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## Effect of Heat Stress on the Expression of GABA Receptor mRNA in the HPG Axis of Wenchang Chickens

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### ■Keywords

Heat Stress; GABA receptor mRNA; HPG axis; Chickens.

### ABSTRACT

We investigated the effect of heat stress (HS) on the expression of the GABA receptor in the hypothalamic-pituitary-gonadal (HPG) axis of Wenchang chickens. Real-time quantitative RT-PCR (qRT-PCR) was used to quantify the GABA receptor mRNA levels along the HPG axis of chickens under HS ( $40 \pm 0.5$  °C) for 1-6 weeks. Our results showed that the expression of GABA<sub>A</sub> and GABA<sub>B</sub> receptor at the mRNAs levels in the tissues of HPG axis exhibited fluctuation and variability. After HS, the mRNA level of GABA<sub>A</sub> receptor was significantly reduced in the hypothalamus of 1-week-old and in the pituitary of 3-week-old chickens, but significantly increased in the pituitary of 1-, 4-, and 5-week-old chickens. The GABA<sub>B</sub> receptor mRNA level significantly declined in the hypothalamus of 1-week-old and in the pituitary of 3-week-old chickens, but was significantly upregulated in the pituitary and testis of 1- and 2-week-old chickens. At other time points, the expressions of GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor showed no significant differences compared with control group. These results indicated that the levels of GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor mRNAs varied in different tissues of the HPG axis in chickens of different ages, displaying temporal and spatial variations. GABA receptor behaved as a positively-regulated gene by HS, i.e., its mRNA was increased by HS; similarly, it was a negatively-regulated gene by HS, when its expression was reduced by HS.

### INTRODUCTION

Heat stress caused by high ambient temperatures adversely affects the growth, development, and reproduction of animals (Rensis & Scaramuzzi, 2003; McMichael & Dear, 2010). It is also one of the main factors restricting the development of intensive large-scale poultry farming. HS affects the behavior, physiology, and gene expression of animals. Under HS, chickens show behavioral changes, including reduced food intake, extended wing-spreading resting, increased water intake, etc. Under high temperature, the bird's body resists to HS by inducing the release of hypothalamic-pituitary hormones, i.e., they regulate their physiological activities, such as metabolism, immunity, and reproduction, etc. via synthesis and release of hormones related to the adrenal axis, thyroid axis, and gonadal axis (Akazome *et al.*, 2002; Elnagar *et al.*, 2010; Sohail *et al.*, 2010; Yin *et al.*, 2011). HS induces the expression of numerous genes, such as heat shock protein 70, growth- and metabolism-related genes (EGFR, SFRP1, and PDK4), immune-related genes (HSF1, Bcl-2, and Bax- $\alpha$ ), reproductive hormone receptor-associated genes (LHR, ESR, PRLR, and FSHR), rhythmic genes (Per2), etc. (Cai *et al.*, 2004; Zhao *et al.*, 2012; Li & Cui, 2013).

To date, the impact of HS on the expression of the GABA (gamma-aminobutyric acid) receptor has not been reported. As an inhibitory



neurotransmitter, GABA is widely present in the central nervous system and peripheral tissues; it can promote food intake in animals, regulate endocrine, autoimmune, hormonal, and reproductive physiology, and improve functions of anti-hypoxia, anti-HS, etc. in animals (Chen *et al.*, 2001; Jonaidi *et al.*, 2002; Chen *et al.*, 2014; Chen *et al.*, 2015). GABA exerts its biological functions via interaction with GABA receptor. Our previous studies showed that prenatal HS significantly increased the density of the GABA receptor protein on the membrane of the testicular and ovarian cells of newborn mice. HS also reduced the positive rate and average densitometric value of the GABA<sub>B</sub> receptor in immunostaining, indicating a delayed development of GABA<sub>B</sub> receptor (Wang *et al.*, 2011; Xie *et al.*, 2011).

Due to the widespread use of fluorescent quantitative PCR (qPCR), the study of the differential expression of a trace amount of gene transcripts is fast and convenient. Knowing the expression pattern of GABA<sub>A</sub> and GABA<sub>B</sub> receptors along the HPG axis in chickens under HS not only provides a theoretical basis for better understanding of the development and the pathological analysis of reproductive system in chickens, but also a reference for intensive poultry farming during hot seasons. Therefore, in the present study, the differential expression of gamma-aminobutyric acid (GABA) A receptor, beta 3(NM\_205346.3) and gamma-aminobutyric acid (GABA) B receptor, 2(XM\_419066.4) along the HPG axis of 1- to 6-weeks old Wenchang chickens was evaluated. The qRT-PCR technique was applied to determine the effects of changes in GABA receptor gene expression on the development of the gonadal axis of chickens and the effects of HS on its development.

## MATERIALS AND METHODS

### Sample collection

Healthy 1-day-old Wenchang chicks, provided by the Hainan Yongji Poultry Co., Ltd. (Hainan, China), were randomly divided into control (CK) and HS groups, with 140 chickens per group. In each group, there were 104 males, used for the collection of hypothalamus, pituitary, and testicular tissue samples, and 36 females, used for harvesting ovarian tissue samples.

Chickens of the HS group were daily placed between 12:00 and 14:00, in an artificial climate chamber at the temperature of 40±0.5 °C and humidity of 70-80%, while the CK birds were kept at room temperature. All birds had free access to water and feed (Chen *et al.*, 2014). At 15:00 of each weekend of weeks 1-6,

appropriate numbers of male and female birds in each group were sacrificed to collect hypothalamus, pituitary, testis, and ovary samples. In each group, triplicate samples of the hypothalamus and pituitary were collected from 3-10 males per group, and testis and ovary samples were collected from 6 birds per group. (Cheng *et al.*, 2012). The tissue samples were frozen in liquid nitrogen immediately after the dissection and stored at -70 °C until subsequent RNA extraction.

This experiment was approved by the Hainan Normal University Animal Experimentation Ethics Committee.

### Total RNA isolation and cDNA synthesis

About 10-20 mg of respective hypothalamus, pituitary, testis, and ovary samples were used to isolate total RNAs using the RNeasy Animal RNA Purification Kit (Qiagen, DP431, Beijing, China). The purity, concentration, and integrity of the extracted RNAs were determined using a spectrometer and agarose gel electrophoresis. Total RNAs were then reverse-transcribed into first strand cDNA using the FastQuant RT Kit (with gDNase) (Tiangen, KR106, Beijing, China). The cDNA products were stored in -20 °C freezer for further use.

### Real-time PCR

The gene sequences of GABA receptors of *Gallus gallus* were retrieved from the NCBI database, and the qPCR primers were designed using the Primer Premier 5.0 software. The primers for GABA<sub>A</sub> receptor  $\beta$ 3 subunit gene (NM\_205346.3) were as follows: forward, 5'-GCCCTCCATACTGATTACCAT-3'; reverse, 5'-GAATACAAAGCAGCCCATAGATA-3'. The primers for GABA<sub>B</sub> receptor subunit GB2 (XM\_419066.4) and internal control  $\beta$ -actin genes (NM\_205518.1) were based on previous report (Cheng *et al.*, 2012). The primers for GABA<sub>B</sub> receptor subunit GB2 were as follows: forward, 5'-TCGGGACCAACCCAACGTGC-3', reverse, 5'-CGTGC TGGCCTGATTGACGCT-3'. The primers for  $\beta$ -actin: forward, 5'-CCGCTCTATGA AGGCTACGC-3'; reverse, 5'-CTCTCGGCTGTGGTGGTGAA-3'.

The qPCR was set up using the Super Real PreMix Plus (SYBR Green) (Tiangen, FP205, Beijing, China) following the manufacturer's instructions, and the annealing temperature and the primer concentrations were optimized by experiment. The reaction (20  $\mu$ L) included 10  $\mu$ L 2 $\times$  SuperReal PreMix Plus, 0.6  $\mu$ L of each forward and reverse primers (10  $\mu$ M), and 1  $\mu$ L cDNA, with volume adjusted with sterile double distilled H<sub>2</sub>O. The amplification was conducted on the Roche



LightCycler® Nano quantitative PCR instrument, and the PCR conditions were as follows: 95 °C denaturation 15 min; 95 °C 10 s, 60 °C 20 s, 72 °C 20 s, 40 cycles; with dissociation curve. Reactions added with sterile H<sub>2</sub>O instead of the template served as negative control, and each sample was with 3 replicates. After PCR, the dissociation curve was used to confirm the specific amplification, and a quantitative analysis was performed based on the Ct values of both the target and internal control genes.

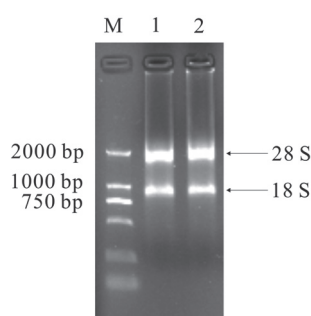
### Data processing

The relative quantification of gene expression was performed using  $2^{-\Delta Ct}$  method [ $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ ] (Jin *et al.*, 2014). Data were statistically analyzed using Microsoft Excel and SPSS 16.0 software, and the relative expression was presented as means $\pm$ SEM. The differences in gene expression between CK and HS groups were analyzed by Student *t*-test.

## RESULTS

### The integrity of the extracted total RNA

The OD<sub>260</sub>/OD<sub>280</sub> values of the extracted total RNAs were between 1.8 and 2.1. The results of 1.5% agarose gel electrophoresis (Figure 1) indicated good RNA integrity, without degradation and other contaminants.



**Figure 1** – Agarose gel electrophoresis of total RNAs. Note: M, Marker DL2000; 1, ovarian RNA of the CK group in week 3; 2, ovarian RNA of the HS group in week 3. Agarose gel electrophoresis of other samples also clearly showed the 28s and 18s bands like these two representative samples.

### The sensitivity and reliability of qPCR

The qPCR amplification curves of GABA<sub>A</sub> receptor, GABA<sub>B</sub> receptor, and  $\beta$ -actin genes of the HPG axis displayed a S-shape, with smooth baseline and large slope rate in the exponential amplification phase. All dissociation curves showed a single peak, without non-specific amplification and primer dimers. The amplification efficiencies were close between both the target and control genes (both in the range of

0.9-1.1). The qPCR products were then cloned and subjected to sequencing, and the sequences showed 100% homology with the original sequence of *Gallus gallus* in GenBank.

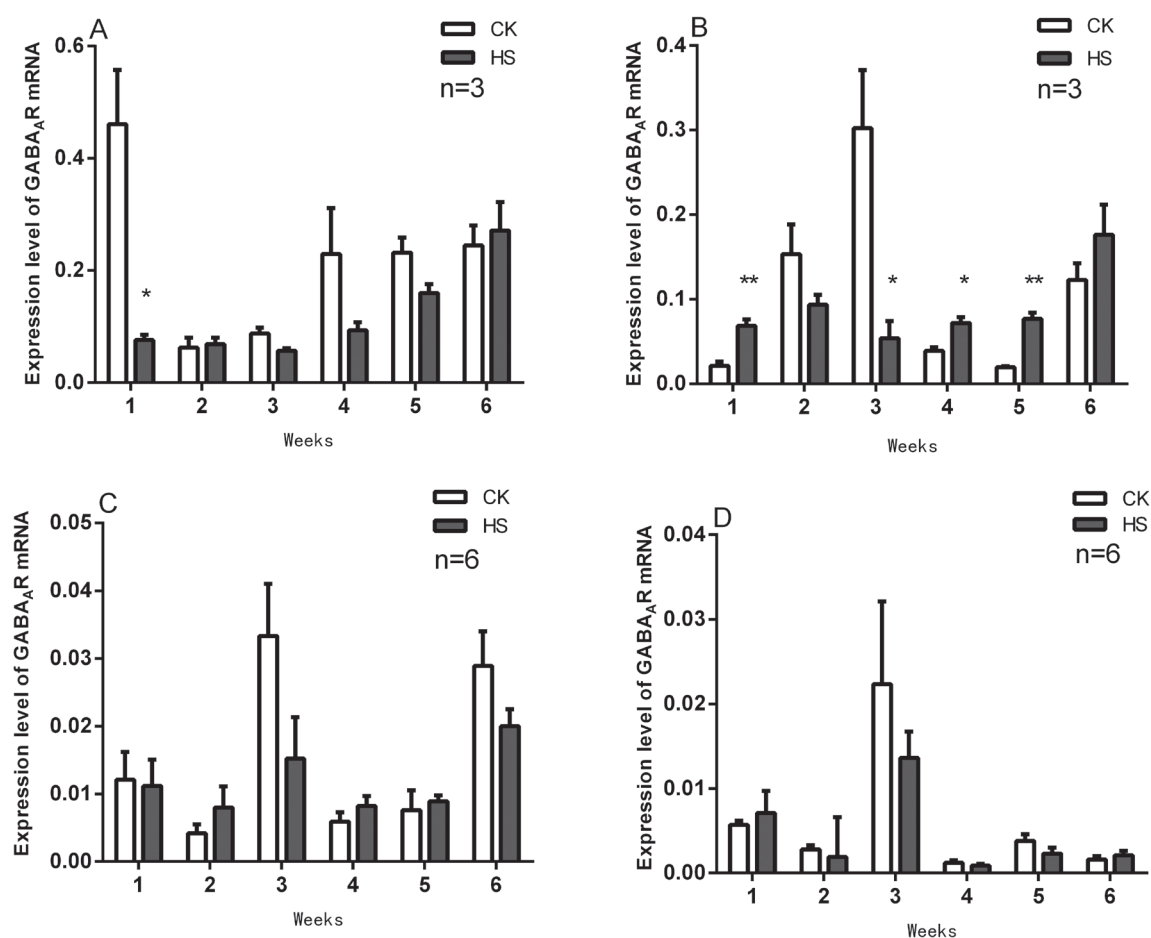
### The effect of HS on the expression of GABA receptor in the HPG axis

The relative expressions of GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor against the internal control  $\beta$ -actin gene in the hypothalamus, pituitary, testis, and ovary of chickens at different week-ages are shown in Figure 2 and 3.

As shown in Figure 2, the levels of GABA<sub>A</sub> receptor mRNA in the tissues of HPG axis of 1- to 6-week-old chicken displayed fluctuation and variability. Its expression in the hypothalamus peaked in the 1<sup>st</sup> week, declined in the 2<sup>nd</sup> week, but exhibited a rising trend during weeks 3-6. The levels of GABA<sub>A</sub> receptor mRNA in the pituitary increased in weeks 1-3, showed a decreasing trend in weeks 4-5, but increased in week 6. Its expression in the testis exhibited a decreasing trend in the first 2 weeks, increased in the 3<sup>rd</sup> week, declined in the 4<sup>th</sup> week, but increased again at 5-6 weeks of age. The expression of GABA<sub>A</sub> receptor in the ovary showed a decreasing trend in the first 2 weeks, increased in weeks 3 and 5, but declined in weeks 4 and 6. The GABA<sub>A</sub> receptor mRNA in the testis and ovary displayed a similar trend of expression, and its maximum level in the pituitary, testicular, and ovarian tissues appeared in the week 3.

HS affected the expression of GABA<sub>A</sub> receptor. One week after HS, the levels of GABA<sub>A</sub> receptor mRNA in the hypothalamus significantly decreased 0.2-fold relative to the control group, whereas its expression in the pituitary increased 3.4-fold relative to the control group. While the levels of GABA<sub>A</sub> receptor mRNA in the pituitary of chickens significantly decreased 0.2-fold of that of the control group three weeks after HS, its expression in the same tissue significantly increased 1.9-fold of that of the control group at 4 weeks of age. Five weeks after HS, the expression of GABA<sub>A</sub> receptor in the pituitary of chickens significantly increased 3.9-fold compared with the control group.

As shown in Figure 3, the expression of GABA<sub>B</sub> receptor in the tissues of HPG axis of 1- to 6-week-old chickens also varied and fluctuated. Its expression in the hypothalamus showed a decreasing trend in the first four weeks, increased in week 5 week, but decreased again in week 6. The expression of GABA<sub>B</sub> receptor mRNA in the pituitary increased in weeks 1-3, but declined in weeks 4-6. Its expression in the



**Figure 2** – Relative levels of GABA<sub>A</sub> receptor mRNA in the hypothalamus, pituitary, testis, and ovary of Wenchang chickens. Note: A, hypothalamus; B, pituitary; C, testis; and D, ovary. The results are expressed as mean±SEM by 2- Ct; \*  $p < 0.05$ .

testis exhibited an increasing trend in the first three weeks, decreased in weeks 4-5, but increased again in week 6. The levels of GABA<sub>B</sub> receptor mRNA in the ovary showed an increasing trend in the first three weeks and in week 5, but decreased in weeks 4 and 6. The GABA<sub>B</sub> receptor mRNA in the testis and ovary displayed a similar trend of expression during the first four weeks, and its maximum level of expression in the pituitary, testicular, and ovarian tissues appeared at 3 weeks of age.

HS affected the expression of GABA<sub>B</sub> receptor in chickens. One week after HS, the levels of GABA<sub>B</sub> receptor mRNA in the hypothalamus and testis of chickens significantly decreased 0.4- and 0.3-fold, respectively, compared with the control group; however, its expression in the pituitary increased 1.7-fold relative to the control group. While the levels of GABA<sub>B</sub> receptor mRNA in the pituitary and testis of chickens significantly increased 4.8- and 2.8-fold relative to the control group two weeks after HS, its expression in the pituitary significantly decreased 0.1-fold relative to the in control group at three weeks

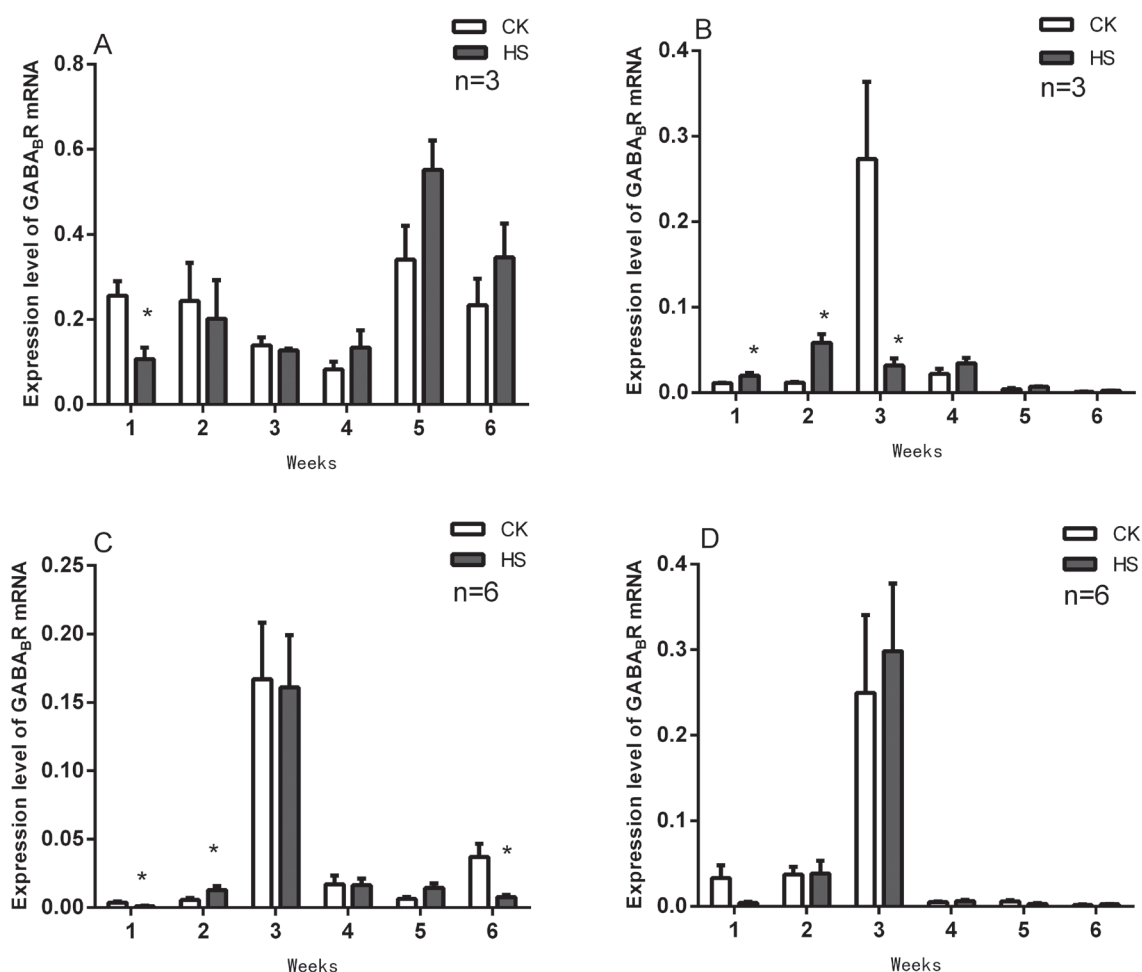
of age. Six weeks after HS, the expression of GABA<sub>B</sub> receptor in the testis of chickens significantly dropped, and corresponded to 0.2-fold of that in control group.

## DISCUSSION

The present study detected low levels of GABA<sub>A</sub> receptor  $\beta 3$  subunit and GABA<sub>B</sub> receptor R2 subunit mRNAs in the hypothalamus, pituitary, testicular, and ovarian tissue samples of Wenchang chickens. The expression of GABA receptor gene in different tissues of Wenchang chickens at different ages showed fluctuation and variability. Its expression in the hypothalamus and pituitary were slightly higher than those in the testis and ovary, which is different from previously reported results that the GABA contents in peripheral tissues, such as fallopian tubes, ovaries, testes, etc. were 2-fold higher than those in the central nervous system (Erdo *et al.*, 1982).

One week after HS, the expression of both GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor were significantly reduced in the hypothalamus, indicating that the normal





**Figure 3** – Relative levels of GABA<sub>B</sub> receptor mRNA in the hypothalamus, pituitary, testis, and ovary of Wenchang chickens. Note: A, hypothalamus; B, pituitary; C, testis; and D, ovary. The results are expressed as mean±SEM by 2- Ct; \* p< 0.05.

expression of receptors in the body was damaged by HS, and the appearance of a stable state thereafter may be a result of the body adaptation to the hot environment. Three weeks after HS, the significantly reduced expressions of both GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor in the pituitary may an early reproductive development or may be accompanied by a potential hormone development cycle, which may also induce the compensatory synthesis of a large amount of heat-shock proteins (HSPs), leading to a reduction in the production of other proteins (Tu *et al.*, 2010). When HS significantly increased the mRNA expression of the GABA receptor, the latter played a positive regulation role, resulting in an enhanced inhibition of GABAergic neurons. However, when HS significantly reduced the expression of GABA receptor mRNA, the GABA receptor functioned as a negative regulator of HS response, leading to a weakened inhibition. Previous studies have shown that treatment of spermatogonial stem cells with GABA increases the expression of GABA receptor, but inhibits cell proliferation, while treating oocytes

with GABA increases the expression of GABA receptor, but reduces estrogen production (Biggs *et al.*, 2013; Du *et al.*, 2013). The animal's body can regulate sexual development and maturation by secreting hormones of the HPG axis, which is the center controlling the development and function of reproductive system in animals. The classic pathway of the HPG axis is as follows: hypothalamus-secreted gonadotropin-releasing hormone (GnRH) reaches the pituitary and stimulates the pituitary gonadotropin (GTH) secreting cells to synthesize and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH); these gonadotropins reach the gonad via circulation and subsequently activate the production of sex hormones, such as testosterone, progesterone, etc. Akinci *et al.* (1999) showed that multiple GABA<sub>A</sub> receptor subunits, including the GABA<sub>A</sub> receptor  $\beta 3$  subunit, are present in the testis and ovary. Upon binding to the GABA receptors, GABA can regulate the synthesis and release of sex and other related hormones in the HPG axis, thereby exerting regulatory effects on the reproductive



development of animals (Akinci & Schofield, 1999). Studies also found that, by affecting the nutrient absorption, HS may cause metabolic disorders and altered progesterone levels, resulting in prolonged calving-to-estrus intervals, decreased estradiol levels, reduced generation of ovarian granulosa cells, and decreased vitality of follicles in cows (Rensis & Scaramuzzi, 2003). The regulatory effects of HS, GABA receptor, and sex hormones on the development of HPG axis are a particularly complex process that warrants further investigations.

In conclusion, the levels of GABA<sub>A</sub> receptor and GABA<sub>B</sub> receptor mRNAs varied in different tissues of the HPG axis in chickens of different ages, displaying temporal and spatial variations. Heat stress affected the expression of GABA receptor mRNA. Under heat stress, the expression of GABA receptor mRNA is significantly increased or decreased in the different tissues of the HPG axis.

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