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RESEARCH ARTICLE

Comparative Study of Reproductive Organs and Egg Quality Characteristics in Hyline and Domestic Layer Breeds

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ABSTRACT

For comparison of the reproductive organs and the egg quality in hens, a case study was undertaken on traditional Chinese Zhenning and commercial Hyline hen. A total of 15 hens from each breed were selected to study lipogenesis, serum lipids, and organ body weight ratios of liver, ovary and oviduct to compare their lipid synthesis and transportation mechanism. Moreover we also examined the histomorphological comparison of the egg forming organs and egg quality parameters between two breeds. Results showed significantly higher values of peroxisome proliferator-activated receptors gamma (PPAR γ), estrogen receptor alpha (ERα) and estrogen receptor beta (ER β) mRNA expressions in the liver of Zhenning than Hyline hen. Same difference was also observed in the vitellogenin (Vtg) protein levels. Triglyceride levels were higher in the Hyline hens. Besides this, liver, ovary and oviduct body weight ratio were significantly higher in Hyline hen than Zhenning hen. Moreover, Hyline hen had significantly higher oviduct weights. Ovary weight and hierarchy was also bigger in the Hyline hen. Finally, egg quality parameter comparison showed significantly lower values in the Zhenning eggs than the White Hyline eggs. These findings suggest that Hyline and Zhenning hen differ significantly due to selection in terms of lipogenesis, increase in body weight ratio of liver, ovary and oviduct morphology, while Hyline hens have also superior egg quality than Zhenning hen eggs.

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INTRODUCTION

Egg quality has been defined by Stadelman and Cotterill (1973) as 'the characteristics of an egg that affect its acceptability to the consumers'. Miscellaneous studies have identified egg quality parameters and the factors affecting egg quality parameters, of which the most important one is genetic make up of an individual bird (Monira *et al.*, 2003; Nys, 2009; Silversides *et al.*, 2006; Wolc *et al.*, 2012; Zita *et al.,* 2014) and age (Chang-Ho *et al*. 2014). It has been established that smaller the size of eggs, higher will be the proportion of yolk and smaller will be the proportion of albumen (Tharrington *et al.*, 1999). Physiologically egg quality depends on the synthesis of yolk from liver and its transportation to the oocytes and then deposition of albumen and shell gland layers around it (Perry *et al.*, 1978). In addition to this, it has been reported that most of the liver transcriptions activity for the yolk precursor synthesis is activated by the estrogens (Flouriot *et al*., 1996) and response of transcription varies in the breeds and strains of birds. Moreover, ovarian hierarchy size decides yolk quality to be deposited in the follicles and released into oviduct. Besides, excluding yolk, 65-70% of total egg mass is accumulated in the oviduct. Avian oviduct is a tubular organ responsible for albumen and shell secretions which encapsulates the yolk and transport the egg until it is laid. Several studies on histological and ultrastructural aspects on oviduct in the avian species have been conducted to understand egg formation process (Bakst, 1998; Mishra *et al*., 2014) and it has been observed that every individual fragments, have some specific roles in egg quality related parameters.

Selection is a useful tool for improving hen's productivity and it has put tremendous pressure on the egg formation system. The respective size of the organs and their physiology has been altered in proportion to their body conformity. In this respect, at this stage it is

imperative to understand comparative changes in organs involved in the egg formation process and their physiology. For this purpose a comparative study has been designed to study the traditional Zhenning hen, which is the cross of the multiple strains of the original Ninghaimeilinji breed commonly found in the Zhejiang province PR China and with commercial Hyline breed to study their reproductive organs and egg quality parameters. This study will provide valuable information to the breeders to focus on the special region for the improvement of their breed and to improve the welfare of the hens.

MATERIALS AND METHODS

Experimental design, birds and management: A total of 30 Zhenning and Hyline hens were procured from Zhenning Animal Husbandry Co., Ltd., China at the 18 weeks of age and 15 hens from each breed were separately placed into the 3 tier wire cages with 4 birds in each nest (0.38×0.32m). Hens were provided *ad libitum* access to layer ration and water, subjected to an 8:16 lighting schedule. No additional supplements or vitamins were provided to the hens. After one week of acclimatization, 8 replicate hens from each breed were weighted and killed by the cervical dislocation. Immediately after killing samples weighing less than 50 mg were directly frozen in the liquid nitrogen and then stored at −80°C until further analysis. Weights of the organs were recorded and liver, ovary and oviduct samples were collected for hematoxylin and eosin (H&E) staining. Remaining 7 hens from each breed were further reared for obtaining the eggs for measuring the egg quality parameter and blood collection. All experimental procedures were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University.

Histological analysis: Samples of the liver, ovary and oviduct were washed with the cold PBS and then fixed in the 4% formaldehyde for 48 hours, dehydrated, embedded in the paraffin. Dewaxed sections with a thickness of 4 μm were stained with H&E. The slides were observed for morphological changes under a Eclipse 80i, Nikon, Japan microscope, and pictures were captured with a digital camera (DS-Fi1, Nikon, Japan).

Morphological measurement of an ovary and oviduct: The follicles were separated according to the size into small white follicles (SWF) (<1 mm), large white follicles (LWF) (>1 mm and <6 mm), small yellow follicles (SYF) (>6 to <8 mm) and large yellow follicles (LYF) (>8 mm to 40 mm). They were individually weighted and sized separately. Total size and weight of oviduct and their regions were measured for individual hen.

Follicular density measurement: The follicular density was calculated by the presence of total number of follicles per unit of the section. For this purpose, ovarian fragments measuring ≈ 7×3×3 mm^3 were sectioned and 10 sections of 5 μm were selected at the interval of 300 μm from each fragment.

Plasma lipids and protein analysis: Blood was collected from brachial vein during morning hours immediately after oviposition. Plasma was immediately separated by centrifugation for 10 min at 1400 *g*. Plasma triglycerides were measured by using Triglyceride reagent kit (Dongus Zhejiang Province, PR China) by enzyme colorimeter method and total cholesterol were measured by using total cholesterol reagent. Proteins were measured with apoprotein assay kit (Beyotime Institute of Biotechnology) and vitellogenin (Vtg) was measured by Vtg assay kit by using colorimeter. Thermo scientific Multiskan MK3 plate reader was used to read the plates.

Determination of the egg quality parameters: A total of 100 eggs from each breed were used to measure the egg quality parameters. The freshly laid eggs were analyzed by the Digital Egg Tester DET6000 made in Japan every day. Egg shell thickness was determined by the Digital Egg shell thickness Gauge. Weight of the eggshell was determined after clearing it from other egg contents and egg yolk was taken separately after removing albumin and others layers from it. Total albumin weight was taken after subtracting egg shell weight and yolk weight from the total egg weight.

RNA extraction and RT-PCR: The extraction of total RNA, preparation of cDNA and the amplification of target genes by PCR were carried out according to the manufacturer's instructions. Briefly, liver tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA in the presence of liquid nitrogen. RNA purity and concentration were determined spectrophotometrically at 260/280 nm in the range of 1.8–2.0 by NanoDrop 2000 (Thermo Scientific, Waltham City, MA, USA). Total RNA (2μg) was reverse transcribed by Fermentas One step RT-PCR kit (MBI Fermentas, Burlington, ON, Canada) and amplified by PCR. The cDNA of genes involved in lipid metabolism such as: PPARα, PPARγ, Sterol regulatory element-binding transcription factor 1 (SREBP-1), fatty acid synthase (FAS), microsomal triglyceride transfer protein (MTP), stearoyl-CoA desaturase 1 (SCD1), apolipoprotein B (apoB), apolipoprotein VLDL-II (apoVLDL-II), vitellogenin gene II (VtgII), microsomal triglyceride transport protein (MTP), malic enzyme, adenosine triphosphate citrate lyase (ACLY)] and estrogen metabolism like estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), with β-actin as a reference, were amplified by real-time quantitative PCR. The primer sequences of the target genes are listed in Table 1.

Statistical analysis: All data are expressed as mean±SEM. The differences between groups were analyzed by Student's *t*-test. $P<0.05$ was used to indicate statistically significant differences. The data was analyzed on the Graph prism 5 software.

RESULTS

Difference in the mRNA expression related to the lipid metabolism: The result revealed that PPARγ messenger RNA expression level was significantly higher in Zhenning hen than Hyline hen ($P<0.5$) (Fig. 1).

Table 1: Primers for PCR analysis

Gene	Forward Primer	Reverse primer	Product length (bp)	Accession No.
PPARα	AGGCCAAGTTGAAAGCAGAA	TTTCCCTGCAAGGATGACTC	155	NM_001001464.1
PPARγ	TGACAGGAAAGACGACAGACA	CTCCACAGAGCGAAACTGAC	164	NM_001001460.1
SREBP-1	CATTGGGTCACCGCTTCTTCGTG	CGTTGAGCAGCTGAAGGTACTCC	145	AY029924
FAS	CACCAGGATTCGCTCAGTG	GCGGTCAACAACAACATCAA	100	NM_205155.1
apoB	ATTCCTGACTTGAAGATACCAGAG	GTTCGCAGATGCTGTAGTATTATG	159	NM_001044633.1
apoVDL-II	CTTAGCACCACTGTCCCTGAAGT	TGCATCAGGGATGACCAGC	81	NM_205483
MTP	CAAGAACCGGATAGCCATGT	AGGAGAGCCAACTCTGTCCA	175	XM_420662.2
ACLY	GGGCCACAAAGAAATCTTGA	CAGCAATAATGGCAATGGTG	167	NM_001030540.1
Malic enzyme	AGTGCCTACCTGTGATGTTG	GGCTTGACCTCTGATTCTCT	101	AF408407
ERα	TAGTTCCGCTCTACGACCTCTT	AGTTGGTTTCGGTTCTCCTCTT	106	NM_205183.2
ER β	AAAGAACAGAAACCCCATTCAG	GCACAGAGGGACATTTTGATT	82	NM_204794.2
VTGII	CAACATATCTTCCGCTTGTAACATTG	TTCACAACAAAGATTTCTCCAGTAGC	147	NM_001031276
SCD1	AGTGGTGTTGCTGTGCTTCA	CTAAGGTGTAGCGCAGGATG	107	NM_204890
β-actin	ACACCCACACCCCTGTGATGAA	TGCTGCTGACACCTTCACCATTC	136	NM_205518

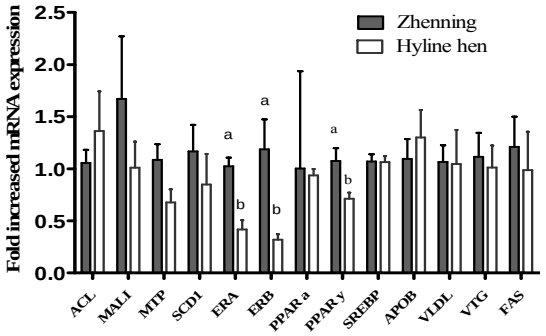

Fig.1. Difference in the mRNA expression related to the yolk precursors formation in the Zhenning and Hyline hens (n=8). Data are presented as mean±SEM and corrected for β-actin. Superscript letters indicate significant difference (P<0.05).

Non-significant difference were observed among PPARα, SREBF1, apoB, VLDL, Vtg, FAS, ACLy, Malic enzyme, MTP, SCD1 and VtgII between two breeds. However, one fold increase was noticed in the ERα levels of Zhenning hen than Hyline hens. Similarly, ER beta gene also showed one fold increase in gene expression of Zhenning hen, when compared to Hyline hen (P<0.05)

Blood chemical contents related to egg production: Comparative blood parameters related with egg production showed that there was a significant increase in triglyceride levels of Hyline hen (7.50±0.84) in comparison to Zhenning hen (4.25±0.54) at P<0.0057. However there were no significant difference between Zhenning and Hyline hens in cholesterol and Apo protein levels, respectively. Furthermore, Vtg-Protein levels were significantly higher in Zhenning hen (89.06±2.27 mg/L**)** in comparison to Hyline hen (83.61±2.05mg/L), P<0.0467.

Egg quality parameters: Significant differences were observed between two breeds with respect to egg quality parameters (P<0.01, Table 2). Hyline hen had significantly higher egg weight and albumin weight. Contrarily yolk weight was highly significant in Zhenning hen. In addition, egg shell weight was significantly higher in Hyline hen. Haugh unit and albumen height were also significantly higher in Hyline hen.

Morphological difference of organs: The mean value of Zhenning hen liver was significantly lower and there was significantly lower liver and body weight ratio between Zhenning and Hyline hen. Oviduct weight was significantly lower in Zhenning breed. While oviduct and body weight ratio percentage was also significantly lower in Zhenning hen. Similarly, significantly low magnum, isthmus and shell gland weight was observed in Zhenning hen.

There was a significant difference in body weight, ovary weight and ovary to body weight ratio between Zhenning and Hyline hens. Number of SWF (<1 mm) was significantly lower in Zhenning hen than Hyline hen, while the number of LWF (2-4 mm) was significantly higher in Zhenning hen. There was also higher number of atretic follicles in local breed. The total number of preovulatory hierarchical follicles were five (F1- F5) in Zhenning hen, whereas, Hyline hen consisted of six to eight follicles (F1-F8). Preovulatory hierarchical follicles weight varied as compared with all individual follicles. In Zhenning hen, irregular incremental change was observed in follicles weight (F5, 0.5842 g; F4, 1.5250 g; F3, 4.1231 g; F2, 7.2496 g; F1, 11.6790 g) in the order of one fold, two and half fold, three fold, and four and half fold in F5, F4, F3, F2 and F1 follicles, respectively. In comparison, Hyline hen showed almost gradual incremental change of nearly 2 fold in F5 to the F1 follicles. Follicular density between two breeds also differed. Zhenning hen showed significantly higher follicular density in the healthy follicles measuring >300-1000 µm per section and it had the significantly higher number of abnormal follicles as shown in Table 3.

Histology of the liver, ovary and oviduct: Histological observation of the liver did not show any marked difference, except that there was some fat deposition in peripheral parenchymal tissues in Zhenning hen (Figure 2B *g-h*). Moreover, a significant atretic follicles formation in ovary of Zhenning hen was observed (Figure 2B e-*f*). Similarly, marked difference was also observed in histology of oviduct folds. Height of magnum mucosal folds, isthmus mucosal folds, and shell gland mucosal fold showed significantly lower size in Zhenning hens (Table 4, Fig. 2A, Fig. 2B).

DISCUSSION

In the present study, comparative gene expression of the genes involved in lipid metabolism and transport in Zhenning and Hyline hens showed a very marginal difference between two breeds. Increase in ERα and ERß

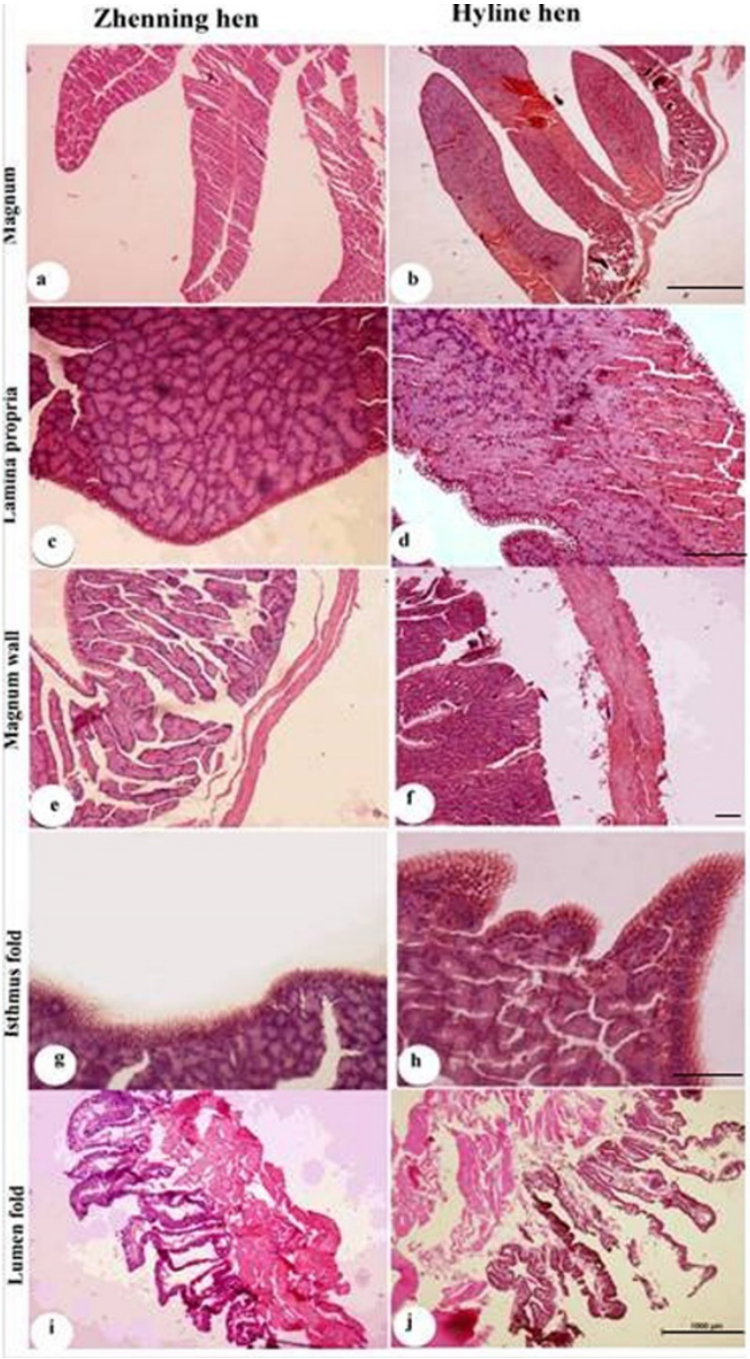

Fig. 2A: Histological slide of the oviduct regions. **a-b)** Magnum folds of the Zhenning and Hyline hens (1000µm bar). **c-d)** Lamina propria of Hyline hen show more secretory activity than the Zhenning hen (20x). **e-f)** Hyline hen shows more uniformity and thickness of walls in the magnum than the Zhenning hens (100 µm bar). **g-h)** Zhenning hen has low surface area in the Isthmus folds than the Hyline hens(100µm bar). **i-j)** Lumen of the Hyline hen has more longer folds than the Zhenning hens.

gene expressions along with PPAR γ that regulate lipid metabolism (Diot and Douaire, 1999; Rakhshandehroo *et al.,* 2010) in Zhenning hen can be related to their increased yolk weights. Contrarily to this, Vtg II and ApoVLDLII II mRNAs expressions were not significantly higher in Zhenning hens under in vivo conditions, which have been reported to rise under *in vitro* conditions when subjected to estrogen stimulation in cultured chicken hepatocyte (Li *et al.*, 2014).

In addition to this, higher triglyceride levels in blood serum of Hyline hens can be due to species response to lipolytic hormones that affect the balance between lipolytic and estrification and due to proportionate amount of carbohydrates in the diet (Schneider *et al.,* 1990) and possibly due to their longer hierarchy.

Ovary and body weight ratio showed marked differences and total preovulatory hierarchical follicle count was much less (F5-F1) in Zhenning hens than Hyline breed (F7-F1). But, higher SYF count in Zhenning hens is comparable with low egg producing hen. Moreover, SWF (2-4 mm) and LWF (4-8mm) count was higher in Zhenning hens than Hyline hens and total counts

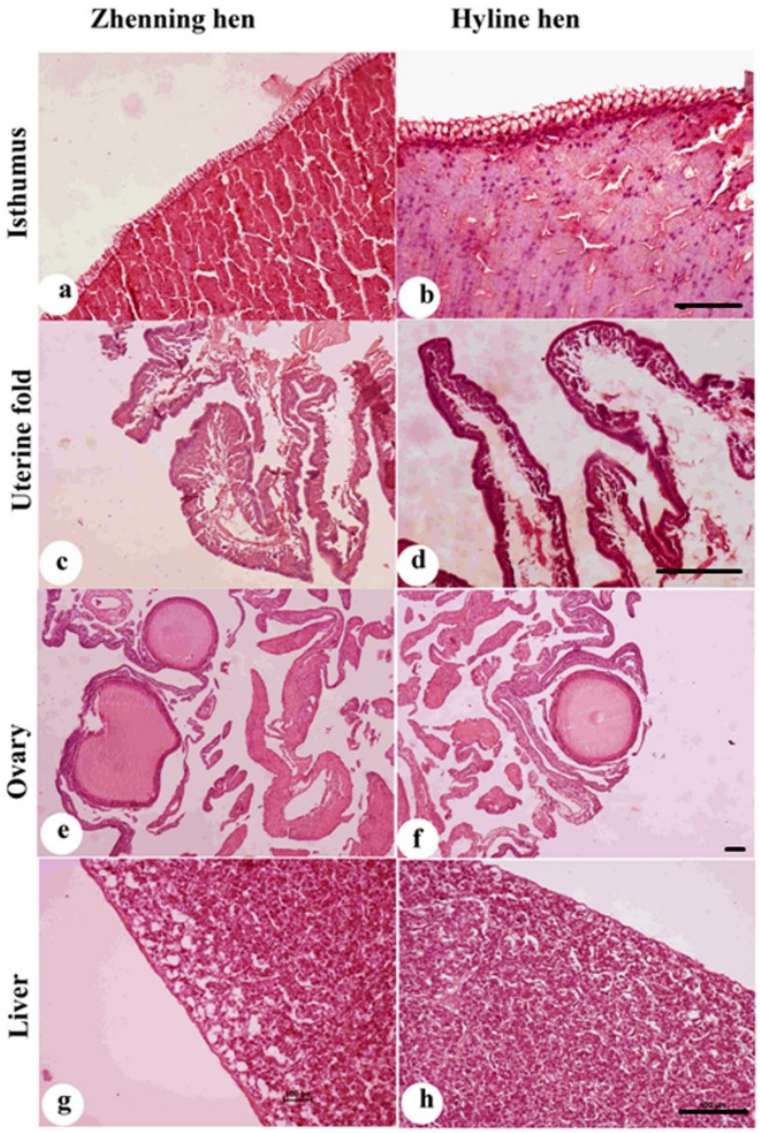

Fig. 2B. Histological slides of the uterine gland fold, ovary and parenchyma. **a-b)** Isthmus shows less secretory area in the Zhenning hen than the Hyline hen. **c-d)** Uterus gland folds are congested in the Zhenning hen and not well organized in comparison to the Hyline hen (4x, 1000 µm bar). **e-f)** Zhenning hen has more atric follicles in comparison to the Hyline hen in the ovary (10x, 100 µm bar). **g-h)** The parenchyma of the liver has more fat deposits then the Hyline hens (100 µm bar).

of atric follicles were also higher in Zhenning than Hyline hens. This might be due to heavy competition for nutrients in the ovary among SYF and the SWF. Additionally, local hens gained more rapid yolk deposition in an irregular manner in an individual follicle due to the limited preovulatory follicular hierarchy as compared to Hyline hens with almost gradual yolk deposition in each follicle at each stage.

Non-significant difference in oviduct size was noticed between two breeds, but oviduct weight of Zhenning hen was significantly lower than Hyline hen and difference also existed in individual segment of oviduct. In addition to this histological structures like the density and height of oviduct regions such as: magnum, isthmus, and Shell gland in Zhenning hens were comparatively lower than Hyline hens. Size and height of mucosal fold epithelium showed marked difference in all segments of oviduct, but difference was significant in magnum region. Additionally, tubular glandular area and size were comparatively abundant in Hyline hens than Zhenning hen. Therefore, it can be considered that Hyline hens have higher secretory activity of albumen than Zhenning hens.

Egg quality traits were significantly lower in Zhenning hens. Our results for Hyline hens for egg quality were in conformity with Giriraj *et al.* (2008); Sreenivas *et al.* (2013) and Shim *et al.* (2013). Moreover, HU values were lower in Zhenning hen which indicates that albumen quality of Zhenning hen is not in equivalence with Hyline hen. It has been reported that albumen quality is affected by the strain of bird and genetic selection (Tharrington *et al.*, 1999; Silversides and Scott, 2001) and also low viscosity of albumen and ovomucin play important role (Li-Chan and Nakai, 1989), while Leeson and Caston (1997) have speculated that low viscosity and thin albumen may result from eggs spending longer than normal time in the oviduct. Findings of this study revealed that there is direct relationship between albumen quantity and magnum weight as evident from size of magnum

Table 2: Comparison of egg quality (mean±SEM) between Zhenning (n=7) and Hyline (n=7) breeds

Egg quality	Zhenning Breed	Hyline breed	P value
Egg weight (g)	42.06±0.5	49.07±0.39	0.0001
Albumin weight (g)	20.23±0.43	32.79±0.38	0.0032
Yolk weight (g)	12.56±0.12	11.10±0.16	0.0001
Egg shell weight (g)	4.74±0.07	5.18±0.08	0.0001
Albumin height (mm)	5.73±0.15	8.19±0.22	0.0001
Haugh Unit (HU)	81.22±0.88	93.75±0.74	0.0001
Albumin weight (g)	25.00±0.40	29.43±1.42	0.0032
Albumin (%)	59.41±0.63	65.14±1.47	0.0003
Yolk (%)	29.28±0.61	22.54±0.57	0.0001

Table 3: Comparison of follicular density (mean±SEM) between (n=8) Zhenning and Hyline (n=8) breeds

Follicular count per section	Zhenning Breed	Hyline Breed	P value
No. of healthy follicles			
<100 µm follicles	7.50±1.00	9.00±0.82	0.26
>100-300 µm follicles	4.00±0.44	3.13±0.51	0.2137
>300-1000 µm follicles	3.63±0.23	2.38±0.23	0.0015
No. of atretic follicles			
<100 µm follicles	8.75±0.88	4.38±0.50	0.0005
>100-300 µm follicles	4.47±0.83	2.13±0.35	0.0191
>300-1000 µm follicles	3.78±0.72	1.88±0.26	0.0247

Table 4: Comparison of the oviduct morphology parameters (mean±SEM) between Zhenning (n=8) and Hyline (n=8) breeds

Characters	Zhenning hen	Hyline hen	P value
Body weight (g)	1.36±0.03	1.26±0.04	0.0453
Ovary weight (g)	29.95±0.89	50.73±1.13	0.0001
Oviduct body weight ratio (%)	2.22±0.10	4.07±0.16	0.0001
Number of SWF (<1 mm)	23.00±1.27	38.14±1.82	0.0001
Number of SWF (2-4 mm)	10.82±0.50	9.10±0.49	0.0235
Total weight of LYF (F6-F1, g)	25.16±0.71	36.88±0.39	0.0001
Atric follicles number	6.64±0.41	4.46±0.37	0.0008
Oviduct weight (g)	41.36±0.56	54.93±1.66	0.0001
Oviduct body weight ratio (%)	3.35±0.11	4.04±0.21	0.0001
Oviduct size (cm)	53.56±0.79	56.94±1.42	0.056
Magnum weight (g)	20.15±0.83	28.50±0.50	0.0001
Isthmus weight (g)	5.74±0.08	5.02±0.14	0.0006
Isthmus size (cm)	11.63±0.32	13.63±0.18	0.0001
Shell gland weight (g)	12.63±0.52	14.62±0.32	0.0055
Height of mucosal fold (mm)	3.05±50.35	3.63±56.05	0.0001
Height of isthmus fold (mm)	1.09±0.08	1.30±0.05	0.0232
Height of shell gland fold (mm)	0.93±0.05	1.31±0.05	0.0001

folds and secretory area. Conversely, yolk weight was higher in Zhenning hen than Hyline hen that might be due to individual preovulatory follicle and also more yolk accumulation in its smaller hierarchy (F5-F1), than Hyline hen. Thus the genetic difference between two breeds has significant role on egg quality parameters which is in congruity with the work of Zita *et al*. (2009), Kocevski *et al.* (2011), Onbasilar *et al.* (2011) and Icken *et al*. (2014).

Conclusion: It is, therefore, concluded that selection has a significant effect on organ morphology, follicular dynamics, body weight ratio, egg quality and to some extent to the lipogenesis mechanism. Furthermore Hyline hens have superior egg quality than Zhenning hen eggs.

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