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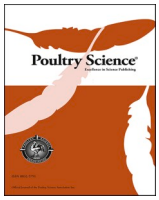

Evaluating ovarian follicles and their steroid hormone gene expression patterns in a high egg-producing research turkey line

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ABSTRACT

Low egg-producing turkeys reduce the profitability of a flock by limiting the number of poults that can be hatched. Understanding the biological mechanics behind egg-production rates will greatly benefit the industry. Two lines with vastly different egg production rates are the Ohio State University E line, and its unselected counterpart, the random-bred control one (**RBC1**). Differences between E Line and RBC1 hens (n = 4 per line) were investigated by measuring egg production traits, ovarian and follicle anatomical characteristics, and gene expression for reproductively important genes within different follicle types. Data were analyzed by an ANOVA mixed model procedure in SAS. The E line hens produced 20% more eggs than the RBC1 hens, even though they had similar numbers of preovulatory follicles in their ovaries. This was accomplished by increasing clutch length and keeping the pause length the same. On the gene expression side small white follicles (**SWF**) within E line hens had less *LHCGR* expression which coincided with downregulation of *CYP11A1* and *CYP17A1*. Along with an upregulation of *PRLR* in small yellow follicles (**SYF**) which also coincided with downregulation of *CYP17A1*. In both cases changes in pituitary hormone receptor transcription levels appeared to affect the steroid hormone synthesis pathway. In SWF from E line hens *ESR2* was downregulated, however in the large white follicles and selected follicles *ESR1* was the estradiol receptor which was downregulated. The similarity in preovulatory numbers suggests that E line hens aren't selecting more follicles to grow, but instead, follicles are growing faster. Based on the gene expression patterns, the reduction of steroid hormone synthesis might hint at the follicles putting more energy into growth and differentiation. At the same time, the decrease in estradiol receptor might limit the negative effects of estradiol on granulosa cells and allow for more rapid growth, suggesting a possible mechanism for the higher egg production trait of the E line.

Introduction

Commercial turkeys are predominantly raised for meat production, with the industry selecting birds that produce a heavy carcass, with the lowest feed conversion ratio. This is also true for the broiler industry and, in both cases, has negatively impacted egg production for these meat-producing birds (McCartney et al., 1968; Decuypere et al., 2010). There are vast variations in egg production rates within a commercial flock with high egg-producing hens (**HEPH**) and low egg-producing hens (**LEPH**), as well as between breeds based on the intensity for which egg production was selected for during the line's development (Nestor et al., 1996; Brady et al., 2020). Although egg production is not the primary concern for the industry, any reduction in egg production reduces the profitability of parent breeder flocks, by increasing the cost to produce each poult that will be raised for consumption. Increasing hen egg production while maintaining the turkey's superior carcass traits would be an ideal way to increase flock profitability.

Egg production is controlled by the hypothalamus-pituitary-gonadal (**HPG**) axis, which regulates follicle and egg formation leading up to and during reproduction. The HPG axis orchestrates this through several protein and steroid hormones, with the cascade starting at the hypothalamus with the secretion of gonadotropin-releasing hormone (**GnRH**) and gonadotropin-inhibitory hormone (**GnIH**), which stimulate and inhibit the production of gonadotropins, respectfully (Bedecarrats et al., 2009). These gonadotropins are follicle stimulating hormone (**FSH**), and luteinizing hormone (**LH**) which are secreted from the anterior pituitary gland, and directly control follicle development and ovulation (Du et al., 2020). Another hormone which is important to follicle development is

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prolactin (**PRL**), which is secreted by lactotrophs within the anterior pituitary gland and usually has a negative effect on follicle development (Wilkanowska et al., 2014). These anterior pituitary hormones influence the granulosa, interna theca, and externa theca cells located within ovary. In response to these anterior pituitary hormones follicular cells produce steroid hormones which are classified as estrogens, androgens, and progesterone (**P_4**). These hormones work in an auto- and paracrine fashion, to modulate follicle development, and the reproductive axis, as a whole.

Within a mature avian ovary, multiple follicle classes exist, from immature dormant primordial follicles, through slow-growing primary and prehierarchal follicles, to mature rapidly growing preovulatory follicles. The prehierarchal follicles can be further broken down into small white follicles (**SWF**) 1-2 mm in diameter, large white follicles (**LWF**) 3-5 mm, and small yellow follicles (**SYF**) 6-10 mm. The SWF are responsible for most of the estrogen production in the major form of estradiol (**E_2**), which is produced by the theca externa cells (Wang and Gong, 2017). Locally, E_2 inhibits the growth and differentiation of granulosa cells, but also affects egg formation at all levels (oviduct, liver, kidneys, bone, and gut) of the laying bird (Davis et al., 2000; Hanlon et al., 2022). E_2 also plays a part in the negative feedback loop by stimulating the production of PRL. PRL can inhibit GnRH, FSH, and LH secretion, and interfere with FSH and LH's abilities to influence follicular cells (Dunn et al., 2003; Tan et al., 2021). The next step is follicle selection, where one prehierarchal follicle is selected to enter the preovulatory follicle pool 10-40 mm. This is marked by further differentiation of the granulosa cell layer, increased androgen and P_4 production, further vascularization, and rapid growth with uptake of yellow yolk. Androgen production peaks in the F5 follicle and is synthesized in the theca interna layer. These androgens can be excreted or shuttled to the theca externa layer where they are converted into estrogens via aromatases (Porter et al., 1989; Magoffin, 2005). On the other hand, P_4 peaks in the F1 follicle, and is produced via the granulosa cell layer. As P_4 levels rise along with LH levels, a certain threshold is reached which triggers ovulation. Although P_4 participates in the positive feedback loop with LH to cause ovulation, it also has a stimulatory effect on PRL, as the bird comes to the end of laying its clutch PRL increases and can trigger broodiness, which inhibits the development of new follicles (Reddy et al., 2022; Wilkanowska et al., 2014).

Understanding the hormonal and cellular differences between hens with varying egg production rates will be key to improving egg production as a whole. In a previous study that looked at HEPH (>145 eggs/cycle) and LEPH (<110 eggs/cycle) within a commercial turkey flock, the HEPH laid more eggs per day, had more eggs per clutch, and had shorter pause lengths between clutches (Brady et al., 2020). However, the HEPH did not have larger ovaries, F1 follicles, or an increased number of preovulatory follicles. The increase in egg production was correlated with increased gene expression related to ovulation and steroidogenesis. Most notably there was increased expression of FSH's receptor (***FSHR)*** in the theca externa layer of both F1 and F5 follicles, but interestingly there was no difference in *FSHR* from SWF. All follicle types saw an increase in genes associated with estrogen production, particularly the SWF, this resulted in higher plasma estradiol concentrations for the HEPH. Although genes associated with P_4 production were upregulated in the F1 follicles, there was no difference in plasma P_4 concentration.

To further investigate the differences between high and low egg-producing hens, two turkey lines selected for a contrast in egg production rates were evaluated. The random-bred control one (**RBC1**) line and E line were created at the Ohio State University during the 1950′s-60′s and were transferred to the Beltsville Agricultural Research Center (**BARC**) in 2017. The RBC1 was founded first and came from a base stock of four breeds of large white turkey breeds (McCartney, 1964). The E-line was developed from this RBC1 population with selection starting in the 1960′s, to see the effect of selecting a bird solely for its egg production (McCartney et al., 1968). After 34 generations the E line hens produced ~ 140 more eggs than the RBC1 during 250 days of egg production (Nestor et al., 1996). The aim of the current study was to further investigate the differences between high and low egg-producing turkey hens, by (1) measuring egg production traits, (2) characterizing ovarian and follicle gross anatomical features, and (3) elucidating gene expression patterns associated with steroidogenesis in RBC1 and E line hens. The genes evaluated were a panel of 3 pituitary hormone receptors: *FSHR*, LH receptor (***LHCGR***), and PRL's receptor (***PRLR***). Along with 5 hormones involved in steroid production: steroidogenic acute regulatory protein (***STAR***), cholesterol side chain cleavage enzyme (***CYP11A1***), 17, 20-lyase (***CYP17A1***), 17-hydroxysteroid dehydrogenase (***HSD17B1***), aromatase (***CYP19A1***). Finally, four steroid hormone receptors were investigated: progesterone receptor (***PGR***), estrogen receptor 1 and 2 (***ESR1*** and ***ESR2***), and androgen receptor (***AR***).

Materials and methods

Hen selection and tissue collection

Four RBC1 and E line hens were randomly selected from a larger cohort of individually housed RBC1 and E line hens (48 birds per line) to be used for this study (n = 4 hens/line). Hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed and water *ad libitum,* according to their nutrient requirement. Daily egg records were used to calculate each hen's age at first egg (**AFE**), number of eggs per day (**EPD**), which was calculated by dividing the total number of eggs produced by the number of days the hen was in production (52 to 62 days), depending on AFE. The weekly average per hen was also calculated by summing the number of eggs laid by each hen during a given week, to illustrate the production curve. Clutch length was defined as the number of consecutive days eggs were laid in sequence, and pause length was the period of no-lay days between clutches. Individual AFE, EPD, weekly average, clutch, and pause lengths were averaged within lines. Hens were sampled at 51 weeks of age, when a hardshell egg was contained within the reproductive tract (Bacon et al., 1983). This meant that hens were in production for ~8-9 weeks before being sampled depending on AFE, which is in line with the previous commercial line turkey studies focusing on gene expression differences in hens with low and high egg production (Brady et al., 2020). Hens were euthanized via cervical dislocation between 0800 hr and 1200 hr, and the ovaries were immediately collected. Hen body and whole ovary weights, as well as follicle weights were recorded, follicle types are as follows: SWF, 1-2 mm; LWF, 3-5 mm; SYF, 6-10 mm; selected follicle (**SF**), is the smallest pre-ovulatory follicle > 11 mm. Ovary weights and SF weights were normalized to body weight, which gave their relative weights. The number of preovulatory follicles (11 to 50 mm) was also recorded. SWF, LWF, SYF, and SF were collected whole using previously established methods (Brady et al., 2020) for subsequent evaluation of gene expression. In brief, the yolk was drained, and the follicle was snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. For gene expression analyses a number of SWF, LWF and SYF were collected per bird and were pooled, whereas only one SF was collected per bird. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC (AUP #19-011).

RT-qPCR

Whole follicle tissues were homogenized with Trizol, and then chloroform was added to separate out the nucleic acids. Total RNA was extracted from homogenates using RNeasy Mini kits (Qiagen, Valencia, CA) with on-column RNase-Free DNase Set (Qiagen, Valencia, CA) treatment and quantified with Quant-iT RiboGreen RNA Quantitation Reagent (Thermo Fisher Scientific, Waltham, MA). Total RNA (1 μg) was reverse transcribed using Superscript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) and random hexamer primers (Thermo

Fisher Scientific, Waltham, MA) in a reaction volume of 20 μL. For each follicle stage, a pool of total RNA was made, and the reverse transcription reaction was performed with nuclease-free water replacing reverse transcriptase (No RT) as a control for genomic DNA contamination. Completed reactions were diluted to 200 μL with nuclease-free water before RT-qPCR analysis.

Primer pairs (Integrated DNA Technologies, Skokie, IL) used in PCR reactions were designed according to parameters previously described (Brady, et al. 2023) using NCBI primer BLAST Software (NCBI, Bethesda, MD) to target a 3′ region of the transcript and to span an intron. Primers were designed to amplify all known transcript isoforms, with a melting temperature ($\mathbf{T_m}$) of 58 to 60°C, a GC content (**GC%**) of 40 to 60%, a length of 18 to 30 nucleotides, and to yield a product length of 90 to 250 nucleotides.

Primer amplification efficiency was determined by analyzing 2-fold serial dilutions of pooled cDNA with RT-qPCR. Efficiency was calculated as the absolute value of the slope of the linear regression line that resulted after plotting Ct versus $\log_2$-transformed cDNA relative concentration. Amplification efficiencies were between 0.90 and 1.15 for each primer pair. Primer pairs were validated by melt curve analysis and for PCR product specificity by gel electrophoresis followed by DNA sequencing. Primer sequences are listed in Table 1.

RT-qPCR reactions (15 μL) contained 2 μL cDNA, 0.4 μM of each primer, and 7.5 μL iTaq Universal SYBR Green Supermix (2X) (Bio-Rad, Hercules, CA). All reactions were performed using CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA) with the following cycling conditions: initial denaturation at 95°C for 3 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melt curve analysis was conducted to ensure that a single PCR product was amplified in each reaction and was absent from the No RT and water negative controls. Data within each follicle stage were normalized to the average of three housekeeping genes and analyzed by the $2^{-\Delta\Delta\ Ct}$ method to quantify relative mRNA levels. For SWF, *TUBB3, TBP*, and *PPIA* were used for normalization. For LWF, *TUBB3, GAPDH*, and *ACTB* were used for normalization. For SYF, *TUBB3, TBP*, and *ACTB* were used for normalization. Finally, for SF, *TUBB3, GAPDH*, and *TBP* were used for normalization. All RT-qPCR reactions for each gene were analyzed in a single run within a 96-well plate with all follicle stages, allowing accurate performance of relative quantification within and between lines.

Statistics

Body weight, ovary weight, and relative follicle weights were averaged within E and RBC1 lines, whereas AFE, EPD, clutch and pause lengths were averaged for each hen and then averaged within then E and RBC1 lines. Anatomical and egg production parameters as well as normalized mRNA levels between E and RBC1 lines were compared by ANOVA using the mixed model procedure (PROC MIXED) in SAS 9.4 software (SAS Institute, Cary, NC). Statistical significance was reached if $P < 0.05$. Gene expression data are presented relative to RBC1 expression for all follicle stages.

Results

Although not statistically significant (p=0.068*)*, the average weight of RBC1 hens was numerically larger than the E line hens (Table 2). The appearance and morphology of the E line hen's ovaries and the RBC1 ovaries were similar. The ovary weights and relative weights also were similar between the two lines. There was also no difference in the number of preovulatory follicles present, with roughly 6-7 follicles per bird. With respect to follicle weights, there was no difference between the E line and RBC1 follicle weights for the SF, SYF, LWF, and SWF. There was a significance difference (p = 0.046) in the AFE with the E line hens starting at 294.5 ± 1.6 days of age, and the RBC1 hens coming into production at 300.2 ± 1.7 days of age. The weekly egg production per line from 41 to 50 weeks of age was visualized in Fig. 1. Egg production during the laying period was significantly higher in E line hens with longer clutch lengths, and more eggs laid per day (p=0.016 and 0.022, respectively), whereas the pause lengths were similar between the two lines (Fig. 2).

For the SWF, the only pituitary hormone receptor showing a difference in gene expression was *LHCGR*, which was downregulated (p=0.011) in E line hens, and roughly half that of the RBC1 hens' expression (Fig. 3). Two genes involved in steroid hormone production, *CYP11A1* and *CYP17A1,* were both downregulated in E line hens

Table 2
Body weight and ovarian morphology for RBC1 and E line hens at 37 weeks of age.

Parameter	RBC1	E line	*P*-value
Body weight (lb)	14.94 ± 0.43	13.14 ± 0.67	0.0675
Ovary weight (g)	103.75 ± 9.86	85.36 ± 4.42	0.1396
Ovary relative weight (%)	*1.54 ± 0.17*	*1.44 ± 0.07*	*0.6055*
Number of preovulatory follicles	7.00 ± 0.71	6.50 ± 0.50	0.5847
SF weight (g)	3.17 ± 0.36	2.90 ± 0.47	0.6627
SF relative weight (%)	*0.05 ± 0.01*	*0.05 ± 0.01*	*0.8946*
SYF weight (g)	0.21 ± 0.02	0.24 ± 0.01	0.2214
LWF weight (g)	0.07 ± 0.01	0.07 ± 0.01	0.6202
SWF weight (g)	0.02 ± 0.00	0.02 ± 0.00	0.7409

Data are presented as means ± SEM, with P values being based on a one-way ANOVA mixed model procedure, with significance set at (*P< 0.05*), which is denoted by an asterisk.

Table 1
Primers for RT-qPCR.

Symbol	Gene ID	Forward Primer	Reverse Primer
FSHR	104909561	TTGCCACCACGGGTAAAAGT	AGCAAACCCTGGTTGGCATA
LHCGR	100303684	GAAGCCGTTCTGACCTACCC	GTACGTGTGCTTTTCCGCTG
PRLR	100126248	TGTGTGGATCGTCTTGGGTG	TGGTCCTGGAACTGGTGGTA
PGR	100545565	TGGCTAATTCCCAGCGTTTCT	CTCGGGGAATTCAACGCTCA
ESR1	100542782	ATCCACCGTGTTCTGGACAAA	TCGTAGAGCGGAACCACATT
ESR2	100543001	TCACAGATGCTCTGGTGTGG	GAGTGTGTGCGCATTCAACA
AR	100540598	GGCAGCTGGTCTACGTTGTT	AAGATCTCCAACCCATGGCA
STAR	100546732	TACCTGGGAACATCGTCGGA	TTAACTTGGTCTGCGAGGGG
CYP11A1	100543260	GCTGAGCTTTGGATTTGGGC	AACTTGGTCCCGACTTCCAC
HSD17B1	100549838	GGGGGCTCATAGTGTTCTGC	CAGGGCCATCCTTGTTCAGA
CYP17A1	100540331	GGTGGTCAACAGTTACAGGC	ACTTTGGATGGAAGGAGAGGG
CYP19A1	100549427	ACCCTGTGAAAAAGGGGACA	AACTTTCCTACACAGCCCCG
TUBB3	100546417	CAGTTTTGGGAGGTGATCAGCGA	CCCGCTCTGACCGAAAATGA
ACTB	100303677	GCCGAGAGAGAAATTGTGCG	GCACTGTGTTGGCATACAGG
PPIA	100538405	GATCATCCCCGGCTTCATGT	TCGTCTTGCCATCCTTGGAG
TBP	100543138	AGCTACGAGCCGGAATTGTT	TCTCGTTACTGCTGTGCCAT
GAPDH	100303685	GGGAGGCTTACTGGAATGGCT	AGTCACAGGAGACAACCTGG

[1]Ensembl turkey genome assembly (https://useast.ensembl.org/Meleagris_gallopavo/Info/Index) gene identification preceded by ENSMGAG000000.

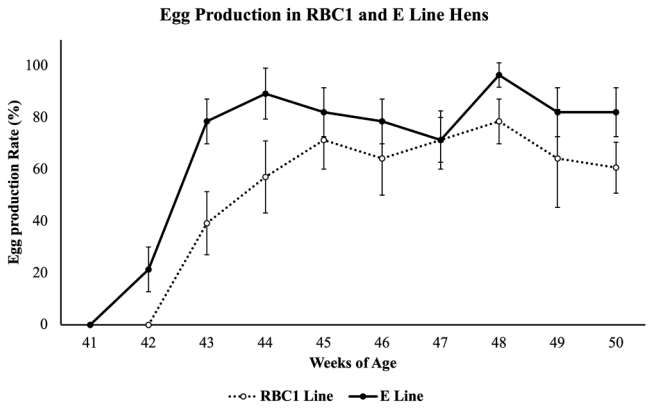

Fig. 1. Weekly egg production rates. E line and RBC1 hens (n=4) from 41 to 50 weeks of age. Data are expressed as eggs/hen on a weekly basis, values are presented as means ± SEM.

(p=0.047 and 0.007, respectively), with *CYP11A1* being roughly half again of the RBC1 hens' expression, and *CYP17A1* being less than half. For the steroid hormone receptors, only *ESR2* transcription levels varied between these two groups with lower (p=0.003) expression in the E line hens, again around half the expression of the RBC1 hens.

For the LWF, the only gene exhibiting differential expression between the two lines was the steroid hormone receptor *ESR1*, which was downregulated in E line hens compared to RBC1 (p=0.024) (Fig. 4). There were no differences between lines for the pituitary hormone receptors or the steroid hormone production genes, although there was a large variation in gene expression values for the E line hens with respect to the *STAR* gene.

In the SYF, the *PRLR* gene expression was elevated (p=0.013) in the E line hens compared to the RBC1, which was the only pituitary hormone receptor to show a significant variation (Fig. 5). For the steroid hormone genes, *CYP17A1* transcription was downregulated in E line (p=0.031). However, there were no significant differences in gene expression for steroid hormone receptors between the two lines.

Finally, for the SF, there was no significant difference in the gene expression for the pituitary hormone receptors or steroid hormone production genes between the two lines (Fig. 6). Although not statistically different, the variation in *HSD17B1* and *CYP19A1* gene expression was wide, with some of the E line hens having gene expression values 4 to 7-fold higher than the other E line hens and all the RBC1 hens. For the steroid hormone receptor genes, the *ESR1* expression was lower (p=0.047) in the E line hens compared to the RBC1.

Discussion

This study is the first to compare ovarian morphology, follicle morphology, and transcription levels of key reproductive genes in two historic turkey lines with vastly different egg production rates. It was demonstrated that the increase in egg production (**EPD**) by the E line hens was achieved by increasing the clutch length and keeping the pause length the same. During the study the E line hens came into production 6 days earlier than the RBC1 hens and produced 20% more eggs, concluded from the EPD data. The increase in egg production might be further explained by the ovarian and follicle morphology. The fact that the number of preovulatory follicles were the same indicates that the E line hens weren't selecting and growing more follicles at the same time. Instead, the follicles appear to be moving faster through the preovulatory hierarchy. Previous studies involving high- and low-egg producing Jinding ducks (Tao et al., 2017) and turkey hens (Brady et al., 2020), also saw no difference in the number of preovulatory follicles between the high and low egg-producing birds, suggesting these hens are also moving follicles through the hierarchy faster. In chickens, however, for

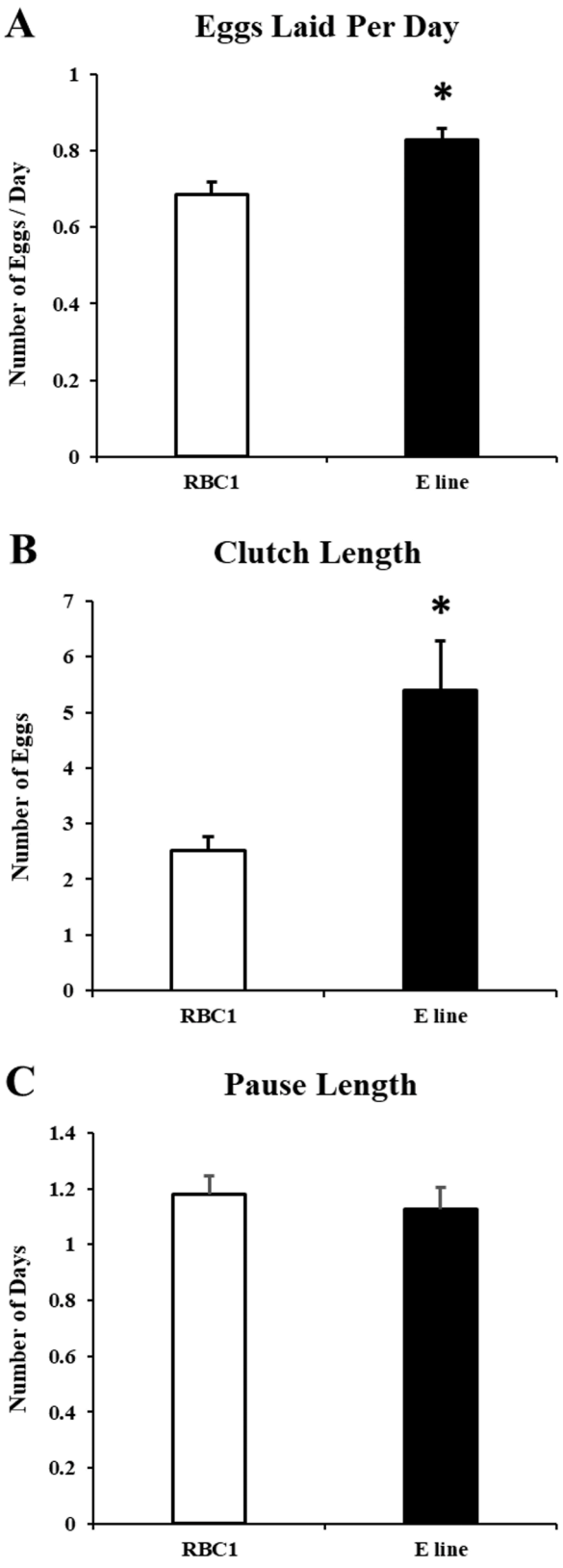

Fig. 2. Egg production traits for RBC1 and E-line hens. (A) eggs laid per day (EPD), (B) average clutch length, and (C) average pause length data are shown. Data are presented as means ± SEM, with *P* values being based on a one-way ANOVA mixed model procedure, with significance set at ($P < 0.05$), which is denoted by an asterisk.

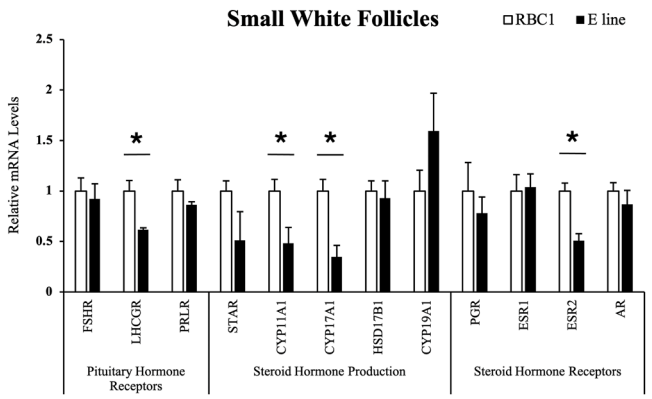

Fig. 3. Small white follicle steroidogenic gene expression. Relative mRNA levels of follicle stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHCGR*), prolactin receptor (*PRLR*), steroidogenic acute regulatory protein (*STAR*), cholesterol side chain cleavage enzyme (*CYP11A1*), 17, 20-lyase (*CYP17A1*), 17-hydroxysteroid dehydrogenase (*HSD17B1*), aromatase (*CYP19A1*), progesterone receptor (*PGR*), estradiol receptor 1 and 2 (*ESR1* and *ESR2*), and aromatase receptor (*AR*) are shown in RBC1 and E line hens at 37 weeks of age. Normalized data are presented relative to RBC1 expression for each gene. Data was analyzed using a one-way ANOVA mixed model procedure, with significance set at $P < 0.05$. Significant expression differences between the two breeds are denoted by a line and asterisk.

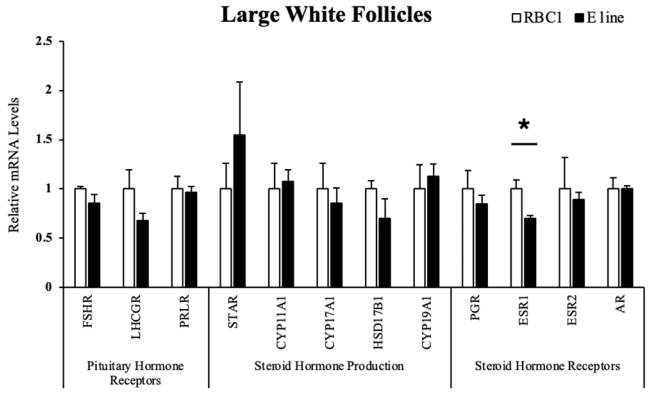

Fig. 4. Large white follicle steroidogenic gene expression. Relative mRNA levels of follicle stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHCGR*), prolactin receptor (*PRLR*), steroidogenic acute regulatory protein (*STAR*), cholesterol side chain cleavage enzyme (*CYP11A1*), 17, 20-lyase (*CYP17A1*), 17-hydroxysteroid dehydrogenase (*HSD17B1*), aromatase (*CYP19A1*), progesterone receptor (*PGR*), estradiol receptor 1 and 2 (*ESR1* and *ESR2*), and aromatase receptor (*AR*) are shown in RBC1 and E line hens at 37 weeks of age. Normalized data are presented relative to RBC1 expression for each gene. Data was analyzed using a one-way ANOVA mixed model procedure, with significance set at $P < 0.05$. Significant expression differences between the two breeds are denoted by a line and asterisk.

breeds that exhibited high (leghorn) and low (silky) egg production, there was a difference in the number of preovulatory follicles (Tai et al., 2022), suggesting that there might be different mechanisms of increasing egg production. One observation from the commercial turkey and duck examples is that these species have historically been selected for meat production. Whereas the layer chicken has been exclusively selected for egg production, this might explain the differences in physiological methods of increasing egg production. To answer how the E line hens might be speeding up follicle development, it is prudent to next turn to the gene expression profiles of the follicles.

Viewing the follicle gene expression patterns wholistically there is a clear trend, with most of the differences in gene expression between the E line and RBC1 hens being lower expression within the E line follicles. Out of the eight differences that were observed, seven were cases with lower expression, whereas only one saw a higher gene expression compared to the RBC1 hens. This follows a similar trend within SWF and the F5 follicles from HEPH and LEPH commercial turkeys, with eight out of the eleven differences seeing a lower expression of similar genes during the basal period for HEPH (Brady et al., 2020). Focusing first on pituitary hormone receptors within follicles, the E line hens had lower mRNA expression of *LHCGR* within their SWF compared to the RBC1 hens, although not significant in LWF, the pattern seems similar. Since granulosa cells within unselected follicles do not express LHCGR, the expression difference seen here must be coming solely from the theca cells (Johnson et al., 1996; You et al., 1996; Johnson, 2014). In response to LH binding to LHCGR, cAMP is activated and triggers steroid hormone production (E_2 in theca externa cells; testosterone (**T**) in theca interna cells). LH also stimulates the proliferation of theca externa cells, as seen in SYF cultures using cells from chicken follicles (Jia et al., 2010). Therefore, here the SWF from E line hens could be reducing steroid hormone synthesis, and at the same time decreasing the proliferation of theca cells. This however is in contrast to a previous study where the *LHCGR* expression was upregulated in HEPH compared to LEPH, during both basal and surge periods for SWF, steroid synthesis genes were also upregulated (Brady et al., 2020). This means that, although the results are different the pattern is similar, steroid synthesis genes upregulated with *LHCGR* expression 'up', or in our case steroid synthesis genes downregulated with 'down' regulation of *LHCGR*.

For the other pituitary hormone receptor, the E line hens saw a higher mRNA expression of *PRLR* within their SYF compared to the RBC1 hens. PRLR is expressed in primordial follicles to the F1 follicles, in the domestic goose, however, the highest levels appear in SYF, compared to 6-8 mm and F5 follicles (Deng et al., 2023). It has been shown that *PRLR* can be regulated by prolactin, as chicken granulosa cells from SYF treated with ovine PRL (oPRL) *in vitro*, exhibited an increase in *PRLR* mRNA expression (Hu et al., 2017a). In prehierarchal 6-8 mm follicles, glycosylated oPRL was able to inhibit E_2 and P_4 synthesis at high concentrations by downregulating *CYP19A1* and *3B-HSD*, however, there was no change in *CYP11A1* expression, and *CYP17A1* expression was not measured (Hu et al., 2017b). Apart from decreasing steroid hormone synthesis, PRL also seems to play a role in follicle progression, as hens immunized against PRL saw an increase in LWF (Li et al., 2011). The conclusion from this previous study was that PRL is required for the transition from LWF to SYF. Therefore, the upregulation of PRLR expression here might be causing the downregulation of the steroid hormone synthesis genes, and at the same time increasing the transition from LWF to SYF. In fact, during the first 62 days of the laying cycle the E line hens do appear to have higher PRL plasma levels than the RBC1 hens (Bacon et al., 1983).

Interestingly in both cases of pituitary hormone receptor expression differences in the SWF and SYF appeared to have potentially led to downregulation of steroid hormone synthesis. Only the follicle types (SWF and SYF) with variations in pituitary hormone receptor expression saw differences in steroid hormone production genes. *CYP11A1* saw lower mRNA expression in SWF and *CYP17A1* saw lower expression in SWF and SYF, for E line hens. It should also be noted again that significance was not met, however, there did appear to be a lot of variation in *HSD17B1* and *CYP19A1* expression in the SF. CYP11A1, also known as cholesterol side-chain cleavage enzyme, is the enzyme responsible for converting cholesterol into pregnenolone, which can then be turned into all of the steroid hormones. Whereas CYP17A1 is a hydroxylase enzyme with multiple functions in early androgen and estrogen synthesis. In the case of SWF, the downregulation of CYP11A1 would affect the synthesis of all the sex steroid hormones. Whereas for SWF and SYF, the downregulation of CYP17A1 would preferentially affect T and E_2 synthesis but would allow for P_4 synthesis, however since these follicle types aren't the main contributors of P_4 it is unlikely this is affecting P_4 plasma levels. The large gene expression variation in HSD17B1 and CYP19A1 in some of the E line hen SF could mean that some of these hens are

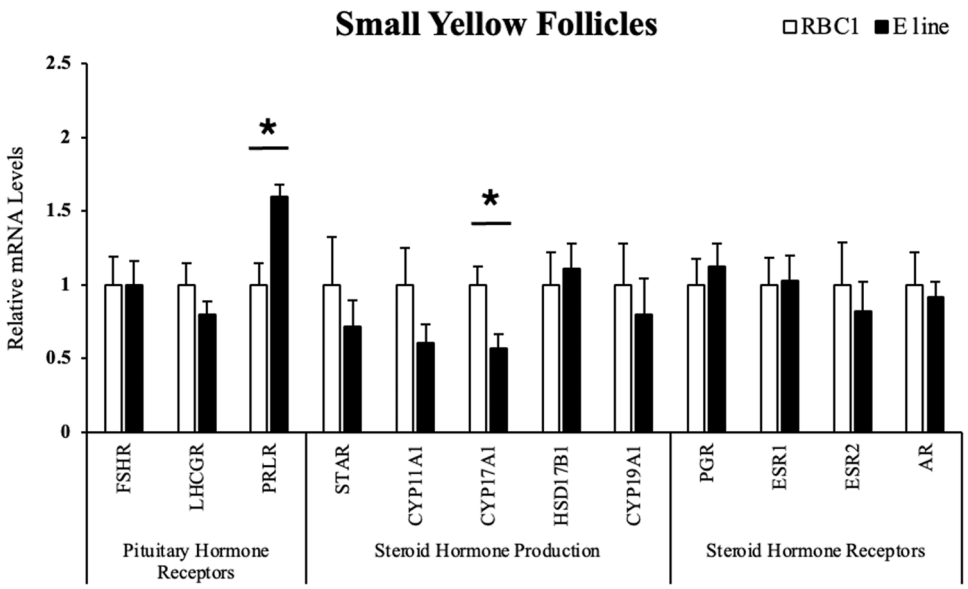

Fig. 5. Small yellow follicle steroidogenic gene expression. Relative mRNA levels of follicle stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHCGR*), prolactin receptor (*PRLR*), steroidogenic acute regulatory protein (*STAR*), cholesterol side chain cleavage enzyme (*CYP11A1*), 17, 20-lyase (*CYP17A1*), 17-hydroxysteroid dehydrogenase (*HSD17B1*), aromatase (*CYP19A1*), progesterone receptor (*PGR*), estradiol receptor 1 and 2 (*ESR1* and *ESR2*), and aromatase receptor (*AR*) are shown in RBC1 and E line hens at 37 weeks of age. Normalized data are presented relative to RBC1 expression for each gene. Data was analyzed using a one-way ANOVA mixed model procedure, with significance set at $P < 0.05$. Significant expression differences between the two breeds are denoted by a line and asterisk.

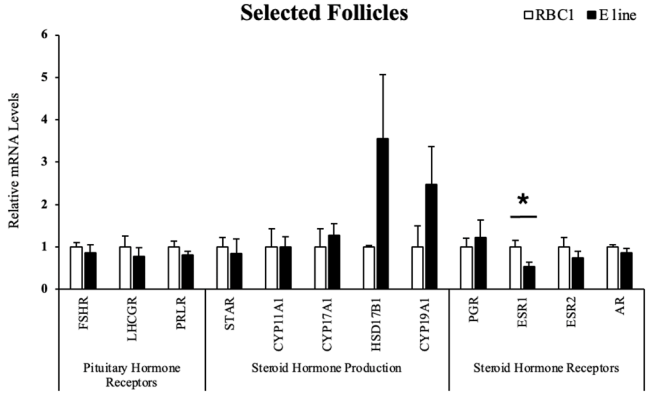

Fig. 6. Selected follicle steroidogenic gene expression. Relative mRNA levels of follicle stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHCGR*), prolactin receptor (*PRLR*), steroidogenic acute regulatory protein (*STAR*), cholesterol side chain cleavage enzyme (*CYP11A1*), 17, 20-lyase (*CYP17A1*), 17-hydroxysteroid dehydrogenase (*HSD17B1*), aromatase (*CYP19A1*), progesterone receptor (*PGR*), estradiol receptor 1 and 2 (*ESR1* and *ESR2*), and aromatase receptor (*AR*) are shown in RBC1 and E line hens at 37 weeks of age. Normalized data are presented relative to RBC1 expression for each gene. Data was analyzed using a one-way ANOVA mixed model procedure, with significance set at $P < 0.05$. Significant expression differences between the two breeds are denoted by a line and asterisk.

producing large amounts of T and E_2, compared to the RBC1 SF. However, only by increasing the number of birds sampled would we be able to answer this question. In the previous turkey study, HEPH saw an increase in *CYP17A1* expression in SWF during the basal period (Brady et al., 2020). This previous finding is also supported by the evidence that overall, in prehierarchal follicles *CYP11A1* expression is higher in high egg-producing chickens versus low egg-producing chicken breeds (Tai et al., 2022). It therefore seems reasonable to suggest that the increase in egg production seen from the E line hens is unique, for steroid hormone synthesis, compared to these two previous studies.

Switching to the steroid hormone receptors, only *ESR1* and *ESR2* expression was different between the E line and RBC1 hens. *ESR1* expression was lower in LWF and SF, whereas *ESR2* expression was lower in SWF. ESR1 and ESR2 also known as (ERα and ERβ, respectively) are the two intracellular estradiol receptors (Hanlon et al, 2022). In chicken preovulatory follicles *ESR1* is more abundant than *ESR2*, and both are expressed to a greater degree in granulosa cells versus theca cells (Hrabia et al., 2008). In SWF to SYF from quails and chickens there was no variation in *ESR1* or *ESR2* expression, however in chicken SWF to SYF there was more *ESR1* than *ESR2* present (Hrabia et al., 2004; 2008). When birds were treated with E_2 or follicles were treated with E_2 *in vitro*, treatment caused an increase in expression of *ESR1* but not *ESR2* (Zhao et al., 2017). Chicken granulosa cells incubated with LH and E_2 see a massive fold increase in P_4 production over cells incubated only with LH or E_2 (Sasanami and Mori, 1999). When granulosa cells from chicken SYF and LWF were cultured for 48h with and without E_2, the untreated cultures saw twice as much proliferation of granulosa cells, then those that were treated with E_2 (Davis et al., 2000). This lack of granulosa cell proliferation within prehierarchal follicles has also been seen in geese, via the ESR1 (Ouyang et al., 2023). Circulating E_2 levels were not measured in this study, however, previously the E_2 plasma levels were measured in the E line and RBC1 hens, with little variation between the two lines observed (Bacon et al., 1983). In the contrary, in the more recent study E_2 plasma concentrations were higher in the HEPH versus the LEPH commercial turkeys (Brady et al., 2020). Additionally, plasma E_2 levels have also been observed higher in high egg-producing chickens versus a breed with lower egg production (Hanlon et al., 2021). Although not from any specific follicle type, when whole duck ovaries were analyzed for mRNA levels *ESR1* expression was downregulated in high egg-producing birds, compared to low egg-producing ones (Tao et a., 2017). If E_2 inhibits granulosa cell growth in prehierarchal follicles to slow down growth in a negative feedback loop, by downregulating *ESR1* and *ESR2*, then the E line hen's follicles might allow for more to grow or to grow more rapidly. By downregulating estradiol receptors, they allow for more granulosa cell proliferation, which might be how they are accelerating development.

In this study, E line hens produced more eggs than RBC1 hens by increasing clutch length and by keeping pause length the same. The ovary weights and number of preovulatory follicles were the same

between lines, suggesting that the E line hens weren't selecting more follicles to grow but instead, they were proceeding through the hierarchy faster than the RBC1 follicles. The gene expression patterns of reproductively important genes were investigated, which suggested that steroid hormone synthesis was decreased in SWF and SYF, and was potentially decreased due to *LHCGR* downregulation, and *PRLR* upregulation respectively. This decrease could be due to more SWF and SYF present, and therefore each follicle has to contribute less steroid production, or by decreasing steroid production the follicle can use more energy to grow and grow faster than the RBC1 follicles. Another possibility is that other follicles are contributing more to certain steroid production than normal, as seen with the large variation in some genes for SF. The decrease in *ESR1* and *ESR2* could also be helping with follicle growth since high levels of E_2 can halt granulosa cell proliferation. Downregulating the receptor might dampen the effects and so allow granulosa cells to proliferate at a rate necessary for quicker follicle development.

Declaration of competing interest

The authors listed do not have any conflicts of interest to report.

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