



■ Author(s)

- Lu BB¹ <https://orcid.org/0000-0003-0868-1333>
Liang W¹ <https://orcid.org/0000-0003-4158-0104>
Liang C¹ <https://orcid.org/0000-0002-0375-257X>
Yu ZQ¹ <https://orcid.org/0000-0003-1746-4058>
Xie XZ¹ <https://orcid.org/0000-0003-1161-6073>
Chen Z¹ <https://orcid.org/0000-0001-8156-8754>

¹ Ministry of Education Key Laboratory for Ecology of Tropical Islands, Hainan Normal University, Haikou 571158, China.

■ Mail Address

Corresponding author e-mail address
Chen Zhong
Hainan Normal University, Long Kun Nan
Road, Haikou City, 571158, P.R. China.
Phone: +86-0898-65883521
Email: zh.chen@hainnu.edu.cn

■ Keywords

Heat stress; hypothalamic-pituitary-gonadal (HPG) axis; reproductive hormone; Wenchang chicks.



Effect of Heat Stress on Expression of Main Reproductive Hormone in Hypothalamic–Pituitary–Gonadal Axis of Wenchang Chicks

ABSTRACT

The hypothalamic–pituitary–gonadal (HPG) axis is a key neuroendocrine regulation system involved in the growth and reproduction of poultry. High-temperature conditions lead to the physiological dysfunction of target organs of the HPG axis of poultry, ultimately affecting the animals' growth and development. In this study, we evaluated the effect of heat stress (HS) on the development of cells secreting major reproductive hormones of the HPG axis (i.e., hypothalamus, pituitary gland, ovary, and testis) of Wenchang chicks. Seventy-two one-day-old healthy Wenchang chicks were randomly divided into control (CK) and HS groups. The HS group was placed in a $40 \pm 0.5^{\circ}\text{C}$ artificial climate chamber for heat-stress treatment from 13:00 to 15:00 daily for six consecutive weeks. As development progressed, compared with the CK group, the gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels in the hypothalamus and testosterone (T) levels in the testes of male chicks in the HS group were significantly decreased at three weeks of age ($p < 0.05$). However, GnRH levels in the hypothalamus and estradiol (E_2) levels in the ovaries of female chicks in the HS group were variable and significantly lower than those of the CK group at four and five weeks of age ($p < 0.05$). In addition, the LH and FSH levels in the pituitary gland were significantly lower than those in the CK group at two and four weeks of age and at four and six weeks of age, respectively ($p < 0.05$). In brief, HS caused dysfunction of the corresponding target organs of the HPG axis in Wenchang chicks, and this affected the normal growth and development of the cells' HPG axis.

INTRODUCTION

With global warming, high-temperature and high-humidity environments in tropical and subtropical regions are increasingly detrimental to the livestock industry. Poultry are highly susceptible to heat stress (HS), because they are covered with feathers and lack sweat glands. When the ambient temperature exceeds the upper limit of the thermoneutral zone of poultry, the body will be under stress, resulting in tissue and endocrine system disorders and suppressed organ development. These issues can result in stunted growth and reproductive performance and may even lead to shock or death of poultry in severe cases (Zhou *et al.*, 2016; Zhu *et al.*, 2017).

In animals, the endocrine and nervous systems work together to form a neuroendocrine–immune network. The hypothalamic–pituitary–gonadal (HPG) axis is an important component of the endocrine system, regulating reproductive functions of the body. The GnRH secreted from the hypothalamus activates specific receptors on the pituitary gland to stimulate the synthesis and secretion of



FSH and LH from gonadotropic cells of the pituitary gland. FSH and LH enter peripheral blood circulation to ultimately promote the secretion of sex hormones, and the sex hormones exert feedback inhibition to affect the synthesis of GnRH, FSH, and LH. This cycle is essential for studying the distributions of reproductive hormones (Chimento *et al.*, 2014). High temperatures affect the synthesis and secretion of several relevant hormones such as FSH, LH, and E₂ (Zhang *et al.*, 2012). Under such environmental circumstances, the body regulates the HPG axis by changing the secretion activities of these hormones. Studies have shown that high temperatures alter the secretion of GnRH in the hypothalamus as well as LH and FSH in the pituitary gland of poultry, leading to the dysregulation of the HPG axis (Elnagar *et al.*, 2010; Chen *et al.*, 2015; Tu *et al.*, 2016). Existing studies on major reproductive hormones of the HPG axis have been mostly reported in mammals (Schams *et al.*, 1980; Kim *et al.*, 2013; Maurya *et al.*, 2016), and related studies of Wenchang chicks, especially those evaluating the effects of high temperature on the synthesis of T and E₂ in the reproductive system and inhibiting the gonad development of poultry, have rarely been reported. Hence, in this study, we examined the developmental changes of related reproductive hormones in the hypothalamus, pituitary gland, ovary, and testis of chicks using immunohistochemistry. We evaluated the effect of HS on the growth and development of cells secreting major reproductive hormones in the HPG axis of Wenchang chicks. The results provide fundamental information concerning the growth and development of chicks in high-temperature environments.

MATERIALS AND METHODS

Experimental animals and heat-stress treatment

One hundred forty-four healthy one-day-old Wenchang chicks (1:1 male-to-female ratio) with no significant differences in body weights were purchased from Hainan YongJi Livestock (Hainan, China). The chicks were randomly divided into two groups: control (CK) and heat-stress (HS) groups, with 72 animals (36 males and 36 females) per group. The chicks were weighed, labeled, and fed *ad libitum* with free access to feed (purchased from Zhanjiang Yilong Feed Mill Co. Ltd., Zhanjiang, China) and sterile water, and were housed in a ventilated and lighted (14 h of light, 10 h of darkness) animal room. The Wenchang chicks in the HS group were placed in an artificial

climate chamber (model LRH-800-GS, Tomorrow Environmental Protection Instrument, Shaoguan, China) daily for HS at 40 ± 0.5°C from 13:00 to 15:00, and the Wenchang chicks in the CK group were simultaneously placed in an unheated artificial climate chamber for 2 h of control treatment at 29.7 ± 2.3°C. After the HS or control treatment, all chicks were returned to their cages for routine feeding (Liang *et al.*, 2016).

Sample collection and tissue sectioning

At the end of 1–6 weeks of age, six chicks were randomly selected from each group for dissection of the hypothalamus, pituitary gland, ovary, and testis. Excess fat and connective tissues were removed and placed on ice followed by removal of blood with saline solution, and the dissected organs were placed in Boiun's fixative solution. Fixed tissues were dehydrated, cleared, embedded in paraffin, and sectioned into 5-μm slices. The experiments were conducted with the approval of the Hainan Normal University Animal Experimentation Ethics Committee.

Immunohistochemistry

The 5-μm tissue sections were deparaffinized, quenched in deionized water containing 3% H₂O₂ for 15 min, blocked for 1 h, and incubated separately with 1:150 polyclonal primary antibodies (anti-GnRH, bs-7419R; anti-FSH, bs-1536R; anti-LH, bs-0952R; anti-T, bs-4535PB, and anti-E₂, bs-4533PB purchased from Biorbyt, Cambridge, UK), then diluted in phosphate-buffered saline (PBS) overnight at 4°C. After washing with PBS, the tissue sections were incubated with biotinylated anti-rabbit secondary antibodies for 30 min at 37°C and further labeled with streptavidin for 30 min at 37°C (Rabbit Immunohistochemistry Kit, SP-0023, Beijing Bioss Biotech, Beijing, China; 36312ES64, Shanghai Yeasen Biotech, Shanghai, China), followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution (36302ES01, Shanghai Yeasen Biotech). The tissue sections were then counter-stained in hematoxylin dye, followed by conventional dehydration, clearing, and mounting with neutral gum.

Six tissue sections stained with different antibodies were observed under an Olympus BX50F light microscope (Olympus Optical, Tokyo, Japan) by selecting 10 different fields of view to capture images using an Mshot MC50 digital camera (Guangzhou Mingmei Photoelectric Technology, Guangdong, China). The sections were analyzed using Image-pro



Plus 6.0 image analysis software (Media Cybernetics, Rockville, MD). We counted the numbers of cells with positive staining of the relevant hormones and recorded their integrated optical density (IOD) in different fields of view per tissue section.

Data processing and statistical analysis

The experimental results were presented as mean \pm standard error mean (Mean \pm SEM). Experimental data analysis was performed using Microsoft Excel (Version 12) and SPSS 16.0 (IBM SPSS, Chicago, IL) software. Analysis of variance (ANOVA) and Duncan's method were used for multiple comparisons. $p<0.05$ was considered a statistically significant difference.

RESULTS

Effect of HS on GnRH expression in hypothalamus of Wenchang chicks

Immunohistochemical analysis revealed that GnRH-positive staining was distributed in the cell bodies and around the nuclei of neurons in the hypothalamic tissues; this is illustrated by a light-brown color and irregular shape (Figure 1). The average IOD of GnRH-

positive cells in the hypothalamus was obtained by quantitative analysis of the positive expression (Table 1). As the chicks developed, the GnRH-positive staining in the hypothalamic tissues of the male Wenchang chicks of the CK and HS groups initially increased and then declined. The GnRH-positive staining of the female HS chicks showed high variability. Compared with the CK group, GnRH-positive staining in the hypothalamic tissues was significantly decreased in 3–6-week-old male chicks and 4–5-week-old female chicks of the HS group (Table 1; $p<0.05$).

Effect of HS on LH and FSH expression in pituitary tissues of Wenchang chicks

According to the LH and FSH immunohistochemistry, the LH- and FSH-positive staining was randomly distributed in the cytoplasm and membranes of the pituitary tissues (Figure 2). Quantitative analysis of the positive staining was performed to obtain the average IODs of the FSH- and LH-positively stained cells in the pituitary gland (Table 2). The LH- and FSH-positive cells in the pituitary tissues of the male chicks of the CK group initially increased and then decreased until the chicks were six weeks old. The LH- and FSH-positive cells in the pituitary tissues of the male chicks of the HS group initially increased and then decreased. Among the female chick pituitary tissues, the numbers of LH-positive cells initially decreased and then increased, and the FSH-positive staining displayed high variability. The numbers of LH-positive cells in male pituitary tissues of the HS group were significantly lower than in the CK group at 2–6 weeks of age. The numbers of FSH-positive cells of male pituitary tissues of the HS group were significantly higher than in the CK group at 1–2 weeks of age, but significantly lower than in the CK group at 3–6 weeks of age. Compared with the CK group, the LH-positive cells of female pituitary tissues of the HS group significantly decreased at two and four weeks of age, and the FSH-positive cells of female pituitary tissues of the HS group significantly decreased at four and six weeks of age (Table 2, $p<0.05$).

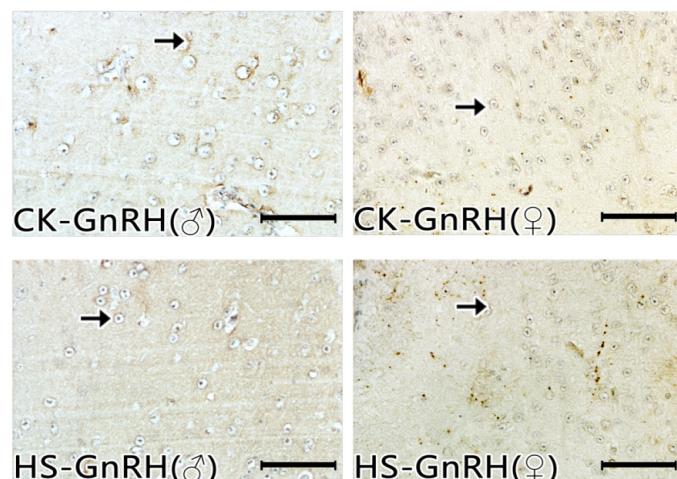


Figure 1 – Effects of heat stress on GnRH on the hypothalamus of Wenchang chicks

CK: Control chicks; HS: Heat stress; ♂: male, ♀: female

The arrowheads indicate positive reactions. Bar=50 μ m.

Table 1 – Effect of HS on average IOD of GnRH in the hypothalamus of Wenchang chicks ($\text{IOD}/\mu\text{m}^2$).

Item	Group	Age (week)					
		1	2	3	4	5	6
GnRH (♂)	CK	1.69 \pm 0.08 ^{aA}	2.01 \pm 0.09 ^{bA}	3.19 \pm 0.51 ^{eB}	2.42 \pm 0.20 ^{dB}	1.78 \pm 0.09 ^{abB}	2.09 \pm 0.13 ^{cB}
	HS	1.87 \pm 0.04 ^{cB}	2.18 \pm 0.07 ^{dB}	2.32 \pm 0.12 ^{eA}	1.81 \pm 0.06 ^{cA}	1.54 \pm 0.08 ^{bA}	1.37 \pm 0.08 ^{aA}
GnRH (♀)	CK	0.17 \pm 0.01 ^{aA}	0.15 \pm 0.03 ^{aA}	0.27 \pm 0.07 ^{bc}	0.37 \pm 0.04 ^{cB}	0.67 \pm 0.23 ^{dB}	0.25 \pm 0.07 ^{abc}
	HS	0.26 \pm 0.09 ^{bB}	0.36 \pm 0.04 ^{bB}	0.25 \pm 0.03 ^{ac}	0.19 \pm 0.04 ^{cA}	0.3 \pm 0.03 ^{aA}	0.28 \pm 0.06 ^a

Notes: CK, control group; HS, heat-stress group; ♂, male chicks; ♀, female chicks; lowercase letters (a–c) represent comparisons between different ages; uppercase letters (A–B) represent comparisons between different groups; different letters represent significant differences ($p<0.05$, $n=6$).



Table 2 – Effect of HS on average IOD of gonadotrophin in pituitary glands of Wenchang chicks ($\text{IOD}/\mu\text{m}^2$).

Item	Group	Age (week)					
		1	2	3	4	5	6
LH (♂)	CK	3.26±0.13 ^{aA}	5.79±0.29 ^{eB}	7.27±0.25 ^{fB}	4.85±0.17 ^{dB}	3.94±0.12 ^{bB}	4.36±0.16 ^{cB}
	HS	3.63±0.16 ^{cB}	4.04±0.17 ^{dA}	4.20±0.15 ^{dA}	3.76±0.14 ^{cA}	3.39±0.14 ^{bA}	2.90±0.12 ^{aA}
LH (♀)	CK	7.94±0.71 ^{cA}	4.13±0.47 ^{bB}	4.12±0.24 ^b	4.46±0.14 ^{bB}	3.38±0.39 ^a	2.6±0.62 ^{aA}
	HS	9.81±1.09 ^{cB}	3.56±0.4 ^{abA}	4.37±0.32 ^b	3.1±0.87 ^{aA}	2.95±0.59 ^a	6.47±0.67 ^{bB}
FSH (♂)	CK	3.72±0.11 ^{aA}	3.92±0.11 ^{dA}	5.28±0.16 ^{dB}	4.32±0.10 ^{cB}	3.90±0.09 ^{bB}	4.48±0.16 ^{dB}
	HS	3.89±0.10 ^{bB}	4.15±0.18 ^{cB}	4.22±0.06 ^{cA}	3.94±0.22 ^{bcA}	3.43±0.21 ^{aA}	3.27±0.15 ^{aA}
FSH (♀)	CK	5.41±0.54 ^{cA}	4.77±0.99 ^{bc}	4.58±0.53 ^b	7.88±0.5 ^{dB}	2.34±0.42 ^{aA}	8.24±0.59 ^{dB}
	HS	6.42±0.99 ^{cB}	5.23±0.71 ^b	4.48±0.46 ^b	3.31±0.32 ^{aA}	3.2±0.68 ^{aB}	5.25±0.64 ^{bA}

Notes: CK, control group; HS, heat-stress group; ♂, male chicks; ♀, female chicks; lowercase letters (a–c) represent comparisons between different ages; uppercase letters (A–B) represent comparisons between different groups; different letters represent significant differences ($p<0.05$, $n=6$).

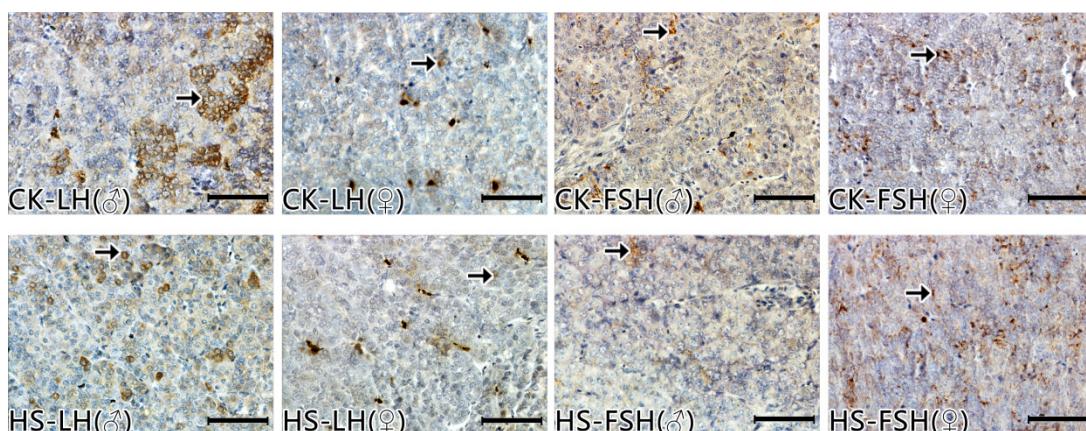


Figure 2 – Effects of heat stress on LH and FSH on the hypophysis of Wenchang chicks

CK: Control chicks, HS: Heat stress; ♂: male, ♀: female

The arrowheads indicate positive reactions. Bar=50 μm .

Effect of HS on respective E2 and T expression in ovaries and testis in Wenchang chicks

Immunohistochemistry of E2 and T showed that E_2 -positive staining was distributed around the follicles in the ovary, and T-positive staining was distributed near the testicular stroma and the germ cells in the testis (Figure 3). The average IODs of E_2 in the ovary and T in the testis were obtained by quantitative analysis of the positive staining (Table 3). As the chicks developed, numbers of T-positive cells in male testicular tissues and E_2 -positive cells in the female ovary tissues of the CK group initially increased and then decreased.

Compared with the CK group, T-positive cells in male testicular tissues significantly declined at 3–6 weeks of age in male chicks, and E_2 -positive cells in the female ovary tissues significantly decreased at 4–5 weeks of age in female chicks of the HS group (Table 3, $p<0.05$).

DISCUSSION

The development and sexual maturation of animal reproductive organs is closely related to the regulatory function of the HPG axis. The hypothalamus promotes the synthesis and secretion of gonadotropin by secreting GnRH to act on the pituitary gland, and the peripheral blood circulation transports gonadotropin to act on

Table 3 – Effect of HS on average IOD of testosterone (T) in testes and E_2 in ovaries of Wenchang chicks ($\text{IOD}/\mu\text{m}^2$).

Item	Group	Age (week)					
		1	2	3	4	5	6
T (♂)	CK	2.28±0.14 ^{aA}	2.67±0.18 ^{bA}	4.78±0.16 ^{eB}	4.15±0.10 ^{dB}	3.06±0.20 ^{cB}	3.14±0.06 ^{bB}
	HS	2.77±0.10 ^{cB}	2.97±0.08 ^{dB}	3.45±0.07 ^{eA}	3.03±0.15 ^{dA}	2.47±0.11 ^{bA}	2.09±0.09 ^{aA}
E2 (♀)	CK	2.21±0.34 ^a	3.14±0.49 ^{bA}	6.96±0.58 ^{dA}	7.36±0.44 ^{dB}	7.03±0.9 ^{dB}	5.99±0.61 ^c
	HS	2.37±0.7 ^a	6.31±0.97 ^{bB}	8.38±0.83 ^{cB}	6.52±0.4 ^{dA}	5.94±0.66 ^{bA}	6.83±0.75 ^b

Notes: CK, control group; HS, heat-stress group; ♂, male chicks; ♀, female chicks; lowercase letters (a–c) represent comparisons between different ages; uppercase letters (A–B) represent comparisons between different groups; different letters represent significant differences ($p<0.05$, $n=6$).

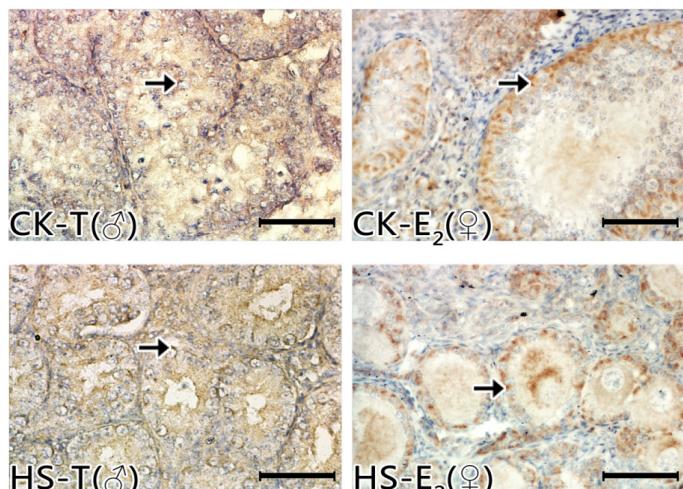


Figure 3 – Effects of heat stress on T in testicle and E₂ in ovary of Wenchang chicks

CK: Control chicks; HS: Heat stress; ♂: male, ♀: female

The arrowheads indicate positive reactions. Bar=50 μ m.

the gonads, thereby completing the regulation of the endocrine and exocrine systems (Qing *et al.*, 2003). The thermoregulatory system of young animals has not yet matured, and overly high ambient temperature easily affects the homeostasis of the body, leading to dysregulation of the HPG axis and ultimately affecting the growth and reproduction of the animals and even causing shock or death (Yu, 2009; Sun *et al.*, 2015; Zhou *et al.*, 2016).

The hypothalamus is a gray-matter structure at the base of the forebrain that is involved in endocrine secretion and maintaining the balance of metabolic energy in the body (Blouet *et al.*, 2010; Wilding, 2010). GnRH is one of the reproductive hormones synthesized by specific hypothalamic cells with bulging axons at their center, and GnRHs are released into the hypophyseal portal system in a pulsed manner. GnRH is important for the development of gonads and the maintenance of sexual maturity (Ye *et al.*, 2003; Zhang, 2017). In this study, the GnRH-positive cells in the hypothalamus of male chicks of the CK group were significantly fewer than in male chicks of the HS group at the early stage of growth and development; this might be due to the high-temperature adaptability of the chicks, or to the high-temperature environment having no negative impact on growth and development of the chicks. After two weeks of age, the chicks entered a rapid growth period that was not conducive to the development of the hypothalamus and that initiated the blockage of neuronal development (Zhuang *et al.*, 2004; Zhou *et al.*, 2015; Lu *et al.*, 2018). Studies have shown that hypothalamic tissue is susceptible to high temperature. Preoptic and anterior hypothalamic

regions are thermoregulatory centers, and HS activates the neurons of the anterior and preoptic-anterior hypothalamic regions to resist stimulation (Jiang *et al.*, 1994; Ishiwata *et al.*, 2002). GnRH is secreted by GnRH neurons in the preoptic, anterior, and arcuate nucleus regions of the hypothalamus; therefore, HS can activate the preoptic area of the thalamus to affect GnRH neuronal development and inhibit GnRH secretion, which is consistent with the results of HS affecting the development of GnRH cells in the hypothalamus in this study. GnRH is a hypothalamic-terminal output signal that plays a key role in regulating the downstream elements of the HPG axis. GnRH concentration in the hypothalamus is closely related to the development of the gonads and plays an important role in regulating the secretion of sex hormones (Asa *et al.*, 2002; Pereyra, 2005; Liu, 2010). Therefore, GnRH was necessary to maintain the reductive functions of the chicks, and HS affected the normal growth and development of hypothalamic GnRH cells, resulting in insufficient secretion of GnRH in the hypothalamus, further causing neuroendocrine dysfunction of the HPG axis and affecting the reproductive system.

The pituitary gland is the most important endocrine gland in the body, and the pulsed release of GnRH from the hypothalamus enters the anterior pituitary gland through the hypophyseal portal system to promote gonadotrophin synthesis and secretion. LH and FSH are both gonadotrophins secreted by basophils in the pituitary gland that act synergistically on gonadal tissues (Mountford *et al.*, 1989; Dahia *et al.*, 2006). In this study, compared with the CK group, the numbers of LH- and FSH-positive cells of the male chicks in the HS group significantly decreased at 2–6 and 3–6 weeks of age, respectively. The LH-positive cells of the female chicks in the HS group were significantly fewer than in the CK group at two and four weeks of age, and the FSH-positive cells of the female chicks in the HS group were significantly fewer than in the CK group at four and six weeks of age. Our previous studies have shown that the development of basophils in the pituitary glands of chicks can be hindered by a high-temperature environment from three weeks of age (Lu *et al.*, 2018; Liang *et al.*, 2018). Further, under HS, the development of GnRH cells in the hypothalamus is inhibited and the concentration of GnRH is decreased, leading to a decline of secretory function of the pituitary gland, further affecting the synthesis and secretion of LH and FSH. These results are consistent with the findings of Yu (2009); their study concluded that HS causes damage to the pituitary glands of rabbits,

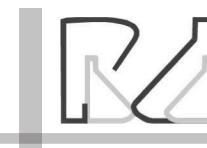


resulting in a significant decrease in the secreted hormone activities. Our previous study showed that changes in LH in the pituitary gland and serum of the male chicks of the HS group are basically consistent. However, serum FSH levels of the 1–6-week-old male chicks of the HS group were significantly higher than in the CK group (Zhang *et al.*, 2014), which was contrary to the FSH levels in the pituitary glands of 3–6-week-old male chicks. Three weeks of age is a rapid growth period of chicks, and HS damages the pituitary gland, thereby lowering the pituitary and serum LH levels. Further studies are needed concerning the mechanism underlying the reduction of the pituitary and serum LH levels and how this affects the synthesis, processing, and secretion of FSH to cause the differences between the serum and pituitary FSH levels. For male chicks, FSH promotes the proliferation of spermatogonia and the development of spermatogenic epithelium in the testes (Zhang *et al.*, 2014). Insufficient LH secretion leads to a decrease in the richness of testicular interstitial tissue, which further affects the secretion and synthesis of T. Spermatogenesis in the testes, which mainly depends on the interaction between FSH and T. If the secretion and synthesis of FSH and T are blocked, the reproductive function of the body will be seriously damaged (Mclachlan *et al.*, 1996; França *et al.*, 2001; Mclachlan *et al.*, 2002). For female chicks, FSH promotes ovarian follicular and follicular granulosa cell development. Once FSH binds to its specific receptor, it not only induces LH receptor formation but also synergizes with LH to promote the synthesis and secretion of estrogen, which continuously synergizes with FSH to promote the growth and development of follicles. After two weeks of age, female chicks enter a period of rapid development, and HS begins to affect the development of the pituitary gland in females. In this study, FSH- and LH-positive cells in the pituitary tissues of female chicks in the HS group were significantly reduced, which interfered with the normal secretion of estrogen. However, at six weeks of age, LH-positive staining of the female chicks in the HS group was significantly higher than that of the CK group, which might be because of LH promoting the maturation and ovulation of follicles to further promote the luteinization of granular cells after ovulation in order to maintain secretion of progesterone from luteal cells. When the LH content is too low, the body regulates the LH content through a compensatory mechanism to maintain normal growth and development (Zhao *et al.*, 2003). Therefore, HS affects the normal development of gonadotrophin cells in the pituitary glands of chicks,

thereby preventing spermatogenesis in the testes and follicle development in the ovaries.

Testosterone (T) is the most active and highest content androgen in the testes. Its locally high concentration in the testes, significantly higher than in plasma, is essential for spermatogenesis. In this study, the changes of T in the testes were consistent with the FSH levels in the pituitary tissues of male chicks, suggesting that both T and FSH might synergistically regulate spermatogenesis. After three weeks of age, T-positive cells in the male testes of the HS group were significantly fewer than in the CK group, suggesting that the high-temperature environment began to suppress the normal development of the seminiferous tubules and had adverse effects on the spermatogenic cells of the male chicks (Lu *et al.*, 2018). However, the high-temperature environment restricted the secretion of GnRH, FSH, and LH synthesis and secretion in the HPG axis of male chicks after three weeks of age, resulting in the absence of proper stimulation in the Leydig cells, thereby affecting their ability to secrete T. These findings are consistent with the previous report of significant reduction of T concentration in the testes of heat-stressed mice (Yang *et al.*, 2013). Our previous study showed that serum T levels of 1–6-week-old male chicks in an HS group were significantly lower than in a CK group (Zhang *et al.*, 2014). Thus, HS severely hinders the development of the testis germ cells, thereby hindering spermatogenesis and sperm maturation in male chicks.

Estrogens are the major hormones promoting the development of female reproductive organs and maintaining the metabolic processes that play a key role in the development of follicles and regulation of the estrous cycle. Estrogens secreted by the ovaries include E₂ and estrone, which are interconvertible. Results of this study showed that E₂-positive staining in the ovaries in both groups was basically consistent; the numbers of positive cells initially increased and then decreased as the chicks developed. On one hand, FSH-positive staining in the pituitary glands of female chicks in the HS group was significantly stronger than that in the CK group before two weeks of age. The relatively high concentration of FSH promoted E₂ synthesis and secretion, resulting in high E₂-positive staining. On the other hand, the high E₂ concentration led to positive feedback on the hypothalamus that promoted the synthesis and release of GnRH but also had a negative feedback on the pituitary that inhibited FSH synthesis. This effect on FSH ultimately led to the early termination of follicular development,



thereby reducing the secretion of E₂ (Chen, 2012). The difference in the E₂-positive staining between the two groups of females was that the peak of E₂-positive cells in the ovaries of the CK group was delayed by one week compared with the HS group, possibly due to heat accumulation in the ovarian tissues suppressing follicle development at all levels from three weeks of age and causing ovarian dysfunction (Liang *et al.*, 2018). Previous studies have shown that when poultry are subjected to HS, a natural compensatory effect will alter the functional abnormality of the HPG axis to become normal (Cangar *et al.*, 2008; Zhao *et al.*, 2003). Since HS damages female reproductive function, the body must secrete large amounts of E₂ to maintain the growth and development of the reproductive system. At five weeks of age, E₂-positive staining of the chicks in the HS group was significantly lower than that in the CK group, suggesting that the chicks tried to alleviate the injury caused by HS. Therefore, HS affected the growth of germ cells in ovaries and hindered the normal development of the reproductive system of female chicks.

CONCLUSIONS

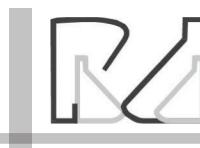
In summary, HS caused the corresponding target organ dysfunction of the HPG axis in Wenchang chicks, affecting the normal development of the cells secreting the major reproductive hormones and the synthesis of steroid hormones. This process inhibited the development and sexual maturity of the reproductive system of chicks, and thus may reduce the breeding rate of Wenchang chickens. Therefore, further studies on reliable factors that improve the development of cells secreting the major reproductive hormones under HS may provide an empirical basis for improving the reproductive success rate of poultry in high-temperature environments.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (NSFC31560680).

REFERENCES

- Asa SL, Ezzat S. The pathogenesis of pituitary tumours. *Nature Reviews Cancer* 2002;2:836-849.
- Blouet C, Schwartz GJ. Hypothalamic nutrient sensing in the control of energy homeostasis. *Behavioural Brain Research* 2010;209:1-12.
- Cangar Ö, Aerts JM, Buyse J, Berckmans D. Quantification of the spatial distribution of surface temperatures of broiler. *Poultry Science* 2008;87:2493-2499.
- Chen L. Function of BDNF and NT-4 in secretion of gonadotrophins and expression of their receptors in the reproductive tissues in rat. Changchun (CN): Jilin University; 2012.
- Chen Z, Zhang JR, Zhou YW, Liang C, Jiang YY. Effect of heat stress on the pituitary and testicular development of Wenchang chicks. *Archives Animal Breeding* 2015;58:373-378.
- Chimento A, Sirianni R, Casaburi I, Pezzi V. Role of Estrogen Receptors and G Protein-Coupled Estrogen Receptor in Regulation of Hypothalamus–Pituitary–Testis Axis and Spermatogenesis. *Frontiers in Endocrinology* 2014;5:1-8.
- Dahia CL, Rao AJ. Demonstration of follicle-stimulating hormone receptor in cauda epididymis of rat. *Biology of Reproduction* 2006;75:98-106.
- Elnagar SA, Scheideler SE, Beck MM. Reproductive hormones, hepatic deiodinase messenger ribonucleic acid, and vasoactive intestinal polypeptide-immunoreactive cells in hypothalamus in the heat stress-induced or chemically induced hypothyroid laying hen. *Poultry Science* 2010;89:2001-2009.
- França LR, Silva Junior VA, Chiarini-Garcia H, Garcia SK, Debeljuk L. Cell proliferation and hormonal changes during postnatal development of the testis in the pig. *Biology of Reproduction* 2001;63:1629-1636.
- Ishiwata T, Hasegawa H, Yazawa T, Otokawa M, Aihara Y. Functional role of the preoptic area and anterior hypothalamus in thermoregulation in freely moving rats. *Neuroscience Letters* 2002;325:167-170.
- Jiang MY, Wang LX, Wang XM. Effect of high ambient temperature on egg production luteinizing hormone and progesterone secretion of laying hens. *Chinese Journal of Animal Science* 1994;30:6-8.
- Kim B, Park K, Rhee K. Heat stress response of male germ cells. *Cellular & Molecular Life Sciences* 2013;70(15): 2623-2636.
- Liang C, Xie XZ, Zhou YW, Jiang YY, Xie LJ, Chen Z. Effects of γ -aminobutyric acid on the thymus tissue structure, antioxidant activity, cell apoptosis, and cytokine levels in chicks under heat stress. *Czech Journal of Animal Science* 2016;61:539-550.
- Liang W, Lu BB, Li QH, Chen Z. Effect of heat stress on the growth and development of pituitary and ovary in Wenchang chicks. *Heilongjiang Animal Science and Veterinary Medicine* 2018;36:20-23.
- Liu HY. Effects and regulatory mechanism of GnRH on secretion function of Leydig cells of testis in rat. Nanjing (CN): Nanjing Normal University; 2010.
- Lu BB, Liang W, Xie XZ, Chen Z. Effects of Heat Stress with Growth and Development of Tissues on HPT axis in Wenchang Chicks. *China Poultry* 2018;40:34-39.
- Maurya VP, Sejian V, Kumar D, Naqvi SMK. Impact of heat stress, nutritional restriction and combined stresses (heat and nutritional) on growth and reproductive performance of malpura rams under semi-arid tropical environment. *Journal of Animal Physiology and Animal Nutrition* 2016;100(5):9.
- Mclachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Progress in Hormone Research* 2002;57:149-179.
- Mclachlan RI, Wreford NG, O'Donnell L, de Kretser DM, Robertson DM. The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. *Journal of Endocrinology* 1996;148:1-9.
- Mountford PS, Bellow PA. Cloning and DNA sequence analysis of the cDNA for the precursor of ovine follicle stimulating hormone beta-subunit. *Nucleic Acids Research* 1989;17:6391-6391.



- Pereyra ME, Sharbaugh SM, Hahn TP. Interspecific variation in photo-induced gnrh plasticity among nomadic cardueline finches. *Brain Behavior and Evolution* 2005;66:35-49.
- Qing SZ, Chen SL, Shen XF. Immunohistochemical localization of oxytocin on hypothalamus-pituitary-ovary axis in dairy goat during estrous cycle. *Chinese Journal of Veterinary Science* 2003;23:300-302.
- Schams D, Stephan E, Hooley RD. The effect of heat exposure on blood serum levels of anterior pituitary hormones in calves, heifers and bulls. *Acta Endocrinologica* 1980;94(3):309.
- Sun H, Jiang R, Xu S, Zhang Z, Xu G, Zheng J, et al. Transcriptome responses to heat stress in hypothalamus of a meat-type chicken. *Journal of Animal Science & Biotechnology* 2015;6(1):6.
- Tu WL, Cheng CY, Wang SH, Tang PC, Chen CF, Chen HH, et al. Profiling of differential gene expression in the hypothalamus of broiler-type Taiwan country chickens in response to acute heat stress. *Theriogenology* 2016;85:483-494.
- Wilding JP. Neuropeptides and appetite control. *Diabetic Medicine A Journal of the British Diabetic Association* 2010;19:619-627.
- Yang M, Qin YH. Androgen concentration changes in heat-stressed mice and its effects on expression of HSPs mRNA in Sertoli Cell. *Chinese Journal of Animal Science* 2013;49:22-25.
- Ye D, Pan JW, Liao MJ, Zhang Z, Zhu M. The Structure and Biological Functions of Gonadotropin-releasing Hormone(GnRH). *Progress in Biochemistry and Biophysics* 2003;30:49-53.
- Yu XL. Studies on the cloning of neuromedins and its receptor in rabbit and effects of heat stress on expression of NMS mRNA in the HPA axis. Nanjing (CN): Agricultural University; 2009.
- Zhang JR. Effect of heat stress on primary reproductive hormones secretion and their receptors development in Pituitary testicular axis of chickens. Hainan (CN): Normal University; 2014.
- Zhang LW. Affecting pathway of melatonin on monochromatic light-induced expressions of GnRH-I and GHRH in chick hypothalamus. Beijing (CN): Agricultural University; 2017.
- Zhang M, Zou XT, Hui LI, Dong XY, Zhao W. Effect of dietary γ -aminobutyric acid on laying performance, egg quality, immune activity and endocrine hormone in heat-stressed roman hens. *Animal Science Journal* 2012;83(2):141-147.
- Zhao H, Tian ZZ, Chen BY. The functional compensation in hypothalamus-pituitary-ovary axis after ovariectomy in rats. *Chinese Journal of Neuroscience* 2003;19:229-233.
- Zhou YW, Cai T, Zhou HJ, Chen Z. Study on weight, visceral organs development and heart rate variation in male wenchang chicks. *Journal of Domestic Animal Ecology* 2015;36:39-43.
- Zhou YW, Liang C, Jiang YY, Chen Z. Effects of GABA on expressions of IL-6, TNF- α and HSP70 mRNA in bursa of fabricius of chicks under heat stress. *China Poultry* 2016;38:27-31.
- Zhu YW, Li WX, Lu L, Zhang LY, Ji C, Lin X, et al. Impact of maternal heat stress in conjunction with dietary zinc supplementation on hatchability, embryonic development, and growth performance in offspring broilers. *Poultry Science* 2017;96:2351-2359.
- Zhuang YC, Wu QB. Effects of temperature on growth performance at the early stage of chicks. *Poultry Husbandry and Disease Control* 2004;22:16-17.