Effects of stocking density on ovarian development and maturation during the rearing period in Shan-ma ducks

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ABSTRACT Stocking density critically affects the growth and subsequent performance of animals in modern poultry production. This study investigated the effects of stocking density on ovarian development, ovarian maturation, and the mRNA expression of key genes in the reproductive axis during the rearing period of Shan-ma ducks. The experiments involved 180 healthy 7-wk-old Shan-ma ducks and randomly divided into low stocking density (**LSD**; n = 30, density = 5 birds/m^2), medium stocking density (**MSD**; n = 60, density = 10 birds/m^2) and high stocking density groups (**HSD**; n = 90, density = 15 birds/m^2), for rearing. After examining ovarian development and measuring hormone levels in the plasma and expression levels of key regulatory genes in the reproductive axis at 19 wk of rearing, analysis of the gonad index analysis, reflecting stocking density, uncovered statistically significant differences. The gonad index of the LSD group was significantly higher than those of the MSD and HSD groups ($P < 0.01$), while no significant difference was observed between the MSD and HSD groups. pre-ovulatory follicles (**POFs**) and small yellow follicles (**SYFs**) development was only apparent in the LSD group, with the large white follicles (**LWFs**) number of this group being significantly higher than that of the MSD group ($P < 0.05$). The blood levels of E2 (estradiol), P4 (progesterone), and T (testosterone) were significantly higher in the LSD group than in the MSD and HSD groups ($P < 0.05$ or 0.01). Also, the levels of both P4 and T were significantly higher in the MSD group than in the HSD group ($P < 0.01$). The gene expression levels of *GnRHR, FSH, AMHR,* and *FSHR* were significantly increased in the LSD group compared to the MSD and HSD groups ($P < 0.05$ or 0.01), while the expression levels of *GnIHR* and *GDF9* were significantly decreased in the LSD and MSD groups compared to the HSD group ($P < 0.05$ or 0.01). Steroid biosynthesis pathway genes such as *StAR, CYP11A1, 3β-HSD, CYP19A1,* and *BMP15* were significantly downregulated at greater stocking densities ($P < 0.05$ or 0.01). Likewise, the protein expression of StAR, 3β-HSD, and CYP19A1 was also significantly decreased ($P < 0.05$ or 0.01). These results demonstrate that both medium and high stocking densities suppressed the expression of the key reproduction-promoting factors, while the expression level of the key reproductive inhibitory factors was enhanced. Therefore, rates of ovarian development and maturation could be reduced by a high stocking density leading to a delay in reproduction performance during the rearing period of Shan-ma ducks.

Key words: stocking density, growing period, ovarian development, reproductive axis

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INTRODUCTION

The egg-laying traits of poultry are mainly affected by the genetics, nutrition, and development of the ovaries. Well-developed ovaries ensure the high egg-laying performance of the poultry. In modern poultry production, stocking density has a major impact on ovarian development and egg production (Cai et al., 2019; Li et al., 2019). Rearing poultry at high stocking densities reduces sex hormone levels, retards ovarian development, and causes follicular atresia, resulting in poor laying performance (Rosenfeld et al., 2001; Miller and Auchus, 2011; Luo et al., 2016; Hou et al., 2018; Krause and Schrader, 2019). Presently, the poultry breeding industry is somewhat focused on the influence of the environment on egg production. Stocking density is an important environmental factor for the layer hen

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(Krause and Schrader, 2019). However, no standard regulations of stocking density have yet been established for the Shan-ma duck in China. A high stocking density is often selected to achieve high production efficiency. However, high stocking densities affect health status, immunity, and oxidation resistance, particularly influencing the reproductive development and laying performance of the ducks (El-Tarabany, 2016; Weimer et al., 2019).

Currently, most studies about the effects of stocking density on gonad development mainly focus on the laying stage; few focus on the rearing period (Geng et al., 2020; Pu et al., 2021). During the growth and development of the poultry ovary and follicle, multiple genes regulate various physiological processes such as the expression of the gonadotropin receptors, steroid biosynthesis, and cell proliferation (Liu and Zhang, 2008). The present study investigated the influences of different stocking densities on ovarian development, hormone levels in the plasma, and key regulatory factors in the reproductive axis of Shan-ma ducks during the growing period. This study reveals the implication of stocking density on ovarian development and maturation during the rearing period of Shan-ma ducks − information that could guide poultry production.

MATERIALS AND METHODS

Ethics Statement

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Zhongkai University of Agriculture and Engineering (NO.2020071109). The protocol was approved by the Committee on the Ethics and Welfare of Animal Experiments of Zhongkai University of Agriculture and Engineering. All efforts were made to minimize suffering of the animals.

Animals and Experimental Design

This study selected a total of 180 seven-wk-old Shan-ma ducks randomly placed into the low stocking density (**LSD**; n = 30), medium stocking density (**MSD**; n = 60), and high stocking density groups (**HSD**; n = 90). The ducks were reared semi-openly on land and water, with a shed: ground: pool area ratio of 6: 6: 3 m^2. The stocking densities for the LSD, MSD, and HSD groups were 5, 10, and 15 birds/m^2, corresponding to 30, 60, and 90 ducks. Each group of ducks was further subdivided equally into 3 repeats. Three duck pens with the same feeding conditions and of the same size were used. The duck pens were built with concrete floors and movable metal enclosures for the poultry. The distribution of each pen was consistent, including duck sheds (3 × 2 m; 6 m^2), sports grounds (3 × 2 m; 6 m^2), and pools (3 × 1 m; 3 m^2). The indoor temperature was 25 ± 3°C and the relative humidity was 75 to 85%. Natural ventilation was adopted in the shed. Natural light was used during the day while incandescent bulbs were used to control lighting at night. Energy-saving lamps were placed 2 m above the ground, generating an average light intensity of 30 lux. In the first week of the experiment, the light controller was set for 10 h illumination, and the illumination was extended for 1 h every other week until the illumination stabilized at 17 h. During the experiment, ducks were fed in accordance with the standard production diet for such poultry. All ducks were managed as per a standardized feeding schedule for 12 wk. Dietary composition and nutrient levels are in accordance with the laboratory's original feeding standards (Wang et al., 2021). The number of feeding troughs and drinking water tanks in each group was controlled to ensure that the feeding level and drinking water area was the same for all ducks.

Sample Collection

Six Shan-ma ducks (n = 6) were randomly selected from each group and weighed at the end of the experiment (19 wk) to determine ovarian development and calculate the gonadosomatic index (ovarian weight/body weight*100%). All follicle types were counted, that is, pre-ovulatory follicles (POFs, ⌀ > 8 mm), small yellow follicles (SYFs, ⌀ = 6−8 mm), and large white follicles (LWFs, ⌀ = 3−6 mm). The hypothalamus, hypophysis, and ovarian tissues (after removal of surface follicles >3 mm in diameter) were collected, flash-frozen in liquid nitrogen and stored at −80°C. Blood samples were also taken.

Measurement of the Serum Hormone

Blood was collected from the wing vein. After standing at room temperature for 2 h, the serum samples were obtained by centrifuging at 4,000 r/min for 10 min and the serum was stored at −20°C. The E2 (estradiol), P4 (progesterone), and T (testosterone) hormones were measured according to the instructions of the ELISA kits, E2 ELISA kit was purchased from CUSABIO (Shanghai, China), P4 and T ELISA kits were purchased from Elabscience (Shanghai, China) Co., Ltd.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted by Trizol (Thermo Fisher, Carlsbad, CA) and reverse transcribed using the ReverTra Ace qPCR RT kit (Toyobo, Japan). The synthesized cDNA was stored at −20°C. The relative expression of the genes *GnRH* and *GnIH* in the hypothalamus, *GnRHR, GnIHR*, and *FSHR* in the hypophysis, and *FSHR, LHR, StAR, CYP11A1, 3β-HSD, CYP19A1, AMH, AMHRII, GDF9*, and *BMP15* in the ovary were quantified. The primers for the quantitative real-time PCR (**RT-qPCR**) were designed using the mRNA sequences of the related genes in ducks (Table 1) and then synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. A 20-μL reaction volume was prepared according to the instructions of the manufacturer of the

Table 1. Primers for real-time quantitative PCR.

Genes	Primer sequence (5'-3')	The length of the product (bp)
β-Actin1	F: CCTCTTCCAGCCATCTTTCTT	109
	R:TGTTGGCATACAGGTCCTTAC	
GAPDH	F: GCTGATGCTCCCATGTTCGTGAT	85
	GTGGTGCAAGAGGCATTGCTGAC	
GnRH	F: CTGGGACCCTTGCTGTTTTG	209
	R:AGGGGACTTCCAACCATCAC	
GnIH	F: AAAGTGCCAAATTCAGTTGCT	128
	R:GCTCTCTCCAAAAGCTCTTCC	
GnRHR	F: TCTGCTGGACCCCCTACTAC	127
	R:TCCAGGCAGGCATTGAAGAG	
GnIHR	F: TGGCCCTCATCGTTGTCA	116
	R:CTTCCTGCGAGACACCTT	
FSH	F: GTGGTGCTCAGGATACTGCTTCA	209
	R:GTGCAGTTCAGTGCTATCAGTGTCA	
FSHR	F: AGCACCTTCCAAGCCTTAGA	209
	R:TGACCATGGAAGGCAGATGT	
LHR	F: GTAACACTGGAATAAGGGAAT	191
	R:GAAGGCATGACTGTGGATA	
StAR	F: CTGCCCATCTCCTACCGCCCAC	263
	R:CTGCTCCACCACCACCTCCA	
CYP11A1	F: ACAGGGAGAAGTTGGGTGTC	145
	R:GTAGGGCTTGTTGCGGTAGT	
3β-HSD	F: AGAAGTGACAGGCCCAAACT	187
	R:ACATGGATCTCAGGGCACAA	
CYP19A1	F: GGATGGGAGTAGGTAATGCC	273
	R:ACAAGACCAGGACCAGACAG	
AMH	F: GACCCTGGCCATCAGACATC	108
	R:GGTGTCATCCTGGTGAAGCA	
AMHRⅡ	F: CTTCCTGTGCGACACCATCA	111
	R:ACGATGTCTCTCATGGTGTC	
GDF9	F: ATCTGTTCCCAAGCCCTCT	141
	R:GGATTCACCCACTACCGAC	
BMP15	F: TCCTCTTCCTCAATGACACTC	133
	R:AGGGAGCAGTCGCTTTTATC	

SYBR Green PCR Master Mix (Thermo Fisher) for real-time fluorescence quantification using the cDNA as templates. The reaction system comprised 10 μL of SYBR Green PCR Master Mix, upstream and downstream primers (0.5 μL each), ddH_2O (8 μL), and cDNA (1 μL). The PCR procedure comprised pre-denaturation at 50°C for 2 min and then at 95°C for 10 min, all for one cycle; 95°C for 15 s and then the annealing temperature for 1 min, all for 40 cycles. Three replicates were undertaken for each sample.

Western Blot Analysis

The total proteins of the ovarian tissues were crushed in liquid nitrogen, dissolved in lysis buffer (Biyotime Biotechnology, China), sonicated, and centrifuged at 4°C for 20 min at 12,000 rpm, and the protein concentrations were quantified by the bicinchoninic acid (**BCA**) method (Thermo Fisher). After denaturing using sodium dodecyl sulfate (**SDS**), the proteins were loaded on 10% SDS-PAGE gels (Epizyme Biomedical Technology, China), and the upper gel was electrophoresed at 80 V for 20 min and the lower gel at 120 V for 60 min. The proteins after electrophoresis were transferred to the PVDF membranes at 200 mA for 60 min and the membranes were then blocked in 5% skimmed milk for 1 h at room temperature. The membranes were then incubated with the primary antibody at 4°C overnight. The primary antibodies used were rabbit polyclonal StAR (Affinity Biosciences, China), 3β-HSD (Affinity Biosciences, China), CYP19A1 (Abcam, UK), and mouse monoclonal β-actin (Affinity Biosciences). Subsequently, the rinsed membranes were incubated with the secondary antibody at room temperature for 1 h and rinsed 5 times with TBST for 3 min each. The secondary antibodies were goat anti-mouse IgG (H+L) HRP (Affinity Biosciences) and goat anti-rabbit IgG (H+L) HRP (Affinity Biosciences). The chemiluminescence images of the membranes were captured using a chemical luminescence immunity analyzer (Tanon, China) after incubation with an ECL kit. The western blot protein bands were analyzed in grayscale using the Image J software (https://imagej.en.softonic.com/).

Statistics and Data Analysis

The RT-qPCR results were analyzed using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). All the experimental data were analyzed by one-way ANOVA using the SPSS 19.0 software (SPSS Inc, Chicago, IL), and significant differences between the stocking density groups were tested for with the LSD multiple comparison method. The results were presented as the mean ± SEM, with $P < 0.05$ and $P < 0.01$ indicating significant differences, and $P > 0.05$ indicating no significant differences.

RESULTS

The Effects of Stocking Density on Ovary Development

Stocking density plays a pivotal role in ovarian follicular development in the Shan-ma duck. The ovaries of the LSD group were fully developed, with numerous developing follicles and dominant follicles, while only small white follicles were observed in the MSD group and no follicle development was apparent in the HSD group (Figure 1). The gonad index was calculated and the number of follicles at different stages in the ovaries was counted (Table 2). The gonad index decreased gradually with increasing stocking density, where the gonad index of the LSD group was significantly higher than those of the MSD and HSD groups ($P < 0.01$), while there was no significant difference between the latter 2 groups. POFs and SYFs were formed only in the LSD group, and the number of LWFs was significantly higher in the LSD group than in the MSD group ($P < 0.01$).

Influence of Stocking Density on Reproductive Hormone Levels

As shown in Figure 2, the serum levels of P_4 and T were significantly higher in the LSD group than in the MSD and HSD groups ($P < 0.01$). Also, the serum levels of P_4 and T in the MSD group were significantly higher

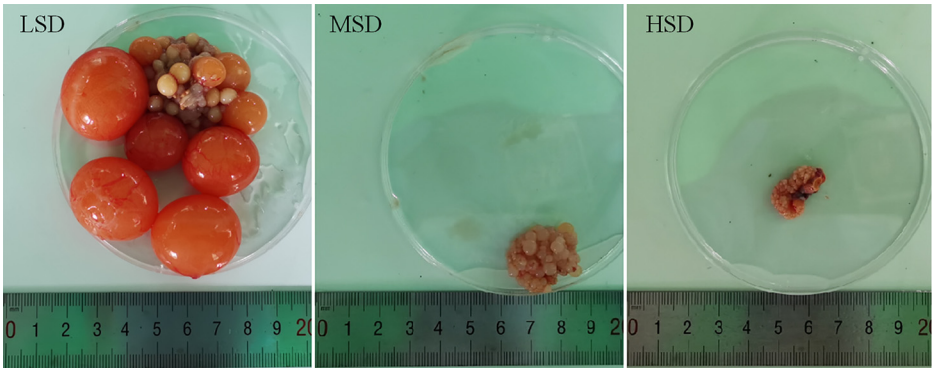

Figure 1. Stocking density affected ovarian development in the Shan-ma duck. (LSD) Ovary morphology in low stocking density group, (MSD) ovary morphology in medium stocking density group, (HSD) ovary morphology in high stocking density group. The LSD group were fully developed and mature for egg production, while the ovaries of the ducks of the MSD group only formed large white follicles, and there was no obvious ovarian development in the ducks of the HSD group.

than in the HSD group ($P < 0.01$). The serum level of E2 was significantly higher in the LSD group than in the MSD ($P < 0.01$) and the HSD groups ($P < 0.05$), while there was no significant difference between MSD group and HSD group.

The Effects of Stocking Density on Gene Expression of Key Reproduction Regulatory Factors

The expression levels of the key regulatory factors in the reproductive axis are illustrated in Figure 3. As observed, the expression levels of *GnRH, GnRHR,* and *FSH* in the hypothalamus-pituitary axis gradually declined as stocking density increased. In the hypothalamus and pituitary, the expression levels of *GnRHR* were significantly higher in the LSD group than in the HSD and MSD groups ($P < 0.01$), and the expression levels of *FSH* were significantly higher in the LSD than in the HSD group ($P < 0.01$). Similarly, the expression levels of *GnIH* and *GnIHR* gradually increased, with the expression levels of *GnIHR* being significantly lower in the LSD group than in the HSD group ($P < 0.05$). At the gonad level, the expression of *AMH, AMHRII, FSHR,* and *LHR* decreased with increasing stocking density, and the expression levels of *AMHRII* and *FSHR* were significantly higher in the LSD group than in the MSD and HSD groups ($P < 0.05$). The increase in the stocking density increased the expression levels of *GDF9* in the ovaries, and the *GDF9* level was significantly lower in the LSD group than in the MSD and HSD groups ($P < 0.01$), with the *GDF9* expression being significantly higher in the HSD group than in the MSD group ($P < 0.01$).

Table 2. Effects of stocking densities on the ovarian index and follicular development of ducks.

Indicators	LSD	MSD	HSD
GSI%	3.94 ± 0.37^{a}	0.25 ± 0.06^{c}	0.19 ± 0.04^{c}
POF	8.00 ± 0.50^{a}	0^{c}	0^{c}
SYF	2.40 ± 0.22^{a}	0^{c}	0^{c}
LWF	23.00 ± 1.35^{a}	2.16 ± 1.25^{c}	0^{c}

Note: Data are presented as the mean ± SEM, n = 6.

Abbreviations: HSD, high stocking density group; LSD, low stocking density group; MSD, medium stocking density group.

[ac]Adjacent letters indicate significant differences ($P < 0.05$) and interphase letters indicate extremely significant differences ($P < 0.01$).

The Effects of Stocking Density on the Steroid Biosynthesis Pathway

The key genes and expressions of proteins involved in the steroid biosynthesis pathway in the ovarian tissues were characterized and determined in Figures 4 and 5. The expression levels of *CYP19A1, CYP11A1, 3β-HSD,* and *StAR* decreased with the increase in stocking density. The expression levels of *CYP19A1, CYP11A1, 3β-HSD,* and *BMP15* in the LSD group were significantly higher than those in the HSD group ($P < 0.05$ or 0.01). The expression of the proteins CYP19A1, 3β-HSD, and StAR were consistent with the expression levels of the corresponding genes and decreased with the increase in stocking density. The protein expression was significantly higher in the LSD group than in the MSD and HSD groups ($P < 0.05$ or 0.01).

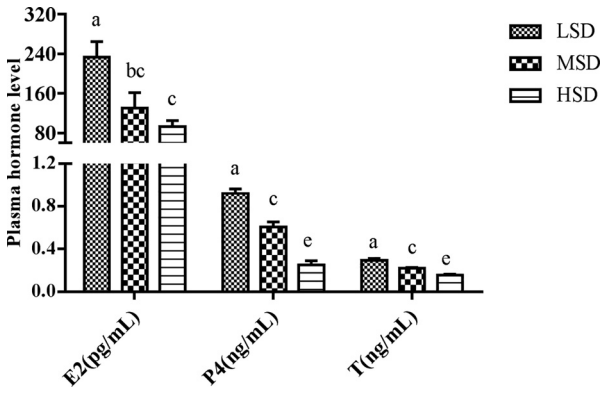

Figure 2. Stocking density effect on serum reproductive hormone levels. (LSD) Serum reproductive hormone levels in low stocking density group, (MSD) serum reproductive hormone levels in medium stocking density group, (HSD) serum reproductive hormone levels in high stocking density group. Data are presented as the mean ± SEM, n = 6. Adjacent letters indicate significant differences ($P < 0.05$) and interphase letters indicate extremely significant differences ($P < 0.01$).

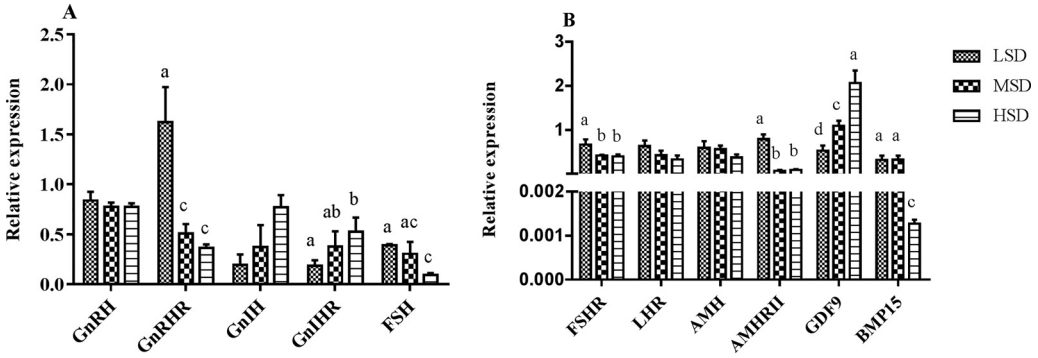

Figure 3. Effects of different stocking densities on reproductive axis gene expression. (A) Relative mRNA expression of GnRH and GnIH in hypothalamus; relative mRNA expression of GnRHR, GnIHR, and FSH in the pituitary. (B) Relative mRNA expression of FSHR, LHR, AMH, AMHRII, GDF9, and BMP15 in ovarian tissue. Data are presented as the mean ± SEM, n = 6. Adjacent letters indicate significant differences ($P < 0.05$) and interphase letters indicate extremely significant differences ($P < 0.01$).

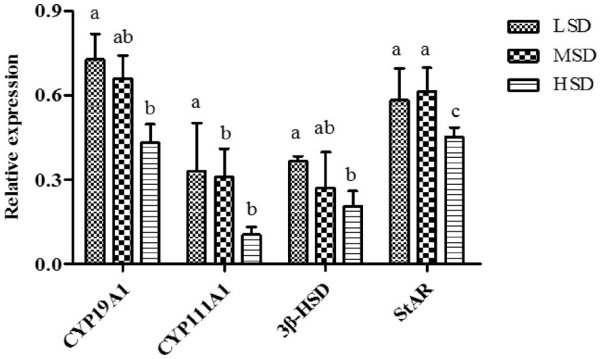

Figure 4. Effects of stocking densities on the expression of genes related to ovarian steroid biosynthesis. Relative mRNA expression of CYP19A1, CYP11A1, 3β-HSD, and StAR in ovarian tissue. Data are presented as the mean ± SEM, n = 6. Adjacent letters indicate significant differences ($P < 0.05$) and interphase letters indicate extremely significant differences ($P < 0.01$).

DISCUSSION

The reproductive activity of poultry is closely related to the development of the reproductive system during the rearing period and to the farm management during the egg-laying period (Shi et al., 2020). Previous studies have found that in poultry rearing, stocking density affects the development of the reproductive system during the brooding period and egg production during the laying period (Lewis et al., 1997). The subsequent ovarian development and egg production of poultry is directly affected by the development of the oviducts, ovaries, stroma, and follicles during the rearing period (Renema et al., 1999; Cui et al., 2019). High stocking densities decrease the level of sex hormones, retarding ovarian development and resulting in follicular atresia and rapid decline in egg-laying performance (Hou et al., 2018). Increasing the stocking density could lead to a decrease in egg production and weight of the quails (Soares et al., 2018). In the present study, the increase in stocking density was found to significantly decrease the ovary weights and gonad index. The ovaries of the LSD group were fully developed and exhibited a high number of POFs, SYFs, and LWFs. In contrast, the ovaries of the MSD group only formed a few LWFs, with no follicular development in the HSD group. Our results indicate that stocking density significantly affected the ovarian development of the egg-laying ducks during the growing period. Both the gonad index and degree of ovarian development of the egg-laying ducks significantly decreased with increasing stocking density, and the ovarian development and maturity were retarded in the medium and high stocking densities.

The ovarian and follicular development of poultry is mainly under the regulation of the reproductive axis (Long et al., 2017; Hanlon et al., 2021). The serum levels of the reproductive hormones of the different groups were detected and the results indicate that increasing the

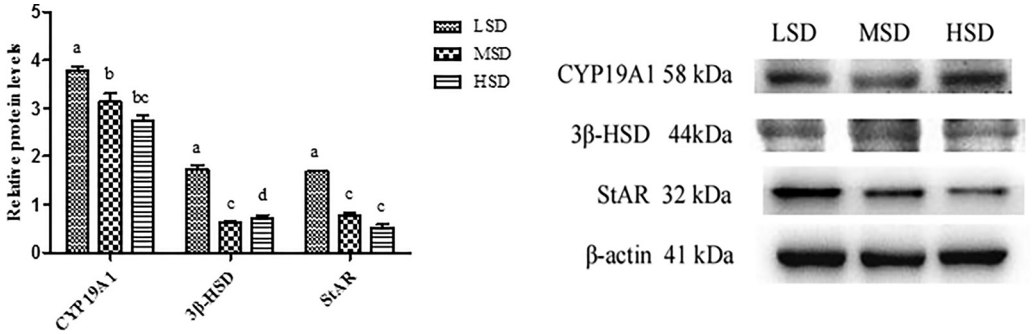

Figure 5. Effects of stocking densities on protein levels of the ovarian steroid biosynthesis pathway. The protein expression levels of CYP19A1, 3β-HSD, and StAR in ovarian tissue. Data are presented as the mean ± SEM, n = 6. Adjacent letters indicate significant differences ($P < 0.05$) and interphase letters indicate extremely significant differences ($P < 0.01$).

stocking density decreased the blood levels of E2, P4, and T, which were significantly higher in the LSD group than in the MSD and HSD groups. Variations in the levels of the reproductive hormones in the blood were consistent with the follicular development in those groups, indicating that the increase in the stocking density regulates ovarian development by affecting the reproductive endocrine system (Zakaria et al., 1983; Baarends et al., 1995; Cai et al., 2015). The expression levels of the key regulatory factors in the reproductive axis were investigated. The gene expression levels of the main reproductive regulatory factors of the reproductive axis were further assessed, and it was found that the gene expression levels of *GnRH, GnRHR*, and *FSH* decreased, while the gene expression levels of *GnIH* increased. These results suggested that increased stocking density can significantly inhibit the activity of the reproductive axis and delay gonad development. Our results were consistent with the findings from previous reports (Maddineni et al., 2008).

FSH and LH secreted by the pituitary regulate the follicular development and synthesis of the steroid hormones by binding to the FSHR and LHR at the gonad level (Johnson and Woods, 2009; Liu et al., 2021). AMH is mainly secreted by ovarian granulosa cells and plays an important role in inhibiting the growth of the primary follicles in the ovary, as well as the follicular recruitment and sensitivity to FSH (Durlinger et al., 2001). The expression levels of *AMH* and *AMHR* have been demonstrated to gradually decrease with the development of the follicles (Johnson et al., 2008). *AMH* and *AMHR* expression in quails were found to significantly decrease because light was found to promote reproductive activity. On the contrary, the *AMH* and *AMHR* expression was found to significantly increase since short light periods inhibit testes development (Otake and Park, 2016). Exogenous administering of *AMH* was found to inhibit follicular development and egg production in hens, while active immunization with *AMH* was found to promote the expression of *FSHR* and *LHR*, steroid production, and follicular development in geese (Chen et al., 2020). The present study reported a significant decrease in the expression levels of *FSHR* and *LHR* with increasing stocking density. The *FSHR* expression level was significantly higher in the LSD group than in the MSD and HSD groups, which was consistent with the results of relevant studies. There was a declining trend in *AMH* and *AMHR* expression as the stocking density increased, which was inconsistent with previous reports. This may be due to the fact that only the rearing period of egg-laying ducks was observed in the present study and not the egg-laying period. Actually, AMH is mainly synthesized and secreted by follicular granulosa cells and is directly related to the degree of ovarian development (Chen et al., 2020). In beef heifers, circulating AMH concentrations were approximately 6- and 2-fold greater in animals with high (25 follicles 3 mm in diameter) or intermediate (16−24 follicles) compared with a low (15 follicles) antral follicle count during follicular waves (Ireland et al., 2008; Mossa et al., 2017). AMH secretion increased in the LSD group due to adequate ovarian development and more follicles (3 mm in diameter), while *AMH* secretion decreased significantly in the MSD and HSD groups due to insufficient ovarian development and fewer granulosa cells. This provides mechanisms explaining why increased stocking density leads to retarded ovarian development.

BMP15 and GDF9 are important intraovarian regulatory factors of the transfer growth factor-β (**TGF-β**) superfamily secreted by the oocyte. They are structurally homologous and functionally similar (Belli and Shimasaki, 2018). *BMP15* regulates follicular development and steroidogenesis either as a homodimer or as a heterodimer (Elis et al., 2007). In the current study, the expression level of *BMP15* gradually decreased with increasing stocking density; *BMP15* was significantly lower in the HSD group than in the LSD and MSD groups. The expression level of *GDF9* tended to increase with increasing stocking density, possibly due to the differences between *BMP15* and *GDF9* in influencing early embryonic development (Spicer et al., 2008). *BMP15* stimulates the granulosa cells and prompts follicular cell development by enhancing proliferation and inhibiting apoptosis of the granulosa cells. Therefore, *BMP15* expression in the LSD group significantly increased with follicular development, whereas *BMP15* expression was significantly decreased in the MSD and HSD groups due to the delayed ovarian development. *GDF9* promotes the development of the original follicles into secondary follicles, particularly in the early stages of follicular development in response to the reproductive hormones. In this study, the ovarian development was delayed in both the MSD and HSD groups compared to the LSD group, where the HSD group was the slowest − the follicles were at the initial stage of development. Therefore, the *GDF9* expression level was found to decrease gradually with the decrease in stocking density and the gradual acceleration in ovarian development. However, many questions about the mechanism by which *BMP15/GDF9* regulates follicular development await resolution. *BMP15/GDF9* affects the ovulation rate in sheep, promoting ovulation in heterozygotes and causing sterility in homozygotes, and the interspecific differences in the ovulation rate were closely related to the *BMP15/GDF9* ratio (Crawford and Mcnatty, 2012). *BMP15/GDF9* ratio could promote the expansion of the cumulus oophorus and the related gene expression by generating the *SMAD2/3* signals via *ALK4/5/7* and *BMPR2* (Peng et al., 2013).

Additionally, the gene and protein expression levels of the steroid hormone biosynthesis pathway factors, like *CYP19A1, CYP11A1, 3β-HSD,* and *StAR*, were investigated. The results of the expression were consistent with the ovarian development and expression levels of the key regulatory factors in the reproductive axis, both of which gradually decreased with the increase in the stocking density. The results further revealed that the stocking density directly influences ovarian development by affecting the reproductive axis, thus affecting the ovarian function, follicular development, and maturity of the egg-laying ducks during the growing period.

CONCLUSIONS

The present study indicated that both medium and high stocking densities may play a key role in inhibiting the expression of reproductive-promoting genes and inducing the expression of reproductive-suppressing genes, which can delay ovarian development and maturation of the Shan-ma duck.

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Author contributions: DJ, XZ, and YH contributed to hypothesis generation, experimental design, data interpretation, and manuscript preparation. YX, SLF, and XF conducted the experiments. XS, DX, and YT contributed to the data interpretation

DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.101809.

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