermatogonia, spermatocytes, round spermatids, and no elongated spermatids (Fig. 2D).

Transcriptome of Angora rabbit testis by RNA sequencing

To explore the transcriptome differences in testis tissues across different seasons, transcriptome sequencing of 12 representative testes was performed using RNA sequencing to determine which genes exhibited alterations. We obtained 36.08–40.53 million raw reads. After filtering the low-quality raw reads, 35.81–40.36 million clean reads were obtained. Approximately 81.70–87.14% of clean reads were uniquely mapped to the rabbit genome (Supplementary Table 3). Then, sample grouping was assessed, and the PCA results showed that the testis samples from spring and autumn were clustered together and differentiated from testis samples from summer in the direction of component 1 (Fig. 3A). Gene expression analysis detected 10,174 DEGs in the testes of Angora rabbits between spring and summer, comprising 5607 genes that were highly expressed in summer and 4567 genes that were highly expressed in spring. In addition, 9784 DEGs were detected between summer and autumn, comprising 4301 genes that were highly expressed in

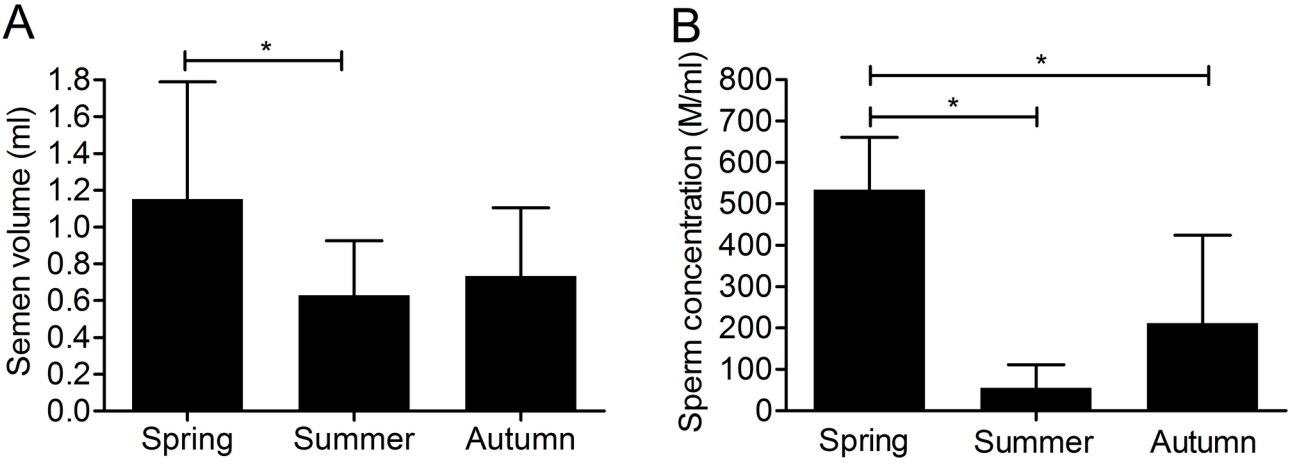

Fig. 1 The effect of season on semen volume and sperm concentration. **A** Semen volume; **B** Sperm concentration. *$P < 0.05$

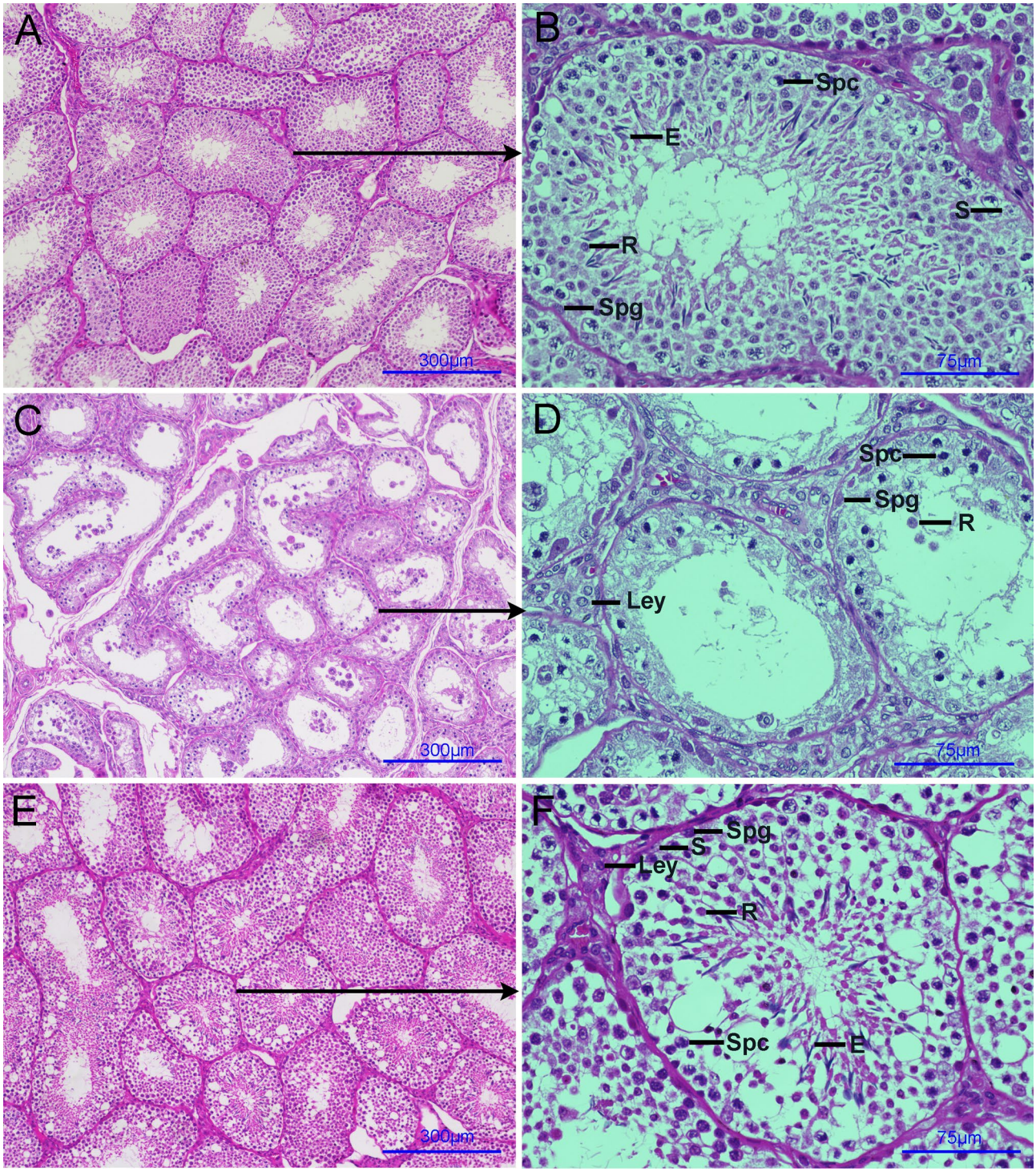

Fig. 2 Morphological characteristics of testis in different seasons. HE-stained testes from spring (**A**, **B**), summer (**C**, **D**), and autumn (**E**, **F**). Spg, spermatogonia; Spc, spermatocyte; R, round spermatid; E, elongated spermatid; S, Sertoli cell; Ley, Leydig cell. Scale bars = 300 μm (**A**, **C**, and **E**) and 75 μm (**B**, **D**, and **F**)

autumn and 5483 genes that were highly expressed in summer. However, only 21 DEGs were detected between autumn and spring (Fig. 3B). In addition, hierarchical clustering showed that the gene expression patterns of the testes in autumn and spring were similar but notably different from those in summer, and more genes were upregulated in summer (Fig. 3C), demonstrating that heat stress in summer changed the gene expression

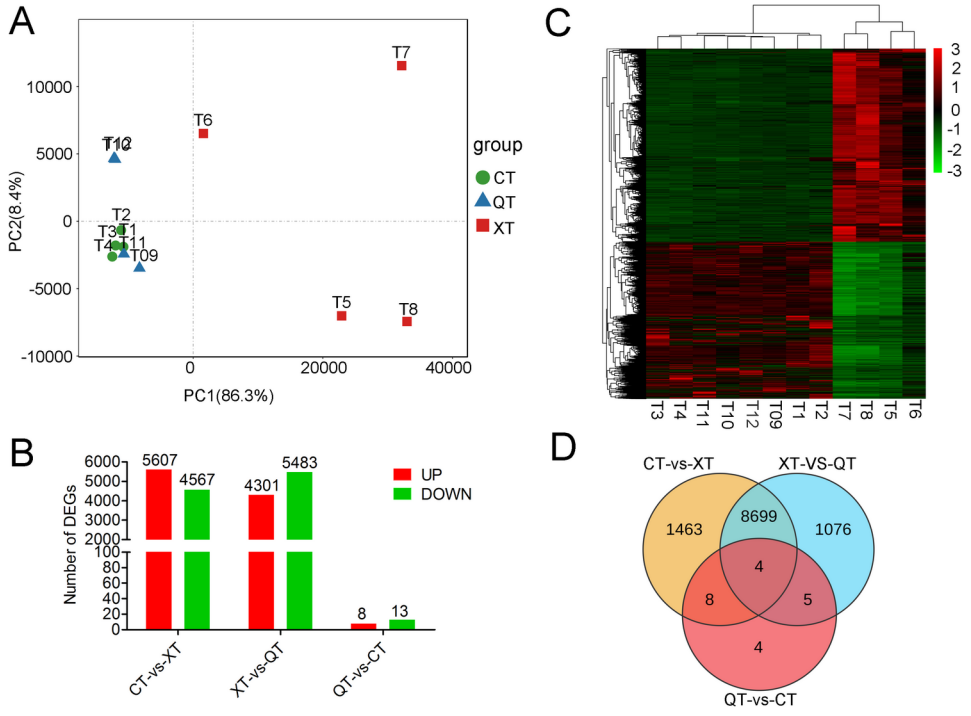

Fig. 3 Difference analyses of testicular genes in spring, summer, and autumn. **A** PCA of DEGs of testes between spring, summer, and autumn. **B** The number of DEGs of testes in different seasons. **C** Cluster analysis of DEGs. The red color indicates increased expression, and the green color indicates decreased expression. **D** Venn diagram representing the common differentially expressed gene distribution. T1–4 represent testes in spring, T5–8 represent testes in summer, and T09–12 represent testes in autumn. CT, XT, and QT represent the testis tissues from spring, summer, and autumn, respectively

pattern in Angora rabbits. Venn analysis showed that 8703 genes were differentially expressed between the two comparison groups (spring vs. summer and summer vs. autumn) (Fig. 3D). Simultaneously, the expression trends of these genes were opposite in the spring/summer and summer/autumn groups. Except for four genes, all other genes underwent significant changes in expression levels after heat stress in summer, but their expression levels recovered to the same level as in spring during autumn, demonstrating that the state of the testis returned to normal in autumn.

Functional enrichment analysis of DEGs from testes between spring, summer, and autumn

GO and KEGG enrichment analyses were performed to identify the biological functions of DEGs from the testes in different seasons. A total of 998 biological processes were significantly enriched in the DEGs between spring and summer. As shown in Supplementary Table 4, some GO terms related to reproductive and heat stress, including spermatogenesis, sperm motility, spermatid development, spermatid differentiation, cell death, regulation of apoptotic processes, and response to external stimuli, were significantly enriched. In addition, 1004 biological processes were significantly enriched in DEGs between summer and autumn. Spermatogenesis, sperm motility, male gamete generation, spermatid development, cell death, the regulation of apoptotic processes, and responses to external stimuli were also significantly enriched. However, no GO terms were significantly enriched by the DEGs in the testes between autumn and spring.

KEGG enrichment analysis showed that 122 signaling pathways were significantly enriched in DEGs from testicular tissues between spring and summer. In addition, 95 signaling pathways were significantly enriched in the DEGs between summer and autumn. As presented in Supplementary Table 5, the MAPK, Rap1, TNF, Ras, Hedgehog, Apoptosis, and Wnt signaling pathways were significantly enriched by DEGs from the two comparisons (spring vs. summer and summer vs. autumn). No signaling pathways were significantly enriched in the DEGs between autumn and spring. This indicates that summer heat stress has a greater impact on testicular tissues.

Gene expression pattern analysis

Gene expression patterns in the testes were analyzed during spring, summer, and autumn (Fig. 4). The eight gene profiles showed eight gene expression patterns during spring, summer, and autumn. Most genes were assigned to Profiles 5 and 2 (Fig. 4A). Gene expression in Profile 5 increased in summer and decreased in autumn, and the opposite trend was observed in Profile 2. Figure 4B

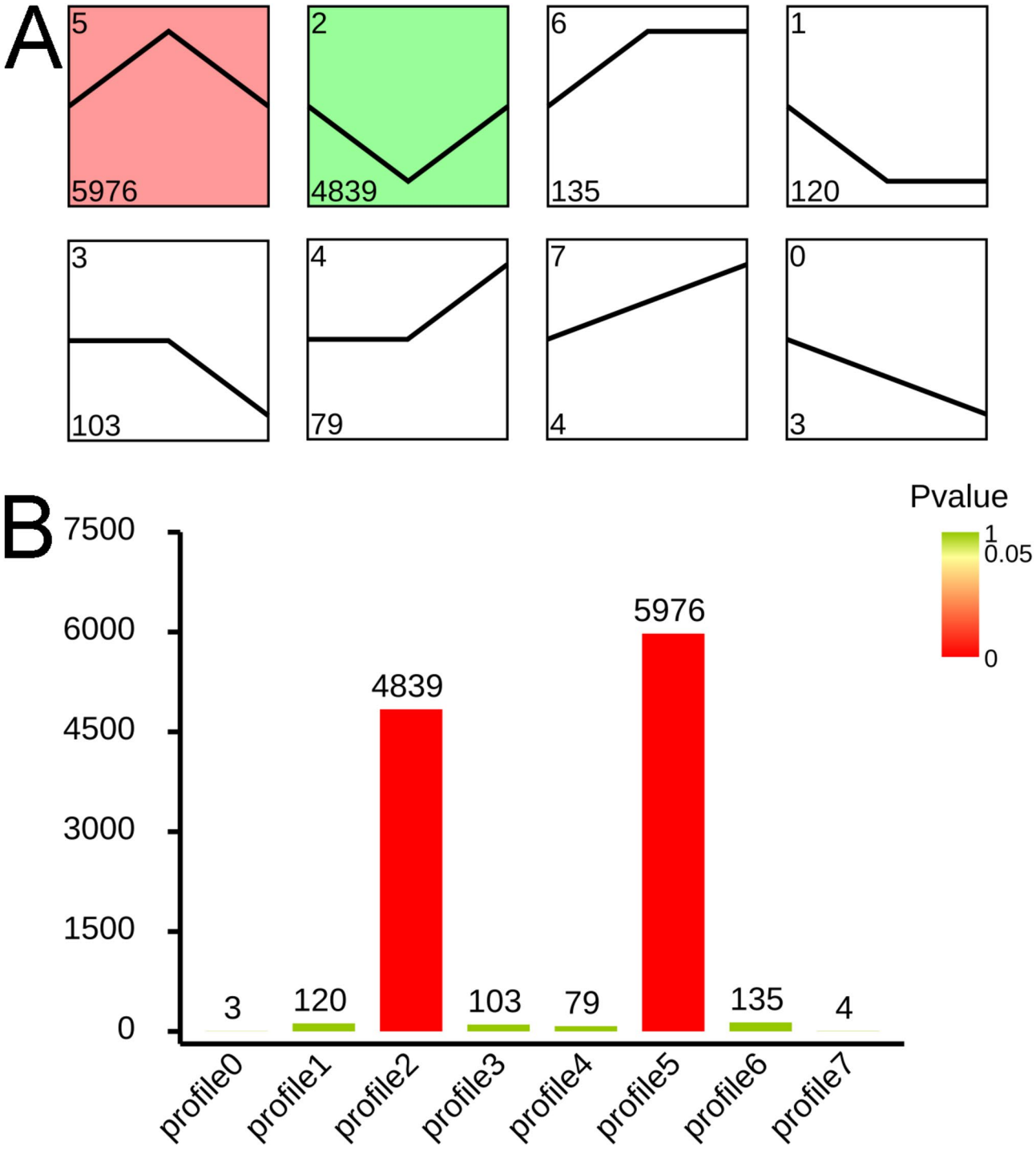

Fig. 4 Gene expression pattern analyses of testes during spring, summer, and autumn. **A** The gene expression trend and gene number; **B** The *P*-value of gene profiles

shows that the *P*-value for Profile 2 was the lowest, followed by Profile 5. Functional enrichment analyses were performed for the genes in Profiles 5 and 2. Figure 5A shows that the DEGs from Profile 5 were significantly involved in biological processes, including the regulation of responses to stimuli, immune system processes, cell motility, and anatomical structure development. DEGs from Profile 2 were significantly involved in sperm motility, sexual reproduction, spermatid differentiation, spermatid development, male gamete generation, and spermatogenesis (Fig. 5B). KEGG analysis showed that 117 signaling pathways were significantly enriched in

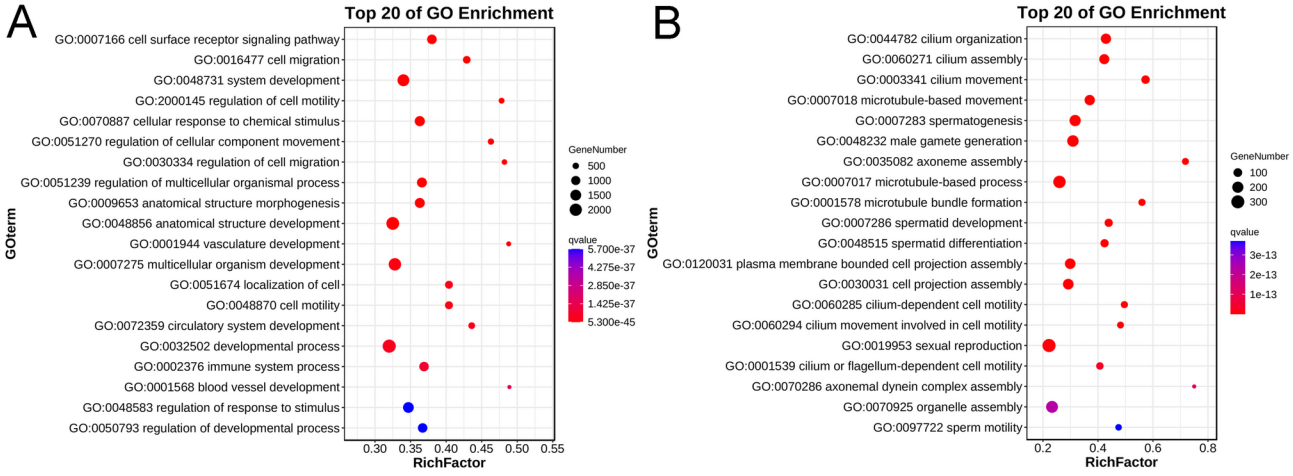

Fig. 5 GO enrichment analysis of the DEGs from Profiles 5 (**A**) and 2 (**B**)

DEGs from Profile 5. Figure 6 shows some signaling pathways related to reproduction and heat stress of summer were filtered, such as MAPK, TNF, PI3K-Akt, Hippo, Endocrine resistance, Rap1, GnRH, Ras, FoxO, Notch, TGF-beta, and HIF-1 signaling pathway. The number of DEGs was highest in the PI3K-Akt signaling pathway. However, only renin secretion and the Hedgehog signaling pathway were significantly enriched in DEGs from Profile 2 (Supplementary Table 6).

Validation of DEGs with qRT-PCR

Eight DEGs were randomly selected to validate our RNA-Seq data, and their expression levels in different seasons were detected using qRT-PCR (Fig. 7). The expression levels of sperm acrosome associated 1 (SPACA1), CFAP97 domain containing 1 (CFAP97D1), SHC binding and spindle associated 1 like (SHCBP1L), polyamine modulated factor 1 binding protein 1 (PMFBP1), sperm equatorial segment protein 1 (SPESP1), L-lactate dehydrogenase C chain (LDHC), and heat shock protein family B (small) member 9 (HSPB9) decreased in summer and increased in autumn, and the expression pattern of cyclin D2 (CCND2) showed the opposite trend, which was similar to the RNA-Seq results.

Discussion

Heat stress negatively affects livestock reproduction, particularly when high temperature is combined with high humidity. Increases in air THI are correlated with poor sperm production [37]. The THI obtained in this study indicated that Angora rabbits suffered from severe heat stress (28.91) in August and no heat stress in spring or autumn [38]. Heat stress impairs spermatogenesis and decreases sperm concentration, leading to decreased sperm production [39]. Cao et al., 2023 reported that heat stress reduces sperm quality mainly because of an imbalance in the testicular microenvironment and disturbed retinol metabolism [40]. Heat stress can also lead to testicular atrophy, which impairs male fertility [41]. In this study, under severe heat stress, semen volume and sperm density decreased in summer compared to those in spring and autumn. In addition, testicular morphological analysis showed apparent atrophy of the seminiferous tubules and an increase in the gap between the seminiferous tubules and a few germ cells during summer. Intact testis morphology, seminiferous tubules, and all germ cell types were observed in spring and autumn. Semen quality improved in autumn but was not significantly different from summer. In rabbits, approximately 44 days are required for spermatogonia to differentiate into spermatozoa in the testis [42], and 10–14 days are required for spermatozoa to pass through the epididymis into the ejaculate [43]. Therefore, at least 54 days are required for spermatogonia to differentiate into mature spermatozoa [44]. In this study, testicular tissue and semen were collected on May 9, August 14, and November 10 in spring, summer, and autumn, respectively. Therefore, we speculated that semen quality had not fully recovered by November 10 and that a longer recovery period would be required. These findings indicated that heat stress during summer impaired testis and spermatogenesis in Angora rabbits, damaged testicular tissues recovered to normal levels in autumn, and the recovery of semen quality was slower than that of testicular tissue.

This study also analyzed the changes in the mRNA profiles of Angora testes in spring, summer, and autumn using high-throughput sequencing. Expression analysis of DEGs showed that heat stress in summer caused differential expression of many genes; however, only 21 genes were differentially expressed between autumn and spring, which was consistent with the results of PCA, hierarchical clustering, and testicular morphological analyses. These results indicated that the expression profiles of testes in autumn and spring were similar, but

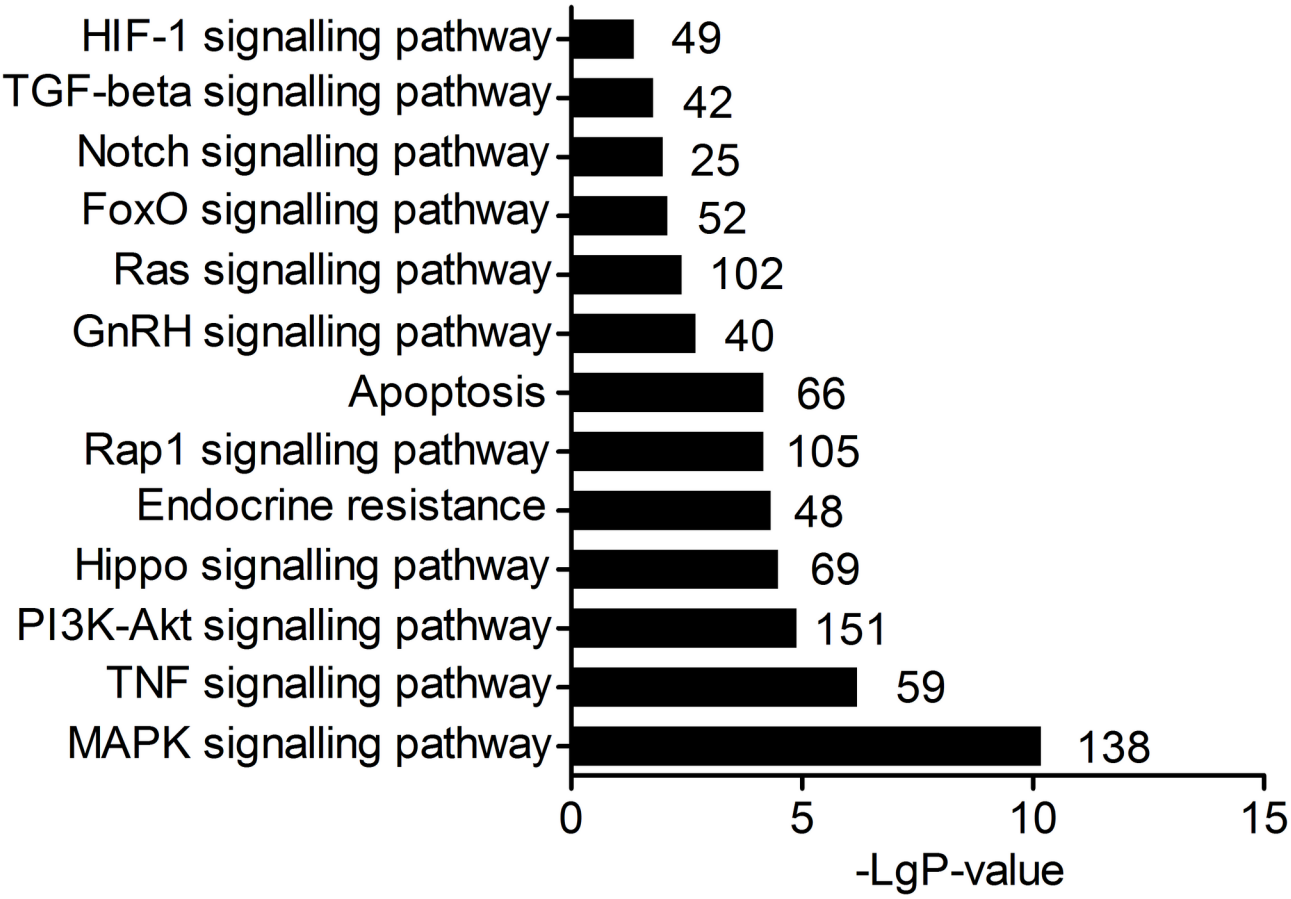

Fig. 6 KEGG signaling pathways enriched by DEGs from Profile 5

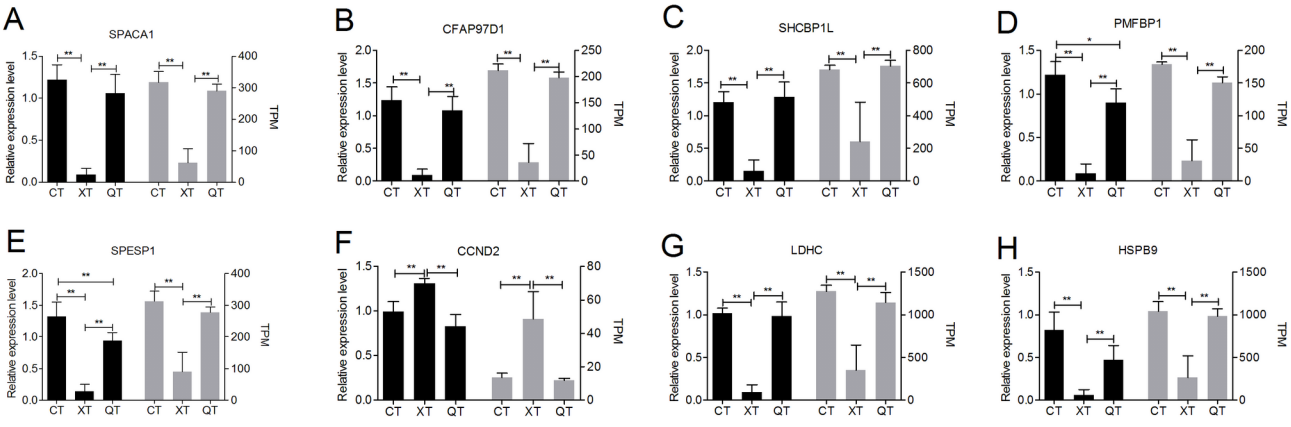

Fig. 7 qRT-PCR validation of the mRNA-seq data. **A** SPACA1; **B** CFAP97D1; **C** SHCBP1L; **D** PMFBP1; **E** SPESP1; **F** CCND2; **G** LDHC; **H** HSPB9. The black and grey columns represent the qRT-PCR and sequencing results, respectively. CT, XT, and QT represent the testis tissues from spring, summer, and autumn, respectively. *$P < 0.05$; **$P < 0.01$

different from those in summer. In addition, 8703 DEGs showed opposite expression trends between spring and summer and summer and autumn, and their expression levels recovered to the same levels as in spring during autumn. Approximately 8 weeks are required before semen quality returns to normal [20], demonstrating that after heat stress in summer, the expression of genes regulating fertility returns to normal levels in the testes in autumn. In addition, eight genes were randomly selected for validation by qRT-PCR, and the results were consistent with the RNA-Seq data, demonstrating the reliability of the RNA-Seq data. Xun et al..(2015) [45] found that

the expression level of HSPB9 was highest in July (summer), higher in September and November (autumn) and lowest in January and March (winter-spring), which was contrary to our findings. HSPB9 is testis-specific heat stress protein and expresses in spermatogonia, spermatocytes, and round spermatids [45]. It is speculated that Angora rabbits are more sensitive to heat stress. In addition, semen quality and testicular morphological analyses showed severe heat stress seriously damaged the spermatogenic epithelium of testis, resulting in temporary loss of spermatogenic function, which resulted the expression of HSPB9 might be inhibited due to severe heat damage.

GO enrichment analysis showed that biological processes related to fertility and apoptosis, including spermatogenesis, sperm motility, spermatid development, cell death, regulation of apoptotic processes, and response to external stimuli, were significantly enriched by DEGs from the two comparisons (spring vs. summer and summer vs. autumn). During spermatogenesis, the differentiation and maturation of spermatocytes and other cells are extremely sensitive to heat stress [19]. This indicates that heat stress during the summer negatively influences spermatogenesis. The main enriched pathways, including the MAPK, Rap1, TNF, Ras, Hedgehog, Apoptosis, and Wnt signaling pathways, were significantly enriched in DEGs from the two comparisons (spring vs. summer and summer vs. autumn). MAPK is a fundamental signaling transduction pathway that regulates transcription and ectoplasmic specialization in the testis, mature spermatozoa flagellar motility, hyperactivation, and acrosome reaction [46]. The MAPK signaling pathway is important in regulating seasonal spermatogenesis [47], and can be activated by Rap1 in multiple cells [48, 49]. The Rap1 signaling pathway is regulated by a wide range of external stimuli [50]. External stimuli may trigger activation of the TNF-α/TNFR1 signaling pathway, which can result in cellular apoptosis [51, 52]. Heat shock protein 70 (HSP70) is crucial in reducing cell death by inhibiting TNF-induced apoptosis, suppressing the activity of MAPKs [53, 54], and protecting cells against stress-related damage [55]. Hedgehog signaling is a crucial regulator of male gonadal development and promotes germ cell survival in rat testes [56, 57]. External stimuli such as heat exposure can activate the Hedgehog signaling pathway [58]. Wnt signaling control is essential for adult spermatogenesis, and heat exposure disrupts the Wnt signaling pathway, which may cause male infertility [59, 60]. Consequently, we hypothesized that when the testes of Angora rabbits were exposed to heat stress in summer, multiple pathways were activated to disrupt spermatogenesis and induce apoptosis. In contrast, some pathways could promote the survival of germ cells to reduce damage in the testes in summer.

Gene expression pattern analysis is a method for clustering genes according to their expression characteristics in multiple continuous samples [61]. In the present study, the expression patterns of genes in the testes in spring, summer, and autumn were divided into eight profiles. Among them, most genes were clustered into Profiles 2 and 5, which showed opposite expression trends, providing a reference for identifying the key regulatory factors affecting spermatogenesis and testis development under heat stress. Enrichment analysis revealed that the genes in Profile 2 were involved in various biological processes, including sperm motility, spermatid differentiation, spermatid development, male gamete generation, and spermatogenesis. The expression of the genes in Profile 2 decreased in summer and increased in autumn. This demonstrates that the genes in Profile 2 were mainly involved in regulating spermatogenesis, and heat stress in summer decreased the expression of these genes and damaged spermatogenesis. Genes in Profile 5 were involved in biological processes related to cell death, apoptosis, immunity, and response to stimuli, as well as the MAPK, TNF, PI3K-Akt, Hippo, Rap1, GnRH, Ras, and HIF-1 signaling pathways. The PI3K/AKT pathway regulates oxidative stress-related processes [62, 63]. In addition, the expression of the genes in Profile 5 increased in summer and decreased in autumn. The results showed that genes from Profile 5 were important regulators of cell apoptosis, immunity, and the response to heat stress.

Conclusion

Our study revealed that heat stress during summer decreased semen quality, damaged the morphological structure of the testes and spermatogenesis, and altered gene expression profiles in Angora rabbits. After a recovery period, the testicular morphology and gene expression levels returned to normal in autumn. The mRNA expression profiles of the testes revealed the potential functional genes and pathways that regulate rabbit reproduction under heat stress. This study provides valuable insights into the molecular mechanisms underlying the response to heat stress in the testes. Further studies are required to investigate the regulation mechanism of crucial genes in reproduction during summer heat stress to improve reproductive performance of rabbits.

Abbreviations

CCND2	Cyclin D2
CFAP97D1	CFAP97 domain containing 1
DEG	Differentially expressed genes
FDR	False discovery rate
GO	Gene Ontology
HSP	Heat shock proteins
HSPB9	Heat shock protein family B (small) member 9
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDHC	L-lactate dehydrogenase C chain
PMFBP1	Polyamine modulated factor 1 binding protein 1

RH	Relative humidity
ROS	Reactive oxygen species
SHCBP1L	SHC binding and spindle associated 1 like
SPACA1	Sperm acrosome associated 1
SPESP1	Sperm equatorial segment protein 1
THI	Temperature and humidity index
TPM	Transcripts Per kilobase Million

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11659-x.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
D.H. and H.D. conceived the study. H.Z., D.H., and Y.W. performed sample collection and total RNA preparation. H.D. and Y.W. performed the qRT-PCR validation. H.D., J.W., and H.Z. conducted the data analysis and prepared figures and tables. H.D. wrote the manuscript. All authors read and approved the final manuscript.

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Data availability
The raw reads data was submitted to the Short Read Archive (SRA) under the accession number SRP562154 and BioProject accession number PRJNA1220349 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1220349).

Declarations

Ethical approval and consent to participate
All experimental protocols of this study were approved by the Animal Care Advisory Committee of the Anhui Academy of Agricultural Sciences (protocol code AAAS2021-29).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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