Ad libitum feeding alters mRNA abundance in the ovarian cortex of broiler breeder hens

Kate Anthony¹, Tyler Bruce Garner¹,²*, Ramesh Ramachandran¹,² and Francisco Javier Diaz¹,²

¹Department of Animal Science, The Pennsylvania State University, University Park, Pennsylvania, USA
²Integrative and Biomedical Physiology Program, Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania, USA

Correspondence should be addressed to F J Diaz: fjd10@psu.edu
*(K Anthony and T B Garner contributed equally to this work)

Abstract

Ad libitum feeding in broiler breeder (BB) hens causes reduced egg production, lower fertility, and improper eggshell deposition. Restricted feeding (RF) is the only effective intervention available to normalize ovarian function and improve reproductive efficiency. This study aimed to assess the transcriptional changes in ovarian cortex of BB hens with free access to feed compared to those on a RF diet. RNA was isolated from the ovarian cortex of Cobb 500 pullets raised to 10 and 16 weeks of age on either a full-feeding (FF) or RF diet. Microarray analysis identified 386 differentially expressed genes between the two feeding groups at 16 weeks of age. Gene ontology enrichment identified overrepresentation of Neuroactive ligand–receptor interaction pathways, Cell adhesion molecules, Steroid hormone biosynthesis, and various KEGG pathways. From these groups, 46 genes were selected for follow-up validation by quantitative PCR. The findings show that 33 of the 46 genes had significantly different abundance by age and/or feeding level. Most of these genes were repressed in RF hens and belonged to the steroid biosynthesis and neuropeptide signaling groups. The VIPR2 receptor was higher in the FF group leading us to hypothesize that vasoactive intestinal peptide (VIP) is an important regulator of small cortical follicles. Culture of hen cortical follicles with VIP increased Star, an indication of increased steroidogenic activity, although did not elevate Cyp11a1. These results offer insights and suggest the possible mechanisms and pathways responsible for the increases in cortical follicle growth associated with excess feed intake in BB hens.

Lay summary

Giving breeder hens unrestricted access to feed can lead to problems with their ovaries, including excessive growth of the ovary and reduced fertility. Giving a limited amount of feed is the only effective way to reduce this growth of the ovaries and improve fertility. This study aimed to assess the changes in the molecules that make proteins in the body in hens fed unrestricted and restricted diets. In the hens fed a limited amount of feed, there were more of one type of molecules, while there were more of another type in the ovaries of hens with unrestricted access to feed. These results show that how much a hen eats can alter the number of these molecules in the ovary and this could help us understand why their ovaries grow excessively and why their eggs are less fertile.

Key Words: ▶ broiler breeder ▶ microarray ▶ ovary ▶ restricted feeding ▶ steroid ▶ VIP

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Introduction

Genetic selection for growth rate, feed intake, feed conversion, and meat yield of broiler breeder (BB) lines has led to an increase in hen growth by over four-fold since 1957 (Zuidhof et al. 2014). Consequently, this genetic selection strategy has diminished reproductive efficiency. When given free access to feed, BBs will exceed the caloric intake required for optimal growth, resulting in metabolic dysfunction, increased lipid deposition, and associated ovarian dysfunction. The exact relationship between the level of feed, changes in metabolism, and reproductive dysfunction is not entirely clear. Currently, the most effective intervention to improve BB reproductive efficiency is through significant caloric restriction (Yu et al. 1992a,b, Robinson et al. 1993, Hocking & McCormack 1995). However, a promising new study suggests that modulating light wavelength can improve BB reproductive performance (Zaguri et al. 2020).


Previous work from our lab has shown that the abundance of the steroidogenic transcripts Cyp11a1 (cytochrome P450 family 11 subfamily A member 1), Cyp19a1 (cytochrome P450 family 19 subfamily A member 1), Hsd3b1 (hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1), and Star (steroidogenic acute regulatory protein) are increased in small follicles 1 mm in diameter compared to 0.5 mm follicles (Diaz et al. 2011) as well as in ovarian cortexes from BB hens given an ad libitum diet compared to those fed restricted diets (Diaz & Anthony 2013). Increased expression of steroidogenic proteins is associated with advanced development of cortical and prehierarchical follicles in full-feeding (FF) hens (Diaz & Anthony 2013).

The purpose of this study was to further explore transcriptional differences between ovarian cortexes of prepubertal BB hens consuming FF or RF diets. Using microarrays, the transcriptomes of 16-week FF and RF hens were probed for differentially expressed genes (DEGs) from which gene ontology (GO) enrichment analysis identified overrepresented GO terms and KEGG pathways. Genes of interest were selected for follow-up quantitative PCR (qPCR) analysis and validation which revealed several genes and pathways differentially regulated between FF and RF prepubertal BB hens, most notably those involved in steroid biosynthesis, extracellular matrix maintenance and remodeling, cell adhesion, and neuroactive signaling pathways including an increase in the vasoactive intestinal peptide (VIP) and its receptor VIPR2. Since VIP is known to regulate steroidogenesis in granulosa cells from larger follicles (Johnson & Tilly 1988, Johnson et al. 1994, Kim & Johnson 2016), we further assessed the effect of VIP on the expression of steroidogenic transcripts in small cortical follicles of prepubertal hens.

Materials and methods

Animals

Day-old Cobb 500 chicks were purchased from Longenecker’s Hatchery (Elizabethtown, PA, USA) for these experiments. For the microarray and qPCR experiments, 20 chicks were raised to 10 weeks of age and 18 were raised to 16 weeks of age in accordance with the Cobb-Vantress breeder management guide (https://www.cobb-vantress.com/). From hatching until 2 weeks of age, chicks were given full access to feed, after which each age group was randomly divided in half. One treatment group continued a FF diet while the second group was placed on RF according to the breeder’s guidelines. The RF group was closely monitored to assure recommended growth rate until ovary collection at 10 or 16 weeks. Feed was purchased from Wenger Feeds (Rheems, PA, USA), and the same starter, grower, and finisher rations were fed to both treatment groups. All BBs remained on 8 h of daylight until the end of the experiment. Hens were weighed and then euthanized by cervical dislocation. Ovaries were extracted, weighed, and processed as described below. Cortical follicles used for the VIP experiments were collected from ovarian cortexes of mature (30–55 weeks) BB hens raised according to growers’ guidelines. All procedures on animals described herein were reviewed and approved by the Pennsylvania State...
University Institutional Animal Care and Use Committee and were performed in accordance with The Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies 2010).

**RNA isolation**

Small sections (<1 cm²) of cortex from each ovary were placed in RNAlater (Thermo Fisher Scientific). Total RNA was isolated using the RNAeasy mini kit (Qiagen) according to the manufacturer’s instructions and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For Affymetrix array hybridization, RNA was extracted from the 16-week-old ovarian cortex samples and delivered to the Genomics Core Facility at the Huck Institutes of the Life Sciences at the Pennsylvania State University for subsequent processing. The sample quality was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies) and only samples with an RNA integrity number (RIN) above 9 were selected from which three samples in each group were chosen randomly for microarray analysis.

RNA processing and chip hybridization were conducted using a 3’ IVT Express Kit (Thermo Fisher Scientific) following the manufacturer’s recommendations. For qPCR validation, independent samples from both 10- and 16-week-old ovarian cortex were analyzed. RNA quality and quantity were assessed on the Experion Automated Electrophoresis System using the Experion RNA StdSens analysis kit (Bio-Rad). The Experion features an RNA quality indicator (RQI) to assess RNA integrity which ranges between 1 (completely degraded) and 10 (intact RNA). An RQI greater than 7 was considered acceptable with all samples ranging between 8.5 and 9.7. Cortex samples from four randomly selected FF and RF hens at both 10 and 16 weeks were analyzed for validation.

**Microarray**

Six Affymetrix GeneChip Chicken Genome Arrays were purchased from Affymetrix. Sequence information is publicly available from GenBank®, UniGene (Build 18; 15 May 2004) and Ensembl (GRCG6a released April 2018). RNA from ovarian cortical tissue from three FF and RF 16-week-old hens were hybridized using a 3’ IVT Express Kit (Thermo Fisher). Arrays were scanned with an Affymetrix GeneChip Scanner 3000 7G, and expression intensities were normalized using the Robust Multiarray Average method commonly used with Affymetrix chips (Irizarry et al. 2003). Unannotated probes were removed, and overrepresented gene ontologies and pathways were identified using the DAVID (Database for Annotation, Visualization, and Integrated Discovery) v6.8 platform (Huang et al. 2009a,b). Processed microarray results are available online on the NCBI GEO database accession number GSE184105.

**qPCR**

For each sample, 1 µg of total RNA was reverse transcribed using the Quantitect RT kit (Invitrogen) according to manufacturer’s instructions, diluted 1:5 with nuclease-free water, and stored at −20°C. Primers (Table 1) were designed with Primer3 (version 0.4.0 University of Tartu, Tartu, Estonia). Amplification reactions were prepared in duplicate, in 10 µL volumes containing 10 ng cDNA, 200 nM of each primer, and 5 µL 2X PerfeCTa SYBR Green FastMix Low Rox (Quanta Biosciences, Inc., Gaithersburg, MD, USA), then run on a 7500 Fast Real-Time PCR System (Applied Biosystems) using default fast conditions: initial hold at 95°C for 20 s, followed by 40 cycles each at 95°C for 3 s and 60°C for 30 s. CT values were calculated using the 7500 software (v.2.0.4) and expression was quantified by the ΔΔCt method (Livak & Schmittgen 2001) (18) with Rpl19 (ribosomal protein L19) mRNA as the normalizer gene (Johnson & Lee 2016). All amplification products were sequenced for gene verification.

**Cortical follicle isolation and culture**

Follicles approximately 0.4 mm in diameter were excised from ovarian cortices by carefully teasing them away from the surrounding connective tissue with fine, sterile forceps and hypodermic needles in DMEM ((HyClone, Thermo Scientific) containing 2.5% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.1 mM non-essential amino acids). Five follicles were used for each of four replicates which were allocated to the following treatments: Fresh (at collection without incubation or treatment), media only (control), and either 1 µM Vasactive Intestinal Octacosapeptide (Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA) or 10 ng/mL rhFSH (National Hormone and Pituitary Program, Torrance, CA, USA). The dose of VIP and follicle-stimulating hormone (FSH) was previously shown to induce a robust increase in steroidogenesis in granulosa cells from prehierarchical follicles (Johnson et al. 1994, Johnson & Lee 2016, Kim & Johnson 2016). The bioactivity of the FSH preparation was confirmed by the induction of STAR mRNA in granulosa cells from 6 to 9 mm follicles (data not shown). Follicles...
were punctured with a 30G hypodermic needle to remove oocyte contents, thus allowing better perfusion of the follicle with treatment media. For each replicate, 200 µL of media-containing follicles was transferred to a free-standing 2 mL microcentrifuge tube and allowed to equilibrate at 40°C with 5% CO₂ for 30 min and then incubated in a gently shaking 40°C water bath overnight (17 h). Transcript abundance for Star, Cyp11a1, Vipr1 (VIP receptor 1), and Vipr2 in VIP-treated samples, or Star and Fshr in FSH-treated samples, was measured via qPCR as detailed above.

**Statistical analysis**

Real-time qPCR ΔΔCt values were analyzed by two-way ANOVA with hen age and feeding level as the two factors and an interaction term. A multiple test correction was performed using the Benjamini-Hochberg (BH) procedure.

### Table 1

<table>
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<th>Gene</th>
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<th>Reverse primer</th>
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<td>Vip2</td>
<td>AGGAAGCAAGAAGTCATGGA</td>
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with a false discovery rate of 0.05 (Benjamini & Hochberg 1995). Transcript abundances for VIP- and FSH-treated follicles were assessed by one-way ANOVA with Tukey’s HSD. All analyses were performed in R and a significance threshold of \( \alpha = 0.05 \) was used for all statistical tests.

**Results**

**Gene functional annotation**

Of more than 38,000 microarray probes, 18,745 remained after removing unannotated probes representing 11,561 unique genes. From this subset, 386 DEGs having a greater than two-fold difference were identified in total with 151 having greater abundance in FF hens and 235 in RF hens. The two DEG lists were uploaded to the DAVID Functional Annotation Tool to annotate related GO terms by cellular components, molecular functions, and biological processes. The GO analyses provided an overall assessment on the differences in cellular and molecular regulation in the ovaries of FF and RF hens. Results from KEGG analysis grouped the DEGs into pathways (Fig. 1). Enriched KEGG pathways determined by DAVID’s Fisher Exact Statistics with BH correction include the neuroactive ligand–receptor interaction, cell adhesion molecules, steroid hormone biosynthesis, focal adhesion, extracellular matrix–receptor interaction, androgen and estrogen metabolism, and MAPK signaling pathways.

**Selection of DEGs for qPCR validation**

To increase confidence in our microarray results and focus on our original hypotheses, genes representative of our functional annotation results were selected for qPCR validation on an independent group of cortical ovary samples \((n = 4\) each of 10- and 16-week-old FF and RF hens). Ultimately, 46 genes were selected for qPCR validation, from which 27 had similar statistical results between the microarray and qPCR results. The remaining genes were not statistically significant in either the qPCR or microarray while no genes had a disagreement between the two methods.

**qPCR results from 10- and 16-week-old hens**

Of the 46 genes selected for qPCR to compare between 10- and 16-week FF and RF hens, 33 had at least one mean \( \Delta \Delta Ct \) value that was significantly different from the other means as identified by two-way ANOVA with BH correction. Of these 33 genes, only *Hsd17b1* (hydroxysteroid 17-beta dehydrogenase 1) had a statistically significant interaction term between the hen age and feed type. Further, 26 genes had statistically different expression based on feed type (RF vs FF) while 21 genes were found to have significantly different abundance based on BB age (10- vs 16 week old).

**Neuropeptide signaling**

Six of the ten genes analyzed by qPCR in the neuroactive ligand–receptor interaction group of genes had significantly different abundance by feed type, of which included *Chrm5* (cholinergic receptor muscarinic 5), *Drd4* (dopamine receptor D4), *Gabra3* (gamma-aminobutyric acid type A receptor subunit alpha3), *Gabra5*, *Nell2* (neural EGFL like 2), and *Vipr2* (Fig. 2). Of these genes, *Nell2* and *Vipr2* were significantly reduced while *Chrm5*, *Drd4*, *Gabra3*, and *Gabra5* were significantly more abundant in RF hens compared to FF hens. Three genes in this pathway, *Grid1* (glutamate ionotropic receptor delta type subunit 1), *Vip*, and *Vipr2*, had their relative expression reduced from 10- to 16-weeks of age. *Gabbr2* (gamma-aminobutyric acid type B receptor subunit 2) and *Vipr1* did not have any statistically significant differences between the groups studied.

**Cholesterol metabolism**

The transcript expression of the two cholesterol transporter genes analyzed by qPCR, *Star* and *Stard4*, were both significantly less abundant from 10- to 16 weeks of age, and expression of both transcripts was reduced in RF

![Figure 1 Heatmap of the significant GO terms (y-axis) and some of the top genes from the microarray (x-axis). Boxes indicate if the gene belongs to the GO term and if it is increased (red) or decreased (blue) compared to RF hens.](https://raf.bioscientifica.com)
Feed restriction and ovarian gene expression

Figure 2 Stripchart of the nine genes belonging to the Neuroactive group. Each point represents a replicate from qPCR analysis while horizontal lines indicate means and vertical lines s.d. FF hens are represented in green and RF hens in blue with the darker shades indicating 16-week-old hens. For the calculation of ddCt values, RPL19 was used as the normalizer gene and the average dCt value from the 10-week RF hens was used as the reference group. Asterisks (*) indicate statistically significant differences by age and crosses (+) indicate statistically significant differences by feed (\( *P < 0.037, **P < 0.01, ***P < 0.001, ****P < 0.0001 \)).

hens (Fig. 3). Dhcr24 (24-dehydrocholesterol reductase) but not Hmgcr (3-hydroxy-3-methylglutaryl-CoA reductase), both of which are involved in cholesterol biosynthesis, also had significantly reduced abundance in RF hens. Conversely, Hmgcr but not Dhcr24 had elevated abundance in 16-week-old hens compared to BBs at 10 weeks of age. Four genes important for steroidogenesis, Cyp11a1, Cyp19a1, Hsd17b1, and Hsd3b2, similarly had significantly reduced abundance in RF hens, and in particular Hsd17b1 and Hsd3b2 saw a sharp increase in abundance from 10- to 16 weeks of age in FF hens that was absent in RF hens. Hsd17b1 had a statistically significant interaction term with increased abundance in 16-week FF hens compared to all other groups.

Extracellular matrix

Of the two matrix metalloproteinases (MMPs) assessed by qPCR, both of which are zinc-binding proteases involved in ECM degradation, expression of Mmp10 but not Mmp9 was significantly less abundant in RF hens (Fig. 4). Similarly, the expression of collagen, type VI, alpha 1 (Col6a1) but not the other three collagen transcripts (collagen, type I, alpha 2 (Col1a2), collagen, type3, alpha3 (Col3a1), and collagen, type IV, alpha2 (Col4a2)) were significantly less abundant in 10- and 16-week RF hens without an age effect. Expression of Anxa2 (annexin A2), Fhl2 (four and a half LIM domains 2), Lama2 (laminin subunit alpha 2), and Vcan (versican) were less abundant in 16-week-old hens though significantly more abundant in FF hens while Fn1 (fibronectin 1) was more abundant in FF hens with no differences by age.

Cell adhesion

Ccdk80 (coiled-coil domain containing 80), Cdhl3 (cadherin 13), Mgl1 (monoglyceride lipase), Myh11 (myosin heavy chain 11), and Pten (phosphatase and tensin homolog) all had significantly lower abundance in 16-week-old hens compared to their 10-week old counterparts with significantly higher abundance in FF hens (Fig. 5). Negr1 (neuronal growth regulator 1) also was less abundant in the older hens but its expression was unaffected by feed type. Conversely, Postn (perioestin) expression was slightly more abundant in FF hens but was not different by hen age. Although Cldh18 (claudin 18) had statistically reduced abundance in 16-week-old hens, there was a great degree of variability in 10-week old hens. Cd36 (cluster of differentiation 36) was the only gene in this group to not have statistical significance.

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Transcription and defense

In the transcription and defense set of genes, only three of the eight quantified by qPCR were differentially expressed with Dlx5 (distal-less homeobox 5) having reduced abundance while Satb2 (SATB homeobox 2) and Supth3 (SPT3 homolog, SAGA and STAGA complex component) had increased abundance at 16-week compared to the 10-week-old hens (Fig. 6). No genes in this group had statistical significance between the RF and FF feed types. A high degree of variance was observed for Cath3 (cathelicidin-3), Gal7 (Lgasl7), and leukocyte cell-derived chemotaxin 2 (leukocyte cell derived chemotaxin 2) in the 16-week-old FF hens.

VIP- and FSH-induced expression of steroidogenic transcripts

Follicles with a diameter of 0.4 mm were selected as they fall within the cut-off for large cortical follicles in BBs and contain both a theca and granulosa layer, thus would be responsive to steroidogenic activators and able to synthesize the cholesterol transporter STAR. After 17 h, VIP-treated follicles maintained Star abundance equal to that of fresh samples while follicles cultured without VIP did not
maintain Star expression (Fig. 7A). The VIP-treated follicles had a lower abundance of Cyp11a1 or Vipr2 expression compared to fresh follicles (Fig. 7B, C, and D). However, VIP treatment increased the abundance of Vipr1 by five-fold (Fig. 7B, C, and D). Conversely, follicles cultured for 17 h with FSH were unable to maintain Star abundance even though the follicles do express the FSH receptor (Fig. 8).

Discussion

The intraovarian defects that cause excessive follicular development in BB hens and how feed level modulates ovarian activity remains to be fully defined. Previously, our lab has shown that ad libitum feeding causes not only increased selection of hierarchical follicles but also increased development of prehierarchical follicles from 9 mm to small (~1 mm) cortical follicles to suggest that unrestricted feeding results in increased follicle activation and/or early growth (Diaz & Anthony 2013). In the present study, the transcriptome of ovarian cortical tissue in 16-week-old FF and RF BB hens was analyzed to identify genetic pathways modulated by the plane of nutrition during early follicular development.

From the >38,000 probes that comprised the Affymetrix microarray chip, 386 DEGs (~3.3% of all annotated, unique probes) were identified based on a >two-fold difference in expression between 16-week-old FF and RF hens. Using functional annotation analysis through DAVID (Huang et al. 2009a,b), DEGs were classified into the neuroactive ligand–receptor interaction, cell adhesion molecules, steroid hormone biosynthesis, focal adhesion, and the transcription and defense categories. Representative transcripts from these groups and specific transcripts previously shown to be differentially expressed in FF and RF hen ovaries were selected for follow-up with qPCR validation and analysis.

The ovary is a well-known source of steroid hormones. In the hen, large prehierarchical and hierarchical follicles are major sites of synthesis for both estrogen and progesterone (Bahr & Johnson 1984, Onagbesan et al. 2009, Johnson 2015). Although not a major source of systemic steroids, the small cortical follicles and ovarian stroma produce steroids locally (Nitta et al. 1991, Lee & Bahr 1994, Lee et al. 1998). Similar to our previous RF study with 17-week-old BB hens (Diaz & Anthony 2013), the steroidogenic transcripts Cyp11a1, Cyp19a1, Hsd3b, and Star were elevated in 10- and 16-week-old FF hens. This finding is in agreement with other studies that found the abundance of Cyp11a1 and Cyp19a1 mRNA, in addition to Cyp17a1 (Cytochrome P450 Family 17 Subfamily A Member 1), to increase from 0.4 to 1.6 mm follicles (Zhou et al. 2020). Further, we observed in this study that three other steroidogenic transcripts, Stard4, Dhhcr24, and Hsd17b1, were elevated in FF hens at 16 weeks of age. These data indicate that pathways involved in steroid production are upregulated prematurely in BB hens fed ad libitum, which corresponds with increased follicular development (Diaz & Anthony 2013), at least 1–2 weeks before sexual maturity and could be a driver of the earlier propensity of FF hens to reach sexual maturity compared to RF hens (Yu et al. 1992b).
Follicular development is associated with dynamic remodeling of the ovarian stroma as follicles continually grow, regress, and ovulate. Consistent with increased follicular development in FF hens, we found that transcripts involved in extracellular maintenance and remodeling were elevated in FF hens compared to their RF counterparts. This finding is sensible as FF hens have an increased number and size of cortical follicles which would require upregulation of genes involved in supporting their expanding extracellular matrix (Diaz & Anthony 2013). Many MMP transcripts are known to be modified following treatment with equine chorionic gonadotropin (eCG), which has FSH-like properties, in chicken ovaries, including the two MMPs studied in our qPCR analysis, MMP9 and MMP10 (Wolak et al. 2021). Importantly, MMP9 is suppressed while MMP10 is elevated in yellow follicles following eCG treatment (Wolak et al. 2021). In our qPCR analysis, MMP10 but not MMP9 was found to be statistically elevated in FF hens and appears to follow a similar trend with the findings from Wolak et al. (2021).

Fhl2, an assembler of extracellular membranes, is expressed in rat GCs where it acts as a transcriptional coactivator that augments inhibin transcriptional expression (Matulis & Mayo 2012). The hyaluron-binding proteoglycan versican (Vcn1) has been relatively unstudied in avian species, although in rodents the protein is expressed along the matrix of the granulosa layer throughout folliculogenesis, particularly during ovulation (Russell et al. 2003). Fibronectin is a component of the ovarian follicle basal lamina which is upregulated during vitellogenesis in salmon follicles (Guzmán et al. 2014). Fibronectin protein abundance also increases in granulosa cells of mature hen follicles (Asem et al. 1992). Our qPCR results follow similarly as Fhl2, Vcn1 and Fnl1 increase in FF hens at the ages studied, indicative of advanced follicle growth and maturity.

Col6a1, but not three other collagen transcripts, Col1a1, Col3a1, and Col4a2, was significantly upregulated in FF hens. Little is known about the Col6a1 collagen transcript in hen ovaries. In separate transcriptomic assessments, Col6a1 abundance and collagen thickness were found to increase from 0.4 to 1.6 mm follicles (Zhou et al. 2020), while conversely Col6a1 is downregulated in granulosa cells from F5 follicles compared to 6 mm prehierarchical follicles (Zhu et al. 2019). Our data appear to be in agreement with Zhou et al. (2020) as prepubescent FF BBs display increased numbers of 0.1–0.5 mm follicles compared to RF hens (Diaz & Anthony 2013), and therefore increased abundance of some collagen transcripts could be expected from cortex samples.

Pten serves to inhibit primordial follicle activation through the inactivation of the PI3K-Akt-Foxo3 pathway (John et al. 2008). qPCR analysis showed a statistically significant increase in Pten abundance in FF hens. This was surprising as one might expect Pten to be suppressed since FF hens have more growing follicles than RF hens at 16 weeks of age (Diaz & Anthony 2013). It may be that other pathways that induce the activation of primordial follicles are overriding the inhibitory actions of Pten and/or that the increase in Pten expression in FF hens is not enough to fully suppress the numbers of growing follicles.

In the neuroactive ligand-receptor interaction group, several interesting changes were noted. First, Grid1 abundance was significantly suppressed in 16-week-old hens. In sheep, GRID1 has been identified as a major candidate gene for affecting reproductive prolificacy (Nosrati et al. 2019), although a specific role for Grid1 in reproductive processes in the ovary is unknown. In two RNA-seq studies, using whole-ovary tissue of low- and high-egg producing Jinghai Yellow Chickens (Zhang et al. 2019) and Jinding Ducks (Tao et al. 2017), KEGG analysis similarly identified the neuroactive ligand-receptor interaction pathway to be highly enriched independent of body weight. In Jinghai Yellow Chickens of similar weight but grouped as either having high or low egg production, expression of Chrm5 was reduced in low egg-producing hens compared to hens that produced a relatively high number of eggs (Zhang et al. 2019). Conversely, the same study identified Vipr2 to have a greater expression in the hens producing a relatively low number of eggs (Zhang et al. 2019). Our results also show that Chrm5 transcript abundance is lower and Vipr2 higher in FF hens which are known to have lower egg production compared to RF hens. Whether the altered abundance of these two genes is a cause or a result of ovarian dysfunction in FF hens remains to be determined.

VIP-positive nerve fibers are located in the theca layer of small cortical (≤ 5 mm) and prehierarchical follicles (6–9 mm) (Johnson et al. 1994). Moreover, VIP added to granulosa cells in vitro from either preovulatory or prehierarchical follicles promotes the expression of steroidogenic enzymes and progesterone production (Johnson & Tilly 1988, Advis et al. 1989, Johnson et al. 1994). In rats, VIP has been reported to promote primordial follicle activation and early growth (Chen et al. 2013). Two VIP receptors, VIP receptor 1 (VIPR1, also known as VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) receptor 1 (VPAC1)) and VIPR2 (also known as VPAC2), are Gas-protein coupled receptors that bind both VIP and PACAP to stimulate adenylyl cyclase activity (Laburthe et al. 2009).
et al. 2002). Both VIP receptors are expressed in ovarian granulosa and theca tissue from early follicle growth up until ovulation (Kim & Johnson 2016). The steroidogenic effects of VIP and expression of VIP receptors in follicles still contained within the hen ovarian cortex, particularly those in prepubertal hens, has yet to be resolved. In our study, Vipr2 but not Vipr1 was more abundant in FF hens at both 10 and 16 weeks of age. Although little is known about these transcripts in the hen ovary, in mouse ovaries both VIPR1 and VIPR2 are present in granulosa cells and residual ovarian tissue where they prevent granulosa cell apoptosis upon stimulation by VIP (Barberi et al. 2007). Additionally, VIP is known to stimulate adenyl cyclase-mediated steroidogenesis in the granulosa cells of hens (Johnson & Tilly 1988). Therefore, we sought to examine if VIP affected steroidogenesis in the immature BB ovary, and indeed VIP does maintain Star transcript abundance in cultured cortical follicles although it did not significantly affect Cyp11a1 expression. Interestingly, the expression of Vipr2 was reduced while Vipr1 increased in follicles following VIP treatment.

While differences were found in the expression of one of the receptors for VIP, the abundance of Vip was not different statistically among the four groups. The expression of Nell2, a peptide growth factor containing EGF-like repeats, was increased ~four-fold in FF hens at 10 and 16 weeks of age compared to their RF counterparts. While little is known about Nell2 in non-neural tissues, particularly in hen ovaries, there is some evidence to speculate that it may be involved in the release of VIP as Nell2 is expressed in glutamatergic neurons (Ha et al. 2008) and glutamate stimulates VIP release in the cat cerebral cortex (Wang et al. 1986). In the same study with cats, antagonists to the neurotransmitter gamma-aminobutyric acid (GABA) also stimulate VIP release (Wang et al. 1986), and findings in our qPCR results identify at least two GABA receptors (Gabra3 and Gabra5) to be suppressed in FF hens. Secondly, the expression of two adenyl cyclase inhibitors, Chrm5 and Dnd4, is reduced in FF hens. While much more experimentation must be done to confidently elucidate the role of VIP and VIP-regulatory genes in the ovarian disruption observed in FF hens, an interesting supposition that VIP may be involved not be transcriptional regulation of its own gene but of a multi-tiered regulatory scheme that involves upregulation of its release (Nell2) and of a receptor (Vipr2) alongside downregulation of VIP release inhibitors (Chrm5, Dnd4, Gabra3, and Gabra5) in FF hens emerges.

In adult hen ovaries, gonadotropins stimulate Star expression and progesterone production only form prehierarchical follicles about ready to enter the hierarchy 6–8 mm in diameter (Johnson et al. 2002). Previous work demonstrated that VIP can also increase progesterone production and/or STAR expression in preovulatory granulosa cells (Johnson & Tilly 1988) and prehierarchical follicles 6–8 mm (Johnson et al. 1994). However, VIP-positive nerve tracts can be found even in small 0.5 mm follicles (Johnson et al. 1994). In the present study, we show that even these small cortical follicles are sensitive to VIP as indicated by increased Star transcript abundance. However, they may not be fully responsive since VIP did not increase the abundance of Cyp11a1.

In our study, VIP was unable to maintain Vipr2 expression while Vipr1 increased over five-fold after 17 h. This finding was surprising as it did not reflect our qPCR results where Vipr2 but not Vipr1 was upregulated in FF hens. It is possible that responsiveness to VIP through changes in expression of its receptors may be influenced by VIP treatment. Culture with VIP did however maintain Star expression in hen ovarian cortexes to a level nearly identical to fresh tissue indicating that, at least in the immature ovary, VIP can modulate gene expression likely through effects on cells other than the granulosa.

Conclusions

Overall, many of the DEGs that were identified are increased in FF vs RF hens, suggesting that these pathways are positively correlated with follicle activation and early growth. Pathways involved in steroidogenesis, cell adhesion, and remodeling of the ECM were particularly upregulated in FF hens, indicating a central role of these pathways in follicle activation and or early growth. Future work could exploit these changes to alter ovarian function in BB hens without the need for severe feed restriction. Finally, this study revealed a role for VIP in maintaining the capacity for steroid production in small cortical follicles and, interestingly, in the transcriptional regulation of its own receptor, Vipr2. How VIP signaling may mediate ovarian dysfunction in BB hens fed ad libitum and the possible interventions that may ameliorate these effects are the focus of future studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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K A collected and analyzed data and wrote the paper, T B G analyzed data and wrote the paper. F J D conceived of the study and wrote the paper. R W wrote the paper.

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