OVARIAN PHYSIOLOGY OF HENS WITH VARYING REPRODUCTIVE EFFICIENCIES: INVESTIGATIONS OF OVARIAN FACTORS AND TRANSCRIPTOMIC DIFFERENCES.

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Laurie Francoeur

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OVARIAN PHYSIOLOGY OF HENS WITH VARYING REPRODUCTIVE EFFICIENCIES: INVESTIGATIONS OF OVARIAN FACTORS AND TRANSCRIPTOMIC DIFFERENCES.

Laurie Francoeur, Ph.D.
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Domestication and intense genetic selection in chickens have resulted in highly efficient meat (broiler) and egg-producing (layer) breeds. The work reported in this dissertation explores differences between hens with different egg-laying rates in two breeds of chickens and factors involved in follicle development.

Intense selection in broiler hens has caused suboptimal reproduction, which can be alleviated by restricting feed intake. In the first study, using RNA-sequencing, we investigated transcriptional differences in granulosa cells of 6-8 mm follicles from broiler breeder hens fed at different levels. RNA-sequencing analysis revealed genes involved in steroidogenesis and FSH regulation were upregulated in broiler breeder hens fed *ad libitum*. IGF1 and FSH were identified as upstream regulators of these genes that could influence genes involved in follicle development. A second study investigated the role of IGF1 treatment *in vitro* in the presence and absence of FSH in granulosa cells of prehierarchal follicles. Several genes upregulated in broiler breeder hens fed *ad libitum* were increased by IGF1, and FSH enhanced the expression of some genes.

Laying hens are extremely reproductively efficient during their first year; however, the average egg-laying rate of a flock decreases after the first year, leading producers to cull hens. Within this average are hens that continue to lay at a high rate and hens with a marked decline of egg-laying rate. Hens exhibiting persistent laying rates (PL, 100% egg-laying rate) and nonpersistent laying rates (NPL, ~80% egg-laying rate) were selected for retrospective and ovarian analysis. PL hens had higher body weights and egg-laying rates earlier in life. At the ovarian level, PL hens had more follicles, lower rates of atresia in <100 μm follicles, and higher AMH and BMP15 mRNA expression. The final study investigated the regulation of AMH by FSH and the functional role of AMH using RNA-interference in granulosa cells of 3-5 mm and 6-8 mm follicles. FSH decreased AMH mRNA expression in 3-5 mm but not 6-8 mm follicles. AMH did not affect mRNA expression of follicle development markers examined in the presence or absence of FSH. The work in this thesis provides new insights into the physiology underlying follicle development and egg-laying rates.

BIOGRAPHICAL SKETCH

Laurie Francoeur was born in Quebec City, Quebec, Canada in 1995. Her family moved to New Jersey when she was five years old. While Laurie had always enjoyed science, her interest in Reproductive Biology began during her senior year of high school in Ms. Reed's AP Biology class.

Laurie began studying Biology at Rutgers University (after a quick detour studying Management and Economics at the University of Toronto), where she had the opportunity to join Dr. Hamilton's pest management laboratory in the Entomology Department. There she was exposed to laboratory and field research for the first time and fell in love with research. In Dr. Hamilton's lab, Laurie had the opportunity to do science outreach in the community and conduct her own research projects. Laurie conducted a few experiments looking at the morphological development of the genital plate in brown marmorated stink bugs and developed a method to sex immature individuals. She also looked at the effects of multiple mating events on fecundity. These experiments resulted in an honor's thesis during her senior year in 2017.

In the Fall of 2017, Laurie started her Ph.D. under the guidance of Dr. Pat Johnson in Cornell's Department of Animal Science. During her time at Cornell, Laurie had the opportunity to research ovarian physiology using the hen as a model. She served on the executive board of the Animal Science Graduate Student Association (ASGSA) as Treasurer and, with the help of her team, hosted the first annual ASGSA symposium for graduate students in the department to showcase their research. She also served as a Teaching Assistant for many classes in the department and enjoyed developing her teaching skills and interacting with undergraduate students. She received the

Outstanding Graduate Teaching Assistant Award from the College of Agriculture and Life Sciences for her work as a teaching assistant. Laurie was fortunate to continue to practice community outreach during her graduate career as a co-leader for the Tompkins County 4-H Poultry Club and through Cornell programs such as Expanding Your Horizons. She also created a mini course on human reproduction for high school students through GRASHOPPER. Laurie developed her mentoring skills by teaching two undergraduate students in the lab and volunteering as a graduate mentor in a program led by Cornell's Office of Academic Diversity Initiatives. As a proud member of the Society for the Study of Reproduction, she attended several annual meetings where she presented four posters and a flash talk and was awarded the 2022 USDA merit award for one of her abstracts. After graduating with her Ph.D., Laurie hopes to enter the pharmaceutical industry to work on drugs aimed at improving women's health.

To my fiancé, Jim, and my parents, Rene, and Lucie. Thank you for the constant source of motivation and support throughout this journey.

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TABLE OF CONTENTS

Biographical sketch ………………………………………………….………………… v

Acknowledgements ……………………………………..……………………… viii

Chapter 1: Literature Review

Breeding History of Chickens ……………………………….………………… 1

Reproduction in the Hen ………………………………..………………… 3

Follicle Selection ………………………………………………………...……… 13

Regulation of Follicle Development ………………………………………… 16

Broiler Breeder Hen Reproduction …………………………………………… 24

Summary ……………………………………………………………………… 29

References …………………………………………………………………… 31

Chapter 2: *Ad Libitum* Feeding in Broiler Breeder Hens Alters the Transcriptome of Granulosa Cells of Prehierarchal Follicles.

Abstract …………………………………………………………………….. 51

Introduction ……………………………………………….…………….. 52

Materials and Methods ……………………………………...……… 53

Results ……………………………………………………………………... 57

Discussion ………………………………………………………………….. 68

Acknowledgments …………………………………………………………. 72

References …………………………………………………...….. 73

Chapter 3: Effect of IGF1 and FSH treatment on the Function of Granulosa Cells of Prehierarchal Follicles in Chickens.

Abstract ………………………………………………………………….. 79

Introduction ……………………………………………….………….. 81

Materials and Methods …………………………………………..…… 83

Results ………………………………………………………………... 88

Discussion ……………………………………………………………….. 98

Acknowledgments ………………………………………………….... 102

References …………………………………………………………... 103

Chapter 4: Characterization of Persistency of Lay in Laying Hens

Abstract ………………………………………………………………… 110

Introduction …………………………………………...………….. 112

Materials and Methods ……………………………………………… 113

Results ……………………………………………………………….. 120

Discussion ………………………………………………………… 140

Acknowledgments ………………………………………………….... 144

References …………………………………………………………... 145

Chapter 5: Investigations of the Regulation and Function of AMH in Granulosa Cells in Hens.

Abstract ………………………………..………………………………….. 150

Introduction ……………………………………………………….... 152

Materials and Methods ……………………………………………… 154

Results …………………………………………………...……….. 158

Discussion ……………………………………………….………….. 168

Acknowledgments ………………………………………………...… 171

References ……………………………………………………………………... 172

Chapter 6: Conclusions and Future Directions ……………………………………… 179

References ……………………………………………………………………... 187

Appendices ……………………………………………………………………………….. 191

LIST OF FIGURES

Figure 1.1 Chicken ovary from a laying hen ..………………………………………. 7

Figure 1.2 Schematic of undifferentiated and differentiated granulosa cells ………... 15

Figure 2.1 Egg production (eggs/hen/day) of RF and FF hens in response to dietary treatment …......................................……………………………………… 59

Figure 2.2 Principle component analysis, hierarchal clustering, and MA plot for RNA-sequencing data of granulosa cells from 6–8-mm follicles from RF and FF hens …… 61

Figure 2.3 Boxplots of normalized counts from RNA-seq analysis comparing FF (n=2) and RF (n=3) gene expression of select DEGs ……………………………………..… 62

Figure 2.4 CYP11A1, STAR, INHA, and INHBB mRNA expression in granulosa cells of 6–8-mm follicles in FF and RF hens …………………………………..……….. 63

Figure 2.5 Upstream regulator analysis of differentially expressed genes in RF and FF hens ……………………………………......…………………………………… 67

Figure 3.1 IGF1R and FSHR mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles ..……………………………………...……………….. 90

Figure 3.2 AMH mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles ………………………………...…………………………………… 92

Figure 3.3 INHA and INHBA mRNA expression in cultured granulosa cells from 6-8 mm follicles ……………………………………...…………………………... 93

Figure 3.4 STAR and CYP11A1 mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles……………………...…………………………………… 95

Figure 3.5 Cell proliferation and progesterone production in cultured granulosa cells from 6-8 mm follicles …………………………………...…………………… 97

Figure 4.1 Egg-laying efficiency and body weights of PL (n=7) and NPL (n=6) hens from 16 to 76 weeks of age ..…………………………………………………….... 122

Figure 4.2 Plasma estradiol concentrations (pg/mL) in PL (n=3) and NPL (n=4) hens from 16 to 76 weeks of age ..…………………………………………………….... 125

Figure 4.3 Ovaries, ovarian weights, and follicle numbers of PL (n=3) and NPL (n=4) hens at 76 weeks of age ..………...…………………………...………………… 126

Figure 4.4 Relative AMH and BMP15 mRNA expression in PL (n=3) and NPL (n=4) hen ovarian tissues at 76 weeks of age ………...……………………………………. 128

Figure 4.5 Histology of ovarian sections of PL and NPL hens at 76 weeks of age … 129

Figure 4.6 Follicle density and rate of atresia of follicles <500 µm in ovarian sections of PL (n=3) and NPL (n=3-4) hens at 76 weeks of age ……...………………………… 131

Figure 4.7 Plasma levels of T3 and T4 at 26 and 76 weeks of age in PL (n=7) and NPL (n=6) hens …………………………...…………………………………….……… 134

Figure 4.8 Principal component analysis and MA plot ……………………………... 135

Figure 4.9 Estrogen receptor upstream regulator network and ESR1 and ESR2 normalized counts and ovarian mRNA expression for PL and NPL hens ………….. 139

Figure 5.1 Relative AMH and FSHR mRNA expression in cultured granulosa cells of 3-5 mm and 6-8 mm treated with FSH (0, 10, 25, 100 ng/mL) ……………………….. 160

Figure 5.2 Relative AMH mRNA expression in granulosa cells of 3-5 mm follicles treated with different concentrations of AMH siRNA ……………………………... 162

Figure 5.3 Relative mRNA expression of AMH in granulosa cells of 3-5 mm and 6-8 mm follicles …………………………...………………………………………….. 163

Figure 5.4 Relative FSHR mRNA expression in granulosa cells of 3-5 mm follicles ……………………...……………………………………………………………… 165

Figure 5.5 Relative STAR and CYP11A1 mRNA expression in granulosa cells of 3-5 mm follicles …………………………...………………………………………….. 166

Figure 5.6 Relative BMPR2 mRNA expression in granulosa cells of 3-5 mm follicle ……………………...………………………………………………………………... 167

Figure 6.1 Proposed model for AMH action on BMP15 pathway and FSH regulation of AMH …………………………...………………………………………………… 186

LIST OF TABLES

Table 2.1 Primer Sequences for RT-qPCR ……………………………...…….……… 57

Table 2.2 Mean body weight, normalized fat pad, liver, and ovary weights, as well as follicle numbers (3–5-mm, 6–8-mm, and follicles >9-mm) and egg production (eggs/hen/day) for RF and FF hens in response to dietary treatment ………………… 58

Table 2.3 Enrichment analysis of upregulated differentially expressed genes in FF hens ……………...…….………………...…….………………...…….……………...…….…… 65

Table 2.4 Enrichment analysis of upregulated differentially expressed genes in RF hens ……………...…….………………...…….………………...…….……………...…….…… 66

Table 3.1 Primer Sequences for RT-qPCR …………………………………………… 87

Table 4.1 Primer Sequences for RT-qPCR ……………………………...………...… 118

Table 4.2 Correlation analysis of egg-laying efficiencies from 16-96 weeks of age .. 123

Table 4.3 Correlation analysis of body weights from 16-96 weeks of age …………. 124

Table 4.4 Enrichment analysis for upregulated genes in ovaries from PL and NPL hens ………...…….……………...…….………………...………………………………….. 136

Table 4.5 Enrichment analysis for downregulated genes in ovaries from PL and NPL hens …………………………...……………………………………………………... 137

Table 4.6 Top 25 Regulators of DEGs in ovaries from PL hens ……………………. 138

Table 5.1 siRNA sequences for RNAi …………………………...……………….…… 156

Table 5.2 Primer Sequences for RT-qPCR ……………………………...……...…… 157

Appendix 1. RNA-Seq Alignment Summary …………...………………...…...…… 191

Appendix 2. Top 50 upregulated differentially expressed genes in FF hens, including the Ensembl gene ID, gene name, log_2(FC), and FDR, and average normalized read counts for FF and RF groups are indicated for each gene ………….........………… 192

Appendix 3. Top 50 upregulated differentially expressed genes in RF hens, including the Ensembl gene ID, gene name, log_2(FC) and FDR, and average normalized read counts for FF and RF groups are indicated for each gene …………...……………… 195

Appendix 4. RNA-Seq Alignment Summary …………...……………………...…... 198

Appendix 5. Top 50 upregulated differentially expressed genes in PL hens. The Ensembl gene ID, gene name, log_2(FC), FDR, and average normalized read counts for PL and NPL groups …………...……………………...……………………...……… 199

Appendix 6. Top 50 downregulated differentially expressed genes in PL hens. The Ensembl gene ID, gene name, log_2(FC), FDR, and average normalized read counts for PL and NPL groups …………...……………………...……………………...……… 202

LIST OF ABBREVIATIONS

ABAM	Antibiotic-antimycotic
AMH	Anti-mullerian hormone
AMHKO	Anti-mullerian hormone knock out
AMHR2	Anti-mullerian hormone receptor type 2
BMP15	Bone morphogenetic protein 15
BMP6	Bone morphogenetic protein 6
BMPR2	Bone morphogenetic protein receptor type 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CEL	Carboxyl ester lipase
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP19A1	Aromatase
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked Immunoassay
ERK	Extracellular signal-regulated kinases
ESR1	Estrogen receptor alpha
ESR2	Estrogen receptor beta

F1	Largest follicle
FBS	Fetal bovine serum
FDR	False discovery rate
FF	Full fed
FSH	Follicle stimulating hormone
FSHb	Follicle stimulating hormone subunit beta
FSHR	Follicle stimulating hormone receptor
GH	Growth hormone
GHR	Growth hormone receptor
GIOT1	Gonadotropin inducible ovarian transcription factor 1
GnIH	Gonadotropin-inhibitory hormone
GnRH-I	Gonadotropin-releasing hormone
H&E	Haemotoxylin and Eosin
HPG	Hypothalamic–pituitary–gonadal
HSD3B1/3βHSD	3beta-hydroxysteroid dehydrogenase
IGF1	Insulin like growth factor 1
IGF1R	Insulin like growth factor 1 receptor
IGF2	Insulin like growth factor 2
IGFBP1	Insulin like growth factor binding protein 1
IGFBP2	Insulin like growth factor binding protein 2
IGFBP5	Insulin like growth factor binding protein 5
INHA	Inhibin subunit alpha
INHBA	Inhibin subunit beta A

INHBB	Inhibin subunit beta B
IPA	Ingenuity pathway analysis
IR	Insulin receptor
L:D	Light:dark
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LYF	Large yellow follicle
mRNA	messenger ribonucleic acid
NPL	Nonpersistent-laying
NR5A1	Nuclear receptor subfamily 5 group A member 1
NR5A2	Nuclear receptor subfamily 5 group A member 2
NT	Non-target
OCLN	Occludin
oFSH	Ovine follicle stimulating hormone
PCA	Principle component analysis
PCOS	Polycystic ovarian syndrome
PL	Persistent-laying
POF	Post-ovulatory follicle
pro-AMH	Anti-mullerian hormone precursor
RF	Restricted fed
rhIGF1	Recombinant human insulin like growth factor 1
RIA	Radioimmunoassay

RIN	RNA integrity number
RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Reverse transcription quantitative real-time PCR
siRNA	Small interfering RNA
STAR	Steroidogenic acute regulatory
SWF	Small white follicle
SYF	Small yellow follicle
T3	Triiodothyronine
T4	Thyroxine
TCM	Testis-conditioned media
TGFβ	Transforming growth factor β
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VLDL	Very low-density lipoprotein

CHAPTER 1

LITERATURE REVIEW

Breeding History of Chickens

Early archeological findings suggest that chickens have been domesticated since as early as 6000 BCE from the wild red junglefowl [1]. Up until the 1900s, domesticated chickens were raised for both egg and meat consumption. Specific breeds for meat (broiler breeds) and egg production (layer breeds) were established in the mid-1900s through intense genetic selection for fast growth and reproductive traits, respectively (reviewed in [2]). Today, broiler hens can reach 2 kg by 34 days of age [3], compared to the red junglefowl which weighs less than 2 kg even as an adult [2]. Laying hens are also considerably more efficient than their ancestors, laying 259 eggs on average by 60 weeks of age [4], compared to approximately 50 eggs annually (see below for broiler laying rates) [2]. Intense genetic selection has resulted in highly efficient and productive birds. In 2020, the poultry industry contributed $35.5 billion to the U.S. economy [5]. Broilers and egg production make up the majority, with broilers contributing $21.7 billion (59.4 billion pounds) and egg production contributing $8.66 billion (112 billion eggs produced) [5]. The remaining $5.2 billion was generated from turkey production and chicken meat (excluding broilers) [5].

Although broiler breeds are highly efficient and develop to market size by five weeks of age, intense selection for rapid growth has resulted in suboptimal reproduction in broiler hens. These hens have aberrant follicle growth, often leading to erratic laying, multiple ovulations, and poor-quality eggs [6]. To overcome reproductive inefficiencies

in broilers, producers maintain a parent stock of females for reproduction termed "broiler breeder hens". One strategy used to improve broiler breeder hen reproductive efficiency is restricted feeding, which results in an ovarian phenotype more like the laying hen's. Broiler breeder hens fed a restricted diet (RF) lay at a higher rate than those fed *ad libitum* (FF), although dietary change is insufficient to reach the egg-laying efficiency seen in layer breeds of hens (154 vs. 259 eggs by 60 weeks) [4,7]. While restricted feeding improves egg-laying, the mechanism by which feeding level affects ovarian function in broiler breeds is not fully understood. Understanding the etiology of ovarian dysfunction could identify potential remedies for this suboptimal reproduction. Moreover, commercial guidelines for restricted feeding increase producers' labor demands and raise welfare concerns for broiler hens.

Laying hens are extremely efficient at egg-laying, and selection for reproductive traits has resulted in hens that lay approximately one egg per day during the first year. The production of an egg from ovulation to oviposition takes approximately 24-28 hours. One way to increase egg-laying efficiency in these hens is by extending their high egg-laying rate past the first year, increasing the persistency of lay. Following the first year of lay, the flock average for egg production decreases [8]. Within this average are hens that continue to lay at a rate of one egg per day (100%) and those that decline to 80% or lower. The commercial goal of egg producers is to produce 500 eggs by 100 weeks of age per bird (reviewed in [9]), [10]. A few commercial farms have achieved this standard to date [11,12]. In the U.K., researchers have estimated that increasing egg production by only 25 eggs per bird could reduce the number of hens per year by 2.5 million [9]. Identifying early differences between persistent-laying (PL) and non-

persistent laying (NPL) hens may allow the development of candidates for genetic selection or tools to identify hens with persistent high egg production.

Domestication and selective breeding have resulted in efficient meat and egg-producing breeds of chickens. However, broiler hens have reproductive dysfunction, and laying breeds could be improved by extending the laying period. The work described in this thesis addresses aspects of ovarian physiology in broiler hens and in laying hens contributing to differences in egg-laying efficiency.

Reproduction in the Hen

Embryonic Development of the Female Reproductive System

Chicken embryonic development takes 21 days from fertilization to hatch and the left and right ovaries begin to develop in chickens early in embryonic development. Only the left ovary will grow and be maintained to adulthood and the right ovary will regress during development, leaving the mature hen with only one functional ovary and oviduct. The right and left oviducts develop at the same rate until embryonic day 10, after which the right oviduct regresses steadily until hatch [13]. In the female chicken embryo, anti-mullerian hormone (AMH) plays a role in the regression of the right oviduct. AMH content is highest in the female embryo when regression of the right Mullerian duct peaks [13]. Although AMH content is high in the female embryo, increased estrogen receptor expression protects the left ovary [14,15] and left oviduct from regression [16] and promotes its development by preventing AMH from binding to its receptor [17].

During the third and fourth days of embryonic development, primordial germ cells invade both ovaries, with significantly more entering the left ovary [18]. During days 9-15 of embryonic development, germ cells are present in nests [19]. The number of germ cells in the left ovary peaks at about 17 days of embryonic development, with an estimated 680,000 germ cells [19]. Near the end of embryonic development and early post-hatch life, oocytes become surrounded by pre-granulosa cells [20]. The germ cell population drastically decreases thereafter, and hens are hatched with a finite number of oocytes of approximately 480,000 [19]. Oocyte numbers slowly decrease throughout a hen's reproductive life as follicles are recruited and either undergo atresia or ovulation. There is currently no evidence suggesting that hens carry out oogenesis after hatch. By one week post-hatch, follicles consisting of an oocyte and a monolayer of granulosa cells can be seen in the left ovary, and by one month post-hatch, follicles with theca cells can be observed [15]. Estradiol promotes the activation of primordial follicles by increasing cell proliferation and ovarian estrogen receptor α mRNA expression in chicks [21].

Sexual Maturity

Hens typically reach sexual maturity at around 18-22 weeks of age, when the first egg is laid. Leading up to the first egg, several changes in estradiol, progesterone, and luteinizing hormone (LH) plasma concentrations occur. Before puberty, estradiol differentiates the oviduct and prepares it for egg production [22]. Estrogen is detectable in plasma from hatch and begins to rise from 6 weeks until it peaks approximately 2-3 weeks before the first egg [23]. Four weeks before the first egg is a rise in LH, which

subsequently decreases leading up to the first egg [24]. One week before the first egg, plasma progesterone concentrations rise [24]. Estrogens also influence secondary sex characteristics in the hen, such as reddening of the comb and wattles, which are observable just before egg-laying begins [25].

Adult Female Reproductive Anatomy

The adult female reproductive system consists of the hypothalamus, pituitary, ovary, and oviduct (Figure 1). The left ovary is developed and functional in the adult hen, while the right ovary is rudimentary, as described above. The oviduct comprises the infundibulum (cranial end), magnum, isthmus, and shell gland (caudal end). Portions of an egg are formed throughout the oviduct: the magnum secretes albumen, the isthmus contributes the outer and inner egg membranes, and the shell gland forms the shell of the egg [20]. During oviposition, the egg will leave the shell gland, enter the vagina, and exit through the cloaca.

The left ovary is composed of medullary tissue and cortical tissue in the adult ovary [26]. Early in follicle development, follicles are found in cortical tissue and project from the ovary on stalks as they develop [26,27]. The oocyte is surrounded by the perivitelline membrane, a structure analogous to the zona pellucida of mammals [28]. Yolk, produced by the liver, is incorporated into the oocyte of the follicle at different stages of development as very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) particles [28]. Surrounding the oocyte is a single layer of granulosa cells that can appear columnar, pseudostratified columnar, or polyhedral [26]. A collagenous basement membrane surrounds the granulosa cell layer and separates it

from the theca layer, which can be further divided into theca interna and theca externa cells [26]. The theca layer is vascularized and provides the follicle access to the blood supply (reviewed in [27]).

Folliculogenesis is a tightly regulated process, and follicles can be categorized by their developmental state. Primordial follicles are in a resting state before being activated at <0.05 mm [21]. Primordial follicles have a single layer of pre-granulosa cells [27]. Upon activation, follicles develop granulosa cells and recruit theca cells [27]. Follicles continue to grow through the prehierarchal stage (< 8 mm) as they begin to accumulate yolk [27]. Growing follicles can be further subdivided by size and color: 1-5 mm (small white follicles, SWF), 6-8 mm (small yellow follicles, SYF), and >9 mm follicles (large yellow follicles, LYF) [27]. Small prehierarchal follicles (1-5 mm) contain white yolk rich in lipoproteins and appear white in color, and large prehierarchal follicles (6-8 mm) have begun to accumulate yolk and appear yellow [27] (Figure 1).

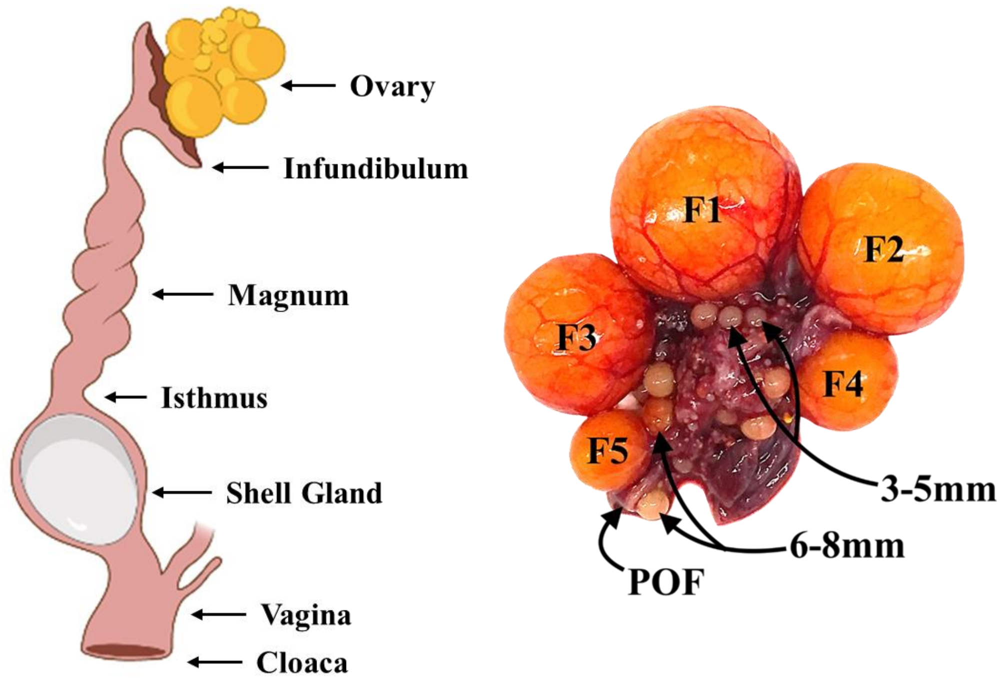


Figure 1. Chicken reproductive tract (left) and ovary (right) from a laying hen. The hen reproductive tract consists of the ovary, infundibulum, magnum, isthmus, shell gland, vagina, and cloaca. 3-5 mm follicles appear white in color, and 6-8 mm follicles appear yellow. Preovulatory follicles are designated as F1-F5, where the F1 is the next follicle to ovulate, and the F2 will ovulate on the following day. The postovulatory follicle (POF) is the remnant of the follicle following ovulation (Created with BioRender.com).

Follicle selection is thought to occur at the 6-8 mm follicle stage [29,30], and it is from this group that one follicle enters the preovulatory or hierarchal stage. Preovulatory follicles are those above 9 mm in diameter and are in a stage of fast growth and rapid yolk accumulation. Preovulatory follicles are described according to their order in the hierarchy, with the largest termed the F1 follicle. Depending on the number of follicles in the cohort, the smallest is labeled the F5 or F6. Ovulation occurs from the F1 follicle, and the F2 is the next follicle to ovulate, typically the following day in an efficient egg-laying hen. In a reproductively efficient hen, one 6-8 mm follicle is selected each day to replenish the preovulatory follicle pool after daily ovulation. It takes approximately 3 days for a follicle to grow from 3 to 5 mm, 2 days to grow from 5 to 8 mm, and 6 days for an 8 mm follicle to reach ovulation [31].

In contrast to mammals, a postovulatory follicle (POF), rather than a corpus luteum, forms following ovulation. The POF produces low amounts of steroid hormones [32,33] and influences the timing of oviposition [34]. Approximately 4-6 days after ovulation, the POF regresses [35] via caspase-mediated apoptosis [36].

Atresia is the degeneration of a follicle through apoptosis. In follicles >1 mm, an atretic follicle has less turgor, appears deformed, and may have hemorrhages on the follicular surface [31]. In laying hens, atresia in follicles >8 mm is rare [31]. Atresia in the larger follicles is likely unfavorable due to the considerable energy and resource demand involved in yolk accumulation. In contrast, in mammalian folliculogenesis, several follicles grow in each wave, and only one is selected to become dominant per ovarian cycle, while the subordinate follicles undergo atresia (reviewed in [37]).

Ovarian Cycle and Hypothalamic-pituitary-gonadal axis

An ovulatory cycle spans from the ovulation to the oviposition of an egg, and hens have a cycle approximately every 24-28 hours. A sequence or clutch consists of consecutive daily ovulations, followed by a pause/skip day when ovulation does not occur. Birds are long-day breeders, allowing birds in the wild to raise their young in an optimal environment with sufficient resources. Commercial settings take advantage of this and rear hens under long-day photostimulation (16L:8D) to maintain high laying rates. Exposure to long days promotes gonadotropin-releasing hormone (GnRH-I) release, increasing gonadotropins and promoting ovarian function (reviewed in [38]).

The ovarian cycle of hens is driven by a series of endocrine events in the hypothalamic-pituitary-gonadal (HPG) axis. In chickens, GnRH-I from the hypothalamus stimulates LH release from the pituitary [39]. GnRH-I does not directly affect follicle-stimulating hormone (FSH) levels [40] and may instead modulate FSH indirectly through activins and inhibins. Gonadotropin-inhibitory hormone (GnIH) was also identified in birds, and GnIH treatment of cultured anterior pituitary glands decreases LH [41,42] and FSH release [42], gonadotropin α-subunit [42,43], and FSHβ-subunit [42]. Incubating hens, out of lay, have lower GnRH-I and higher GnIH than laying hens [42].

As in mammals, ovulation occurs in response to an LH surge, although a rise in progesterone [44], not estradiol, triggers the LH surge. The largest follicle of the hierarchy produces the most progesterone [45] and stimulates the LH surge through positive feedback [44]. The progesterone surge occurs approximately 4-6 hours before ovulation, followed by the LH surge [44,46]. Although estrogen does not cause the

preovulatory LH surge in birds, estrogen peaks 4-6 hours ahead of ovulation [47]. Estrogen is thought to prime the hypothalamus for ovulation as ovariectomized hens not treated with estrogen failed to produce an LH surge [48].

Steroidogenesis

One of the main functions of the hen ovary is to produce steroid hormones, including estradiol, progesterone, and testosterone. Steroid hormones are derived from cholesterol first transported into the inner mitochondria by steroidogenic acute regulatory protein (STAR). Cholesterol is then converted to pregnenolone by Cytochrome P450scc (CYP11A1). Pregnenolone can be further converted to androstenedione through either the Δ^4 or Δ^5 steroidogenic pathway (reviewed in [49]).

Steroidogenesis in the hen depends on a three-cell model, consisting of granulosa cells, theca interna, and theca externa, as was proposed by Nitta *et al.* [50]. Granulosa cells primarily produce progesterone and testosterone, while theca cells produce estradiol and testosterone [51]. Theca cells require progesterone from granulosa cells to produce testosterone [51], and only the theca externa cells express aromatase to convert testosterone to estradiol [50]. In the hen, steroidogenesis is accomplished primarily through the Δ^5 pathway in theca cells and the Δ^4 pathway in granulosa cells [49]. In contrast, mammalian steroidogenesis works through a two-cell model involving theca interna and granulosa cells [52].

Steroidogenesis in the hen ovary is regulated by gonadotropins, FSH and LH. Both FSH and LH act through their respective G-protein coupled receptors, follicle-stimulating hormone receptor (FSHR), and luteinizing hormone receptor (LHR). FSH

and LH stimulate their second messenger, cyclic AMP (cAMP), in the theca and granulosa cells of follicles [53]. In preovulatory follicles, FSH-stimulated cAMP production decreases, and LH-stimulated cAMP production increases as follicles progress through the hierarchy [53]. In contrast to steroidogenesis in mammalian follicles, the small follicles (<5 mm) of birds produce the most estrogen [54]. Small follicles are unable to produce progesterone [55] due to a lack of STAR [56] and CYP11A1 [57] in the granulosa cell layer. Granulosa cells of preovulatory follicles produce progesterone in response to both FSH and LH [51,53]. Progesterone production in response to LH increases with increasing follicle size, with the F1 producing the most progesterone [51] and granulosa cells of the F1 produce increasing amounts of progesterone as the time to ovulation approaches [32].

Molting

Molting occurs in domestic birds approximately one year after egg-laying initiation, and results in the replacement of feathers. During this time, the left ovary and oviduct regress, and hens stop laying. During molting there are significant decreases in plasma estradiol, progesterone, and LH and significant increases in plasma T3 and T4 compared to hens in lay [58]. Molting can be induced by restricting food, water, or light or by injecting progesterone [59]. Hens that undergo forced molts have increased egg-laying rates upon return to lay [60], and molting can be used to increase the length of lay. Although forced molts increase the length of lay, egg production ceases for approximately 4-6weeks during the molt.

Aging

As hens age, egg production decreases [61]. Older hens have fewer follicles, and the incidence of atresia increases with age [62]. This may result in reduced egg production because fewer follicles mature to ovulation. Older hens also lay larger eggs due to more yolk accumulating into fewer follicles, and the diameter of the F1 also increases with age [61]. Hens exposed to natural seasonal photostimulation begin to decrease egg production after 4 years [63]. In commercially reared hens, continuous long-day photostimulation may deplete ovarian reserves prematurely compared to hens exposed to regular seasonal photostimulation that are not cycling daily.

Ovulation is also less efficient in older hens. At the HPG axis, older hens have lower plasma LH levels when injected with GnRH or progesterone than younger hens [8]. F1 and F2 follicles from older hens are also less responsive to LH and produce less cAMP when LH-stimulated [64]. Basal plasma progesterone and estradiol levels do not differ in young and old hens [64]. Plasma progesterone concentrations during the preovulatory progesterone surge have not been compared between the two ages.

One concern with increasing the length of lay or persistency in hens is the increase in age-related health issues. Laying hens are one of the only known animals to spontaneously develop ovarian cancer, other than humans. Hens have a 30-35% chance of developing ovarian cancer after 2.5 years [26]. Hens from a mutant strain with decreased ovulation [65] and hens given progestin and estrogen to mimic combination oral contraceptives have a lower cancer incidence [66]. Another concern is the risk for osteoporosis in older hens [67]. The eggshell contains 2.2 g of calcium, of which 2/3 is acquired from the diet and 1/3 from the medullary bone of the hen (reviewed in [9,69]).

Estrogen and progesterone stimulate the gut to absorb calcium from the diet (reviewed in [9]). A recent study investigating the correlations between bone density and egg production from 62-64 weeks of age found no evidence that increased egg production leads to bone loss [68], though this has not been studied in older birds.

Follicle Selection

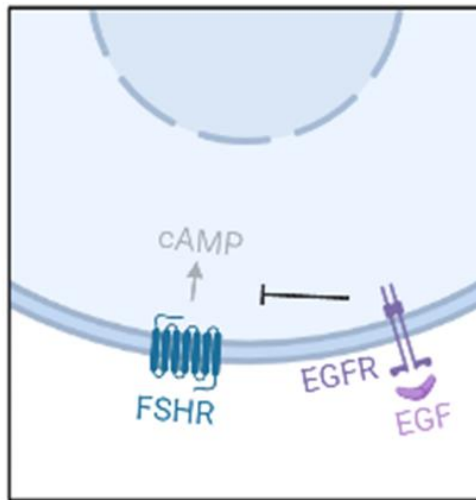
Folliculogenesis is a highly regulated process. One key event in folliculogenesis is follicle selection. Follicle selection is the event where one follicle from a pool of growing follicles is selected to become dominant and continue maturation until it ovulates [70]. In monovulatory species, several follicles grow per cycle in waves, and only one is selected to reach dominance, while subordinate follicles undergo atresia [37]. In the hen, follicle selection is thought to occur daily at the 6-8 mm stage [70]. Follicle selection is characterized by two critical events: granulosa cell differentiation and steroidogenic competency [71].

Before selection, granulosa cells are said to be undifferentiated and steroidogenically incompetent [72]. Undifferentiated granulosa cells express low levels of STAR, LHR, CYP11A1, and FSHR ([56,73–75], and reviewed in [27]). Low expression of STAR and CYP11A1 make these granulosa cells unable to produce progesterone from cholesterol; however, the addition of pregnenolone increases progesterone production, suggesting these cells express 3βHSD [28]. Although granulosa cells at this stage express FSHR, there is no increase of cAMP or progesterone production in response to FSH [29].

At the time of follicle selection, one follicle from the 6-8 mm pool expresses significantly more FSHR, and interestingly, this is not always the largest of the group [73]. The remaining unselected follicles from the 6-8 mm cohort remain in an undifferentiated state rather than undergoing atresia and are selected in subsequent cycles. In response to FSH, mRNA expression of STAR [73,76], CYP11A1 [57], and LHR [56,75] increases in granulosa cells. With increased STAR and CYP11A1 mRNA expression (Figure 2), granulosa cells gain steroidogenic competency and initiate progesterone production [29]. After selection, granulosa cells transition from FSH to LH dependence [53], and FSHR decreases [77]. LH signaling further increases STAR mRNA expression [56], and production of progesterone increases as follicles develop and approach ovulation [51].

Recently, the transcriptomes of granulosa cells from 6 mm and preovulatory (F5) follicles were sequenced to investigate differences around the time of follicle selection [79]. This study revealed 963 differentially expressed genes between granulosa cells before and after follicle selection, depicting a large shift in transcriptomes during the transition of follicle selection [79]. Among genes upregulated in granulosa cells of the selected follicle were genes involved in steroidogenesis (NR5A2, STAR, CYP11A1) and genes involved in FSH regulation (INHA, INHBB) [79].

Undifferentiated **Differentiated**



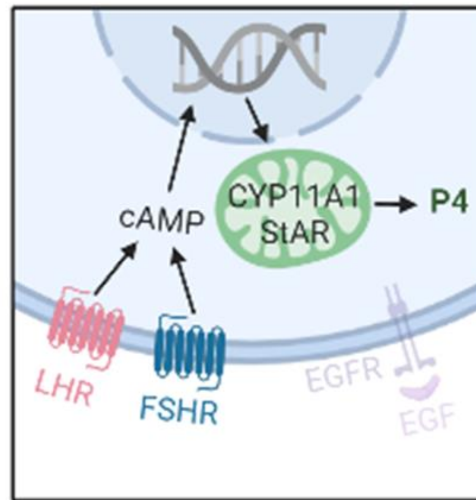


Figure 2. Schematic of undifferentiated and differentiated granulosa cells. Adapted Johnson and Woods 2015 [78], (Created with BioRender.com).

Regulation of Follicle Development

Several factors regulate follicle development, including FSH, LH, growth factors (IGF1), epidermal growth factor (EGF), and factors from the TGFβ family (ex: AMH, BMPs, inhibins, activins). As mentioned above, FSH and LH play a significant role in regulating steroidogenesis. The oocyte, through BMP15, likely also has a role in follicle selection as it increases FSHR and decreases tight-junction protein to allow yolk uptake (occludin, OCLN) and decreases AMH in cultured granulosa cells of 3-5 mm and 6-8 mm follicles [80]. EGF, a key regulator in prehierarchal follicles, restricts granulosa cell differentiation by keeping CYP11A1 and FSHR expression low [81]. EGF also prevents FSH-stimulated effects through EGF-receptor and PKC signaling and decreases LHR mRNA expression, preventing granulosa cell-differentiation [82,83].

Inhibin and Activin

Inhibins and activins regulate FSH at the pituitary level, with inhibins having an inhibitory role and activins having a stimulatory role. Inhibins are a combination of an alpha subunit and a β_A or β_B subunit. The alpha subunit dimerizes with a β_A subunit to make inhibin A (α-β_A) and with the β_B subunit to make inhibin B (α-β_B). Activin lacks the alpha subunit and is composed of only beta subunits. Activin A is a homodimer of β_A subunits (β_A-β_A), Activin B is a homodimer of β_B subunits (β_B-β_B), and Activin AB is a heterodimer of β_A and β_B subunits (β_A-β_B). Inhibin B is produced primarily by small follicles of hens, and inhibin A is produced by preovulatory follicles [84]. Inhibin B is secreted at low levels in follicles <4 mm and subsequently increases before peaking in

7-9 mm follicles [84]. Secretion then decreases to low to undetectable levels in preovulatory follicles [84]. Inhibin A begins to be produced in 9 mm follicles and rises to its peak level in the F1 follicle [84].

The expression of inhibin and activin mRNA subunits has previously been investigated at different stages of follicle development. Inhibin α subunit begins to be expressed at the 5-12 mm follicle stage [85]. Expression peaks in the F5 follicle and then gradually declines [85]. Inhibin/activin β_A subunit expression is not detectable until the preovulatory stage of follicle development and is most highly expressed at the F1 stage [85]. In contrast, the inhibin/activin β_B subunit begins to be expressed at the 5-12 mm follicle stage, where it is at its highest expression, and expression declines until it is no longer detectable, starting at the F4 follicle stage [85].

IGF1

Chicken insulin-like growth factor 1 (IGF1) is a 7 kDa protein of 70 amino acids and is primarily secreted by the liver (reviewed in [86]). IGF1 and IGF2 are also expressed and produced in the ovary at a lower level than in the liver [87,88]. In the ovary, IGF1 and IGF2 mRNA are expressed in the theca and granulosa cell layers of preovulatory and prehierarchal follicles [87,88]. In culture, IGF1 is secreted by granulosa cells of 3-10 mm follicles and preovulatory follicles (F1-F4) [87,89]. Local production of IGF1 suggests that it may act in a paracrine or autocrine action in the ovary [89].

A cell surface receptor, insulin-like growth factor type 1 receptor (IGF1R), is present in both the theca and granulosa cells of preovulatory and prehierarchal follicles

and is highest in the granulosa cell layer [88,90,91]. Its expression increases with increasing follicle size [88,90]. As in mammals, chickens have IGF1 and IGF2 receptors; however, IGF2 does not bind to the IGF2 receptor and instead acts through IGF1R binding [92,93]. IGF1 was found to have the highest affinity for IGF1R, followed by IGF2 and insulin [91]. Upon ligand binding, IGF1 phosphorylates IGF1R beta subunit, ERK, and Akt in granulosa cells of preovulatory follicles [90].

Binding proteins primarily regulate IGF1 bioavailability. In mammals, there are six IGF1 binding proteins (IGFBP 1,2,3,4,5, and 6) (reviewed in [86,87]). IGF1 binding proteins differ by species, and in chickens, seven IGF1 binding proteins have been annotated in the Galgal6 chicken genome assembly (IGFBP 1,2,3,4,5, 6, and 7), and two (IGFBP2 and 5) have been characterized [94,95]. IGFBP2 and 5 are expressed in chicken ovarian tissue, suggesting that binding proteins likely regulate locally produced IGF1 and IGF2 availability [87].

IGF1 plays a significant role in mammalian follicle development, as evidenced by IGF1 and IGF1R knockout mouse models. IGF1 global knockout mice are infertile and have reduced ovarian volumes [96]. While small follicles are present in IGF1 knockout mice, preovulatory follicles are rare compared to wild-type mice [96]. These mice also have reduced basal and estradiol-stimulated cell proliferation [97]. IGF1R granulosa cell knockout mice are sterile, and follicle development is arrested at the secondary follicle stage [98]. These mice also have decreased STAR, CYP11A1, LHR, INHA, INHBA, and INHBB expression [98]. This implies that IGF1 is essential for follicle function in mice. IGF1 treatment in cultured bovine granulosa cells increases

cell number, estrogen production, and expression of FSHR, CYP11A1, CYP19A1, and 3beta-hydroxysteroid dehydrogenase (HSD3B1/3βHSD) [99].

The function of IGF1 in chicken follicles has not been studied as extensively as in mammals. In theca and granulosa cells of preovulatory follicles, IGF1 increases cell proliferation [90,100,101]. IGF1 treatment of granulosa cells from preovulatory follicles increases STAR, CYP11A1, 3βHSD protein expression, INHA mRNA [102], and progesterone and inhibin A production [90,100,101,103]. In cultured theca cells of preovulatory follicles, IGF1 inhibits basal and LH-stimulated estrogen production [101].

A few studies have investigated the effect of IGF1 on follicle function in hens at the prehierarchal stage, yet its role is not fully understood, and some results are contradictory. In whole cultured prehierarchal follicles (1-4 mm and 6-8 mm), IGF1 has been shown to increase estradiol production in culture, and the addition of growth hormone (GH) did not provide an additive effect on estradiol [104]. This is in contrast to results that indicated that IGF1 inhibited basal and LH stimulated estrogen production in cultured theca cells of prehierarchal follicles [101] and unpublished research from our lab (Francoeur and Johnson), showing no effect on estradiol production in IGF1-treated whole 1 mm and 3 mm follicles. The effect of IGF1 on estrogen production in prehierarchal follicles of the hen remains unclear.

The effect of IGF1 on markers of follicle selection in prehierarchal follicles has had limited attention. IGF1 treatment in cultured granulosa cells of 6-8 mm follicles has not been shown to increase the mRNA expression of genes important for follicle selection (STAR, CYP11A1, or LHR) or affect progesterone production [56,105].

Interestingly, IGF1 or FSH treatment of granulosa cells of 6-8 mm follicles did not affect FSHR expression; however, co-treatment of IGF1 and FSH significantly increased FSHR expression [106]. One study found that treatment with IGF1 in granulosa cells of prehierarchal follicles (3-10 mm) increased cell proliferation, as observed in preovulatory follicles [89]. While this is interesting, the large range of follicles used in the prehierarchal pool for this study makes interpretation difficult. Granulosa cells from follicles in the upper range of this pool are likely already differentiated and may not be representative of prehierarchal follicles. Limited research has been done on the role of IGF1 in granulosa cells of small prehierarchal follicles (<6-8 mm).

In mammalian granulosa cells from antral follicles, IGF1 and FSH have been shown to act synergistically to increase IGF1R [99], STAR [107], CYP19A1 [99] and CYP11A1 expression [108,109], aromatase activity [110], and production of progesterone [108] and estrogen [110]. In the hen, IGF1 has additive effects on INHA mRNA expression [102] and inhibin A production with LH or FSH in granulosa cells of preovulatory follicles [103]. IGF1 also has an additive effect on progesterone and inhibin A production in the presence of either LH or FSH [100,103].

AMH

Anti-mullerian hormone (AMH), a TGFβ-like homodimer glycoprotein, was first discovered in the 1940s by Jost [111]. Initially, AMH was found to cause the regression of Mullerian ducts in male embryos and was aptly named for its function [111]. Since its discovery, it has been shown to be essential in regulating follicle

development in adult female ovaries. In mammalian and avian species, AMH is expressed in the granulosa cell layer of growing follicles [112,113]. AMH is transcribed from the AMH gene, which codes for pro-AMH. Once translated, pro-AMH is then cleaved to its biologically active form, $AMH_{N,C}$ [114]. Like many TGF-β factors, AMH binds to its specific heterodimeric receptor, AMHR2, and then phosphorylates a nonspecific type I receptor. Downstream phosphorylation of SMAD proteins produces transcriptional changes [115].

The AMH knock out (AMHKO) mouse model has been influential in discerning the role of AMH in ovarian function. AMH null mice, although fertile, show an early depletion of their ovarian follicle pool [116] and are more sensitive to FSH [117]. AMHKO mice have more large preantral and small antral follicles and have increased follicle numbers when stimulated by FSH compared to wild-type mice [117]. In mammals, AMH treatment prevents primordial follicle activation [116], decreases FSH sensitivity [117], and FSH-induced steroidogenesis [118,119]. In cultured granulosa cells, AMH inhibits FSH-induced estradiol production [118,119], intracellular cAMP accumulation [118] and expression of FSHR [120], aromatase [118,120,121], STAR [119], and CYP11A1 [121]. AMH administration in mice decreases STAR, LHR, and aromatase expression and estradiol levels [122]. In cultured human granulosa-lutein cells, AMH blocked the increase of CYP19A1 [121,123], CYP11A1 [121], and estradiol by FSH [119,123]. AMH also inhibited CYP19A1 and CYP11A1 mRNA expression by LH [121].

AMH has been a valuable diagnostic tool in clinical settings. In women, low levels of AMH are observed in women with premature ovarian insufficiency [124,125]

and high levels in women with polycystic ovarian syndrome (PCOS) [126]. AMH levels are inversely associated with aging and are used in a clinical setting to estimate the ovarian reserve [127]. AMH has not been studied in aging hens, but it may be involved in decreased egg-laying associated with aging.

In the hen, AMH expression is highest in small follicles and decreases with follicle development until the time of selection [128]. While little is known about the role of AMH in the chicken, its expression pattern suggests that it plays a similar role in chicken and mammalian folliculogenesis. AMHR2 is most highly expressed in the oocytes of hens [129]. A current obstacle in the field is a lack of commercially available bioactive AMH. Chicken AMH is biologically active in mammals, as demonstrated by the regression of the rat Mullerian ducts when cultured with chick embryonic testes; however, mammalian AMH is not active in chickens [130]. Due to the high AMH content [13,131], we have previously used testis-conditioned media (TCM) from male chicken embryos as a source of chicken AMH [132]. Treatment with TCM in granulosa cells from 3-5 mm and 6-8 mm follicles increases cell proliferation, and this effect can be blocked by treatment with AMH antibody [132].

A recent paper reported results using chicken AMH from a prokaryotic expression system to investigate the role of AMH in prehierarchal follicles [133]. Granulosa cells from 6-8 mm follicles treated with AMH showed a decrease in CYP11A1, STAR, and HSD3B1 mRNA expression at most doses and a reduction in progesterone production [133]. The recombinant protein produced for this paper was not validated or described, and it is unclear which portion of AMH was made. More research is needed to understand the role of AMH on steroidogenesis in the hen.

AMH mRNA expression is regulated by several factors in chicken granulosa cells. In chickens, oocyte-conditioned media [128], BMP15 [134], and Vitamin D3 [135] decrease expression, TGFβ + FSH and BMP6 [80] increase expression, and estradiol and progesterone [128] have no effect on AMH expression. BMP6 and AMH mRNA are both highly expressed in 1-2 mm follicles [80]. BMP6 likely functions to keep AMH expression high in small follicles to regulate follicle development and prevent early recruitment of small follicles [80].

One known regulator of AMH in mammals is FSH [136–142]. FSH has been found to have different effects on AMH depending on experimental design and species. *In vitro*, FSH treatment decreases AMH mRNA expression and AMH secretion in bovine granulosa cells [136,137], increases expression in human granulosa-luteal cells [138], and has no effect on expression in ovine granulosa cells [137]. FSH treatment *in vivo* decreases plasma AMH levels in goats and humans [139,140] and increases concentrations in cows [142]. FSH beta subunit (FSHb) knockout mice have higher levels of AMH, and the introduction of a human FSHb transgene causes a decrease in ovarian AMH expression [141], suggesting that FSH decreases AMH. *In vivo* FSH treatment of laying hens with low egg production increased AMH mRNA and AMHR2 mRNA in SWF but had no effect on plasma AMH levels [143]. As stated by the authors, increased AMH expression following FSH treatment in hens may be a mechanism that decreases FSH sensitivity [143]. More research is needed to understand how AMH is regulated in other follicle stages.

There is growing evidence that AMH plays a vital role in influencing the HPG axis [144–146]. Recently, AMHR2 protein has been shown to be expressed in the

hypothalamus of heifer brains, which suggests that AMH influences the HPG axis [144]. In mice, AMH treatment increased GnRH neuron firing, and intracerebroventricular administration of AMH increased LH secretion [145]. Furthermore, one study in geese showed that autoimmunization against AMH increased FSHb mRNA expression [146], suggesting AMH influences the HPG axis in avian species as well. Based on these studies, it is likely that AMHR2 mRNA may be expressed in the hypothalamus of chickens [144–146]. AMH levels seen in hens could regulate the HPG axis by signaling in the hypothalamus. Understanding the relationship between AMH levels and changes in the HPG axis may explain ovarian phenotypes such as those seen in FF broiler breeder hens.

Broiler Breeder Hen Reproduction

Broiler breeds have been selected for high feed efficiency and fast growth, but the intense genetic selection has negatively impacted reproduction in these hens. Broiler breeder hens display reproductive deficiencies when fed *ad libitum*, including erratic laying, egg defects [147], internal ovulation [148], and multiple ovulations [6]. In comparison, commercially reared laying hens are provided with *ad libitum* feed and are efficient egg layers [4]. *Ad libitum*-fed broiler breeder hens, however, are not able to regulate feed intake, leading to overfeeding and weight gain [149]. To improve egg production, producers restrict the amount of feed given to broiler breeder hens [7]. Restricted feeding in broiler breeder hens reduces the incidence of reproductive defects and improves egg production. In broiler breeder hens, sexual maturity is also influenced

by the feeding level [6,150,151]. Feed restriction during rearing significantly delays oviduct development and the onset of sexual maturity by 4-5 weeks [6,150,151].

At the ovarian level, broiler breeder hens fed *ad libitum* have significantly more preovulatory follicles [6,43,152,153] arranged in multiple hierarchies [154,155]. Restricted feeding decreases the number of large follicles and atresia [148,149]. More than one 6-8 mm follicle is likely selected into the preovulatory pool daily from the multiple hierarchies of FF hens [154,155]. This increased selection may be attenuated by restricted feeding, as evidenced by the decrease in preovulatory follicles [6,43,152,153]. The impact of increased feeding on prehierarchal follicle numbers is variable, with some studies finding increased numbers [152], lower numbers [149], and others finding no differences [43,156].

Ad libitum feeding also affects the HPG axis in broiler breeder hens. *Ad libitum* feeding increases GnRH-I and gonadotropin α-subunit mRNA expression but does not affect GnIH mRNA expression [43]. The effect of feeding in broiler breeder hens on plasma gonadotropin and steroid hormones is variable. Plasma LH levels increase in response to increased feed intake [43,150], although, this is dependent on strain [150]. Another study found no difference in plasma LH levels in FF and RF hens [157]. Dietary intake effect on plasma FSH levels is unclear as some studies find no difference [155], elevated levels [150,158,159], or decreased plasma levels in FF compared to RF hens [43]. Some studies have found either lower plasma estradiol [150,160] or no difference in plasma estradiol levels [155,157]. Plasma testosterone increases in response to increased feeding in broiler breeder hens [160], and plasma progesterone levels do not seem to be affected by feeding levels [157,160]. Studies differ in their

experimental design, and some begin feeding treatment before the onset of sexual maturity. *Ad libitum* feeding accelerates the onset of egg-laying, and therefore, FF and RF hens in these studies may be at different points in their reproductive lifetime. To our knowledge, there is currently no research outlining a thorough endocrine profile for FF and RF hens treated after the onset of lay.

Feeding levels also affect follicle function in broiler breeder hens. Preovulatory follicles from broiler breeder hens produce abnormal steroid hormone levels, specifically in the two largest preovulatory follicles, signifying irregular development [161]. Granulosa cells of preovulatory follicles also respond differently to LH in FF and RF hens [148]. In FF hens, granulosa cells of the three largest follicles produce similar levels of progesterone when treated with LH, yet restricted feeding establishes more of a hierarchy with progesterone production increasing with follicle size [148]. LH and progesterone surges have previously been measured, and no difference was observed in the amplitude or length of the surge in FF or RF hens *in vivo* [157]. *In vitro* work suggested that multiple preovulatory follicles respond to LH and produce high levels of progesterone in FF hens, which results in multiple ovulations and decreased egg production. In the *in vivo* study, researchers sampled blood over 36 hours and captured one double ovulation in FF hens during this time, but not the LH or progesterone surge associated with these ovulations [157]. More data are needed to assess preovulatory surges associated with double ovulations.

Differences in follicle development markers important for selection have not yet been studied in broiler breeder hens in response to dietary treatment. Dietary intake likely affects follicle selection, however, based on the observation of multiple

hierarchies [154,155]. *Ad libitum* feeding in broiler breeder hens also disrupts follicle function as the largest two follicles produce similar amounts of progesterone [161].

In addition to reproductive deficiencies, broiler breeder hens fed *ad libitum* have increased plasma glucose, nonesterified fatty acids, triacylglycerol, cholesterol, insulin, and leptin compared to those fed restricted diets [149,152,160]. Overfed broiler breeder hens have increased body weights, liver weights, and abdominal fat weight [149,150,152]. The link between metabolic and reproductive concerns in these hens is not fully understood.

One plausible connection between dietary intake and reproductive function is IGF1. In adult broiler breeder hens, IGF1 mRNA is more highly expressed in the livers of FF compared to RF hens [152]. IGF1 binding protein mRNA expression (IGFBP1 and IGFBP2) is also highest in RF hen livers, suggesting more bioavailable IGF1 in FF hens [152]. In contrast to these findings, it was reported that immature hens coming into lay had higher plasma IGF1 levels in the restricted-fed state compared to *ad libitum*-fed broiler breeder hens [162]. The different findings from these studies may be attributed to differences in experimental design as one study investigated IGF1 in adult hens treated after sexual maturity, while the second study reported IGF1 results from young hens. To our knowledge, no work has been done to compare plasma IGF1 levels in restricted and *ad libitum*-fed broiler breeder hens following sexual maturity.

Genes involved in the IGF1 system have also been investigated in prepubertal growing broiler breeder hens. Ovarian mRNA expression of genes involved in the IGF1 system (IGF1, IGF2, IGFR, IGFBP2, IGFBP5, GHR, or IR) did not differ between FF and RF hens [163]. One significant limitation is that only 2 samples, each representing 3

hens, were analyzed per group. The small sample size is likely insufficient to capture these birds' physiology. Additionally, evaluating the ovary before puberty may not be the best reflection of the effect of the IGF1 system on reproduction in broiler breeder hens.

Progesterone production in response to *in vitro* IGF1 treatment in granulosa cells of preovulatory follicles differs in broiler breeder hens fed at different levels. Cultured granulosa cells of the F1, F2, and F3 preovulatory follicles from FF hens produce similar amounts of progesterone when treated with IGF1, although this effect varies by strain [148]. However, in restricted-fed hens, IGF1 increases progesterone production with increasing follicle size [148]. Furthermore, the addition of LH or FSH in cultured granulosa cells of preovulatory follicles in RF hens further increased progesterone production when co-treated with IGF1 [148]. There was, however, no additive effect on progesterone production in cells of FF hens cotreated with IGF1 and LH or FSH [148]. It is plausible that increased IGF1 availability in hens provided with *ad libitum* feed [152] may desensitize granulosa cells to IGF1, making them less responsive than those in restricted-fed hens.

Interestingly, the phenotype of FF hens also shares similarities with women diagnosed with PCOS, including increased follicle development, irregular ovulation, anovulatory cycles, metabolic syndrome, and obesity [164]. Women with PCOS also have increased AMH levels [126]. Previous research from our lab has found elevated ovarian AMH mRNA levels in granulosa cells from prehierarchal follicles in broiler breeder hens compared to layer hens [132]. Increased AMH mRNA expression is

further intensified by *ad libitum* feeding [132]. AMH is an essential regulator of follicle development; however, its role in the FF hen phenotype has not been investigated.

Metformin is widely used to treat insulin resistance, and in women with PCOS, it improves reproductive outcomes [165]. In RF hens, metformin treatment improves egg production, decreases the number of preovulatory follicles, decreases body weight and adiposity, and decreases testosterone levels [166]. Interestingly, metformin treatment of granulosa cells from 3-5, 6-8, and 9-12 mm follicles decreases FSH-induced FSHR, STAR, and CYP11A1 mRNA expression [167]. Metformin also decreases FSH-induced progesterone production in granulosa cells of 6-8 mm, 9-12 mm, and F5 follicles [167]. These results suggest that reproductive insufficiencies in the hen may stem from metabolic issues.

The link between feeding level and reproductive efficiency in broiler breeder hens is not fully understood. IGF1 is a known regulator of follicle development in mammals, and increased IGF1 availability in response to increased feeding might affect follicle development in FF hens. IGF1 has been studied in preovulatory follicles, although limited attention has been given to prehierarchal follicles in the hen. A greater understanding of IGF1 function in prehierarchal follicles and its role in follicle selection could provide insight into the reproductive inefficiencies observed in FF hens with more preovulatory follicles [43,152,153] and increased IGF1 [152].

Summary

Follicle development is a highly regulated process in hens and is essential for optimal egg production. The work presented in this dissertation summarizes differences

in hens with varying egg-laying efficiencies in two breeds of hens: broilers and layers. Hens have been selected to be highly efficient meat (broiler) or egg producers (layer). Broiler breeder hens face reproductive disruptions when fed *ad libitum*, compared to laying hens which can regulate feed intake and are reproductively efficient when fed *ad libitum*. Restricted feeding improves the ovarian phenotype in broiler breeder hens to resemble the ovarian phenotype of laying hens more closely. Follicle selection is likely disrupted by *ad libitum* feeding as these hens have increased numbers of preovulatory follicles. Laying hens differ in egg-laying efficiencies later in life; however, not much is known about the physiology underlying these differences.

In Chapter 2, we investigated transcriptional differences in granulosa cells of 6-8 mm follicles (the stage from which selection is thought to occur) in broiler breeder hens fed at different levels. We hypothesized that genes, such as STAR or CYP11A1 which contribute to follicle selection, might be disrupted in hens fed *ad libitum*. In Chapters 3 and 5, we investigated the role of IGF1 and AMH, respectively, in prehierarchal follicles. Both factors are differentially expressed in broiler breeder hens fed at different levels; however, not much is known about their function in chicken follicles. In Chapter 4, we identified differences between hens exhibiting variable persistency in egg-laying rates.

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CHAPTER 2

AD LIBITUM FEEDING IN BROILER BREEDER HENS ALTERS THE TRANSCRIPTOME OF GRANULOSA CELLS OF PREHIERARCHAL FOLLICLES[ab]

Abstract

Intense selective breeding of chickens has resulted in suboptimal egg production in broiler breeder hens. This reproductive phenotype is exacerbated by *ad libitum* feeding, which leads to excessive and disorganized follicular growth. One strategy used to improve broiler breeder hens' reproductive efficiency is restricted feeding. In this study, we sought to identify transcriptional changes, which translate the level of dietary intake into increased follicle selection. Broiler breeder hens (n = 16 per group) were raised according to commercial guidelines until 28 weeks of age and then randomly assigned to an *ad libitum* diet (FF) or continued on a restricted diet (RF) for 6 weeks. Following dietary treatment, FF hens (n = 2) with excessive follicle selection and RF hens (n = 3) with normal follicle selection were selected for RNA-sequencing. Transcriptomes of granulosa cells from 6–8 mm follicles were sequenced to identify transcriptional differences in the follicle population from which selection was made for the preovulatory stage. Differential expression analysis identified several genes known to play a role in follicle development (CYP11A1, STAR, INHA, and INHBB) that are upregulated in FF hens. These changes in gene expression suggest earlier granulosa cell differentiation and steroidogenic competency in the granulosa layer from FF hens.

a. Francoeur L, Stephens CS, Johnson PA. Ad Libitum Feeding in Broiler Breeder Hens Alters the Transcriptome of Granulosa Cells of Pre-Hierarchal Follicles. Animals 2021, Vol 11, Page 2706 2021; 11:2706 (Reprint, copyrights retained by authors after publication).

b. In collaboration with Claire. S. Stephens who conducted the animal experiment and tissue collection.

Introduction

Broiler chickens are selected for fast growth and feed efficiency, and laying hens are selected for optimal egg production. Selection pressure for ideal production traits has resulted in vastly different reproductive efficiencies between broiler breeders and laying hens. Although laying hens can lay almost one egg per day, broiler hens have aberrant follicle growth, which often leads to erratic laying, multiple ovulations, and poor-quality eggs [1]. These events contribute to suboptimal reproduction in these hens. One strategy used to improve broiler breeder hens' reproductive efficiency is restricted feeding, which results in an ovarian phenotype more like that of the laying hen. While restricting feed (RF) results in improved egg production compared to *ad libitum* feeding (FF), dietary change alone is not sufficient to reach the egg-laying efficiency seen in layer breeds. At the ovarian follicle level, FF broiler breeder hens have multiple hierarchies and significantly more preovulatory follicles than hens on a restricted diet [2,3]. Despite excessive follicle development, ovulation is erratic, resulting in low egg production. It is not known how feeding level directly impacts follicle selection and growth.

Follicle selection occurs when one follicle from a pool of growing follicles begins to become dominant and continues maturation until it ovulates [4]. In the hen, follicle selection is characterized by two important events: granulosa cell differentiation and the initiation of progesterone synthesis. Prior to selection, granulosa cells are said to be undifferentiated and steroidogenically incompetent [5]. Undifferentiated granulosa cells express low levels of the steroidogenic acute regulatory protein (STAR), luteinizing hormone receptor (LHR), cytochrome P450scc (CYP11A1), and follicle-stimulating hormone receptor (FSHR) [6,7](reviewed in [8]). At the time of selection, granulosa cells undergo transcriptional changes in key regulators and receptors. STAR and CYP11A1 expression increases [6,9], and follicles

begin to produce progesterone [10], with the largest preovulatory follicle producing the highest amount of progesterone and stimulating the LH surge for ovulation [11,12]. In a reproductively efficient hen, the largest preovulatory follicle will ovulate each day, and one 6–8 mm follicle will be selected to replace it and replenish the preovulatory follicle pool (reviewed in [8]and [4]). In the FF broiler breeder hen, there is a large number of unorganized preovulatory follicles [2], which contributes to the reproductive inefficiency of these birds. Although factors associated with follicle selection in laying hens have been identified [4,6,9], differences in prehierarchal follicle transcriptomes have not yet been studied in broiler breeder hens in response to dietary treatment.

In this study, we investigated transcriptional changes in granulosa cells of 6–8 mm follicles to identify factors that may be disrupting normal follicle selection in FF broiler breeder hens. Specifically, we hypothesized that the increased feed intake in these hens disrupts important regulators of follicle development, resulting in increased follicle numbers.

Materials and Methods

Animals

One-day-old broiler breeder chicks (Cobb 700, n = 32) were donated by Cobb-Vantress and raised in floor pens in Cornell University's Poultry Facility according to commercial guidelines [13]. Birds were kept on a light cycle of 15 h of light and 9 h of dark. At 28 weeks of age, hens were randomly assigned to one of two pens and fed either an *ad libitum* diet (FF, n = 16) or were continued on a restricted feed diet of 146 g/day/bird (RF, n = 16) for an additional 6 weeks according to commercial guidelines [13] and as previously described in Stephens and Johnson, 2017 [14]. Throughout the length of the experiment, egg production per pen was measured daily. During the sixth

week of the dietary treatment, hens were weighed, euthanized using CO_2, and samples were collected. Egg production was calculated as eggs/hen/days in a one-week period. Egg production during weeks 1–5 is reported as these represented full weeks for both treatment groups. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University (protocol number 2009-0036).

Sample Collection

The liver, fat pad, and ovary were removed from the hen and weighed. Organ weights were normalized to the body weight. Upon collection, the ovary was placed in ice-cold Krebs-Ringer bicarbonate buffer, and follicles were collected and separated by size. Follicles from the 3-5 mm, 6-8 mm, and >9 mm size categories were counted, and 6–8 mm follicles were removed. Granulosa cells were collected and pooled following the procedure outlined in Wang et al. [15] and stored in RLT lysis buffer at −80 °C until further processing.

RNA Extraction

Total RNA was extracted from granulosa cells using an RNeasy Mini kit with optional on-column DNase treatment (Qiagen Inc., Valencia, CA, USA). The quantity and purity of the samples were analyzed using spectrophotometry (Implen, Munich, Germany). RNA integrity was then determined by a Fragment Analyzer (Advanced Analytical, Ames, IA, USA). All samples had an RQN of >9.9.

RNA Sequencing and Quality Control

Samples were selected for RNA-sequencing based on the number of preovulatory follicles (>9 mm). For the FF group (n = 3), hens with more than 10 preovulatory follicles were selected to represent an excessive follicle selection

phenotype. For the RF group (n = 3), hens with 6–7 preovulatory follicles were selected to represent a normal follicle selection phenotype. The granulosa cell layer from 6-8 mm follicles of these two phenotypes was collected as described above and used for RNA sequencing.

RNA samples were submitted to Cornell's Transcriptional Regulation and Expression Facility for cDNA library preparation. Samples were enriched by PolyA+ RNA isolation using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). Libraries were then generated using the NEBNext Ultra II [Directional] RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Before sequencing, libraries were quantified using a Qubit 2.0 (dsDNA HS kit; Thermo Fisher, Waltham, MA, USA). cDNA libraries were sequenced on Illumina's NextSeq500 (Illumina, San Diego, CA, USA) at a depth of 75 bp for a minimum of 31M reads per sample. FastQ files were first processed through trim-galore (Barbraham Institute, Cambridge, UK) as a quality control step to trim adaptors and filter for low-quality reads. The sample files were then aligned to the Galgal6 genome using the RNA-seq aligner STAR [16]. A minimum of 91.7% reads were mapped to the genome in each sample (Appendix 1).

To verify for sample clustering by biological replicates, hclust in R was used [17]. This analysis identified one outlier in the FF group, which was discarded in further analyses for a final sample of n = 2 in this group. A principle component analysis (PCA) was utilized to visualize the variance among samples.

RNA Sequencing Analysis

Differential expression analysis was conducted to identify differentially expressed genes (DEGs) between FF and RF hens using DeSEQ2. Criteria for DEGs were a false discovery rate (FDR) of < 0.05, a log fold change of >1, and a minimum

read count of 200. Gene ontology enrichment analysis with an FDR cutoff of 0.05 using ShinyGo v0.61 [18] was conducted on DEGs upregulated in FF and RF hens and generated the ten most significant terms. Additionally, Qiagen's Ingenuity Pathway Analysis (IPA) was used to identify the predicted upstream regulators [19,20] of DEGs.

cDNA Synthesis and Real-Time qPCR

Gene expression of select DEGs (CYP11A1, STAR, INHA, and INHBB) was quantified using real-time qPCR to validate RNA-sequencing results. Total RNA from granulosa cells was extracted as described above. One µg of total RNA from each sample was reverse transcribed to cDNA in a 20 µL reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

The AB StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for quantitative PCR. Each sample was analyzed in a 25 µL volume reaction with a final concentration of 1X for the Power SYBR Green (Applied Biosystems, Foster City, CA, USA) and 300 nM for the primers. Each reaction consisted of 1 µL of cDNA and samples, standards, no template controls, and reactions lacking reverse transcriptase were assessed in duplicates. Sample expression for each gene was determined from the standard curve and normalized to 18S expression. To measure the housekeeping gene, 18S primers from Ambion® QuantumRNA™ 18S Internal Standard (ThermoFisher Scientific, Waltham, MA, USA) were used. Previously published primer sequences were used for CYP11A1 and STAR [21]. Primers for INHA and INHBB were designed to span exon-exon junctions using Primer-BLAST [22]. Primer sequences for gene targets can be found in Table 1.

Table 1. Primer Sequences for RT-qPCR.

Target Gene	Primer Sequence
CYP11A1	F 5′-ACTTCAAGGGACTGAGCTTTGGGT-3′ R 5′ AGTTCTCCAGGATGTGCATGAGGA 3′
STAR	F 5′-TGCCTGAGCAGCAGGGATTTATCA- 3′ R 5′- TGGTTGATGATGGTCTTTGGCAGC-3′
INHA	F 5′-TCTTCCCTTCCACAGACGTG- 3′ R 5′- CTGTAGAACCAGAGCTGGGC-3′
INHBB	F 5′-TTCGCCGAGACAGACGAT- 3′ R 5′- TTACTTTTCGCCTGCTGCCT-3′

Statistical Analysis

All body parameters and mRNA expression data were compared between the RF and FF groups using PROC GLM of the SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Week was included as a fixed effect in the generalized linear model for egg production.

Results

Body Parameters and Ovarian Morphology

Following 6 weeks of dietary treatment, FF hens had significantly higher live body weights than RF hens ($p < 0.001$, Table 2). Both fat pad and liver weights were also significantly higher in FF hens compared to RF hens when normalized to body weight ($p < 0.001$, Table 2).

FF hens had significantly more follicles greater than 9 mm in diameter compared to RF hens ($p = 0.002$, Table 2), indicating an increase in the number of follicles selected into the preovulatory hierarchy. Ovarian weight normalized to body

weight was not different between RF and FF hens ($p = 0.101$, Table 2). Egg production was significantly higher for RF hens than FF hens throughout the course of the experiment ($p = 0.013$, Table 2 and Figure 1).

Table 2. Mean body weight, normalized fat pad, liver, and ovary weights, as well as follicle numbers (3-5 mm, 6-8 mm, and follicles >9 mm) and egg production (eggs/hen/day) for RF and FF hens in response to dietary treatment (n = 15–16 per group). Means are presented as means ± SD.

Parameter	RF	FF	p-Value
Body weight (g)	3389 ± 162	4483 ± 359	<0.0001
Fat pad weight/BW	0.015 ± 0.007	0.030 ± 0.008	<0.0001
Liver weight/BW	0.028 ± 0.005	0.048 ± 0.010	<0.0001
Ovary weight/BW	0.018 ± 0.003	0.021 ± 0.005	0.101
3-5 mm follicles	32.2 ± 11.0	36.3 ± 12.0	0.332
6-8 mm follicles	11.6 ± 3.9	10.7 ± 3.7	0.519
Follicles > 9 mm	6.3 ± 0.9	8.2 ± 2.0	0.002
Eggs/hen/day	0.862 ± 0.048	0.678 ± 0.039	0.013

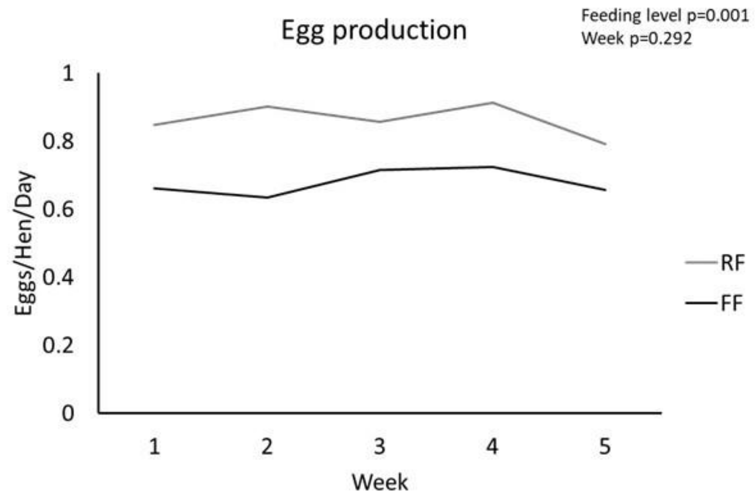

Figure 1. Egg production (eggs/hen/day) of RF and FF hens in response to dietary treatment (n = 16 per group, p = 0.013). P-values for individual fixed effects are in the top right corner.

RNA Sequencing and Differential Gene Expression

PCA revealed that samples clustered with biological replicates along the first component (Figure 2A), and this was confirmed by hierarchal clustering (Figure 2B). Differential gene expression analysis resulted in 350 DEGs, of which 207 and 143 were upregulated in FF hens and RF hens, respectively (Figure 2C). The top 50 upregulated genes in FF and RF hens are listed in Appendices 2 and 3, respectively. Of the genes upregulated in the granulosa cells of 6-8 mm follicles of FF hens, several are known to be important for steroidogenesis (CYP11A1, STAR, NR5A1, and NR5A2) and in regulating FSH (INHA and INHBB) (Figure 3). CYP11A1, STAR, INHA, and INHBB mRNA expression were measured using qPCR to validate RNA-sequencing results. CYP11A1, INHA, and INHBB expression were significantly higher ($p < 0.01$), and STAR expression tended to be higher in granulosa cells of 6-8 mm follicles of FF hens ($p = 0.08$, Figure 4).

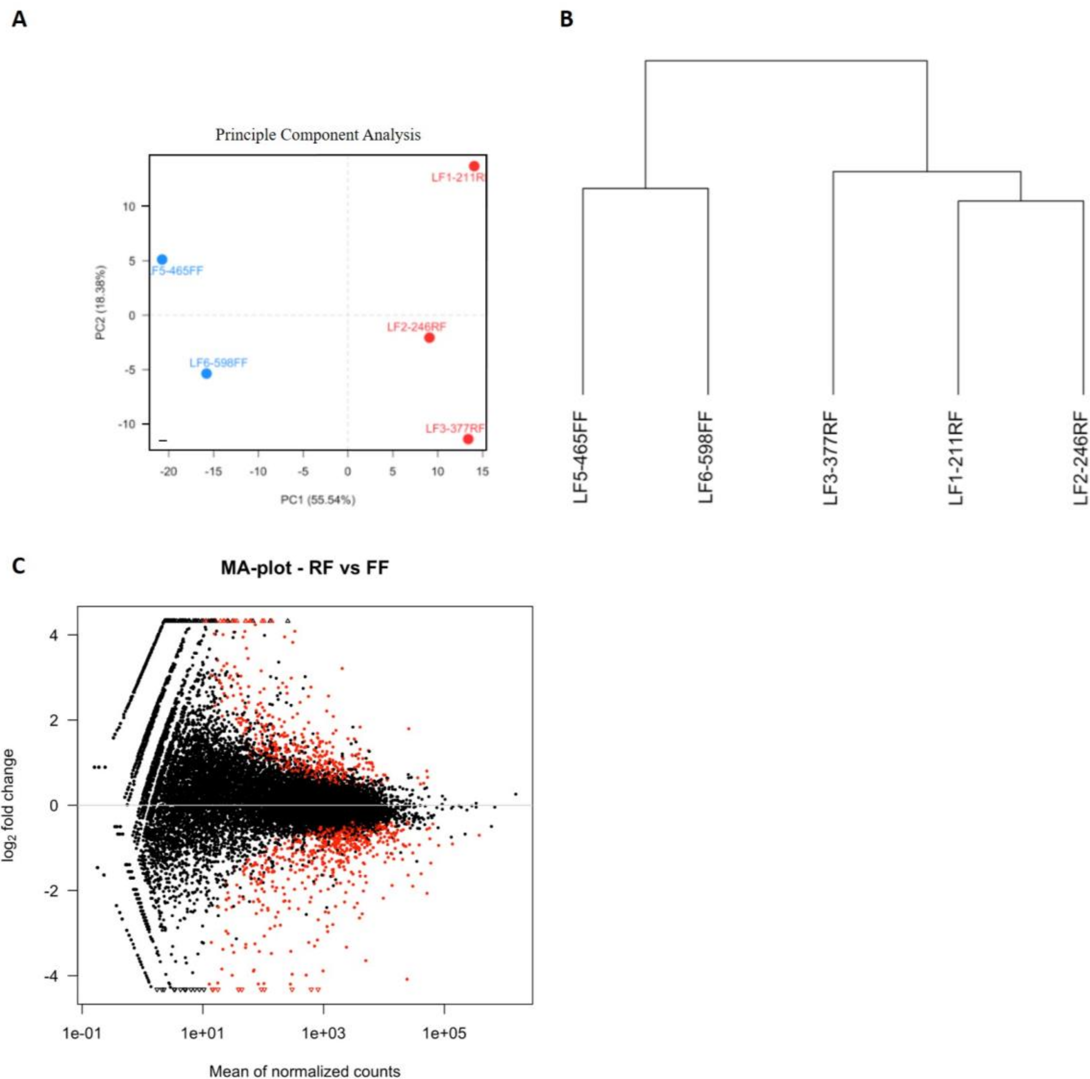

Figure 2. Principle component analysis, hierarchal clustering, and MA plot for RNA-sequencing data of granulosa cells from 6-8 mm follicles from RF and FF hens. (A) Principle component analysis comparing FF (n = 2, blue) and RF (n = 3, red) transcriptomes. (B) Hierarchal clustering of FF (n = 2) and RF (n = 3) samples. (C) MA plot comparing normalized counts in FF and RF hens. Black dots represent expressed genes and red dots indicate DEGs. Genes located below the x-axis represent genes more highly expressed in FF hens and genes located above the x-axis represent genes more highly expressed in RF hens.

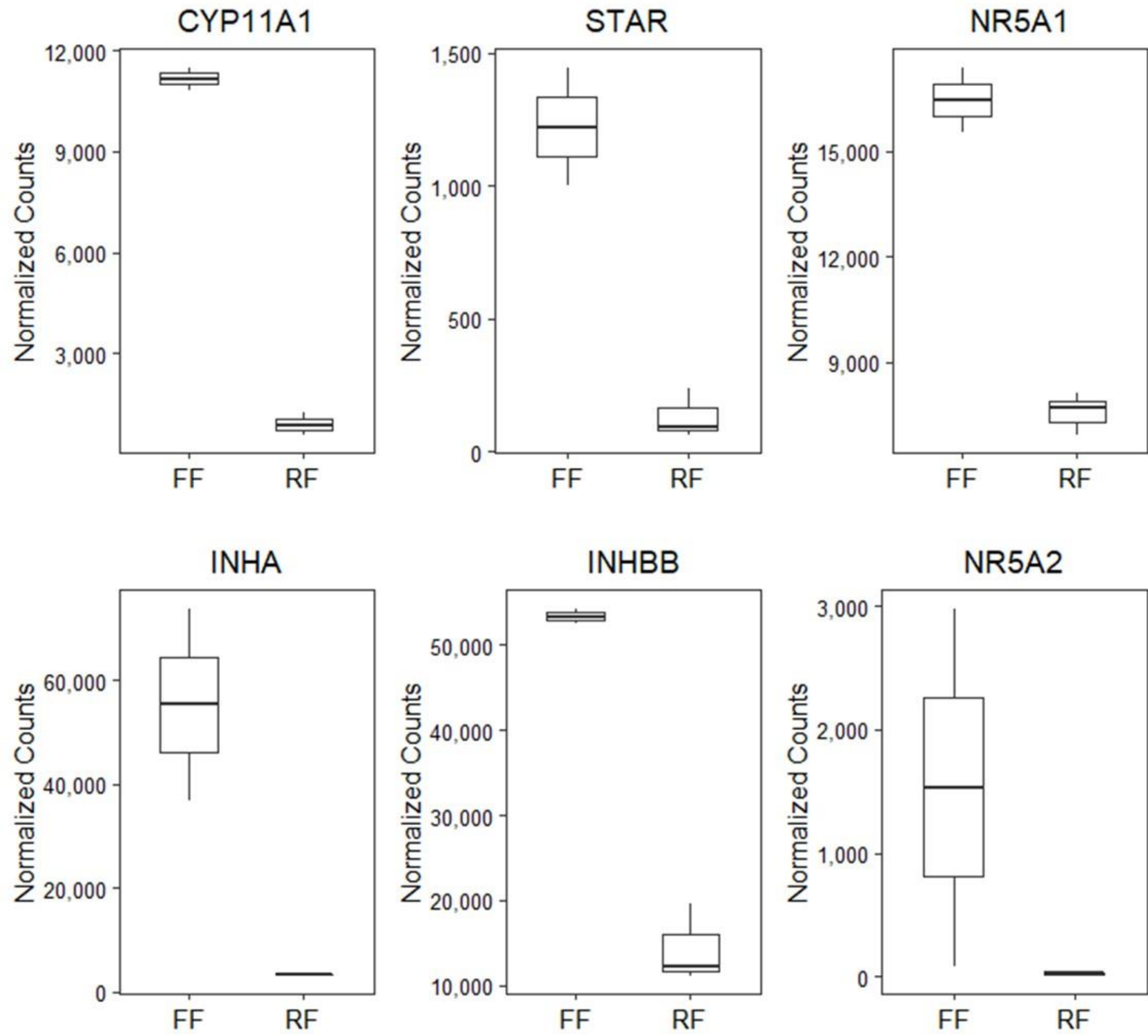

Figure 3. Boxplots of normalized counts from RNA-seq analysis comparing FF (n = 2) and RF (n = 3) gene expression of select DEGs (CYP11A1, STAR, NR5A1, INHA, INHBB, and NR5A2).

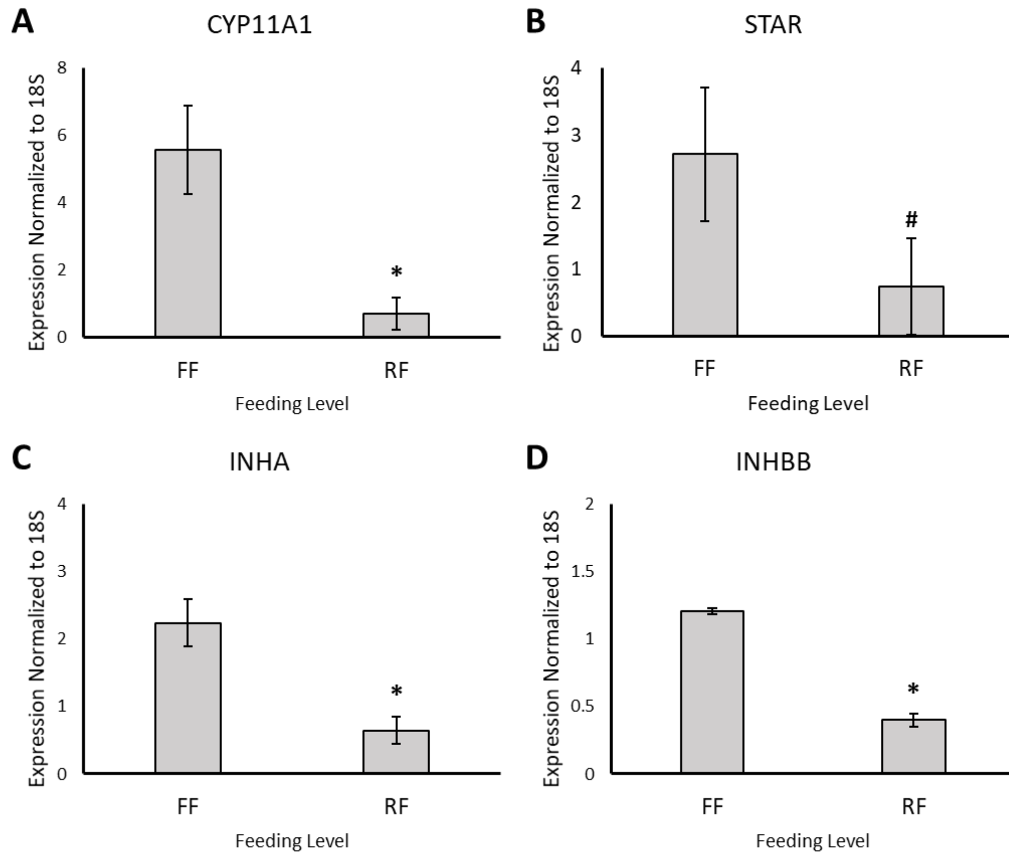

Figure 4. CYP11A1, STAR, INHA, and INHBB mRNA expression in granulosa cells of 6-8 mm follicles in FF and RF hens. Bars represent the mean expression normalized to 18S ± SD in granulosa cells of 6-8 mm follicles in FF (n = 2) and RF hens (n = 3) (p > 0.01, *; p = 0.08, #). (A) CYP11A1 mRNA expression (p < 0.01); (B) STAR mRNA expression (p = 0.08); (C) INHA mRNA expression (p < 0.01); and (D) INHBB mRNA expression (p < 0.01).

Enrichment Analysis

Enrichment analysis categorized DEGs by functional category. The top ten significant terms associated with DEGs upregulated in FF and RF hens are listed in Tables 3 and 4, respectively. Several DEGs upregulated in FF hens are associated with terms related to lipid metabolism, such as “lipid catabolic process” and “cellular lipid catabolic process” (Table 3). The term “transmembrane transport” categorized the most upregulated DEGs in FF hens (Table 3). Other terms of note reflect cellular reorganization in the granulosa cells of 6-8 mm follicles in FF hens, including “positive regulation of actin filament bundle assembly” and “positive regulation of cellular component biogenesis” (Table 3). DEGs upregulated in RF hens are associated with cellular homeostasis. Several of the top functional terms are associated with ion transport: “ion transport”, “cation transport”, “metal ion transport”, and “ion transmembrane transport” (Table 4). Other terms are associated with maintaining cell physiology, such as “regulation of system process” and “positive regulation of cell size” (Table 4).

Upstream Regulators

Using IPA’s upstream regulator analysis, several transcriptional regulators were predicted to be upstream of DEGs. Among them are gonadotropins (LH, CG, and FSH), and growth factors (TGFB1, BMP6, AGT, BMP4, GDF9, HGF, IGF1, and BMP7) (Figure 5A). Two predicted upstream regulators of note are FSH with 23 downstream DEGs (Figure 5B) and Insulin-like Growth Factor 1 (IGF1) with 20 downstream DEGs (Figure 5C). Of the DEGs, seven genes (CYP11A1, EDN, IGFBP4, INHA, NR5A, STAR, and WT1) are predicted by IPA to be regulated by both FSH and IGF1.

Table 3. Enrichment analysis of upregulated differentially expressed genes in FF hens. This table shows the top ten significant terms associated with DEGs found to be upregulated in FF granulosa cells of 6-8 mm follicles. The functional category, number of genes found in the DEG list, total number of genes found in the database for the specific functional category term, and the FDR are listed.

Functional Category	Genes	Total Genes	FDR
Lipid catabolic process	10	186	2.17×10^{-4}
Heme export	2	2	1.65×10^{-2}
Positive regulation of actin filament bundle assembly	4	44	3.44×10^{-2}
Cellular lipid catabolic process	6	126	3.44×10^{-2}
Proteoglycan biosynthetic process	4	48	3.81×10^{-2}
Inositol trisphosphate biosynthetic process	3	21	3.81×10^{-2}
Positive regulation of cellular component biogenesis	9	345	3.81×10^{-2}
Heme transport	2	6	4.43×10^{-2}
Inositol trisphosphate metabolic process	3	24	4.43×10^{-2}
Transmembrane transport	17	1122	4.43×10^{-2}

Table 4. Enrichment analysis of upregulated differentially expressed genes in RF hens. This table shows ten significant terms associated with DEGs found to be upregulated in RF granulosa cells of 6-8 mm follicles. The functional category, number of genes in the DEG list, total number of genes in the database for the specific functional category term, and the FDR are listed.

Functional Category	Genes	Total Genes	FDR
Ion transport	12	1117	3.26×10^{-2}
Cation transport	10	757	3.26×10^{-2}
Muscle contraction	5	171	3.26×10^{-2}
Nitric oxide mediated signal transduction	2	11	3.26×10^{-2}
Regulation of heart contraction	4	112	3.26×10^{-2}
Regulation of nitric oxide mediated signal transduction	2	6	3.26×10^{-2}
Metal ion transport	8	545	3.26×10^{-2}
Ion transmembrane transport	10	790	3.26×10^{-2}
Regulation of system process	6	291	3.26×10^{-2}
Positive regulation of cell size	2	6	3.26×10^{-2}

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Upstream Regulator	Genes	Total genes	z-score	p-value
LH	21	123	-0.021	<0.001
CG	25	130	-0.54	<0.001
FSH	23	131	-1.723	<0.001
AR	26	117	0.768	<0.001
TGFB1	53	169	-0.722	<0.001
VEGF	24	-	-0.419	<0.001
HIF1A	22	145	-0.295	<0.001
TNF	52	148	1.103	<0.001
PAX3	14	-	-	<0.001
TCF7L2	20	86	0.642	<0.001
BMP6	10	-	-1.691	<0.001
PPARGC1A	18	100	-1.422	<0.001
AGT	29	133	0.091	<0.001
KLF2	12	88	0.666	<0.001
BMP4	13	132	-1.237	<0.001
LHCGR	6	71	-0.537	<0.001
GDF9	6	77	-0.132	<0.001
HGF	20	141	-0.595	<0.001
NR0B1	6	70	2.224	<0.001
IGF1	20	142	-1.175	<0.001
PKA catalytic subunit	5	107	-1.213	<0.001
HNRNPA2B1	10	-	-	<0.001
BMP7	11	108	0.241	<0.001
POR	11	88	2.414	<0.001

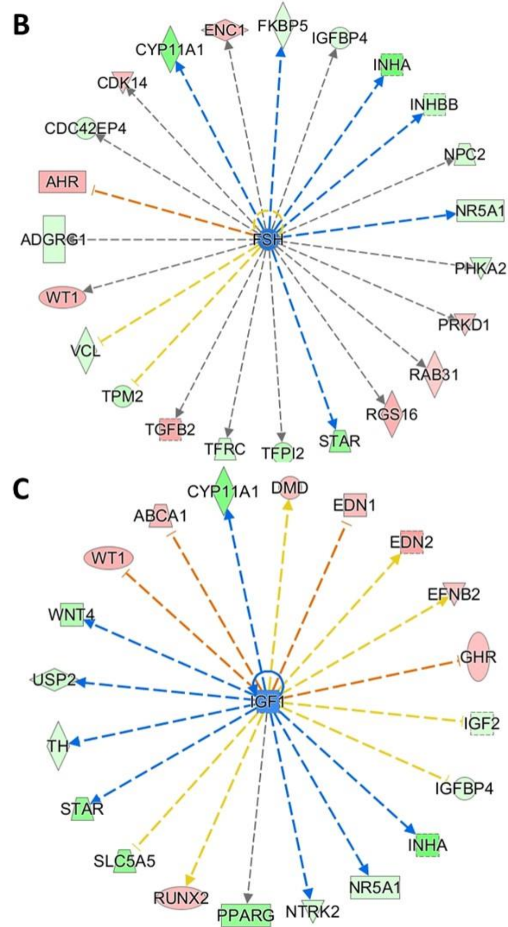

Figure 5. Upstream regulator analysis of differentially expressed genes in RF and FF hens. (A) The top 25 predicted upstream regulators of DEGs from the granulosa cells of 6-8 mm follicles from FF and RF hens are shown. The upstream regulator, number of gene targets in the DEG dataset, total number of genes found in the IPA network for the specific regulator, z-score of activation, and p-value of overlap are indicated. (B) FSH network wheel of gene targets in dataset. Green shapes are upregulated and red shapes are downregulated in FF hens (z-score = −1.723). Line colors indicate the state of activation (blue = activation; orange = inhibition; yellow = findings inconsistent with downstream gene state; and grey = unpredicted effects). (C) IGF1 network wheel of gene targets in dataset (z-score = −1.175). Colors of the shapes and lines have the same meaning as in panel B.

Discussion

Broiler breeder hens fed *ad libitum* have increased follicle selection and excessive follicular growth, which results in decreased egg production. Commercial producers can increase egg production by restricting the dietary intake in these hens, resulting in a more regulated follicle hierarchy. In this study, we sought to generate hypotheses for the increased follicle development observed in FF hens by identifying differences in the transcriptomes of granulosa cells from 6-8 mm follicles, the stage of follicle selection.

Consistent with a previous study in our lab, we found that the preovulatory follicle number was significantly higher in FF hens when compared to RF hens [23]. Although we have previously found a significantly higher number of 3-5 mm and 6-8 mm follicles in FF hens compared to RF hens [23], we did not observe this in the present study. Another group using a similar experimental protocol also indicated no difference in numbers in these follicle size categories [24]. Our finding of a higher number of preovulatory follicles in FF hens is consistent with the hypothesis of increased follicle selection in response to increased dietary intake. At the time of follicle selection, granulosa cells become differentiated and gain the capacity to produce progesterone [25]. In the laying hen, one 6-8 mm follicle is selected approximately each day. This follicle enters the preovulatory stage to replace the recently ovulated follicle and this permits both an organized follicle hierarchy and efficient egg production. Given the increased number of preovulatory follicles observed in FF hens, the process of follicle selection is likely increased. We found transcriptional changes in the granulosa cell layer of 6-8 mm follicles, the stage at which follicle selection occurs.

In laying hens, the transcriptomes of granulosa cells from a 6 mm follicle and those from the most recently selected preovulatory follicle (F5) have been sequenced

to investigate the transcriptional changes during the transition from unselected to selected follicles [26]. Of the top 50 DEGs identified in the granulosa cells of the recently selected follicle of the laying hen, 22 overlap with those we identified in the granulosa cells (6-8 mm follicles) of the FF hen. Among the notable genes in common between these two groups are CYP11A1 and STAR. CYP11A1 and STAR are important for the production of progesterone, an important functional change acquired at the time of follicle selection [10]. Given the strong overlap in gene expression between these two populations, it is possible that granulosa cells of 6-8 mm follicles of FF hens are more differentiated than those of RF hens. This early differentiation may occur in the granulosa layers of multiple 6-8 mm follicles, thereby disrupting the follicle hierarchy.

In the laying hen, granulosa cells begin to produce progesterone from cholesterol following selection into the preovulatory follicle stage [10]. This steroidogenic competency is associated with an increase in STAR and CYP11A1 expression [6,9]. Given the increased expression of STAR and CYP11A1 in granulosa cells from 6-8 mm follicles of the FF hen, progesterone production may increase earlier in follicle development than in laying hens or RF hens. Furthermore, two transcriptional activators, namely NR5A1 (synonym SF-1) and NR5A2 (synonym LRH-1), were found to be upregulated in the FF hen. NR5A1 binds to the promotor of CYP11A1 to increase its transcription and the production of progesterone in the granulosa cells of rats [27]. A second transcription factor, NR5A2, also increases CYP11A1-promoter activity in human granulosa cells [28] and in the presence of FSH, increases CYP11A1 mRNA and progesterone synthesis in cultured rat granulosa cells [29]. The increased STAR, CYP11A1, NR5A1, and NR5A2 expression in granulosa cells of 6-8 mm follicles of FF hens suggests that the production of progesterone may be initiated at this stage of follicle development, earlier than

observed in laying hens. It has previously been proposed that production of progesterone may be initiated earlier in follicle development in FF hens [30]. Both the F1 and F2 follicles in FF hens have been shown to secrete higher progesterone in FF hens compared to RF hens, where only the F1 follicle secretes high progesterone levels [30]. Alternatively, these hens may be gaining the machinery for steroidogenesis during the 6-8 mm follicle stage and producing higher levels of progesterone once they enter the preovulatory stage. Dysregulation in the production of progesterone may be stimulating multiple ovulations and therefore be contributing to the double ovulations that are often observed in FF hens.

In addition to changes in the ovary, we found that full-feeding increased body weight, liver weight/BW, and fat pad weight/BW, as we have previously shown [23]. Increased dietary intake results in increased adiposity, with FF hens showing increased fat pad weight and increased plasma triglyceride and cholesterol levels compared to RF hens [23]. Enrichment analysis showed "lipid catabolic process" and "cellular lipid catabolic process" as some of the most significant terms for granulosa cells of 6-8 mm follicles from FF hens. This suggests that granulosa cells of 6-8 mm follicles may adjust their physiology to accommodate the increased amount of plasma lipids. Interestingly, the DEG with the highest fold change in both the granulosa cells of the most recently selected follicle of the laying hen [26] and the granulosa cells of 6-8 mm follicles from FF broiler breeder hens is Carboxyl Ester Lipase (CEL). CEL is known to be excreted from the pancreas and has effects on lipid absorption in the intestine (reviewed in [31]). Among its roles, CEL functions in cholesterol absorption, in the reverse transport to the liver, and potentially in the cholesterol uptake by cells (reviewed in [31]). In the chicken, CEL is mainly expressed in the pancreas [32]. CEL expression in the pancreas has been shown to be influenced by dietary cholesterol in rats [33]; however, to our knowledge, our study is the first demonstrating that diet

influences CEL expression in gonads. Although the role of CEL has not yet been investigated in the ovary, it may facilitate cholesterol transport in the cell. Two other lipid transporters, namely ABCA12 and SLC25A1, a mitochondrial transporter important for downstream endogenous cholesterol synthesis (summarized in [34]), are also increased in granulosa cells of 6-8 mm follicles of FF hens. Upregulation of CEL, ABCA12, and SLC25A1 may be increasing cholesterol availability to granulosa cells of 6-8 mm follicles in FF hens with higher levels of plasma cholesterol and facilitating enhanced progesterone production.

Although dietary intake affects the transcriptomes of granulosa cells of 6-8 mm follicles, the factors directly contributing to these changes remain unclear. The upstream regulator analysis predicted FSH and IGF1 to have effects on several DEGs upregulated in FF hens. In the hen, granulosa cells become responsive to FSH at the time of selection and one of the 6-8 mm follicles shows an increase in FSHR mRNA [7]. FSH increases cAMP levels to stimulate STAR and CYP11A1 transcription [6,9]. In the current study, INHBB, INHA, STAR, and CYP11A1 were upregulated in granulosa cells of 6–8-mm follicles from FF hens and predicted to be downstream of FSH. Reported plasma FSH levels in RF and FF hens are variable as some studies found no difference [2], elevated levels [35–37], or decreased FSH plasma levels in FF compared to RF hens [24]. More research is needed to determine the effect of increased dietary intake on FSH plasma levels.

In a previous study, we investigated liver transcriptome differences between FF and RF hens and showed that FF hens have elevated liver IGF1 mRNA and protein compared to RF hens [23]. At the preovulatory follicle stage, IGF1 can increase progesterone production and expression of STAR, CYP11A1, and 3βHSD in chicken granulosa cells [38]. In granulosa cells of prehierarchal follicles, IGF1 increases cell proliferation [39]. In mammals, FSH and IGF1 have been shown to increase

CYP11A1 and STAR expression synergistically [40–43]. Using upstream regulator analysis, we found seven genes (CYP11A1, EDN, IGFBP4, INHA, NR5A, STAR, and WT1) downstream of both FSH and IGF1. These genes could be targets for synergistic effects of FSH and IGF1 in the hen, and elevated IGF1 in FF hens [23] may synergize with FSH in FF hens to enhance follicle development.

This is the first study to investigate differences in the transcriptome of prehierarchal follicles between broiler breeder hens fed at different levels. Three hundred and fifty genes were found to be differentially expressed between FF and RF hens in granulosa cells from 6-8 mm follicles. Several genes involved in follicle selection were upregulated in prehierarchal follicles of FF hens, suggesting an ovarian effect of dietary treatment at early stages in follicle development. Findings from this study suggest that granulosa cells of 6-8 mm follicles may mature earlier in FF hens than in RF hens, particularly with respect to capacity for progesterone synthesis. In addition, increased plasma cholesterol levels, FSH, and IGF1 may be involved in some of these transcriptional changes. These hypotheses have opened new research avenues which we are actively pursuing. Ultimately, this research helps clarify the processes contributing to the reproductive inefficiencies observed in broiler breeder hens. A greater understanding of the etiology of these reproductive inefficiencies can provide targets for treatment and genetic selection to improve the reproductive health and welfare of these hens.

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CHAPTER 3

EFFECT OF IGF1 AND FSH TREATMENT ON THE FUNCTION OF GRANULOSA CELLS OF PREHIERARCHAL FOLLICLES IN CHICKENS[a]

Abstract

IGF1 is an essential regulator of mammalian follicle development and has been shown to synergize with FSH to amplify its effects. In avian preovulatory follicles, IGF1 increases the expression of genes involved in steroidogenesis and the production of progesterone and inhibin A. The role of IGF1 in prehierarchal follicles has not been well studied in chickens. This study aimed to investigate the role of IGF1 in granulosa cells of 3-5 and 6-8 mm prehierarchal follicles and evaluate whether IGF1 and FSH synergize to promote follicle development. Granulosa cells of 3-5 and 6-8 mm prehierarchal follicles were cultured with IGF1 (0, 10, 100 ng/mL) in the presence or absence of FSH (0, 10 ng/mL). Expression of genes important in follicle development (FSHR, IGF1R, AMH, STAR, CYP11A1, INHA, and INHBA), cell proliferation, and progesterone production were evaluated. IGF1 treatment alone significantly increased STAR, CYP11A1, and INHBA mRNA expression and cell proliferation in granulosa cells of 6-8 mm follicles. IGF1 and FSH synergized to increase STAR mRNA expression in 6-8 mm follicles. IGF1 and FSH co-treatment were necessary to increase CYP11A1 mRNA expression in 3-5 mm follicles and INHA mRNA expression in 6-8 mm follicles. Although the expression of genes involved in steroidogenesis was significantly increased following IGF1 treatment, IGF1 did not increase progesterone production in granulosa cells of 6-8 mm follicles; however, FSH treatment was

a. In collaboration with Deena M. Scoville who collected and cultured granulosa cells for the first experiment and participated in RT-qPCR analysis.

sufficient to stimulate progesterone production in these cells. IGF1 did not affect AMH mRNA expression, although FSH significantly decreased AMH expression in granulosa cells of 3-5 mm follicles. These results suggest that IGF1 may act with FSH to promote follicle selection at the small prehierarchal follicle stage.

Introduction

Follicle development in the laying hen is a tightly regulated process. Primordial follicles are activated at <0.05 mm [1] and transition to primary follicles (<1 mm) before eventually reaching the prehierarchal follicle pool (1-8 mm) (reviewed in [2]). The prehierarchal follicle pool consists of small white follicles (1-3 mm), large white follicles (3-5 mm), and small yellow follicles (6-8 mm). From the 6-8 mm stage, one follicle will be selected and join the hierarchy of preovulatory follicles, a stage of fast growth with rapid yolk accumulation (reviewed in [2,3]). This high level of organization facilitates a predictable ovulation pattern and results in a laying rate of approximately one egg per day.

At the time of follicle selection, several changes occur at the granulosa cell level of the follicle as the cells transition from an undifferentiated to a differentiated state. Among these changes are an increased FSHR expression [4], a gain in steroidogenic capacity [5], a shift from inhibin B to inhibin A production [6], and a decrease in AMH expression [7]. Before selection, granulosa cells are steroidogenically incompetent and unable to produce progesterone [8]. These cells lack expression of STAR (steroidogenic acute regulatory protein) [9] and the enzyme responsible for cholesterol side-change cleavage (Cytochrome P450 Family 11 Subfamily A Member 1; CYP11A1) [10]. At the time of selection, one follicle of the 6-8 mm cohort has a significant increase in FSHR expression compared to other follicles in this stage [4]. In response to FSH, STAR [4,11], and CYP11A1 [10] mRNA expression increase in granulosa cells, resulting in the initiation of progesterone production [5].

Insulin-like growth factor 1 (IGF1) is a 7 kDa protein of 70 amino acids and is primarily secreted by the liver (reviewed in [12]). IGF1 is also locally produced in granulosa cells [13]. The function of IGF1 is mediated through a cell surface receptor, insulin-like growth factor type 1 receptor (IGF1R). IGF1R is present on hen theca and granulosa cells of preovulatory follicles, and its expression increases with follicle size [14]. In mammals, IGF1 is essential for folliculogenesis. Mice with inactivated IGF1R in granulosa cells are sterile due to arrest in follicle development before the preovulatory stage [15]. These mice also have reduced STAR, CYP11A1, aromatase (CYP19A1), luteinizing hormone receptor (LHR), inhibin α-subunit (INHA), inhibin βA-subunit (INHBA), and inhibin βB-subunit (INHBB) expression [15]. In cultured bovine granulosa cells from antral follicles (2-8 mm), IGF1 increases cell number, estrogen production, and expression of FSHR, CYP11A1, CYP19A1, and 3beta-hydroxysteroid dehydrogenase (HSD3B1/3βHSD) [16].

In chicken preovulatory follicles, IGF1 has been shown to increase granulosa cell STAR, CYP11A1, and 3βHSD protein expression [14], INHA mRNA expression [17], production of progesterone [14,18,19], inhibin A [19], and cell proliferation [14,18]. IGF1 has also been shown to have an additive effect with LH or FSH on progesterone and inhibin A production [18,19]. In prehierarchal follicles (3-10 mm), IGF1 increased granulosa cell proliferation, which could be blocked by immunoneutralization [13]. There has been limited research on the effect of IGF1 in prehierarchal follicles and its role in follicle selection.

An understanding of the role of IGF1 in avian follicle development could elucidate reproductive inefficiencies seen in broiler breeder hens (meat-producing

breed). Broiler breeder hens have increased follicle growth, leading to reproductive complications such as erratic laying, multiple ovulations, and poor-quality eggs [20]. These hens grow very fast and, unless feed-restricted, have poor follicle development. Broiler breeder hens fed *ad libitum* have a higher liver expression of IGF1 and lower liver expression of IGF1 binding proteins (IGFBP1 and IGFBP2) than hens on a restricted diet, suggesting more bioavailable IGF1 [21]. Furthermore, RNA-sequencing analysis revealed IGF1 as an upstream regulator of transcriptomic differences in granulosa cells of 6-8 mm follicles in broiler breeder hens fed at different levels [22]. Increased IGF1 availability in broiler breeder hens fed *ad libitum* [21] may disrupt follicle development in the ovaries of these hens leading to decreased reproductive efficiencies.

The aim of this study was to investigate the role of IGF1 in granulosa cells of prehierarchal follicles using laying hens which have well-organized patterns of follicle selection. We predicted that IGF1 would affect expression of markers of follicle selection and development such as FSHR, AMH, inhibin A subunits, STAR, and CYP11A1 and stimulate steroidogenesis in granulosa cells of prehierarchal follicles. We anticipated that the addition of FSH would potentiate the effects of IGF1.

Materials and Methods

Animals and Granulosa Cell Collection

Single-comb White Leghorn hens were housed in individual cages on a 16L:8D light schedule with *ad libitum* food and water. Egg production was recorded daily, and hens in their first year of lay with a regular laying pattern were used. Hens

were selected at mid-sequence and euthanized with CO2 within 1-2 hours of oviposition. All animal procedures were approved by The Institutional Animal Care and Use Committee of Cornell University (IACUC protocol number 2009-0036).

Granulosa cell collection and culture

For each cell culture replicate, ovaries from 2-3 hens were collected immediately after euthanasia and placed in ice-cold Krebs-Ringer bicarbonate buffer. Follicles (3-5 mm and 6-8 mm) were removed from the ovary and separated by size classification. Granulosa cells were collected from follicles and pooled by size before dispersion and washing using a protocol from Wang *et al.* [23] with modifications. Briefly, granulosa cells were digested in 0.2% collagenase solution (Thermo Fisher Scientific, Waltham, MA, USA) + 0.4% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) + 0.02% trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and were inverted between three 10-15 min incubations at 37° C. Cells were washed three times in M199 + 5% fetal bovine serum (FBS) (Cytiva, Marlborough, MA, USA) + 2% antibiotic antimycotic (ABAM) (Bioworld, Dublin, OH, USA) and separated by centrifugation (1600 rpm, 5 minutes). Cells were counted using trypan blue exclusion with a hemocytometer. Granulosa cells were plated in 6-well culture dishes at a density of 3.2-4 x 10^6 cells per well in a 1.5 mL volume, according to Stephens *et al.* [24], but with media containing 5% FBS instead of 10%. Following the initial 24 hours of culture, media were removed and replaced with 2 ml of treatment media which lacked serum [24].

In the first experiment, the effect of IGF1 in the presence or absence of FSH on gene expression was investigated in granulosa cells from 3-5 mm and 6-8 mm follicles (n = 6-7 replicate cultures). Cells were treated with recombinant human IGF1 (rhIGF1, BioVision, Milpitas, CA, USA) at 0, 10, or 100 ng/ml in the presence or absence of ovine FSH (oFSH; NIDDK-oFSH-19-SIAFP, AFP4117A; 0, 10 ng/ml) and cultured for 24 hours. We validated the bioactivity of IGF1 to increase proliferation of granulosa cells from prehierarchal follicles, as was previously reported by Ahumada-Solórzano *et al.* [13] and FSH bioactivity has been previously shown [25]. After 24-hour culture, cells were scraped from wells and stored in RLT buffer at -80°C until further processing.

In the second experiment, the effect of IGF1 treatment (± FSH) on the production of progesterone and cell proliferation in granulosa cells of 6-8 mm follicles was examined (n = 7-8 replicate cultures). Granulosa cells from 6-8 mm follicles were collected as described above but were cultured in a 1 ml volume. A smaller volume was used to estimate the expected low progesterone concentration. After an initial 24 hours, cells were cultured in serum-free media with IGF1 in the presence and absence of FSH, as described above. Following 24 hours of treatment, media were collected and stored at -20°C for progesterone assays. For each replication, treated cells were plated in duplicate in a parallel 96-well plate at a density of 50,000 cells in 100 µl to evaluate cell proliferation for each treatment.

Total RNA extraction and Quantitative Real-Time PCR

Total RNA from granulosa cells was extracted using an RNAeasy Mini Kit with optional on-column DNase treatment (Qiagen Inc., Valencia, CA). RNA quantity and purity were analyzed using spectroscopy (Implen, Westlake Village, CA, US). For cDNA synthesis, 1 μg of total RNA from each sample was reverse transcribed to cDNA in a 20 μl reaction using a high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA). The mRNA expression of selected genes of interest was assessed using quantitative real-time PCR (Applied Biosystems StepOnePlus Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). PCR was carried out in a 25 μl volume reaction using a 300 nM primer concentration with a 1X final concentration of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). One μl of cDNA was used per reaction. Reactions for samples, standards, no-template controls, and reactions lacking reverse transcriptase were performed in duplicate. mRNA expression for each gene was determined from a standard curve (made from a pool of granulosa cells) and normalized to 18S expression. Primer sequences for gene targets are in Table 1.

Progesterone Concentration

Progesterone concentration was assessed in media (in duplicate or triplicate) from cultured granulosa cells of 6-8 mm follicles according to the manufacturer's protocol using a commercial RIA kit (catalog no. 582601, Cayman Chemicals). The intra-assay CV was 9.7%, and the inter-assay CV was 13.1%. Average progesterone concentrations (pg/ml) were normalized to the control for each replicate culture.

Table 1. Primer Sequences for RT-qPCR.

Target Gene	Forward	Citation
FSHR	F: 5’-GCACCTTCCAAGCCTCAGATAT-3’ R: 5’ -CCCTATGGACGACGGGTAAA-3’	Stephens *et al*. [24]
IGF1R	F: 5’-AGTACAACTACCGCTGCTGA-3’ R: 5’-CTCTCTTGCCACATGAGC-3’	Stephens (unpublished)
AMH	F: 5’-CCCCTCTGTCCCTCATGGA-3’ R: 5’-CGTCATCCTGGTGAAACACTTC-3’	Stephens *et al*. [24]
STAR	F: 5′-TGCCTGAGCAGCAGGGATTTATCA-3′ R: 5′-TGGTTGATGATGGTCTTTGGCAGC-3′	Johnson and Lee [26]
CYP11A1	F: 5′-ACTTCAAGGGACTGAGCTTTGGGT-3′ R: 5′-AGTTCTCCAGGATGTGCATGAGGA-3′	Johnson and Lee [26]
INHA	F: 5′-TCTTCCCTTCCACAGACGTG- 3′ R: 5′- CTGTAGAACCAGAGCTGGGC-3′	Francoeur *et al.* [22]
INHBA	F: 5’-GTGGGAGATGATGGCTATGTGG-3’ R: 5’-GCTGCCGCTGCTGTTGAAAC-3’	Chen *et al.* [27]

Cell proliferation

For each replicate of granulosa cell culture, a parallel 96-well plate was used to assess cell proliferation in response to treatment. Following the cell culture protocol described above, 20 µl of CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, US) was added to each well. Plates were incubated according to the manufacturer’s protocol for 1 hour, and absorbance was evaluated at 492 nm. Average absorbance values were normalized to the absorbance of the control for each replicate culture to produce fold-change.

Statistical analysis

Gene expression of granulosa cells, progesterone concentration, and cell proliferation were analyzed with a mixed model using JMP Pro 16 software (SAS Institute Inc., Cary, NC, USA). Each model consisted of a fixed effect of treatment and a random effect of culture replicate. Post-hoc Tukey test was done with $\alpha=0.05$ when appropriate. Statistical analyses were conducted on log-transformed values for gene expression and progesterone concentrations to improve the normality of the residuals. Means and 95% confidence intervals were back-transformed to the linear form for simplicity of presentation.

Results

IGF1 and FSH effect on receptor expression in granulosa cells of 3-5 mm and 6-8 mm follicles

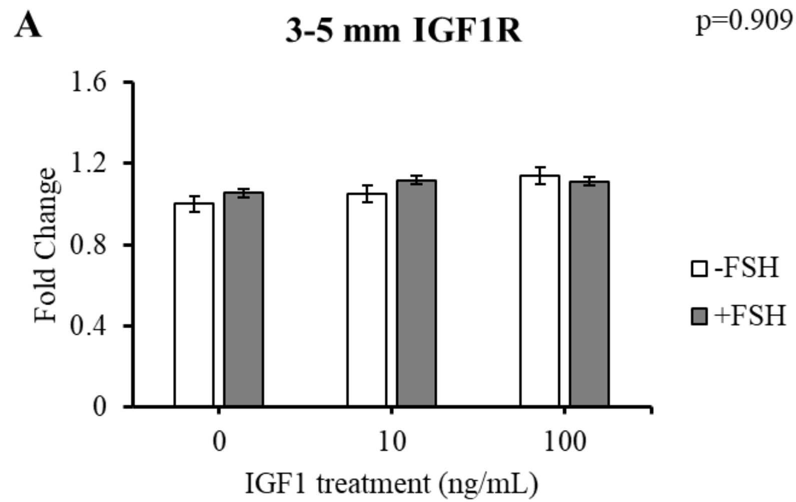
IGF1 in the presence or absence of FSH in granulosa cells of 3-5 mm follicles did not affect IGF1R expression (Figure 1A, $p=0.909$). At the 6-8 mm follicle stage, the high dose of IGF1 in the absence of FSH decreased IGF1R expression (Figure 1B, $p=0.009$); in the presence of FSH, however, IGF1R expression was not affected by IGF1. There was no effect on FSHR expression by IGF1 or FSH in granulosa cells from either 3-5 mm follicles (Figure 1C, $p=0.681$) or 6-8 mm follicles (Figure 1D, $p=0.197$).

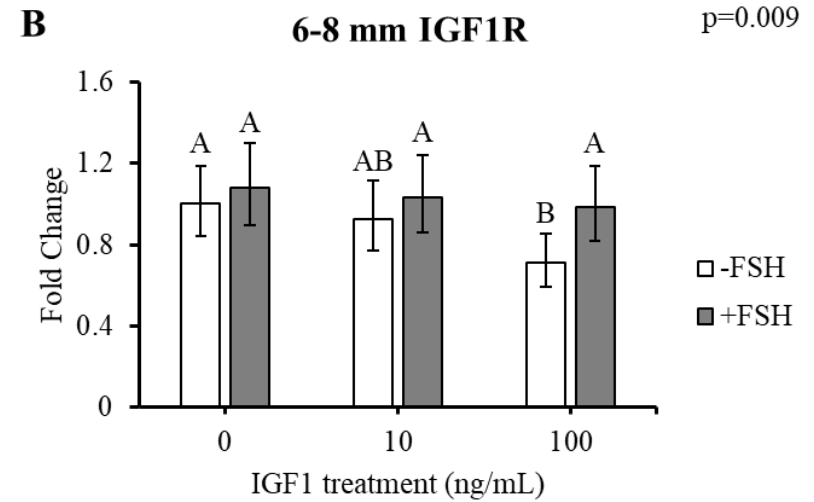
IGF1 and FSH effect on AMH in granulosa cells of 3-5 mm and 6-8 mm follicles

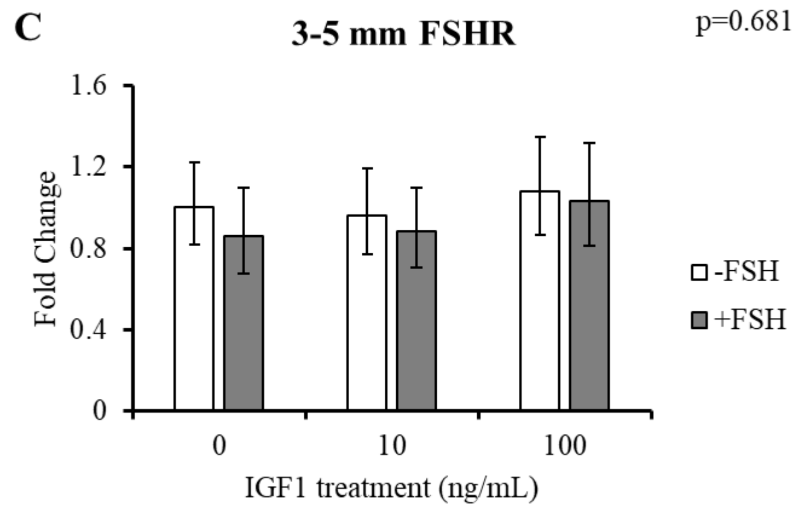
While IGF1 did not affect AMH mRNA expression in either follicle class, FSH significantly decreased AMH mRNA expression in granulosa cells from 3-5 mm follicles (Figure 2A, $p<0.001$). In granulosa cells of 6-8 mm follicles, FSH treatment did not decrease AMH expression compared to the control (Figure 2B).

IGF1 and FSH effect on inhibin and activin subunit expression in granulosa cells of 6-8 mm follicles

In granulosa cells of 6-8 mm follicles, IGF1 or FSH treatment alone did not affect the expression of INHA; in the presence of FSH, however, IGF1 at both doses significantly increased INHA mRNA expression (Figure 3A, $p=0.011$). IGF1 treatment significantly increased INHBA mRNA expression at both doses in the presence or absence of FSH (Figure 3B, $p<0.001$).


A
3-5 mm IGF1R
p=0.909
Fold Change
1.6
1.2
0.8
0.4
0
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH


B
6-8 mm IGF1R
p=0.009
Fold Change
A
A
AB
A
B
A
1.6
1.2
0.8
0.4
0
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH


C
3-5 mm FSHR
p=0.681
Fold Change
1.6
1.2
0.8
0.4
0
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH

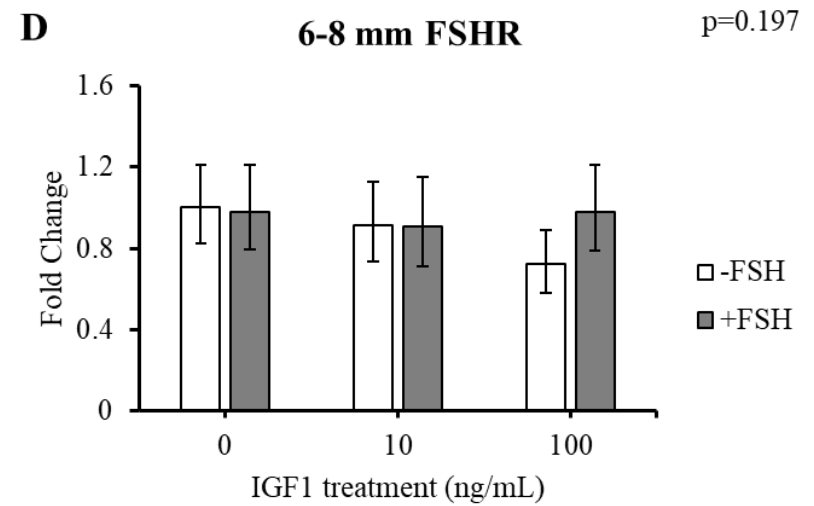

D
6-8 mm FSHR
p=0.197
Fold Change
1.6
1.2
0.8
0.4
0
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH

Figure 1. IGF1R and FSHR mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles. Bars represent means, and error bars represent 95% confidence intervals. Post hoc Tukey's HSD tests with $\alpha=0.05$ were performed to show group designation. Groups with different letters are significantly different from one another ($p<0.05$). **A.** Relative mRNA expression of IGF1R in granulosa cells of 3-5 mm follicles ($p= 0.909$, $n=5-7$). **B.** Relative mRNA expression of IGF1R in granulosa cells of 6-8 mm follicles ($p= 0.009$, $n=5-6$). **C.** Relative mRNA expression of FSHR in granulosa cells of 3-5 mm follicles ($p= 0.681$, $n=5-7$). **D.** Relative mRNA expression of FSHR in granulosa cells of 6-8 mm follicles ($p= 0.197$, $n=4-6$).

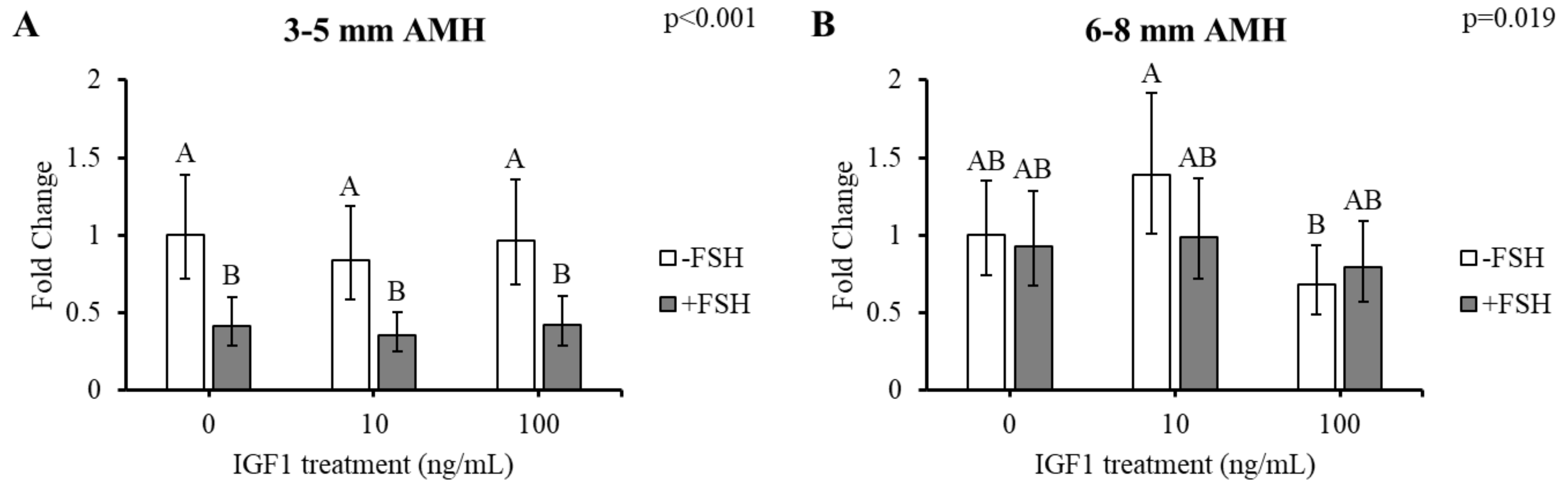


Figure 2. AMH mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles. Bars represent means, and error bars represent 95% confidence intervals. Post hoc Tukey's HSD tests with $\alpha=0.05$ were performed to show group designation. Groups with different letters are significantly different from one another ($p<0.05$). **A.** Relative mRNA expression of AMH in granulosa cells of 3-5 mm follicles ($p<0.001$, n=5-7). **B.** Relative mRNA expression of AMH in granulosa cells of 6-8 mm follicles ($p=0.019$, n=5-6).

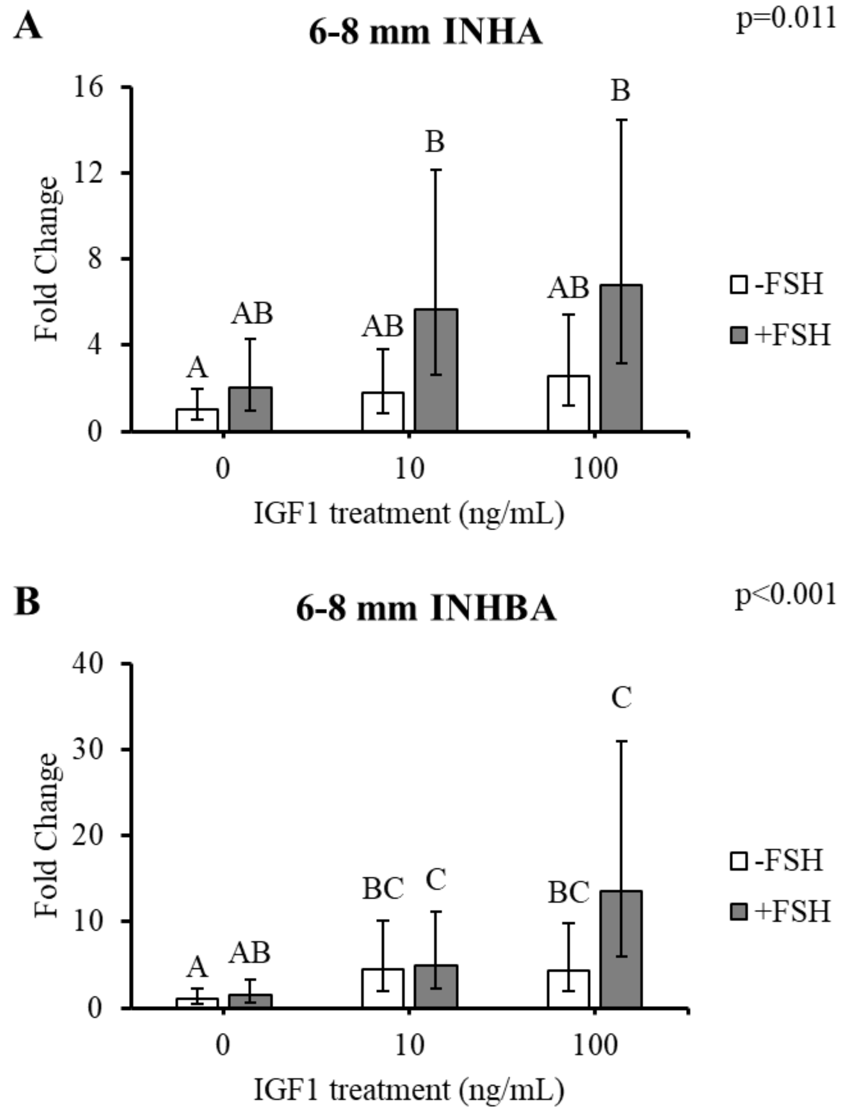
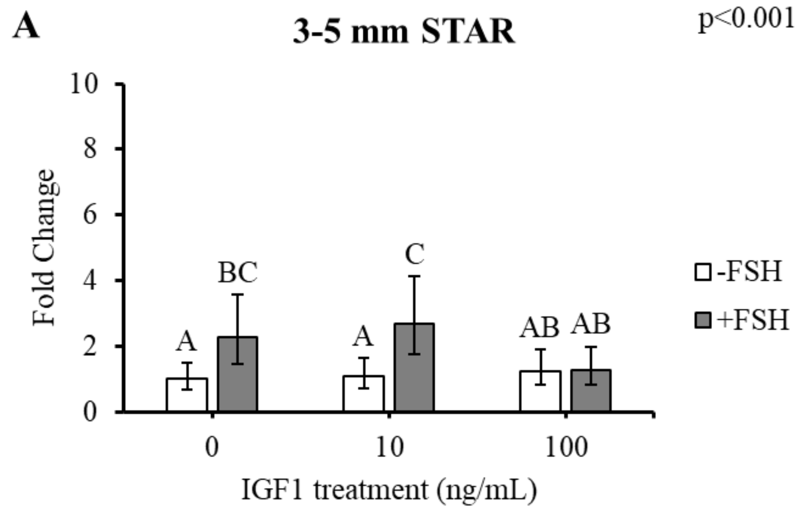


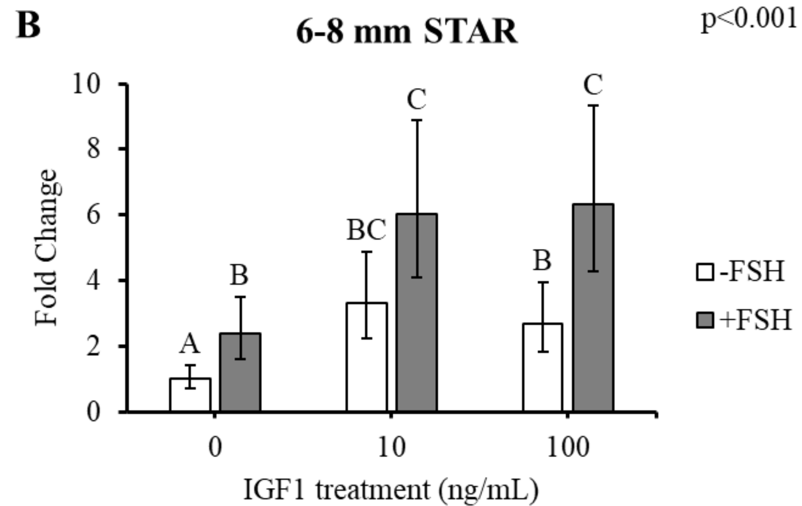
Figure 3. INHA and INHBA mRNA expression in cultured granulosa cells from 6-8 mm follicles. Bars represent means, and error bars represent 95% confidence intervals. Post hoc Tukey's HSD tests with $\alpha=0.05$ were performed to show group designation. Groups with different letters are significantly different from one another ($p<0.05$). **A.** Relative mRNA expression of INHA in granulosa cells of 6-8 mm follicles ($p=0.011$, $n=4$-5). **B.** Relative mRNA expression of INHBA in granulosa cells of 6-8 mm follicles ($p<0.001$, $n=5$-6).

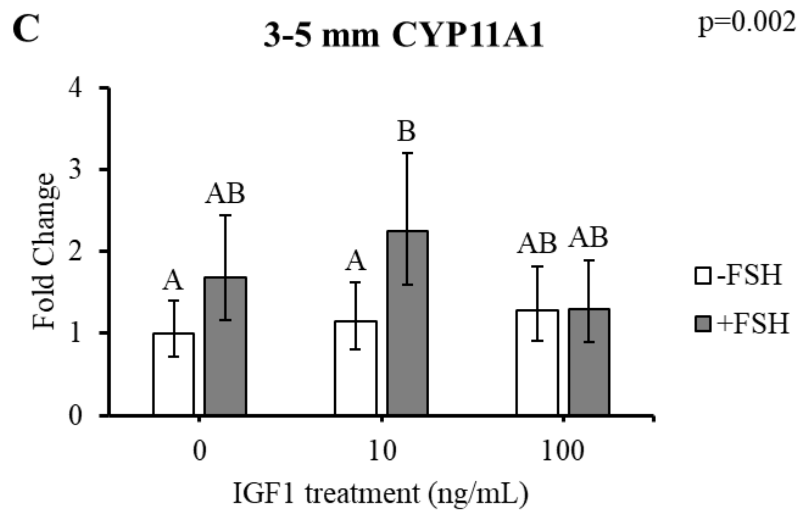
IGF1 and FSH effect on STAR and CYP11A1 expression in granulosa cells of 3-5 mm and 6-8 mm follicles

At the 3-5 mm follicle stage, IGF1 alone did not affect STAR mRNA expression (Figure 4A). FSH, however, alone and in the presence of low dose IGF1 ($p<0.001$), increased STAR mRNA expression. FSH did not increase STAR expression in combination with the high dose of IGF1 (Figure 4A) in granulosa cells of 3-5 mm follicles. In granulosa cells of 6-8 mm follicles, IGF1 alone significantly increased STAR mRNA expression (Figure 4B, $p<0.001$), and FSH treatment alone also increased STAR (Figure 4B, $p<0.001$). IGF1 in the presence of FSH significantly increased STAR mRNA expression, and this effect was synergistic at the high dose of IGF1 (Figure 4B, $p<0.001$).

At the 3-5 mm follicle stage, IGF1 did not increase CYP11A1 mRNA expression in the absence of FSH (Figure 4C). While FSH treatment alone did not affect CYP11A1 mRNA expression in granulosa cells of 3-5 mm follicles, the combination of FSH and low dose IGF1 significantly increased mRNA levels (Figure 4C, $p=0.002$). At the 6-8 mm follicle stage, low dose IGF1 but not high dose IGF1 significantly increased CYP11A1 mRNA levels in the absence of FSH (Figure 4D, $p<0.001$). FSH treatment alone significantly increased CYP11A1 mRNA expression at this stage of follicle development (Figure 4D, $p<0.001$). IGF1 at both doses in the presence of FSH significantly increased CYP11A1 mRNA expression, but this was not synergistic (Figure 4D, $p<0.001$).


A
3-5 mm STAR
p<0.001
Fold Change
10
8
6
4
2
0
A
BC
A
C
AB
AB
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH


B
6-8 mm STAR
p<0.001
Fold Change
10
8
6
4
2
0
A
B
BC
C
B
C
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH


C
3-5 mm CYP11A1
p=0.002
Fold Change
4
3
2
1
0
A
AB
A
B
AB
AB
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH

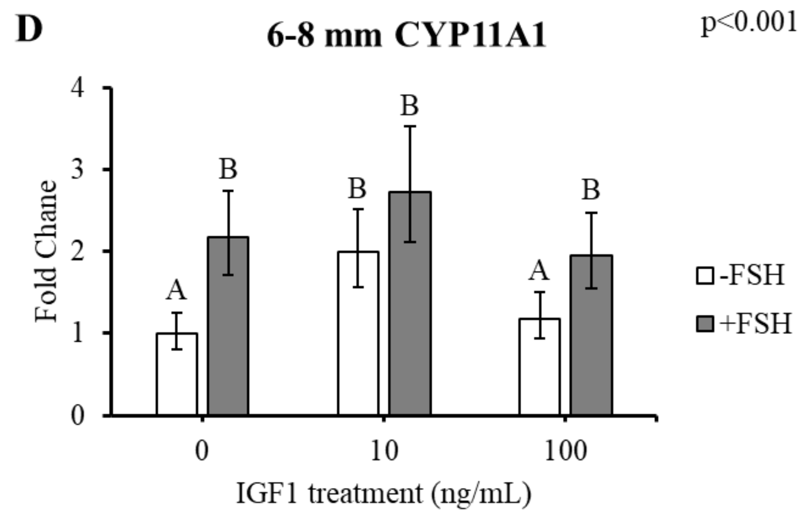

D
6-8 mm CYP11A1
p<0.001
Fold Chane
4
3
2
1
0
A
B
B
B
A
B
0
10
100
IGF1 treatment (ng/mL)
-FSH
+FSH

Figure 4. STAR and CYP11A1 mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles. Bars represent means, and error bars represent 95% confidence intervals. Post hoc Tukey's HSD tests with $\alpha=0.05$ were performed to show group designation. Groups with different letters are significantly different from one another ($p<0.05$). **A.** Relative mRNA expression of STAR in granulosa cells of 3-5 mm follicles ($p<0.001$, n=5-7). **B.** Relative mRNA expression of STAR in granulosa cells of 6-8 mm follicles ($p<0.001$, n=5-6). **C.** Relative mRNA expression of CYP11A1 in granulosa cells of 3-5 mm follicles ($p=0.002$, n=5-7). **D.** Relative mRNA expression of CYP11A1 in granulosa cells of 6-8 mm follicles ($p<0.001$, n=4-6).

IGF1 and FSH effect on cell proliferation and steroidogenesis in granulosa cells of 6-8 mm follicles

Treatment of granulosa cells from 6-8 mm follicles with IGF1 increased cell proliferation at low and high doses (Figure 5A, $p<0.001$). While FSH treatment alone did not affect cell proliferation, treatment with FSH and the high dose of IGF1 significantly increased cell proliferation (Figure 5A).

IGF1 did not increase progesterone production at either dose (Figure 5B). FSH treatment significantly increased the production of progesterone alone and in the presence of both doses of IGF1 (Figure 5B, $p<0.001$).

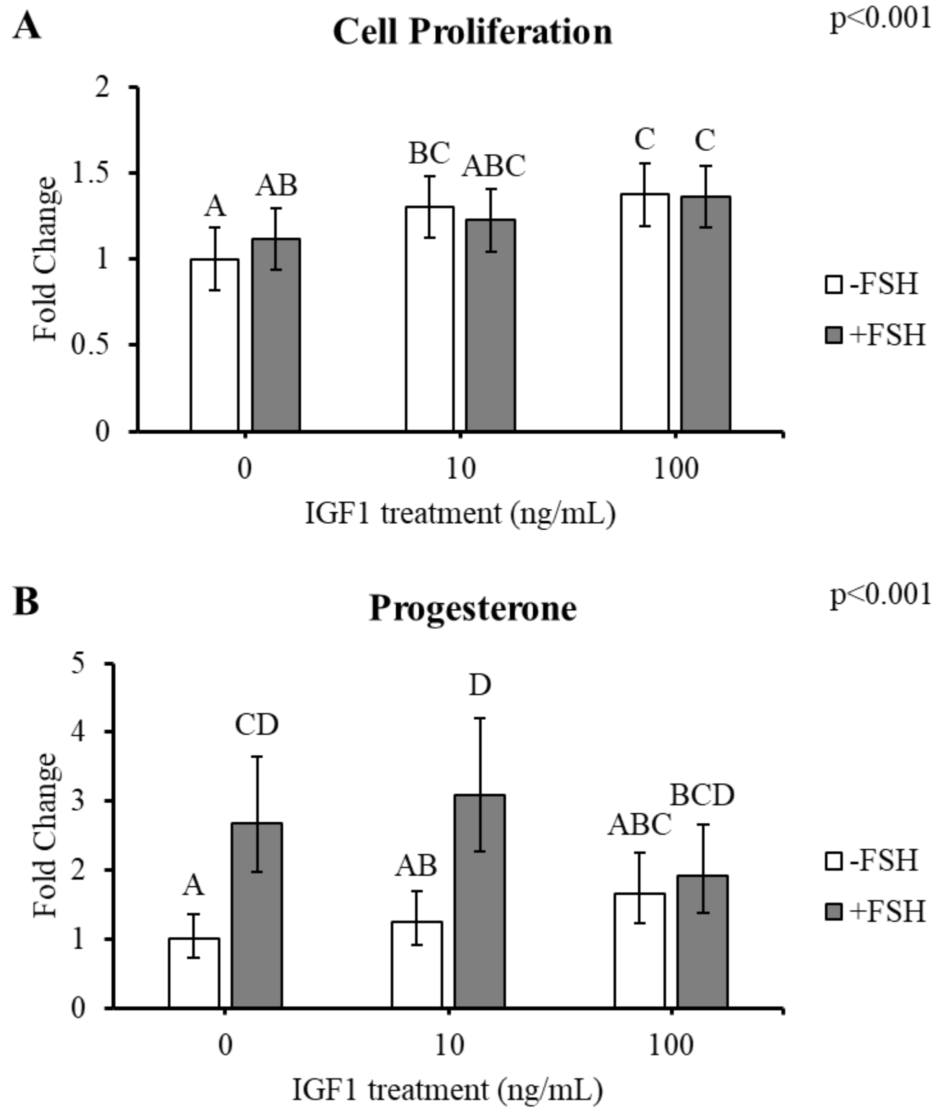


Figure 5. Cell proliferation and progesterone production in cultured granulosa cells from 6-8 mm follicles. Bars represent means, and error bars represent 95% confidence intervals. Post hoc Tukey's HSD tests with $\alpha=0.05$ were performed to show group designation. Groups with different letters are significantly different from one another ($p<0.05$). **A.** Cell proliferation is represented by average absorbance normalized to the control within each replicate ($p<0.001$, n=8). **B.** Average progesterone concentration (pg/ml) normalized to the control within each replicate ($p<0.001$, n=6-7).

Discussion

IGF1 is a well-known regulator of mammalian follicle development [15,16,28–31]. Its role in preovulatory avian follicle development has received limited attention [14,18,19]; however, little is known about its effect on prehierarchal follicles. This study aimed to investigate the role of IGF1 on prehierarchal follicle function in the laying hen and as a model for broiler breeder hens that have increased IGF1 bioavailability when fed *ad libitum* [21]. Granulosa cells from prehierarchal follicles of laying hens were treated with IGF1 in the presence or absence of FSH. Broiler breeder hens fed *ad libitum* have higher levels of liver IGF1 expression [21], increased numbers of preovulatory follicles [21,22,32], and decreased reproductive efficiencies [21,22] compared to restricted-fed hens. IGF1 was identified as an upstream regulator of differentially expressed genes in granulosa cells of 6-8 mm prehierarchal follicles in broiler breeder hens [22]. We hypothesized that IGF1 might affect gene expression of factors around the time of follicle selection which could lead to the increased number of preovulatory follicles observed in broiler breeder hens fed *ad libitum* [21,22,32].

Our results demonstrated that levels of markers of follicle development (STAR, CYP11A1, INHA, and INHBA) are enhanced by IGF1, indicating that IGF1 (along with FSH in the case of STAR and INHA) may be promoting follicle selection in granulosa cells of 6-8 mm follicles. STAR, CYP11A1, and INHA were upregulated and predicted to be downstream of IGF1 and FSH in granulosa cells of 6-8 mm follicles from *ad libitum*-fed broiler breeder hen [22]. In the present study, we confirmed that IGF1 and FSH regulate expression of these genes and synergistically affect STAR expression. The effect of increased feeding on FSH plasma levels in

broiler breeder hens is not well-defined as some studies find increased [33–35], decreased [32], or unchanged [36] plasma FSH levels in response to feeding levels. Increased IGF1 bioavailability in broiler breeder hens fed *ad libitum* [21] may be sufficient to advance steroidogenesis and potentially disrupt the follicle hierarchy, regardless of any change in FSH levels. Future research in an *in vivo* model is required to confirm that increased IGF1 leads to an increased number of selected follicles in broiler breeder hens.

At the time of follicle selection in hens, FSHR expression increases in one 6-8 mm follicle [4], facilitating an increase in STAR [4,9] and CYP11A1 expression [10] to promote steroidogenic competency [4,5]. We had anticipated that IGF1 and FSH might increase FSHR expression, as previously shown in chicken granulosa cells of 6-8 mm follicles [4]. In the present study, however, neither IGF1 nor FSH affected FSHR expression in 3-5 mm or 6-8 mm follicles. A different dose (50 ng/mL) of IGF1 was used in the previous experiment, which could explain the differences between these studies. It is unclear whether IGF1 and FSH affect STAR and CYP11A1 expression through increased expression of FSHR.

IGF1 treatment in granulosa cells from chicken preovulatory follicles increases STAR and CYP11A1 expression to promote progesterone production [14], and in mammals, IGF1 synergizes with FSH to produce these effects [28–30]. In the current study, we found that IGF1 treatment alone significantly increased STAR expression at the 6-8 mm stage but not at the 3-5 mm stage. FSH alone, however, was sufficient to increase STAR expression at both stages of prehierarchal follicles. Interestingly, in 6-8 mm follicles, IGF1 and FSH co-treatment increased STAR expression synergistically,

as also occurs in mammalian granulosa cells [28]. CYP11A1 expression increased only by the combination treatment of the low dose of IGF1 and FSH at the 3-5 mm follicle stage and by either the low dose of IGF1 or FSH at the 6-8 mm follicle stage. The high dose of IGF1 failed to affect CYP11A1 expression at the 6-8 mm follicle stage, and this may be due to the lower expression of IGF1R at this dose. Although IGF1 increased STAR and CYP11A1 expression, we did not find any effect on progesterone production. Our results are consistent with a previous study that showed that IGF1 treatment of cultured 6-8 mm granulosa cells did not increase progesterone production [37]. It is possible that IGF1 increases STAR and CYP11A1 and prepares the cell to become steroidogenically competent, but progesterone production requires further differentiation. Additionally, 24 hours of culture may not be sufficient time to observe the effects on progesterone production driven by IGF1.

Inhibins play an essential role in the regulation of FSH. Inhibin A is produced primarily by preovulatory follicles, and inhibin B is produced by small follicles in hens [6]. In chicken granulosa cells from preovulatory follicles, IGF1 stimulates INHA mRNA expression [38] and increases inhibin A production, an effect that is amplified in the presence of FSH [19]. In our experiment, INHBA expression in granulosa cells from 6-8 mm follicles increased significantly with IGF1 alone and with IGF1 and FSH co-treatment. In hens, INHBA mRNA normally is expressed in follicles greater than 12 mm and is highest in the F1 follicle [39]. It was intriguing that IGF1 increased INHBA in granulosa cells of 6-8 mm follicles, considering that inhibin A production usually begins following follicle selection [6]. Preliminary data from our lab showed that IGF1 significantly decreased INHBB mRNA expression, (inhibin βB-

subunit), in granulosa cells of 6-8 mm follicles (unpublished). An increase in INHBA expression likely signifies a transition from inhibin B to inhibin A production. As inhibin B has been shown to have a stronger negative effect on FSH than inhibin A [40], increased IGF1 expression in *ad libitum* broiler breeder hens [21] may make these hens less effective at maintaining the strict follicle hierarchy as follicles transition prematurely from inhibin B to inhibin A production.

In our experiment, IGF1 did not affect AMH expression in either 3-5 mm or 6-8 mm follicles. Interestingly, FSH decreased AMH levels in cultured granulosa cells of 3-5 mm follicles but did not affect levels in granulosa cells of 6-8 mm follicles. AMH expression decreases with follicle size, and levels may have already been too low to detect a difference in 6-8 mm follicles [7]. Similar results were found in cultured bovine granulosa cells where FSH decreased AMH expression in 3-5 mm but not in 5-10 mm follicles [41]. While the function of AMH in chicken folliculogenesis is unknown, it likely has a similar role in avian and mammalian species, given its expression pattern. In mammals, AMH restricts folliculogenesis by inhibiting primordial follicle activation [42], decreasing FSH sensitivity [43], and inhibiting FSH-induced steroidogenesis [44,45]. If AMH plays a similar role in chicken follicle development, the decrease in AMH expression by FSH may remove a restraint on follicle development. In a human ovarian granulosa-like tumor cell line, FSH decreased AMH expression by increasing gonadotropin inducible ovarian transcription factor 1 (GIOT1), which represses AMH transcription [46]. Although the mechanism of AMH regulation by FSH remains to be explained in chicken granulosa cells, it is

possible that FSH directly regulates AMH through its second messenger or other factors within the pathway.

In conclusion, IGF1 may promote follicle development by increasing the expression of genes involved in steroidogenesis (STAR and CYP11A1) and FSH regulation (INHA and INHBA) in prehierarchal follicles. Our findings of an increased expression of genes associated with follicle selection suggest that increased IGF1 in broiler breeder hens fed *ad libitum* [21] may contribute to increased follicle selection of the small growing follicles. High IGF1 bioavailability could explain the disruptions observed in follicle development in these hens and provide a link between increased feeding levels and reproductive efficiency.

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CHAPTER 4

CHARACTERIZATION OF PERSISTENCY OF LAY IN LAYING HENS[a]

Abstract

Intense selective breeding in laying hens has produced a hen that can lay at a highly efficient rate of approximately one egg per day during the first year of lay. The flock's egg-laying average declines after the first year, leading producers to cull hens. There is considerable variability within the flock, with some hens continuing to lay at a high rate while others decline below the flock average. In this study, we sought to understand physiological and transcriptional differences between hens with high egg-laying rates after the first year (persistent layers=PL, egg-laying rate = 100%) and those with decreased rates (non-persistent layers=NPL, egg-laying rate ≤ 82%). Forty laying hens were reared from 16 to 96 weeks of age. Egg-laying data were collected daily, and plasma samples and body weights were taken twice monthly from 16 to 28 weeks of age and then monthly. Persistency was evaluated at 76 weeks, and 7 PL hens and 6 NPL hens were identified. Egg-laying efficiency, body weight, and plasma hormone concentrations were analyzed retrospectively to identify physiological differences. Ovarian tissue was collected from a subset of hens (PL n=3 and NPL n=4) at this time for gene expression analysis, RNA-sequencing, and histological analysis. Daily egg-laying data and monthly body weights were collected for the remaining hens until 96 weeks. PL hens had significantly higher egg-laying rates from 68 weeks of age and higher body weights from 32 weeks of age compared to NPL hens. Based

a. In collaboration with Deena M. Scoville who participated in collecting egg-laying and body weight data, and plasma and tissue samples and Jaclyn G. Ahern who participated in conducting the Apoptag assay and histological analysis.

on correlation analysis, egg-laying efficiency, and body weight earlier in life had predictive value for persistency. At the time of euthanasia, PL hens had significantly more 3-5 mm and >12 mm follicles than NPL hens. PL hens had higher AMH and BMP15 mRNA expression in ovarian samples, suggesting they may have a larger ovarian reserve than NPL hens. From histological analysis, PL and NPL hens had similar numbers of <500 µm follicles per cortex area, but PL hens had lower rates of follicle atresia in follicles <100 µm. RNA-sequencing analysis of ovarian samples revealed 279 differentially expressed genes between PL and NPL hens. Although plasma estradiol levels did not differ between PL and NPL at any point during the experiment, upstream regulator analysis using Qiagen's Ingenuity Pathway Analysis revealed the estrogen receptor as a predicted upstream regulator of DEGs. A greater understanding of the physiology of persistent hens could enhance production efficiency, producer profit, and animal welfare by allowing for the identification of persistent hens to maintain in the flock or by leading to strategies for genetic selection.

Introduction

Laying hens have been bred to have highly efficient egg-laying rates and lay approximately one egg per day during their first year. In 2020, the U.S. poultry industry produced 112 billion eggs, equivalent to $8.66 billion [1]. The production of an egg from ovulation to oviposition takes approximately 24-28 hours, suggesting little room for improvement in reproductive efficiency during the first year of lay. After the first year, the average egg-laying rate of a flock declines [2,3], and there is significant variability within the flock as some hens continue to lay at 100% efficiency (1 egg per day) and others decline to ~80% or lower. This variability leads many egg producers to cull hens when egg production declines. Prolonging the egg-laying period would increase environmental sustainability, increase producer profit, and improve animal welfare by requiring fewer hens to be raised for the same number of eggs and decreasing replacement costs.

Efficient egg-laying depends on several factors, including nutrition, photostimulation, and follicle development. An efficient laying rate is contingent on a hen having an organized follicle hierarchy. Hens are hatched with a finite number of oocytes, estimated at approximately 480k oocytes [4], which are maintained for the length of a hen's reproductive lifetime. Many follicles will degenerate through follicle atresia, typically before the 8 mm stage [5]. To sustain an egg-laying rate of one egg per day, the largest follicle in the ovarian hierarchy ovulates, and one 6-8 mm follicle is selected and enters the preovulatory hierarchy (reviewed in [6,7]). As hens age, the number of follicles in the hierarchy decreases [3], and the incidence of atresia increases [2,8], resulting in decreased egg production.

The source of variation between persistent-laying (PL) and non-persistent laying (NPL) hens is unknown. Subtle endocrine or genetic differences among hens could cause differences in production. PL hens may also have a larger ovarian reserve or have lower rates of follicle atresia than NPL hens. In this study, we characterized hens as PL or NPL after having monitored the hens from hatch to 76 or 96 weeks of life. We then retrospectively evaluated egg production, body weight, plasma estradiol, and thyroid hormones throughout their life. Ovaries from PL and NPL hens were analyzed at 76 weeks of age to identify differences in ovarian weight, follicle numbers, atresia, and gene expression that might explain differences observed in persistency. Finally, we performed an RNA-sequencing analysis of the ovary to identify transcriptional differences associated with persistency. Ultimately, a greater understanding of persistency could lead to developing decision-making tools for producers to identify persistent layers to maintain in a flock.

Materials and Methods

Animals

Single-comb White Leghorn hens (Shaver; n = 40) were reared in individual cages. Hens were given feed and water *ad libitum* and maintained on a 16L:8D cycle. Egg-laying data were collected daily starting at 16 weeks of age, and age at sexual maturity (first egg) was noted. Blood samples from the brachial vein and body weights were taken from hens every 2 weeks from 16 to 28 weeks of age and then monthly until 96 weeks of age. Egg-laying efficiency was determined as the number of eggs per number of days in a set period, with 100% efficiency meaning daily oviposition.

This was calculated every two weeks from 16 weeks until 28 weeks and then monthly until 96 weeks of age. Hens that had molted (8 hens), were diagnosed with ovarian cancer at necropsy (3 hens) or died before the completion of the experiment (4 hens) were excluded from the analysis. One hen was excluded due to late onset of sexual maturity. The final population evaluated at 76 weeks consisted of 24 hens. At this time, we stratified the hens according to egg-laying efficiency, and hens were characterized as persistent laying (PL, n=7, egg-laying rate= 100%) or non-persistent laying (NPL, n=6, egg-laying rate ≤82%), based on upper and lower quartiles respectively. A subset of this group (n = 3 PL; n = 4 NPL) was selected for tissue collection and RNA-sequencing analysis (see below). Hens were euthanized using CO_2, and tissue samples were collected using the methods described below. The remaining 17 hens were followed until 96 weeks of age with continued collection of daily egg-laying data and monthly body weight data.

All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University (IACUC protocol number 2009-0036).

Tissue collection

Following euthanasia at 76 weeks of age, ovaries from the subset of PL (n=3) and NPL hens (n=4) were removed and placed in ice-cold Krebs-Ringer bicarbonate buffer. Ovaries were weighed, and follicles were removed from the ovary, separated by size (3-5, 6-8, 9-12, and >12 mm), and counted. Granulosa cells from five to six follicles from the 3-5 mm and 6-8 mm follicle pools were collected and pooled within their size category following the procedure outlined in Wang *et al.* [9]. Samples were

stored in RLT lysis buffer at -80°C until further processing. Ten 1 mm follicles from each hen were collected, snap-frozen, and stored at -80°C until subsequent mRNA extraction. One piece of the ovary was snap-frozen and stored at -80°C until further processing, and a second piece was fixed in 10% formalin and embedded in paraffin for histological analysis.

Primary follicle counts and Apoptag

Three 5 µm sections (50 µm apart), stained with hematoxylin-eosin stain (H&E; n = 3 PL; n = 4 NPL), and three adjacent unstained slides per hen (n = 3 PL; n = 3 NPL) were prepared by the Cornell University Histology Lab. H&E slides were imaged at 20X using Aperio ScanScope (Leica BioSystems, Wetzlar, Germany), and images were analyzed with Aperio ImageScope (Leica BioSystems, Wetzlar, Germany) using histology descriptions from Apperson *et al.* as a guide [10]. The ovarian cortex area for each section was demarcated from the medulla and measured in ImageScope. Follicles with a visible oocyte nuclear membrane were counted in each section (Figure 4E), and follicle diameters were estimated by averaging the diameter of the follicle at the two longest perpendicular lengths. Follicles were characterized by size (<100, 100-200, 200-300, 300-400, and 400-500 µm), and the larger follicles (300-400 and 400-500 µm) were only counted from the first ovarian section to prevent double counting. Follicles from three sections were summed and normalized to the cortex area of the first section to obtain the follicle density for each hen.

Atretic follicles were identified using the adjacent unstained sections with a TUNEL assay (ApopTag Peroxidase In Situ Apoptosis Detection Kit; S7101, Millipore Sigma, Burlington, MA, USA), according to the manufacturer's protocol. Follicles having >30% apoptotic granulosa cells were characterized as atretic as defined in Yu *et al*., 2004 [11]. The rate of atresia per follicle size category (number of atretic follicles per size category/total number of follicles in each category) was assessed in ovarian sections from PL and NPL hens (n=3 hens per group).

Plasma hormone assay

Blood samples were collected from the brachial vein using heparinized needles and placed on ice until processing. Samples were centrifuged for 10 minutes at 4 °C at 2000 rpm. Plasma was collected, and samples were stored at -20 °C with 30 µL of sodium citrate until assayed. Plasma estradiol was measured in PL (n=3) and NPL (n=4) samples from 16 to 76 weeks of age using an Estradiol Coated Tube Radioimmunoassay Kit (MP Biomedicals, Santa Ana, CA, USA). Plasma samples were diluted 1:4 with a rooster plasma pool (endogenously low in estradiol) to dilute samples to the assay range. The intra-assay CV was 13.9%. Plasma triiodothyronine (T3) and thyroxine (T4) concentrations were assessed from PL (n=7) and NPL hens (n=6) by Cornell University College of Veterinary Medicine Animal Health Diagnostic Center Endocrinology lab.

RNA extraction, Reverse Transcription, and Real-Time qPCR

Total RNA was extracted from 1 mm whole follicles and granulosa cells from 3-5 and 6-8 mm follicles using an RNeasy Mini kit with optional on-column DNase treatment (Qiagen Inc., Valencia, CA). Total RNA was extracted from a ~10 mg sample of the ovarian body using Trizol reagent (ThermoFisher Scientific, Waltham, MA, USA). Ovarian RNA was cleaned using an RNeasy Mini kit with optional on-column DNase treatment. The quantity and purity of the samples were analyzed using spectrophotometry (Implen, Munich, Germany).

Reverse transcriptase reactions were performed using 1 µg of total mRNA in a 20 µL volume using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative polymerase chain reactions (RT-qPCR) were performed using the AB StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). One µg of cDNA was added to each reaction. Reactions for unknown samples, standards, no template controls, and reactions lacking reverse transcriptase were analyzed in duplicate in a 25 µL volume. Reactions contained a final concentration of 1X Power SYBR Green (Applied Biosystem, Foster City, CA, USA) and 300 nM primers. Unknown sample expression was determined from the standard curve (constructed from 1 mm and 3 mm whole follicles, or 3-8 mm granulosa cells) and normalized to 18S. 18S primers from Ambion® (QuantumRNA™ 18S Internal Standard; ThermoFisher Scientific, Waltham, MA, USA) were used to determine 18S expression, and other primer sequences used are listed in Table 1.

Table 1. Primer Sequences for RT-qPCR

Target Gene	Primer Sequence	Citation
AMH	F: 5’-CCCCTCTGTCCCTCATGGA-3’ R: 5’-CGTCATCCTGGTGAAACACTTC-3’	Stephens *et al.* [12]
BMP15	F: 5’-ACATGCTGGAGCTGTACCAA-3′ R: 5′-GACACGGAGAAGGTGCTCA-3′	Stephens *et al.* [12]
ESR1	F: 5’-TGCAACGACTATGCTTCAGG-3’ R: 5’-CTGGTTAGTGGCAGGACACA-3’	Stephens *et al.* [13]
ESR2	F: 5’-AATTTTCGCACCAGACCTTG-3’ R: 5’-GGCCAGGAGCATATCAAAGA-3’	Stephens *et al.* [13]

RNA sequencing and quality control

RNA integrity from extracted ovarian RNA (n =3 PL; n =4 NPL) was determined by Fragment Analyzer (Advanced Analytical, Ames IA) and all samples had an RNA integrity number (RIN) >7.5. RNA samples were submitted to Cornell University Transcriptional Regulation & Expression Facility for cDNA library preparation, RNA-sequencing, and differential expression analysis. Samples were enriched by PolyA+ RNA isolation using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA). Libraries were generated using the NEBNext Ultra II [Directional] RNA Library Prep Kit (New England Biolabs, Ipswich, MA). Before sequencing, cDNA libraries were quantified using a Qubit 2.0 (dsDNA HS kit; Thermo Fisher, Waltham MA), and libraries were sequenced on Illumina’s NextSeq500 (Illumina, San Diego, CA) at a depth of 75 bp for a minimum of 32M reads per sample. FastQ files were first processed through

trim-galore (Barbraham Institute, Cambridge, UK) to trim adaptors and filter for low-quality reads as a quality control step. The sample files were then aligned to the Galgal6 genome using STAR [14]. A cluster analysis was run using hclust in R to verify sample clustering by biological replicate. A principal component analysis (PCA) was performed to visualize variance among samples.

RNA sequencing analysis

Differential expression analysis was conducted to identify differentially expressed genes (DEGs) between ovaries from PL and NPL hens using DeSEQ2. Criteria for differentially expressed genes were a false discovery rate (FDR) < 0.05, a $\log_2$ fold change ($\log_2$(FC)) >1, and a minimum read count of 50. Enrichment analysis with an FDR cutoff of 0.05 was conducted using ShinyGo v0.61 [15] to generate the ten most significant terms. Differentially expressed genes were analyzed using Qiagen's Ingenuity Pathway Analysis (IPA) to identify predicted upstream regulators [16].

Statistical Analysis

Total eggs, age at first egg, ovarian weights, follicle numbers, and gene expression were compared between PL and NPL selected at 76 weeks of age with linear regression analysis using PROC GLM in SAS v9.4. An analysis of simple effects was performed for weights, egg-laying efficiencies, and plasma estradiol concentrations comparing PL and NPL each week from 16-76 weeks of age using PROC MIXED with repeated measures and a slice statement in SAS v9.4. T3 and T4

plasma concentrations at 26 and 76 weeks of age in PL and NPL hens were compared with a linear regression analysis using PROC MIXED with repeated measures. A Post-hoc Tukey test was done with α=0.05 for T3 plasma concentrations. Correlation analysis of egg-laying efficiencies and body weights from ages 16 to 96 weeks of age compared with egg-laying efficiency and total eggs laid at age 76 and 96 weeks of age was conducted using PROC CORR in SAS v9.4.

Results

PL hens laid more total eggs and had higher egg-laying efficiencies and body weights than NPL hens

PL and NPL hens reached sexual maturity at the same age (142 ± 12.34 vs. 143 ± 10.49 days, p=0.88, n=6-7). PL hens laid 385.1 ± 8.9 total eggs by 76 weeks of age compared to 356.7 ± 17.2 total eggs laid by NPL hens (p<0.01, n=6-7). Starting at 68 weeks of age, PL hens had significantly higher egg-laying efficiencies than NPL hens, and this trend continued until 76 weeks of age (Figure 1A, p<0.05, n=6-7). PL hens were significantly heavier than NPL hens from 32 to 76 weeks of age (Figure 1B, p<0.05, n=6-7).

A correlation analysis revealed that egg-laying efficiency at 76 weeks of age was highly correlated with egg-laying efficiencies at 32, 40, 48, 52, and 60-72 weeks of age. In comparison, egg-laying efficiency at 96 weeks of age was highly correlated with egg-laying efficiency at 52, 76, 80, and 92 weeks of age. Total eggs laid at 76 weeks of age correlated with egg-laying efficiencies from 20-24, 36, 40, and 52-76 weeks of age. Total eggs laid at 96 weeks of age correlated with egg-laying

efficiencies at 22, 24, 36, 40, 52, and 64-96 weeks of age. Results are summarized in Table 2.

Body weight at 26-64 and 76 weeks of age was significantly correlated with egg-laying efficiency at 76 weeks of age. Body weight at 16-20, 26, and 32-76 weeks of age was also highly correlated with total eggs laid by 76 weeks, and body weight at 18-20, 32-68, and 76 weeks of age was highly correlated with total eggs laid by 96 weeks. Results are summarized in Table 3.

Plasma estradiol concentrations did not differ between PL and NPL hens from 16 to 76 weeks of age

From 16 to 76 weeks of age, plasma estradiol did not differ between PL and NPL hens at any timepoint (Figure 9, $p>0.05$, $n=3-4$).

PL hens had more visible follicles than NPL hens at 76 weeks of age

A representative picture of a PL and NPL ovary is in Figures 3A and 3B, respectively. Ovaries of PL hens did not differ in weight from those of NPL hens (Figure 3C, $p=0.08$, $n=3-4$). PL hens had significantly more 3-5 mm ($p=0.04$, $n=3-4$) and preovulatory follicles (>12 mm, $p=0.01$, $n=3-4$); however, the number of 6-8 mm follicles and 9-12 mm follicles did not differ between PL and NPL hens (Figure 3D, $p>0.05$, $n=3-4$).

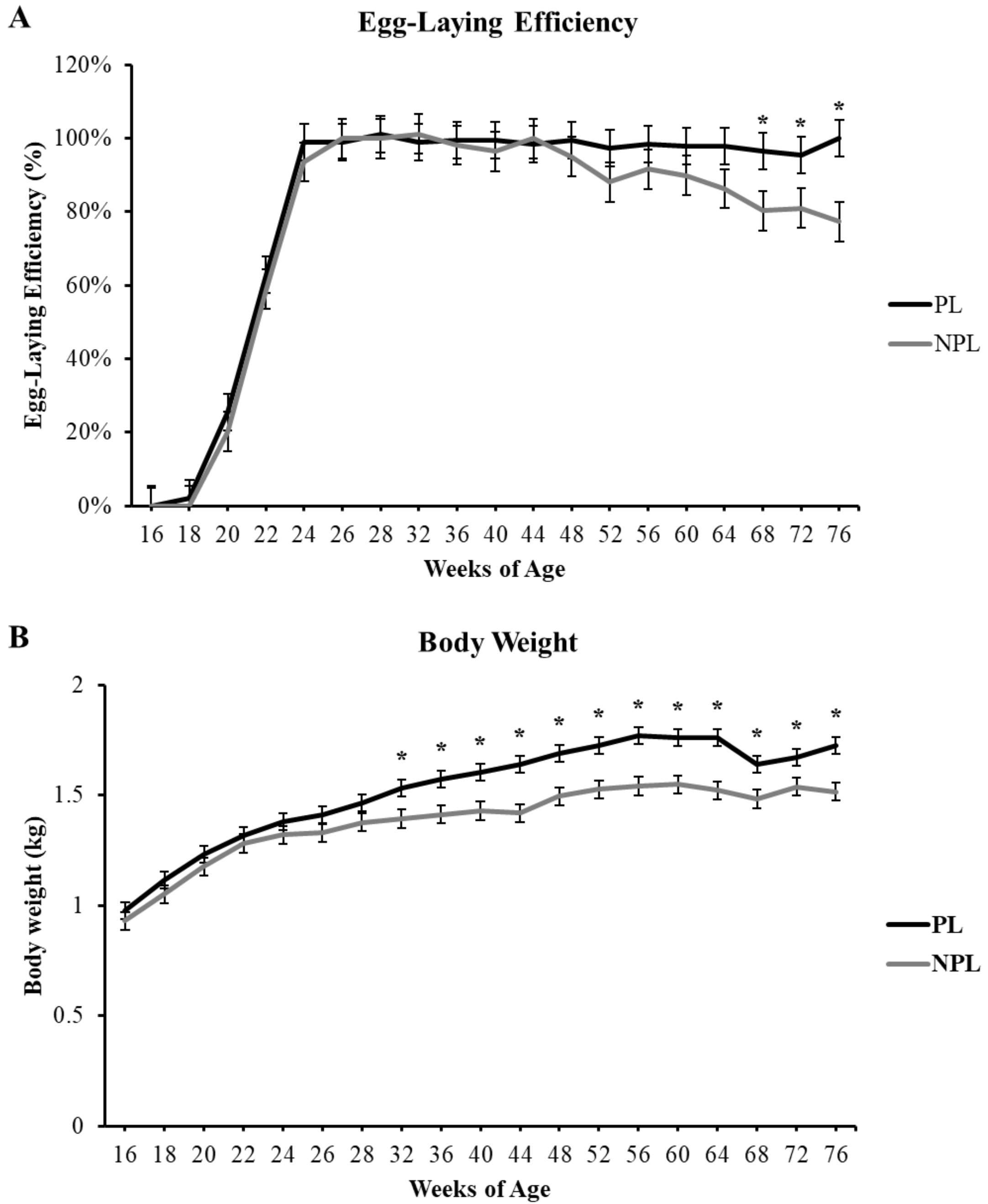


Figure 1. Egg-laying efficiency and body weights of PL (n=7) and NPL (n=6) hens from 16 to 76 weeks of age. **A.** Egg-laying efficiency for PL and NPL hens. **B.** Body weights for PL and NPL hens. Data points represent means ± SE; p-values for comparisons are indicated as *, $p<0.05$.

Table 2. Correlation analysis of egg-laying efficiencies from 16 to 96 weeks of age. Correlation coefficients and p-values for egg-laying efficiencies compared with egg-laying efficiency and total eggs laid at 76 (n=24) and 96 weeks of age (n=17).

	Egg-laying Efficiency at 76 weeks (n=24)		**Egg-laying Efficiency at 96 weeks (n=17)**		**Total eggs laid at 76 weeks (n=24)**		**Total eggs laid at 96 weeks (n=17)**	
Age (weeks)	**Correlation coefficient**	**p-value**	**Correlation coefficient**	**p-value**	**Correlation coefficient**	**p-value**	**Correlation coefficient**	**p-value**
16	.	.	.	.	.	.	.	.
18	0.20	0.345	.	.	0.30	0.155	.	.
20	0.02	0.919	0.10	0.708	0.49	**0.015**	0.41	0.107
22	0.07	0.757	-0.15	0.558	0.65	**<0.001**	0.48	**0.052**
24	0.29	0.173	0.45	0.070	0.51	**0.012**	0.77	**<0.001**
26	-0.16	0.451	0.29	0.257	-0.28	0.182	-0.34	0.178
28	0.06	0.770	0.00	1.000	0.06	0.798	0.07	0.786
32	-0.42	**0.043**	-0.29	0.261	-0.22	0.300	-0.28	0.272
36	0.27	0.208	0.09	0.736	0.49	**0.016**	0.53	**0.028**
40	0.54	**0.006**	0.35	0.163	0.74	**<0.001**	0.68	**0.003**
44	-0.24	0.257	0.03	0.912	-0.23	0.289	-0.37	0.149
48	0.54	**0.006**	0.17	0.503	0.36	0.083	0.29	0.257
52	0.60	**0.002**	0.76	**<0.001**	0.61	**0.001**	0.57	**0.016**
56	0.35	0.092	0.13	0.629	0.45	**0.026**	0.34	0.188
60	0.48	**0.019**	-0.08	0.746	0.50	**0.012**	0.05	0.836
64	0.76	**<0.001**	0.25	0.329	0.75	**<0.001**	0.67	**0.003**
68	0.61	**0.002**	0.45	0.069	0.80	**<0.001**	0.70	**0.002**
72	0.71	**<0.001**	0.25	0.332	0.70	**<0.001**	0.65	**0.005**
76	1.00	.	0.57	**0.016**	0.67	**<0.001**	0.78	**<0.001**
80	.	.	0.64	**0.005**	.	.	0.58	**0.016**
84	.	.	0.31	0.229	.	.	0.69	**0.002**
88	.	.	0.32	0.216	.	.	0.69	**0.002**
92	.	.	0.53	**0.030**	.	.	0.81	**<0.001**
96	.	.	1.00	.	.	.	0.57	**0.018**

Table 3. Correlation analysis of body weights from 16 to 96 weeks of age. Correlation coefficients and p-values for body weights compared with egg-laying efficiency and total eggs laid at 76 (n=24) and 96 weeks of age (n=17).

Age (weeks)	Egg-laying Efficiency at 76 weeks (n=24)		Egg-laying Efficiency at 96 weeks (n=17)		Total eggs laid at 76 weeks (n=24)		Total eggs laid at 96 weeks (n=17)	
	Correlation coefficient	p-value	Correlation coefficient	p-value	Correlation coefficient	p-value	Correlation coefficient	p-value
16	0.16	0.467	0.07	0.799	0.55	**0.006**	0.45	0.068
18	0.24	0.267	0.17	0.509	0.66	**<0.001**	0.60	**0.011**
20	0.26	0.225	0.13	0.624	0.65	**<0.001**	0.61	**0.009**
22	0.28	0.182	0.22	0.401	0.22	0.293	0.31	0.222
24	0.38	0.068	0.19	0.476	0.33	0.115	0.36	0.155
26	0.45	**0.028**	0.13	0.610	0.45	**0.028**	0.40	0.112
28	0.47	**0.020**	0.23	0.374	0.39	0.061	0.41	0.104
32	0.58	**0.003**	0.19	0.475	0.57	**0.004**	0.48	**0.049**
36	0.57	**0.004**	0.19	0.457	0.59	**0.002**	0.49	**0.048**
40	0.58	**0.003**	0.18	0.478	0.61	**0.002**	0.49	**0.043**
44	0.61	**0.002**	0.25	0.333	0.63	**<0.001**	0.59	**0.013**
48	0.53	**0.008**	0.20	0.452	0.65	**<0.001**	0.56	**0.020**
52	0.50	**0.012**	0.19	0.473	0.65	**<0.001**	0.57	**0.016**
56	0.52	**0.010**	0.21	0.409	0.64	**<0.001**	0.54	**0.024**
60	0.48	**0.019**	0.19	0.476	0.64	**<0.001**	0.56	**0.019**
64	0.50	**0.012**	0.20	0.438	0.74	**<.0001**	0.58	**0.015**
68	0.38	0.071	0.19	0.458	0.57	**0.003**	0.53	**0.028**
72	0.36	0.084	0.13	0.629	0.49	**0.016**	0.42	0.092
76	0.46	**0.025**	0.14	0.589	0.63	**<0.001**	0.50	**0.041**
80	.	.	0.16	0.547	.	.	0.47	0.060
84	.	.	0.22	0.398	.	.	0.45	0.071
88	.	.	0.16	0.532	.	.	0.44	0.075
92	.	.	-0.02	0.930	.	.	0.25	0.327
96	.	.	0.23	0.383	.	.	0.44	0.078

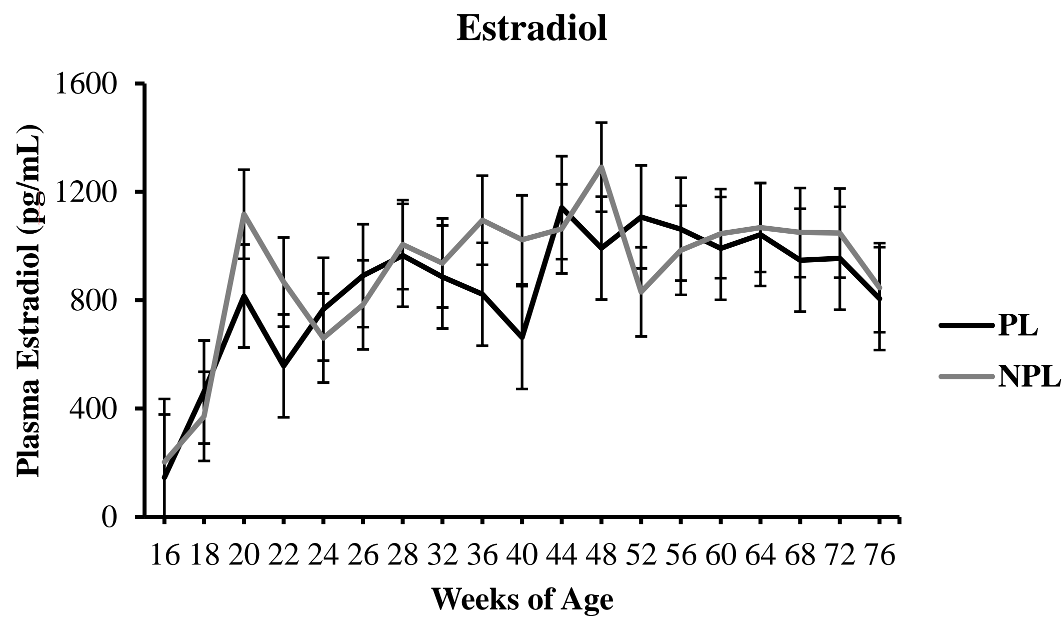


Figure 2. Plasma estradiol concentrations (pg/mL) in PL (n=3) and NPL hens (n=4) from 16 to 76 weeks of age. Data points represent means ± SE.

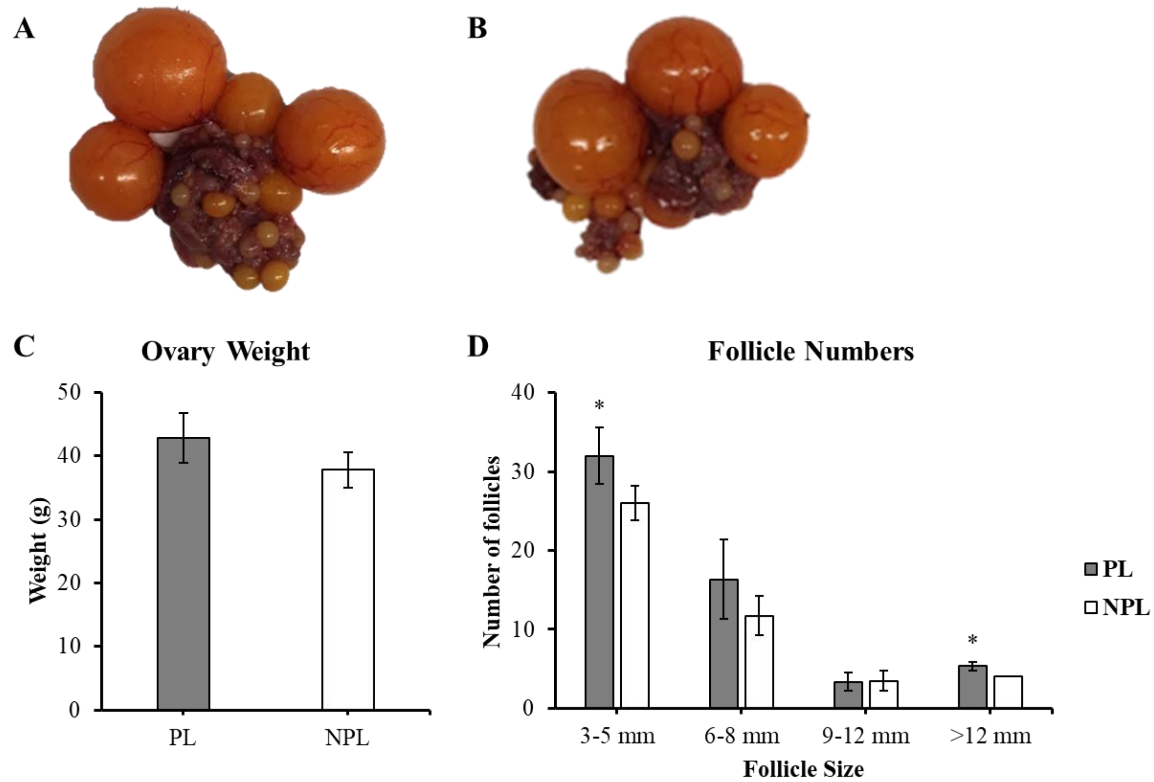


Figure 3. Ovaries, ovarian weights, and follicle numbers of PL (n=3) and NPL (n=4) hens at 76 weeks of age. **A.** Representative picture of an ovary from a PL hen. **B.** Representative picture of an ovary from an NPL hen. **C.** Ovarian weights for PL and NPL hens. **D.** Number of 3-5 mm, 6-8 mm, 9-12 mm, and >12 mm follicles from PL and NPL hen ovaries. Bars represent means ± SD; p-values for comparisons are indicated as *, $p<0.05$.

PL hens had higher ovarian AMH and BMP15 mRNA expression at 76 weeks of age

AMH expression was significantly higher in whole ovaries ($p=0.03$, $n=3-4$) of PL hens compared to NPL hens (Figure 4A). AMH mRNA expression was not different in 1 mm whole follicles or granulosa cells of 6-8 mm follicles. BMP15 expression was significantly higher in whole ovaries of PL hens ($p=0.03$, $n=3-4$) but not in 1 mm whole follicles (Figure 4B).

PL and NPL hens did not differ in follicle densities <500 µm, and NPL hens had increased rates of atresia in <100 µm follicles at 76 weeks of age

A representative H&E section of a PL and NPL hen is in Figures 5A and 5B, respectively, and a representative apoptag section of a PL and NPL hen is in Figures 5C and 5D, respectively. An H&E-stained follicle from a PL hen (Figure 5E), an apoptag-stained healthy follicle from a PL hen (Figure 5F), an H&E-stained follicle from an NPL hen (Figure 5G), and an apoptag-stained atretic follicle from an NPL hen (Figure 5H) are in Figure 5. Follicle densities of <500 µm follicles did not differ between PL and NPL hens (Figure 6A, $p=0.09$). NPL hens, however, have a significantly higher rate of atresia in follicles <100 µm compared to PL hens (Figure 6B, $p=0.01$). Rates of atresia did not differ between PL and NPL hens in other intraovarian size categories (<500 µm).

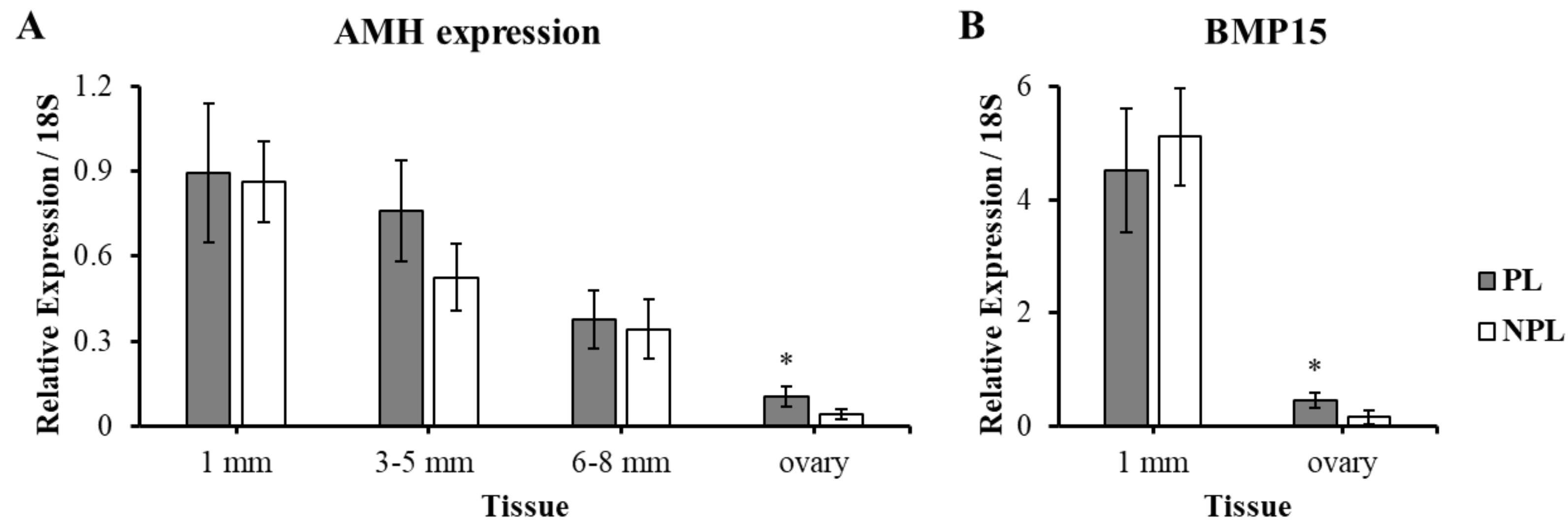
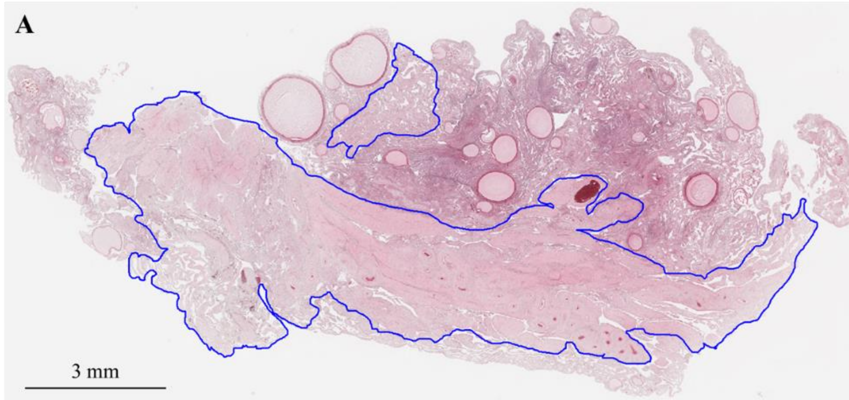


Figure 4. Relative AMH and BMP15 mRNA expression measured by RT-qPCR in PL (n=3) and NPL (n=4) hen ovarian tissue at 76 weeks of age. **A**. Relative expression of AMH mRNA in 1 mm whole follicles, granulosa cells of 3-5 mm and 6-8 mm follicles, and the ovarian body from PL and NPL hens. **B**. Relative expression of BMP15 mRNA in 1 mm whole follicles and ovarian body from PL and NPL hens. Bars represent means ± SD. P-values for comparisons are indicated as *, $p<0.05$.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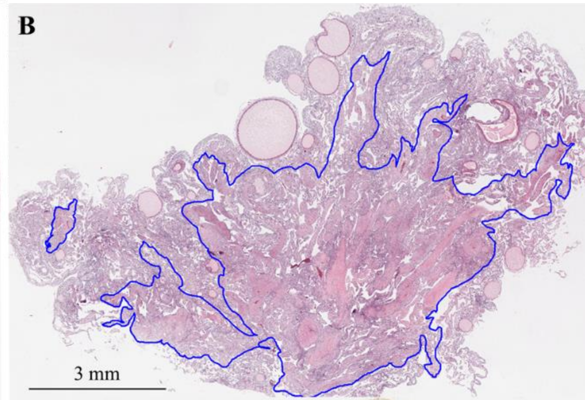
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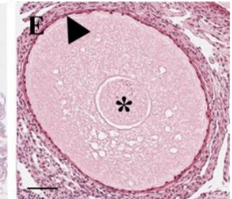
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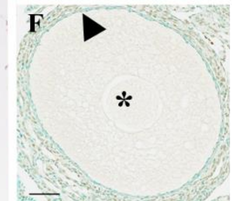
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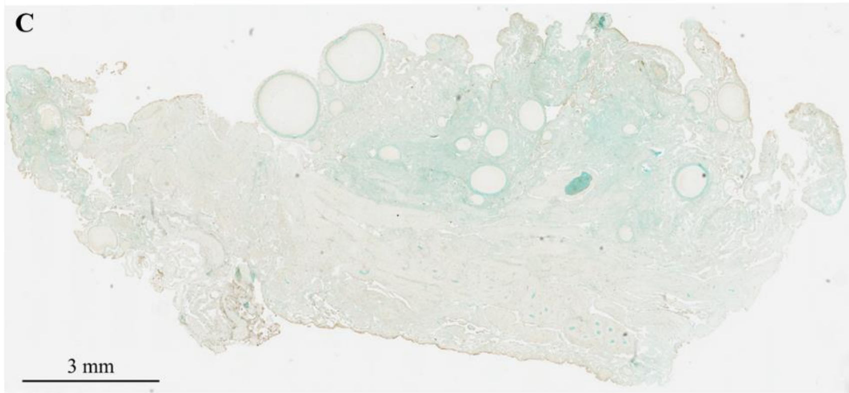
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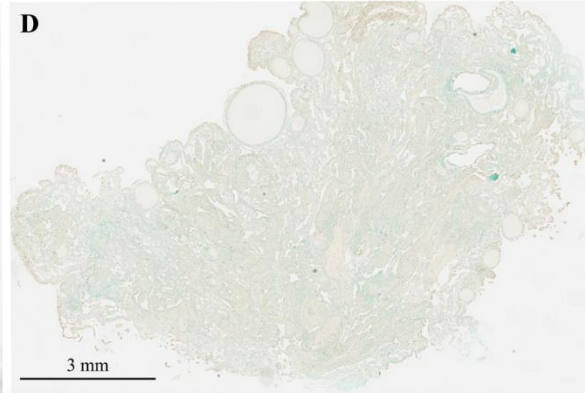
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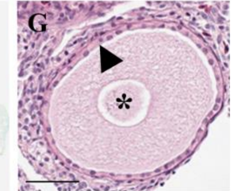
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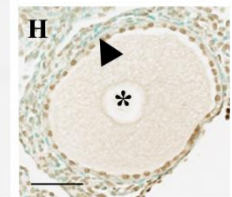
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Figure 5. Histology of ovarian sections of PL and NPL hens at 76 weeks of age. **A.** Representative H&E staining of a PL hen ovarian section. **B.** Representative H&E staining of an NPL hen ovarian section. **C.** Representative Apoptag staining of a PL hen ovarian section. **D.** Representative Apoptag staining of an NPL hen ovarian section. In H&E-stained slides, the medulla is demarcated in blue. In Apoptag-stained ovarian sections, green nuclei indicate live cells, and brown nuclei indicate apoptotic cells. **E.** H&E staining of a healthy follicle from a PL hen. **F.** Apoptag staining of a healthy follicle from a PL hen. **G.** H&E staining of an atretic follicle from an NPL hen. **H.** Apoptag staining of an atretic follicle from an NPL hen. Arrowheads point to the granulosa cell layer, and asterisks represent oocyte nuclei. Scale bars are in the bottom left corners of images (A-D, 3 mm; E-H, 50 µm).

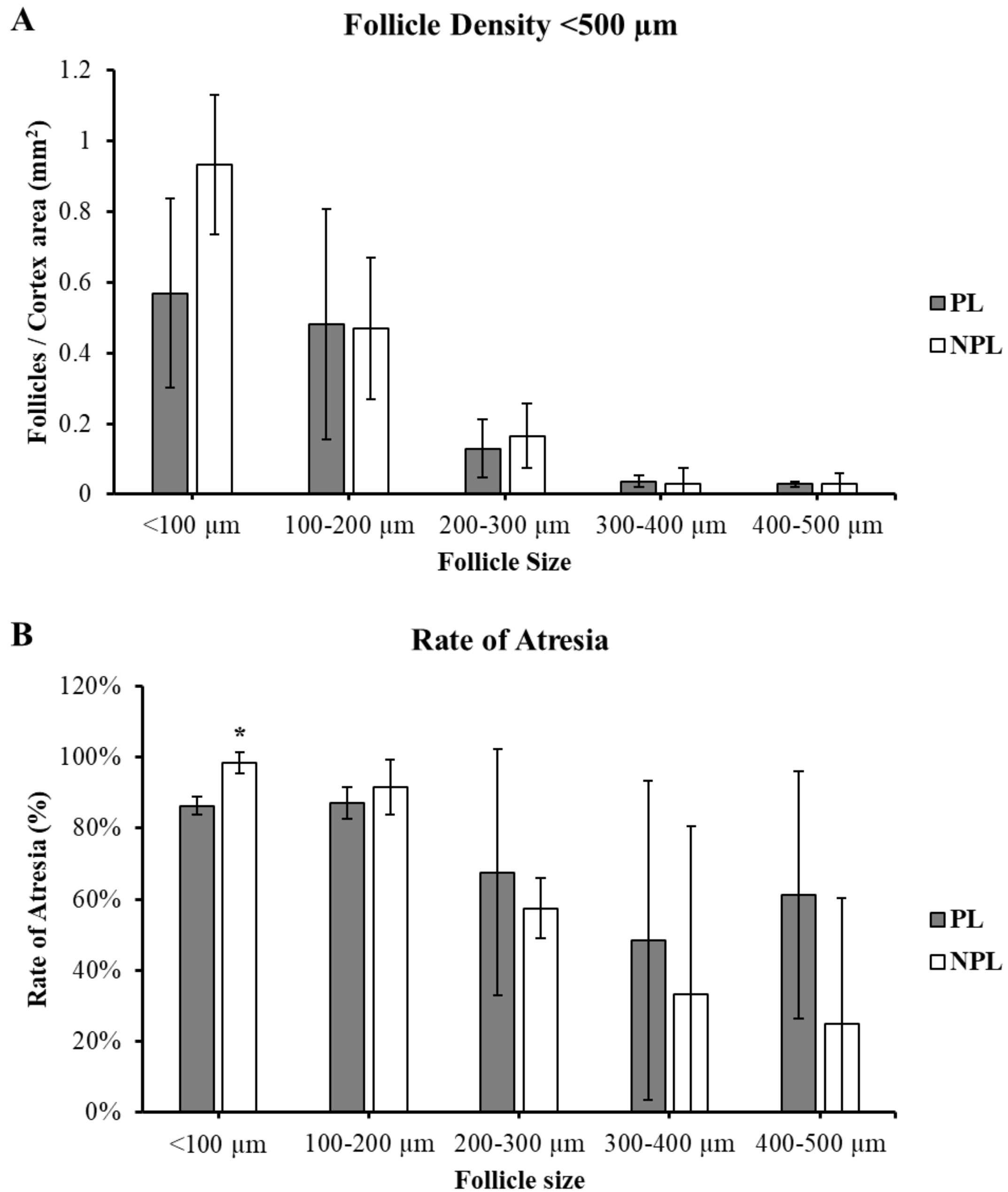

Figure 6. Follicle density and rate of atresia of follicles <500 µm in ovarian sections of PL (n=3) and NPL (n=3-4) hens at 76 weeks of age. **A.** Follicle density of follicles <500 µm in PL (n=3) and NPL (n=4) ovarian sections. **B.** Rate of Atresia of follicles <500 µm in PL (n=3) and NPL (n=3) ovarian sections. Bars represent means ± SD; p-values for comparisons are indicated as *, p<0.05.

Plasma T3 and T4 concentrations did not differ between PL and NPL hens at 26 and 76 weeks of age

Plasma T3 and T4 concentrations did not differ between PL and NPL hens at either 26 or 76 weeks of age (Figure 7, $p>0.05$, n=6-7). Plasma T3 concentrations significantly decreased in PL hens from 26 weeks to 76 weeks of age (Figure 7A, $p=0.01$, n=6-7).

RNA Sequencing and Differential Gene Expression

RNA-sequencing resulted in a minimum of 30M mapped reads per sample (Appendix 4). Principal Component Analysis (PCA) revealed one PL sample as an outlier, and this sample was removed from downstream analyses for a final sample size of n=2 for PL and n=4 for NPL (Figure 8A). Differential expression analysis revealed 66 upregulated genes and 213 downregulated genes in PL compared to NPL (Figure 8B). The top 50 upregulated genes and top 50 downregulated genes in PL ovaries are listed in Appendices 5 and 6, respectively. Gene Ontology analysis was performed to categorize DEGs by biological function. Enriched pathways in genes upregulated in PL hen ovaries include response to pain, defense response to fungus, regulation of pH, various terms relating to ion homeostasis, chemical homeostasis, and homeostatic process (Table 4). Enriched pathways in genes downregulated in PL hen ovaries include vestibulocochlear nerve development, collagen fibril organization,

cellular response to amino acids, several terms involved in extracellular remodeling, blood vessel development, vascular development, and heart development (Table 5).

Estrogen Receptor was predicted as an upstream regulator in PL hens at 76 weeks of age

Upstream regulator analysis identified several upstream regulators shown in Table 6. Although ESR1 and ESR2 did not meet DEG criteria (ESR1, log(FC)= -0.8, FDR= 0.07; ESR2 log(FC)=0.2, FDR= 0.70), The estrogen receptor was one of the most significant upstream regulators of DEGs. ESR1 and ESR2 counts are shown in Figure 9B. DEGs downstream of the estrogen receptor pathway included CXCR4, CALB1, PCDH7, PCDH10, and LOXL2, among others shown in the upstream regulator wheel in Figure 9A. ESR1 and ESR2 mRNA expression did not differ in 1 mm whole follicles or whole ovary samples in PL and NPL hens (Figure 9C, $p<0.05$).

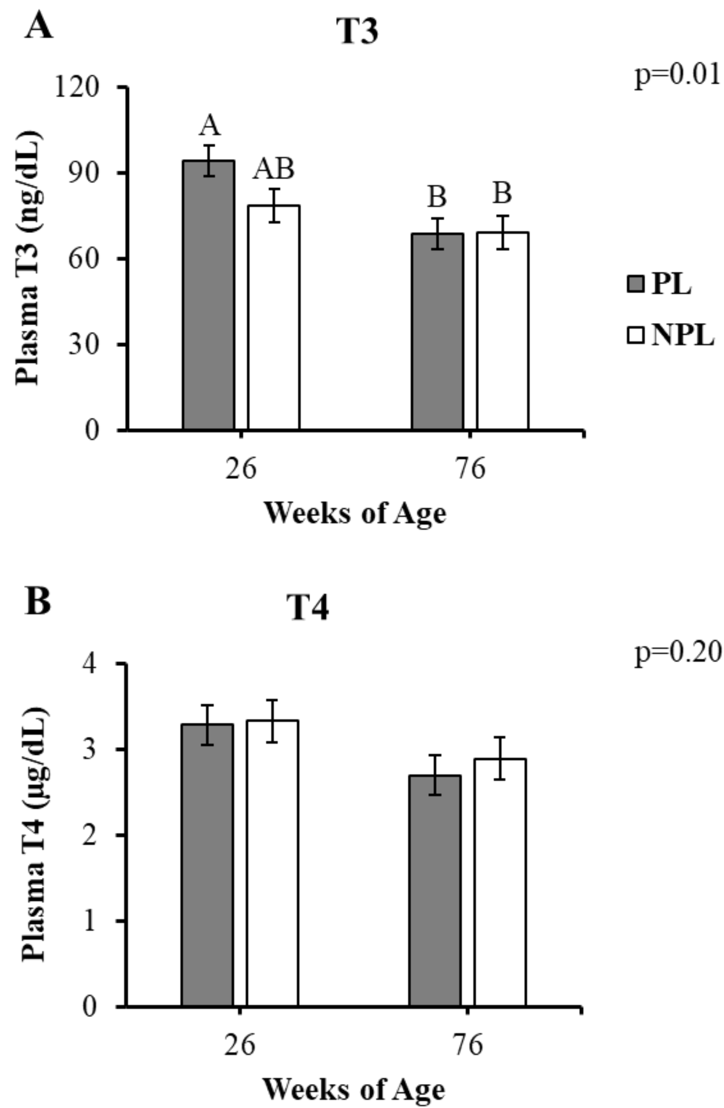

Figure 7. Plasma levels of T3 and T4 at 26 and 76 weeks of age in PL (n=7) and NPL hens (n=6). **A.** T3 plasma levels (p=0.01). **B.** T4 plasma levels (p=0.20). Bars represent mean ± SE. Means with different letters are significantly different from one another (p<0.05). P-values are in the top right corner of each panel.

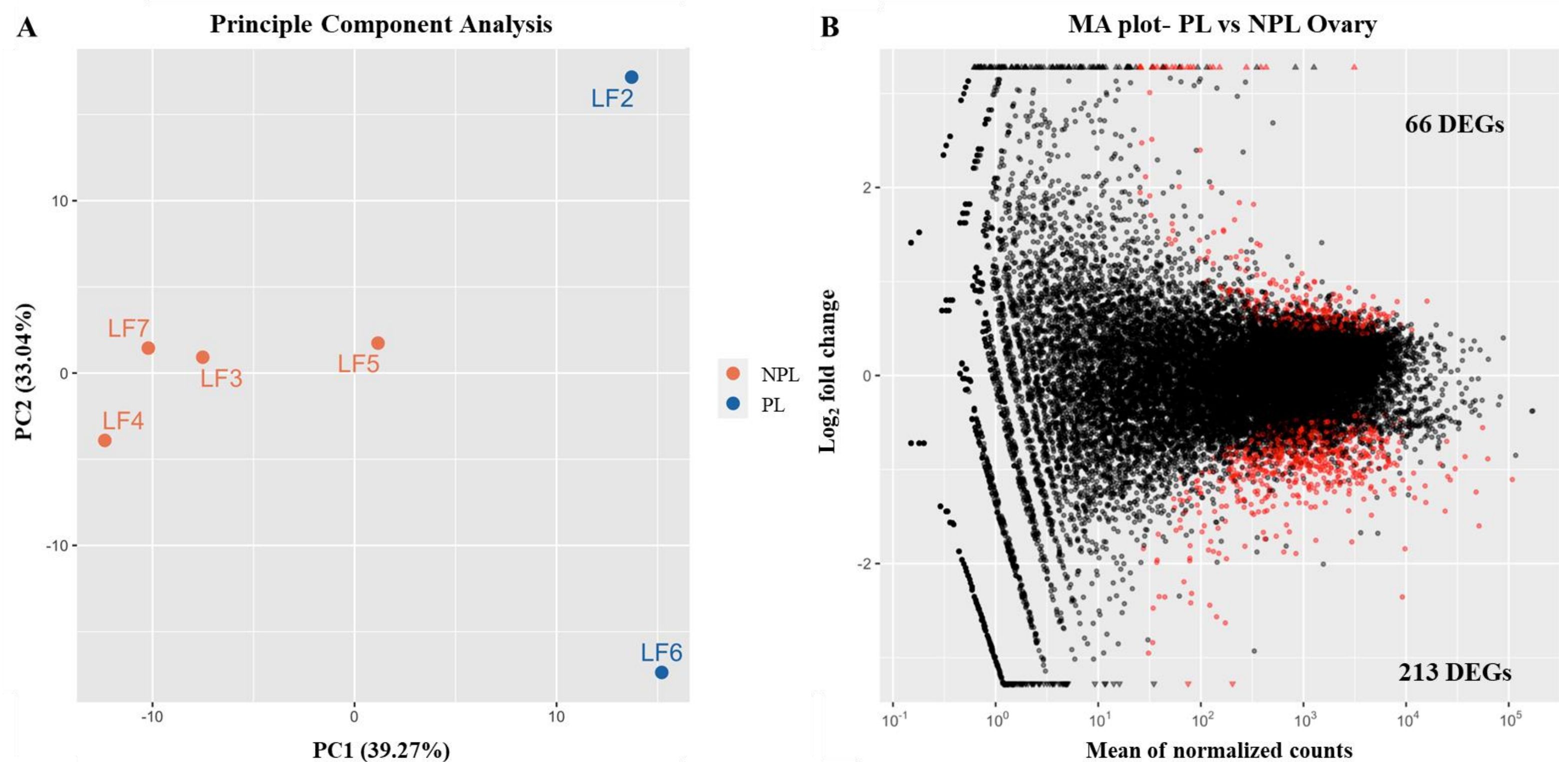

Figure 8. Principal component analysis and MA plot. **A**. PCA shows sample variation along principal component 1 (PC1) and 2 (PC2). **B.** MA plot depicting differentially expressed genes (DEGs) with FDR <0.05 and absolute log(FC) >1. Red dots represent DEGs. Genes with a positive log(FC) are upregulated in PL hens (66 genes), and ones with a negative log(FC) are downregulated in PL hens (213 genes).

Table 4. Enrichment analysis for upregulated genes in ovaries from PL compared to NPL hens. The biological pathway, fold enrichment of the pathway, FDR, number of genes found in the DEG list, and the total number of genes found in the database for the specific pathway are listed.

Pathways	Fold Enrichment	FDR	Genes	Pathway Genes
Response to pain	61.0	4.50E-02	2	11
Defense response to fungus	55.9	4.90E-02	2	12
Regulation of pH	23.1	3.30E-03	4	58
Monovalent inorganic cation homeostasis	20.5	7.60E-04	5	82
Inorganic ion homeostasis	8.2	3.60E-04	9	369
Ion homeostasis	7.6	4.70E-04	9	395
Cation homeostasis	7.4	1.50E-03	8	362
Chemical homeostasis	7.2	1.40E-05	13	606
Homeostatic process	5.0	1.70E-04	14	941
Ion transport	4.1	6.30E-03	11	908

Table 5. Enrichment analysis for downregulated genes in ovaries from PL compared to NPL hens. The biological pathway, fold enrichment of the pathway, FDR, number of genes found in the DEG list, and the total number of genes found in the database for the specific pathway are listed.

Pathway	Fold Enrichment	FDR	Genes	Pathway Genes
Vestibulocochlear nerve development	44.6	3.74E-03	3	6
Collagen fibril organization	14.1	8.28E-04	6	38
Cellular response to amino acid stimulus	12.1	7.10E-03	5	37
Response to amino acid	10.1	1.41E-02	5	44
Extracellular matrix organization	8.1	1.19E-07	16	176
External encapsulating structure organization	8.1	1.19E-07	16	176
Extracellular structure organization	8.0	1.19E-07	16	178
Blood vessel development	4.1	1.10E-04	19	418
Vasculature development	3.8	1.83E-04	19	442
Heart development	3.8	3.33E-03	14	331

Table 6. Top 25 Upstream Regulators of DEGs in ovaries from PL hens. The upstream regulator, z-score of activation, and p-value of overlap are indicated.

Upstream Regulator	z-score	p-value of overlap
AGT	-0.83	2.01E-14
FKBP10	2.53	3.12E-11
estrogen receptor	0.41	1.24E-10
L-triiodothyronine	-0.06	1.95E-09
beta-estradiol	-0.80	2.31E-09
Alpha catenin	2.56	2.34E-09
MRTFA	-2.79	2.08E-08
TGFB1	-4.02	4.13E-08
CPXM1	-	4.73E-08
corticosterone	-0.19	6.28E-08
IRS1	-	6.81E-08
MRTFB	-2.62	8.49E-08
NMDA Receptor	-0.15	1.87E-07
IL1B	-1.26	2E-07
CCR2	-2.43	2.2E-07
GLIS1	-1.70	2.81E-07
D-glucose	-1.33	2.96E-07
TGFB3	-1.70	3.42E-07
NR4A3	1.96	4.24E-07
FOS	0.44	5.48E-07
PD98059	2.40	5.61E-07
SP1	-2.17	5.75E-07
SRF	-2.42	5.83E-07
ESR2	-0.76	6.06E-07
FOXA1	0.63	6.14E-07

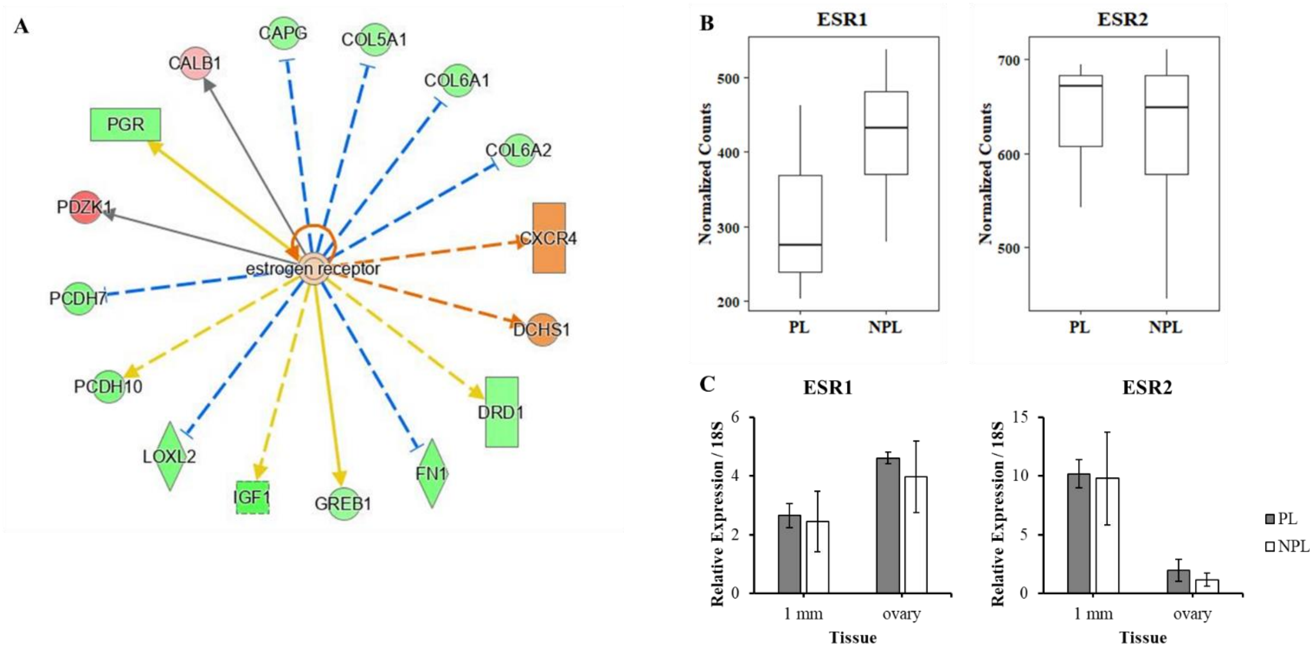

Figure 9. Estrogen receptor upstream regulator network and ESR1 and ESR2 normalized counts and ovarian mRNA expression for PL and NPL hens. **A.** Estrogen receptor network wheel (red shape=increased expression, green shape=decreased expression, orange shape=predicted activation, orange line=predicted activation, blue line=predicted inhibition, yellow lines=findings inconsistent with the state of the downstream molecule, gray lines=effect not predicted). **B.** Normalized counts for ESR1 and ESR2 in ovaries of PL and NPL (n=2-4; FDR>0.05). **C.** Relative mRNA expression of ESR1 and ESR2 measured by RT-qPCR in 1 mm whole follicles and ovaries of PL and NPL (n=2-4; p>0.05).

Discussion

Laying hens are extremely reproductively efficient and lay approximately one egg per day during their first year of production. The egg-laying rate of the flock decreases after the first year of lay [2,3], but there is variability among hens within the flock. In this study, we sought to characterize physiological and transcriptional differences between PL and NPL hens underlying the differences in egg-laying persistency. PL and NPL hens were selected and characterized based on their egg-laying efficiencies at 76 weeks of age, and data were analyzed retrospectively. PL hens had significantly higher egg-laying efficiencies starting at 68 weeks of age and laid more total eggs than NPL hens by 76 weeks of age. The duration of the laying period was similar between PL and NPL hens, as hens reached sexual maturity at approximately the same age.

Efficient laying hens have organized follicle hierarchies, allowing daily ovulation. Ovaries from PL hens had significantly more 3-5 mm and more preovulatory (>12 mm) follicles than NPL hens. The increased number of follicles (3-5 mm and preovulatory) could contribute to persistency of egg-laying rates due to more follicles being available for development and, ultimately, for ovulation. It is not known whether these follicles are selected at an increased rate or whether there is an increased number of follicles available for daily selection.

We had hypothesized that PL and NPL hens might also differ in the size of the ovarian reserve. We used two approaches to assess this: ovarian gene expression and histological assessment of ovarian sections. Ovarian gene expression analysis revealed that PL hens have higher expression of AMH and BMP15 mRNA, suggesting that PL

hens have more small follicles (<1 mm). AMH is produced by small growing follicles [17,18], and serum levels can be used to estimate the size of the ovarian reserve [19–22]. BMP15 is an oocyte-specific factor expressed in growing follicles [12]. In a second approach, we estimated small (<100 µm) follicle numbers per cortex area of PL and NPL hens and found no difference. Our histological analysis only covers 150 µm of a ~40 g ovary, and it is possible that follicle distribution is not uniform throughout the ovary. Evaluation of small follicle health using a TUNEL assay revealed that NPL hens had significantly higher rates of atresia in <100 µm follicles.

Atretic follicles express less AMH in rodents [18,23]. Although PL and NPL hens have similar numbers of small follicles, ovarian AMH expression may be lower in NPL hens due to the increased number of atretic follicles. Higher rates of atresia in follicles <100 µm may explain the lower number of 3-5 mm and preovulatory follicles observed in NPL hens. In our RNA-sequencing analysis, AMH met the FDR cutoff but not the fold-change criteria for DEGs (log(FC)=0.8, FDR=0.02). AMH has previously been shown to be upregulated in the ovaries of high egg-producing chickens [24], suggesting that AMH may play a role in regulating egg-laying efficiencies in hens later in life. AMH may also protect follicles of PL hens against atresia, as has been shown in mammals [25].

Interestingly, PL hens were significantly heavier than NPL hens starting at 32 weeks of age. It was previously reported that hens with higher egg-laying rates weighed significantly more starting from 20-22 weeks of age, continuing until 68-90 weeks of age, depending on the strain [26]. Although we did not measure feed intake in these hens, the difference in body weight may be attributed to consumption. In

broiler breeder hens, feed intake greatly influences follicle development [13,27,28], and it may also impact the persistency of lay. Body weight was correlated with egg-laying rates at 76 weeks of age and with total eggs laid at 76 and 96 weeks of age as early as 16 weeks of age. Body weight had the highest correlation coefficient (0.74) with total eggs at 76 weeks and at 64 weeks of age. Correlation coefficients between body weight and total eggs laid at 76 and 96 weeks were also high between weeks 18 and 20, ranging from 0.60-0.66. Though more research with larger sample sizes is needed to make a recommendation to producers, hen body weights early in the laying phase could be used by producers to predict which hens will exhibit persistency later in life.

Thyroid hormones are known to affect reproduction and metabolism in hens. We measured plasma T3 and T4 concentrations as an indication of the metabolic rate at week 26 (before hens diverged in body weight) and at week 76 (when PL hens were significantly heavier and had higher egg-laying efficiencies). Plasma T3 and T4 levels did not differ between PL and NPL hens, although PL hens showed a decrease in plasma T3 levels from 26 to 76 weeks of age. T3 treatment in hens is associated with decreased egg-laying rates and plasma LH and steroid hormone levels [29]. The decrease in plasma T3 levels with age in PL hens may contribute to the maintenance of high egg production.

RNA-sequencing revealed 279 DEGs in PL versus NPL ovarian samples. Upstream regulator analysis identified estrogen receptors as potential regulators of DEGs. Neither ESR1 nor ESR2 was differentially expressed in the RNA-sequencing analysis, and mRNA expression was not different in 1 mm whole follicles or ovarian

samples in PL and NPL hens. Plasma estradiol levels did not differ between PL and NPL hens from 16 to 76 weeks of age. Although the expression of estrogen receptors and plasma estradiol levels are not different between PL and NPL hens, there may be sequence differences in ESR1 or ESR2, causing enhanced receptor signaling in PL hens. ESR1 and ESR2 may be suitable candidate genes to explore genetic differences associated with persistency which could be used for breeding selection.

One potential concern with increasing persistency of lay in hens is the potential for an increase in ovarian cancer in hens. Laying hens are one of the only known animals to spontaneously develop ovarian cancer other than humans [30], and estrogen exposure is associated with ovarian cancer [31]. Several genes downstream of estrogen receptors in our RNA-sequencing analysis are known to be involved in cancer, including upregulation of CXCR4 [32] and CALB1 [33] and downregulation of PCDH10 [34], PCDH7 [35], and LOXL2 [36]. Expression of these genes in PL hens could indicate an increased risk of developing ovarian cancer. After 2.5 years of age, hens have a 30-35% chance of developing ovarian cancer [37], and hens with decreased ovulations, either due to genetic strain [38] or hormone treatment have a lower cancer incidence [39]. Because the risk for ovarian cancer increases with advanced age [37] and ovulations in hens [38,39], PL hens may have a higher risk for ovarian cancer, an issue potentially requiring mitigation.

In conclusion, PL hens differ from NPL hens in egg-laying efficiency, body weight, and in ovarian follicle number and gene expression. PL hens have more 3-5 mm and >12 mm follicles, increased ovarian expression of AMH and BMP15, and a decreased rate of atresia. Through RNA-sequencing analysis, we identified 279 DEGs

in PL hens. We found that body weight and egg-laying efficiency earlier in life were strongly correlated with total eggs laid and egg-laying efficiencies at 76 and 96 weeks of age. These variables have the potential to be developed for decision-making tools for producers to predict persistency early in the life of the hen. Ultimately, a greater understanding of the physiology of persistent hens can lead to improved welfare and productivity in laying hens.

Acknowledgments

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CHAPTER 5

INVESTIGATIONS OF THE REGULATION AND FUNCTION OF AMH IN GRANULOSA CELLS IN HENS[a]

Abstract

Anti-mullerian hormone (AMH) is an important follicle regulator in mammals that prevents premature primordial follicle activation and restricts follicle development by reducing FSH sensitivity and inhibiting FSH-induced increase of steroidogenic enzymes. AMH is produced by granulosa cells of growing follicles in mammalian and avian species. In chickens, expression declines at the time of selection, similar to the expression pattern of mammalian follicles. The role of AMH in chicken granulosa cells and how it might affect follicle selection is not known. There is currently no commercially available chicken AMH, which has made studying its role difficult in the chicken model. In this study, we investigated the role of AMH on markers of follicle development by knocking down the expression of AMH mRNA using RNA interference in cultured granulosa cells of 3-5 mm and 6-8 mm follicles, the stage where follicle selection is thought to occur. We also investigated FSH regulation of AMH mRNA expression in cultured granulosa cells of 3-5 mm and 6-8 mm follicles. FSH treatment significantly decreased AMH mRNA expression in granulosa cells of 3-5 mm but not 6-8 mm follicles. AMH mRNA expression was significantly reduced in granulosa cells of 3-5 mm and 6-8 mm follicles when transfected with an AMH-specific siRNA. Knockdown of AMH did not affect markers of follicle development

a. In collaboration with Deena M. Scoville who performed cell culture and RT-qPCR of the first experiment.

(FSHR, STAR, CYP11A1, BMPR2) or FSH responsiveness in granulosa cells of 3-5 mm follicles. These data suggest that AMH does not regulate follicle development directly by affecting markers of steroidogenesis, FSHR or BMPR2 at this stage of follicle development in chickens.

Introduction

In chickens, anti-mullerian hormone (AMH) mRNA is expressed in the granulosa cell layer of growing follicles, and its expression is highest in 1 mm follicles and declines with increasing follicle size [1]. The AMH receptor, AMHR2, is expressed predominantly in the granulosa cells and ooplasm of small follicles and its expression is higher in the ooplasm [2]. Although the role of AMH in the hen follicle is unknown, its expression pattern is similar to that of mammals [1,3]. In chicken granulosa cells, oocyte conditioned media [1], bone morphogenic protein 15 (BMP15) [4], vitamin D3 [5], and TGFβ decrease AMH expression [6]. Bone morphogenic protein 6 (BMP6) has been shown to increase AMH expression [6], while neither estradiol [1], progesterone [1], nor insulin-like growth factor- 1 (IGF1) [7] affected the expression of AMH mRNA. In mammals, FSH regulates AMH, although the effect differs by species and model [8–14].

In the laying hen, follicles are organized in a hierarchy, resulting in approximately one ovulation per day. Follicle selection is thought to occur in the 6-8 mm follicle pool when the granulosa cells of one follicle express significantly more follicle-stimulating hormone receptor (FSHR) [15]. Before follicle selection, granulosa cells do not produce progesterone [16] due to lack of steroidogenic acute regulatory protein (STAR) [17] and the enzyme responsible for cholesterol side-change cleavage (Cytochrome P450 Family 11 Subfamily A Member 1; CYP11A1) [18]. When steroidogenically competent, the granulosa cells have increased expression of STAR and CYP11A1 [15,17,19] and begin producing progesterone [20]. AMH

mRNA expression declines during development from the 3-5 mm to the 6-8 mm follicle stage [1], the stage of follicle selection.

In mammals, AMH prevents primordial follicle activation [21], decreases FSH sensitivity [22], and FSH-induced steroidogenesis [23,24]. AMH may function to restrict follicle selection in avian ovaries based on its expression pattern [1], and immunization against AMH in geese significantly increases the number of preovulatory follicles [25]. Chick testis-conditioned media (TCM), a rich source of AMH [26,27], increased granulosa cell proliferation, an effect which could be blocked by pretreatment with AMH antibody [28]. Interestingly, similar to what is observed in women with polycystic ovarian syndrome (PCOS) [29], broiler breeder hens have increased ovarian AMH mRNA expression [28] and numbers of preovulatory follicles [30–32]. A greater understanding of AMH function in chicken folliculogenesis, could help elucidate the etiology of the reproductive inefficiencies observed in broiler breeder hens.

Mammalian AMH is not bioactive in chickens [33], and the function of AMH has been challenging to study in chickens due to the lack of a commercially available chicken AMH. Although TCM has been informative in studying AMH function, we sought a more specific approach. In the current study, we investigated the role of AMH by reducing AMH mRNA expression using RNA interference (RNAi). We hypothesized that decreased AMH mRNA expression would accelerate the expression of markers of follicle development in granulosa cells of prehierarchal follicles. We examined FSH regulation of AMH, the function of AMH in granulosa cells of prehierarchal follicles, and the effect of AMH on FSH responsiveness.

Materials and Methods

Animals

Single-comb White Leghorn hens were housed in individual cages, and egg-laying was recorded daily. Hens had access to *ad libitum* feed and water and were exposed to a 16L:8D light cycle. Hens within their first year of lay which had consistent laying patterns were euthanized by CO_2, and ovaries were immediately removed and placed in ice-cold Krebs-Ringer Bicarbonate buffer. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University (IACUC protocol number 2009-0036).

Granulosa Cell Culture

Granulosa cells from 3-5 mm and 6-8 mm follicles were isolated from 1-3 laying hens for each culture replicate and pooled by size in ice-cold Krebs buffer according to Wang *et al.* [34]. Granulosa cells were dispersed, washed, and cultured as previously described by Francoeur *et al*., (unpublished) [7].

In the first experiment, granulosa cells from 3-5 mm (n=5 replicate cultures) and 6-8 mm follicles (n=6 replicate cultures) were plated in 6-well culture dishes at a density of 4 x 10^6 cells per well in a 1.5 mL volume and cultured for 22-23 hours [7]. Media were replaced with serum-free media and cultured 24 hours before treatment with ovine FSH (oFSH; NIDDK-oFSH-19-SIAFP, AFP4117A) at 0, 10, 25, or 100 ng/mL in 2 mL. Cells were collected after an additional 24 hours and stored in RLT buffer at -80 °C until processing.

In the second experiment, granulosa cells from 3-5 mm (n=7 replicate cultures) and 6-8 mm follicles (n=6 replicate cultures) were plated in 24-well culture dishes at a density of 2 x 10^5 cells per well in 0.5 mL of media [7] for 24 hours. Media were replaced with treatment media lacking serum and ABAM. Cells were transfected according to the protocol below and treatment media consisted of either a non-target (NT) siRNA, AMH siRNA, or no treatment. Twenty-four hours after transfection, cells were treated with oFSH at 0 or 10 ng/mL. After an additional 24 hours of incubation, cells were collected and stored in RLT buffer at -80 ̊C until processing.

Knockdown of AMH by RNAi

A specific siRNA targeting AMH was designed using Horizon's siDESIGN Center tool (Horizon Discovery, Cambridge, UK). A previously described non-target siRNA (NT) was used as a negative control [35]. All siRNAs were blasted against the Galgal6 chicken genome to verify that there were no off-target genes (Table 1). An additional group was left untreated to compare endogenous levels of AMH in cultured granulosa cells.

Transfection was done using Lipofectamine RNAiMax Transfection Reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, granulosa cells were cultured for 24 hours in a 24-well plate until cells were at ~60-80% confluency. Lipofectamine RNAiMax (1.5 µL) and 10 pmol of siRNA were individually diluted in 25 µL of Opti-MEM media (Thermo-Fisher Scientific, Waltham, MA, USA). The diluted siRNA and Lipofectamine RNAiMax were then combined (1:1) and incubated at room temperature for 5 minutes. Treatment

(50 µL) was added to cells in a 0.5 mL volume, and 50 µL of Opti-MEM media was added to the untreated control. Each siRNA and untreated group was plated in duplicate (n = 6 or 7 replicates). After 24 hours, wells were treated with oFSH (0 or 10 ng/mL), cultured for an additional 24 hours, and then collected and stored in RLT buffer at -80 ̊C until processing.

Table 1. siRNA sequences for RNAi.

Target Gene	siRNA sequence	Start position	Reference
AMH	**Sense:** 5' CCACAGCUCUGCCGAGAAA-dTdT 3' **Antisense:** 5' UUUCUCGGCAGAGCUGUGG-dTdT 3'	100	NM_205030
Non-Target	**Sense:** 5' UUCUCCGAACGUGUCACGU-dTdT 3' **Antisense:** 5' ACGUGACACGUUCGGAGAA-dTdT 3'	NA	Xu *et al.*, 2018 [35]

RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA was extracted using the RNeasy Mini Kit and RNeasy Micro Kit with on-column DNase treatment (Qiagen Inc., Valencia, CA) for the first and second experiments, respectively. The quantity and purity of RNA were measured using spectrophotometry (Implen, Munich, Germany). Reverse transcriptase reactions were performed using 1 µg of total mRNA in a 20 µL volume using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative polymerase chain reactions (RT-qPCR) were performed using the AB StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). One µg of cDNA was added to each reaction. Reactions for unknown samples, standards, no-template controls, and reactions lacking reverse transcriptase

were analyzed in duplicate in a 25 µL volume. A final concentration of 1X for Power SYBR Green (Applied Biosystem, Foster City, CA, USA) and 300 nM for primers were used in the reaction. Unknown sample expression was determined from the standard curve (constructed from pooled granulosa cells) and normalized to 18S. 18S primers from Ambion® QuantumRNA™ 18S Internal Standard (ThermoFisher Scientific, Waltham, MA, USA) were used to determine 18S expression, and all other primer sequences can be found in Table 2.

Table 2. Primer Sequences for RT-qPCR.

Target Gene	Primer Sequence	Citation
AMH	F: 5'-CCCCTCTGTCCCTCATGGA-3' R: 5'-CGTCATCCTGGTGAAACACTTC-3'	Stephens *et al.* [36]
FSHR	F: 5'-GCACCTTCCAAGCCTCAGATAT-3' R: 5'-CCCTATGGACGACGGGTAAA-3'	Stephens *et al.* [36]
STAR	F: 5′-TGCCTGAGCAGCAGGGATTTATCA-3′ R: 5′-TGGTTGATGATGGTCTTTGGCAGC-3′	Lee and Johnson [37]
CYP11A1	F: 5′-ACTTCAAGGGACTGAGCTTTGGGT-3′ R: 5′-AGTTCTCCAGGATGTGCATGAGGA-3′	Lee and Johnson [37]
BMPR2	F: 5'-GGAATCAGCGAGAGCCGAAT-3' R: 5'-TGGGTCAGGAGGTGGGAAGT-3'	Stephens, unpublished

Statistical Analysis

Gene expression was compared between treatment groups with a mixed model consisting of the fixed effect of treatment and the random effect of culture replicate using JMP Pro 16 software (SAS Institute Inc., Cary, NC, USA). Post-hoc Tukey test was done with $\alpha=0.05$ when appropriate.

Results

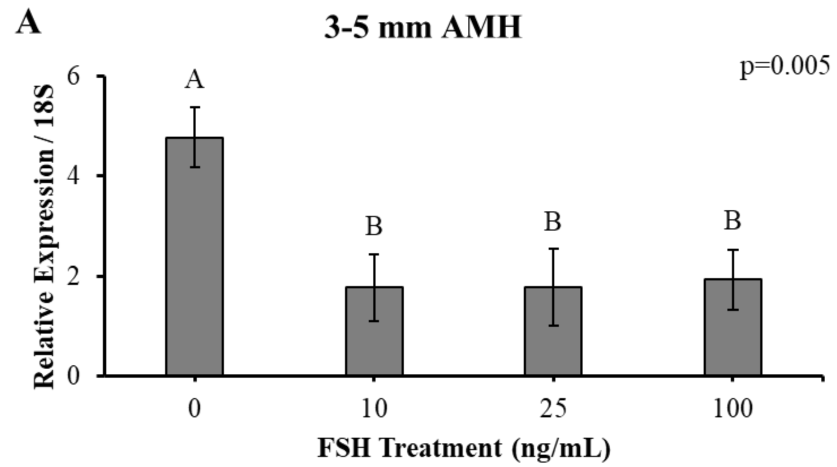
FSH decreases AMH mRNA expression in granulosa cells of 3-5 mm follicles

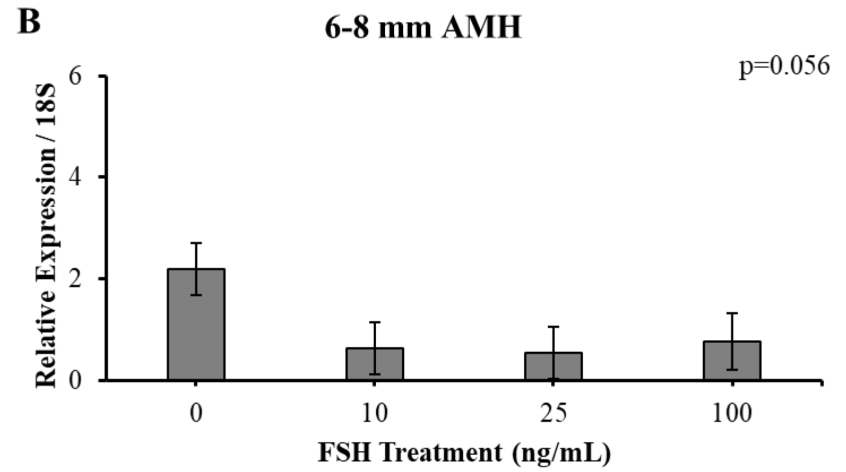
In granulosa cells from 3-5 mm follicles, FSH treatment significantly decreased AMH mRNA expression with maximal effect at the dose of 10 ng/mL (Figure 1A, $p=0.005$). FSH treatment did not affect AMH expression in cultured granulosa cells of 6-8 mm follicles compared to the control (Figure 1B, $p=0.056$). FSH treatment did not affect FSHR mRNA expression in granulosa cells of either 3-5 (Figure 1C, $p=0.085$) or 6-8 mm follicles (Figure 1D, $p=0.112$).

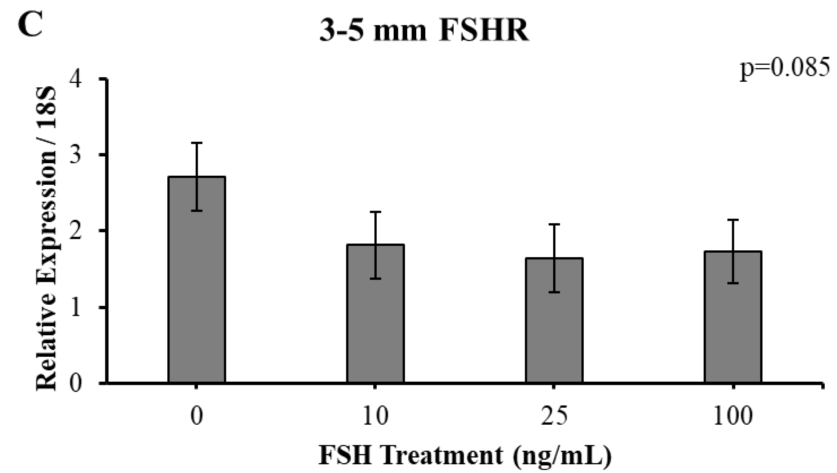
AMH mRNA expression can be knocked down using RNAi in 3-5 mm and 6-8 mm follicles

The effectiveness of transfection using an siRNA targeting AMH was tested at two different doses (5 and 10 pmol) in granulosa cells of 3-5 mm follicles to determine the most effective treatment. In this preliminary test, both doses decreased AMH mRNA expression from the untreated and NT controls (Figure 2). The 5 pmol dose reduced expression by 63%, and the 10 pmol dose decreased expression by 70%. The higher dose was used for AMH knockdown in subsequent cultures.

AMH mRNA expression in granulosa cells treated with the NT siRNA was not different from the untreated control in either 3-5 mm or 6-8 mm follicles. Transfection of the AMH siRNA significantly decreased AMH mRNA expression in granulosa cells from 3-5 mm (Figure 3A, $p<0.001$) and 6-8 mm follicles (Figure 3B, $p<0.001$) compared to the untreated and NT siRNA controls. In granulosa cells of 3-5 mm follicles, FSH treatment significantly reduced AMH mRNA expression in the untreated and NT siRNA controls (Figure 3A, $p<0.001$) although not in granulosa cells of 6-8 mm follicles (Figure 3B). In granulosa cells of 3-5 mm follicles, treatment with the AMH siRNA resulted in a 78% average reduction from the untreated and NT controls. In granulosa cells of 6-8 mm follicles, treatment with the AMH siRNA resulted in an 85% average reduction from the untreated control and NT control, respectively.


A
3-5 mm AMH
p=0.005
A
B
B
B
Relative Expression / 18S
0
2
4
6
0
10
25
100
FSH Treatment (ng/mL)


B
6-8 mm AMH
p=0.056
Relative Expression / 18S
0
2
4
6
0
10
25
100
FSH Treatment (ng/mL)


C
3-5 mm FSHR
p=0.085
Relative Expression / 18S
0
1
2
3
4
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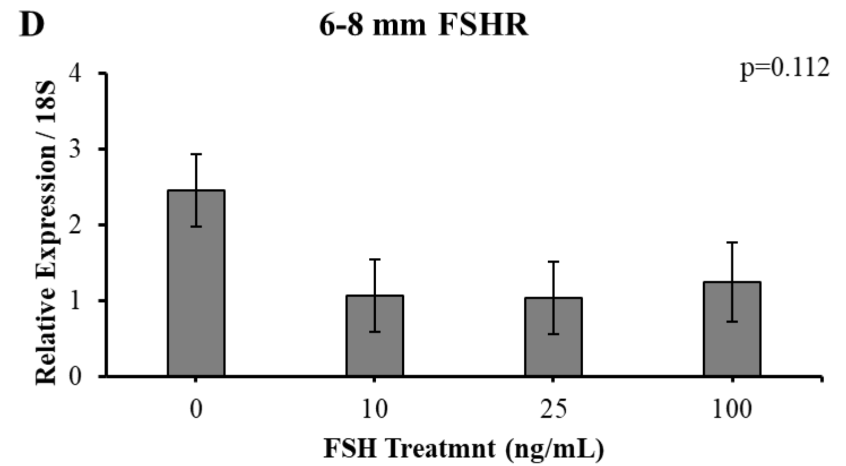
10
25
100
FSH Treatment (ng/mL)


D
6-8 mm FSHR
p=0.112
Relative Expression / 18S
0
1
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25
100
FSH Treatmnt (ng/mL)

Figure 1. Relative AMH and FSHR mRNA expression in cultured granulosa cells of 3-5 mm and 6-8 mm follicles treated with FSH (0, 10, 25, 100 ng/mL). Bars represent means ± SE, means with different letters are significantly different from one another ($p>0.05$), and p-values are in the top right corners of the panels. **A.** Relative mRNA expression of AMH in cultured granulosa cells of 3-5 mm follicles (n=3-5; p=0.005). **B.** Relative mRNA expression of AMH in cultured granulosa cells of 6-8 mm follicles (n=5-6; p=0.056). **C.** Relative mRNA expression of FSHR in cultured granulosa cells of 3-5 mm follicles (n=4-5; p=0.085). **D.** Relative mRNA expression of FSHR in cultured granulosa cells of 6-8 mm follicles (n=5-6; p=0.112).

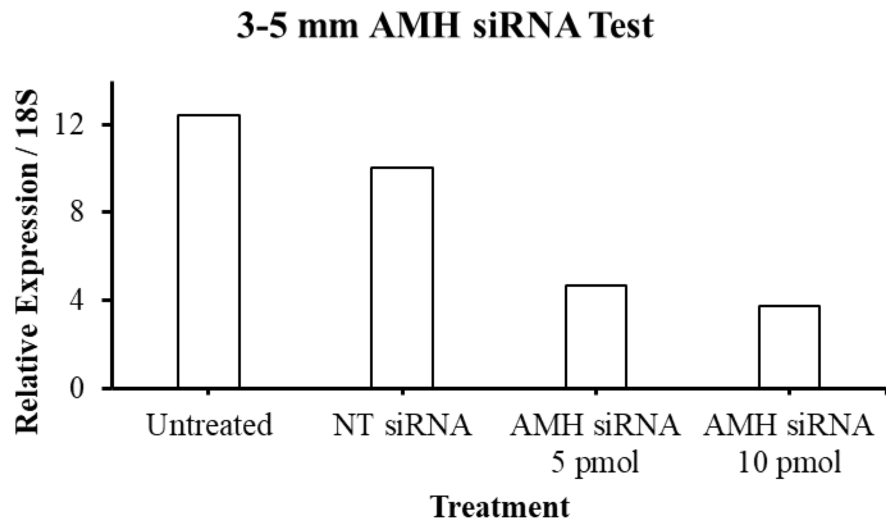

Figure 2. Relative mRNA expression of AMH in granulosa cells of 3-5 mm follicles treated with different concentrations of AMH siRNA. Granulosa cells were untreated or treated with either a NT or AMH siRNA at a concentration of 5 or 10 pmol (n=1).

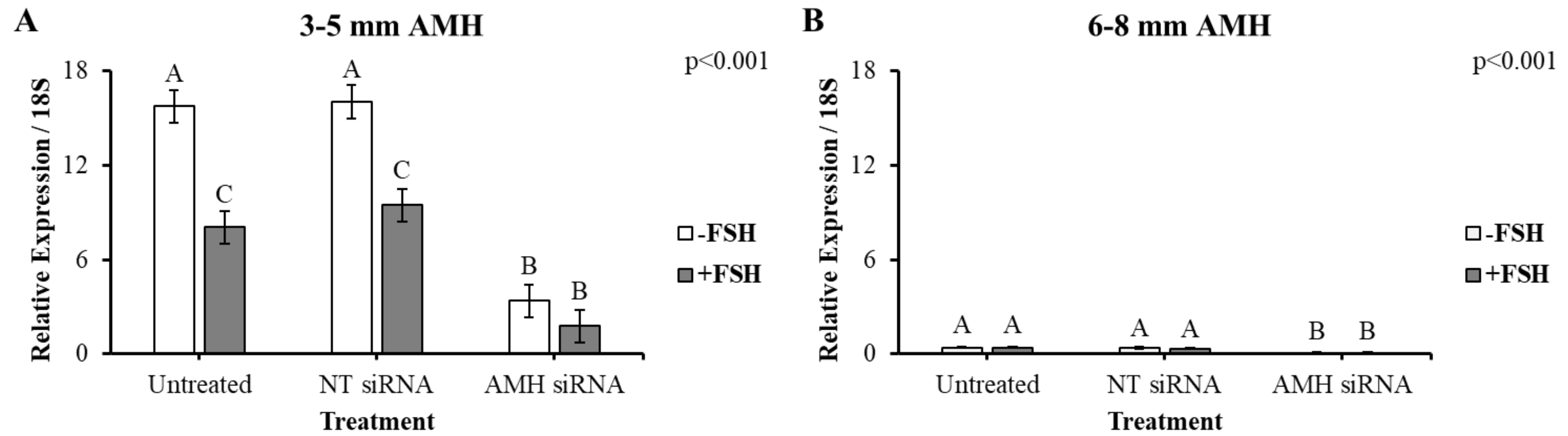

Figure 3. Relative AMH mRNA expression in cultured granulosa cells from 3-5 mm and 6-8 mm follicles. Bars represent means ± SE, means with different letters are significantly different from one another (p>0.05), and p-values are in the top right corners of the panels. Granulosa cells were untreated or treated with an NT or AMH siRNA in the presence and absence of FSH (0, 10 ng/mL). **A.** Relative mRNA expression of AMH in 3-5 mm cultured granulosa cells (n=7; p<0.001). **B.** Relative mRNA expression of AMH in 6-8 mm cultured granulosa cells (n=5-6; p<0.001).

AMH knockdown does not affect FSHR mRNA expression

AMH knockdown did not affect FSHR mRNA expression compared to the untreated and NT siRNA controls in cultured granulosa cells of 3-5 mm follicles and the addition of FSH did not affect expression (Figure 4, $p=0.234$).

AMH knockdown does not affect STAR or CYP11A1 expression in the presence or absence of FSH

In granulosa cells of 3-5 mm follicles, treatment with the NT or AMH siRNA did not affect STAR mRNA expression compared to the untreated control. FSH treatment significantly increased STAR expression in NT siRNA-treated cells compared to the untreated control (Figure 5A, $p<0.001$). Treatment with NT and AMH siRNAs did not affect CYP11A1 mRNA expression compared to the untreated control in the presence or absence of FSH in granulosa cells of 3-5 mm follicles (Figure 5B).

AMH knockdown does not affect BMPR2 expression in the presence or absence of FSH

In granulosa cells of 3-5 mm follicles, transfection of the NT or AMH siRNA did not affect BMPR2 expression. The addition of FSH to the NT siRNA group significantly increased BMPR2 expression compared to the untreated control, but this was not different from the NT siRNA control treated without FSH (Figure 6, $p=0.010$).

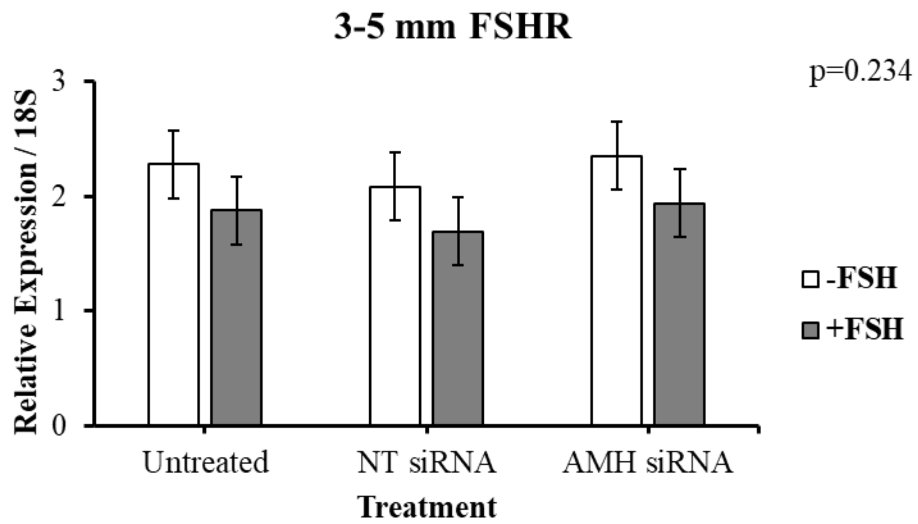

Figure 4. Relative FSHR mRNA expression in cultured granulosa cells from 3-5 mm follicles. Bars represent means ± SE, and the p-value is in the top right corner of the panel. Granulosa cells were untreated or treated with an NT or AMH siRNA in the presence and absence of FSH (0, 10 ng/mL; n=7; p=0.234).

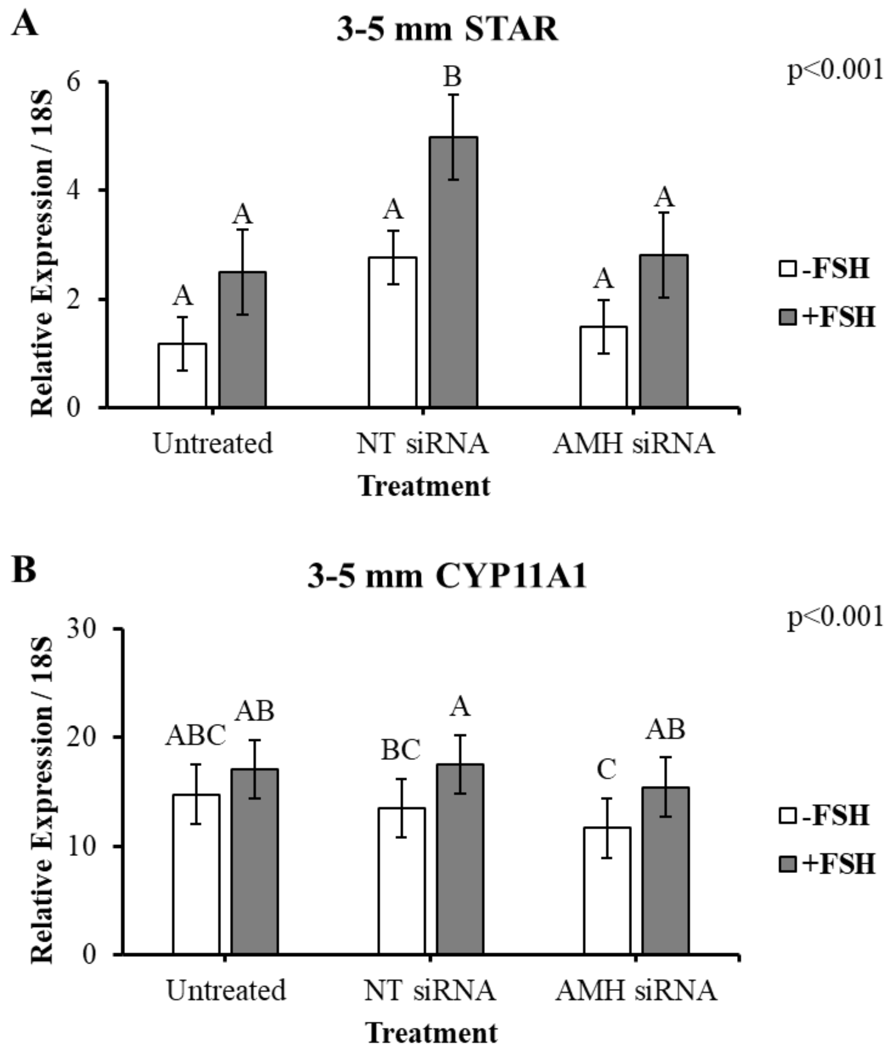

Figure 5. Relative STAR and CYP11A1 mRNA expression in cultured granulosa cells from 3-5 mm follicles. Bars represent means ± SE, means with different letters are significantly different from one another ($p>0.05$), and p-values are in the top right corners of the panels. Granulosa cells were untreated or treated with an NT or AMH siRNA in the presence and absence of FSH (0, 10 ng/mL). **A.** Relative mRNA expression of STAR in 3-5 mm cultured granulosa cells ($n=7$; $p<0.001$). **B.** Relative mRNA expression of CYP11A1 in 3-5 mm cultured granulosa cells ($n=7$; $p<0.001$).

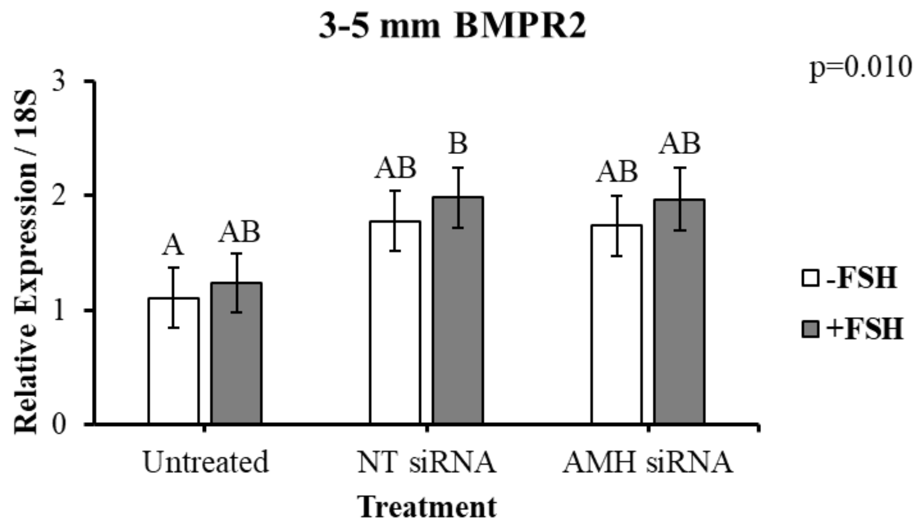

Figure 6. Relative BMPR2 mRNA expression in cultured granulosa cells from 3-5 mm follicles (n=6-7; p=0.010). Bars represent means ± SE, means with different letters are significantly different from one another (p>0.05), and the p-value is in the top right corner of the panels. Granulosa cells were untreated or treated with an NT or AMH siRNA in the presence and absence of FSH (0, 10 ng/mL).

Discussion

In this study, we investigated the effect of FSH on AMH mRNA expression at different doses and the role of AMH in granulosa cells of prehierarchal follicles using RNAi. We found that FSH decreases AMH expression in granulosa cells of 3-5 mm follicles. We successfully knocked down AMH using RNAi by ~80-85% in granulosa cells from 3-5 and 6-8 mm follicles. We chose not to investigate the role of granulosa cells in 6-8 mm follicles further due to very low AMH expression in the controls. To our knowledge, this is the first attempt to study the function of AMH in chicken follicles using RNAi.

Several factors have been shown to regulate AMH in the hen [1,4–6], including FSH [7]. In the current experiments, we found that FSH treatment in granulosa cells significantly decreases AMH in 3-5 mm follicles but not in 6-8 mm follicles. This corroborates results from our previous study, where FSH decreased AMH mRNA expression at the 3-5 mm but not at the 6-8 mm follicle stage [7].

FSH has been shown to regulate AMH in several species, although the effect is controversial as different studies have found an increase [10,13,38], decrease [8,9,11,12], or no change [9] in AMH expression with FSH treatment. In the cow, FSH treatment *in vivo* increases plasma AMH [13] but decreases expression *in vitro* [8,9]. Similarly, *in vivo* FSH administration in low-yielding chickens increased AMH and AMHR2 mRNA expression in 2-4 mm follicles [38], although we found a decrease when granulosa cells from similar sized follicles were treated with FSH *in vitro*. In the hen, exogenous FSH administration also increases the number of growing follicles [38,39] and may increase plasma AMH levels simply due to the increased

number of follicles present. Studying the effect of FSH on AMH in an *in vitro* culture system may therefore be more appropriate to understand the direct result of FSH on AMH in the ovary, as has been previously suggested by Taieb *et al.* [10].

While AMH regulation has been studied in chicken granulosa cells [1,4–7], the function of AMH in chickens has not yet been elucidated due to a lack of commercially available bioactive AMH. We had hypothesized that knocking down AMH would promote follicle development by increasing markers of follicle selection such as FSHR, STAR, and CYP11A1 and that FSH might potentiate these effects. AMH knockdown did not affect FSHR, STAR, or CYP11A1 mRNA expression in granulosa cells of 3-5 mm follicles in the presence or absence of FSH. Previous reports in the hen have shown an inverse relationship between AMH and FSHR during follicle development [6] and after Vitamin D3 treatment of granulosa cells of prehierarchal follicles [5]. Our data suggest that AMH does not affect FSHR mRNA expression directly, as has been shown in steroidogenic human ovarian granulosa-like tumor cells [40]. Additionally, it was recently demonstrated that granulosa cells of 6-8 mm follicles treated with a recombinant chicken AMH showed decreased expression of STAR, CYP11A1, and HSD3B1 at most doses [41]. Our data are inconsistent with these results as we saw no effect on STAR or CYP11A1 following AMH reduction. No validation of the recombinant protein used in the previous study [41] was presented, making interpretation of these results challenging.

Several studies have shown that AMH does not directly affect STAR and CYP11A1 expression but acts indirectly to decrease FSH-induced effects on these genes [24,42]. In our study, FSH did not affect expression of these markers in

untreated controls or in AMH reduced granulosa cells. STAR mRNA expression increased, however, in response to NT siRNA transfection and FSH treatment. Although blasting the sequence of our non-target control returned no hits, the non-target siRNA may affect genes involved in STAR regulation, which we could not anticipate.

The oocyte-specific hormone [43], BMP15, has been shown to decrease AMH and occludin (OCLN) expression and increase FSHR expression, promoting follicle selection [4]. We had hypothesized that AMH might be restricting follicle selection by inhibiting BMP15 signaling through BMPR2 expression in granulosa cells of 3-5 mm follicles. We found that knockdown of AMH had no effect on BMPR2 mRNA expression in granulosa cells. AMHR2 expression is higher in the ooplasm compared to granulosa cells of 3-5 mm follicles. The effects of AMH on the BMP15 pathway may be observable in the oocyte rather than granulosa cells, although we have not examined this.

Although AMH expression was decreased by almost 80% in siRNA-treated granulosa cells of 3-5 mm follicles, we did not observe effects on selected gene expression in response to AMH reduction. It is possible that AMH may not have been knocked down enough to cause changes in mRNA levels of our target genes. Alternatively, other compensatory mechanisms at this stage of follicle development may be regulating steroidogenic gene expression. Finally, although AMH mRNA was significantly reduced, 48 hours may not be sufficient time to reduce AMH at the protein level and affect downstream gene expression. AMH serum half-life ranges

from 2-4 hours in mice [44], 28 hours in ovariectomized women [45], and 48 hours in the cow [46]. AMH half-life has not yet been assessed in chickens.

Although reducing AMH mRNA did not affect the expression of the genes of interest, this is the first study in chickens to knock down AMH using RNAi. RNAi provides an alternative method to study AMH function in the hen, given the lack of commercially available chicken AMH. This study showed that FSH decreases AMH in granulosa cells of 3-5 mm follicles. We showed that knockdown of AMH does not affect markers of follicle selection (FSHR, STAR, CYP11A1, and BMPR2) and that AMH does not seem to affect FSH responsiveness in these genes. We propose that AMH may act by binding to its receptor, primarily expressed in the oocyte [2], and inhibit BMP15 action. This would inhibit follicle selection by preventing BMP15 from decreasing OCLN and AMH and increasing FSHR expression in granulosa cells [36]. FSH may be promoting follicle selection by decreasing AMH and thus removing the restriction on the BMP15 pathway. Future studies could use RNA-sequencing techniques in granulosa cells treated with AMH or NT siRNAs to identify genes downstream of AMH in the hen in a high-throughput method.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Domestication and intense genetic selection in chickens have resulted in highly efficient meat (broiler) and egg-producing (layer) breeds. Intense selection in broiler breeder hens, however, has caused suboptimal reproduction, which can be alleviated by restricting feed intake. The link between feed intake and egg production remains unclear. Laying breeds have been selected for efficient egg-laying and can lay approximately one egg per day during their first year. Following this first year, the flock's average egg-laying rate declines [1,2], leading many producers to cull hens. One way to increase the efficiency of laying breeds is to increase the length of laying past the first year. The aims of this dissertation were to 1) characterize transcriptional differences in follicles of broiler breeder hens fed at different levels, 2) investigate the roles of potential factors (IGF1 and AMH) influencing follicle development in hens, and 3) characterize differences between persistent-laying (PL) and non-persistent laying (NPL) hens.

The first study characterized transcriptional differences in granulosa cells of 6-8 mm follicles between restricted and *ad libitum*-fed broiler breeder hens (Chapter 2). Broiler breeder hens fed *ad libitum* had lower egg production rates throughout the experiment and increased numbers of preovulatory follicles, as previously described [3–6]. RNA-sequencing analysis revealed transcriptional differences in granulosa cells of 6-8 mm follicles of *ad libitum*-fed compared to restricted fed hens, including genes essential for steroidogenesis and genes involved in FSH regulation. Progesterone

production is similar from the two largest preovulatory follicles of broiler breeder hens fed *ad libitum* as compared to different levels of production from these follicles of restricted fed hens [7]. The increase in steroidogenic gene expression observed in granulosa cells of broiler breeder hens fed *ad libitum* suggests that the gain of steroidogenic capacity may occur earlier in development in these hens. Previous research has indicated that broiler breeder hens fed *ad libitum* have multiple hierarchies potentially due to increased follicle selection [8]. It is also plausible that the increased expression of steroidogenic genes in broiler breeder hens fed *ad libitum* represents multiple selected follicles in the 6-8 mm follicle pool rather than one selected follicle, observed in laying hens [9]. The gene expression profile of granulosa cells of 6-8 mm follicles of broiler breeder hens fed *ad libitum* resembled that of the granulosa cells of recently selected follicles in laying hens [10]. This aligns with the hypothesis that *ad libitum* feeding in broiler breeder hens may have increased follicle selection, contributing to multiple hierarchies [8].

IGF1 and FSH were identified as potential upstream regulators of differentially expressed genes. Although the effect of dietary intake on FSH is inconclusive [3,11–14], *ad libitum* feeding in broiler breeder hens increases liver IGF1 mRNA expression [4]. To investigate the potential role of IGF1 in follicle regulation of broiler breeder hens, we examined the effect of IGF1 treatment on cultured granulosa cells from prehierarchal follicles (3-5 mm and 6-8 mm) using the laying hen as a model (Chapter 3). IGF1 increased STAR, CYP11A1, INHA, and INHBA mRNA expression in 6-8 mm follicles. In the previous study, IGF1 was predicted to affect gene expression

(STAR, CYP11A1, and INHA) in broiler breeder hens fed *ad libitum*. This study validated that IGF1 increases the expression of these genes in an *in vitro* system.

Future studies could investigate the effects of exogenous IGF1 administration *in vivo* in restricted-fed broiler breeder hens to test the hypothesis that increased IGF1 availability in broiler breeder hens fed *ad libitum* increases follicle selection and the number of preovulatory follicles. Gene expression analysis confirming the differences we observed in granulosa cells of 6-8 mm follicles could help elucidate the role of IGF1 in the reproductive dysfunction of these hens. Although IGF1 mRNA expression is greater in the livers of *ad libitum*-fed broiler breeder hens compared to restricted-fed hens, suggesting increased levels [4], plasma IGF1 levels have not been evaluated in adult broiler breeder hens. Future studies should confirm the effects of dietary intake on plasma IGF1 levels.

In addition, we investigated transcriptomic differences in broiler breeder hens using granulosa cells pooled from several 6-8 mm follicles. It is not clear however, whether all follicles at this stage have increased expression or whether a few follicles are being selected and contributing to the multiple hierarchies in broiler breeder hens fed *ad libitum*. Studies investigating gene expression patterns in individual follicles may provide a clearer picture of the effects of increased feed intake on follicle selection.

In the third study, we characterized differences between PL and NPL hens (Chapter 4). Laying hens are extremely efficient egg-layers in their first year of lay, but the flock's average rate decreases after the first year. There is variation, however, within the flock after the first year, with some hens continuing to lay at a high rate of

one egg per day (100%), while others decline to rates of approximately 80% or lower. We identified PL and NPL hens based on egg-laying rates (PL= 100%, NPL ≤82%) at 76 weeks of age. Retrospective analysis of PL and NPL hens revealed that PL hens had higher body weights starting at 32 weeks of age and higher egg-laying rates from 68 weeks of age compared to NPL hens. At the ovarian level, PL hens had significantly more follicles (3-5 mm and >12 mm) than NPL hens. PL hens also likely have more extensive ovarian reserves than NPL hens based on increased AMH and BMP15 mRNA expression and lower rates of atresia in <100 µm follicles.

RNA-sequencing analysis showed transcriptional differences between PL and NPL hens, and estrogen receptors were identified as upstream regulators. Although ESR1 and ESR2 mRNA expression or plasma estradiol levels did not differ between PL and NPL hens, differences in egg-laying rates may be associated with changes in estradiol signaling. Future studies should investigate genetic differences in estradiol receptor sequences. Genetic differences identified between PL and NPL hens could be used to identify hens for breeding programs to select for high egg-laying persistency.

This study revealed physiological differences associated with the persistency of lay. Egg producers aim to reach an average of 500 eggs in 100 weeks in a flock [15,16], and some have achieved this benchmark [17,18]. Retrospective analysis of egg-laying efficiencies and body weights identified that these variables could be utilized as predictive tools for producers to decide which hens to maintain in a flock. In large egg-production facilities using cage systems, egg counters could be utilized to identify high egg producing hens. Measuring body weights is already common practice by egg producers to ensure that the flock growth curve is uniform. Producers

could use body weight data to identify persistent layers. More research with larger sample sizes is needed to further develop predictive tools of persistency using body weight and egg-laying rates; however, the research presented in this dissertation, suggests that body weight and egg-laying rates early in life are informative for persistency. Increasing persistency in layers could improve animal welfare, environmental sustainability, and producer profit.

In the final study (Chapter 5), we investigated the role of AMH in prehierarchal follicles (3-5 mm) using RNA-interference. We confirmed that FSH decreased AMH mRNA expression in granulosa cells of 3-5 mm but not 6-8 mm follicles (as shown in our previous study, Chapter 3). In chickens, AMH decreases around the time FSH receptor expression increases [9,19] and FSH may decrease AMH, promoting follicle development. The mechanism by which FSH regulates AMH is not known. In mammals, a transcription factor (GIOT1) known to repress AMH expression was upregulated by FSH [20], but this has not been confirmed in hens. Future studies could investigate the mechanism by which FSH regulates AMH.

Using a specific AMH siRNA, we successfully knocked down AMH by approximately 80-85% in granulosa cells of 3-5 mm and 6-8 mm follicles. In mammals, AMH has been shown to inhibit FSH-induced STAR and CYP11A1 mRNA expression [21,22], although we did not find this. A second hypothesis was that AMH might affect follicle selection by regulating the BMP15 pathway. Knock down of AMH did not affect BMP15 receptor mRNA expression in granulosa cells. Given the high expression of the AMH receptor (AMHR2) in the oocyte [23], it would be better to study the role of AMH using the whole follicle. Although RNAi was

efficient in granulosa cells, RNAi may not be the best approach to study AMH function in a whole follicle system. RNAi takes approximately 24 hours to knock down expression, according to the manufacturer's protocol and our lab currently does not have an effective method to culture whole follicles long term, while maintaining viability. In addition, the multiple cell layers in the follicle may reduce RNAi efficiency. A purified bioactive chicken AMH may be required to investigate AMH function in the whole follicle. BMP15 promotes follicle selection by decreasing AMH and OCLN and increasing FSHR [24]. AMH may restrict follicle selection by inhibiting BMP15 in the oocyte. If this is the case, FSH may decrease AMH mRNA expression in granulosa cells to remove the restriction on follicle selection. The proposed mechanism is outlined in Figure 1. Future experiments should investigate the role of AMH on BMP15 expression in the oocyte, using a whole follicle culture system.

The work presented in this dissertation provides new insights into the reproductive physiology of hens selected for different egg-laying rates. Follicle selection is thought to be disrupted in broiler breeder hens fed *ad libitum*. The present work provides RNA-sequencing data supporting this hypothesis. Previous research has indicated that increased IGF1 bioavailability may be contributing to the ovarian dysfunction. We showed evidence that IGF1 increases markers of follicle development in prehierarchal follicles which may be promoting follicle selection in these hens. Results from another experiment identified differences associated with egg-laying persistency. Hens with higher egg-laying efficiencies later in life had more follicles and less atresia in small follicles (<100 μm), which likely contributes to increased egg-

laying efficiencies. In the last study we decreased AMH mRNA using RNAi and generated a new hypothesis for how AMH may be affecting follicle development through interactions with the oocyte.

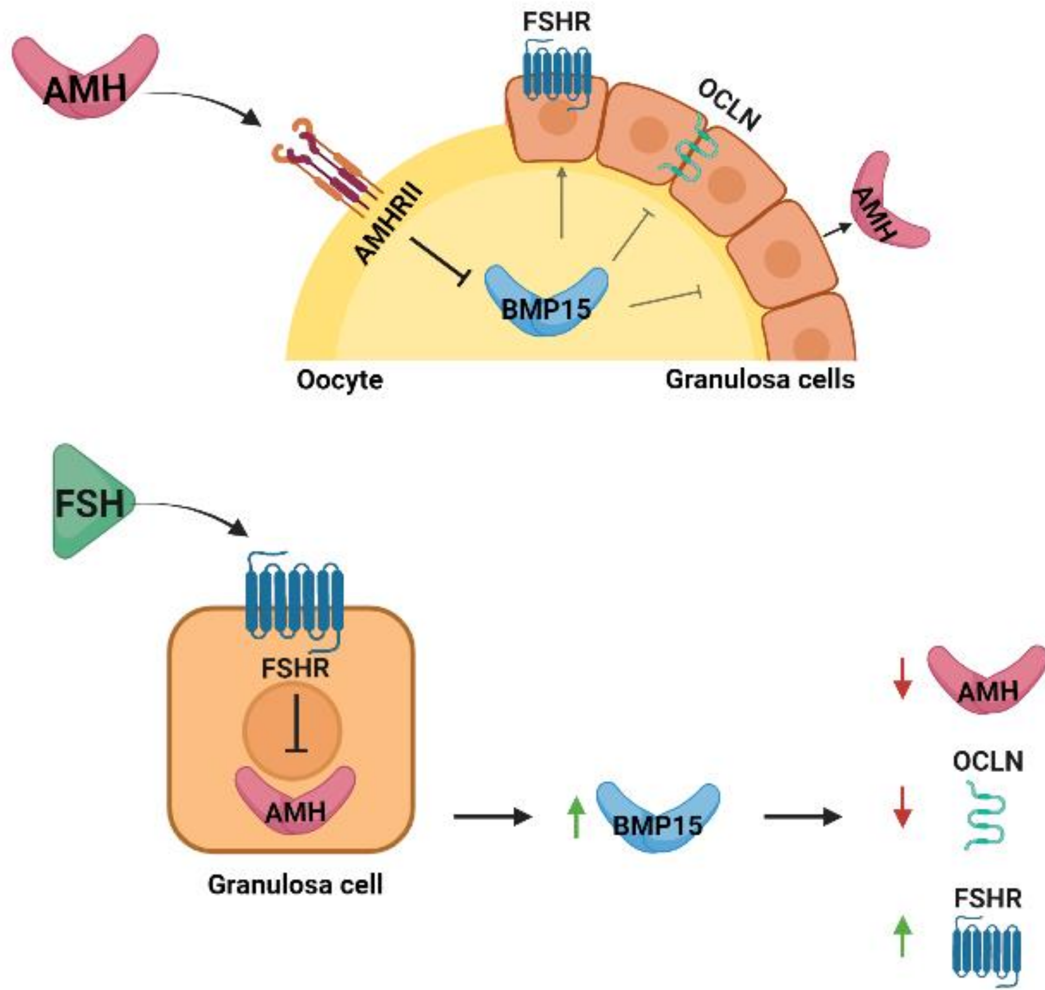

Figure 1. Proposed model for AMH action on BMP15 pathway and FSH regulation of AMH (Created with BioRender.com).

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APPENDICES

Appendix 1: RNA-Seq Alignment Summary. Summary of mapped reads to the Galgal6 genome assembly.

cDNA Library	Total Reads	Mapped Reads	% Aligned
211RF	31302807	28332671	90.5%
246RF	39972051	36131559	90.4%
377RF	49634581	45080210	90.8%
465FF	36996330	33194095	89.7%
598FF	45763471	40732555	89.0%

Appendix 2. Top 50 upregulated differentially expressed genes in FF hens, including the Ensembl gene ID, gene name, log_2(FC), and FDR, and average normalized read counts for FF and RF groups are indicated for each gene.

GeneID	Gene Name	log_2(FC)	FDR	FF	RF
ENSGALG00000034436	CEL	−8.6	0.01	749	2
ENSGALG00000002182	NR5A2	−6.4	<0.01	1531	18
ENSGALG00000038884	SRL	−4.9	<0.01	250	8
ENSGALG00000045327	-	−4.9	<0.01	222	8
ENSGALG00000026808	TMEM72	−4.8	<0.01	1922	70
ENSGALG00000006440	-	−4.2	<0.01	652	35
ENSGALG00000050830	SV2A	−4.2	<0.01	237	13
ENSGALG00000054770	INHA	−4.1	<0.01	55,417	3271
ENSGALG00000010269	KCNAB1	−4.0	<0.01	1174	75
ENSGALG00000042836	KCNH2	−4.0	<0.01	688	44
ENSGALG00000047771	PGF	−3.9	<0.01	2220	150
ENSGALG00000034982	CYP11A1	−3.6	<0.01	11,165	893
ENSGALG00000029968	GADD45B	−3.5	<0.01	300	27
ENSGALG00000001207	PLCH2	−3.4	0.01	630	59
ENSGALG00000006598	SORL1	−3.3	<0.01	5205	517
ENSGALG00000041932	SLC5A5	−3.3	<0.01	1534	155
ENSGALG00000003242	STAR	−3.2	<0.01	1222	132
ENSGALG00000010364	AADAC	−3.2	0.01	1694	189
ENSGALG00000040355	TCF24	−3.1	<0.01	220	26

ENSGALG00000004974	PPARG	−3.0	<0.01	2454	314
ENSGALG00000005884	MAPKKK3L	−2.9	<0.01	270	37
ENSGALG00000011242	OBSL1	−2.8	<0.01	2359	328
ENSGALG00000042607	RSPO3	−2.8	<0.01	321	45
ENSGALG00000014938	ABHD3	−2.8	<0.01	396	56
ENSGALG00000038399	PLEKHA6	−2.8	<0.01	2921	420
ENSGALG00000050611	-	−2.7	<0.01	1650	257
ENSGALG00000041143	UMOD	−2.6	<0.01	7916	1266
ENSGALG00000011537	PDE10A	−2.6	<0.01	325	53
ENSGALG00000008815	LRRN4	−2.5	<0.01	8678	1484
ENSGALG00000011608	INF2	−2.5	<0.01	2394	418
ENSGALG00000011803	EMP1	−2.5	<0.01	347	61
ENSGALG00000019077	-	−2.5	<0.01	2109	372
ENSGALG00000043234	HBA1	−2.5	<0.01	236	43
ENSGALG00000042929	-	−2.4	<0.01	746	139
ENSGALG00000010326	FLVCR2	−2.4	<0.01	530	98
ENSGALG00000006453	TF	−2.4	<0.01	232	43
ENSGALG00000037603	SESN2	−2.4	<0.01	6298	1195
ENSGALG00000003750	PLCG1	−2.4	<0.01	3056	590
ENSGALG00000033683	PHOSPHO1	−2.3	<0.01	6924	1408
ENSGALG00000016415	MAP7D2	−2.3	<0.01	4248	882
ENSGALG00000044649	P2RX2	−2.3	<0.01	1134	235

ENSGALG00000016954	RGCC	−2.3	<0.01	416	87
ENSGALG00000008537	EPHB3	−2.3	<0.01	516	108
ENSGALG00000036728	PRDM16	−2.2	<0.01	436	92
ENSGALG00000041708	WNT4	−2.2	<0.01	18,324	4040
ENSGALG00000014734	-	−2.2	<0.01	1166	260
ENSGALG00000018803	-	−2.1	<0.01	415	98
ENSGALG00000009512	TFPI2	−2.1	<0.01	94,942	22,674
ENSGALG00000049157	DOK4	−2.0	<0.01	4274	1034
ENSGALG00000038458	LOXL1	−2.0	<0.01	1624	401

Appendix 3. Top 50 upregulated differentially expressed genes in RF hens, including the Ensembl gene ID, gene name, log_2(FC) and FDR, and average normalized read counts for FF and RF groups are indicated for each gene.

GeneID	Gene Name	log_2(FC)	FDR	FF	RF
ENSGALG00000051980	-	6.5	0.01	2	229
ENSGALG00000036798	COL4A1	4.1	0.02	32	535
ENSGALG00000007819	PDZRN3	4.0	<0.01	24	361
ENSGALG00000015908	COL12A1	3.8	<0.01	34	491
ENSGALG00000043754	GLUL	3.2	<0.01	340	3158
ENSGALG00000010858	LRP2	3.0	0.03	56	446
ENSGALG00000040755	ANGPT4	2.9	<0.01	44	320
ENSGALG00000048104	-	2.8	<0.01	54	367
ENSGALG00000003670	MAFB	2.8	<0.01	137	938
ENSGALG00000016843	COL4A2	2.8	0.01	40	271
ENSGALG00000033338	GPT2	2.6	<0.01	60	374
ENSGALG00000030065	TENM3	2.6	<0.01	306	1805
ENSGALG00000009405	GRIA2	2.6	<0.01	143	839
ENSGALG00000034453	SAMD11	2.4	<0.01	113	604
ENSGALG00000050840	APCDD1	2.2	<0.01	355	1641
ENSGALG00000006172	ABCC8	2.1	0.01	120	534
ENSGALG00000038364	NOV	2.1	<0.01	492	2083
ENSGALG00000016820	GAS6	2.1	<0.01	49	207
ENSGALG00000013697	CNDP1	2.1	<0.01	106	443

ENSGALG00000011200	THBS2	2.0	<0.01	65	264
ENSGALG00000014178	-	2.0	<0.01	82	318
ENSGALG00000011623	ADAMTS3	1.9	<0.01	208	803
ENSGALG00000031916	ZP1	1.8	0.01	78	283
ENSGALG00000000667	EDN2	1.8	<0.01	1580	5639
ENSGALG00000026055	PALM	1.8	<0.01	60	210
ENSGALG00000009687	KCNK2	1.8	<0.01	10,362	35,972
ENSGALG00000004812	FAM129A	1.8	0.02	58	203
ENSGALG00000007268	-	1.8	<0.01	192	669
ENSGALG00000002671	-	1.8	<0.01	660	2230
ENSGALG00000032836	-	1.7	<0.01	137	445
ENSGALG00000012595	AGTPBP1	1.7	<0.01	96	310
ENSGALG00000001768	TENM2	1.7	0.01	340	1095
ENSGALG00000011994	SYNPO2	1.7	0.02	66	215
ENSGALG00000036883	MET	1.7	<0.01	619	1988
ENSGALG00000009612	TGFB2	1.7	<0.01	188	600
ENSGALG00000045776	CPN2	1.7	0.01	90	282
ENSGALG00000002081	MMP28	1.6	<0.01	112	348
ENSGALG00000015542	PLPPR1	1.6	<0.01	105	324
ENSGALG00000017065	-	1.6	0.05	84	259
ENSGALG00000011145	TRIL	1.6	0.01	99	301
ENSGALG00000034085	-	1.6	0.01	207	627

ENSGALG00000031534	ARID5B	1.6	<0.01	360	1081
ENSGALG00000026981	NHSL1	1.6	0.01	370	1102
ENSGALG00000027514	-	1.6	<0.01	1168	3445
ENSGALG00000016251	-	1.6	<0.01	1485	4369
ENSGALG00000012115	WT1	1.5	<0.01	1514	4420
ENSGALG00000010902	CERS6	1.5	0.05	153	448
ENSGALG00000051001	-	1.5	0.01	294	844
ENSGALG00000009495	FGFR2	1.5	<0.01	229	648
ENSGALG00000012834	AKR1D1	1.5	<0.01	460	1297

Appendix 4. RNA-Sequencing Alignment Summary. Summary of mapped reads to the Galgal6 genome assembly.

cDNA Library	Total reads	Mapped reads	Mapped Reads
8 PL	35502510	32390352	91.2%
9 PL	37044982	34595905	93.4%
20 PL	32960069	30385312	92.2%
10 NPL	42417486	38609015	91.0%
12 NPL	37249947	33213184	89.2%
13 NPL	39723999	36753685	92.5%
42 NPL	33914634	30530949	90.0%

Appendix 5. Top 50 upregulated differentially expressed genes in PL hens. The Ensembl gene ID, gene name, log_2(FC), FDR, and average normalized read counts for PL and NPL groups.

GeneID	Gene Name	log2(FC)	FDR	PL	NPL
ENSGALG00000003075	SLC34A1	10.2	0.02	366	0
ENSGALG00000016667	AvBD10	9.7	<0.01	9365	11
ENSGALG00000016491	APOB	9.6	0.03	236	0
ENSGALG00000000309	-	7.6	<0.01	99	0
ENSGALG00000051283	-	7.6	0.01	452	2
ENSGALG00000015492	PDZK1	7.3	<0.01	74	0
ENSGALG00000006635	RHCG	6.4	0.04	124	2
ENSGALG00000040666	ATP6V0A4	6.0	0.01	190	3
ENSGALG00000000919	PIGR	5.9	<0.01	75	1
ENSGALG00000040045	DBH	5.9	<0.01	76	1
ENSGALG00000051882	-	5.1	0.05	1224	37
ENSGALG00000006717	-	4.6	<0.01	146	6
ENSGALG00000002957	-	4.6	0.01	206	8
ENSGALG00000016020	CLIC6	4.5	<0.01	127	6
ENSGALG00000024031	CYB561	4.4	0.01	72	4
ENSGALG00000053694	OVST	4.2	0.04	229	12
ENSGALG00000032282	CALB1	3.7	0.04	723	55
ENSGALG00000007320	FXYD2	3.7	0.01	118	10
ENSGALG00000016791	SLC9A2	3.6	0.01	90	8

ENSGALG00000019553	SERPINB10B	3.6	<0.01	338	28
ENSGALG00000024272	S100A9	3.6	0.05	985	84
ENSGALG00000040424	SCNN1A	3.5	0.01	96	8
ENSGALG00000034698	TMEM52B	3.3	0.01	142	14
ENSGALG00000004196	UGT1A1	3.0	0.04	76	10
ENSGALG00000010983	NPY	2.5	<0.01	74	13
ENSGALG00000051710	-	2.5	0.03	58	10
ENSGALG00000049250	-	2.4	<0.01	214	40
ENSGALG00000008332	F2	2.1	0.03	60	14
ENSGALG00000007636	PCK1	2.0	0.03	124	30
ENSGALG00000048197	-	2.0	<0.01	254	63
ENSGALG00000049800	-	1.9	0.05	50	13
ENSGALG00000030493	ENTPD3	1.9	0.01	62	16
ENSGALG00000030025	FABP4	1.8	<0.01	456	128
ENSGALG00000005621	CAMK2A	1.8	<0.01	624	177
ENSGALG00000052439	-	1.7	0.02	60	18
ENSGALG00000007596	-	1.7	0.01	390	124
ENSGALG00000016144	TMPRSS2	1.6	0.04	95	31
ENSGALG00000050992	-	1.5	0.01	295	101
ENSGALG00000033535	RGS16	1.5	0.03	95	33
ENSGALG00000048762	-	1.5	0.03	295	102
ENSGALG00000023953	-	1.5	0.02	236	82

ENSGALG00000051136	-	1.5	0.01	85	31
ENSGALG00000051161	-	1.4	0.02	98	36
ENSGALG00000027986	NIPAL1	1.4	0.01	168	62
ENSGALG00000008980	VWA2	1.4	0.02	603	228
ENSGALG00000021341	ADAM20	1.4	0.02	88	33
ENSGALG00000009737	TAC1	1.4	0.01	642	246
ENSGALG00000017168	SLN	1.3	0.01	410	164
ENSGALG00000009266	FGA	1.3	0.05	198	79
ENSGALG00000034567	POU4F3	1.3	0.04	362	150

Appendix 6. Top 50 downregulated differentially expressed genes in PL hens. The Ensembl gene ID, gene name, log_2(FC), FDR, and average normalized read counts for PL and NPL groups.

GeneID	Gene Name	log2(FC)	FDR	PL	NPL
ENSGALG00000039205	-	-3.7	<0.01	22	294
ENSGALG00000043914	-	-3.4	<0.01	10	108
ENSGALG00000054062	-	-2.6	<0.01	39	240
ENSGALG00000052892	-	-2.6	<0.01	34	196
ENSGALG00000054480	-	-2.4	<0.01	31	168
ENSGALG00000005613	-	-2.4	<0.01	20	109
ENSGALG00000009601	NELL2	-2.4	<0.01	2452	12547
ENSGALG00000052843	-	-2.4	<0.01	12	61
ENSGALG00000032980	DLGAP3	-2.4	<0.01	10	54
ENSGALG00000046947	-	-2.3	0.03	22	111
ENSGALG00000017308	CHRDL2	-2.2	<0.01	20	92
ENSGALG00000014726	RAB3C	-2.0	<0.01	26	104
ENSGALG00000017790	RF00001	-2.0	0.02	13	50
ENSGALG00000030151	LUZP2	-1.9	<0.01	61	236
ENSGALG00000007217	BEST1	-1.9	<0.01	26	104
ENSGALG00000015167	PRUNE2	-1.9	<0.01	300	1116
ENSGALG00000048283	-	-1.9	<0.01	54	198

ENSGALG00000010583	VIT	-1.9	<0.01	232	848
ENSGALG00000048880	-	-1.9	<0.01	48	175
ENSGALG00000017197	CNTN5	-1.9	0.03	43	156
ENSGALG00000041634	ACTG2	-1.8	0.04	3580	12842
ENSGALG00000029264	PI15	-1.8	<0.01	100	353
ENSGALG00000012755	IGF-I	-1.8	<0.01	30	103
ENSGALG00000006575	VIPR2	-1.8	<0.01	1134	3867
ENSGALG00000052853	-	-1.8	0.01	48	163
ENSGALG00000037131	-	-1.8	<0.01	512	1723
ENSGALG00000044866	-	-1.7	<0.01	164	548
ENSGALG00000049067	-	-1.7	<0.01	216	706
ENSGALG00000042570	-	-1.7	0.02	16	50
ENSGALG00000030932	NKX2-3	-1.7	<0.01	166	527
ENSGALG00000030687	LGI3	-1.7	0.01	298	945
ENSGALG00000046861	-	-1.7	<0.01	42	133
ENSGALG00000008727	-	-1.6	0.01	24	74
ENSGALG00000007269	GABRA3	-1.6	<0.01	62	190
ENSGALG00000050414	-	-1.6	0.03	26	82
ENSGALG00000041555	COL1A1	-1.6	<0.01	21832	66150
ENSGALG00000027809	INSYN2B	-1.6	<0.01	46	139
ENSGALG00000010200	TRHDE	-1.6	<0.01	292	879

ENSGALG00000025958	RF00012	-1.6	0.02	76	222
ENSGALG00000009219	C3H1orf95	-1.5	<0.01	28	80
ENSGALG00000040342	ADAMTS1	-1.5	<0.01	2086	5844
ENSGALG00000019361	FAM19A2	-1.5	<0.01	149	416
ENSGALG00000051101	-	-1.5	<0.01	146	400
ENSGALG00000005524	NTSR1	-1.5	0.03	44	123
ENSGALG00000047639	-	-1.5	<0.01	33	89
ENSGALG00000015630	EDIL3	-1.4	<0.01	169	462
ENSGALG00000016904	SLITRK6	-1.4	0.02	194	530
ENSGALG00000009719	PCDH10	-1.4	<0.01	63	172
ENSGALG00000003578	FN1	-1.4	<0.01	11188	30400
ENSGALG00000054718	FLNA	-1.4	0.01	1286	3488