ABSTRACT

Title of Dissertation: IDENTIFICATION OF THE MOLECULAR NETWORKS GOVERNING OVULATION FREQUENCY IN LOW AND HIGH EGG PRODUCING TURKEY HENS

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Selection for carcass traits has negatively impacted and varied the reproductive efficiency of the commercial turkey hen, creating overall lowered egg production as well as distinct groups of low egg producing hens (**LEPH**) and high egg producing hens (**HEPH**). Ovulation frequency correlates with egg production and differs between LEPH and HEPH. Ovulation is governed primarily by the hypothalamo-pituitary-gonadal (**HPG**) axis through a preovulatory surge (**PS**) of luteinizing hormone (**LH**) and progesterone (**P4**) but ovulation can be influenced by the hypothalamo-pituitary-thyroid (**HPT**) axis. Dysregulation of the thyroid and reproductive axes, disrupting the PS, leads to lowered egg production, leaving the poultry industry to compensate with larger breeding flocks. LEPH exhibited hypothalamic and pituitary expression consistent with decreased ovulation stimulation and increased ovulation inhibition as well as decreased steroid synthesis in follicle cells. Neuroendocrine and ovarian tissues from HEPH showed a

higher sensitivity and response to *in vitro* HPG axis stimulation. Moreover, cells from HEPH responded positively to HPG axis inhibition while cells from LEPH responded negatively, indicating functional differences in HPG axis regulation. RNA sequencing results reinforced the higher expression of HPG axis genes in HEPH and showed higher expression of HPT axis genes in LEPH. Estradiol (**E2**) was identified as an upstream regulator activated in HEPH. Hens with average egg production (**AEPH**) also showed upregulation of E2 receptors during the PS, suggesting involvement in positive feedback loops. Supporting the role of E2 in neuroendocrine feedback, higher plasma concentrations of E2 were seen during the PS in HEPH. Looking into the HPT axis, LEPH displayed lower plasma concentrations of triiodothyronine (**T3**) and higher levels of thyroxine (**T4**) outside of the PS while HEPH displayed lower levels of T3 and higher levels of T4 inside of the PS. The T3 and T4 levels surrounding the PS in HEPH were consistent with levels seen in AEPH. At a molecular, cellular, and endocrine level, the reproductive physiology of LEPH and HEPH differs, ultimately leading to egg production differences. Studies tying the noted differences to egg production rates will allow for identification of genetic markers to increase the reproductive efficiency of commercial breeding hens.

IDENTIFICATION OF THE MOLECULAR NETWORKS GOVERNING OVULATION FREQUENCY IN LOW AND HIGH EGG PRODUCING TURKEY HENS

by

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TABLE OF CONTENTS

LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF ABBREVIATIONS xiii

CHAPTER 1: Literature Review 1
Introduction 1
The Turkey Industry 2
The Hypothalamo-Hypophyseal Complex 6
The HPG Axis 7
Function and Overview 7
Hypothalamic Releasing Factors and Receptors 8
Pituitary Gonadotropins and Receptors 10
Steroid Hormones and Receptors 10
HPG Axis Signaling 13
GPCRs 13
Steroid Hormone Receptors 16
Steroidogenesis 17
Follicle Development 20
Ovarian Structure 20
Folliculogenesis 22
Activation 22
Recruitment 23
Selection 23
Ovulation 24
Egg Production and Ovulation Frequency 24
The HPT Axis 25
Function and Overview 25
Hypothalamic Releasing Factors and Receptors 26
Pituitary Thyrotropin and Receptor 26
Thyroid Hormones and Receptors 26
The Avian Genome 28

Rationale and Objectives 31

CHAPTER 2: Characterization of gene expression in the hypothalamo-pituitary-gonadal axis during the preovulatory surge in the turkey hen 32

Abstract 33

Introduction 33

Materials and Methods 35

Hen Selection 35

RIAs 37

Cell Isolation 37

qRT-PCR 39

Statistics 41

Results 43

Plasma Hormone Levels 43

Hypothalamus and Pituitary Gene Expression 43

Follicle Gene Expression 47

Discussion 51

CHAPTER 3: Characterization of the hypothalamo-pituitary-gonadal axis in low and high egg producing turkey hens 56

Abstract 57

Introduction 57

Materials and Methods 59

Hen Selection and Cell Isolation 59

RIAs 60

qRT-PCR 60

Statistics 61

Results 61

Production and Plasma Parameters 61

Hypothalamic Gene Expression 65

Pituitary Gene Expression 65

F1 Follicle Gene Expression 69

F5 Follicle Gene Expression 71

SWF Gene Expression 73
Discussion 75

CHAPTER 4: Differences in *in vitro* responses of the hypthalamo-pituitary-gonadal hormonal axis between low and high egg producing turkey hens 80
Abstract 81
Introduction 81
Materials and Methods 84
Hen Selection 84
Cell Isolation and Culture 84
qRT-PCR 86
RIAs 87
Statistics 87
Results 87
GnRH Treatment in Pituitary Cells 87
GnIH Treatment in Pituitary Cells 90
LH Treatment in F1G 93
FSH Treatment in SWF 93
Discussion 96

CHAPTER 5: Transcriptome analysis of the hypothalamus and pituitary of turkey hens with low and high egg production 100
Abstract 101
Background 102
Results and Discussion 103
Transcriptome Alignment and Mapping 103
Overview of DEGs 104
RNAseq Confirmation 108
Overview of Network Analysis 112
The HPG Axis 112
The HPT Axis 117
Upstream Analysis 124
Effect of T3 and E2 on Pituitary Gonadotropin Production 127

Conclusions 132
Materials and Methods 132
Hen Selection and Tissue Collection 132
RIA 133
RNA Isolation, cDNA Library Construction, and Sequencing 134
Bioinformation Analysis of Sequencing Data 136
Ingenuity Pathway Analysis 137
Culture of Pituitary Cells 137
RT-qPCR 138
RNA Sequencing Confirmation 138
Statistics 139

CHAPTER 6: Transcriptome analysis during follicle development in turkey hens with low and high egg production.. 147
Abstract 148
Background 149
Results and Discussion 151
Transcriptome Alignment and Mapping 151
Overview of DEGs 151
RNAseq Confirmation 158
F1G Network Analysis 161
F5G Network Analysis 163
F5I Network Analysis 165
SWF Network Analysis 167
Upstream Analysis 169
Effect of T3 on SWF E2 Production 171
Conclusions 174
Materials and Methods 175
Hen Selection and Tissue Collection 175
Follicle Wall Cell Isolation 176
RNA Isolation, cDNA Library Construction, and Sequencing 177
Bioinformation Analysis of Sequencing Data 178
Ingenuity Pathway Analysis 178

Culture of SWF Cells 179
RIAs 179
RNA Sequencing Confirmation 180
Statistics 180

CHAPTER 7: Characterization of hypothalamo-pituitary-thyroid axis in the hypothalamus, pituitary gland, and ovarian follicles of turkey hens during the preovulatory surge and in hens with different levels of egg production 183
Abstract 184
Introduction 185
Materials and Methods 186
Hen Selection and Cell Isolation 186
RIAs 188
qRT-PCR 188
Statistics 189
Results 189
Plasma Hormone Levels 189
Hypothalamic Gene Expression 193
Pituitary Gene Expression 195
F1G Gene Expression 197
F5E Gene Expression 197
Discussion 200

CHAPTER 8: Conclusions and Future Directions 205
Conclusions 206
Future Directions 216

REFERENCES 219

LIST OF TABLES

Table 2.1	Primers for RT-qPCR	42
Table 3.1	Ovarian and Plasma Parameters in LEPH and HEPH	62
Table 5.1	Significant HPG axis gene expression changes in the hypothalamus and pituitary between LEPH and HEPH, outside and during the PS	117
Table 5.2	Significant HPT axis gene expression changes in the hypothalamus and pituitary between LEPH and HEPH, outside and during the PS	123
Table 5.3	Significant upstream regulators in the hypothalamus and pituitary between LEPH and HEPH, outside and during the PS	126
Supplemental Table 5.1	Significant HPG axis gene expression changes in the hypothalamus and pituitary during the PS, in LEPH and HEPH	140
Supplemental Table 5.2	Significant HPT axis gene expression changes in the hypothalamus and pituitary during the PS, in LEPH and HEPH	141
Supplemental Table 5.3	DEGs in the hypothalamus and pituitary between LEPH and HEPH, outside and during the PS that are predicted to be target genes of beta-estradiol	142
Supplemental Table 5.4	DEGs in the hypothalamus and pituitary during the ovulatory cycle individually in LEPH and HEPH that are predicted to be target genes of beta-estradiol	143
Table 6.1	Significant upstream regulators between LEPH and HEPH in the F1G, F5G, F5I, SWF	170

LIST OF FIGURES

Figure 1.1	Flock egg production	5
Figure 1.2	HPG axis signaling pathways	15
Figure 1.3	The three-cell model of steroidogenesis	19
Figure 1.4	Follicle development and ovulation in avian follicles	21
Figure 2.1	Timing of blood and tissue sampling	36
Figure 2.2	Plasma P4 and E2 hormone profiles in during the PS	44
Figure 2.3	Hypothalamic expression of HPG axis genes in during the PS	45
Figure 2.4	Pituitary expression of HPG axis genes in during the PS	46
Figure 2.5	Follicle granulosa layer expression of HPG axis genes in during the PS	48
Figure 2.6	Follicle theca interna layer expression of HPG axis genes in during the PS	49
Figure 2.7	Follicle theca externa layer expression of HPG axis genes in during the PS	50
Figure 2.8	Summary of the HPG axis gene expression changes in during the PS	55
Figure 3.1	Production parameters in LEPH and HEPH	63
Figure 3.2	Plasma P4 and E2 hormone profiles in LEPH and HEPH in during the PS	64
Figure 3.3	Hypothalamic expression of HPG axis genes in LEPH and HEPH in during the PS	67
Figure 3.4	Pituitary expression of HPG axis genes in LEPH and HEPH in during the PS	68
Figure 3.5	F1 follicle expression of HPG axis genes in LEPH and HEPH in during the PS	70
Figure 3.6	F5 follicle expression of HPG axis genes in LEPH and HEPH in during the PS	72

Figure 3.7	SWF expression of HPG axis genes in LEPH and HEPH in during the PS	74
Figure 4.1	Pituitary expression of gonadotropin production genes after GnRH treatment in LEPH and HEPH	89
Figure 4.2	Pituitary expression of gonadotropin production genes after GnIH treatment in LEPH and HEPH	92
Figure 4.3	P4 production in F1G cells from LEPH and HEPH after LH treatment	94
Figure 4.4	E2 production in SWF cells from LEPH and HEPH after FSH treatment	95
Figure 5.1	Characteristics of DEGs in the hypothalamus and pituitary of LEPH and HEPH in during PS	106
Figure 5.2	Confirmation of RNA sequencing data in the hypothalamus and pituitary in LEPH and HEPH in during PS	110
Figure 5.3	Network analysis in the hypothalamus and pituitary of LEPH and HEPH in during the PS	114
Figure 5.4	Network analysis in the hypothalamus and pituitary of LEPH and HEPH in during the PS	120
Figure 5.5	Activation z-score for beta-estradiol based on DEGs	125
Figure 5.6	Pituitary expression of gonadotropin production genes after T3/E2 pretreatment followed by GnRH treatment in LEPH and HEPH	129
Supplemental Figure 5.1	Number of reads and aligned obtained for each sample in the hypthalamus and pituitary	144
Supplemental Figure 5.2	Network analysis in the hypothalamus of LEPH and HEPH in during the PS	145
Supplemental Figure 5.3	Network analysis in the pituitary of LEPH and HEPH in during the PS	146
Figure 6.1	Number of DEGs in the F1G, F5G, F5I, and SWF of LEPH and HEPH	153
Figure 6.2	Characteristics of DEGs in the F1G, F5G, F5I, and SWF of LEPH and HEPH	156

Figure 6.3	Confirmation of RNA sequencing data in the F1G, F5G, F5I, and SWF in LEPH and HEPH	159
Figure 6.4	Network analysis in the F1G of LEPH and HEPH	162
Figure 6.5	Network analysis in the F5G of LEPH and HEPH	164
Figure 6.6	Network analysis in the F5I of LEPH and HEPH	166
Figure 6.7	Network analysis in the SWF of LEPH and HEPH	168
Figure 6.8	E2 production in SWF cells from LEPH and HEPH after T3 pretreatment followed by FSH treatment	172
Supplemental Figure 6.1	Number of reads and aligned obtained for each sample in the F1G, F5G, F5I, and SWF	182
Figure 7.1	Plasma T3 and T4 hormone profiles in AEPH, LEPH, HEPH in during the PS	191
Figure 7.2	Plasma T3 to T4 ratio and combined T3 and T4 in AEPH, LEPH, HEPH in during the PS	192
Figure 7.3	Hypothalamic expression of HPG axis genes in AEPH, LEPH, and HEPH in during the PS	194
Figure 7.4	Pituitary expression of HPG axis genes in AEPH, LEPH, and HEPH in during the PS	196
Figure 7.5	Follicle granulosa layer expression of HPG axis genes in AEPH, LEPH, and HEPH in during the PS	198
Figure 7.6	Follicle theca interna layer expression of HPG axis genes in AEPH, LEPH, and HEPH in during the PS	199
Figure 8.1	Summary of Chapter 2 HPG axis results between AEPH outside and inside of the PS	207
Figure 8.2	Summary of Chapter 3 HPG axis results between LEPH and HEPH, outside and inside of the PS	209
Figure 8.3	Summary of Chapter 4 HPG axis results between cells isolated from LEPH and HEPH	211
Figure 8.4	Summary of transcriptome results of LEPH and HEPH from Chapters 5 and 6	214

Figure 8.5 Summary of Chapter 7 HPT axis results of AEPH, LEPH, and HEPH, outside and inside of the PS.. 215

LIST OF ABBREVIATIONS

General Abbreviations

Abbreviations	**Name**
%GC	GC content
AC	adenylyl cyclase
AD	androgens
AEPH	average egg producing hens
ANOVA	analysis of variance
ATF1	activating transcription factor 1
BARC	Beltsville Agricultural Research Center
BSA	bovine serum albumen
Ca2+	Calcium
cAMP	cyclic adenosine monophosphate
CBP	cyclic CREB-binding protein
cDNA	complementary cDNA
CGA	glycoprotein hormone alpha-subunit
CPs	chaperone proteins
CREB	cAMP-response element-binding protein
CREM	cAMP-responsive modulator
CREs	cAMP-response elements
CRH	corticotropin-releasing hormone
CYP11A1	cholesterol side chain cleavage enzyme
CYP17A1	17, 20-lyase
CYP19A1	aromatase
D2	iodothyronine deiodinase 2
DAG	diacyl-glycerol
DEGs	differentially expressed genes
DIT	diiodotyrosines
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium: F12 Nutrients
DNA	deoxyribonucleic acid

E2	estradiol
EPD	eggs per day
ERK	extracellular-signal-regulated kinase
ESRa	estradiol receptor alpha
ESRb	estradiol receptor beta
F1G	granulosa layer of the largest preovulatory follicle
F5	largest preovulatory follicle
F5	fifth largest preovulatory follicle
F5G	granulosa layer of the fifth largest preovulatory follicle
F5I	theca interna layer of the fifth largest preovulatory follicle
FSH	follicle stimulating hormone
FSHR	FSH receptor
G-proteins	guanine nucleotide binding proteins
GDP	guanosine diphosphate
GHRH	Growth hormone releasing hormone
GnIH	gonadotropin inhibitory hormone
GnIHR (GNRHR1)	GnIH receptor 1
GnIHR2	GnIH receptor 2
GnRH (GnRH1)	gonadotropin releasing hormone 1
GnRH2	gonadotropin releasing hormone 2
GnRHR (GnRHR1)	GnRH receptor 1
GnRHR3	GnRH receptor 3
GPCRs	G-protein-coupled receptors
GTP	guanosine triphosphate
HEPH	high egg producing hens
HPG	hypothalamo-pituitary-gonadal
HPT	hypothalamo-pituitary-thyroid
HSD17B1	17β-hydroxysteroid dehydrogenase
HSD3B1	3β-hydroxysteroid dehydrogenase
HSPs	heat shock proteins
IP3	inositol triphosphate
IPA	Ingenuity® Pathway Analysis

LEPH	low egg producing hens
LH	luteinizing hormone
LHCGR	LH receptor
MAPK	mitogen-activated protein kinase
ME	median eminence
MIT	monoiodotyrosines
NADH	Nicotinamide adenine dinucleotide
NFAT	nuclear factor of activated T-cells
NGS	next generation sequencing
NPT	no pretreatment
P/S	penicillin G and streptomycin sulfate
P4	progesterone
PCR	polymerase chain reaction
PGR	progesterone receptor
PIP2	phosphatidyl inositol diphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POA	preoptic area
PS	preovulatory surge
PVN	paraventricular nucleus
RIA	radioimmunoassay
RNA	ribonucleic acid
RNAseq	RNA sequencing
RPKM	reads per kilobase million
RT-qPCR	quantitative reverse transcription PCR
SMEM	minimum essential medium, spinner modification
SNPs	single nucleotide polymorphisms
SON	supraoptic nucleus
SST	somatostatin
STAR	steroidogenic acute regulatory
SWF	small white follicles

T3	triiodothyronine
T4	thyroxine
THR	thyroid hormone receptor
THRa	thyroid hormone receptor alpha
THRb	thyroid hormone receptor beta
T_m	melting temperature
TRE	thyroid response element
TRH	thyrotropin releasing hormone
TRHR	TRH receptor
TSH	Thyroid stimulating hormone
TSHR	TSH receptor
VIP	vasoactive intestinal poly-peptide

Gene Names

Abbreviations	Gene Name
ACTA2	alpha-actin-2
ACTB	beta-actin
ALB	albumen
ANXA2	annexin A2
APOA1	apolipoprotein A1
AR	androgen receptor
ARHGAP18	Rho GTPase activating protein 18
BLOC1S4	biogenesis of lysosomal organelles complex-1, subunit 4
CERS2	ceramide synthase 2
CGA	glycoprotein hormone alpha-subunit
CLTB	clathrin light chain B
COX2	cyclooxygenase-2
CRYBB1	crystallin beta B1
CSNK2A2	casein kinase 2 alpha 2
CSNK2A2	casein kinase 2 alpha 2
CYP11A1	cholesterol side chain cleavage enzyme
CYP17A1	17, 20-lyase
CYP19A1	aromatase
DENND2D	DENN domain containing 2D
DIO1	iodothyronine deiodinase 1
DIO2	iodothyronine deiodinase 2
ESR1	estradiol receptor alpha
ESR2	estradiol receptor beta
ESRRB	estrogen related receptor beta
ESYT3	extended synaptotagmin 3
FA2H	fatty acid 2-hydroxylase
FABP6	fatty acid binding protein 6
FHL2	four and a half LIM domain 2
FSHB	follicle stimulating hormone beta-subunit
FSHR	FSH receptor

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GNIH (NPVF)	gonadotropin inhibitory hormone (neuropeptide VF precursor)
GNIHR1 (GNIHR)	gonadotropin inhibitory hormone receptor 1
GNIHR2	gonadotropin inhibitory hormone receptor 1
GNRH	gonadotropin releasing hormone
GNRHR	gonadotropin releasing hormone receptor
HSD17B1	17β-hydroxysteroid dehydrogenase 1
HSD17B11	17β-hydroxysteroid dehydrogenase 11
HSD17B2	17β-hydroxysteroid dehydrogenase 2
HSD3B1	3β-hydroxysteroid dehydrogenase 1
IGF1	insulin like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
INHA	inhibin subunit alpha
INHBB	inhibin subunit beta B
INSIG1	insulin induced gene 1
ITG2A	integral membrane protein 2A
ITGAV	integrin subunit alpha V
ITGB3	integrin beta chain beta 3
LHB	luteinizing hormone beta-subunit
LHCGR	LH receptor
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
MANEA	mannosidase endo-alpha
ND4	NADH dehydrogenase 4
NFKB2	nuclear factor kappa beta-subunit 2
PAPPA2	pappalysin 2
PGK1	phosphoglycerate kinase 1
PGR	progesterone receptor
PRLR	prolactin receptor
PRPF19	pre-mRNA pocessing factor 19
PSMA2	proteasome subunit alpha 2
PSMD2	proteasome 26S subunit, non-ATPase 2
RBBP8	RB binding protein 8 endonuclease

RSAD2	radical s-adenosyl methionine domain containing 2
SHISA2	shisa family member 2
SLC16A12	solute carrier family 16 member 12
SLC7A5	solute carrier family 7 member 5
SLCO1C1	solute carrier organic anion transporter family member 1C1
SST	somatostatin
STAR	steroidogenic acute regulatory
TGFBR1	transforming growth factor beta receptor 1
TRH	thyrotropin releasing hormone
TRHR	TRH receptor
TSHB	thyrotropin stimulating hormone beta-subunit
TSHR	TSH receptor
TTR	transthyretin
TUBB2B	tubulin beta 2B class IIb
U2SURP	U2 snRNP associated SURP domain containing
ZFPM1	zinc finger protein, FOG family member 1

CHAPTER 1

Literature Review

Introduction

Egg production is primarily regulated by the hypothalamo-pituitary-gonadal (**HPG**) axis. However, hormonal inputs from other neuroendocrine axes can influence HPG axis function (Pollock et al., 2002). Hen egg production level is ultimately defined by ovulation frequency, with ovulation rates and egg production rates highly correlated (Wolford et al., 2012). Ovulation is triggered by a preovulatory surge (**PS**) of luteinizing hormone (**LH**) and progesterone (**P4**) that occurs 8-10 hours prior to ovulation in the turkey hen (Yang et al., 1997). The PS is highly correlated with ovulation as well, with nearly each PS resulting in proper follicle ovulation and subsequent egg production (Liu et al., 2001b). Coordinated hormone production from the hypothalamus, pituitary, and ovarian follicles as well as positive and negative feedback on the hypothalamus and pituitary is required for the PS and ovulation to occur, yet these mechanisms are not fully understood in avian species.

The genetic control of ovulation and, moreover, the differential regulation associated with decreased or increased ovulation frequency is poorly understood in avian species (Scanes, 2017). HPG axis changes associated with the PS were previously unexamined and are imperative to understanding differences in ovulation frequency. Additionally, the impact of other neuroendocrine axes, particularly the hypothalamo-pituitary-thyroid (**HPT**) axis, on HPG axis function is not well characterized (Siopes et al., 2010). Understanding the external influences during the PS may help to clarify differences in HPG axis function seen in hens with differential egg production levels. Additionally, a deeper understanding of how the HPG axis is regulated may allow for the identification of additional genetic markers for genetic selection in poultry breeding programs.

The Turkey Industry

The turkey industry focuses on meat production and has selected heavily for carcass traits over the past forty years, with body weight as the most prominent selection trait due to the high impact on carcass value and high degree of heritability (Nestor et al., 2008). Selection for carcass traits has negatively impacted egg production, causing a reduction in the number of poults per turkey breeding hen (Nestor et al., 1996). While breeding programs try to balance advancements in growth parameters without compromising other factors, such as reproductive performance, the negative correlation of growth and reproduction, coupled with positive economic impact of increasing growth parameters, has left the turkey industry with reproductive issues in breeding flocks (Velleman et al., 2007). Reproductive issues, such as reduced egg production, fertility, hatchability, greatly influence the number of turkey poults that can be reared for meat production purposes. Moreover, these reproductive issues influence the number of hens that need to be maintained to produce the number of poults needed.

The reduction in reproductive capacity is a common occurrence seen in poultry selected for meat production, such as chicken broilers (Hocking, 2014). Numerous studies have demonstrated the negative correlation between carcass traits and reproductive traits, and, furthermore, have quantified the impact of selection in the broiler industry through lost egg production (Decuypere et al., 2010). While fewer studies have explored the impact of selection for carcass traits on reproductive efficiency in turkey hens, the conducted studies had similar findings to the studies involving broiler hens (Nestor et al., 2000). While meat production is the priority of the turkey industry, the number of eggs that are laid per hen greatly impacts the profitability of the turkey industry. Selection for egg production traits, such as total number of egg laid, onset of lay, or duration of lay, in turkeys decreased growth parameters as is also seen

in chicken layers and did not prove to be efficient to increase reproductive traits from an industry perspective (Emmerson et al., 2002). A greater understanding of the turkey hen reproductive system may allow for identification of genetic markers associated with increased reproductive parameters that have minimal impact on growth parameters. Enhancement of turkey hen reproduction without compromising growth will beneficial for improving the productivity of the turkey industry.

In addition to lower overall egg production, there is a large amount of variation in egg production within a single commercial flock. Flock egg production exhibits a bell-shaped distribution, creating distinct levels of egg production. Egg production, or eggs per day (**EPD**), can be classified as low egg producing hens (**LEPH**) (EPD<0.6), average egg producing hens (**AEPH**) (0.6<EPD<0.8), and high egg producing hens (**HEPH**) (EPD>0.8) (**Figure 1.1**). HEPH lay nearly twice as many eggs when compared to LEPH. From an industry standpoint, HEPH would provide breeding farms with larger numbers of poults for rearing for meat production, ultimately making the industry more productive and profitable. However, the LEPH have below average egg production, costing the industry to house and feed additional hens to meet poult production demands. Increasing the number of eggs that LEPH lay in a given cycle would be extremely beneficial to the turkey industry, driving down the cost of housing additional hens to compensate for the lower egg production rates. Additionally, the large distribution in egg production decreases flock uniformity, with breeder flock performance and uniformity positively correlated (Aviagen, 2018).

Flock Egg Production

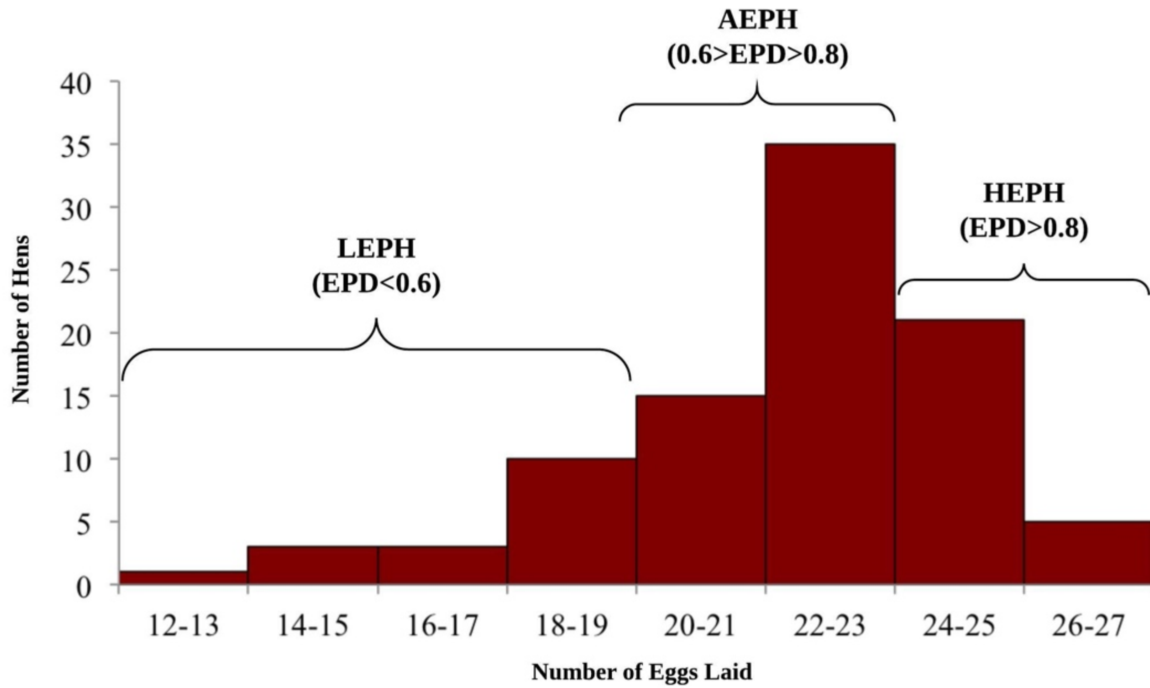


Figure 1.1 Flock egg production over one month during peak production in a flock of 200 hens. The distribution of LEPH, AEPH, and HEPH classifications of egg production exhibits a bell-shaped curve.

The Hypothalamo-Hypophyseal Complex

The hypothalamo-hypophyseal complex provides a link between the nervous and endocrine systems for the five neuroendocrine axes and regulates physiological functions (Krisch, 1989). The complex is composed of the hypothalamus and pituitary, with the median eminence (**ME**) of the hypothalamus centrally located in the complex (Advis and Contijoch, 1993). Formation of the hypothalamo-hypophyseal complex is divided into the adenohypophysis and the neurohypophysis (Takor and Pearse, 1975). The adenohypophysis is derived from oral ectoderm and Rathke's pouch and forms the pars distalis (anterior pituitary) and the pars tuberalis in avian species. The neurohypophysis is derived from neuroectoderm and forms the pars nervosa (posterior pituitary), the infundibular stalk, and the ME.

The hypothalamus is part of the diencephalon of the forebrain and arises from neuroectoderm tissue. Neurosecretory terminals from the preoptic area (**POA**) and neurons of the supraoptic nucleus (**SON**) and paraventricular nucleus (**PVN**) extend into the ME of the hypothalamus (Kuenzel, 2018). Neurons of the PVN also extend into the pituitary, specifically the posterior pituitary. Hypothalamic neurons produce releasing factors induce or inhibit pituitary hormone production. The hypothalamus is responsible for the production of gonadotropin releasing hormone (**GnRH**), gonadotropin inhibitory hormone (**GnIH**), thyrotropin releasing hormone (**TRH**), Vasoactive intestinal poly-peptide (**VIP**), growth hormone releasing hormone (**GHRH**), somatostatin (**SST**), and corticotropin-releasing factor (**CRH**), among other hormones (Shahid and Singh, 2018).

The pituitary gland is divided into the anterior and posterior pituitary, with the two lobes connected to each other and to the ME by hypophyseal portal blood vessels. The anterior pituitary is composed of two types of cells: secretory cells, namely gonadotroph, thyrotroph,

lactotroph, somatotroph, and corticotroph cells, which produce and secrete hormones, and folliculo-stellate cells, which are of neuroectodermal origin and have been implicated in paracrine control of secretory cells (Scanes, 2014). The anterior pituitary is further divided into cephalic and caudal lobes and is responsible for production of gonadotropins, LH and follicle stimulating hormone (**FSH**), by gonadotroph cells, thyrotropin stimulating hormone (**TSH**) by thyrotroph cells, prolactin by lactotroph cells, growth hormone by somatotroph cells, and adrenocorticotropic hormone by corticotroph cells in response to hypothalamic releasing factors from the ME (Scanes et al., 2005). In avian species, somatotrophs are predominantly in the caudal lobe, whereas corticotrophs, lactotrophs, and thyrotrophs are predominantly in the cephalic lobe (Hansen and Hansen, 1977; Berghman et al., 1992; Berghman et al., 1993). Gonadotrophs, on the other hand, are found in both lobes (Mikami, 1986). The posterior pituitary is comprised of neurosecretory terminals responsible for the release of mesotocin or arginine vasotocin, while synthesis of these hormones occurs in the hypothalamus (Robinzon et al., 1988).

The HPG Axis

Function and Overview

The HPG axis governs the hen reproductive system and directly regulates ovulation, ultimately impacting egg production (Sharp et al., 1984). The HPG axis is composed of the hypothalamus, the pituitary, and a single ovary in the hen. Neurons in the hypothalamus are responsible for the production of gonadotropin releasing hormone (**GnRH**), which stimulates the HPG axis, and for the production of gonadotropin inhibitory hormone (**GnIH**), which negatively regulates the HPG axis (Bédécarrats, 2015). The gonadotroph cells in the pituitary are responsible for the production of the gonadotropins, LH and FSH. Gonadotroph cells contain

GnRH and GnIH receptors (**GnRHR** and **GnIHR**, respectively) to regulate the synthesis and production of LH and FSH. The ovary is responsible for steroid production, namely P4, androgens (**AD**), and estradiol (**E2**). Three different cell types compose the wall of avian follicles: granulosa cells, theca interna cells, and theca externa cells. These cell types are responsible for steroidogenesis, with the granulosa cells producing P4, the theca interna cells producing AD, and the theca externa cells producing E2 (Porter et al., 1989a).

Hypothalamic Releasing Factors and Receptors

Hypothalamic releasing factors are synthesized and released in the hypothalamus and act on the anterior pituitary to impact hormone production and secretion. The HPG axis is positively and negatively regulated by GnRH and GnIH, respectively. Both hypothalamic factors are lipophobic decapeptides released from neurosecretory terminals that act on gonadotroph cells of the anterior pituitary to regulate gonadotropin production and release (Tsutsui et al., 2006). Furthermore, release can be stimulated or inhibited by additional neurotransmitters or neuropeptides. For instance, norepinephrine stimulates GnRH release, whereas GnIH can inhibit GnRH release (Contijoch et al., 1990).

Two GnRH peptides exist in the hypothalamus of avian species. GnRH1 (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2) and GnRH2 (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2) are encoded by separate genes, *GNRH1* and *GNRH2*, respectively (Scanes, 2014). Chicken GnRH1 shows a 90% sequence identity with mammalian GnRH and chicken GnRH2 shows a 70% sequence identity with mammalian GnRH (Dubois et al., 2002). In avian species, GnRH1 neurons are located at the POA and septal areas of the hypothalamus, whereas GnRH2 neurons are localized at the mesencephalon (Mikami et al., 1988; Millam et al., 1993). Neuron terminals containing GNRH extend into the external layer of the ME for neuropeptide release into the

hypophysial portal vascular system. Furthermore, GnRH1 neurons were more prominent in the ME than GnRH2 neurons (van Gils et al., 1993). Both GnRH1 and GnRH2 have been shown to stimulate the synthesis and release of pituitary gonadotropins in the chicken and quail, with GnRH2 showing a more potent effect (Hattori et al., 1986). GnRH1 is hypothesized to be the main stimulatory factor in the hypothalamus since immunization against GnRH1, but not GnRH2, resulted in gonadal regression (Sharp et al., 1990). Immunization against GnRH1 also displayed a more prominent reduction in LH (Sharp et al., 1990).

The GnRH receptors, GnRHR1 and GnRHR3, are G-protein coupled receptors that are capable of binding both GnRH1 and GnRH2. GnRHR1 expression is detected in the hypothalamus, pituitary, gonads, spleen, and heart but GnRHR3 expression is localized primarily in the pituitary (Shimizu and Bédécarrats, 2006). GnRHR3 is hypothesized to be the main receptor responsible for gonadotropin production and secretion because expression of GnRHR3 is greater than expression of GnRHR1 in the pituitary (Joseph et al., 2009).

GnIH neurons are mainly localized in the PVN, with nerve terminals at the ME, but have also been found in the POA and septal area in quail and chickens (Ukena et al., 2003; Tsutsui and Ukena, 2006). GnIH neurons also have direct contact with GnRH neurons, indicating regulation of GnRH synthesis and release by GnIH (Tsutsui et al., 2006). GnIH nerve terminals at the ME, indicate that GnIH is released into the hypophyseal portal blood vessels to regulate gonadotropin synthesis and secretion. GnIH treatment *in vitro* has been shown to reduce hypothalamic GnRH expression in addition to pituitary GnRH receptor and gonadotropin expression (Ubuka et al., 2012).

Two receptors are capable of GnIH binding and are also G-protein coupled receptors. GnIHR1 and GnIHR2, encoded by genes *NPFFR1* and *NPFFR2*, respectively, have been

identified in the chicken. GnIHR1 was identified as the primary GnIH receptor after *in vitro* studies demonstrated that cells transfected with chicken GnIHR1 showed increased response to GnIH treatment when compared to cells transfected with chicken GnIHR2 (Ikemoto and Park, 2005).

Pituitary Gonadotropins and Receptors

The pituitary gonadotropins, LH and FSH, act on the ovary to induce ovulation and follicle development, respectively. Both LH and FSH are glycoprotein hormones roughly 30 kDa in molecular weight in the turkey (Burke et al., 1979). Both gonadotropins consist of two glycoprotein subunits, with an α-subunit common to LH, FSH, and TSH and a unique β-subunit for each hormone. *LHB* encodes the β-subunit of LH, *FSHB* encodes the β-subunit of FSH, and *CGA* encodes the common α-subunit. LH induces P4 production in the large follicles of the ovary and induces ovulation through a PS of LH and P4. FSH induces E2 production in the smaller follicles of the ovary and induces follicular development and differentiation (Ritchie, 2014). While LH plasma levels change substantially during the ovulatory cycle, FSH plasma levels remain relatively constant (Mashaly et al., 1976; Vanmontfort et al., 1994). LH and FSH receptors, encoded by *LHCGR* and *FSHR,* respectively, are G-protein coupled receptors and are present in ovarian follicles. As a follicle matures, *LHCGR* mRNA levels increase, whereas *FSHR* mRNA levels decrease, which coincides with gonadotropin responsiveness trends in maturing follicles (You et al., 1996; Johnson et al., 1996).

Steroid Hormones and Receptors

Steroid hormones are derivatives of cholesterol and are characterized by 17 carbon atoms arranged in four rings (Bentley, 2001). Steroid hormones encompass a wide range of lipophilic hormones produced and secreted by the adrenal cortex, testes, and ovaries. Ovarian follicles in

the hen produce P4, AD, and E2, as well as their intermediates. Steroid hormones produced by the ovarian follicles have local effects in the ovary on steroidogenesis and global effects on hypothalamus and pituitary function (Caicedo Rivas and Paz-Calderón Nieto, 2016; Rangel et al., 2014; Ottinger and Bakst, 1995). Ovarian steroids also play a large role in initiation of egg production, with plasma levels peaking 3-4 week before onset of lay (Wineland and Wentworth, 1975; Williams and Sharp, 1977)

P4 is produced in the follicle granulosa cells and is responsible for the PS that triggers ovulation. P4 is one of the two hormones involved in the PS but also exhibits positive feedback on the hypothalamus and pituitary to stimulate synthesis and release of the other hormone involved in the PS, LH (Robinson and Etches, 1986). Cholesterol is converted to P4 by steroidogenic acute regulatory protein (**STAR**), cholesterol side chain cleavage enzyme (**CYP11A1**), and 3β-hydroxysteroid dehydrogenase (**HSD3B1**), encoded by *STAR*, *CYP11A1*, and *HSD3B1*, respectively.

AD, namely dihydrotestosterone and testosterone, are produced in the follicle theca interna and generally regarded as male hormones; however, testosterone influences ovulation and the production of P4 in the follicle granulosa cells (Rangel et al., 2009). Testosterone exhibits a peak prior to ovulation, in which the testosterone peak is correlated with the occurrence of the PS (Massa et al., 1979). Furthermore, treatment of preovulatory granulosa cells isolated from laying hens with testosterone influenced P4 production in a dose and time dependent manner, with short term exposure to high levels and low levels of testosterone decreasing and not effecting P4 production, respectively, whereas longer term cultures with testosterone increased P4 production (Johnson et al., 1988; Sasanami and Mori, 1999). Testosterone is produced by 17, 20-lyase

(**CYP17A1**) and 17β-hydroxysteroid dehydrogenase (**HSD17B1**), encoded by *CYP17A1* and *HSD17B1,* respectively.

E2 is produced in the follicle theca externa and is responsible for development of the reproductive tract and secondary sex characteristics in females. E2 also plays a role in ovulation as a primer for hypothalamic P4 binding (Kawashima et al., 1981). E2 exerts paracrine effects on ovarian steroidogenesis, decreasing granulosa P4 production (Johnson et al., 1988). Testosterone is converted to E2 by aromatase (**CYP19A1**), encoded by *CYP19A1*.

The P4, AD, and E2 receptors are part of the nuclear receptor family and are transcription factors (Evans, 1988). Intracellular steroid hormone receptors are composed of four domains: the variable domain, the DNA binding domain, the hinge region, and the hormone binding domain (Kumar and Thompson, 1999). The DNA binding domain interacts with the DNA at the hormone response element, which is a short sequence in the promotor region of a gene that is unique for each of the ovarian steroid hormones. Upon binding, the steroid hormones elicit genomic effects on transcription of target genes. The P4 receptor is encoded by *PGR* and has two isoforms in the chicken (Jeltsch et al., 1990). There are two E2 receptors, ERα and ERβ, encoded by *ESR1* and *ESR2* (Kon et al., 1980). P4, AD, and E2 receptors are present in the hypothalamus, pituitary, and ovarian follicles. In the hypothalamus and pituitary, binding of receptors for all three steroid hormones fluctuated in relation to the ovulatory cycle (Kawashima et al., 1979; Kawashima et al., 1989; Kawashima et al., 1993). P4 receptor expression increases with follicle maturation, whereas E2 receptor expression decreases with follicle maturation (Yoshimura and Bahr, 1991; Yoshimura et al., 1995). Androgen receptor expression was detected in various follicle types but did not change throughout follicle development (Yoshimura et al., 1993).

HPG Axis Signaling

GPCRs

GNRHR, GNIHR1, GNIHR2, LHCGR, and FSHR are G-protein coupled receptors (**GPCRs**), a receptor family that couples to guanine nucleotide binding proteins (**G-proteins**) (**Figure 1.2**). There are three subtypes of G-proteins: α, β, and γ. Upon receptor binding, guanosine diphosphate (**GDP**) is converted to guanosine triphosphate (**GTP**) to dissociate the Gα subunit from Gβγ. The activated Gα subunit is responsible for initiating downstream signaling through secondary messengers. There are three types of Gα subunits, $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$, which initiate different downstream pathways (Milligan and Kostenis, 2006).

Activated GnRHR1 and GnRHR2 has been shown to couple to $G_{\alpha s}$ and $G_{\alpha q}$ (Shimizu and Bédécarrats, 2006). Coupling to $G_{\alpha s}$ results in activation of adenylyl cyclase (**AC**), leading to the accumulation of intracellular cyclic adenosine monophosphate (**cAMP**) levels. Binding of cAMP activates protein kinase A (**PKA**), allowing phosphorylation of the transcription factors cAMP-response element-binding protein (**CREB**), cAMP-responsive modulator (**CREM**), and activating transcription factor 1 (**ATF1**) (Sassone-Corsi, 2012). Phosphorylation of these proteins allows for transcriptional regulation of target genes through interaction with cAMP-response elements (**CREs**) and the transcriptional coactivators CREB-binding protein (**CBP**) (Naor, 2009). Examples of target genes for GnRH action are *LHB*, *FSHB*, and *CGA*. Increased transcription of *LHB* and *CGA*, specifically, allow for increased synthesis of LH. Coupling to $G_{\alpha q}$ results in stimulation of phospholipase C (**PLC**), causing the production of inositol triphosphate (**IP3**) and diacyl-glycerol (**DAG**) from phosphatidyl inositol diphosphate (**PIP2**). IP3 activates calcium (**Ca^{2+}**) release from intracellular stores while DAG activates protein kinase C (**PKC**). PKC activates mitogen-activated protein kinase (**MAPK**) cascades to drive extracellular-signal-

regulated kinase (**ERK**) dependent transcription via Sf-1 and Egr-1 transcription factors. The elevated cytoplasmic Ca^{2+} concentration drives the activation of calmodulin and nuclear factor of activated T-cells (**NFAT**) transcription factor (Thompson and Kaiser, 2014). The activated transcription factors play a role in initiating the transcription of *LHB*, *FSHB*, and *CGA*, among others. Elevated cytoplasmic Ca^{2+} coupled with the action of PKC induces release of LH, through increased exocytosis (Durán-Pastén and Fiordelisio, 2013).

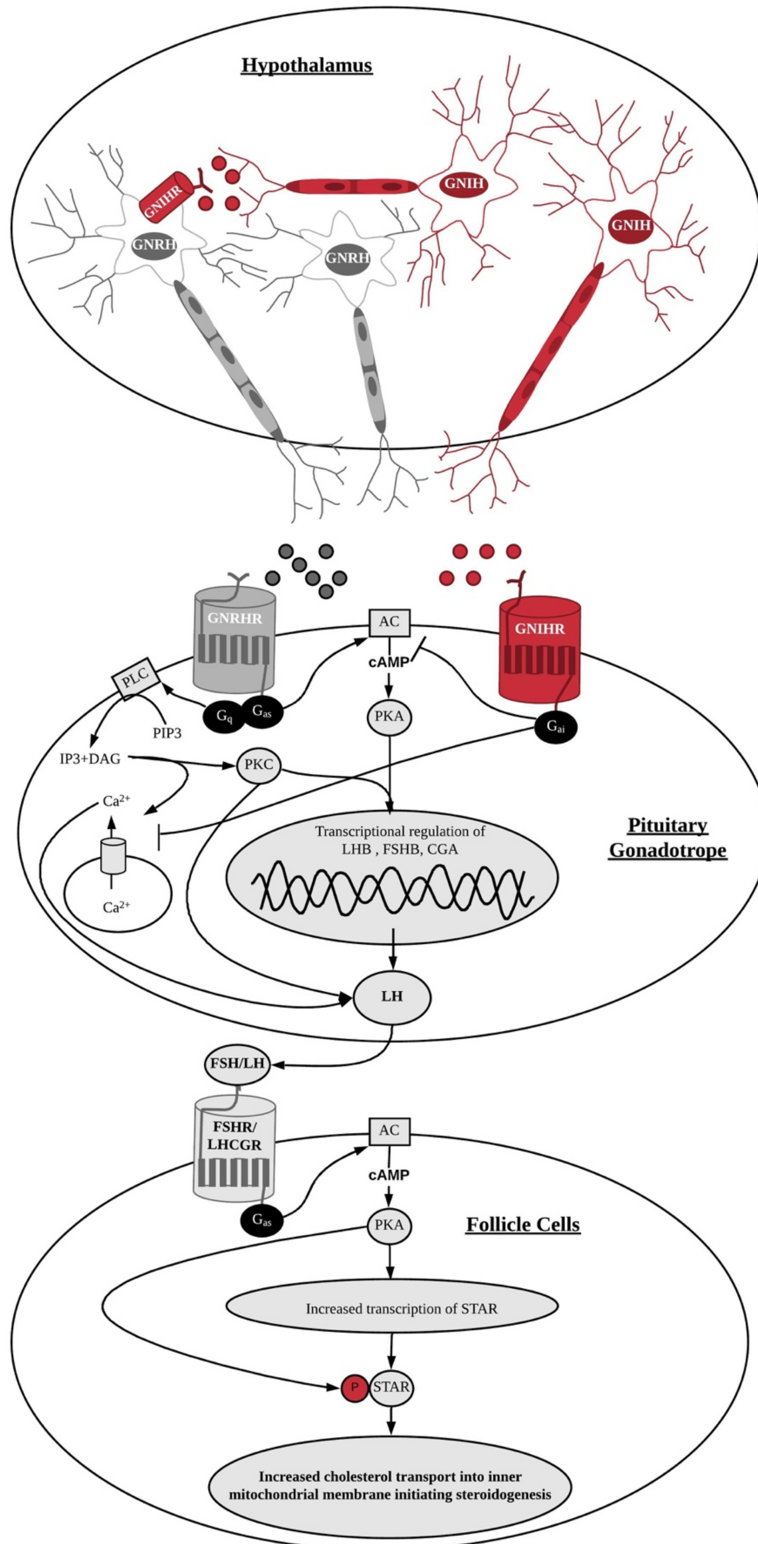


Figure 1.2 HPG axis signaling pathways in the hypothalamus, pituitary, and follicle cells.

GnIHR has been shown to couple to $G_{\alpha i}$ (Bédécarrats et al., 2009). Activation of chicken GnIHR in transfected GH3 cells did not affect PLC activity or IP3 concentration but, after co-transfection with chicken GnRHR and GnIHR, GnIH treatment reduced GnRH stimulation (Shimizu and Bédécarrats, 2010). These results support GnIHR coupling to $G_{\alpha i}$ only. Coupling to $G_{\alpha i}$ results in inhibition of AC and downstream pathways that were activated by $G_{\alpha s}$ coupling (Watts and Neve, 2005).

In the ovary, LHCGR and FSHR also couple to $G_{\alpha s}$ to increase the transcription of steroidogenic genes, through the cAMP-signaling pathway (Li et al., 2014). LHCGR and FSHR activation of PKA specifically allows for the up-regulation of *STAR* transcription and the phosphorylation of STAR to activate the transporter to bind cholesterol (Manna et al., 2009). STAR is responsible for the first step of steroidogenesis and is the rate-limiting step of steroidogenesis. However, LHCGR and FSHR binding also increases transcription of additional steroidogenic genes as well as phosphorylation of transcription factors that regulate steroidogenesis.

Steroid Hormone Receptors

Steroid hormones can elicit genomic and non-genomic actions. In the classical genomic model of steroid hormone action, steroid hormone receptors signal through common mechanisms to interact with hormone response elements of target gene promotors to ultimately impact transcription (Mangelsdorf et al., 1995). Steroid hormones diffuse through the plasma membrane to bind to their respective intracellular receptors located in the cytoplasm. Without steroid hormone binding, receptors are complexed with several heat shock proteins (**HSPs**) and chaperone proteins (**CPs**) that maintain folding and stability of the receptor. Upon steroid hormone binding, the receptor undergoes a conformational change which allows for dissociation

from HSPs/CPs and receptor dimerization. The receptor/ligand complex is translocated to the nucleus to interact with hormone response elements in target genes to impact gene transcription. Steroid hormone non-genomic action does not target gene transcription directly but instead involves GPCRs, cell membrane ion channels, and protein kinase signaling pathways to influence transcription indirectly through protein phosphorylation events, protein translocation events, and opening ion channels (Simoncini and Genazzani, 2003).

Steroidogenesis

Avian ovarian steroidogenesis occurs via a the three-cell model of steroidogenesis (**Figure 1.3**), where granulosa cells produce P4, theca interna cells produce AD, and theca externa cells produce E2 (Porter et al., 1989a). Within the ovary, the granulosa layer of the largest follicle (**F1**) is responsible for the majority of the P4 production, the theca interna layer of the fifth largest follicle (**F5**) is responsible for the majority of the androgen production, and the small white follicles (**SWF**) are responsible for the majority of the E2 production (Porter et al., 1989a; Lee and Bahr, 1994). Steroidogenesis is regulated by gonadotropin signaling via the AC/cAMP signaling pathway, with gonadotropin-stimulated steroidogenesis shifting from FSH responsiveness in less mature follicles to LH responsiveness in more mature follicles. Early in follicle development, only the theca interna and theca externa layers have steroidogenic activity (Johnson and Woods, 2009). Upon follicle selection, the granulosa layer also attains steroidogenic activity.

During steroidogenesis, cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane by STAR. Transportation of cholesterol by STAR is the rate limiting step of steroidogenesis. Cholesterol is converted to pregnenolone by CYP11A1. Pregnenolone can be metabolized to androstenedione through two pathways, the Δ4

and Δ5 steroidogenic pathways. The granulosa layer prefers the Δ4 steroidogenic pathway while the thecal layer prefers the Δ5 steroidogenic pathway (Lee et al., 1998). In the Δ4 steroidogenic pathway pregnenolone is converted to P4 by HSD3B1. P4 can be further converted to adrostenedione by CYP17A1. In the Δ5 steroidogenic pathway pregnenolone is converted to dihydroepiandrosterone by CYP17A1 then dihydroepiandrosterone is converted to adrostenedione by HSD3B1. Adrostenedione is metabolized to testosterone by HSD17B1. Lastly, testosterone can be converted to E2 by CYP19A1.

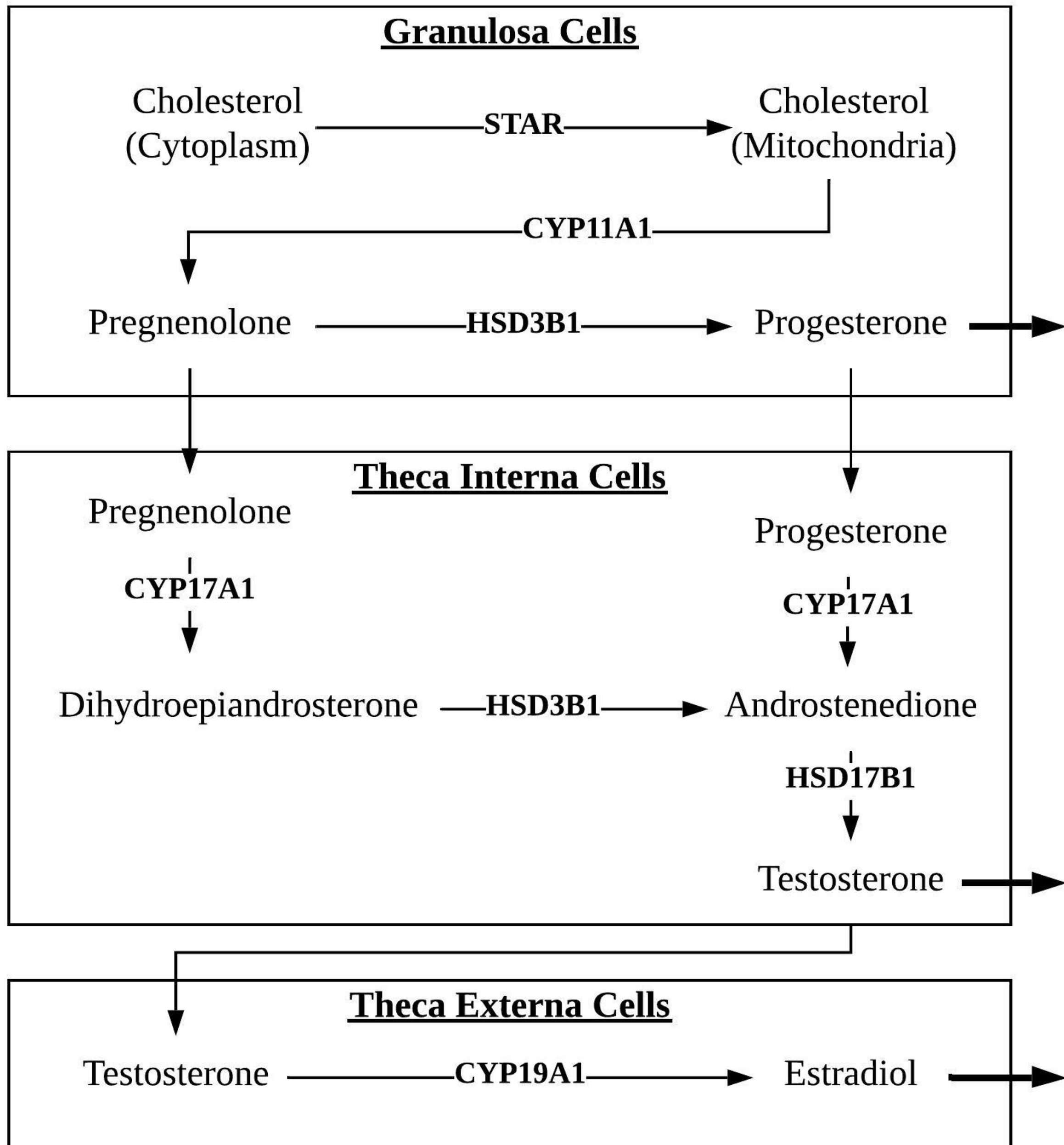


Figure 1.3 The three-cell model of steroidogenesis involved in the production of P4, AD, and E2 from the granulosa, theca interna, and theca externa of avian follicles, respectively.

Follicle Development

Ovarian Structure

The avian ovary is composed of follicles in varying states of maturation. At any given time, there are four different types of follicles: primordial, primary, prehierarchical, and preovulatory follicles. All of the aforementioned follicle types are present at the same time in avian species. An organized follicular hierarchy is maintained by activation of primordial follicles, recruitment of primary follicles, selection of prehierarchical follicles, and ovulation of preovulatory follicles (**Figure 1.4**) (Etches and Petitte, 1990). Each follicle is composed of layers surrounding the oocyte and yolk. From the inside layer to the outmost layer are the oocyte plasma membrane, the perivitelline membrane, the granulosa cells, the basement membrane, the theca interna cells, and the theca externa cells (Apperson et al., 2017).

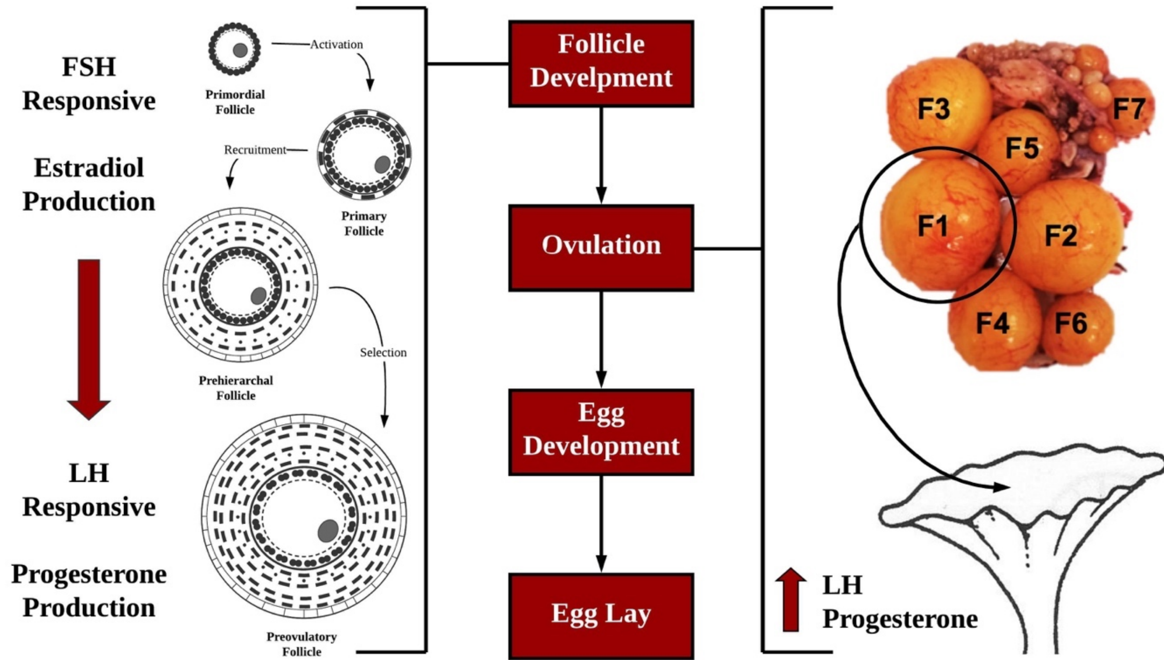


Figure 1.4 Follicle development and ovulation in avian follicles.

Folliculogenesis

During embryonic development, primordial germ cells migrate to the gonad. Following regression of the right ovary and oviduct, a large wave of apoptosis of primordial germ cells occurs (Tagami et al., 2017). Germ cell cysts remaining in late embryonic development and early post hatch are broken down to form individual oocytes. Primary follicles are assembled when individual oocytes are surrounded by a layer of granulosa cells. All oocytes are transformed into primordial oocytes or undergo a second wave of apoptosis, leaving no stem germ cells behind (Johnson, 2014a). An inner perivitelline membrane also forms between the oocyte and the granulosa cell layer (Beyo et al., 2007). Primordial follicles remain in a quiescent state in the ovary until they are activated at sexual maturation and develop into primary follicles. Primordial follicles are less than 80 µm in size. There is evidence that E2 and P4 may promote and inhibit primordial follicle activation in chickens, respectively, as was seen in *in vitro* culture of E2 and P4 with primordial follicles (Zhao et al., 2017; González-Morán, 2007).

Activation

The granulosa cells surrounding the oocyte change morphology from flattened to cuboidal upon activation to primary follicles. Activated primary follicles can remain quiescent in the ovary or can progress to growing primary follicles, which develop a basement membrane and thecal layer of cells surrounding the granulosa cells (Johnson, 2014a). The transition from a quiescent activated primary follicle to a growing primary follicle and the factors involved in the transition are poorly understood. Over a period of weeks, activated follicles integrate white protein rich yolk to slowly increase size (Guraya, 1976). Follicles during this stage range from 0.08 to 1 mm in size.

Recruitment

Primary follicles are recruited to grow into the unselected group of prehierarchical follicles. This process is accompanied by further differentiation of the thecal layer into the theca interna and theca externa layers. Vasculature and nerves are established in the thecal layers as well. This process is also accompanied by an increase in lipid rich yolk accumulation (Johnson, 2014a). Early prehierarchical follicles contain mainly white protein rich yolk and are termed SWF (Johnson, 1992). Prehierarchical follicles range from 1 to 8 mm in size and remain arranged by size in the prehierarchical follicle hierarchy until selection. Previous studies have found that granulosa layer FSH-responsiveness of pre-recruitment follicles increases with follicle size and hypothesized that level of FSH-responsiveness established the order of follicle recruitment (Ghanem and Johnson, 2019).

Selection

Finally, prehierarchical follicles are selected to join the cohort of 6-10 preovulatory follicles that are awaiting ovulation. This process of selection is associated with granulosa cell differentiation, allowing the granulosa cells to become competent for steroidogenesis (Ghanem and Johnson, 2018). Prior to selection, only the thecal layers are capable of steroidogenesis. It is hypothesized that FSHR-responsiveness initiates granulosa cell differentiation. In undifferentiated granulosa cells, FSHR-responsiveness is inhibited by β-arrestin and MAPK signaling (Johnson and Woods, 2009). During selection, FSHR signaling is resumed and granulosa cell differentiation occurs. Once selected, preovulatory follicles rapidly acquire lipid rich yolk and grow substantially in size. The preovulatory follicles are termed F1 through F6 to F10 based on follicle size, with the F1 follicle next in line to respond to the PS and ovulate. Preovulatory follicles range in size from 9-40 mm. In contrast to mammals, once a follicle is

selected to join the preovulatory follicles, the likelihood of follicle atresia is very rare (Johnson, 2003). With each ovulation, a prehierarchical follicle will be selected to join the preovulatory cohort to maintain the follicular hierarchy.

Ovulation

Follicle ovulation is triggered by a PS of LH and P4 that occurs roughly 8-10 hours before ovulation in the turkey and 6-8 hours before ovulation in the chicken (Yang et al., 1997; Liu et al., 2004). Increased LH levels are the result of GNRH stimulation of gonadotroph cells of the pituitary. The F1 follicle, in response to increased LH stimulation, mainly produces increased P4 levels seen during the PS. P4 receptors in the hypothalamus and pituitary allow for a positive feedback loop to increase LH secretion and subsequent P4 production (Johnson et al., 1985). In response to the PS, the F1 follicle will rupture along the avascular stigma, leaving behind the follicle wall for regression. The ovulated follicle is caught by the infundibulum of the oviduct for further egg development.

Egg Production and Ovulation Frequency

Egg production is restricted to a 10-12 hour period when the hen is exposed to light called the "open period" (Cunningham et al., 1984). If the next egg in the sequence would occur outside of the "open period" then the hen will take a rest period, called a pause, to reset the sequence. Poultry will lay several eggs in a row, called a clutch, before taking a pause. Clutch size or length is the number of eggs successively laid before the rest period and varies extensively by species. Pause length is the number of days that make up the rest period and also varies by species.

Egg production takes 26 hours from ovulation to oviposition. Egg production begins with follicle ovulation from the ovary. The ovulating follicle ruptures at the stigma to release the

ovum, leaving behind the follicle wall structure to regress. The ovum is released into the oviduct, which is comprised of 5 distinct segments. The infundibulum is funnel shaped and is responsible for catching the ovulated ovum. The magnum is responsible for surrounding the yolk with albumen. The isthmus forms the inner and outer shell membranes. The shell gland deposits the shell. The vagina adds the outer cuticle to the egg and plays a role in oviposition. In average producing hens, the ovary ovulates the next ovum 30 to 75 minutes after a hen lays an egg. Ovulation interval, or ovulation frequency, is the time between ovulations. Ovulation frequency decreases with the reproductive period but is not affected by hen age (Liu et al., 2002; Liu et al., 2001a). Longer ovulation frequencies have been established in turkey hens selected for carcass traits in comparison to hens selected for egg production (Liu et al., 2005).

The HPT Axis

Function and Overview

The HPT axis is comprised of the hypothalamus, pituitary, and thyroid. However, thyroid hormone receptors are present in the tissues of the HPG axis. The HPT axis is necessary for ovulation to occur, yet the exact mechanisms behind HPT axis control of ovulation are not understood (Lien and Siopes, 1989a). The HPT axis also plays a role in the initiation of egg production but excessive levels of thyroid hormones result in molt (Siopes et al., 2010; Queen et al., 1997). Studies on the HPT axis in terms of reproduction have focused on the role of the HPT axis in seasonal reproduction, with deep brain receptors recognizing long day stimulus to induce TSH and thyroid hormone production, which feedback on the hypothalamus to initiate GnRH production and HPG axis stimulation (Tamai and Yoshimura, 2017). The HPT axis in regard to the ovulatory cycle or egg production levels has yet to be examined in avian species.

Hypothalamic Releasing Factors and Receptors

TRH is a lipophobic tripeptide (p-Glu-His-Pro-NH2) that is produced by neurons localized in the PVN and projected into the ME (Geris et al., 1999). TRH acts on pituitary thyrotrophs to induce TSH production. TRH also has stimulatory effects on prolactin release. Aside from TRH, SST and CRH have inhibitory and stimulatory effects, respectively, on TSH synthesis (De Groef et al., 2003). TRH is originally translated as precursor polypeptide with repetitions of the TRH tripeptide sequence for cleavage. TRH receptors (**TRHR**) are present in both the cephalic and the caudal lobe of anterior pituitary (Harvey and Baidwan, 1989).TRHR is expressed in thyrotroph cells but has also been shown in somatotroph cells (De Groef et al., 2005). TRHR is a GPCR that couples to $G_{\alpha q}$ to elicit TSH synthesis and release (Hsieh and Martin, 1992).

Pituitary Thyrotropin and Receptor

TSH is produced by thyrotroph cells in the pituitary. The thyrotrophs are located in the cephalic lobe of the anterior pituitary (Sharp et al., 1979). In addition, some thyrotrophs express the AR, through the effect of androgen feedback on TSH production is not well established (Sun et al., 2012). TSH is a glycoprotein hormone, with a common α subunit shared with LH and FSH (encoded by *CGA*) and a unique β subunit (encoded by *TSHB*). TSH receptor (**TSHR**), encoded by *TSHR* is expressed in the thyroid gland and is a GPCR that couples to $G_{\alpha s}$ to regulate thyroid hormone production by the thyroid gland (Szkudlinski et al., 2002).

Thyroid Hormones and Receptors

In response to TSH, the thyroid produces thyroid hormones: triiodothyronine (**T3**) and thyroxine (**T4**). The avian thyroid is organized similarly to mammals with spherical follicles with epithelial cell walls. Inside of the follicles is the lumen filled with colloid, where thyroglobulin

and stored thyroid hormone reside (McNabb, 2007). Thyroid hormone synthesis begins with iodide uptake into the thyroid gland from the blood, followed by iodination of tyrosine residues of thyroglobulin. Iodination of tyrosine residues by thyroperoxidase results in the formation of monoiodotyrosines (**MIT**) and diiodotyrosines (**DIT**). Two DIT residues combined together results in T4 formation, whereas the combination of MIT and DIT residues results in T3 formation (Rousset et al., 2000). Iodinated thyroglobulin is broken down through proteolytic cleavage of T3/T4 and the thyroid hormones are released through exocytosis. T3 exerts a negative feedback on TRH and TSH synthesis. The avian thyroid releases mainly T4 rather than T3, requiring outer ring deiodination of T4 by thyroid hormone deiodinase 2 (**D2**) (encoded by *DIO2*) to form T3 (Darras et al., 2006).

Thyroid hormones require thyroid hormone transporters to aid in crossing the plasma membrane of the cell, since thyroid hormones have lipophilic and hydrophilic properties (Bourgeois et al., 2016). Upon entry into the cell, thyroid hormones bind to thyroid hormone receptors (**THRs**). THRs have a high affinity for T3 and a much lower affinity for T4 (Decuypere et al., 2005). Thyroid hormones can bind to nuclear THRs, THRα and THRβ, or to the integrin receptor located in the plasma membrane. Nuclear thyroid receptors are encoded by *THRA* and *THRB,* whereas the integrin receptor subunits are encoded by *ITGAV* and *ITGB3*. Nuclear thyroid hormone receptors elicit genomic effects through binding of thyroid response elements (**TREs**) (Cheng et al., 2010). Unlike steroid hormone receptors, unligated nuclear THRs can bind TREs to regulation transcription and ligated nuclear THRs can bind TREs as homodimers or heterodimers (Decuypere et al., 2005). The integrin receptor elicits non-genomic effects through the phosphorylation and translocation of proteins (Davis et al., 2008). THRs present in the hypothalamus and pituitary regulate *TRH* and *TSHB* expression, synthesis, and

release. Furthermore, thyroid hormone is implicated in regulating steroidogenesis, with T3 treatment increasing P4 and decreasing E2 production in ovarian follicles (Sechman, 2012).

The Avian Genome

Next generation sequencing (**NGS**) technology has allowed for the assembly and comparison of over 75 avian genome drafts (Dodgson, 2014). However, many avian genomes remain incomplete and are not well annotated (Jax et al., 2018). The two most agriculturally relevant avian genomes are the chicken and the turkey genomes. The chicken genome was the first to be sequenced in 2004, followed by the sequencing of the turkey genome in 2010 (Wallis et al., 2004; Dalloul et al., 2010). The chicken and turkey genomes are both comprised of 39 pairs of autosomes and 1 pair of sex chromosomes (Griffin et al., 2008).

Comparisons between the chicken and turkey genomes revealed high similarity between the two genomes, with the most common rearrangements involving intra-chromosomal inversions (Zhang et al., 2011). Specifically, compared to the chicken genome, the turkey genome has 2 inter-chromosomal and 57 intra-chromosomal rearrangements (Aslam et al., 2010). Though a high degree of similarity between the chicken and turkey genomes exists (89% identity), the amount of similarity decreases when the chicken and turkey are compared from a proteomic or phosphoproteomic regard (83% and 70% identity, respectively) (Arsenault et al., 2014). In addition, the turkey genome was found to have a lower frequency of heterozygous single nucleotide polymorphisms (**SNPs**) when compared to the chicken genome, indicating a loss of genetic diversity (Aslam et al., 2012). Further studies revealed that the commercial turkey genome exhibited large sweep regions, which are regions showing lower genomic variation, when compared to heritage genomes, with growth related genes heavily enriched in these regions as a result of intensive genetic selection (Aslam et al., 2014).

Common trends seen across the avian genomes include reduced genome size, a low density of transposable elements, a high stability of karyotype, avian-specific gene losses that are conserved in other species, and a significant number of genes with a high sequence GC content that were previously improperly sequenced or assembled (Gao et al., 2017; Botero-Castro et al., 2017). Avian genomes, on average, are 1/3 of the size of mammalian or reptile genomes (~1.2 billion base pairs). The reduced genome size is due, in part, to two features: clusters of "missing" genes in avian genomes and problems with sequencing and assembly due to high GC content.

Comparison of avian genomes along with comparison between avian genomes and genomes of other vertebrates revealed many genes that were not present in avian genomes yet highly conserved in other vertebrates. Moreover, these "missing" genes tend to be arranged into clusters, where genes on either side of the cluster are highly conserved across avian genomes. Additionally, a majority of the "missing" genes are found in crocodiles, inferring that the loss of these genes occurred after the evolutionary split of dinosaurs and birds from crocodiles (Lovell et al., 2014). Previous studies found that a large number of the "missing" genes are involved in endocrine function.

Advances in NGS technology, specifically Pacbio-based technology, has allowed for more accurate sequencing and assembly of GC rich areas, leading to the discovery of several genes thought to be previously missing in avian species (Hron et al., 2015). The addition of multi-tissue transcriptomic data has shown to be instrumental in identifying genes with strong tissue specific expression patterns that have been improperly identified as "missing" genes in avian genomes (Yin et al., 2019). Even with the discovery of genes thought to be previously missing in avian species, clusters of "missing" genes are still present and the size of the avian

genome is still considerably smaller than that of mammalian and reptile species (Lovell et al., 2015).

The initial sequencing of the turkey genome covered 89% of the genome sequence with 17x coverage depth, whereas the most recent build covers 95% of the genome sequence with greater than 30x coverage depth (Dalloul et al., 2014). Even with improvements in coverage and depth, annotation of the turkey genome is limited, severely impacting transcriptomic and proteomic analysis in the turkey (Porter, 2014; Kunec and Burgess, 2014). As a result, a majority of avian transcriptomic and proteomic studies have been conducted in the highest quality avian genome assembly to date, the chicken (Warren et al., 2017). Further refinement and analysis of the turkey genome will be imperative in expanding transcriptomic and proteomic analysis in the turkey, to identify genetic markers that can used in genomic selection in the turkey breeding programs (Wolc et al., 2016).

Rationale and Objectives

The inner workings of the turkey hen reproductive axis are not consistent within a commercial flock. A portion of the hens in the flock will lay significantly less eggs per cycle while a portion of the hens in the flock will considerably outperform the average number of eggs laid. Moreover, these LEPH are ovulating less frequently than the HEPH, leading to the hypothesis that differences in the function of the hen's HPG axis are ultimately impacting the hen's reproductive performance. Further understanding of HPG axis and external regulation of ovulation as well as how this regulation is distorted by level of egg production is necessary to increase the reduced reproductive performance seen in turkey breeding hens. Improvement of the reproductive efficiency of LEPH would greatly decrease the cost housing additional breeding hens to meet poult production needs as well as decrease the amount of money lost to reduced poult production. Such improvements would increase the efficiency and productivity of the turkey industry, ultimately reducing consumer costs. In an effort to further understand the regulation of ovulation in hens with differential egg production, this research project had the following objectives:

1. To characterize the HPG axis in AEPH, LEPH, and HEPH, inside and outside of the PS that triggers ovulation.
2. To distinguish hormone response differences in pituitary and follicle cells *in vitro* between LEPH and HEPH, outside of the PS that triggers ovulation.
3. To identify novel genes and gene networks involved in ovulation and ultimately, egg production rates.
4. To characterize the HPT axis in AEPH, LEPH, and HEPH, inside and outside of the PS that triggers ovulation.

CHAPTER 2

Characterization of gene expression in the hypothalamo-pituitary-gonadal axis during the preovulatory surge in the turkey hen

ABSTRACT

A PS of LH and P4 triggers follicle ovulation, which is the first step of egg production and is orchestrated by the HPG axis. In the HPG axis, hypothalamic peptides, GnRH and GnIH, control the production of FSH and LH by the pituitary, which subsequently regulate ovarian production of E2 and P4, respectively. The goal of this study was to characterize the HPG axis function of AEPH by assessing plasma hormone profiles and hypothalamic, pituitary, and follicle gene expression outside and during the PS (n=3 per group). Results were analyzed by a one-way ANOVA using the mixed models procedure of SAS. Plasma E2 was not affected by the PS ($p>0.05$), but plasma P4 levels increased 8-fold during the PS when compared to basal P4 levels ($p<0.05$). HPG axis gene expression related to ovulation stimulation (e.g. *GNRH*, *GNRHR*, and *LHB*) was down-regulated during the PS; whereas gene expression related to follicle development (e.g. *FSHB*) was up-regulated during the PS. Additionally, in the hypothalamus and pituitary, E2 receptor expression was up-regulated during the PS, whereas P4 receptor expression was down-regulated during the PS. In the follicle cells, gene expression pertaining to P4 (e.g. *STAR*), AD (e.g. *HSD17B1*), and E2 (e.g. *CYP19A1*) production was up-regulated during the PS. Prior to this study, the HPG axis had yet to be characterized during the PS in the turkey hen. This study showed that the PS significantly impacted gene expression in the hypothalamus, pituitary, and ovarian follicles. These results provide a foundation for further research into the regulation of ovulation and egg production in turkey hens.

Introduction

Egg production in birds begins with follicle ovulation from the ovary followed by the addition of the albumen, shell membranes, and shell within the specialized segments of the oviduct. Hormonal inputs from the hypothalamus, pituitary, and ovary control the timing of

follicle ovulation, and ultimately the timing of egg production (Johnson, 2014). While a PS of LH and P4 is necessary for each daily ovulation, additional hormonal inputs play a role in follicle development and steroid production (Yang et al., 1997). The main neuroendocrine axis that regulates reproductive activity is the HPG axis; however, this axis has yet to be characterized during the PS in the turkey hen.

The HPG axis consists of the hypothalamus, pituitary, and a single ovary in avian species (Decuypere et al., 2002). The hypothalamus regulates pituitary gonadotropin production through the release of either GnRH or GnIH to stimulate or inhibit production, respectively. GnRH action is mediated through GNRHR, whereas GnIH action is elicited through GNIHR1 and GNIHR2, both of which are present on pituitary gonadotrophs (Bédécarrats et al., 2016). In response to hypothalamic releasing factors, the pituitary secretes the gonadotropins, FSH and LH. The ovarian follicles express FSHR and LHCGR. In response to pituitary gonadotropins, ovarian follicles respond with the production of steroid hormones. Steroid hormones, specifically P4 and E2, can prompt various feedback loops through binding of PGR or ESR1/ESR2, present in the hypothalamus and pituitary (Kawashima et al., 1987).

Follicles within the ovary range in maturation from immature primordial follicles to mature preovulatory follicles, with the preovulatory follicles arranged in a hierarchical ovulation order (termed F1-F10 based on follicle size) (Johnson and Woods, 2009). The follicle wall of preovulatory follicles contains three cell types that play specialized roles in steroid production: 1) granulosa cells producing P4; 2) theca interna cells producing AD; and 3) theca externa cells producing E2 (Porter et al., 1989). During follicle maturation through the preovulatory hierarchy, P4 production increases, while androgen and E2 production decreases (Porter, Hargis, Silsby, and El Halawani, 1991). During steroidogenesis, cholesterol is converted to P4 through the

actions of STAR, CYP11A1, and HSD3B1. P4 can be further converted to two AD, androstenedione and testosterone, by the enzymes CYP17A1 and HSD17B1. Lastly, AD can be converted to E2 by CYP19A1 (Robinson and Etches, 1986).

Despite the central role of the PS in egg production, there is little information regarding HPG axis function during the PS in turkey hens. This study examined turkey hens sampled outside or during the PS to (1) define the plasma concentrations of P4 and E2 and (2) characterize the expression of key HPG axis genes in the reproductive tissues, namely the hypothalamus, pituitary, and the follicular cells of the F1 and F5 ovarian follicles.

Materials and Methods

Hen Selection

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at the Beltsville Agricultural Research Center (**BARC**) in individual cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards. Hens were sampled at 35 weeks of age. Daily egg records were used to calculate each hen's number EPD by dividing the total number of eggs produced by the number of days in production. Females with an EPD between 0.68 and 0.72 were classified as AEPH and were used for sampling. The timing of the PS was determined for each hen using hourly egg records. Using the time of egg lay, the prior PS was estimated to occur 34 hours (8 hours from the PS to follicle ovulation and 26 hours from ovulation to egg lay) before the egg was laid (**Figure 2.1**). During a laying sequence, hens will maintain an ovulation interval of 26 hours. Based on the calculated timing of the PS, the subsequent PS could be predicted for the next egg of the sequence.

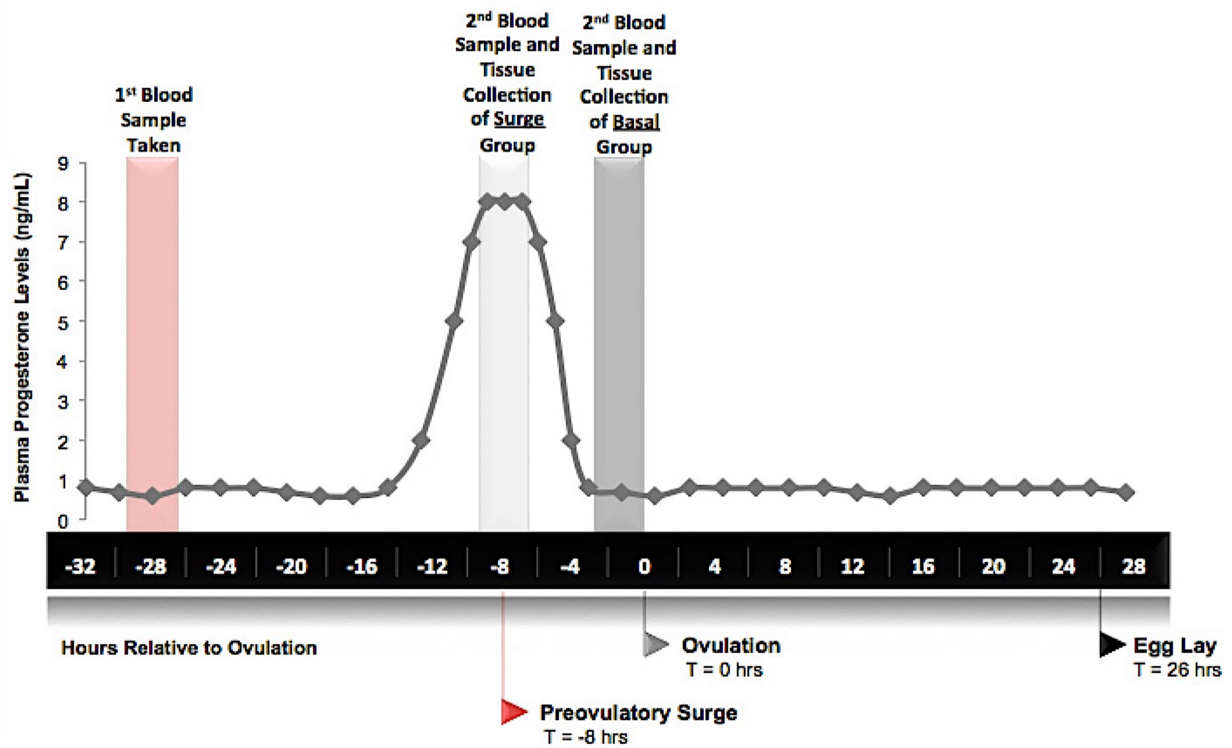


Figure 2.1 An overview of plasma progesterone levels during the PS, relative to the timing of egg lay. The timing of blood sampling and tissue sampling, relative to the PS, are denoted with colored blocks. The PS is denoted with a red triangle, ovulation is denoted with a grey triangle, and egg lay is denoted with a black triangle.

All hens were sampled during the second day of the hen's sequence and were sampled with a hard-shelled egg in the reproductive tract. The hypothalamus, pituitary, F1 follicle, and F5 follicle were isolated from three AEPH outside of the PS and from three AEPH during the PS for a total of six hens. Hens were confirmed to be outside or during the PS by plasma P4 levels. Blood samples were taken from the wing vein immediately before tissue sampling and fractionated by centrifugation. Plasma samples were stored at -20°C prior to assessment through radioimmunoassays (**RIAs**) as described below. The hypothalamus and pituitary were snap frozen in liquid nitrogen as whole tissues for RNA extraction, whereas the F1 and F5 follicles were processed to isolate the three cell types from the follicle wall as described below. Isolated tissues and cells were stored at -80°C prior to RNA extraction. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland.

RIAs

The RIAs used for P4 and E2 were coated tube kits (MP Biomedicals, Solon, OH). For the P4 and E2 RIAs, plasma samples were ether extracted prior to the assay. All protocols were performed as directed by the manufacturer. All samples were assayed in duplicate. The standard curve was assessed for linearity as well as parallelism using serial plasma dilutions. The intraassay coefficients of variation were determined by pools run every 30 samples and were 4.45% for P4 and 6.05% for E2. All samples were measured in a single RIA for each hormone.

Cell Isolation

All cell isolation procedures were performed using Minimum Essential Medium, Spinner modification (**SMEM**) supplemented with 0.1% bovine serum albumen, 100-U/mL penicillin G, and 100-µg/mL streptomycin sulfate (**0.1% BSA and P/S**).

Follicle cell type isolation was achieved using an adapted published method (Porter et al., 1989a). Briefly, the F1 and F5 follicles were drained of yolk through a small incision made at the stigma of each follicle. After the yolk was drained, the follicle wall was inverted. The granulosa layer was peeled off using curved forceps and placed into 20 mL of SMEM (0.1% BSA and P/S) with 1 mg/mL of trypsin. The granulosa layer was incubated for 15 minutes at 37°C in a shaking water bath and triturated every 5 minutes using a flame-polished, siliconized Pasteur pipet. At the end of incubation, the granulosa cells were centrifuged (20 x g for 10 minutes), re-suspended in 10 mL of SMEM (0.1% BSA and P/S), filtered through nylon mesh (70 µm), and held at 37°C prior to Percoll suspension.

The theca interna layer was isolated by clamping the inverted follicle wall across the stigma opening to close off access to the theca externa layer. The clamped follicle was placed into 20 mL of SMEM (0.1% BSA and P/S) with 1 mg/mL of trypsin and incubated for 15 minutes at 37°C in a shaking water bath. The clamped follicle was removed from the SMEM and placed in a glass petri dish, where the theca interna cells were gently scraped from the follicle wall using a scalpel blade. The separated cells were returned to the 20 mL of SMEM (0.1% BSA and P/S) with 1 mg/mL of trypsin and incubated at 37°C for an additional 10 minutes, with trituration every 5 minutes. At the end of incubation, the theca interna cells were centrifuged (20 x g for 10 minutes), re-suspended in 10 mL of SMEM (0.1% BSA and P/S), filtered through nylon mesh (70 µm), and held at 37°C prior to Percoll suspension.

The theca externa layer was dispersed by mincing the remaining clamped follicle and placing the minced pieces into 20 mL of SMEM (0.1% BSA and P/S) with 1 mg/mL of trypsin. The theca externa pieces were incubated at 37°C in a shaking water bath for 30 minutes, with trituration using Pasteur pipettes of decreasing inlet sizes every 5 minutes to dissociate the tissue

pieces. At the end of incubation, the theca externa cells were centrifuged (20 x g for 10 minutes), re-suspended in 10 mL of SMEM (0.1% BSA and P/S), filtered through nylon mesh (70 μm), and held at 37°C prior to Percoll suspension.

Percoll suspensions (50%) were used to remove red blood cells and extra material from each of the three cell-type suspensions. For each cell type, the dispersed cells were layered onto a Percoll suspension and centrifuged (20 x g for 15 minutes). After centrifugation, the follicle cells migrated at the interface of the Percoll suspension, while red blood cells and extra material migrated to the bottom of the Percoll suspension. The follicle cells were collected from the interface using a Pasteur pipette and suspended in 10 mL of SMEM (0.1% BSA and P/S). Cells were washed twice in 10 mL of SMEM (0.1% BSA and P/S) to remove Percoll (20 x g for 10 minutes), pelleted (1000 x g for 10 minutes at 4°C), and snap frozen in liquid nitrogen for RNA extraction.

RT-qPCR

Total RNA was isolated from hypothalamus, pituitary, and the granulosa, theca interna, and theca externa cells from the F1 and F5 follicles with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion, and quantified with Quant-iT RiboGreen RNA Quantitation Reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions (20 μl) for the hypothalamus and pituitary samples were performed on 1 μg total RNA with M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) and an oligo dT primer (Thermo Fisher Scientific. Waltham, MA). Reactions were diluted to 100 μl prior to PCR analysis. Reverse transcription reactions (20 μl) for the granulosa, theca interna, and theca externa samples of the F1 and F5 follicles were performed on 200 ng total RNA with SuperScript III (Thermo Fisher Scientific, Waltham, MA) and an oligo-dT primer (5′-

CGGAATTCTTTTTTTTTTTTTTTTTTTTV-3′). Reactions for the granulosa and theca externa cells were diluted to 200 µl prior to PCR analysis, while reactions for theca interna cells were not diluted prior to PCR analysis. For all reverse transcription reactions, a pool of total RNA was made and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination.

Expression levels of primary genes of the HPG axis were quantified by RT-qPCR. Primers (Integrated DNA Technologies, Skokie, IL) used in the PCR reactions were designed using NCBI primer BLAST Software (NCBI, Bethesda, MD) to target a 3' region of the transcript and to span an intron. Primers were also designed to amplify all known splice variants. Primers were designed to have a melting temperature (**T_m**) of 58-60°C, a GC content (**GC%**) of 40-60%, a length of 18-30 nucleotides, and to yield product of a length of 90-250 nucleotides. Amplification efficiencies between 0.9 and 1.1 were attained for each primer pair. To determine amplification efficiency, 2-fold serial dilutions of pooled cDNA were analyzed by RT-qPCR. Amplification efficiency was calculated as the absolute value of the slope of the linear regression line that resulted after plotting Ct versus $\log_2$-transformed template. Additionally, primers were validated by dissociation curve analysis, gel electrophoresis, and sequencing of PCR products. Primer sequences are listed in **Table 2.1**.

PCR reactions (15 µL) contained 1 µL of cDNA, 0.4 µM of each primer, PCR buffer (50 mM KCl, 10 mM Tris–HCl, 0.1% triton-X-100), 0.12 U/µL Taq Polymerase, 0.2 µM dNTPs, 40 nM fluorescein (Invitrogen, Waltham, MA), and SYBR Green I Nucleic Acid Gel Stain diluted 1:10,000 (Invitrogen). All reactions were carried out using CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA). The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30

seconds, and 72°C for 30 seconds. Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reaction and was absent from the genomic DNA and water controls. Data were normalized to housekeeping genes and analyzed by the $2^{-\Delta\Delta Ct}$ method. For the hypothalamus, *GAPDH* was used for normalization. For the pituitary, *PGK1* was used for normalization. For all of the follicle cell types, *BTUBB2* was used for normalization. For each tissue type, several genes were examined as potential housekeeping genes. The housekeeping gene used for each tissue type was selected based on the smallest range of Ct values among the all of the samples of the tissue type. All PCR reactions for each gene in a given tissue were analyzed in a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data were $\log_2$ transformed before statistical analysis. An ANOVA using the mixed models procedure was conducted to compare the plasma hormone concentrations and $\log_2$ transformed gene expression between the two hen groups (e.g. outside and inside of the PS). The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$. Data are presented relative to basal expression for the hypothalamus and pituitary. For the granulosa, theca interna, and theca externa layers, data are presented relative to basal expression of the F1 follicle to visualize mRNA expression differences due to the PS as well as mRNA expression differences across the F1 and F5 follicles.

Table 2.1 Primers for RT-qPCR

Symbol	Forward Primer	Reverse Primer
GNRH1	TGGCAATCTGCTTGGCTCA	CCAGGGCATTCAGCCTTC
GNIH	CAGTGGCGTTTCTAACACC	ACTCCTCTGCTTTTCCTCC
GNRHR	TCCCAGGAGGGAACTTCAC	TTCATGCGTGCCTTGGAG
GNIHR1	TCTGTCTCCGCCTCTGTTTT	GACAGTTAGGGTGATGGC
GNIHR2	ACCTGGCTGTCAGCGATTTA	TCCTTGGACCATCCCACTC
LHB	GGAGAAGGACGAATGTCCC	CCCCATAAGTGCAGGACG
FSHB	GTGGTGCTCAGGATACTGCT	AGATTCAGGATGGTCACC
CGA	CACACACCAAGGACAGCTC	CTCCCCTAGCTTGCACTCT
PGR	ACCAAGTTCCTTGCTGACC	CCTGGTAGCAATTTTGACC
ESR1	ATCCACCGTGTTCTGGACA	TCGTAGAGCGGAACCACA
ESR2	TCACAGATGCTCTGGTGTG	GAGTGTGTGCGCATTCAA
LHCGR	ATCCACAGCCATGCCTTCAA	TTTATCCAGAGGCGGCAG
FSHR	ACATTCCCACCAATGCCACA	ATCTGAGGCTTGGAAGGT
STAR	ATCTCCTACCAACACCTGCG	GGACATCTCCATCTCGCTG
CYP11A1	GTTGGGTGTCTACGAGAGC	CTCCTTGTTCAGGGTCAG
HSD3B1	TGCTGGAAGAAGATGAGGC	TCACGTTGACTTCCCAGA
CYP17A1	GCTGAAGAAGGGGAAGGCT	GAAGGAGAGGGGCAGTG
HSD17B1	CTGCCACTACTGCGGAAAT	TTTGGAAAGCTCCTGCCT
CYP19A1	TGGATCAGCGGTGAAGAAA	CTTCCAGTGTGCTGGGTT
GAPDH	GGACACTTCAAGGGCACTG	TAACACGCTTAGCACCAC
PGK1	CAAAGGCCCTTGAGAGTCC	ATGCCATTCCACCACCAAT
TUBB2B	GATCTTCCGACCCGACAAC	GAGTGGGTCAACTGGAAG

Results

Plasma Hormone Levels

An 8-fold increase in plasma P4 levels was detected for hens sampled during the PS compared to hens sampled outside of the PS (**Figure 2.1A**); however, plasma E2 levels were similar for both hen groups (**Figure 2.1B**).

Hypothalamic and Pituitary Gene Expression

Hypothalamic expression of the main stimulatory releasing factor and of two of the steroid hormone receptors differed significantly between hens sampled outside and during the PS (**Figure 2.2**). For hens sampled during the PS, *GNRH,* the main stimulatory releasing factor of the HPG axis, was down-regulated. Additionally, *ESR1* and *ESR2,* which encode both E2 receptors, were up-regulated in comparison to hens sampled outside of the PS. Pituitary genes encoding releasing factor receptors, steroid hormone receptors, and gonadotropin subunits were differentially expressed between the two groups of hens (**Figure 2.3**). Both *GNRHR* and *PGR,* which encode receptors for GnRH and P4, respectively, were down-regulated during the PS. Moreover, *GNIHR1* and *ESR1,* which encode receptors for GnIH and E2, respectively, were up-regulated during the PS. In regard to gonadotropin subunit mRNA levels, hens exhibited higher *FSHB* expression and lower *LHB* expression during of the PS compared to outside of the PS.

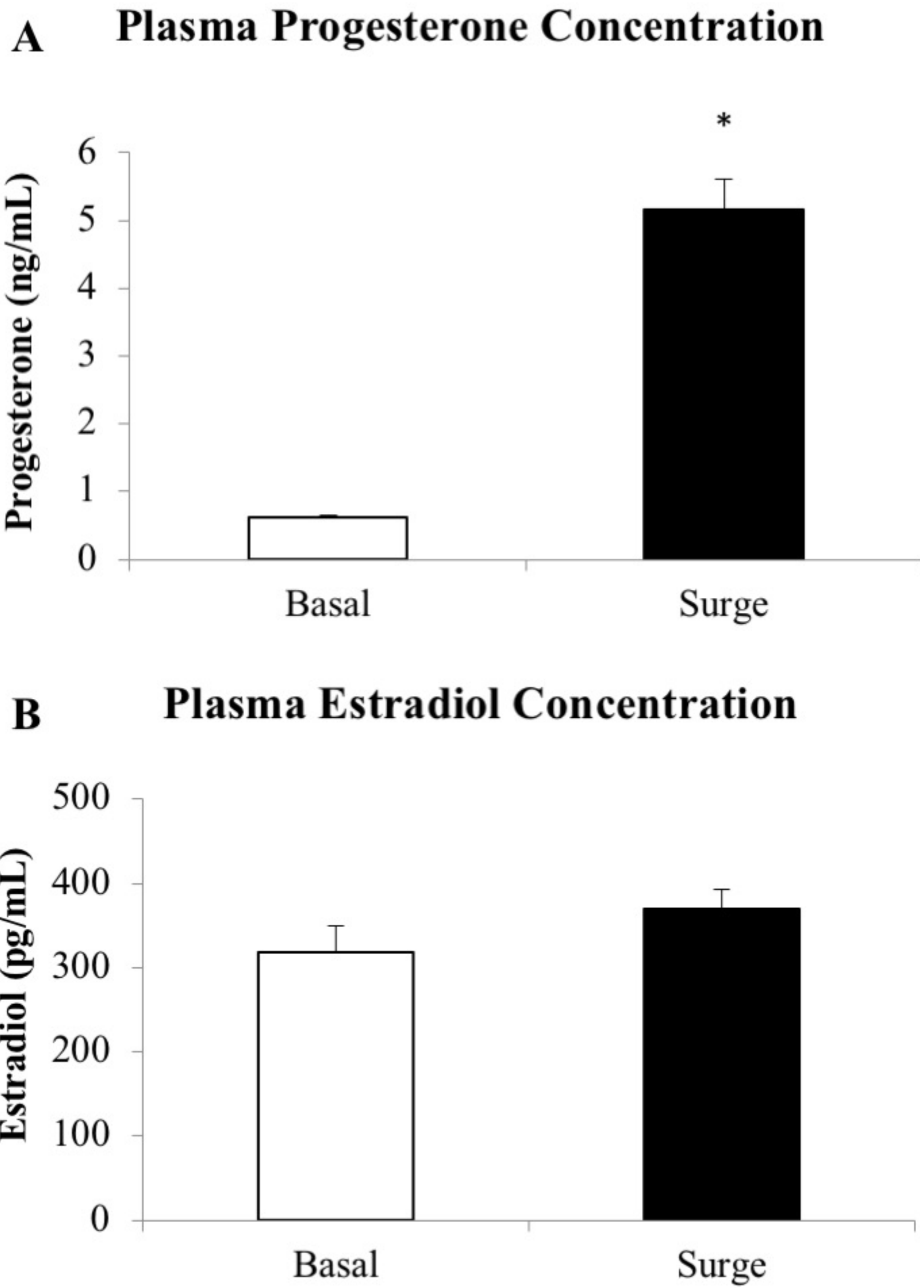


Figure 2.2 Plasma P4 and E2 hormone profiles in hens sampled outside (basal) and inside (surge) of the PS. Significant steroid plasma concentration differences are denoted with an asterisk.

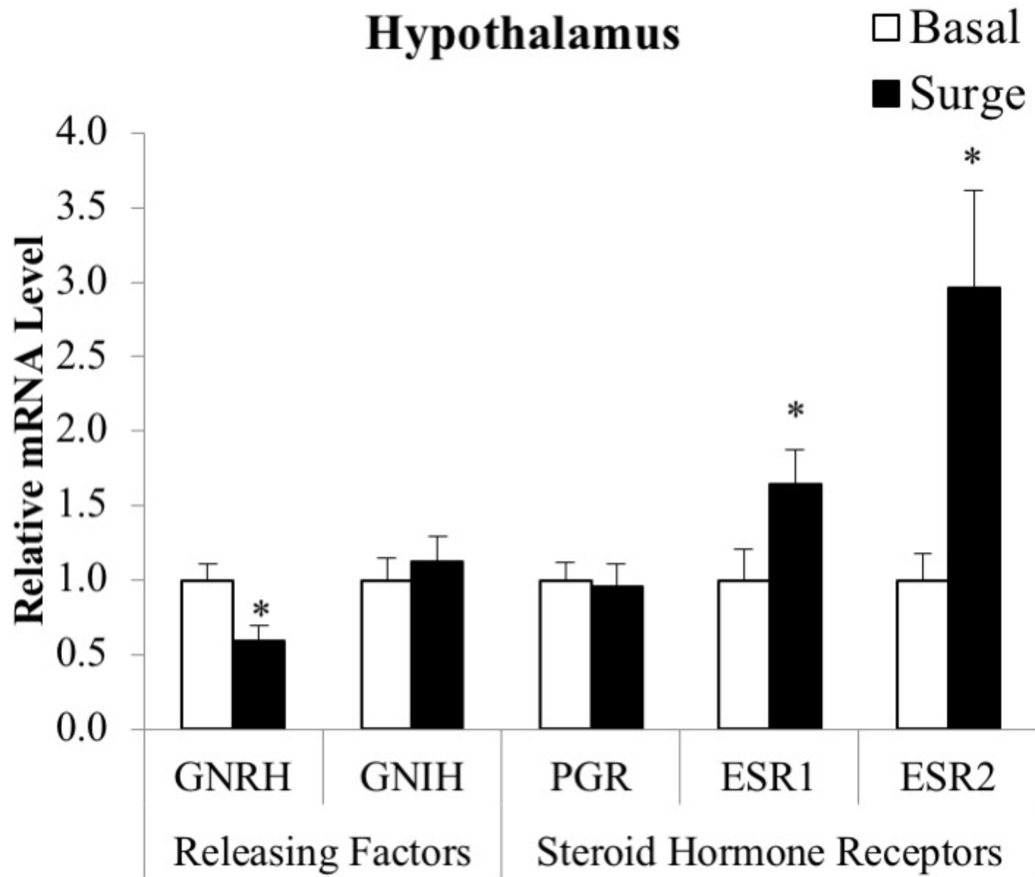


Figure 2.3 Hypothalamic gene expression of HPG axis releasing factors and steroid hormone receptors in hens samples outside (basal) and inside (surge) of the PS. Normalized data are presented relative to basal expression for each gene. Significance is denoted with an asterisk.

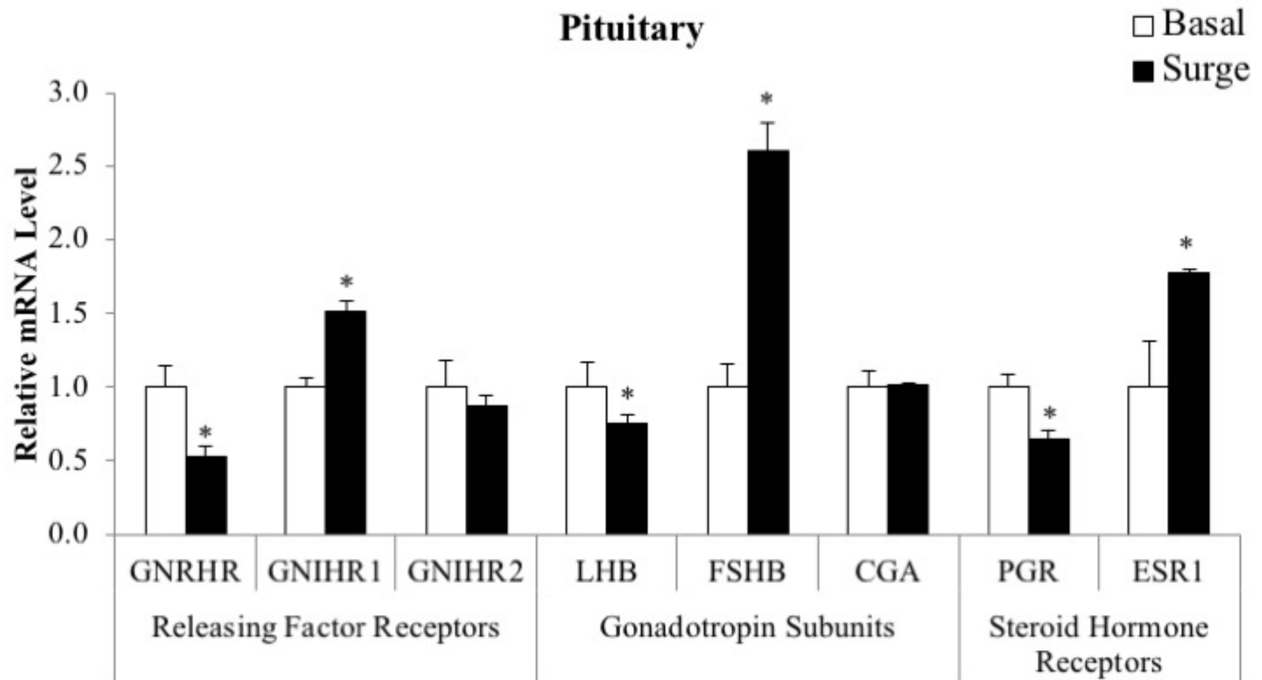


Figure 2.4 Pituitary gene expression of HPG axis releasing factor receptors, gonadotropin subunits, and steroid hormone receptors in hens samples outside (basal) and inside (surge) of the PS. Normalized data are presented relative to basal expression for each gene. Significance is denoted with an asterisk.

Follicle Gene Expression

In the granulosa cell layer, expression of gonadotropin receptors and of key genes involved in P4 production were altered significantly during the PS (**Figure 2.4**). During the PS, expression of *LHCGR*, encoding the LH receptor, was down-regulated in the F1 granulosa cells but was up-regulated in the F5 granulosa cells. Expression of *STAR,* which is involved in the first step of P4 production, was increased during the PS in the F1 granulosa cells, in comparison to expression outside of the PS. In contrast, expression of *CYP11A1,* which is involved in P4 production, was decreased during the PS in the F1 granulosa cells, when compared to expression outside of the PS.

The PS impacted the theca interna cell layer expression of gonadotropin receptors and of genes involved in AD production (**Figure 2.5**). In the F1 theca interna cells, expression of *FSHR* and *LHCGR*, which encode the FSH and LH receptors, respectively, were upregulated during the PS. Expression of *HSD3B1* in the F1 theca interna cells as well as of *HSD17B1* in the F5 theca interna cells, both of which encode enzymes involved in AD production, were increased during the PS in comparison to expression outside of the PS.

In the theca externa layer, expression of gonadotropin receptors and of genes involved in E2 production differed during the ovulatory cycle (**Figure 2.6**). In the F1 theca externa cells, *FSHR* expression was up-regulated during the PS. In the F5 theca externa cells, *LHCGR* expression was up-regulated during the PS, when compared to expression outside of the PS. Regarding the expression of the enzymes involved in E2 production, no significant differences were seen in the theca externa cells of the F1 follicle between the two hen groups, but in the F5 theca externa cells, significant differences were seen in the expression of *CYP19A1*, with *CYP19A1* showing up-regulation during the PS in comparison to expression outside of PS.

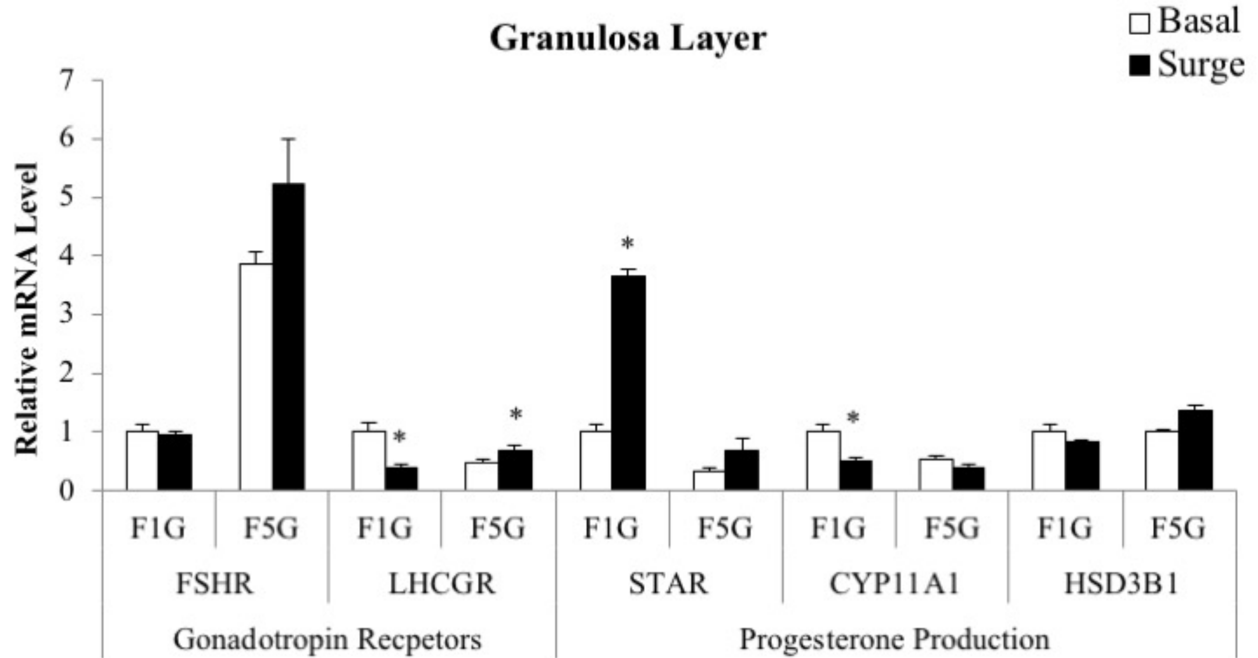


Figure 2.5 Granulosa layer gene expression related to gonadotropin action and P4 production in the HPG axis comparing the expression of hens sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to basal expression of the F1 follicle. Significance is denoted with an asterisk.

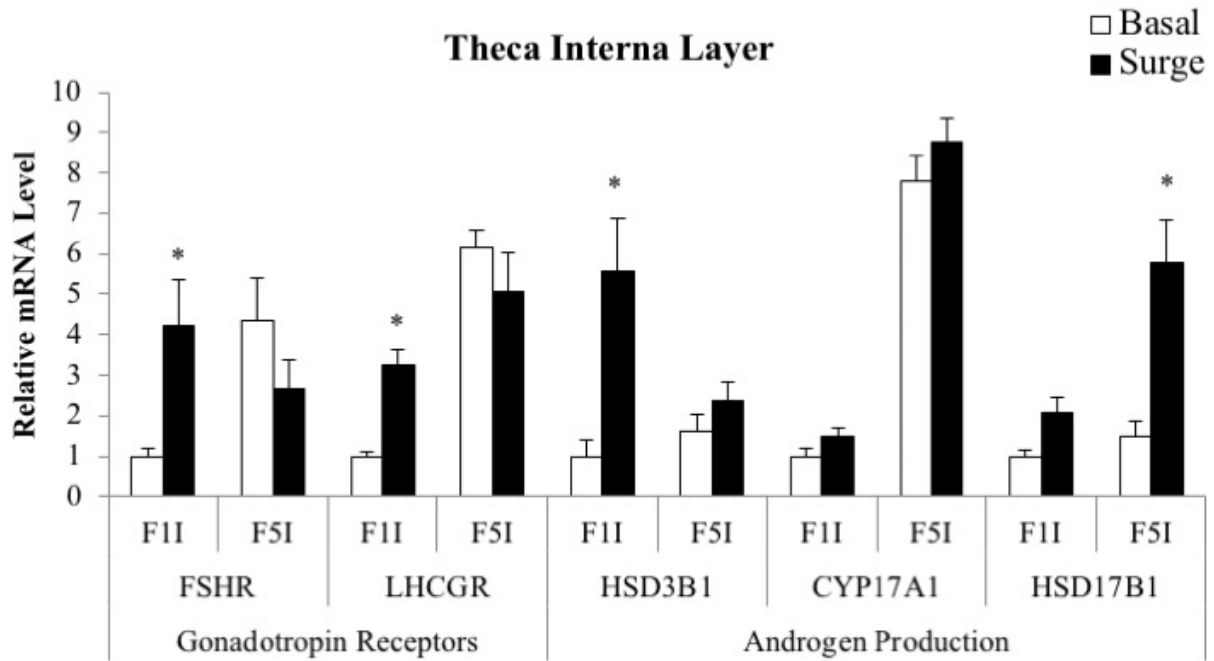


Figure 2.6 Theca interna layer gene expression related to gonadotropin action and androgen production in the HPG axis comparing the expression of hens sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to basal expression of the F1 follicle. Significance is denoted with an asterisk.

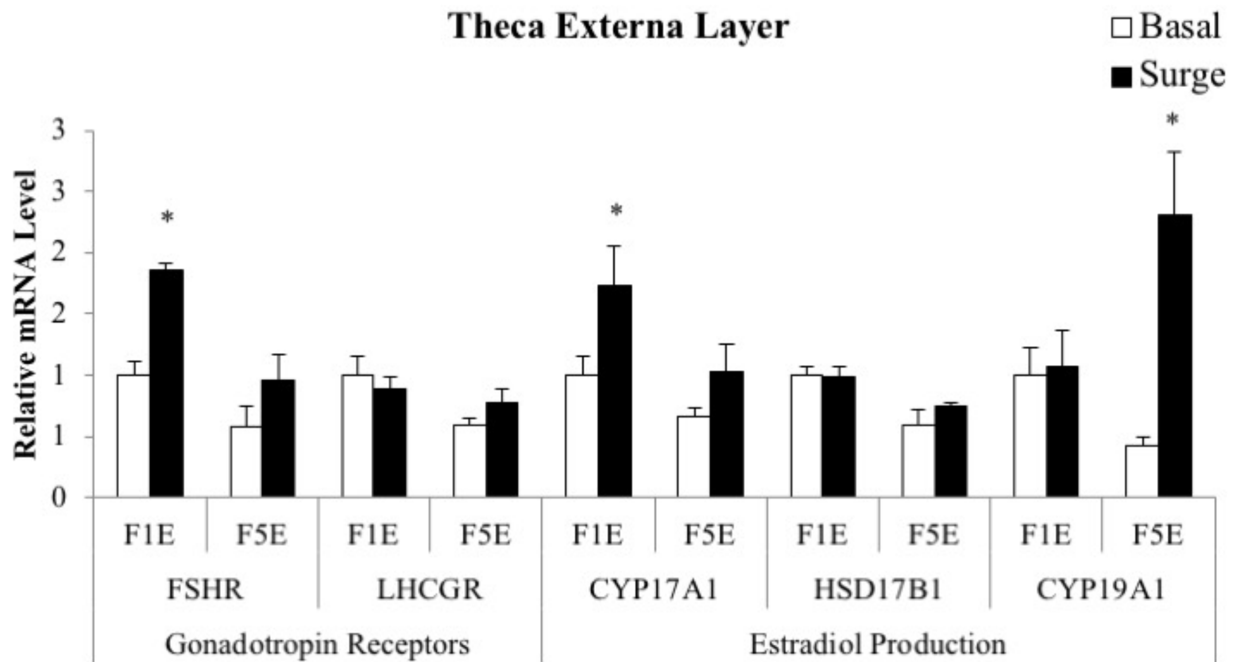


Figure 2.7 Theca externa layer gene expression related to gonadotropin action and E2 production in the HPG axis comparing the expression of hens samples outside (basal) and inside (surge) of the PS. Normalized data are presented relative to basal expression of the F1 follicle. Significance is denoted with an asterisk.

Discussion

This study provided novel insights regarding the mRNA expression of key HPG axis genes during the PS of hormones that triggers ovulation. In each of the tissues examined, the PS impacted the HPG axis. The PS altered HPG axis gene expression, unveiling aspects of ovulation regulation at the molecular level. The gene expression results are summarized in **Figure 2.7**.

Plasma concentrations of P4 both outside and inside of the PS were consistent with previous studies conducted in turkey hens (Yang et al., 1997). Plasma levels of P4 and E2 were also similar to levels seen in studies in laying chickens (Johnson and Tienhoven, 1980) as well as in broiler chickens (Liu et al., 2004). As in previous studies, the PS did not impact E2 levels. An 8-fold increase from 0.6 ng/mL to 5.2 ng/mL in P4 levels confirmed that hens were sampled correctly outside and during the PS. P4 levels in turkeys were consistent with levels seen in chickens; however, several studies have pointed out functional differences in the role of P4 between these two species. For instance, exogenous P4 in chicken hens was capable of inducing ovulation, whereas exogenous P4 was not capable of inducing ovulation in turkey hens (Yang et al., 1998; Johnson and Tienhoven, 1985). This insight indicates that the regulation and feedback for ovulation may include different factors in chickens and in turkeys.

In the hypothalamus, *GNRH* mRNA levels were decreased during the PS. This study is the first to quantify *GNRH* mRNA expression during the PS in any avian species. A previous study demonstrated that plasma levels of GNRH in chicken hens did not change during the PS (Contijoch and Advis, 1993); however, given the apparent differences in the feedback loop for ovulation between the chicken and the turkey, this contrast is not surprising. Similar studies in mammals showed increased expression levels of *GNRH* mRNA as well as increased GNRH plasma concentrations during the PS (Christian and Moenter, 2010). Physiological reproductive

differences between mammalian and avian species most likely account for the discrepancies in *GNRH* mRNA levels seen during the PS.

Another important finding of this study is that expression of E2 receptors within the hypothalamus and pituitary were upregulated during the PS. E2 receptors have been shown to be capable of steroid hormone binding in the hypothalamus and pituitary of chickens (Kawashima et al., 1993). Increased expression of estrogen receptors in the hypothalamus and pituitary during the PS may indicate a feedback mechanism in neuropeptide or gonadotropin regulation. Transcripts for pituitary gonadotropins also were affected by the PS. For example, *LHB* mRNA levels decreased during the PS but *FSHB* mRNA levels increased. High plasma LH concentrations are associated with the PS; however, transcript regulation does not always coincide with plasma concentrations. While a decrease in pituitary mRNA levels for *LHB* during the PS was also seen in chickens, an increased in *GNRHR* mRNA and a decreased in *FSHB* mRNA was also reported (Lovell et al., 2005). Those data are in direct contrast with the current findings for the turkey, where a decrease in *GNRHR* mRNA and a two-fold increase in *FSHB* mRNA were observed. Several key functional differences have been established between chicken and turkey HPG axis P4 feedback activity for ovulation to occur that may be contributing to the inconsistencies of pituitary mRNA expression between species (Bacon and Liu, 2004).

For the turkey, minimal information was previously known in regard to the mRNA expression of gonadotropin receptors and steroid metabolism genes in the F1 and F5 follicles during the PS. Previous studies have focused on granulosa layer gene expression changes during movement through the follicular hierarchy. The expression changes between the F1 and F5 follicles in the current study are consistent with these previous studies (Li and Johnson, 1993;

Woods and Johnson, 2005; Johnson and Bridgham, 2001). In the follicle cells, mRNA levels during the PS were consistent with increased P4 production in the F1 granulosa, increased AD production in the F5 theca interna, and increased E2 production in the F5 theca externa. The F1 granulosa, F5 theca interna, and F5 theca externa are responsible for P4, androgen, and E2 production, respectively, and their steroidogenic related gene expression was significantly impacted by the PS. Increased expression of *STAR* mRNA during the PS had been previously reported in chicken F1 granulosa cells (Johnson et al., 2002) and were also seen in the present study. Transport of cholesterol into the mitochondrial membrane is the rate-limiting step of steroidogenesis (Bauer et al., 2000). Upregulation of *STAR* during the PS is indicative of an increased potential for P4 production. *HSD17B1* and *CYP19A1* mRNA were also upregulated in the F5 theca interna and theca externa, respectively, signifying an increased potential for AD and E2 production.

Each component of the HPG axis showed differential expression during the PS, indicating that gene regulation is globally impacted at both the central and ovarian components of the axis. In the hypothalamus, the PS coincided with a decrease in mRNA expression of the main HPG axis stimulatory factor and with an increase in estrogenic receptor mRNA expression. In the pituitary, lower mRNA levels were seen during the PS for receptors that stimulate the HPG axis and higher mRNA levels were seen for receptors that inhibit the HPG axis. Additionally, in the pituitary, gonadotropin subunit expression showed opposite trends, with *LHB* expression decreasing during the PS and *FSHB* expression increasing during the PS. Follicle cells showed increased expression of *STAR*, *HSD17B1*, and *CYP19A1* in the F1 granulosa, F5 theca interna, and F5 theca externa, which are associated with P4, AD, and E2 production, respectively. Increased pituitary *FSHB* expression in combination with increased F5

theca interna *HSD17B1* and F5 theca externa *CYP19A1* expression during the PS might indicate that the PS initiates increased responsiveness of the smaller preovulatory follicles to pituitary FSH that, in turn, would potentially stimulate their maturation. Understanding the gene expression changes during the PS in the hypothalamus, pituitary, and ovarian cells allows for further studies examining the role of these gene expression changes in the regulation of ovulation. Determining the impact of HPG axis dysregulation on egg production levels would also be instrumental in improving the reproductive efficiency of the turkey hen.

Gene Expression

Tissue	Classification	Gene	Expression	
Hypothalamus	Releasing Factors	*GNRH*	*	
		GNIH		
	Steroid Hormone Receptors	*PGR*		
		ESR1	*	
		ESR2	*	
Pituitary	Releasing Factor Receptors	*GNRHR*	*	
		GNIHR1	*	
		GNIHR2		
	Gonadotropin Subunits	*LHB*	*	
		FSHB	*	
		CGA		
	Steroid Hormone Receptors	*PGR*	*	
		ESR1	*	
Follicle Granulosa	Gonadotropin Receptors	*FSHR*	F1	F5
		LHCGR	F1 *	F5 *
	Progesterone Production	*STAR*	F1 *	F5
		CYP11A1	F1 *	F5
		HSD3B1	F1	F5
Follicle Theca Interna	Gonadotropin Receptors	*FSHR*	F1 *	F5
		LHCGR	F1 *	F5
	Androgen Production	*HSD3B1*	F1 *	F5
		CYP17A1	F1	F5
		HSD17B1	F1	F5 *
Follicle Theca Externa	Gonadotropin Receptors	*FSHR*	F1 *	F5
		LHCGR	F1	F5
	Estradiol Production	*CYP17A1*	F1 *	F5
		HSD17B1	F1	F5
		CYP19A1	F1	F5 *

HPG Axis Fold Change:

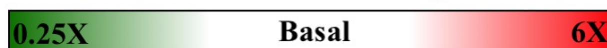


Figure 2.8 Summary of relative HPG axis gene expression changes during the PS. Red, white, and green represent increased expression, no change in expression, and decreased expression during the PS, respectively. The legend at the bottom of the figure displays how the color intensity relates to the fold change difference seen during the PS. Significant changes in gene expression are denoted by an asterisk.

CHAPTER 3

Characterization of the hypothalamo-pituitary-gonadal axis in low and high egg producing turkey hens

Abstract

Variation in egg production exists in commercial turkey hens, with LEPH costing more per egg produced than HEPH. Egg production correlates with ovulation frequency, which is governed by the HPG axis. Ovulation is stimulated by a PS of P4 and LH, triggered by GnRH release and inhibited by GnIH. Differences between LEPH and HEPH were characterized by determining HPG axis plasma hormone profiles and mRNA levels for key genes, both outside and inside of the PS (n=3 per group). Data were analyzed with a two-way ANOVA using the mixed models procedure of SAS. In the HPG axis, plasma P4 levels were not affected by egg production level but were elevated during the PS. In contrast, plasma E2 levels were higher in HEPH than in LEPH but were not affected by the PS. LEPH exhibited decreased gene expression associated with ovulation stimulation and increased gene expression associated with ovulation inhibition in the hypothalamus and pituitary. In ovarian follicle cells, LEPH displayed decreased gene expression associated with P4, AD, and E2 production in the F1 follicle granulosa cells, F5 theca interna cells, and SWF cells, respectively. Different degrees of stimulation and inhibition within all tissues of the HPG axis were noted between LEPH and HEPH turkey hens, with HEPH showing higher expression of genes related to ovulation and steroidogenesis.

Introduction

The turkey industry focuses on meat production and has selected heavily for carcass traits over the past forty years (Nestor el at., 2008). Selection for carcass traits has stunted egg production, causing a reduction in the number of poults per turkey breeding hen (McCartney et al., 1968). While meat production is the priority of the turkey industry, the number of eggs laid per hen greatly impacts the number of turkey poults that can be reared. In addition to lower overall egg production, there is a large amount of variation in egg production within a single

commercial flock, creating two distinct levels of egg production, LEPH and HEPH. LEPH cost more per egg produced than HEPH, which strains the turkey industry.

Egg production begins with follicle ovulation from the ovary, making ovulation frequency and egg production highly correlated. The HPG axis governs the hen's reproductive system and directly regulates ovulation, ultimately regulating egg production. Ovulation is triggered by a PS of P4 and LH roughly 8-10 hours before each ovulation (Yang et al., 1997). The HPG axis can be negatively or positively regulated at the level of the hypothalamus, pituitary, or ovary to impact ovulation timing (Bédécarrats et al., 2016). Within the hypothalamus, GnRH and GnIH, both acting through their respective G-protein coupled receptors on pituitary gonadotroph cells, regulate gonadotropin production (Bédécarrats et al., 2009). Within the ovary, steroid hormone feedback loops regulate gene expression locally as well as in the hypothalamus and pituitary (Rivas and Nieto, 2016; Ottinger and Bakst, 1995). The F1 follicle is responsible for the majority of P4 production, the F5 follicle is responsible for the majority of AD production, and the SWF are responsible for the majority of E2 production (Lee and Bahr, 1994). Avian ovarian steroidogenesis occurs via the three-cell model of steroidogenesis, where granulosa cells produce P4, theca interna cells produce AD, and theca externa cells produce E2 (Porter et al., 1989). P4 production increases with follicle maturation, while AD and E2 production decrease with follicle maturation (Porter et al., 1991).

Previous studies examining AEPH found that the PS significantly impacts the HPG axis steroid hormone profiles and gene expression (Chapter 2). The PS increased plasma P4 levels but did not impact plasma E2 levels. In the hypothalamus and pituitary components of the HPG axis, the PS coincided with a decrease in in mRNA levels for genes associated with ovulation stimulation, an increase in expression of genes associated with ovulation inhibition, and an

increase in mRNA expression for E2 receptors. In the follicle cells, increased expression of genes associated with P4, AD, and E2 production in the F1 granulosa, F5 theca interna, and F5 theca externa, respectively, was seen in response to the PS.

The inner workings of the turkey hen reproductive axis are not consistent within a commercial flock, ultimately resulting in a wide distribution of egg production. While HPG axis plasma steroid hormone levels and gene expression have been characterized in average egg producing hens, these features remain unknown in hens with poor egg production and with superior egg production. This study sought to characterize the P4 and E2 plasma profiles as well as the expression of key HPG axis genes in LEPH and HEPH, both inside and outside of the PS that triggers ovulation. Understanding the perturbations to normal function of the HPG axis that are leading to different egg production levels will be instrumental in improving the egg production capabilities of LEPH.

Materials and Methods

Hen Selection and Cell Isolation

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual wire cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards. Turkey hens were sampled at 37 weeks of age. Daily egg records were used to calculate each hen's EPD by dividing the total number of eggs produced by the number of days in production. Hens were classified as LEPH when EPD<0.6 and as HEPH when EPD>0.8. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland. All hens were sampled on the second day of the hen's sequence. The hypothalamus, pituitary, F1 follicle, and F5 follicle were isolated from six LEPH

and six HEPH, half outside of the PS and half during the PS, creating four experimental groups. The timing of the PS was predicted as previously described (Chapter 2). The entire hypothalamus, pituitary, and SWF were snap frozen for RNA extraction, while the F1 and F5 follicles were subjected to isolation of the three cell types from the follicle wall.

The granulosa, theca interna, and theca externa cells were isolated from the F1 and F5 follicles as previously described (Porter et al., 1989a). Briefly, the yolk was drained from each follicle and the follicle was inverted to peel off the granulosa layer. The theca interna layer was scraped from the inverted follicle and the remaining theca externa layer was minced. All follicle layers were subjected to trypsin dispersion (1 mg/mL) followed by layering onto a Percoll suspension (50%) to remove debris and red blood cells. Isolated cells were snap frozen for RNA extraction.

RIAs

The RIAs used for P4 and E2 were coated tube kits (MP Biomedicals, Solon, OH). For the P4 and E2 RIAs, plasma samples were ether extracted prior to the assay. All protocols were performed as directed by the supplier. All samples were assayed in duplicate. The standard curve was assessed for linearity as well as parallelism using serial plasma dilutions. The intraassay coefficients of variation determined by pools run every 30 samples were 4.26% for P4 and 2.48% for E2. All samples were measured in a single RIA for each hormone.

RT-qPCR

Total RNA was isolated from the hypothalamus, pituitary, and ovarian granulosa, theca interna, and theca externa cell from the F1 and F5 follicles with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA, reverse transcription reactions, and RT-qPCR were performed as previously described (Chapter

2). A pool of total RNA was made, and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. Reactions were diluted by tissue type as previously described prior to PCR analysis (Chapter 2). Primers (IDT, Skokie, IL) were designed and used with cycling parameters described previously (Chapter 2). Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reaction and was absent from the genomic DNA and water controls. Data were normalized to housekeeping genes and analyzed by the $2^{-\Delta\Delta Ct}$ method. For the hypothalamus, *GAPDH* was used for normalization. For the pituitary, *PGK1* was used for normalization. For all of the follicle cell types, *GAPDH* was used for normalization. All PCR reactions for each gene in a given tissue were analyzed in a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data were $\log_2$ transformed before statistical analysis. A two-way ANOVA using the mixed models procedure was conducted to compare the plasma hormone concentrations and $\log_2$ transformed gene expression between LEPH and HEPH, taking the PS into account. The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$.

Results

Production and Plasma Parameters

Significant production differences were noted in egg production, clutch length, and pause length between LEPH and HEPH (**Figure 3.1**). As expected, HEPH exhibited a higher number of eggs laid per day when compared to LEPH (**Figure 3.1A**). Clutch length, which is the number

of eggs laid consecutively, was also higher in HEPH than LEPH (**Figure 3.1B**). Moreover, pause lengths, which is the number of days between clutches, was lower in HEPH in contrast to LEPH (**Figure 3.1C**).

Ovarian morphology did not differ between LEPH and HEPH (**Table 3.1**). The ovaries of LEPH and HEPH did not differ in the number of preovulatory follicles, the weight of the F1 follicle, or the weight of the F5 follicle. Furthermore, the ovary and oviduct weights were not different between LEPH and HEPH.

Table 3.1 Ovarian and Plasma Parameters

Parameter	**LEPH**	**HEPH**
Number of Preovulatory Follicles	10.6	11.6
F1 Follicle Weight (g)	26.5	25.5
Small White Follicle Size (mm)	2-6	2-6
Ovary Weight (g)	197.8	191.4
Oviduct Weight (g)	102.6	110.5
Basal Plasma P4 (ng/mL)	0.85	0.54
PS P4 (ng/mL)	4.87	5.19

LEPH and HEPH did not differ in plasma P4 concentrations, either outside or during the PS (**Figure 3.2A**). Both LEPH and HEPH showed a significant increase in plasma P4 levels during the PS when compared to basal levels. Plasma E2 levels were significantly different between LEPH and HEPH (**Figure 3.2B**). HEPH exhibited higher plasma E2 levels both outside and during the PS. Neither LEPH nor HEPH displayed a change in plasma E2 levels due to the PS.

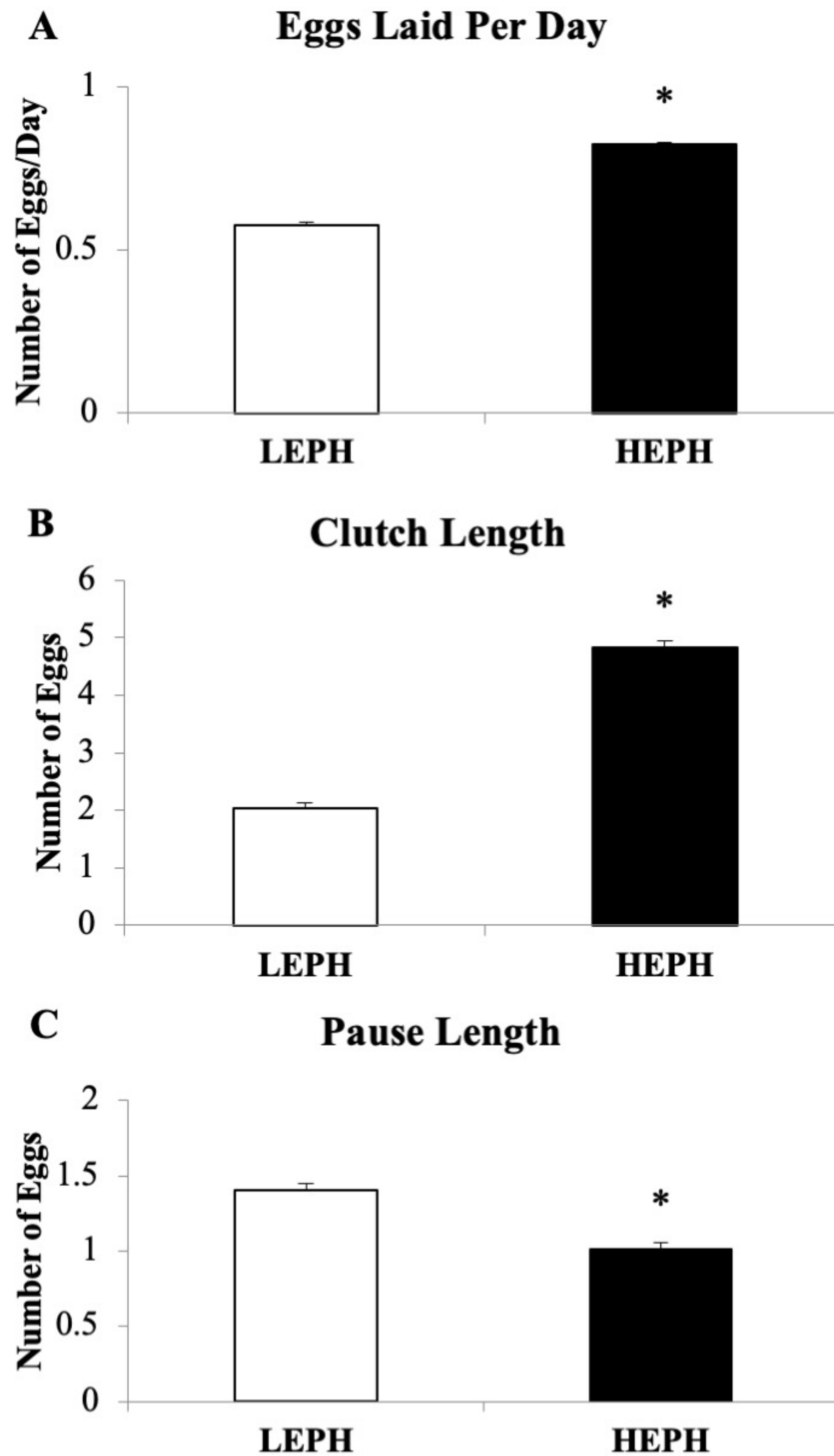
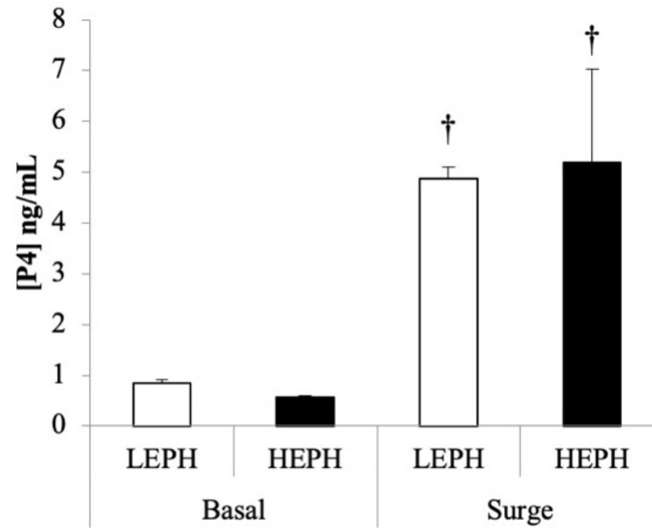


Figure 3.1. Production parameters showing (A) egg production level, (B) clutch length, and (C) pause length in LEPH and HEPH. Significance is denoted with an asterisk.



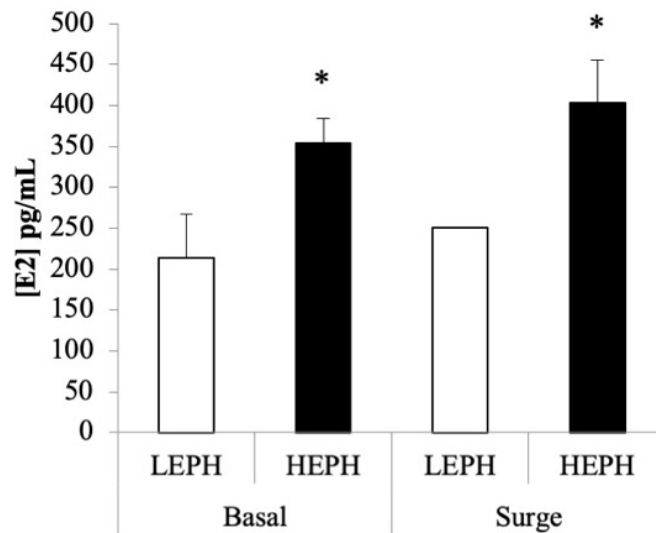


Figure 3.2 Plasma P4 and E2 hormone profiles in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Significant steroid plasma concentration differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge plasma steroid hormone concentrations for a given egg production group are denoted with a dagger.

Hypothalamic Gene Expression

In the hypothalamus, differences in LEPH and HEPH were seen in the mRNA levels of *GNIH, PGR,* and *ESR1* (**Figure 3.3**). LEPH exhibited higher mRNA levels for *GNIH,* the main inhibitory releasing factor of the HPG axis, outside and inside of the PS. LEPH also showed an increase in *GNIH* expression during the PS. Additionally, LEPH showed higher mRNA levels than HEPH for *PGR,* which encodes the P4 receptor, during the PS, whereas, HEPH showed higher mRNA levels than HEPH for *ESR1,* which encodes one of the E2 receptors, during the PS. HEPH also displayed down-regulation of *PGR* during the PS, while LEPH displayed down-regulation of *ESR1* during the PS.

Pituitary Gene Expression

In the pituitary, differences were seen in mRNA levels of LEPH and HEPH for *GNRHR*, *GNIHR1*, *GNIHR2, ESR1, FSHB,* and *LHB* (**Figure 3.4**). LEPH displayed higher mRNA levels for genes associated with the inhibitory pathways of the HPG axis when compared to HEPH. For example, LEPH showed higher expression of *GNIHR1* both inside and outside of the PS and higher expression of *GNIHR2* outside of the PS, both of which encode receptors for GnIH. However, only HEPH showed down-regulation of *GNIHR2* during the PS. LEPH also exhibited lower mRNA levels for genes associated with HPG axis stimulation in comparison to HEPH. For example, LEPH showed decreased expression of *LHB,* the unique subunit of LH, outside of the PS and decreased expression of *FSHB,* the unique subunit of FSH, during the PS. Interestingly, HEPH showed decreased expression of *GNRHR* during the PS compared to levels seen in LEPH and exhibited down-regulation of *GNRHR* expression in response to the PS, while LEPH did not display significant expression changes of *GNRHR* due to the PS. Additionally, only HEPH showed reduced expression of *LHB* in response to the PS. HEPH showed increased mRNA

levels for *ESR1* outside of the PS when compared to LEPH levels and also showed decreased mRNA levels for *ESR1* during the PS when compared to levels outside of the PS. Both LEPH and HEPH displayed down-regulation of *PGR* during the PS, which was seen previously in AEPH (Chapter 2).

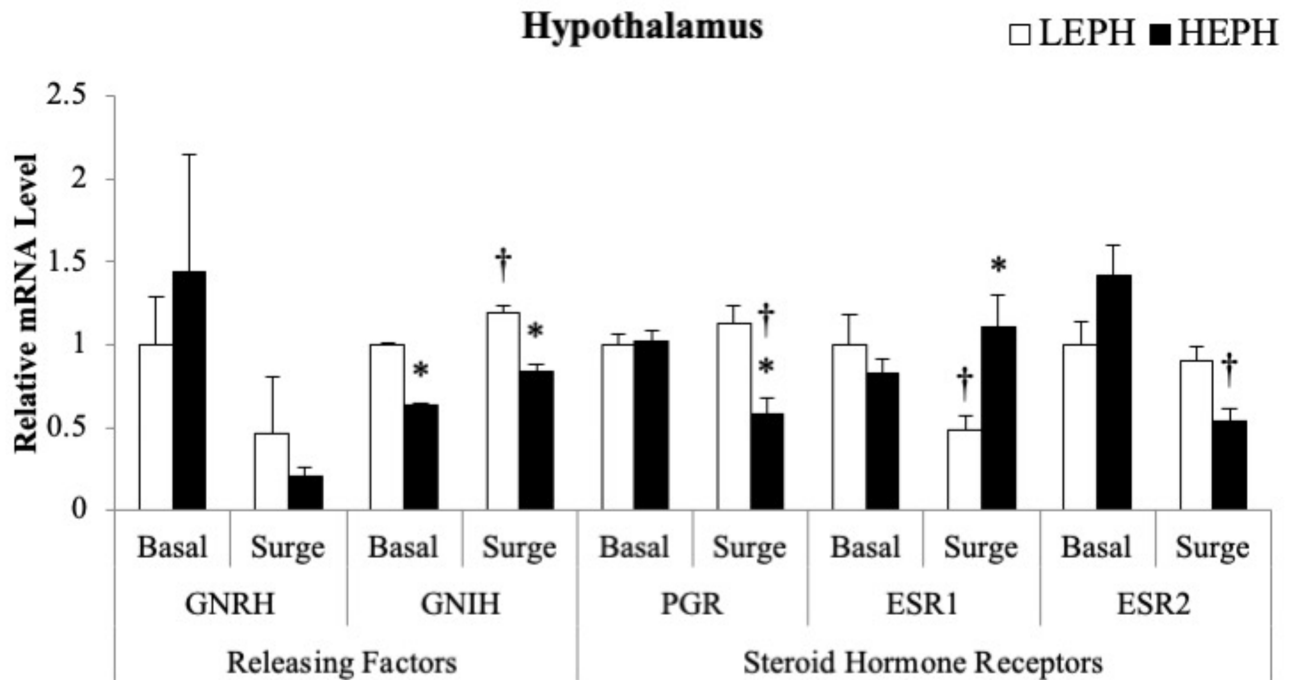


Figure 3.3 Hypothalamic gene expression of HPG axis releasing factors and steroid hormone receptors in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

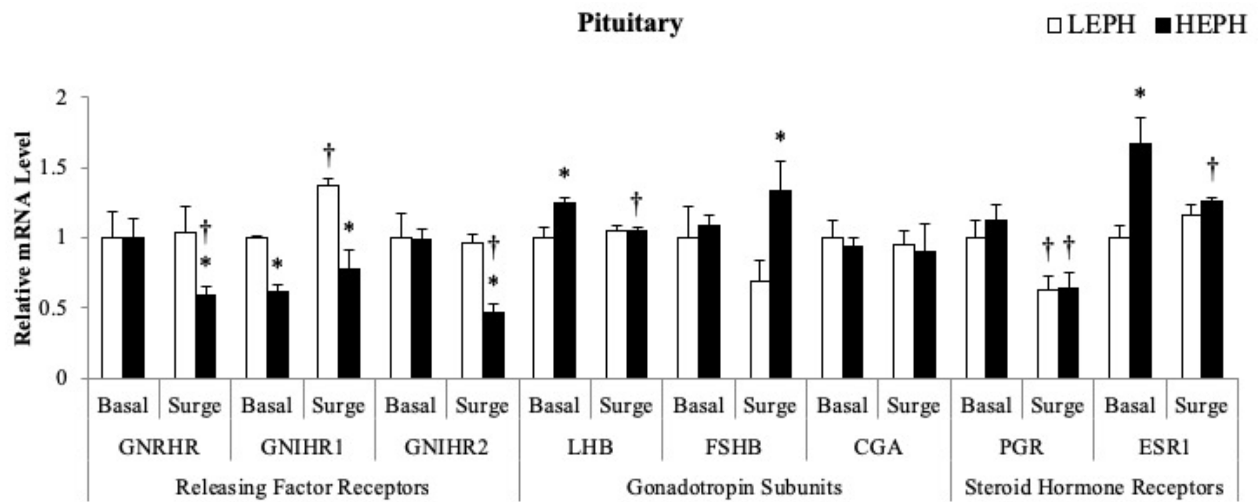


Figure 3.4. Pituitary gene expression of HPG axis releasing factor receptors, gonadotropin subunits, and steroid hormone receptors in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

F1 Follicle Gene Expression

F1 follicle gene expression by cell type is presented in **Figure 3.5**. In the F1 granulosa cells, no significant differences were seen in the expression of genes encoding either gonadotropin receptor but significant expression differences were seen in two of the three genes required for P4 production, specifically *STAR* and *CYP11A1* (**Figure 3.5A**). LEPH demonstrated decreased mRNA levels when compared to HEPH for the genes encoding *STAR* and *CYP11A1*. Both LEPH and HEPH responded to the PS by increasing *STAR* expression but only LEPH responded to the PS by increasing *CYP11A1* expression.

In the F1 theca interna cell layer, differences in mRNA levels were significant for *CYP17A1* (**Figure 3.5B**). *CYP17A1,* which encodes one of the enzymes involved in AD production, showed higher expression in HEPH under basal conditions but showed higher expression in LEPH during the PS. Moreover, LEPH demonstrated up-regulation of *CYP17A1* in response to the PS while HEPH demonstrated down-regulation in response to the PS.

In the F1 theca externa cell layer, differences in mRNA levels were significant for *FSHR*, *LHCGR,* and *CYP19A1* (**Figure 3.5C**). *FSHR* exhibited lower expression in HEPH under basal conditions but showed higher expression in HEPH during the PS. Furthermore, HEPH demonstrated up-regulation of *FSHR* in response to the PS while LEPH did not demonstrate a response to the PS in regards to *FSHR* expression. In addition, HEPH showed decreased expression of *LHCGR* during basal conditions with up-regulation of *LHCGR* expression during the PS. Expression levels of *CYP19A1,* which encodes the enzyme responsible for the conversion of testosterone to E2, were significantly higher in HEPH during the PS when compared to LEPH and HEPH showed up-regulation of *CYP19A1* during the PS when compared to basal levels.

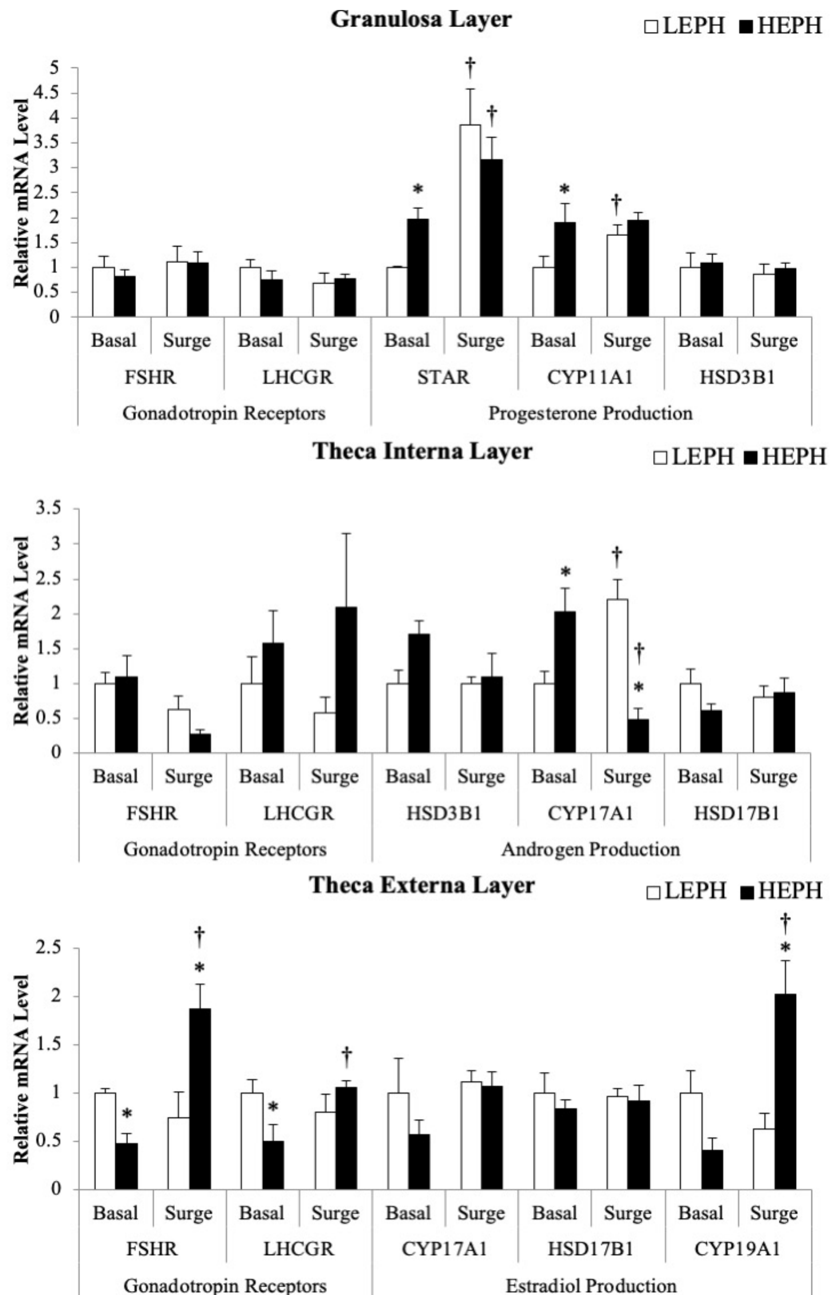


Figure 3.5 F1 gene expression of HPG axis gonadotropin receptors and genes involved ovarian steroid hormone production in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

F5 Follicle Gene Expression

F5 follicle gene expression by cell type is presented in **Figure 3.6**. In the granulosa cells, significant differences were seen in the expression of both gonadotropin receptors and of the enzymes required for P4 production (**Figure 3.6A**). HEPH showed increased mRNA levels for *FSHR* but decreased levels for *LHCGR* outside of the PS when compared to LEPH. Additionally, HEPH responded to the PS by reducing *FSHR* expression and increasing *LHCGR* expression, where LEPH did not change expression of either receptor significantly due to the PS. In regards to P4 production, LEPH showed increased mRNA levels for the genes encoding *STAR*, *CYP11A1*, and *HSD3B1*. Furthermore, HEPH displayed increased expression of these genes in response to the PS while LEPH did not respond to the PS.

In the F5 theca interna cell layer, differences in mRNA levels were significant for *HSD3B1*, *CYP17A1,* and *HSD17B1,* all of which encode enzymes involved in AD production (**Figure 3.6B**). LEPH exhibited higher mRNA levels for *HSD3B1*, *CYP17A1,* and *HSD17B1* under basal conditions when compared to HEPH. Additionally, LEPH displayed higher mRNA levels than LEPH for *HSD17B1* during the PS. Down-regulation of *HSD3B1* and *CYP17A1* were seen in LEPH during the PS, while only down-regulation of *HSD3B1* was seen in HEPH during the PS.

In the F5 theca externa cell layer, differences in mRNA levels were significant for *FSHR* and *CYP19A1* (**Figure 3.6C**). HEPH exhibited higher mRNA levels for *FSHR* during the PS while LEPH showed down-regulation of *FSHR* expression during the PS. LEPH exhibited down-regulation of *CYP19A1* during the PS. HEPH showed lower *CYP19A1* levels when compared to LEPH during basal conditions but showed up-regulation of *CYP19A1* during the PS, resulting in higher mRNA levels than LEPH during the PS.

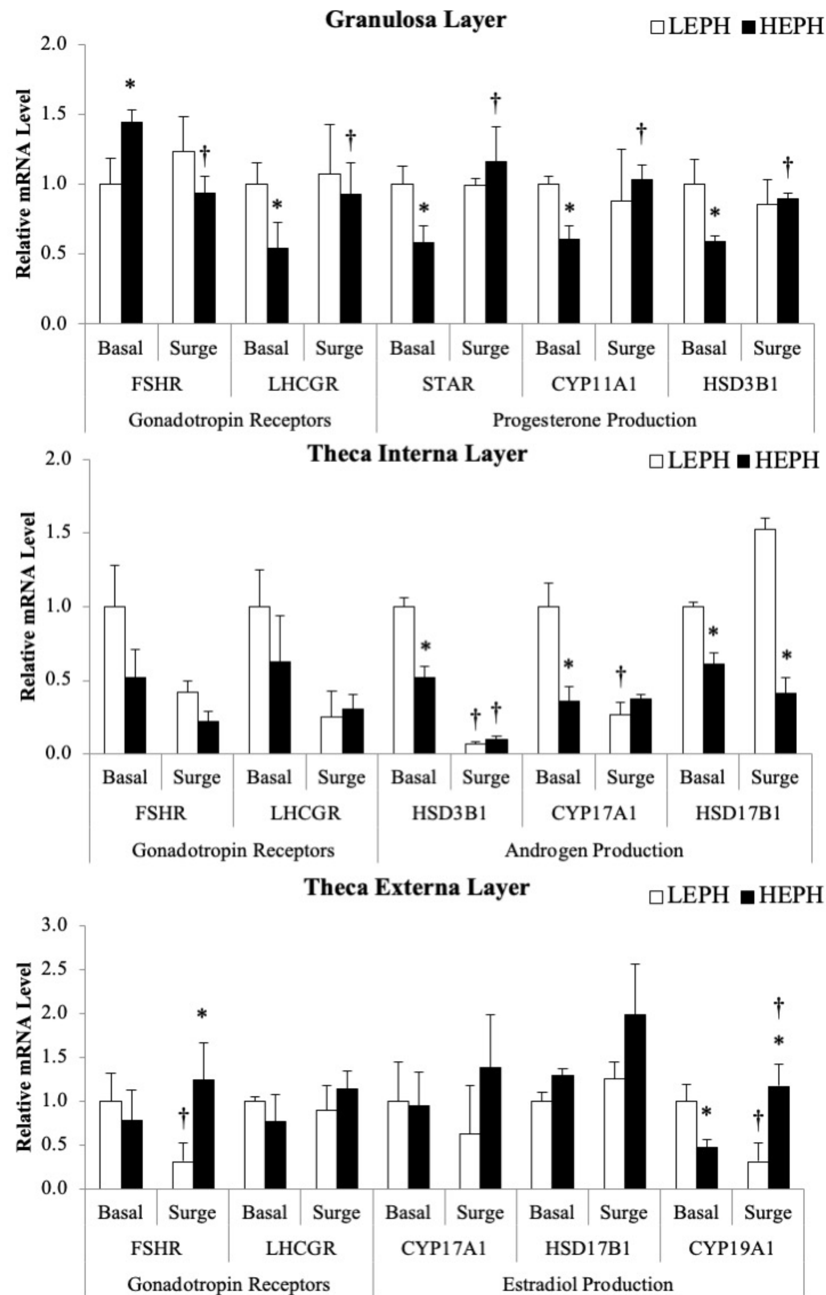


Figure 3.6 F5 follicle gene expression of HPG axis gonadotropin receptors and genes involved ovarian steroid hormone production in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

SWF Gene Expression

SWF gene expression is presented in **Figure 3.7**. Significant gene expression differences between LEPH and HEPH were seen in *LHCGR* and in all three of the genes involved in E2 production. *LHCGR* mRNA levels were higher in HEPH than in LEPH, both outside and during the PS. LEPH exhibited down-regulation of *LHCGR* in response to the PS whereas expression in HEPH did not change. Additionally, HEPH displayed higher gene expression of *CYP17A1* than in LEPH, both outside and inside of the PS. However, both LEPH and HEPH showed decreased mRNA levels for *CYP17A1* during the PS when compared to basal levels. *HSD17B1* and *CYP19A1* expression only differed between LEPH and HEPH during the PS, with both *HSD17B1* and *CYP19A1* mRNA levels higher in HEPH than LEPH. *HSD17B1* expression did not change significantly in HEPH during the PS. In contrast, *HSD17B1* expression decreased significantly in LEPH during the PS. *CYP19A1* expression, however, was down regulated in LEPH during the PS, whereas expression was up-regulated in HEPH during the PS.

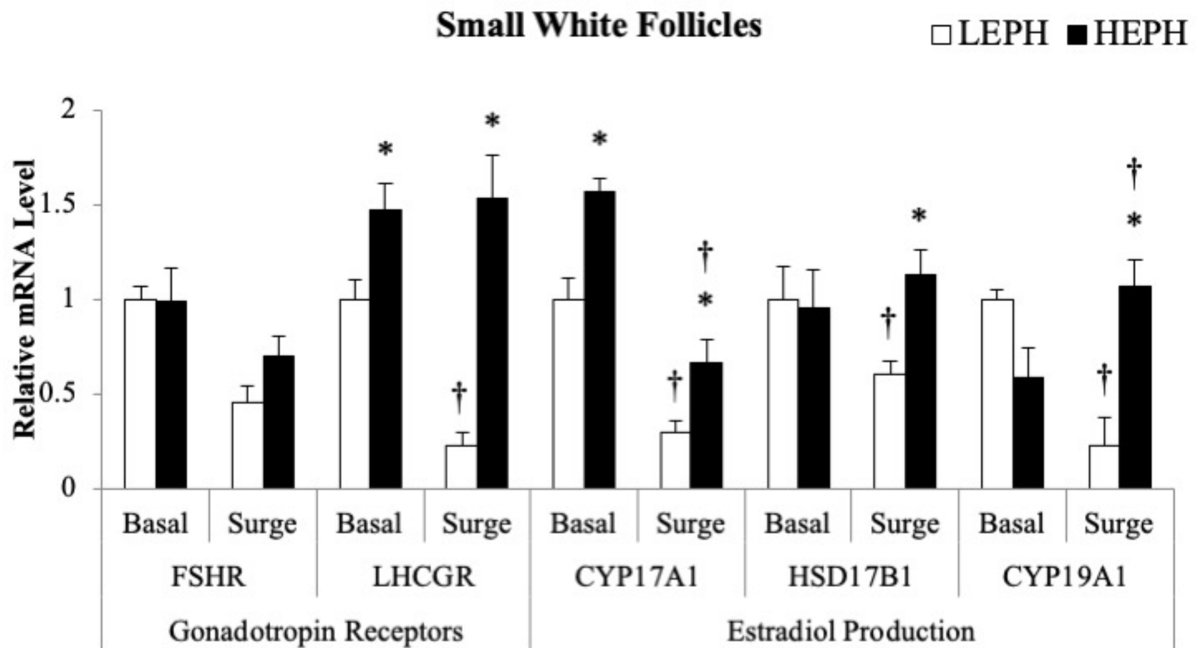


Figure 3.7 SWF gene expression of HPG axis gonadotropin receptors and genes involved estradiol production in LEPH and HEPH sampled outside (basal) and inside (surge) of the PS. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

DISCUSSION

This is the first study to compare production and ovarian parameters, steroid hormone profiles, and HPG axis mRNA levels in LEPH and HEPH of the same breed, strain, and age. Previous studies have examined the impact of genetic selection on HPG axis function through examination of production parameters of chicken and turkey lines divergently selected for meat production and egg production (Nestor et al., 2007). Additionally, there have been studies that examined gene expression changes in chicken strains with low and high egg production (Chen et al., 2007, Yang et al., 2008). Understanding HPG axis function at the macroscopic and molecular levels, as well as how this axis is perturbed in hens with differential egg production is imperative to improving the egg production rates in birds selected for meat production.

Though significant differences were seen in egg production rates, clutch length, and pause length in LEPH and HEPH, these differences were not explained by the morphological structure of the reproductive tract. LEPH did not show reduced follicle numbers, signs of follicle atresia, or an abnormal follicular hierarchy. LEPH also did not appear to have issues attaining the hormone levels for the PS to occur or issues with ovulation. Lastly, the development of the reproductive tract, in terms of individual follicle weight, ovary weight, and oviduct weight, did not appear to be impeded in LEPH when compared to HEPH. In broiler breeding hens, which are also selected predominantly for meat production causing lowered reproductive success, follicular hierarchy issues are common and lead to decreased egg production (Decuypere et al., 2010). Broiler breeding hens have increased numbers of internal ovulations, evidence of which, was not seen in turkey hens with lowed egg production (Hocking, 1993). Ovarian morphology issues common to birds heavily selected for meat production purposes were not seen in turkey hens

with lowered egg production, indicating that selection for meat production is impacting turkey and broiler hens to different degrees and possibly through different mechanisms.

In the present study, both groups of hens displayed basal and peak P4 levels similar to previously reported levels (Yang et al., 1997). Additionally, both groups of hens exhibited a roughly 6-fold increase in plasma P4 levels, with no apparent differences between LEPH and HEPH in plasma P4 concentration during the ovulatory cycle. Plasma E2 concentrations were not affected by the PS in either LEPH or HEPH, but HEPH did display higher plasma E2 levels both outside and during the PS when compared to LEPH. The role of E2 in the regulation of egg production is not fully understood; however, E2 has been shown to bind in the hypothalamus, pituitary, ovary, and oviduct. In laying hens, E2 binding affinity changes in the neurohypophysis during the ovulatory cycle and induces P4 receptor expression in gonadotrophs of the pituitary, implicating a role in ovulation regulation (Takahashi and Kawashima, 2009; Gasc and Baulieu, 1988). Additionally, E2 injection in laying hens resulted in increased binding affinity of P4 in the oviduct, indicating that E2 regulates the action of other sex steroid hormones (Kawashima et al., 1996).

In the hypothalamus and pituitary, LEPH showed higher mRNA levels for *GNIH, GNIHR1,* and *GNIHR2,* as well as lower mRNA levels for *FSHB* and *LHB*, both consistent with increased ovulation inhibition and decreased follicular stimulation. Studies examining low and high prolific goat breeds found that *FSHB* and *LHB* expression was also up-regulated in high prolific breeds (Zi et al., 2013). LEPH also showed up-regulation of the P4 receptor in the hypothalamus during the PS relative to HEPH, whereas, HEPH showed up-regulation of E2 receptor, *ESR1*, in the hypothalamus and pituitary. Higher plasma E2 levels coupled with increased gene expression of E2 receptors in the hypothalamus and pituitary of HEPH suggests

that E2 feedback mechanisms may differ in LEPH and HEPH. Down-regulation of *PGR* in the hypothalamus and *GNRHR* in the pituitary during the PS was previously seen (Chapter 2); however, only HEPH exhibited down-regulation of both receptors during the PS, whereas LEPH showed no expression changes of the receptors during the PS. *PGR* and *GNRHR* stimulate the HPG axis and have been shown to decrease receptor binding and gene expression, respectively, during the PS in chickens (Kawashima et al., 1994; Lovell et al., 2005). Down-regulation of these receptors may serve to prime the HPG axis for the next ovulation to occur.

The F1 follicle is responsible for P4 production, which occurs in the granulosa cells. In the granulosa layer, HEPH showed higher basal mRNA levels of *STAR* and *CYP11A1*, indicating a greater capacity for P4 production. Movement of cholesterol from the outer mitochondrial membrane to the inner membrane by STAR is the rate-limiting step of steroidogenesis (Stocco, 2001). Higher expression of *STAR* in HEPH may allow for increased initiation of steroidogensis. Increased expression of *STAR* and *CYP11A1* in preovulatory follicles were also seen in high prolific goat breeds when compared to low prolific breeds (Zi et al., 2018). Both LEPH and HEPH up-regulated *STAR* during the PS, which is consistent with previous studies (Johnson et al., 2002); however, HEPH, but not LEPH, down-regulated *CYP11A1* during the PS, which was seen previously studies (Chapter 2). In the theca interna and theca externa layer of the F1 follicle, HEPH also showed up-regulation of genes involved in androgen and E2 production, such as *HSD3B1*, *CYP17A1*, and *CYP19A1* when compared to LEPH. Despite the priority of the F1 follicle being P4 production, theca interna and externa layers of the F1 follicle in HEPH may be contributing significantly to total androgen and E2 concentrations. At the transcript level, the F1 follicle of HEPH is more capable of steroidogenesis.

The F5 follicle is responsible for AD production, which occurs in the theca interna cells. In the theca interna layer, LEPH showed up-regulation of *HSD3B1*, *CYP17A1*, and *HSD17B1*, indicating a greater capacity for AD production. AD are necessary for normal reproductive function and have been shown to have positive and negative action on the HPG axis (Rangel and Gutierrez, 2014). Testosterone injections increased the number of internal ovulations in broiler breeders, ultimately decreasing egg production (Navara et al., 2015). On the other hand, testosterone treatment increased P4 production and related gene expression in chicken granulosa cells (Rangel et al., 2009). Interestingly, LEPH also showed up-regulation of all three genes involved in P4 production in the F5 granulosa layer when compared to HEPH. In the F5 theca externa layer, LEPH exhibited higher expression of CYP19A1, the key enzyme involved in E2 production, outside of the PS but inverse gene expression trends were seen during the PS. Overall, the F5 follicle of LEPH is more capable of steroidogenesis at the transcript level.

The SWF are responsible for the majority of E2 production. In the SWF cells, HEPH showed higher mRNA levels of *CYP17A1*, *HSD17B1*, and *CYP19A1*, indicating a greater capacity for E2 production in HEPH than in LEPH. In addition, to up-regulation of E2 production genes at the follicle level, plasma E2 levels and E2 receptor gene expression in the hypothalamus and pituitary were also increased in HEPH compared to LEPH. Declined levels of *CYP17A1* and *CYP19A1* expression in the SWF along with decreased plasma E2 levels have been associated with incubation behavior and follicle atresia in the turkey hen (Tabibzadeh et al., 1994). While signs of follicle atresia were not seen in present study, decreased egg production may exhibit molecular mechanisms similar to follicle atresia. LH receptor expression was also up-regulated in HEPH, both outside and during the PS, compared to levels in LEPH. Studies comparing low and high prolific sheep breeds also found up-regulation of *LHCGR* in early

development follicle from high producers (Abdennebi et al., 1999). Similar to the F1 follicle, HEPH displayed an increased ability for steroid production at the transcript level in the SWF.

In the current study, LEPH and HEPH exhibited clutch and pause length differences but did not exhibit differences in ovarian or oviduct morphology. Differences were not seen in plasma P4 levels but plasma E2 levels were higher in HEPH compared to LEPH. Gene expression differences were established in each tissue of the HPG axis, with LEPH and HEPH displaying different degrees of stimulation and inhibition in all of the tissues of the HPG axis at the mRNA level. Increased egg production was associated with mRNA levels consistent with increased ovulation stimulation, decreased ovulation inhibition, increased P4 synthesis in the F1 follicle granulosa layer, decreased AD synthesis in the F5 follicle theca interna layer, and increased E2 synthesis in the SWF. This study has provided novel insights into the interworkings of the HPG axis in turkey hens. The influence of egg production levels on HPG axis function has been defined through production, morphological and gene expression parameters and lays the foundation for future research to improve the reproductive efficiency of breeding hens in a meat focused industry.

CHAPTER 4

Differences in *in vitro* responses of the hypothalamo-pituitary-gonadal hormonal axis between low and high egg producing turkey hens

Abstract

Ovulation in birds is trigged by a preovulatory surge of LH and P4. Pituitary gonadotroph production of LH is stimulated by GnRH and inhibited by GnIH. Granulosa cells from the largest follicle (**F1G**) respond to LH to produce P4 while SWF respond to FSH to produce E2. LEPH ovulate less frequently than HEPH and exhibit differences in expression of mRNA for components of the HPG axis, suggesting differential responsiveness to trophic stimulation. To test this hypothesis, pituitary cells from LEPH and HEPH were subjected to both GnRH and GnIH treatment followed by expression analysis of mRNA levels for HPG axis genes related to ovulation. Additionally, F1G and SWF from LEPH and HEPH were subjected to LH and FSH treatment, respectively, followed by RIAs for P4 and E2 production. Results were analyzed by a two-way ANOVA using the mixed models procedure of SAS. In response to GnRH and GnIH treatment, HEPH pituitary cells showed up-regulation of genes associated with ovulation stimulation, whereas LEPH cells showed up-regulation of genes associated with inhibition of ovulation. HEPH F1G and SWF cells displayed a higher sensitivity and responsiveness to LH and FSH treatment, respectively. Level of egg production impacted ovulation-related gene expression in pituitary cells after neuropeptide treatment as well as steroid hormone production of F1G and SWF cells after gonadotropin treatment, with HEPH displaying a greater positive response to stimulation. These findings indicate that differences in egg production among turkey hens likely involve differential responsiveness of the cells within the HPG axis.

INTRODUCTION

Differences in egg production rates among turkey hens in a flock result in LEPH and HEPH (Chapter 3). Low egg production in breeding hens costs the industry in lost poult production and is correlated with decreased ovulation frequency (Liu et al., 2005). Follicle

ovulation in avian species is controlled by the HPG axis, which is composed of the hypothalamus, pituitary, and a single ovary. A PS precedes each ovulation and consists of increased P4 and LH, produced by the ovary and pituitary, respectively (Paster, 1991). Steroid hormones, E2 and P4, feedback on the HPG axis to regulate ovulation timing (Ottinger and Bakst, 1995).

The HPG axis can be stimulated by GnRH or inhibited by GnIH, both produced in the hypothalamus with the anterior pituitary as their target tissue. Neuron terminals containing GnRH extend into the external layer of the ME for neuropeptide release into the hypophysial portal vascular system (Bédécarrats, 2015). Neuron terminals containing GnIH also extend into the ME but also have direct contact with GnRH neurons, suggesting the capability of GnIH regulation of GnRH synthesis and release (Bédécarrats et al., 2016). GnRH or GnIH regulate pituitary gonadotropin production by binding to either GNRHR or GNIHR, both located on pituitary gonadotrophs. GNRHR and GNIHR are GPCRs present on pituitary gonadotroph cells. GNRHR has been shown to couple to $G_{\alpha s}$ and $G_{\alpha q}$ whereas GNIHR has been shown to couple to $G_{\alpha i}$ (Tsutsui et al., 2006). There are two receptors for GnIH, however GNIHR1 is considered the primary receptor and GNIHR2 will not be examined in this study.

The ovary is composed of follicles in varying states of maturation, developing from quiescent primordial follicles to preovulatory follicles awaiting ovulation. Steroidogenesis occurs in ovarian follicles, with primary steroid production varying with follicle development and with follicle cell type (Porter et al., 1989). The majority of the ovarian E2 production occurs in the SWF, which are slow growing follicles that have yet to enter the preovulatory hierarchy (Johnson, 1992). Ovarian P4 production primarily occurs in the F1G, which is the next follicle in line to ovulate (Bahr et al., 2005). SWF are mainly responsive to FSH, while F1G are

responsive to LH. FSHR and LHCGR are also GPCRs that couple to $G_{\alpha s}$ to increase the transcription of genes involved in steroidogenesis through the cAMP-signaling pathway, such as STAR and CYP19A1 (Li et al., 2014).

Previous studies comparing HPG axis gene expression of LEPH and HEPH found that HEPH displayed gene expression levels consistent with increased ovulation stimulation and decreased ovulation inhibition in the hypothalamus and pituitary. Additionally, HEPH showed upregulation of genes related to P4 production in the F1G and related to E2 production in SWF (Chapter 2). HEPH showed decreased gene expression of *GNIH* in the hypothalamus, increased expression of both *LHB* and *FSHB* in the pituitary, increased gene expression of *STAR* and *CYP11A1* in the F1G, and increased gene expression of *HSD17B1* and *CYP19A1* in the SWF (Chapter 2).

Based on the existing gene expression differences between LEPH and HEPH, it was hypothesized that HEPH would show an increased sensitivity and responsiveness to GnRH and GnIH treatment in the pituitary, to LH treatment in the F1G, and to FSH treatment in the SWF. This study sought to compare LEPH and HEPH in the responsiveness of isolated pituitary cells to GnRH and GnIH stimulation as well as in the responsiveness of the two follicle cell types responsible for estradiol and progesterone production, SWF and F1G, to FSH and LH stimulation, respectively. Based on previous studies, LEPH were hypothesized to be more responsive to GnIH stimulation while HEPH were hypothesized to be more responsive to GnRH, FSH, and LH stimulation.

Materials and Methods

Hen Selection

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual wire cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards. Hens were sampled at 35 weeks of age. Daily egg records were used to calculate each hen's number of EPD by dividing the total number of eggs produced by the number of days in production. Hens were classified as LEPH when EPD<0.6 and as HEPH when EPD>0.8. Blood samples were taken from the wing vein immediately before sampling and fractionated by centrifugation. Plasma samples were stored at -20°C prior to assessment through RIAs as described below. The pituitary, F1 follicle, and SWF were isolated from four LEPH and four HEPH. All hens were sampled outside of the PS and on the second day of the hen's sequence. Plasma P4 levels were examined to confirm correct sampling time during the ovulatory cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland.

Cell Isolation and Culture

All cell isolation procedures were performed using SMEM or Dulbecco's Modified Eagle Medium (**DMEM**) as noted below. Media was supplemented with 0.1% bovine serum albumen, 100-U/mL penicillin G, and 100-µg/mL streptomycin sulfate (**0.1% BSA and P/S**).

Isolated pituitaries were dispersed in SMEM (0.1% BSA and P/S) using trypsin and collagenase (1 mg/mL of each). After dispersion, cells were filtered through 70 µm nylon mesh and washed twice with DMEM (0.1% BSA and P/S). Cells were diluted to a concentration of 200,000 cells/mL and plated in serum free medium, Dulbecco's Modified Eagle Medium:

Nutrient Mixture F-12 (**DMEM/F12**), supplemented with 0.1% bovine serum albumen, 5-μg/mL human insulin, 100-U/mL penicillin G, and 100-μg/mL streptomycin sulfate. Cells were plated in 24 well poly-L lysine coated plates (Corning Life Sciences, Lowell, MA) at 100,000 cells/well and were allowed to attach for 2 hours before treatment. Pituitary cells were treated with chicken GnRH or GnIH (Phoenix Pharmaceuticals, Burlingame, CA) at basal, 10^{-9}, 10^{-8}, or 10^{-7} M for 6 or 24 hours.

The F1 follicle was removed from the ovary and placed in ice cold SMEM (0.1% BSA and P/S) until isolation of the granulosa cell layer. The follicle was drained of yolk, inverted, and the granulosa cell layer was peeled off of the follicle wall. The granulosa cell layer was dispersed in SMEM (0.1% BSA and P/S) using trypsin (1 mg/mL) as previously described (Chapter 2). After dispersion, cells were filtered through 70 μm nylon mesh and layered on a 50% Percoll solution to remove remaining yolk particles. Cells were washed twice with SMEM (0.1% BSA and P/S) and diluted to a density of 10,000 cells/mL for culture. F1G cells were cultured in SMEM (0.1% BSA and P/S) in 12x75 mm polypropylene tubes ($1x10^5$ cells per tube). Cells were treated with ovine LH (National Hormone & Peptide Program, Torrance, CA) at 0, 1, 10, 100, or 1000 ng/mL for 5 hours.

SWF were minced and dispersed in SMEM (0.1% BSA and P/S) using trypsin (1 mg/mL). After dispersion, cells were filtered through 70 μm nylon mesh and layered on a 50% Percoll solution to remove remaining red blood cells. SWF cells were washed twice with SMEM (0.1% BSA and P/S) and diluted to a density of 10,000 cells/mL for culture. SWF cells were cultured in SMEM (0.1% BSA and P/S) in 12x75 mm polypropylene tubes ($1x10^5$ cells per tube). Cells were treated with porcine FSH (National Hormone & Peptide Program, Torrance, CA) at 0, 1, 10, 100, or 1000 ng/mL for 5 hours.

Cells were maintained in a 37.5°C, 5% CO_2 atmosphere during incubation. Pituitary cells were harvested at the completion of each incubation by retrypsinization, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. The media from the F1G and SWF cell cultures was recovered and stored at -20 °C for P4 and E2 RIAs, respectively.

RT-qPCR

Total RNA was isolated from pituitary cell cultures with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA, RT, and RT-qPCR were performed as previously described (Chapter 2) with the following exception. Reverse transcription reactions were performed on 50 ng total RNA with SuperScript III (Thermo Fisher Scientific, Waltham, MA) and an anchored oligo-dT primer (5′-CGGAATTCTTTTTTTTTTTTTTTTTTTTV-3′) (Integrated DNA Technologies, Skokie, IL). A pool of total RNA was made and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. Reactions were diluted to 40 µl prior to PCR analysis. PCR reactions (15 µL) were carried out as previously described using a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA) (Chapter 2). Data were normalized to *PGK1* and analyzed by the $2^{-\Delta\Delta Ct}$ method, as described previously (Chapter 2). All PCR reactions for each gene in a given tissue were analyzed in a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates. Primers (Integrated DNA Technologies, Skokie, IL) for turkey *PGK1*, *GNRHR*, *GNIHR* (specifically *GNIHR1*), *LHB*, *FSHB*, and *CGA* mRNA were designed and used with cycling parameters described previously (Chapter 2). Data are presented as fold increase over levels in basal cells for each hormone treatment and time point.

RIAs

The RIAs used for P4 and E2 were coated tube kits (MP Biomedicals, Solon, OH). All protocols were performed as directed by the supplier. All samples were assayed in duplicate. All samples were measured in a single RIA for each hormone. Plasma samples were ether extracted and analyzed for progesterone to determine that hens were sampled outside of the preovulatory surge. Culture media from the F1G and SWF cell cultures were assayed for P4 and E2 content, respectively. The standard curve was assessed for linearity as well as dilutional parallelism using serial plasma or culture media dilutions. The intraassay coefficients of variation determined by pools run every 30 samples were 5.61% for P4 and 6.63% for E2.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data were $\log_2$ transformed before statistical analysis. A three-way ANOVA using the mixed models procedure was conducted to compare the $\log_2$ transformed pituitary gene expression between LEPH and HEPH. A two-way ANOVA using the mixed models procedure was used to compare plasma hormone concentrations and culture media hormone concentrations between LEPH and HEPH. The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$.

Results

GnRH Treatment of Pituitary Cells

GNRHR expression in response to GnRH treatment is presented in **Figure 4.1a**. After GnRH treatment for 6 hours, pituitary cells from HEPH showed higher *GNRHR* expression relative to cells from LEPH at lower GnRH concentrations, while cells from LEPH showed higher *GNRHR* expression relative to cells from HEPH at higher GnRH concentrations. Cells

from LEPH showed increased *GNRHR* expression relative to basal (0 M GnRH) expression in response to GnRH treatment only at the highest GnRH concentration. Cells from HEPH showed increased *GNRHR* expression relative to basal expression in response to GnRH treatment only at 10^{-8} M. *GNRHR* expression was not affected by GnRH treatment for 24 hours.

GNIHR expression in response to GnRH treatment is presented in **Figure 4.1b**. After GnRH treatment for 6 hours, *GNIHR* expression was significantly affected by egg production level but a response to GnRH treatment was not seen. After GnRH treatment for 24 hours, pituitary cells from LEPH showed higher *GNIHR* expression relative to cells from HEPH under basal conditions. Cells from LEPH showed decreased *GNIHR* expression relative to basal expression in response to all GnRH treatments. Cells from HEPH did not show a response in *GNIHR* expression to GnRH treatment.

LHB expression in response to GnRH treatment is presented in **Figure 4.1c**. After GnRH treatment for 6 hours, pituitary cells from HEPH showed higher *LHB* expression relative to cells from LEPH at all GnRH treatment concentrations. Cells from LEPH did not show a response in *LHB* expression after GnRH treatment for the concentrations examined. Cells from HEPH showed increased *LHB* expression relative to basal (0 M GnRH) expression in response to GnRH treatment at 10^{-8} M, but decreased expression relative to basal expression at 10^{-7} M GnRH. After GnRH treatment for 24 hours, pituitary cells from HEPH showed higher *LHB* expression relative to cells from LEPH at all GnRH treatment concentrations. Rather than the expected increase in *LHB* mRNA levels in response to GnRH treatment, cells from LEPH showed decreased *LHB* expression after GnRH treatment at 10^{-9} M. On the other hand, cells from HEPH showed increased *LHB* expression relative to basal expression, as would be expected following GnRH treatment.

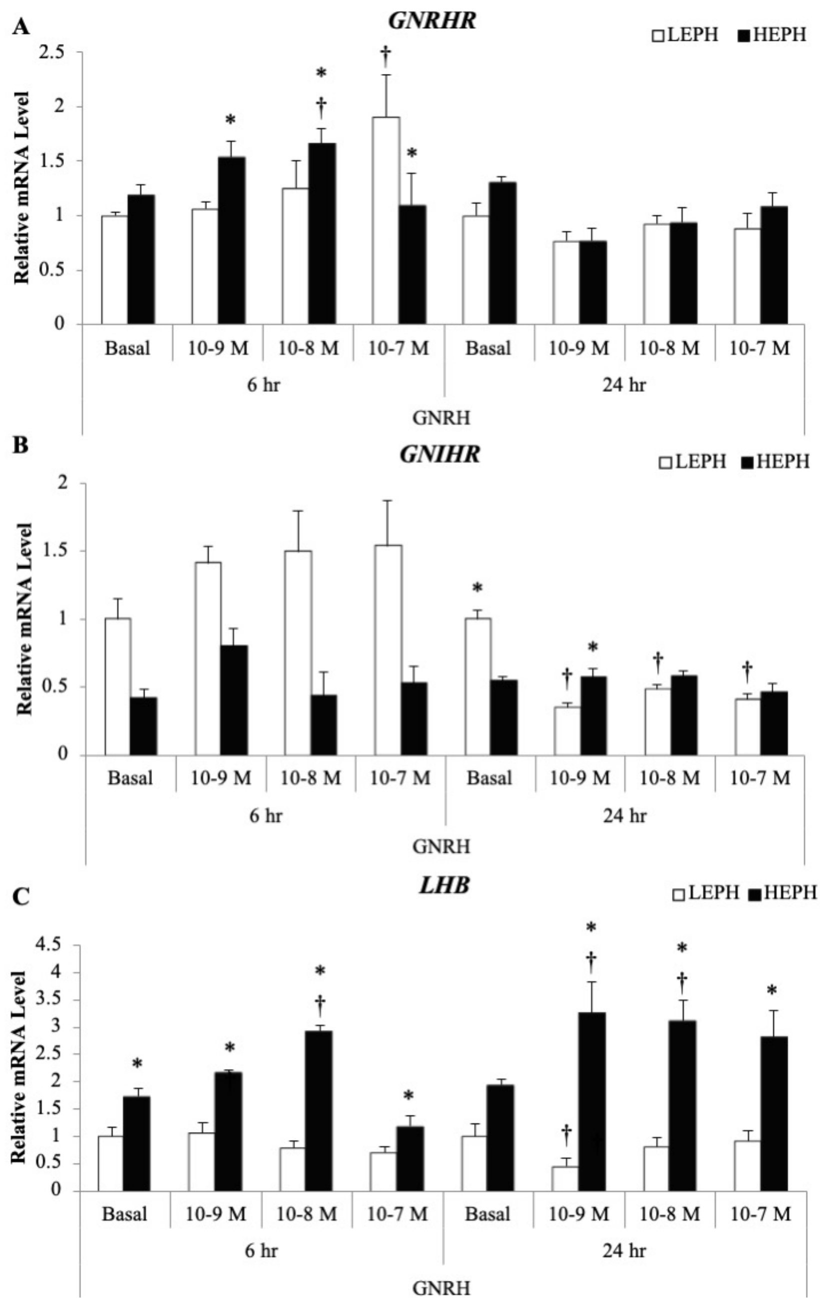


Figure 4.1. Relative pituitary expression of *GNRHR*, *GNIHR*, *LHB* after GnRH treatment in LEPH and HEPH. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and a specific GnRH treatment for a given egg production group are denoted with a dagger.

GnIH Treatment of Pituitary Cells

GNRHR expression in response to GnIH treatment is presented in **Figure 4.2a**. After GnIH treatment for 6 hours, pituitary cells from HEPH showed higher *GNRHR* expression relative to cells from LEPH at lower GnIH treatment concentrations, while cells from LEPH showed higher *GNRHR* expression relative to cells from HEPH at higher GnIH treatment concentrations. Cells from LEPH showed increased *GNRHR* expression relative to basal (0 M GnIH) expression only at the highest GnIH treatment concentration. Cells from HEPH showed increased *GNRHR* expression relative to basal expression in response to GnIH treatment at 10^{-9} and 10^{-8} M, before returning to basal expression. After GnIH treatment for 24 hours, pituitary cells from HEPH showed higher *GNRHR* expression relative to cells from LEPH under basal conditions, while cells from LEPH showed higher *GNRHR* expression relative to cells from HEPH only at the highest GnIH treatment concentration. Cells from LEPH showed increased *GNRHR* expression relative to basal expression in response to all the GnIH concentrations.

GNIHR expression in response to GnIH treatment is presented in **Figure 4.2b.** After GnIH treatment for 6 hours, pituitary cells from LEPH showed higher *GNIHR* expression relative to cells from HEPH at all but the highest GnIH treatment concentration. Cells from LEPH showed increased *GNIHR* expression relative to basal expression in response to GnIH treatment at 10^{-9} and 10^{-8} M, before returning to basal expression. Cells from HEPH showed increased *GNIHR* expression relative to basal expression only at the highest GnIH treatment concentration. After GnIH treatment for 24 hours, pituitary cells from LEPH showed higher *GNIHR* expression relative to cells from HEPH at all but the highest GnIH treatment concentration. Cells from LEPH showed decreased *GNIHR* expression relative to basal expression in response to higher concentrations of GnIH. Cells from HEPH showed decreased

GNIHR expression relative to basal expression before returning to basal expression in response to GnIH treatment at 10^{-9} M.

LHB expression in response to GnIH treatment is presented in **Figure 4.2c**. After GnIH treatment for 6 hours, pituitary cells from HEPH showed higher *LHB* expression relative to cells from LEPH at lower concentrations of GnIH, while LEPH showed higher *LHB* expression at the highest treatment concentration of GnIH. Cells from LEPH showed decreased *LHB* expression relative to basal expression in response to lower concentrations of GnIH, before returning to basal expression. Cells from HEPH showed increased *LHB* expression relative to basal expression in response to lower concentrations of GnIH, before reducing expression at 10^{-7} M GnIH. After GnIH treatment for 24 hours, pituitary cells from HEPH showed increased *LHB* expression relative to cells from LEPH at all treatment concentrations of GnIH. Cells from LEPH showed decreased *LHB* expression relative to basal expression in response to GnIH treatment at lower GnIH concentrations before returning to expression levels consistent with those seen under basal conditions. Cells from HEPH showed increased *LHB* expression relative to basal expression in response to all GnIH treatments.

FSHB and *CGA* expression was not affected by GnRH or GnIH treatment for 6 or 24 hours, and results are not presented.

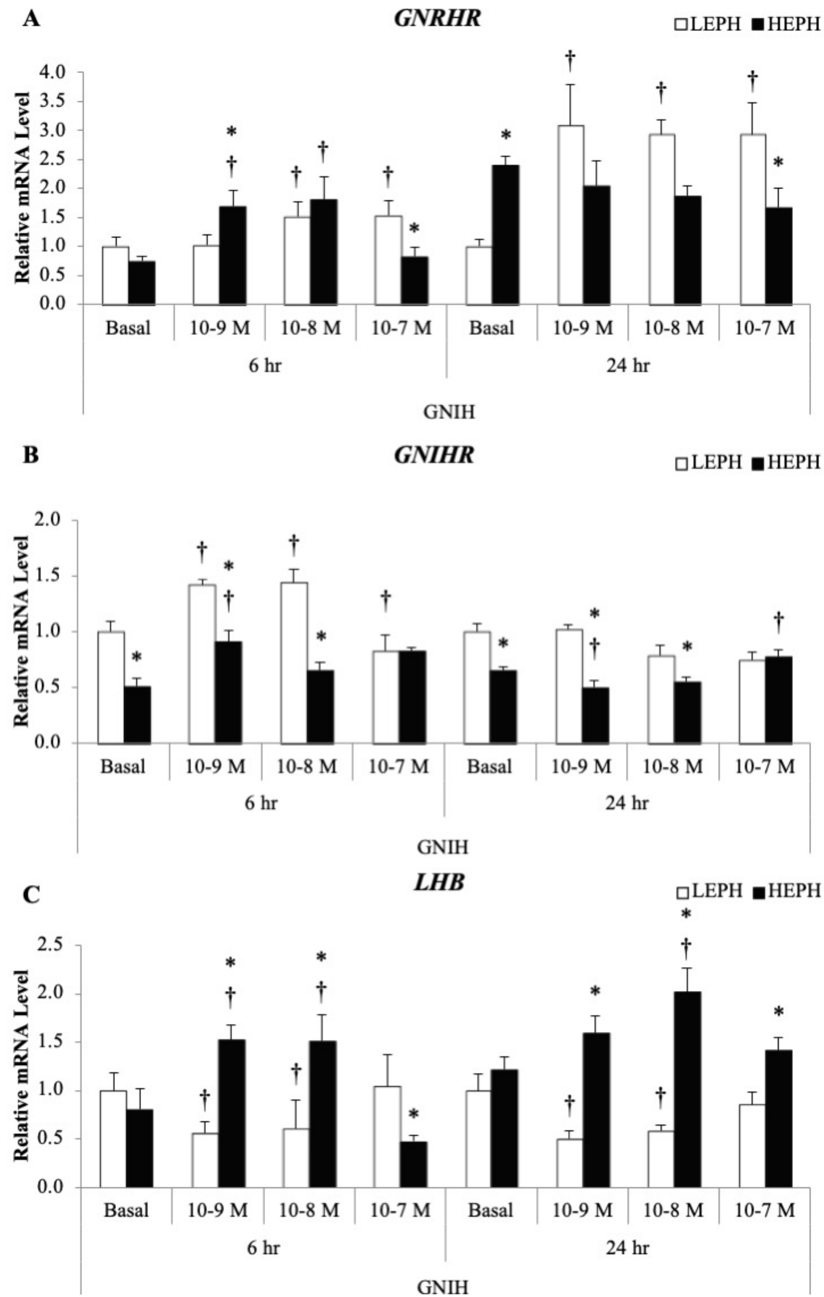


Figure 4.2 Relative pituitary expression of *GNRHR*, *GNIHR*, and *LHB* after GnIH treatment in LEPH and HEPH. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and a specific GNRH treatment for a given egg production group are denoted with a dagger.

LH Treatment of F1G Cells

F1G cell P4 production in response to LH treatment is presented in **Figure 4.3**. Basal P4 production from F1G cells from LEPH and HEPH did not differ significantly. F1G cells from HEPH responded to LH treatment at 10, 100, and 1000 ng/mL, whereas F1G cells from LEPH did not respond to LH treatment at the four experimental concentrations. P4 production differed significantly between F1G cells from LEPH and HEPH after treatment with 10, 100, and 1000 ng/mL of LH.

FSH Treatment of SWF Cells

SWF cell E2 production in response to FSH treatment is presented in **Figure 4.4**. Basal E2 production from SWF cells from LEPH and HEPH did not differ significantly. SWF cells from HEPH responded to FSH treatment at 10, 100, and 1000 ng/mL, whereas SWF cells from LEPH only responded to FSH treatment 100 and 1000 ng/mL. E2 production differed significantly between SWF cells from LEPH and HEPH after treatment with 10, 100, and 1000 ng/mL of FSH.

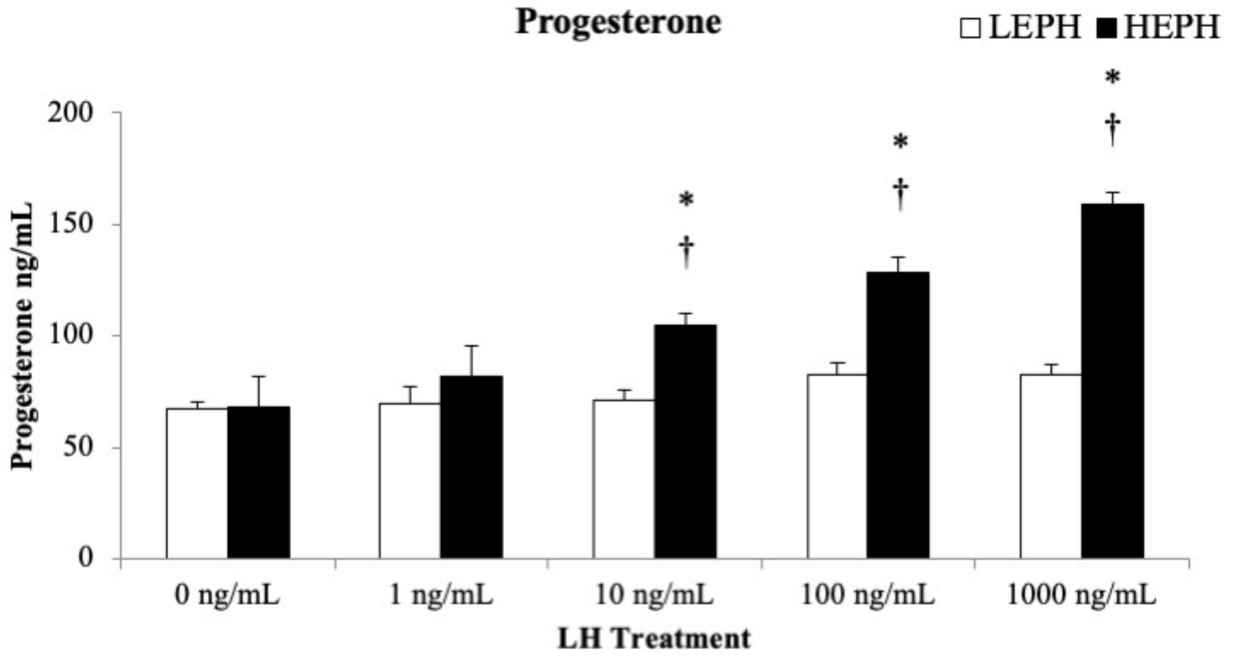


Figure 4.3 P4 production in F1G from LEPH and HEPH after LH treatment. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and a specific LH treatment for a given egg production group are denoted with a dagger.

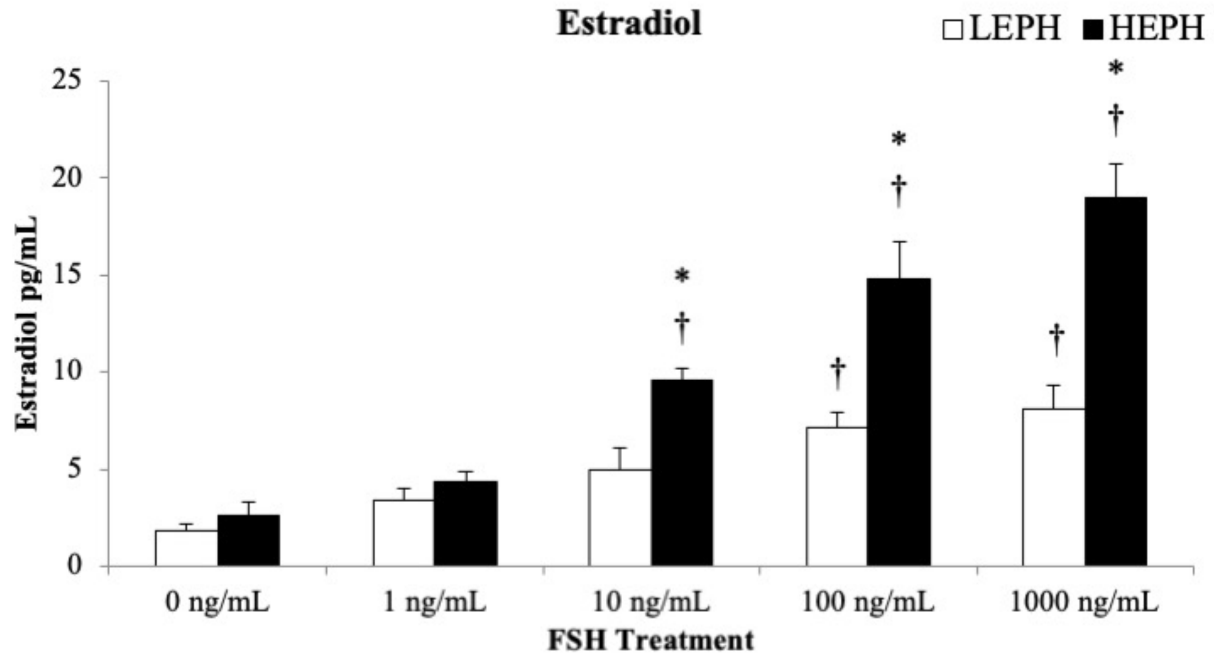


Figure 4.4 E2 production in SWF from LEPH and HEPH after FSH treatment. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and a specific FSH treatment for a given egg production group are denoted with a dagger.

Discussion

The current study showed that pituitary, F1G, and SWF cells from LEPH and HEPH respond differently to HPG axis hormone stimulation and inhibition. Previous studies have focused on HPG axis hormone responses in pituitary and ovarian cells during the initiation of egg production or during gonadal regression in both chicken and turkey hens (Guémené and Williams, 1999; Porter et al., 1991). Additionally, previous studies have compared ovarian response to gonadotropin stimulation in broiler and layer line chicken hens (Hocking and McCormack, 2004). This is the first study to examine HPG axis hormone response differences in hens with differential egg production from the same flock. Differences in *in vitro* responses to stimulation and inhibition coupled with previously identified HPG axis gene expression differences suggest core differences in the regulation of HPG axis function between LEPH and HEPH.

GnRH treatment positively impacted the expression of *GNRHR*, *GNIHR*, and *LHB* in the pituitary cells from HEPH. Receptor gene expression changes occurred during short-term GnRH treatment, while *LHB* gene expression changes were seen during short-term and long-term GnRH treatment. Cells from LEPH displayed minimal increased expression of *GNRHR*, decreased expression of *GNIHR*, and no changes in expression of *LHB* in response to GnRH treatment. Expression of *GNRHR* and *GNIHR* after GnRH treatment has not been previously examined in avian species; however, studies in a mammalian gonadotroph cell line showed up-regulation of *GNRHR* and *GNIHR* following GnRH treatment (Turgeon et al., 2014; Sukhbaatar et al., 2014). Injection of GnRH in chickens resulted in increased plasma LH in previous studies (Wilson et al., 1989). While the results from the current study examine the transcriptional changes due to GnRH treatment, the increased *LHB* expression is consistent with studies at the

protein level. *LHB* expression decreased to basal levels at the highest short-term GnRH treatment; Similar desensitization has been shown in prior studies (King et al., 1986). *FSHB* expression was not affected by GnRH treatment in LEPH or HEPH, which is consistent with prior studies (Proudman et al., 2006).

While GnIH treatment up-regulated *GNRHR* expression in both groups of hens, *GNIHR* expression was only up-regulated in cells from LEPH, and *LHB* expression was only up-regulated in cells from HEPH following GnIH treatment. *In vitro*, activation of *GNIHR* through GnIH binding has been shown to reduce *GNRHR* gene expression in chicken pituitary cells (Bédécarrats et al., 2009). Cells from LEPH and HEPH initially displayed up-regulation of *GNRHR* after GnIH treatment, though long-term treatment resulted in a further increase in *GNRHR* expression in cells from LEPH and in mRNA levels similar to basal expression in cells from HEPH. GnIH treatment has been shown to increase *GNIHR* expression in the chicken (Maddineni et al., 2008). Cells from LEPH and HEPH both showed short-term up-regulation of *GNIHR* after GnIH treatment. However, cells from LEPH displayed higher overall mRNA levels of *GNIHR* for each GnIH treatment. GnIH has also been shown to decrease LH synthesis and release in the chicken, both *in vivo* and *in vitro* (Bédécarrats et al., 2009). This phenomenon was seen in cells from LEPH after GnIH treatment but up-regulation in response to GnIH treatment was seen in cells from HEPH. This may indicate that GnIH signaling in LEPH and HEPH operates differently to control gonadotropin production. Studies in mammalian gonadotroph cell lines indicated that GnIH treatment decreased the expression of *FSHB* and *CGA*, both of which were not seen in the current study (Son et al., 2012). Differences in GnIH regulation may be attributed to the reproductive physiology differences between mammalian and avian species.

A dose-dependent P4 production response to LH treatment was seen in F1G from HEPH, whereas cells from LEPH did not respond to LH treatment in terms of P4 production. Results seen in HEPH cells are consistent with previously published studies in chicken and turkey hens (Bakst et al., 1983; Porter et al., 1991). P4 production from the F1G layer is imperative for the PS to occur and induced ovulation. Lack of response to LH stimulation in F1G cells from LEPH may contribute to decreased ovulation rates seen in this group of hens. An E2 production response to FSH treatment was seen in SWF cells from LEPH and HEPH; however, SWF cells from HEPH responded at a lower dose of FSH treatment and responded with significantly higher E2 production when compared to cells from LEPH. The SWF are the follicle cell type with the greatest FSH binding. Results seen in HEPH were also consistent with previous studies in chicken and turkey SWF (Etches and Cheng, 1981; Porter et al., 1989). Furthermore, F1G and SWF results from the current study are consistent with previous results showing up-regulation of genes in cells from HEPH when compared to cells from LEPH that are involved in P4 and E2 production (Chapter 3). Follicles from LEPH and HEPH appear to respond differently to gonadotropin stimulation, ultimately impacting the steroid hormone production capabilities in these groups of hens.

In summary, HEPH displayed increased responsiveness to GnRH in pituitary cells, to LH in F1G cells, and to FSH in SWF cells. On the other hand, pituitary cells from LEPH displayed increased responsiveness to the inhibitory properties of GnIH, whereas pituitary cells from HEPH responded positively to GnIH treatment. These findings demonstrate that HPG axis responsiveness is different in LEPH and HEPH, with LEPH favoring the inhibitory pathways of the axis and HEPH favoring the stimulatory pathways of the axis. Understanding the impact that

HPG axis hormone responsiveness plays in egg production level would be imperative to improve the reproductive efficiency of LEPH in the turkey industry.

CHAPTER 5

Transcriptome analysis of the hypothalamus and pituitary of turkey hens with low and high egg production

Abstract

Background: HEPH show increased hypothalamic and pituitary gene expression related to HPG axis stimulation as well as increased *in vitro* responsiveness to GnRH stimulation in the pituitary when compared to LEPH. Transcriptome analysis was performed on hypothalamus and pituitary samples from LEPH and HEPH to identify novel regulators of HPG axis function.

Results: In the hypothalamus and pituitary, 4644 differentially expressed genes (**DEGs**) were identified between LEPH and HEPH, with 2021 genes up-regulated in LEPH and 2623 genes up-regulated in HEPH. In LEPH, up-regulated genes showed enrichment of the HPT axis. Beta-estradiol was identified as an upstream regulator regardless of egg production level, timing of the ovulatory cycle, and tissue. When LEPH and HEPH samples were compared, beta-estradiol was activated in HEPH in 3 of the 4 comparisons, which correlated to the number of beta-estradiol target genes up-regulated in HEPH. In *in vitro* pituitary cell cultures from LEPH and HEPH, T3 pretreatment negatively impacted gonadotropin subunit mRNA levels in cells from both LEPH and HEPH, with the effect being more prominent in HEPH cells. Additionally, the effect of E2 pretreatment on gonadotropin subunit mRNA levels in HEPH cells was negative, whereas E2 pretreatment increased gonadotropin subunit mRNA levels in LEPH cells.

Conclusions: Up-regulation of the HPT axis in LEPH and upstream beta-estradiol activation in HEPH may play a role in regulating HPG axis function, and ultimately ovulation rates. Furthermore, T3 and E2 pretreatment impacted gonadotropin mRNA levels following GnRH stimulation, with the inhibitory effects of T3 being more detrimental in HEPH and E2 stimulatory effects being more prominent in LEPH. Differential responsiveness to T3 and E2 pretreatment may be due to desensitization of target genes to T3 and E2 in LEPH and HEPH, respectively, in response general up-regulation of the HPT axis in LEPH and of the HPG axis in

HEPH. Further studies will be necessary to identify possible target gene desensitization mechanisms and elicit the full role that the HPT axis and beta-estradiol upstream regulation play in egg production rates in turkey hens.

Background

Egg production within the turkey industry is necessary for hatching poults for meat production and has be negatively impacted by intensive selection for body weight traits. Egg production varies within commercial flocks, with LEPH being more expensive per egg produced than HEPH. At the neuroendocrine level, egg production is regulated by the HPG axis. Proper function of the HPG axis involves gonadal steroid hormone feedback loop mechanisms and can be impacted by inputs from other neuroendocrine axes, such as the HPT axis.

Within the HPG axis, feedback mechanisms of P4 and E2 are instrumental for follicle ovulation to occur. P4 feedback on the hypothalamus and pituitary triggers a PS of LH and P4, resulting in follicle ovulation, but the role of E2 feedback during the PS is not well characterized in the turkey hen. In the chicken, E2 reduces GnIH production and exerts positive and negative feedback on GnRH production in the hypothalamus, indicating that E2 feedback may play a role in ovulation timing (Ubuka et al., 2013; Li et al., 1994).

In addition to the HPG axis, proper function of the HPT axis is necessary for egg production to occur. The full impact of the HPT axis on reproductive function is not well understood, but studies have shown that increased activity of the HPT axis is associated with gonadal regression (Lien and Siopes, 1989b). On the other hand, studies have shown that HPT axis activity is necessary for the initiation of egg production (Lien and Siopes, 1989a). Additional studies examined the role of the HPT axis in the regulation of reproductive cycles in seasonally reproductive species, however, the HPT axis has not been characterized in

commercial chicken or turkeys during peak egg production and has not been examined in regard to the regulation of the PS (McNabb, 2007b).

LEPH and HEPH exhibited differential expression of genes within the HPG axis, with LEPH showing higher mRNA levels for genes involved in ovulation inhibition and HEPH showing higher mRNA levels for genes involved in ovulation stimulation (Chapter 3). Furthermore, during *in vitro* culture of isolated pituitary cells, LEPH displayed an increased responsiveness to GnIH treatment, whereas HEPH displayed an increased responsiveness to GnRH treatment (Chapter 4). To further understand the mechanisms regulating the differential gene expression and *in vitro* responses seen in these two groups of hens, transcriptome analysis was performed in the hypothalamus and pituitary of LEPH and HEPH, both under basal conditions (outside of the PS) and during HPG axis stimulation (during the PS).

Results and Discussion

Transcriptome Alignment and Mapping

A total of 852,343,043 sequence reads were obtained from the hypothalamus and pituitary, with an average of 35,514,293 reads per sample (**Supplemental Figure 5.1A**). A significantly higher number of reads were obtained from the pituitary samples when compared to the hypothalamus samples, but no differences in the percentage of reads mapped to the turkey genome were seen. On average, 79.9% of reads mapped to the turkey reference genome (Ensembl Turkey_2.01). For each sample, read pairs were aligned with minimal discordant pairs or pairs with multiple alignments (average of 0.58% and 2.29% respectively) (**Supplemental Figure 5.1B**). The number of reads per sample, the number of mapped reads per sample, and the number of properly aligned pairs per sample did not differ significantly between egg production or ovulatory cycle groups in either of the tissue examined.

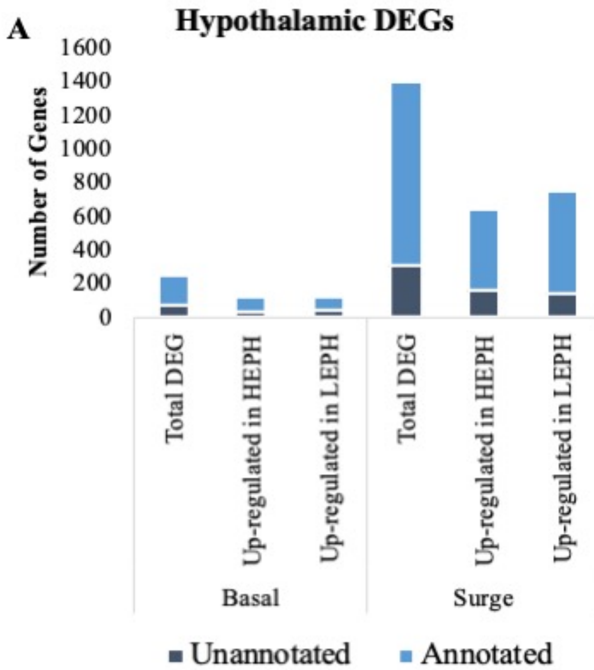
Overview of DEGs

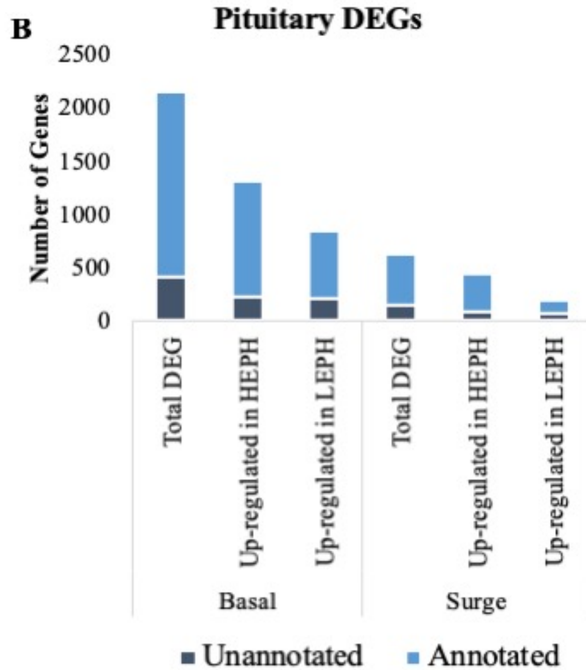
A total of 1641 and 2778 DEGs were identified in the hypothalamus and pituitary, respectively. Analysis of the genes differentially expressed between LEPH and HEPH revealed a significantly higher number of DEGs in the hypothalamus during the PS and in the pituitary outside of the PS. In the hypothalamus, 248 DEGs were identified outside of the PS, whereas 1393 DEGs were identified during the PS (**Figure 5.1A**). The pituitary showed the opposite trend, with 2155 DEGs outside of the PS and 623 DEGs during the PS (**Figure 5.1B**). In the hypothalamus, equal numbers of genes were seen up-regulated in LEPH and HEPH outside of the PS, though a higher number of genes were up-regulated in LEPH during the PS. In the pituitary, both outside and during the PS, a higher number of genes were up-regulated in HEPH compared to LEPH. In the hypothalamus and pituitary, under both ovulatory cycle conditions, unannotated genes accounted for roughly 20-30% of the DEGs, indicating that further progress annotating the turkey genome may reveal additional genes involved in egg production rates or in triggering ovulation.

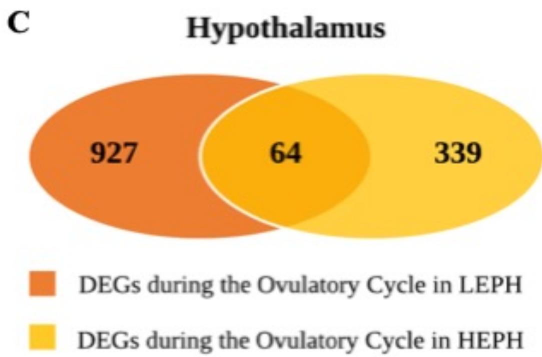
When comparing each hen group during the ovulatory cycle, LEPH displayed twice as many DEGs in the hypothalamus and pituitary between basal and PS conditions when compared to HEPH (**Figure 5.1C and 5.1D**). Of the genes differentially expressed in the hypothalamus during the ovulatory cycle, unannotated genes accounted for 26% of the DEGs unique to LEPH and 47% of the DEGs unique to HEPH. Lower fractions of unannotated DEGs were seen in the pituitary during the ovulatory cycle, with unannotated genes accounting for 21% of the DEGs unique to LEPH and 27% of the DEGs unique to HEPH. In total, LEPH and HEPH shared 64 genes in the hypothalamus and 210 genes in the pituitary that were differentially expressed

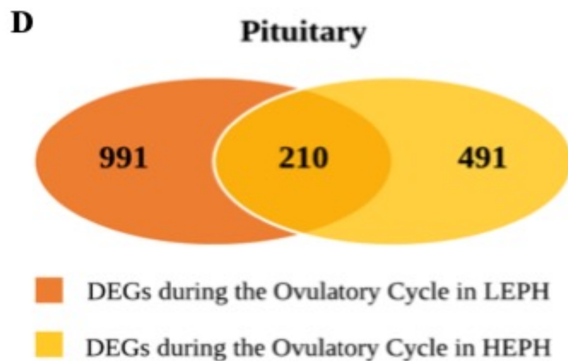
during the ovulatory cycle. Roughly one-fourth of the common DEGs in the hypothalamus and pituitary were unannotated as well.

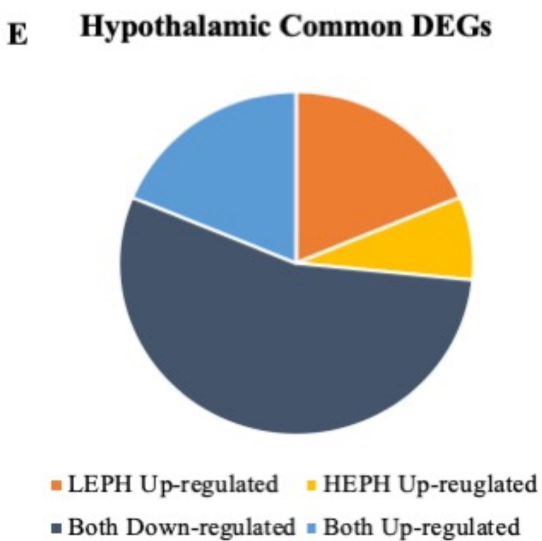
Of the DEGs common to both groups of hens during the ovulatory cycle, a majority showed similar expression patterns in LEPH and HEPH (73% of common DEGs in the hypothalamus and 93% of common DEGs in the pituitary) (**Figure 5.1E and 5.1F**). A larger percentage of the common DEGs showed down-regulation in both groups of hens in the hypothalamus and pituitary compared to the percentage of DEGs that showed up-regulation in both groups of hens. Among the genes in the hypothalamus showing similar expression patterns during the PS in both groups of hens was fatty acid 2-hydroxylase (***FA2H)*** and somatostatin (***SST***). *FA2H*, which was up-regulated in both groups of hens during the PS, is involved in myelin production, which is essential for proper nerve conduction (Jahn et al., 2009). *SST,* which was also up-regulated in both groups of hens during the PS, is the main inhibitory hormone of the somatropic axis but has been shown to inhibit GnRH neuron activity in mice (Bhattarai et al., 2010). Among the genes in the pituitary showing similar expression patterns during the PS in both groups of hens was pre-mRNA processing factor 19 (***PRPF19)***. *PRPF19*, which was down-regulated in both groups of hens during the PS, has been shown in mouse models to impact the splicing of gonadotropin subunits (Feng et al., 2008). Common DEGs with similar expression patterns during the ovulatory cycle in both LEPH and HEPH could indicate a potential role for these genes in the regulation of ovulation.


A
Hypothalamic DEGs
Number of Genes
1600
1400
1200
1000
800
600
400
200
0
Total DEG
Up-regulated in HEPH
Up-regulated in LEPH
Total DEG
Up-regulated in HEPH
Up-regulated in LEPH
Basal
Surge
Unannotated
Annotated


B
Pituitary DEGs
Number of Genes
2500
2000
1500
1000
500
0
Total DEG
Up-regulated in HEPH
Up-regulated in LEPH
Total DEG
Up-regulated in HEPH
Up-regulated in LEPH
Basal
Surge
Unannotated
Annotated


C
Hypothalamus
927
64
339
DEGs during the Ovulatory Cycle in LEPH
DEGs during the Ovulatory Cycle in HEPH


D
Pituitary
991
210
491
DEGs during the Ovulatory Cycle in LEPH
DEGs during the Ovulatory Cycle in HEPH


E
Hypothalamic Common DEGs
LEPH Up-regulated
HEPH Up-reuglated
Both Down-regulated
Both Up-regulated

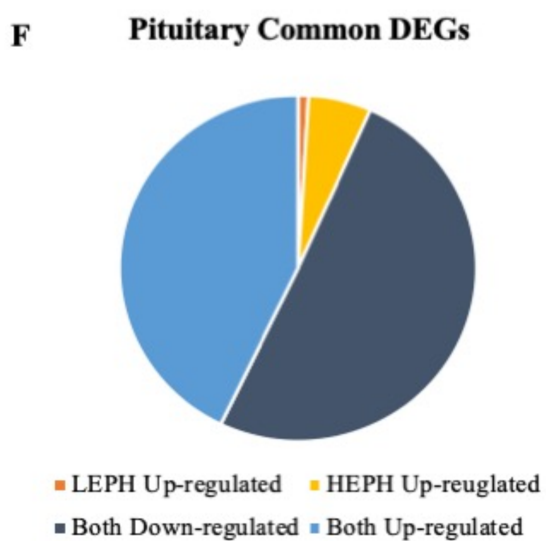

F
Pituitary Common DEGs
LEPH Up-regulated
HEPH Up-reuglated
Both Down-regulated
Both Up-regulated

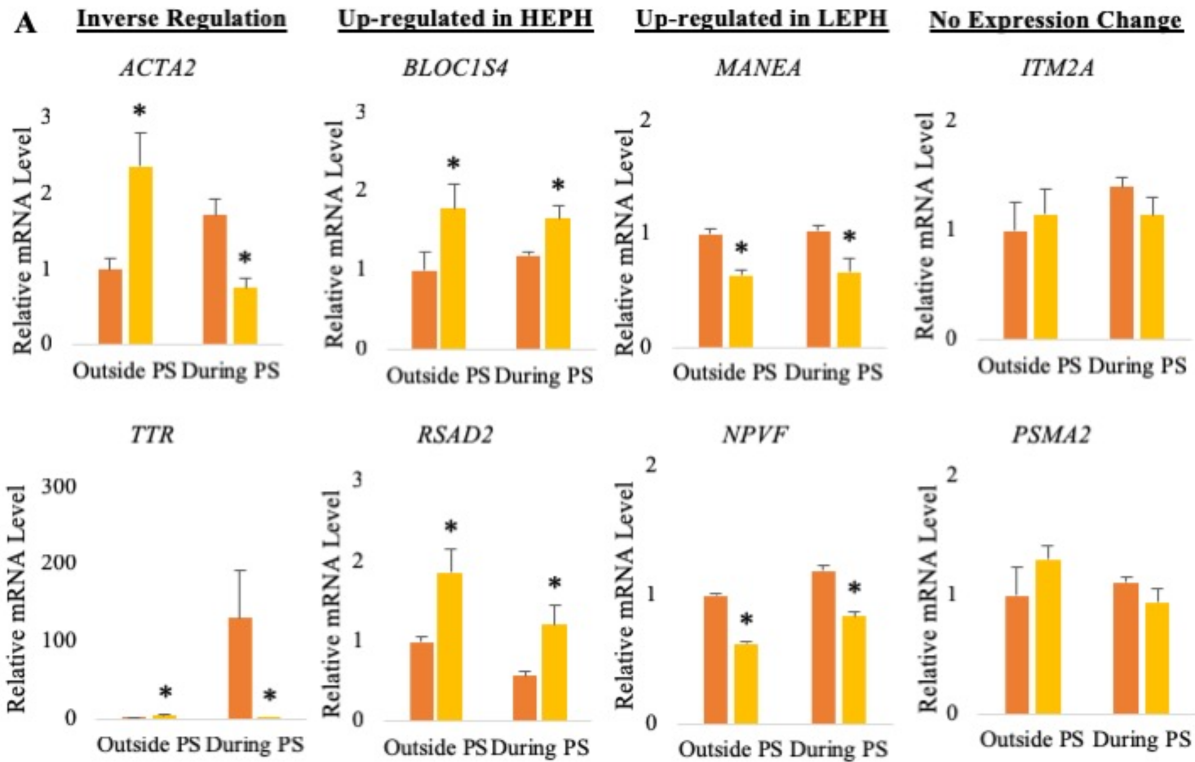
Figure 5.1. (A) Numbers of total, up-regulated in HEPH, and up-regulated in LEPH DEGs in the hypothalamus of LEPH and HEPH sampled outside (basal) and during (surge) the PS (RPKM>0.2, P<0.05). The portion of genes that are unannotated in the turkey genome are represented in dark blue and the portion of gene that are annotated in the turkey genome are represented in light blue. (B) Numbers of total, up-regulated in HEPH, and up-regulated in LEPH DEGs in the pituitary of LEPH and HEPH sampled outside (basal) and during (surge) the preovulatory surge (RPKM>0.2, P<0.05). The portion of genes that are unannotated in the turkey genome are represented in dark blue and the portion of gene that are annotated in the turkey genome are represented in light blue. (C) Venn diagram showing the number of DEGs in the hypothalamus during the ovulatory cycle unique to LEPH and HEPH as well as the number of DEGs during the ovulatory cycle common to both groups of hens (RPKM>0.2, P<0.05). (D) Venn diagram showing the number of DEGs in the pituitary during the ovulatory cycle unique to LEPH and HEPH as well as the number of DEGs during the ovulatory cycle common to both groups of hens (RPKM>0.2, P<0.05). (E) Common hypothalamic DEGs during the ovulatory cycle in both LEPH and HEPH broken down by expression pattern during the PS (RPKM>0.2, P<0.05). (F) Common pituitary DEGs during the ovulatory cycle in both LEPH and HEPH broken down by expression pattern during the PS (RPKM>0.2, P<0.05).

A small percentage of the common DEGs showed inverse expression patterns in LEPH and HEPH (27% of common DEGs in the hypothalamus and 7% of common DEGs in the pituitary). Of the hypothalamic common DEGs showing inverse expression patterns between LEPH and HEPH, proteasome 26S Subunit, Non-ATPase 2 (***PSMD2***) displayed up-regulation in HEPH and down-regulation in LEPH during the PS. In mice, mutations in *PSMD2* have been associated with decreased thyroid hormone production (McCabe and Dattani, 2014). Of the pituitary common DEGs showing inverse expression patterns between LEPH and HEPH, NADH dehydrogenase 4 (***ND4***) and cyclooxygenase-2 (***COX2***) have been previously associated with reproductive functions (Bertani et al., 2004; Lim et al., 1997). Both *ND4* and *COX2* showed up-regulation in HEPH and down-regulation in LEPH during the PS. Swine selected for high ovulation rates displayed higher pituitary *ND4* gene expression when compared to control lines (Bertani et al., 2004). *COX2* encodes the rate limiting enzyme in prostaglandin production, and deletion of *COX2* in mice results in decreased ovulation (Lim et al., 1997). Common DEGs during the ovulatory cycle with inverse expression patterns in LEPH and HEPH could signify a possible role in the regulation of egg production rates.

RNA Sequencing Confirmation

A total of 8 genes per tissue were confirmed through RT-qPCR. Confirmation genes were equally distributed to have one of four expression profiles: genes showing up-regulation in HEPH compared to LEPH (both outside and during the PS), genes showing up-regulation in LEPH compared to HEPH (both outside and during the PS), genes showing up-regulation in one hen group outside of the PS and up-regulation in the other hen group during the PS, and genes showing no changes in expression between hen groups (both outside and during the PS). Each of

the confirmation genes examined in the hypothalamus (**Figure 5.2A**) and pituitary (**Figure 5.2B**) showed expression profiles similar to those obtained through RNA sequencing.


A
Inverse Regulation
Up-regulated in HEPH
Up-regulated in LEPH
No Expression Change
ACTA2
BLOC1S4
MANEA
ITM2A
TTR
RSAD2
NPVF
PSMA2
Relative mRNA Level
Outside PS
During PS

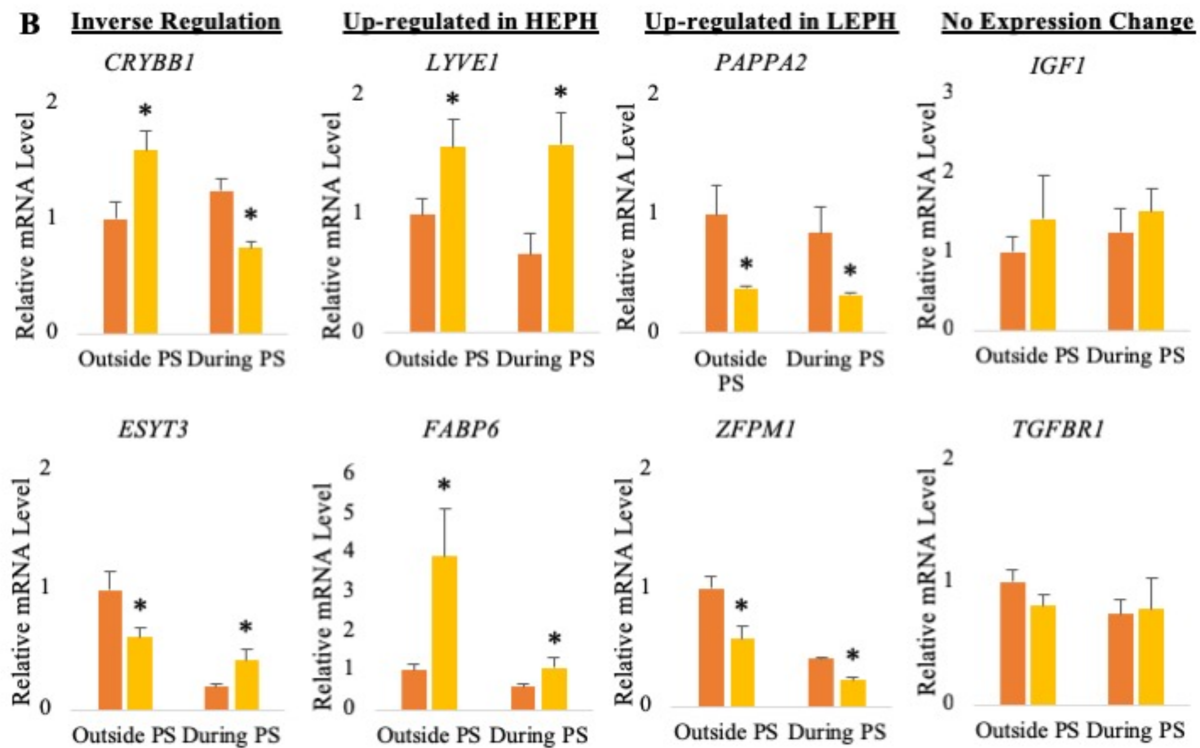

B
Inverse Regulation
Up-regulated in HEPH
Up-regulated in LEPH
No Expression Change
CRYBB1
LYVE1
PAPPA2
IGF1
ESYT3
FABP6
ZFPM1
TGFBR1
Relative mRNA Level
Outside PS
During PS

Figure 5.2 (A) Confirmation of hypothalamic RNA sequencing results. Six DEGs, with expression patterns that showed inverse regulation in LEPH and HEPH outside and during the PS [alpha-actin-2 (***ACTA2***) and transthyretin (***TTR***)], up-regulation in HEPH both outside and during the PS [biogenesis of lysosomal organelles complex-1, subunit 4 (***BLOC1S4***) and radical s-adenosyl methionine domain containing 2 (***RSAD2***)], and up-regulation in LEPH both outside and during the PS [mannosidase endo-alpha (***MANEA***) and neuropeptide VF precursor (***NPVF***)], as well as two genes that were not differentially expression in LEPH and HEPH either outside or during the PS [integral membrane protein 2A (***ITG2A***) and proteasome subunit alpha 2 (***PSMA2***)] were confirmed through RT-qPCR. Normalized data are presented relative to LEPH expression outside of the PS. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk (*). (B) Confirmation of pituitary RNA sequencing data. Six DEGs, with expression patterns that showed inverse regulation in LEPH and HEPH outside and during the PS [crystallin beta B1 (***CRYBB1***) and extended synaptotagmin 3 (***ESYT3***)], up-regulation in HEPH both outside and during the PS [lymphatic vessel endothelial hyaluronan receptor 1 (***LYVE1***) and fatty acid binding protein 6 (***FABP6***)], and up-regulation in LEPH both outside and during the PS [pappalysin 2 (***PAPPA2***) and zinc finger protein, FOG family member 1 (***ZFPM1***)], as well as two genes that were not differentially expression in LEPH and HEPH either outside or during the PS [insulin like growth factor 1 (***IGF1***) and transforming growth factor beta receptor 1 (***TGFBR1***)] were confirmed through RT-qPCR. Normalized data are presented relative to LEPH expression outside of the PS. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk (*).

Overview of Network Analysis

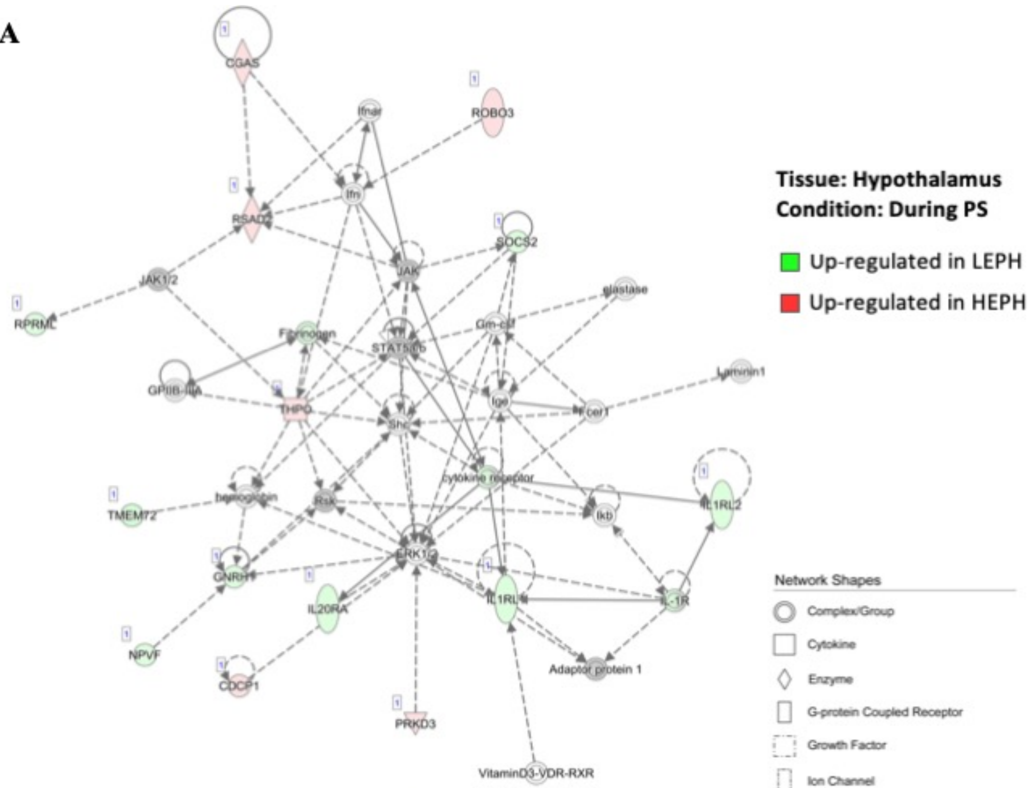
All DEGs between LEPH and HEPH with an absolute fold change greater than 1.5 and a P-value less than 0.05 were submitted for Ingenuity® Pathway Analysis (IPA) (RPKM>0.2). Hypothalamic transcriptome differences between LEPH and HEPH included 160 genes outside of the PS and 305 genes during the PS. Pituitary transcriptome differences between LEPH and HEPH included 1626 genes outside of the PS and 438 genes during the PS. IPA analysis of the DEGs revealed two common themes in the hypothalamus and pituitary: up-regulation of the HPG axis and estradiol signaling in HEPH and up-regulation of the HPT axis in LEPH.

The HPG Axis

In the hypothalamus during the PS, LEPH displayed up-regulation of genes associated with ovulation inhibition as well as an abnormal up-regulation of ovulation stimulation genes when compared to HEPH (**Figure 5.3A**). LEPH exhibited up-regulation of *NPVF* (also called *GNIH*), which encodes avian GNIH and of *GNRH*. GnIH negatively regulates the HPG axis to decrease gonadotropin production in the pituitary (Bédécarrats et al., 2009). Up-regulation of *NPVF* may play a role in reduced ovulation rates seen in LEPH. *GNRH* mRNA levels were previously shown to decrease during the PS in hens with average egg production, whereas in the present study, LEPH showed increased expression relative to HEPH (Chapter 2). In the same study, no expression changes in *NPVF* were seen during the PS in average egg producing hens, whereas in the present study, LEPH showed up-regulation of *NPVF*. Up-regulation of *GNRH* during the PS may prevent hormone levels from returning to basal levels, prolonging the interval between ovulations.

When comparing HEPH outside and during the PS, HEPH showed up-regulation of estrogen related receptor beta (***ESRRB)*** and down-regulation of FSH and LH during the PS

(**Figure 5.3B)**. Estrogen related receptors are ligand-dependent transcription factors capable of E2 binding. Though the function of estrogen related receptors in avian reproduction have not been characterized, function analysis of estrogen related receptors in knock-out mice and zebrafish models indicate that estrogen related receptors are essential for female reproduction (Lu et al., 2017). Decreased LH during the PS is consistent with decreased mRNA levels for *LHB* seen in AEPH during the PS (Chapter 2). Additionally, in this network, casein kinase 2 alpha 2 (***CSNK2A2)*** is down-regulated in the pituitary of HEPH during the PS. *CSNK2A2* encodes an uncharacterized protein in avian species but this protein was shown to be decreased in laying geese pituitaries when compared to non-laying geese, indicating a possible role in egg production or ovulation, (Luan et al., 2017).


A
CGAS
ROBO3
Ifnar
Ifn
RSAD2
SOCS2
JAK1/2
JAK
elastase
RPRML
Fibrinogen
STAT5a/b
Gm-csf
Laminin1
GPIIB-IIIA
THPO
Ige
Fcer1
Shc
cytokine receptor
IL1RL2
TMEM72
hemoglobin
Rsk
Ikb
ERK1/2
GNRH1
IL20RA
IL1RL1
IL-1R
NPVF
CDCP1
Adaptor protein 1
PRKD3
VitaminD3-VDR-RXR
Tissue: Hypothalamus
Condition: During PS
Up-regulated in LEPH
Up-regulated in HEPH
Network Shapes
Complex/Group
Cytokine
Enzyme
G-protein Coupled Receptor
Growth Factor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter

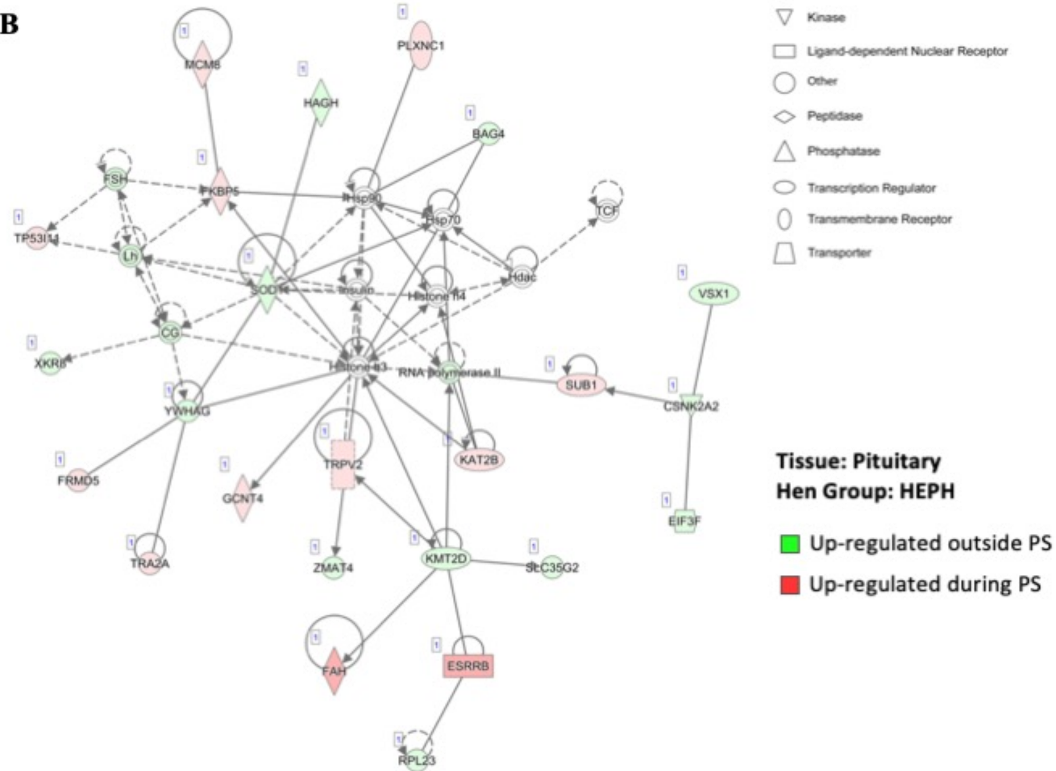

B
MCM8
PLXNC1
HAGH
BAG4
FSH
FKBP5
Hsp90
Hsp70
TCF
TP53I11
Lh
SOD1
Insulin
Histone h4
Hdac
VSX1
CG
XKR6
Histone h3
RNA polymerase II
SUB1
CSNK2A2
YWHAG
TRPV2
KAT2B
FRMD5
GCNT4
EIF3F
TRA2A
ZMAT4
KMT2D
SLC35G2
FAH
ESRRB
RPL23
Tissue: Pituitary
Hen Group: HEPH
Up-regulated outside PS
Up-regulated during PS

Figure 5.3 (A) IPA network analysis in the hypothalamus comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (B) IPA network analysis in the pituitary comparing HEPH gene expression outside and during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated outside of the PS, whereas red represents genes up-regulated during the PS.

Examination of the expression changes of DEGs related to the HPG axis revealed differential regulation of the HPG axis during the ovulatory cycle in LEPH and HEPH (**Table 5.1**). Outside of the PS, LEPH showed up-regulation of genes involved in prolactin signaling and androgen signaling. During the PS, LEPH showed up-regulation of genes involved in stimulatory and inhibitory HPG axis signaling, whereas HEPH showed up-regulation of E2 and prolactin signaling. When LEPH and HEPH were compared individually outside and during the PS, LEPH displayed further increased expression of HPG axis inhibition and prolactin signaling (**Supplemental Table 5.1**). HEPH displayed decreased expression of HPG axis stimulatory genes and increased expression of AD and prolactin signaling. Prolactin signaling showed inverse trends in LEPH and HEPH and was impacted by the PS. Prolactin signaling has been shown to impact LH release in mammals and was up-regulated in HEPH during the PS, indicating a possible role in the shortened ovulation intervals seen in HEPH (Anderson et al., 2008). Both LEPH and HEPH showed down-regulation of *GNRHR* during the PS, which was also seen in AEPH during the PS (Chapter 2). Generally, HEPH displayed down-regulation of the HPG axis during the PS, whereas LEPH displayed up-regulation of both genes that stimulate and inhibit the HPG axis during the PS, presumably leading to a longer ovulation interval in LEPH.

Table 5.1. Significant gene expression changes in the HPG axis between LEPH and HEPH, outside and during the PS (RPKM>0.2, P<0.05).

Outside Preovulatory Surge

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	PRL	prolactin signaling	-2.65	0.0245
	CYP19A1	steroid hormone biosynthesis	-1.52	0.0292
	CYP1A1	steroid hormone biosynthesis	1.92	0.0092
	HSD11B1	steroid hormone biosynthesis	1.67	0.0448
Pituitary	CGA	HPG axis signaling	1.59	0.0217
	AR	steroid hormone signaling	-1.59	0.0126
	STAR	steroid hormone biosynthesis	-16.73	0.0000

During Preovulatory Surge

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	GNRH1	HPG axis signaling	-2.54	0.0362
	NPVF	HPG axis signaling	-1.78	8.39E-06
	FSHR	HPG axis signaling	-7.03	0.0002
	ESR2	steroid hormone signaling	1.35	0.0218
	PRL	prolactin signaling	1.87	0.0299

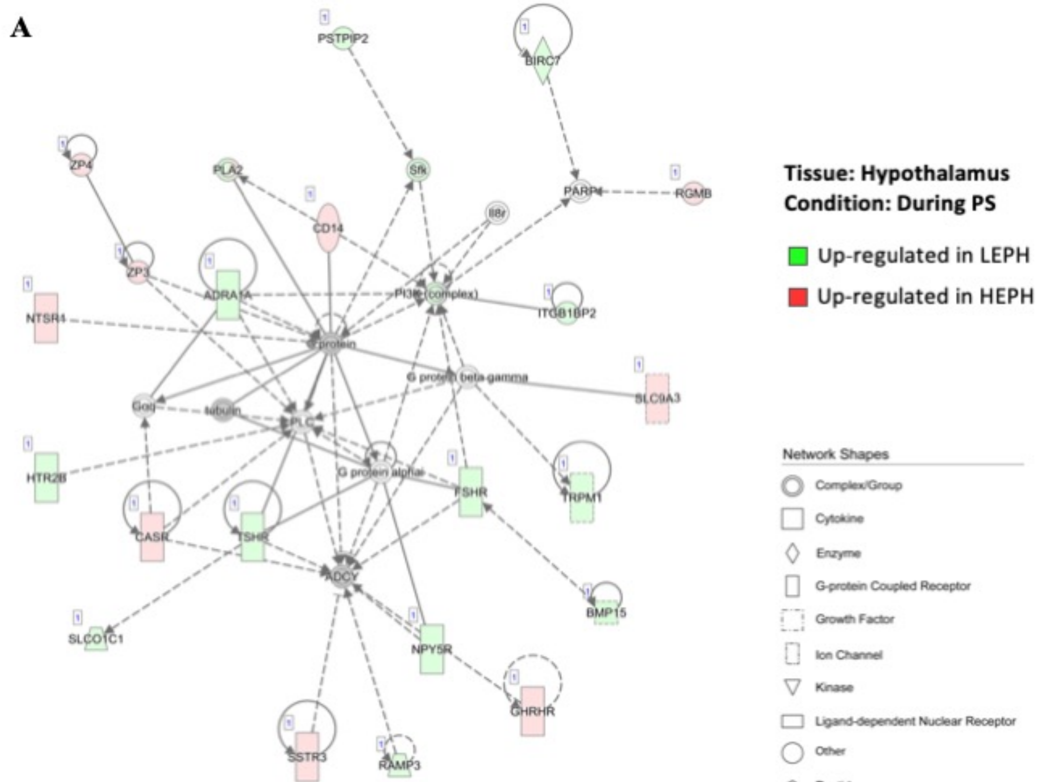
The HPT Axis

DEGs up-regulated in LEPH compared to HEPH were associated with HPT axis expression in each tissue and condition examined (**Figure 5.4 and Supplemental Figures 5.2, and 5.3**). In the hypothalamus during the PS, LEPH displayed increased expression of *TSHR* and solute carrier organic anion transporter family member 1C1 (***SLCO1C1***) relative to HEPH (**Figure 5.4A**). In the pituitary during the PS, LEPH displayed increased expression of *TSHB* in contrast to HEPH (**Figure 5.4B**). *TSHR* expression in the hypothalamus is related to short loop feedback control on thyrotropin releasing hormone signaling (Prummel et al., 2004). Retrograde regulation of *TSHB* on the hypothalamus has also been implicated in increased GnRH production in response to a changing photoperiod in seasonally reproductive birds (Korf, 2018). It is plausible that retrograde *TSHB* feedback on the hypothalamus could also be involved in the timing of ovulation, due to the role of *TSHB* in GnRH signaling initiation coupled with the finding that molecular clockwork components impact *TSHB* pituitary expression in several mammalian species (Unfried et al., 2009). *SLCO1C1* is a thyroid hormone transporter that participates in transporting thyroid hormone across the blood-brain barrier (Bernal, 2010). Up-regulation of *SLCO1C1* in LEPH during the PS would allow greater thyroid hormone concentrations in the hypothalamus, which could ultimately have genomic effects on ovulation rates (Lechan and Fekete, 2007).

Additionally, in the hypothalamus during the PS, LEPH showed up-regulation of solute carrier family 16 member 12 (***SLC16A12***) and integrin (encoded by *ITGAV* and *ITGB3*) relative to HEPH (**Supplemental Figures 5.2A and 5.2B**). *SLC16A12* encodes a thyroid hormone transporter similar to *SLCO1C1,* allowing greater transport of thyroid hormone past the blood brain barrier in LEPH rather than HEPH (Bernal, 2010). Integrin is a plasma membrane receptor

capable of binding thyroid hormones to elicit non-genomic actions of thyroid hormone, such as protein translocation and phosphorylation (Cheng et al., 2010). Up-regulation of integrin in the hypothalamus of LEPH relative to HEPH during the PS, infers possible non-genomic implications of thyroid hormone in the hypothalamus of LEPH (Hammes and Davis, 2015).

In the pituitary during the PS, HEPH showed up-regulation of iodothyronine deiodinase 1 (***DIO1***) relative to LEPH (**Supplemental Figure 5.4A**). *DIO1* is capable of converting thyroid hormone to the biologically active form but is also capable for thyroid hormone deactivation (Visser, 1988). Increased thyroid hormone deactivation could mitigate the effect of thyroid hormone on the tissues of the HPG axis in HEPH. When comparing HEPH outside and during the PS, HEPH showed down-regulation of *TSHB* in the pituitary during the PS (**Supplemental Figure 5.4B**). TSH acts on the thyroid gland to promote the synthesis of thyroid hormones (McNabb, 2007a). Down-regulation of *TSHB* during the PS in HEPH could indicate lower circulating levels of TSH, ultimately impacting circulating thyroid hormones.


A
PSTPIP2
BIRC7
ZP4
PLA2
Sfk
PARP4
RGMB
CD14
Il8r
ZP3
ADRA1A
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ITGB1BP2
NTSR1
G protein
G protein beta gamma
SLC9A3
Gαq
tubulin
PLC
G protein alpha
HTR2B
CASR
TSHR
FSHR
TRPM1
ADCY
SLCO1C1
NPY5R
BMP15
GHRHR
SSTR3
RAMP3
Tissue: Hypothalamus
Condition: During PS
Up-regulated in LEPH
Up-regulated in HEPH
Network Shapes
Complex/Group
Cytokine
Enzyme
G-protein Coupled Receptor
Growth Factor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter

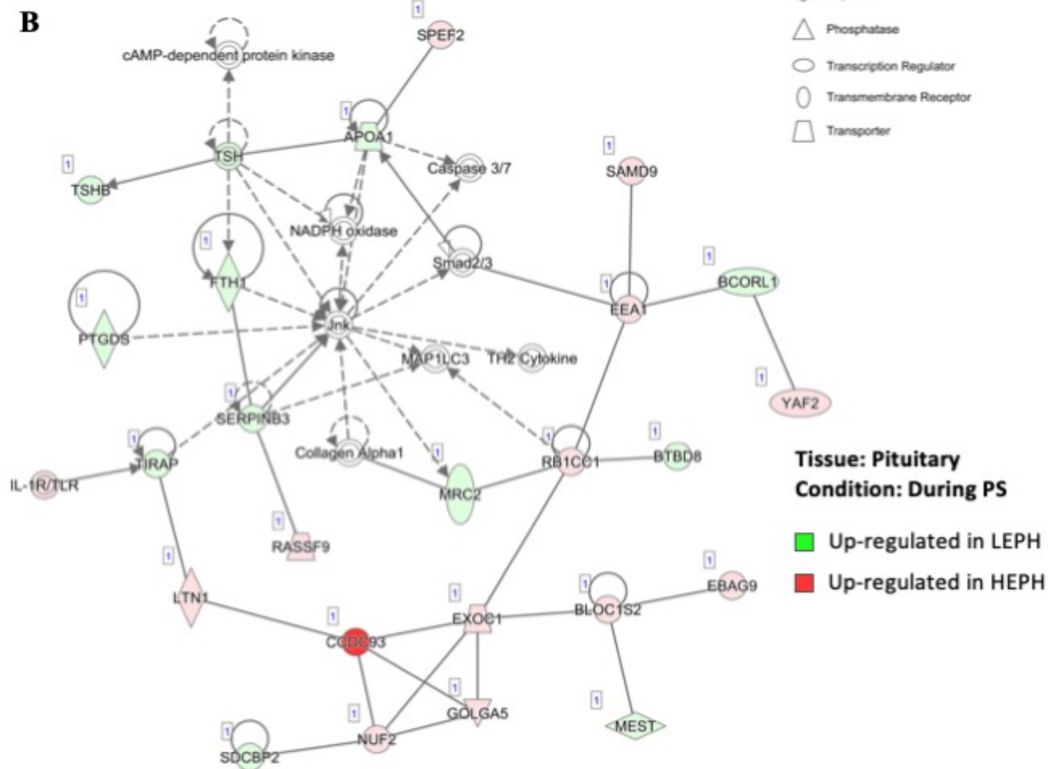

B
cAMP-dependent protein kinase
SPEF2
APOA1
TSH
Caspase 3/7
SAMD9
TSHB
NADPH oxidase
FTH1
Smad2/3
EEA1
BCORL1
PTGDS
Jnk
MAP1LC3
TH2 Cytokine
YAF2
SERPINB3
Collagen Alpha1
RB1CC1
BTBD8
IL-1R/TLR
TIRAP
MRC2
RASSF9
LTN1
EXOC1
BLOC1S2
EBAG9
CCDC93
GOLGA5
MEST
SDCBP2
NUF2
Tissue: Pituitary
Condition: During PS
Up-regulated in LEPH
Up-regulated in HEPH

Figure 5.4. (A) IPA network analysis in the hypothalamus comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (B) IPA network analysis in the pituitary comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.

Examination of the expression changes of DEGs related to HPT axis revealed that LEPH exhibited up-regulation of a majority of the key genes of the HPT axis when compared to HEPH (**Table 5.2**). Outside and during the PS, LEPH displayed higher expression of genes related to HPT axis signaling, thyroid hormone receptors, thyroid hormone transporters, thyroid hormone metabolism, and thyroid hormone synthesis when compared to HEPH. During of the PS, LEPH displayed further increased expression thyroid related genes. When LEPH and HEPH were compared individually outside and during the PS, LEPH displayed increased expression of HPT axis genes during the PS, whereas HEPH displayed decreased expression of HPT axis genes during the PS (**Supplemental Table 5.2**). HEPH during the PS showed down regulation of thyroid hormone transporters and genes involved in HPT axis signaling. Generally, LEPH displayed higher expression of HPT axis genes both outside and during the PS compared to HEPH and displayed further up-regulation of the HPT axis during the PS when compared to levels outside of the PS. HEPH, on the other hand, displayed down-regulation of the HPT axis during the PS and lowered HPT axis expression both outside and during the PS when compared to LEPH.

Table 5.2 Significant gene expression changes in the HPT axis between LEPH and HEPH, outside and during the PS (RPKM>0.2, P<0.05).

Outside Preovulatory Surge

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	TRHR	HPT axis signaling	-1.71	0.0150
	TSHB	HPT axis signaling	-10.79	0.0368
	THRA	thyroid hormone receptor	-2.28	0.0274
	TTR	thyroid hormone transporter	3.58	0.0166
	SLO1C1	thyroid hormone transporter	-2.45	0.0197
Pituitary	CGA	HPT axis signaling	1.59	0.0217
	DIO2	thyroid hormone metabolism	-2.14	0.0012
	SLC5A5	thyroid hormone synthesis	6.48	0.0109
	ATP1B4	thyroid hormone synthesis	-3.17	0.0425
	SLC7A5	thyroid hormone transporter	-1.58	0.0088

During Preovulatory Surge

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	TSHR	HPT axis signaling	-2.07	0.0108
	SLC5A5	thyroid hormone synthesis	3.43	0.0002
	DOUX	thyroid hormone synthesis	2.44	0.0121
	SLC26A4	thyroid hormone synthesis	-1.62	0.0186
	TTR	thyroid hormone transporter	-76.13	1.11E-15
	SLC7A5	thyroid hormone transporter	1.21	0.0359
	SLO1C1	thyroid hormone transporter	-2.83	1.34E-07
Pituitary	TSHB	HPT axis signaling	-1.76	0.0261
	DIO2	thyroid hormone metabolism	-1.61	0.0436
	TTR	thyroid hormone transporter	-11.21	0.0004

Upstream Analysis

Analysis of the predicted upstream regulators for each comparison made also showed a common theme: the involvement of beta-estradiol. While the calculated Z-score varied for the comparisons examined, beta-estradiol was the only upstream regulator common to all of the comparisons (**Figure 5.5**). Additionally, beta-estradiol was among the top five upstream regulators in the pituitary both outside and during the PS (**Table 5.3**). The predicted involvement of beta-estradiol across all conditions examined with target genes involved in the HPG and HPT axes supports the hypothesis that beta-estradiol feedback on the hypothalamus and pituitary impacts the ovulatory process and possibly egg production rates.

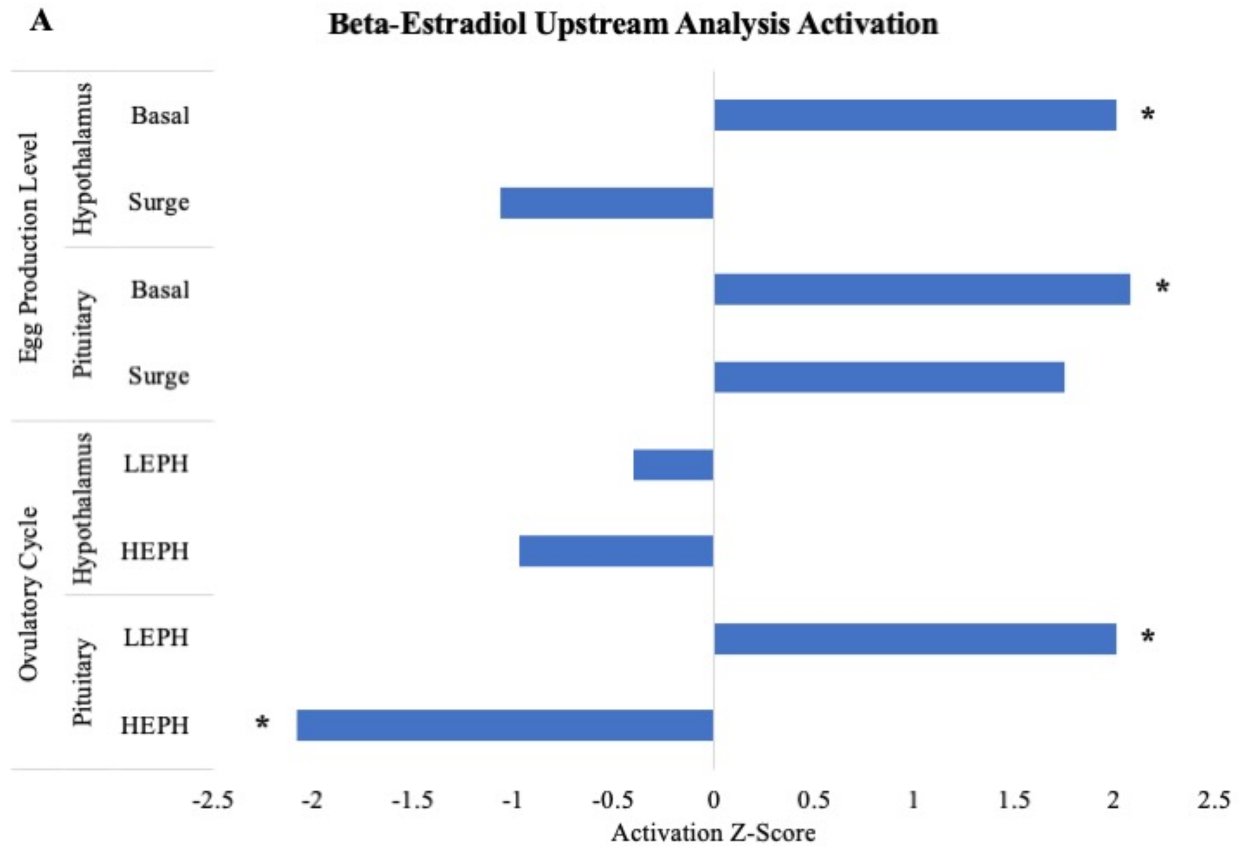


Figure 5.5 Activation z-score calculated for beta-estradiol based on DEGs (RPKM>0.2, P<0.05, |fold change|>1.5). The top panel shows the calculated z-score for beta-estradiol based on DEGs between LEPH and HEPH, both outside (basal) and during (surge) the PS. The bottom panel shows the calculated z-score for beta-estradiol based on DEGs between outside and during the PS in LEPH and HEPH individually. Significant predicted activation (z-score ≥ 2) or inhibition (z-score ≤ -2) of beta-estradiol is denoted with an asterisk (*).

Table 5.3 Significant upstream regulators between LEPH and HEPH, outside and during the PS (RPKM>0.2, P<0.05, |fold change|>1.5).

Outside Prevoulatory Surge

Tissue	Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
Hypothalamus	cyclosporin A	biologic drug	0.678	1E-06	10
	MAPK8	kinase	0.889	2E-06	6
	Pkc(s)	group	0.119	3E-06	7
	FOXF2	transcription regulator	-2	5E-06	4
	FOXA2	transcription regulator	1	6E-06	7
Pituitary	DAP3	other	3	4E-08	7
	actinonin	chemical reagent	-3	1E-06	7
	ALKBH1	enzyme	2.449	5E-06	4
	NSUN3	enzyme	2.449	5E-06	4
	SIRT3	enzyme	-1.952	0.0003	8

During Prevoulatory Surge

Tissue	Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
Hypothalamus	LOXL2	enzyme	-1.406	2E-06	3
	FGF2	growth factor	-0.307	3E-06	15
	beta-estradiol	chemical-endogenous	-1.064	2E-05	39
	Mek	group	1.315	2E-05	9
	BMP2	growth factor	-1.014	3E-05	8
Pituitary	ESR1	nuclear receptor	0.991	4E-06	41
	beta-estradiol	chemical-endogenous	1.749	2E-05	49
	ESR2	nuclear receptor	-0.842	0.0001	17
	CDH2	other	-1.103	0.0002	4
	cholic acid	chemical-endogenous	0.761	0.001	6

For the comparisons between LEPH and HEPH, beta-estradiol was significantly more active in HEPH in the hypothalamus (z-score = 2.011) and pituitary (z-score = 2.079) outside of the PS. Differentially expressed target genes of beta-estradiol in the hypothalamus outside of the PS included *TRHR*, *TSHB*, *TTR*, *PRL*, *HSD17B1*, and *CYP19A1*, while differentially expressed target genes of beta-estradiol in the pituitary outside of the PS included the androgen receptor (***AR***), *CGA*, *STAR*, and solute carrier family 7 member 5 (***SLC7A5***) (**Supplemental Table 5.3**). Beta-estradiol tended to be more active in HEPH in the pituitary during the PS (z-score = 1.75), though not significantly.

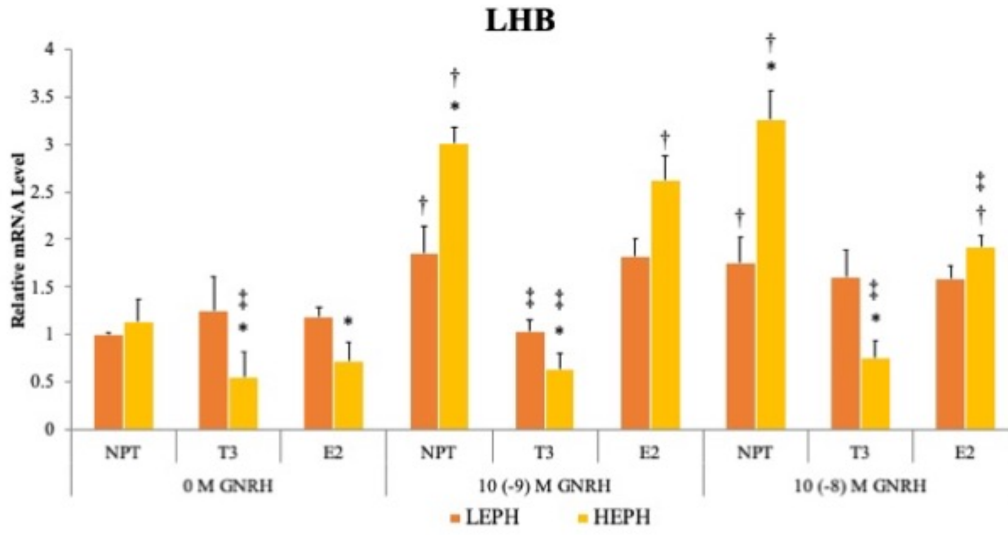
For the comparisons during the ovulatory cycle for each hen group, in the pituitary beta-estradiol was significantly more active during the PS for LEPH (z-score = 2.014) and significantly more active outside of the PS for HEPH (z-score = -2.079). Differentially expressed target genes of beta-estradiol in the pituitary of LEPH included albumen (***ALB)***, prolactin receptor (***PRLR***), *STAR*, and *TTR,* whereas differentially expressed target genes of beta-estradiol in the pituitary of HEPH included *CGA* and *TSHB* (**Supplemental Table 5.4**).

Effect of T3 and E2 on Pituitary Gonadotropin Production

To further examine the impact of T3 and E2 on HPG axis function, gonadotropin subunit mRNA levels were measured in pituitary cells from LEPH and HEPH after no pretreatment (**NPT**), T3, or E2 pretreatment combined with GnRH treatment. Pituitary cells from LEPH and HEPH responded differently to each pretreatment in terms of gonadotropin subunit mRNA levels, indicating functional differences in the response of the HPG axis to thyroid hormone and estradiol that could be related to differences seen in egg production levels between the two groups of hens (**Figure 5.6**). The *in vitro* effects of T3 and E2 pretreatment were seen both with

and without GnRH treatment, indicating that both hormones could be capable of pituitary gonadotropin regulation outside and during the PS.

A



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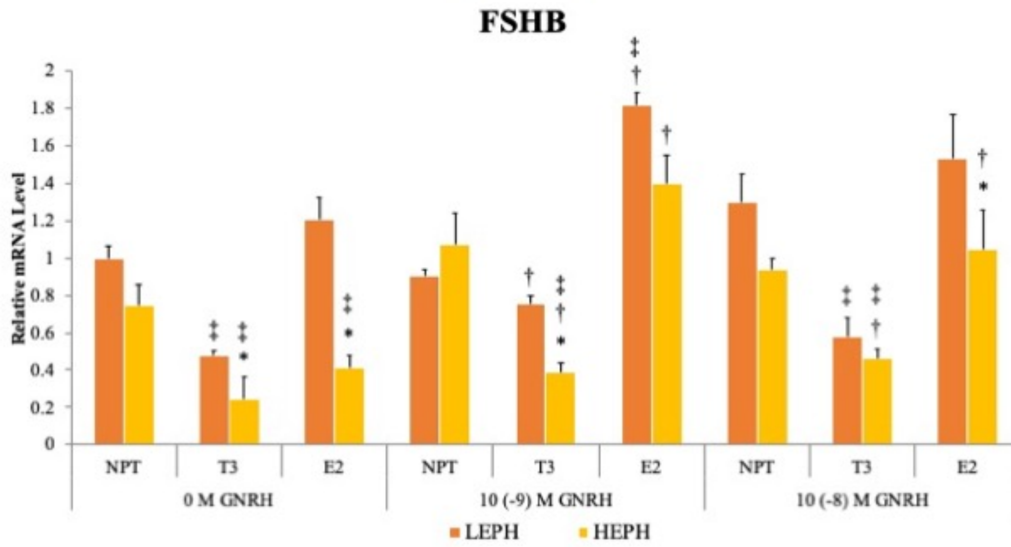


C

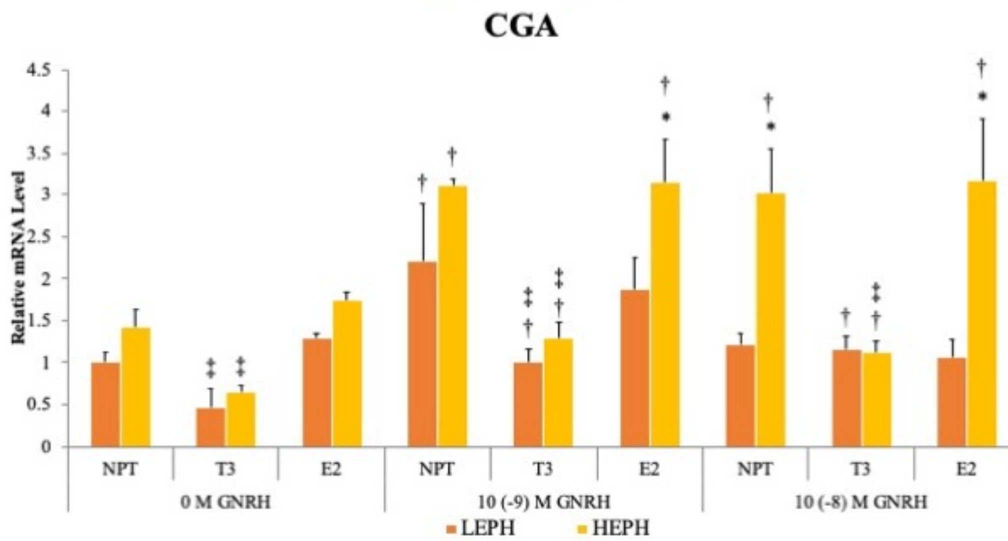


Figure 5.6 Relative pituitary expression of *LHB*, *FSHB*, and *CGA* after pretreatment with NPT, T3, or E2 followed by GnRH treatment in LEPH and HEPH. Normalized data are presented relative to LEPH basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk (*). Significant differences between GnRH treatments for a given egg production group are denoted with a dagger (†). Significant differences between pretreatments for a given egg production group are denoted with a double dagger (‡).

T3 pretreatment decreased *LHB*, *FSHB*, and *CGA* mRNA levels compared to NPT in HEPH pituitary cells, regardless of GnRH treatment concentration. T3 pretreatment also decreased *LHB*, *FSHB*, and *CGA* mRNA levels in LEPH pituitary cells, but only at 10^{-9} M GnRH for *LHB*, 0 M and 10^{-9} M GnRH for *FSHB*, and 0 M and 10^{-8} M GnRH for *CGA*. T3 pretreatment negatively impacted *LHB, FSHB,* and *CGA* mRNA levels in cells from LEPH and HEPH, however the effect was more prominent in HEPH cells. T3 negative regulation of LHB, FSHB, and CGA were also reported in male rats (Bargi-Souza et al., 2019; Bargi-Souza et al., 2015). One possible mechanism for response differences to T3 treatment between LEPH and HEPH is desensitization or down-regulation of THRs in LEPH due to the general up-regulation of the HPT axis seen in the hypothalamus and pituitary of LEPH. THR desensitization in the liver has been documented after T3 injections in mice and *in vitro* T3 treatment decreased THR expression in rat pituitary cells (Ohba et al., 2016; von Overbeck and Lemarchand-Béraud, 1983). Generally, T3 pretreatment negatively regulated gonadotropin production, independent of GnRH treatment concentration, with a higher negative response from HEPH. These findings suggest that HEPH are more sensitive to the effect of T3 treatment on gonadotropin production, whereas LEPH are more resistant to the effects of T3 treatment.

E2 pretreatment decreased *LHB* mRNA levels in HEPH pituitary cells compared to NPT at 10^{-8} M GnRH. E2 pretreatment also decreased *FSHB* mRNA levels in HEPH pituitary cells relative to NPT at 0 M GnRH and increased *FSHB* mRNA levels in LEPH pituitary cells at 10^{-9} M GnRH. E2 pretreatment in HEPH pituitary cells decreased *FSHB* mRNA levels at lower GnRH treatment concentrations but decreased *LHB* mRNA levels at higher GnRH treatment concentrations. Previous studies in chickens have shown E2 to inhibit pituitary LH production (Terada et al., 1997). In contrast, E2 pretreatment upregulated *FSHB* in pituitary cells from

LEPH at 10^{-9} M GnRH. E2 treatment effects on FSHB mRNA levels have not been examined in avian species but E2 injections in quail did not impact FSH plasma levels, which is consistent with the mRNA levels seen in HEPH (Çiftci, 2012). Overall, E2 pretreatment had varied impacts on gonadotropin production, depending on the rate of egg production of the hens.

Conclusions

Hypothalamic and pituitary transcriptome analysis of LEPH and HEPH provided insights into the involvement of the HPT axis and E2 signaling on egg production rates. LEPH displayed higher expression of genes related to the HPT axis in both the hypothalamus and pituitary when compared to HEPH, regardless of timing in the ovulatory cycle. During the PS, further up-regulation of the HPT axis was seen in LEPH, whereas the opposite was seen in HEPH. Beta-estradiol was activated as an upstream regulator in the hypothalamus and pituitary of HEPH compared to LEPH under basal conditions. Additionally, beta-estradiol was activated in LEPH and inhibited in HEPH in the pituitary during the PS. These observations support the hypothesis of beta-estradiol playing a role in the regulation of the PS and possibly in HEPH exhibiting decreased ovulation intervals. Furthermore, T3 and E2 treatment *in vitro* impacted gonadotropin production to different degrees in LEPH and HEPH, inferring that LEPH and HEPH respond differently to T3 and E2 feedback on the pituitary gland.

Materials and Methods

Hen Selection and Tissue Collection

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual wire cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards. Hens were sampled at 37 weeks of age. Daily egg records were used to calculate

each hen's number of EPD. Hens were classified as LEPH when EPD<0.6 and as HEPH when EPD>0.8. EPD cutoffs for LEPH and HEPH were based on previous studies examining average flock egg production and egg production distribution (Chapter 2). The hypothalamus and pituitary were isolated from six LEPH and six HEPH, half sampled outside of the PS and half sampled during the PS. Additionally, three LEPH and HEPH were sampled outside of the PS for pituitary isolation and culture. The timing of the PS was predicted using hourly egg records as previously described (Chapter 2). All hens were sampled on the second day of the hen's sequence. Blood samples were taken from the wing vein immediately before sampling and fractionated by centrifugation. Plasma samples were stored at -20°C prior to assessment through RIA as described below. Plasma P4 levels were examined to confirm correct sampling time during the ovulatory cycle. Whole hypothalamus and pituitary samples from LEPH and HEPH sampled outside and during the PS were snap frozen in liquid nitrogen and stored at -20°C prior to assessment through RNAseq and RT-qPCR as described below. Pituitary samples from LEPH and HEPH exclusively sampled outside of PS were placed in ice cold DMEM until dispersion and cell culture as described below. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland.

RIA

The RIA used to measure plasma P4 levels was a coated tube kit (MP Biomedicals, Solon, OH). All protocols were performed as directed by the supplier. All samples were assayed in duplicate in a single RIA. Plasma samples were ether extracted and analyzed for P4 to determine that hens were sampled at the correct time during the ovulatory cycle, based on experimental group assigned. Hypothalamus and pituitary samples taken from a hen with plasma P4 levels less than 1 ng/dL were considered to be sampled outside of the PS, while hypothalamus

and pituitary samples taken from a hen with plasma P4 levels greater than 4 ng/dL were considered to be sampled during of the PS. The plasma P4 cutoffs for outside and during the PS were based on previous studies determining average plasma P4 levels during the ovulatory cycle (Chapter 2).

RNA Isolation, cDNA Library Construction, and Sequencing

Total RNA was isolated from whole tissue hypothalamus and pituitary samples with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA was performed as previously described (Chapter 2). Amplified cDNA was generated using a SMART-Seq Ultra Low Input RNA kit (Takara Bio, Kusatsu, Japan) following the manufacturer's procedure. Briefly, for each sample 1µl of 10X Reaction Buffer was added to 10ng of total RNA (in 9.5µl nuclease-free water). 10X Reaction Buffer: 19µl of 10X Lysis Buffer and 1µl of RNase Inhibitor. Then, 2µl of 3' SMART-Seq CDS Primer II A (12µM) was added and gently vortexed, samples were incubated at 72°C for 3 min and then placed on ice for 2 min. Then, 7.5µl of RT Master Mix was added to each sample (total reaction volume is 20 µl) and incubated at 42°C for 90 min, followed by 72°C for 10 min then placed on ice. Master Mix (per reaction) consisted of 4µl 5X Ultra Low First-Strand Buffer; 1µl SMART-Seq v4 Oligonucleotide (48µM); 0.5µl RNase Inhibitor (40 U/µl); 2µl of SMARTScribe Reverse Transcriptase (100U/µl). Next, cDNA was amplified by long distance PCR as follows.

To each 20µl cDNA sample, 30µl of PCR Master Mix was added and amplification was carried out using the following conditions: 95°C for 1 minute, followed by 8 cycles of: 98°C for 10 sec, 65°C for 30 sec and 68°C 3 min, 72°C for 10 min. PCR Master Mix (per reaction) consisted of 25µl 2X SeqAmp CB PCR Buffer; 1µl PCR Primer II A (12µM); 1µl SeqAmp DNA Polymerase; 3µl nuclease-free water. Amplified cDNA was then purified using Agencourt

AMPure XP beads (Beckman Coulter, Indianapolis, IN) as follows. To each PCR reaction 50µl of AMPure XP beads was added, vortexed and incubated at room temperature for 8 min, briefly centrifuged, and placed on a MagWell Magnetic Separator 96 (EdgeBio, San Jose, CA) for 5 min. Supernatant was discarded and the beads were washed twice with 200µl of 80% ethanol. Beads air dried (~2 min) and 17µl of Elution Buffer was added to each sample, vortexed and incubated at room temperature for 2 min. The amplified cDNA was quantified using an 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Santa Clara, CA).

To generate sequencing libraries, a Nextera XT DNA library kit (Illumina, San Diego, CA), following the manufacturer's procedure, and 150pg of amplified cDNA per library was used, as recommended by Takara Bio. For each sample two libraries were produced (from the same amplified cDNA), each library had a unique index pairing. To 5µl (30pg/µl) of amplified cDNA, 10µl of Tagment DNA Buffer was added, mixed by pipetting and incubated at 55°C for 5 min then brought to 10°C. Tagmentation was then neutralized by adding 5µl of Neutralize Tagment Buffer and incubating at room temperature for 5 min. To each reaction 5µl of the appropriate index 1 (N7) and 5µl of appropriate index 2 (S5) was added followed by 15µl of Nextera PCR Master Mix, for a total reaction volume of 50µl. PCR conditions were as follows: 72°C for 3 min; 95°C for 30 sec; 12 cycles of: 95°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec; then 72°C for 5 minutes. Libraries were purified using Agencourt AMPure XP (Beckman Coulter, Indianapolis, IN) beads as follows. To each PCR reaction, 30µl AMPure XP bead was added and shook at 1800 rpm for 2 min, then incubated at room temperature for 5 min, briefly centrifuged, and placed on a MagWell Magnetic Separator 96 (EdgeBio, San Jose, CA) for 5 min. Supernatant was discarded, and the beads were washed twice with 200µl of 80% ethanol. Beads were air dried for 15 min. To each library 52.5µl of Resuspension Buffer was added and

shook at 1800 rpm for 2 min then incubated at room temperature for 2 min. Tubes were then placed on a MagWell Magnetic Separator 96 (EdgeBio, San Jose, CA) until liquid was clear (~5min), then 50µl of each library was transferred to a new tube. The libraries were quantified using a 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Santa Clara, CA). For sequencing 24 libraries (2 tissues) were pooled (10nM). Libraries were pooled so that set 1 for each tissue was sequenced in a different pool than set 2. Pools were submitted to North Carolina State University's GSL facility for paired-end sequencing (75 bp reads) on an Illumina NextSeq 500.

Bioinformatic Analysis of Sequencing Data

Processing and analysis of sequencing data was performed using CLC genomics workbench (Qiagen, Valencia, CA). Adapter sequences and low quality sequences (Phred < 20) were removed from FASTQ files using the NGS trim tool. Trimmed reads were mapped to the *Meleagris gallopavo* reference genome (Turkey_2.01). The RNA-seq analysis suite was used to analyze mRNA libraries. DEGs were determined using the "Differential Expression for RNA-Seq" tool. Pairwise comparisons were made between LEPH and HEPH for each timepoint in the ovulatory cycle as well as between timepoints in the ovulatory for each egg production group. Due to poor annotation of the turkey genome, the protein sequences for DEGs that were unannotated in the turkey were subjected to orthologous comparisons in human, mouse, and chicken protein sequences using BIOMART (Ensembl). Unannotated DEGs were assumed orthologous if greater than 50% identity to the human, mouse, and chicken was seen at the protein level.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, Valencia, CA) was performed on the differential expression data. IPA was used to construct gene networks as well as to predict upstream biological regulators for each pairwise comparison. Only DEGs with RMPK>0.2 were used for IPA analysis. The RPKM threshold was selected based on the distribution of log_2 transformed RPKM values across all of the comparisons examined. The threshold of DEGs was set at $P < 0.05$ and absolute fold change ≥1.5. Pathways and predicted upstream regulators with P-value < 0.05 (Fischer's exact test) were considered to be statistically significant. For upstream regulators, published findings in the Ingenuity knowledge database were used to calculate the activation z-score to infer activation or inhibition of transcriptional regulators. Upstream regulators with a z-score greater than 2 or less than -2 and $P<0.05$ were considered to be significantly activated or inhibited.

Culture of Pituitary Cells

All cell isolation procedures were performed using SMEM or DMEM as noted below. Media was supplemented with 0.1% bovine serum albumen, 100-U/mL penicillin G, and 100-μg/mL streptomycin sulfate (**0.1% BSA and P/S**). Dispersion pituitary cells was performed as previously described (Chapter 4). Briefly, isolated pituitaries were dispersed in SMEM (0.1% BSA and P/S) using trypsin and collagenase (1 mg/mL of each) and filtered through 70 μm nylon mesh.

Cells were diluted to a concentration of 200,000 cells/mL and plated in DMEM/F12 supplemented with 0.1% bovine serum albumen, 5-μg/mL human insulin, 100-U/mL penicillin G, and 100-μg/mL streptomycin sulfate. Cells were plated in 24 well poly-L lysine coated plates (Corning Life Sciences, Lowell, MA) at 100,000 cells/well and were allowed to attach for 2

hours before pretreatment. Pituitary cells were pretreated with either NPT (10 µl DMEM/F12 added), 1.5 ng/mL of T2, or 1.5 ng/mL of E2 for 12 hours, followed by treatment with chicken GnRH (Phoenix Pharmaceuticals, Burlingame, CA) at 0, 10^{-9}, or 10^{-8} M for 6 hours. Cells were maintained in a 37.5°C, 5% CO_2 atmosphere during incubation. Pituitary cells were harvested at the end of incubation by retrypsinization, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

RT-qPCR

Total RNA was isolated from pituitary cell cultures with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA, RT, and RT-qPCR were performed as previously described (Chapter 2). A pool of total RNA was made and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. PCR reactions (15 µL) were carried out as previously described using a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA) (Chapter 2). Pituitary data were normalized to *PGK1* and were analyzed by the $2^{-\Delta\Delta Ct}$ method. All PCR reactions for each gene in a given tissue were analyzed in a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates. Primers (Integrated DNA Technologies, Skokie, IL) for turkey *PGK1*, *LHB*, *FSHB*, and *CGA* mRNA were designed and used with cycling parameters described previously (Chapter 2). Data are presented as fold increase over levels in LEPH basal cells for each pretreatment/treatment combination and time point.

RNAseq Confirmation

RNA extracted and quantified from whole tissue hypothalamus and pituitary samples for RNAseq was reverse transcribed as described previously (Chapter 2), with controls for genomic

DNA contamination. PCR reactions were carried out as described above. Hypothalamic data were normalized to GAPDH, whereas pituitary data were normalized to *PGK1*. Data were analyzed as described above. For each tissue, mRNA levels for 12 genes total were determined. DEGs selected for RNAseq confirmation fit the following parameters: $P<0.05$, absolute fold change greater or equal to 1.5, annotated in the turkey genome, and encoded by a single transcript. DEGs fitting these parameters were selected with the following RNAseq expression profiles: 3 DEGs up-regulated in LEPH both outside and during the PS, 3 DEGs up-regulated in HEPH both outside and during the PS, 3 DEGs which showed up-regulation in one egg production group outside of the PS and up-regulation in the other egg production group during the PS, and 3 control genes which did not show expression changes between egg production levels or during the ovulatory cycle. Primers were designed as described above. Data are presented as fold increase over mRNA levels for LEPH outside of the PS for each gene.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data were log_2 transformed before statistical analysis. A three-way ANOVA using the mixed models procedure was conducted to compare the log_2 transformed gene expression between LEPH and HEPH pituitary cell culture samples subjected to different pretreatment and treatment combinations. A two-way ANOVA using the mixed models procedure was used to compare the log_2 transformed gene expression between LEPH and HEPH from hypothalamus and pituitary samples used for RNAseq confirmation. The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$.

Supplemental Table 5.1 Significant gene expression changes in the HPG axis during the PS, in LEPH and HEPH (RPKM>0.2, P<0.05).

LEPH

Tissue	Gene	Function	Fold Change	P-Value
Hypothalamus	NPVF	HPG axis signaling	1.38	0.0470
	FSHR	HPG axis signaling	4.85	0.0033
	HSD17B1	steroid hormone biosynthesis	9.63	0.0358
	HSD11B1	steroid hormone biosynthesis	1.66	0.0341
Pituitary	GNRHR	HPG axis signaling	-1.72	0.0273
	PRLR	prolactin signaling	1.65	0.0008
	STAR	steroid hormone biosynthesis	-9.64	9.11E-07
	BMP6	steroid hormone biosynthesis	-1.71	0.0240

HEPH

Tissue	Gene	Function	Fold Change	P-Value
Hypothalamus	GNRH1	HPG axis signaling	-3.63	0.0005
	AR	steroid hormone signaling	1.56	0.0374
	PRL	prolactin signaling	2.34	0.0118
Pituitary	NPFFR2	HPG axis signaling	2.71	0.0428
	GNRHR	HPG axis signaling	-1.78	0.0496
	CGA	HPG axis signaling	-1.63	0.0139

Supplemental Table 5.2 Significant gene expression changes in the HPT axis during the PS, in LEPH and HEPH (RPKM>0.2, P<0.05).

LEPH

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	TRHR	HPT axis signaling	-1.71	0.0041
	TTR	thyroid hormone transporter	61.89	4.76E-14
	SLC16A1	thyroid hormone transporter	1.35	0.0101
Pituitary	TRHR	HPT axis signaling	1.42	0.0123
	ATP1B4	thyroid hormone synthesis	-2.68	0.0088
	SLC26A4	thyroid hormone synthesis	3.19	4.49E-05
	TTR	thyroid hormone transporter	4.56	0.0118
	ALB	thyroid hormone transporter	5.57	0.0163
	SLC7A5	thyroid hormone transporter	-1.40	0.0380

HEPH

Tissue	Gene	Function	Fold	P-Value
Hypothalamus	THRA	thyroid hormone receptor	2.13	0.0424
	TTR	thyroid hormone transporter	-4.40	0.0049
	SLC16A10	thyroid hormone transporter	-1.56	0.0226
Pituitary	TSHB	HPT axis signaling	-1.94	0.0210
	CGA	HPT axis signaling	-1.63	0.0139
	SLC5A5	thyroid hormone synthesis	-23.57	0.0002

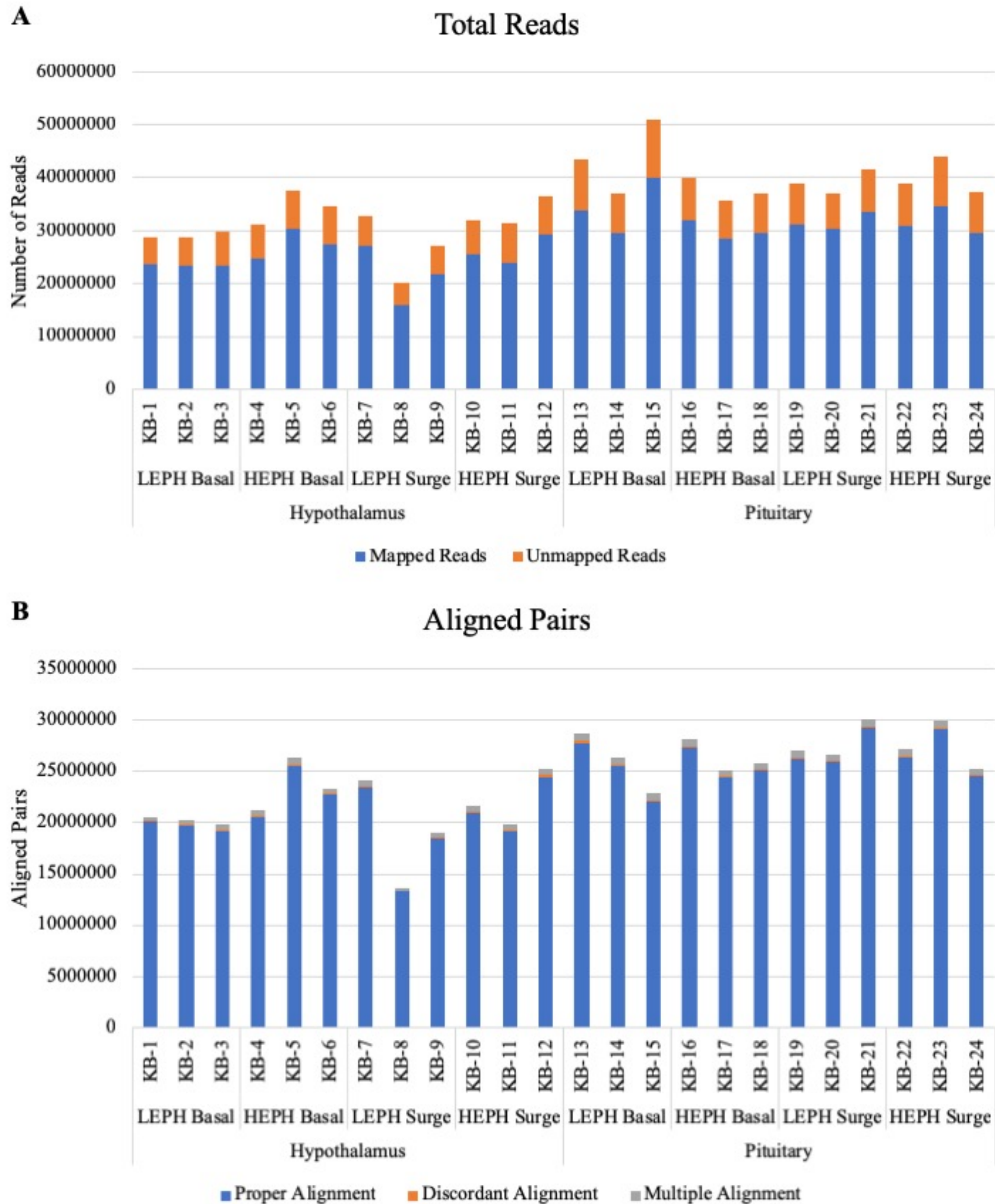
Supplemental Table 5.3 DEGs between LEPH and HEPH, outside and during the PS, that are predicted to be target genes of beta-estradiol (RPKM>0.2, P<0.05, |fold change|>1.5).

Tissue	Condition	Differentially Expressed Target Genes of Beta-Estradiol between LEPH and HEPH
Hypothalamus	Outside PS	ACTA2, ACTC1, ADORA2A, CD74, CREB1, CRLF1, CYBB, CYP19A1, GABRP, HSD17B2, MB, MGLL, MPEG1, OXTR, PRL, SMPDL3A, SRC, TLR2, TRHR, TSHB, TTR
	During PS	ACKR3, ACTA2, ADGRG2, ASB9, ATAD2, CAD, CCNA1, COL1A2, CRIM1, DNMT3B, F3, FABP4, FHL1, GNRH1, GRIK4, KIF3C, KRT19, LYZ, MAP3K8, MMP9, MPEG1, NPVF, PAPSS2, PLEKHA6, PRL, RAMP3, SEMA3A, SHISA2, SLC6A20, SOCS2, SRC, SSTR3, THSD4, TNNC2, TPD52L1, TPH2, TSHR, TTR, USP53
Pituitary	Outside PS	ADCY9, ADGRG2, ADRB1, ADRB3, ANK1, ANXA1, AP1B1, APOA4, AR, ARMT1, ATRX, BAG1, BLOC1S6, BNIP2, BRCA2, CAD, CASP3, CCNE1, CDC45, CDKL2, CGA, CSF3R, CTNND1, CUL4A, CXCL12, CYP26B1, CYTH3, DERL1, DNAJC3, DRD2, DUSP4, EIF3J, ERH, F2RL1, F3, FAS, FEZ2, FHL2, FN1, GALNT1, GCH1, GDNF, GHRHR, GHSR, GREB1, GRIK4, GUCY1A1, GYG1, HADH, HAPLN2, HSP90B1, IL17RD, IL18, INPP5J, KCNAB1, KCNN2, KIF3C, KPNA4, KRAS, LARP6, LRP8, MAL2, MAPK8IP1, MCM5, MMP13, MMP9, MTMR4, MXD1, MYLK, NCOA1, NET1, NRIP3, NRP2, NUCB2, OGDH, OGN, PAK5, PAPSS2, PFKL, PGRMC1, PI4KB, PLEKHA6, PMM2, POLA1, PPARG, PPL, PPRC1, PRKCH, PROS1, PSAT1, PSD, PSMA2, PTPRN, PTPRU, RAB9A, RALA, RER1, RHOQ, RMND1, ROCK1, SEZ6, SLC2A1, SLC38A2, SLC6A20, SLC7A5, SNED1, SRP54, SSR1, STAR, SUCLA2, TANK, TNFSF13B, TRAPPC2B, TRNT1, TUFT1, UBE2B, UBL3, USP19, USP53, YWHAZ
	During PS	ACTA2, APOA1, APOA4, ARC, ATF3, ATP2B1, ATRX, BAG1, BMPR2, C3, CCDC170, CCNA1, CRLF1, CYP26B1, CYTH3, DNAJC3, EDN2, FHL1, FMR1, GALNT1, GJB1, HAS2, HSP90B1, HSPA4, HTR2C, IGFBP2, IL17RD, LUM, MGP, MMP9, MMRN1, MYOF, NELL2, NUCB2, PADI1, PMM2, RAB31, RALA, RGS3, RP2, SNCG, SST, TRIB2, TRNT1, TSHB, TTR, UBL3, USP8, VAV3

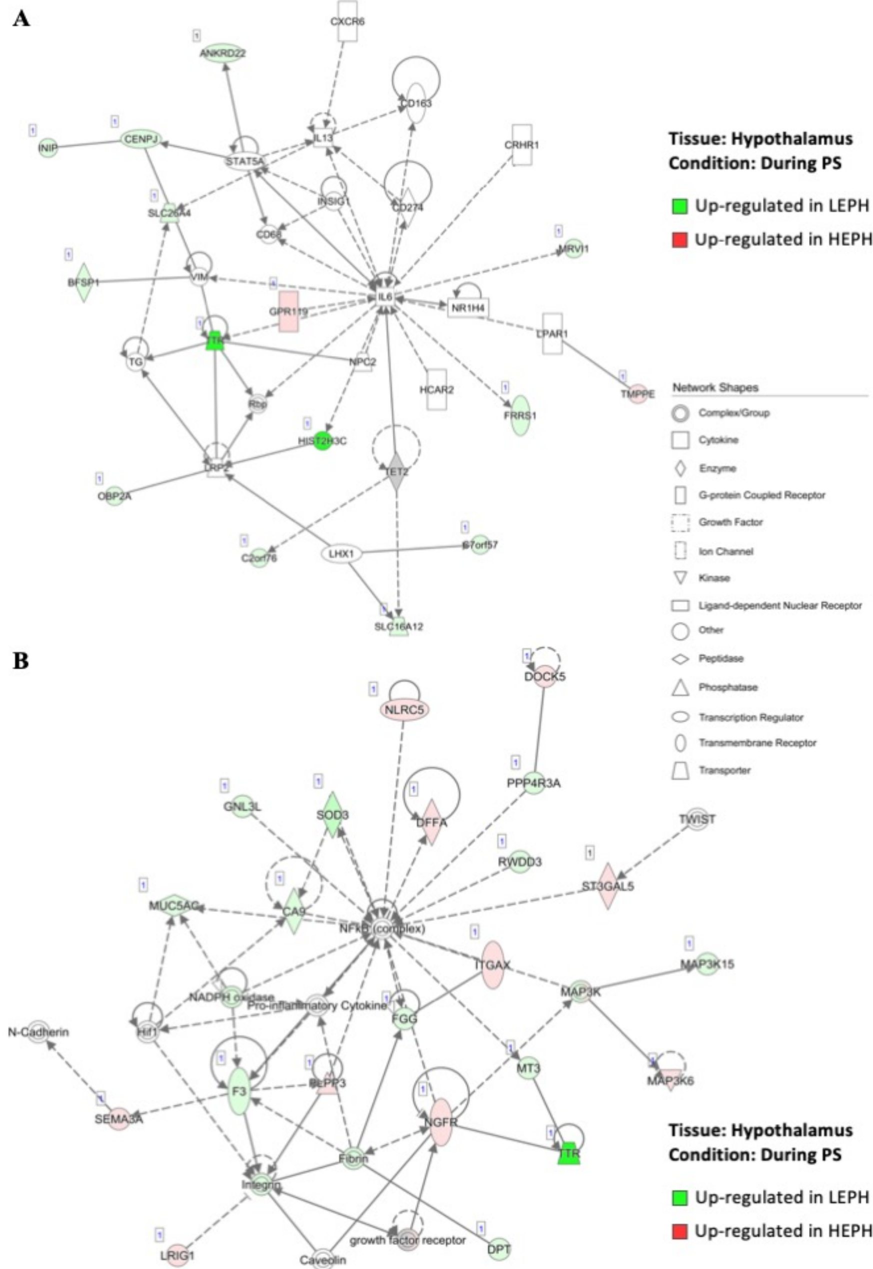
Supplemental Table 5.4. DEGs during the ovulatory cycle individually in LEPH and HEPH, that are predicted to be target genes of beta-estradiol (RPKM>0.2, P<0.05, |fold change|>1.5).

Tissue	Group	Differentially Expressed Target Genes of Beta-Estradiol Outside and During the PS
Hypothalamus	LEPH	ACKR3, ACTA2, ATAD2, CAD, CHAT, CHRM3, CRIM1, EGFR, F2RL1, FADD, FGD6, FOXN3, GABRP, HSD11B2, KITLG, KRT19, LEPR, MMP9, POLA1, PTGS2, RASGRP1, RUNX2, SEMA3A, SEMA3C, SLC12A4, SLC6A20, SLIT2, SMPDL3A, SP4, SRC, SST, TACSTD2, THSD4, TRHR, TTR, USP53
	HEPH	ABCA1, ACTA2, ACTC1, AR, ARC, AURKA, EDN2, GNRH1, HSD11B2, MATN2, NR5A2, ORC1, PDYN, PLEKHA6, PPP1R1B, PRL, RASGRP1, RNF4, SRC, TNNC2, TPD52L1, TTR, VAV3
Pituitary	LEPH	ACTA2, ACTC1, ADAMTS5, ADGRG2, ADRB1, ALB, ARMT1, ASB9, BAG1, BRCA2, CCNE1, CDKL2, CLCA2, CSF3R, CYTH3, DDC, F3, FABP3, FAS, FBN1, FMOD, GALR1, GHSR, GPR12, GUCY1A1, HSD11B2, HTR2C, IL17RD, IL18, IP6K3, KCND3, KCNN2, KRAS, LRP8, MMP9, NRP1, PDK4, PMM2, POMC, PPARG, PPM1K, PREP, PRLR, PROM1, PSD, PTGS2, PTPRN, RMND1, SHISA2, SNCG, SPRY2, SRP54, SST, SSTR1, STAR, THSD4, TNNC2, TP53INP1, TSKU, TTR, TUFT1, UBE2B
	HEPH	ACTC1, ADAMTS1, ADIPOQ, AGTR1, ANK1, APOA1, ARC, ATAD2, ATP5ME, CAMK2A, CGA, COX5A, CRABP2, CYP24A1, EGF, ELOVL2, EREG, ERH, FABP3, GCHFR, GREB1, GRHL2, GRIN2C, HBEGF, HSD11B2, HSPB1, IGFBP2, IL17RD, IL18, LGALS3, MGP, MMP9, MMRN1, MYC, NEDD9, NET1, NIPSNAP1, NPTX1, NRIP3, OPRK1, PAPSS2, PCBD1, PDK4, PDZK1IP1, PLEKHA6, PPL, PPM1K, PPP1R1B, RBPMS, RDH10, RNF4, RPL21, S100A6, SHISA2, SIN3B, SPRY2, SQSTM1, SRC, STC1, THSD4, TMEM258, TMOD2, TP53I11, TP53INP1, TPR, TRIB2, TSHB, UCK2, VAV3

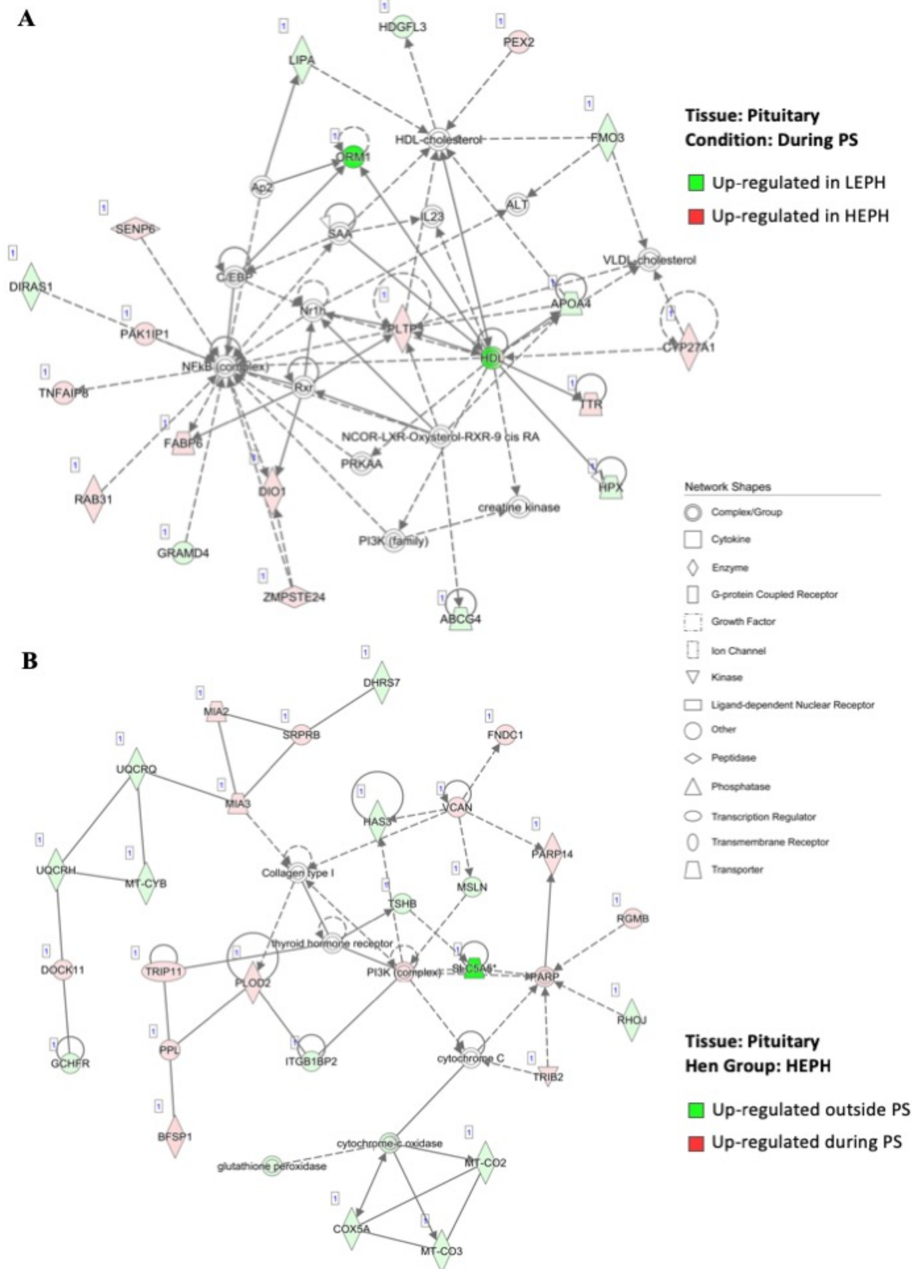
Supplemental Figures



Supplemental Figure 5.1 (A) The number of reads obtained for each sample. The portion of mapped reads for each sample is represented in blue, whereas the portion of unmapped reads for each sample is represented in orange. (B) The number of aligned pairs obtained for each sample. The portion of aligned pairs with proper alignment is represented in blue, with discordant alignment in orange, and with multiple alignments in gray.



Supplemental Figure 5.2 (A) IPA network analysis in the hypothalamus comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (B) IPA network analysis in the hypothalamus comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.



Supplemental Figure 5.3 (A) IPA network analysis in the pituitary comparing LEPH and HEPH gene expression during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (B) IPA network analysis in the pituitary comparing HEPH gene expression outside and during the PS (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated outside of the PS, whereas red represents genes up-regulated during the PS.

CHAPTER 6

Transcriptome analysis during follicle development in turkey hens with low and high egg production

Abstract

Background: LEPH and HEPH exhibit gene expression differences related to steroidogenesis in ovarian follicles. HEPH display increased expression of genes related to P4 and E2 production, in the F1G and SWF, respectively, whereas LEPH display increased expression of genes related to P4 and AD production in the granulosa of the fifth largest follicle (**F5G**) and theca interna layer of the fifth largest follicle (**F5I**), respectively. Transcriptome analysis was performed on F1G, F5G, F5I, and SWF samples from LEPH and HEPH to identify novel regulators of ovarian steroidogenesis that could ultimately impact egg production rates.

Results: In total, 12,221 DEGs were identified between LEPH and HEPH, with 6,212 genes up-regulated in LEPH and 6,009 genes up-regulated in HEPH. Consistent with previous results, HEPH displayed enrichment of steroidogenic genes in the F1G and SWF, whereas LEPH displayed enrichment of steroidogenic genes in the F5G and F5I. Pathway analysis inferred differential regulation of the HPT axis, particularly thyroid hormone transporters and THRs, and of E2 signaling in LEPH and HEPH. The HPT axis showed up-regulation in HEPH in less mature follicles but up-regulation in LEPH in more mature follicles. E2 signaling exclusively exhibited up-regulation in HEPH and beta-estradiol was identified as a top upstream regulator in three of the four tissues examined. Treatment with T3 in SWF cells from LEPH and HEPH *in vitro* decreased E2 production from HEPH cells to the levels seen in LEPH cells, whereas T3 treatment did not impact E2 production in LEPH cells.

Conclusions: Transcriptome analysis of the major cell types involved in ovarian steroid hormone production inferred the involvement of the HPT axis and estradiol signaling in the regulation of differential steroid hormone production seen in LEPH and HEPH. Differential

regulation of steroid hormone production may contribute to differences in egg production rates seen in LEPH and HEPH.

Background

Avian species display an ovarian hierarchy, with follicles in all stages of development present in the ovary at any given time during egg production (Johnson, 2014b). Four types of follicles exist during follicle development: primordial follicles, primary follicles, prehierarchical follicles, and preovulatory follicles. Maintenance of the follicular hierarchy is achieved by coordinated activation of primordial follicles to grow into primary follicles, recruitment of primary follicles to develop into prehierarchical follicles, selection of prehierarchical follicles to join the cohort of preovulatory follicles, and ovulation of preovulatory follicles to undergo egg formation in the oviduct (Johnson, 2014a). The prehierarchical follicles and preovulatory follicles are responsible for the majority of ovarian steroid hormone production. Throughout follicle maturation, steroid production shifts from E2 production in less mature follicles, to AD production in follicles in the middle stages of development, then to P4 production in more mature follicles (Porter et al., 1991a). Along with this shift, a change in gonadotropin sensitivity is seen as well, shifting from FSH responsiveness in less mature follicles to LH responsiveness in more mature follicles (Johnson and Woods, 2009).

Ovarian steroidogenesis occurs through a three-cell model, in which the three cell types of the follicle wall, the granulosa cells, the theca interna cells, and the theca externa cells, produce P4, AD, and E2, respectively (Porter et al., 1989a). SWF, a type of prehierarchical follicle, produce the majority of E2 in the ovary (Johnson, 1992). The F5I, which is part of the preovulatory follicles, produces the majority of AD in the ovary (Porter et al., 1991a). The F1G, which is also part of the preovulatory follicles, produces the majority of P4 in the ovary (Bahr et

al., 1983). Steroid hormones produced in the ovary feedback on the hypothalamus and pituitary to regulate the activity of the HPG axis (Johnson et al., 1985; Li et al., 1994).

The HPG axis is the main regulator of ovulation rates in avian and mammalian species (Paster, 1991). Among commercial turkey breeding hens, variation in ovulation rates are observed within a single flock (Chapter 3). LEPH and HEPH are observed in the top and bottom 15% of the flock. LEPH significantly impact the number of poults produced that can be raised for meat production, and cost more to maintain per egg laid than HEPH. Despite differences in ovulation rates, macroscopic morphology of the ovary does not differ between LEPH and HEPH, inferring that differences in the function of the HPG axis may be responsible for differential egg production levels (Chapter 3). Targeted gene expression approaches in LEPH and HEPH revealed differential expression of steroidogenic genes in the ovary (Chapter 3). LEPH showed up-regulation of genes consistent with increased AD production in the F5I relative to HEPH. HEPH showed up-regulation of genes consistent with increased P4 and E2 production in the F1G and SWF, respectively, relative to LEPH. Interestingly, LEPH also showed up-regulation of genes consistent with increased P4 production in the granulosa layer of the F5G compared to HEPH, suggesting that movement through the follicular hierarchy may occur at a slower rate in LEPH. Increased P4 and E2 production in the F1G and SWF cells from HEPH was also seen *in vitro* following LH and FSH treatment (Chapter 4).

Transcriptome analysis was performed in the F1G, F5G, F5I, and SWF of LEPH and HEPH to obtain a more global understanding of the regulation of steroid hormone production and follicle development in LEPH and HEPH. Both steroid hormone production and follicle development are influenced by the HPT axis and by paracrine effects of steroid hormones in the ovary (Rangel et al., 2009; Sechman, 2012). Steroid hormone receptors and THRs are expressed

in ovarian follicles at each stage of development (González-Morán et al., 2013; Sechman et al., 2009). Furthermore, *in vitro* steroid hormone and T3 treatment impacts steroidogenesis, with E2 generally increasing steroidogenesis and T3 generally decreasing steroidogenesis (Caicedo Rivas et al., 2016; Sechman et al., 2009).

Results and Discussion

Transcriptome Alignment and Mapping

A total of 788,763,171 sequence reads were obtained from the four follicle cell types examined, with an average of 32,865,132 reads per sample (**Supplemental Figure 6.1A**). On average, 77.1% of reads mapped to the turkey reference genome (Ensembl Turkey_2.01). For each sample, read pairs were aligned with minimal discordant pairs or pairs with multiple alignments (average of 0.82% and 3.84% respectively) (**Supplemental Figure 6.1B**). The number of reads per sample, the number of mapped reads per sample, and the number of properly aligned pairs per sample did not differ significantly between LEPH and HEPH in any of the tissues examined.

Overview of DEGs

A total of 1824, 1654, 8163, and 580 DEGs between LEPH and HEPH were identified in the F1G, F5G, F5I, and SWF respectively. A significantly higher number of DEGs between LEPH and HEPH were seen in the F5I, whereas a significantly lower number of DEGs between LEPH and HEPH were seen in the SWF. In the F1G, a larger percentage of DEGs were up-regulated in HEPH, whereas in the F5G a larger percentage of DEGs were up-regulated in LEPH (**Figure 6.1A and 6.1B**). In both the F1G and F5G, only a small percentage of genes were unannotated in the turkey genome (5.56% and 5.35%, respectively). In the F5I, roughly equal numbers of the DEGs were up-regulated in LEPH and HEPH (**Figure 6.1C**). In the SWF,

slightly more DEGs were up-regulated in HEPH compared to those up-regulated in LEPH (**Figure 6.1D**). A larger percentage of DEGs in the F5I and SWF were unannotated in the turkey genome compared to the DEGs in the F1G and F5G (26.72% and 40.78%, respectively). A majority of previous studies examining ovarian steroidogenesis and follicle development have focused on the granulosa layer of the follicle wall and may account for the high percentage of unannotated DEGs seen in the F5I and SWF. Additionally, the heterogenous cell populations found in the F5I and SWF, compared to the homogenous cell population found in the granulosa layer, may contribute to the high percentage of unannotated DEGs seen in the F5I and SWF.

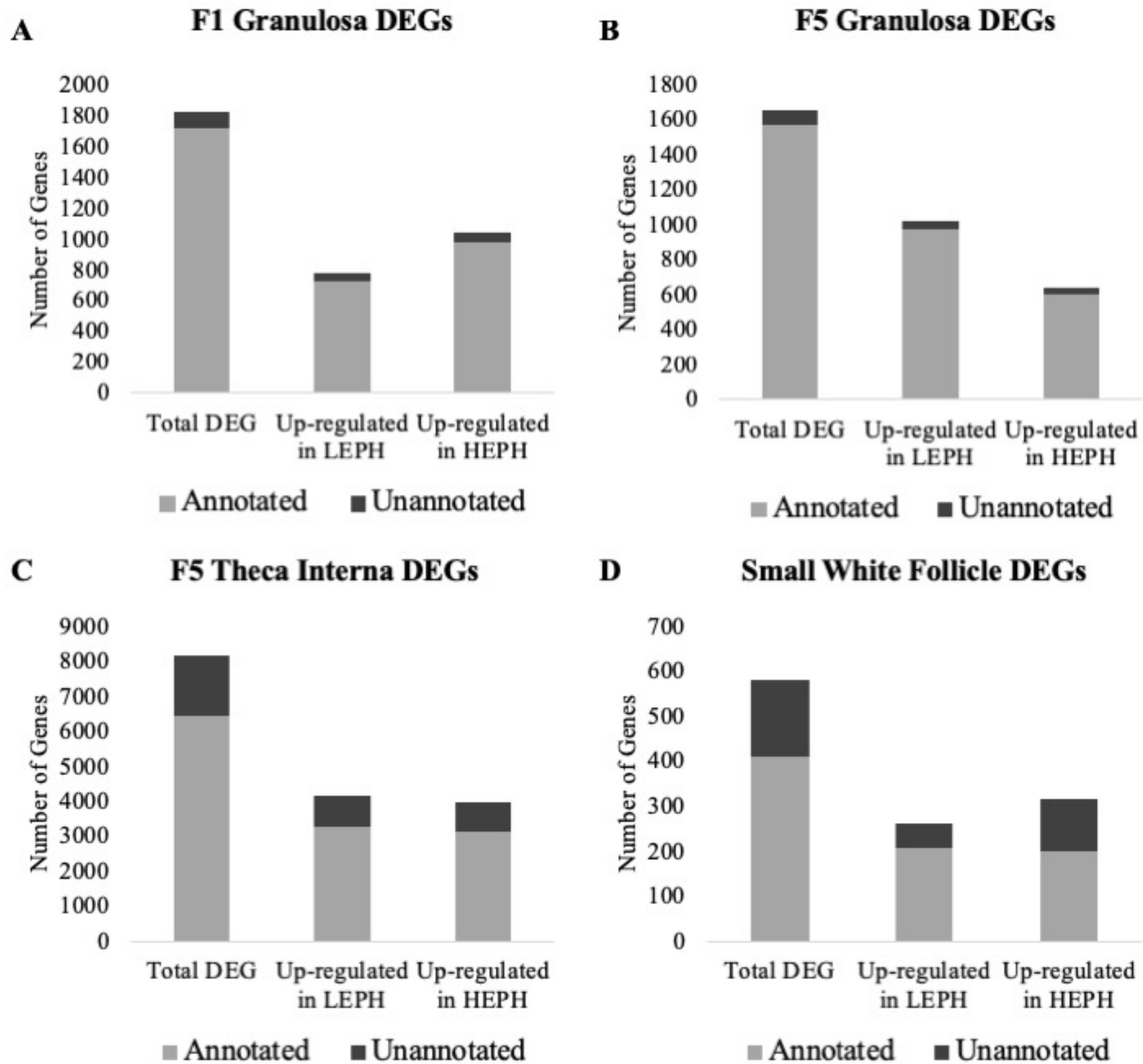


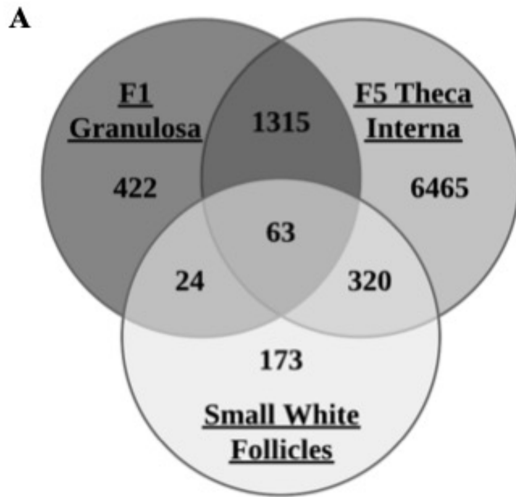
Figure 6.1 Numbers of total, up-regulated in HEPH, and up-regulated in LEPH DEGs between LEPH and HEPH in (A) the F1G, (B) the F5G, (C) the F5I, and (D) the SWF (RPKM>0.2, P<0.05). The portion of genes that are unannotated in the turkey genome are represented in dark grey and the portion of gene that are annotated in the turkey genome are represented in light grey.

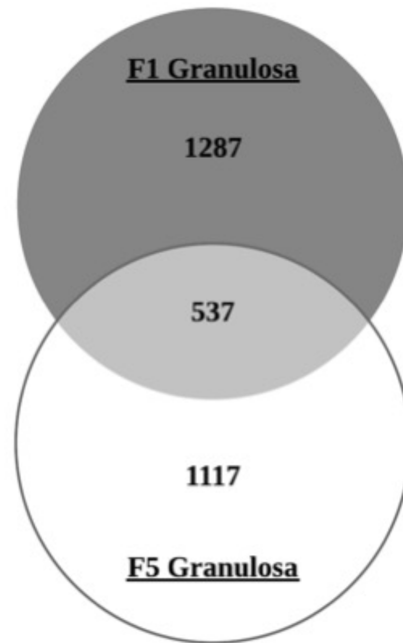
Of the three cell types primarily responsible for P4, AD, and E2 production, 422 DEGs were unique to the F1G, 6465 DEGs were unique to the F5I, and 173 DEGs were unique to the SWF (**Figure 6.2A**). The F1G and F5I had 1315 DEGs in common, the F5I and SWF had 320 DEGs in common, and the F1G and SWF had 24 DEGs in common. The three cells types had 63 DEGs in common, which displayed differential regulation in LEPH and HEPH across the cell types examined (**Figure 6.2B**). Of the DEGs between LEPH and HEPH that were common to the F1G, F5I, and SWF, several have been previously identified as coactivators in the regulation of steroidogenesis. For example, knockdown of four and a half LIM domain 2 (***FHL2***) in mice granulosa cells resulted in decreased expression of *CYP11A1*, which is involved in P4 production (Matulis and Mayo, 2012). Additionally, nuclear factor kappa beta-subunit 2 (***NFKB2***) was identified as an upstream regulator active in bovine thecal cells (Hatzirodos et al., 2017). Of the DEGs common to the F1G, F5I, and SWF, a majority of the genes displayed inverse expression trends in LEPH and HEPH across the follicle cell types. Higher expression of the common DEGs in HEPH was generally seen in the F1G and SWF, indicating possible roles in the regulation of P4 and E2 production. On the other hand, higher expression of the common DEGs in LEPH was generally seen in the F5I, indicating possible roles in the regulation of AD production. Further studies will be necessary to identify possible roles of the common DEGs in steroidogenesis.

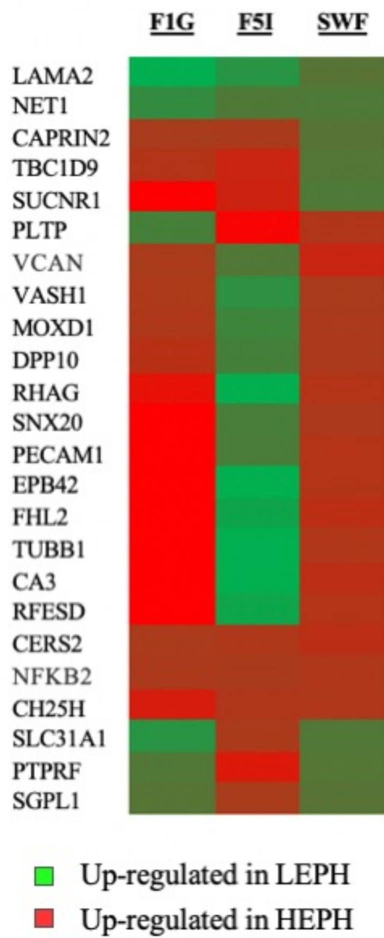
In previous studies, LEPH displayed up-regulation of P4 regulated genes in the F5G, whereas HEPH displayed up-regulation of P4 regulated genes in the F1G (Chapter 3). Moreover, P4 production increases with the amount of time spent in the preovulatory follicle cohort (Johnson and Bridgham, 2001). Comparison of DEGs between LEPH and HEPH in the F1G and F5G, may highlight key differences in movement through the follicular hierarchy. When comparing the F1G and F5G DEGs, 1287 DEGs between LEPH and HEPH were unique to the

F1G, whereas 1117 DEGs between LEPH and HEPH were unique to the F5G (**Figure 6.2C**). Of the DEGs between LEPH and HEPH, 537 DEGs were common to both the F1G and F5G. Of the DEGs between LEPH and HEPH common to both the F1G and F5G, a small percentage displayed up-regulation in HEPH in both the F1G and F5G (5%) or up-regulation in LEPH in both the F1G and F5G (12%). Annexin A2 (***ANXA2***), which plays a role in signal transduction pathways, displayed up-regulation in LEPH in the F1G as well as the F5G and was previously identified as up-regulated in bovine atretic follicles compared to healthy follicles (Hatzirodos et al., 2014a). While atretic follicles where not observed in LEPH, mechanisms similar to those seen in follicle atresia could impact the rate of follicle development in LEPH.

A majority of the DEGs between LEPH and HEPH common to both the F1G and F5G showed inverse regulation in the F1G and F5G (83%) (**Figure 6.2D**). Apolipoprotein A1 (***APOA1***) and clathrin light chain B (***CLTB***) displayed up-regulation in LEPH in the F1G but up-regulation in HEPH in the F5G. *APOA1* encodes the major protein component of high density lipoprotein and was previously shown to up-regulated in less mature follicles rather than more mature follicles in high egg producing ducks, which is consistent with the expression profile seen in HEPH (Wu et al., 2016). *CLTB* encodes two proteins believed to act as regulatory elements and was previously shown to be down-regulated in the granulosa cells swine follicles just prior to ovulation, which is in contrast to expression seen in LEPH (Bonnet et al., 2008). Additionally, *CLTB* was identified to be up-regulated in preovulatory follicles in low egg producing ducks (Tao et al., 2017). Further investigation into the overlapping DEGs between LEPH and HEPH, especially those with inverse expression patterns in the F1G and F5G, may provide insight into the regulatory mechanisms governing the P4 production differences seen in LEPH and HEPH.


A
F1 Granulosa
F5 Theca Interna
1315
422
6465
63
24
320
173
Small White Follicles


C
F1 Granulosa
1287
537
1117
F5 Granulosa

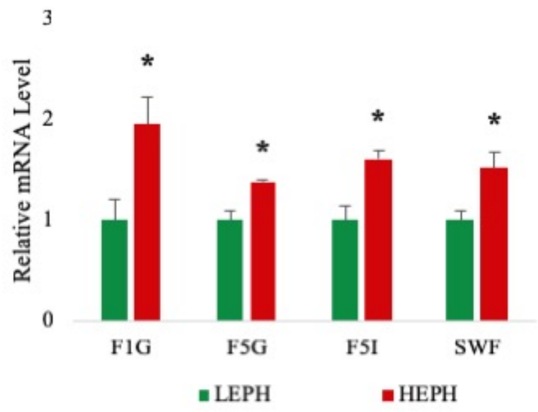

B
F1G
F5I
SWF
LAMA2
NET1
CAPRIN2
TBC1D9
SUCNR1
PLTP
VCAN
VASH1
MOXD1
DPP10
RHAG
SNX20
PECAM1
EPB42
FHL2
TUBB1
CA3
RFESD
CERS2
NFKB2
CH25H
SLC31A1
PTPRF
SGPL1
Up-regulated in LEPH
Up-regulated in HEPH

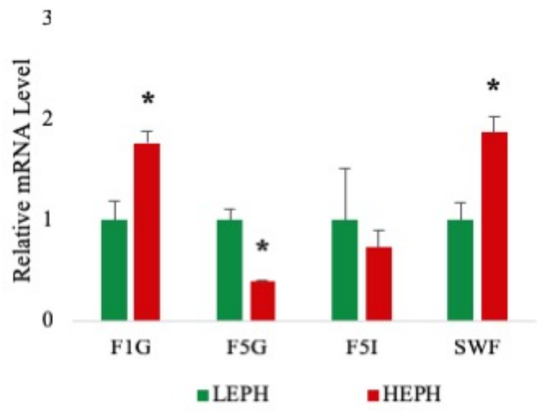
D DEGs Common to F1G and F5G
Higher espression in HEPH in F1G and F5G
Inverse Regulation in F1G and F5G
Higher espression in LEPH in F1G and F5G

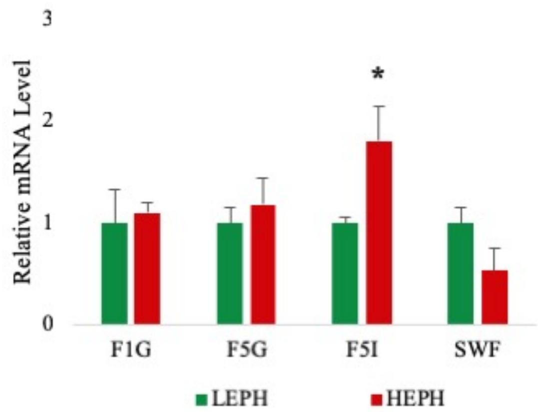
Figure 6.2 (A) Venn diagram showing the number of DEGs between LEPH and HEPH that are unique to the F1G, F5I, and SWF as well as the number of DEGs common to one or more follicle cell types (RPKM>0.2, P<0.05). (B) Heat map showing the expression profiles of the DEGs common to the F1G, F5I, and SWF displaying high expression in each of the follicle cell types examined (RPKM>10, P<0.05). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (C) Venn diagram showing the number of DEGs in unique to the F1G and F5G as well as the number of DEGs common both the F1G and F5G (RPKM>0.2, P<0.05). (D) DEGs common to both the F1G and F5G displaying high expression in both follicle cell types broken down by expression pattern in LEPH and HEPH (RPKM>10, P<0.05).

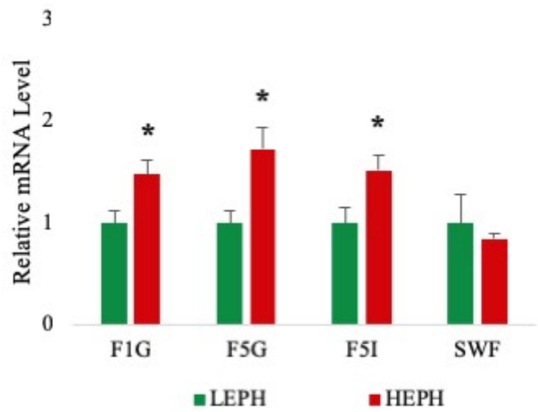
RNA sequencing confirmation

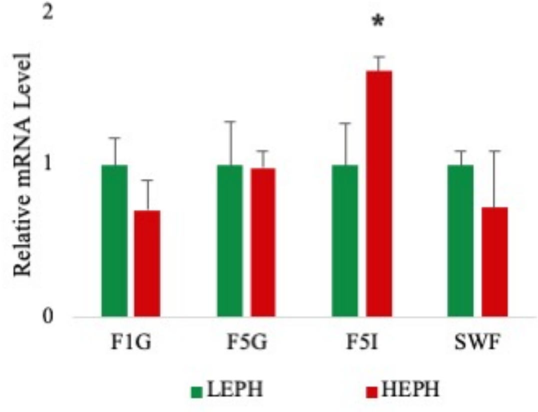
A total of 6 genes common to all four tissues that displayed high expression and different expression patterns among the four tissues were selected for confirmation in each tissue through RT-qPCR. RT-qPCR results confirmed increased expression of ceramide synthase 2 (***CERS2***) in HEPH in all four tissues, increased expression of insulin-like growth factor 1 receptor (***IGF1R***) in HEPH in the F1G and SWF as well as in LEPH in the F5G), increased expression of *ESYT3* in HEPH in the F5I, increased expression of *BLOC1S4* in HEPH in the F1G, F5G, and F5I, increased expression of beta-actin (***ACTB***) in HEPH in the F5I, and increased expression of *PGK1* in HEPH in the F5G and F5I (**Figure 6.3**). Each of the confirmation genes examined in the F1G, F5G, F5I, and SWF showed expression profiles similar to those obtained through RNA sequencing.


A
CERS2: ↑ in HEPH (all tissues)
3
2
1
0
Relative mRNA Level
*
*
*
*
F1G
F5G
F5I
SWF
LEPH
HEPH


B
IGF1R: ↑ in HEPH (F1G and SWF)
↑ in LEPH (F5G)
3
2
1
0
Relative mRNA Level
*
*
*
F1G
F5G
F5I
SWF
LEPH
HEPH


C
ESYT3: ↑ in HEPH (F5I)
3
2
1
0
Relative mRNA Level
*
F1G
F5G
F5I
SWF
LEPH
HEPH


D
BLOC1S4: ↑ in HEPH (F1G, F5G, F5I)
3
2
1
0
Relative mRNA Level
*
*
*
F1G
F5G
F5I
SWF
LEPH
HEPH


E
ACTB: ↑ in HEPH (F5I)
2
1
0
Relative mRNA Level
*
F1G
F5G
F5I
SWF
LEPH
HEPH

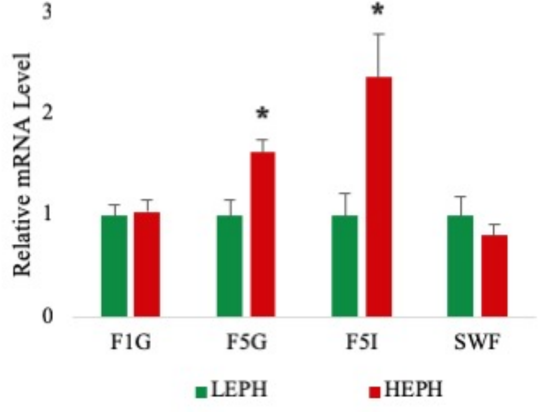

F
PGK1: ↑ in HEPH (F5G and F5I)
3
2
1
0
Relative mRNA Level
*
*
F1G
F5G
F5I
SWF
LEPH
HEPH

Figure 6.3 Confirmation of RNA sequencing results in the F1G, F5G, F5I, and SWF. A total of 6 genes common to all four tissues that displayed high expression and different expression patterns among the four tissues were selected for confirmation in each tissue through RT-qPCR. The expression differences between LEPH and HEPH for each gene seen in RNA sequencing results are listed above each graph. Normalized data are presented relative to LEPH expression for each follicle type for each gene. Significant expression differences between LEPH and HEPH for a given follicle type are denoted with an asterisk (*). (A) Relative mRNA levels for *CERS2*, which was up-regulated in HEPH in all four tissues in RNA sequencing results. (B) Relative mRNA levels for *IGFR1*, which showed increased expression in HEPH in the F1G and SWF and increased expression in LEPH in the F5G in RNA sequencing results. (C) Relative mRNA levels for *ESYT3*, which was up-regulated in HEPH in the F5I in RNA sequencing results (D) Relative mRNA levels for *BLOC1S4*, which showed increased expression in HEPH in the F1G, F5G, and F5I in RNA sequencing results. (E) Relative mRNA levels for *ACTB*, which showed up-regulation in HEPH in the F5I in RNA sequencing results. (F) Relative mRNA levels for *PGK1*, which showed up-regulation in HEPH in the F5G and F5I in RNA sequencing results.

F1G network analysis

The F1G is mainly responsive to stimulation by LH, yet, LEPH displayed up-regulation of *FSHR* when compared to HEPH (**Figure 6.4A**). In previous studies, FSHR expression was shown to decrease in chicken follicles during maturation (Johnson and Woods, 2009). Up-regulation of *FSHR* may interfere with the actions of LH in the mature follicle. Additionally, HEPH displayed up-regulation of RB binding protein 8 endonuclease (***RBBP8***) and U2 snRNP associated SURP domain containing (***U2SURP***) relative to LEPH, which were also found to be up-regulated in healthy bovine follicles (Hatzirodos et al., 2014a). LEPH exhibited up-regulation of *TTR*, a thyroid hormone transporter, which could play a role in eliciting the effects of thyroid hormone on the F1G (**Figure 6.4B**). Additionally, LEPH displayed increased expression of shisa family member 2 (***SHISA2***), which was also up-regulated in atretic bovine follicles (Hatzirodos et al., 2014a). HEPH showed increased expression of insulin induced gene 1 (***INSIG1***), which has been shown to up-regulate *LHCGR* in mouse granulosa cells (Menon et al., 2018). *INSIG1* up-regulation of *LHCGR* in HEPH, coupled with the increased expression of *FSHR* in LEPH, could be responsible for the increased responsiveness of F1G cells from HEPH to LH treatment while F1G cells from LEPH do not respond to LH treatment (Chapter 4).

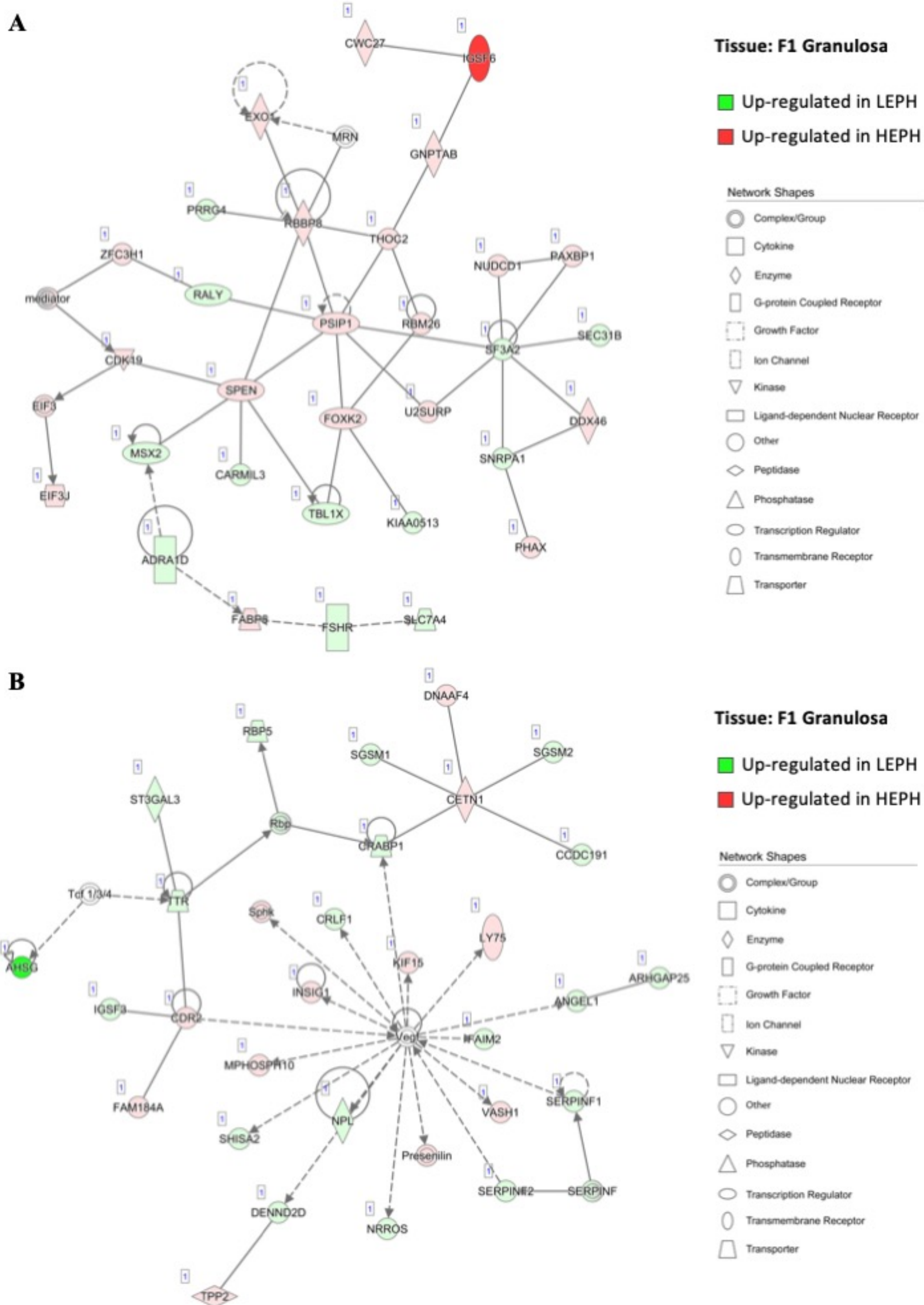


Figure 6.4 IPA network analysis in the F1G comparing LEPH and HEPH gene expression (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.

F5G network analysis

In the F5G, HEPH exhibited increased expression of inhibin subunit alpha (***INHA***) and inhibin subunit beta B (***INHBB***) compared to LEPH (**Figure 6.5A**). Ovarian inhibins feedback on the pituitary as well as exhibit local effects in the ovary related to follicle development (Welt et al., 2002). *INHA* and *INHBB* mRNA levels increase in the granulosa cells during follicle development, suggesting that the F5G from HEPH may be more developed than the F5G from LEPH due to a more rapid follicle development rate to keep up with increased ovulation rates in HEPH (Lovell et al., 1998). LEPH exhibited increased expression of the integrin receptor in the F5G when compared to HEPH (**Figure 6.5B**). The integrin receptor binds thyroid hormones to elicit non-genomic actions of thyroid hormone in target issues. The mRNA levels of the two genes encoding the subunits of the integrin receptor (*ITGAV* and *ITGB3*) decrease significantly with follicle maturation (Sechman, 2012). Up-regulation of the integrin receptor in LEPH is consistent with expression profiles seen in less mature follicles, possibly indicating that LEPH follicles are moving slower through the follicular hierarchy than in HEPH.

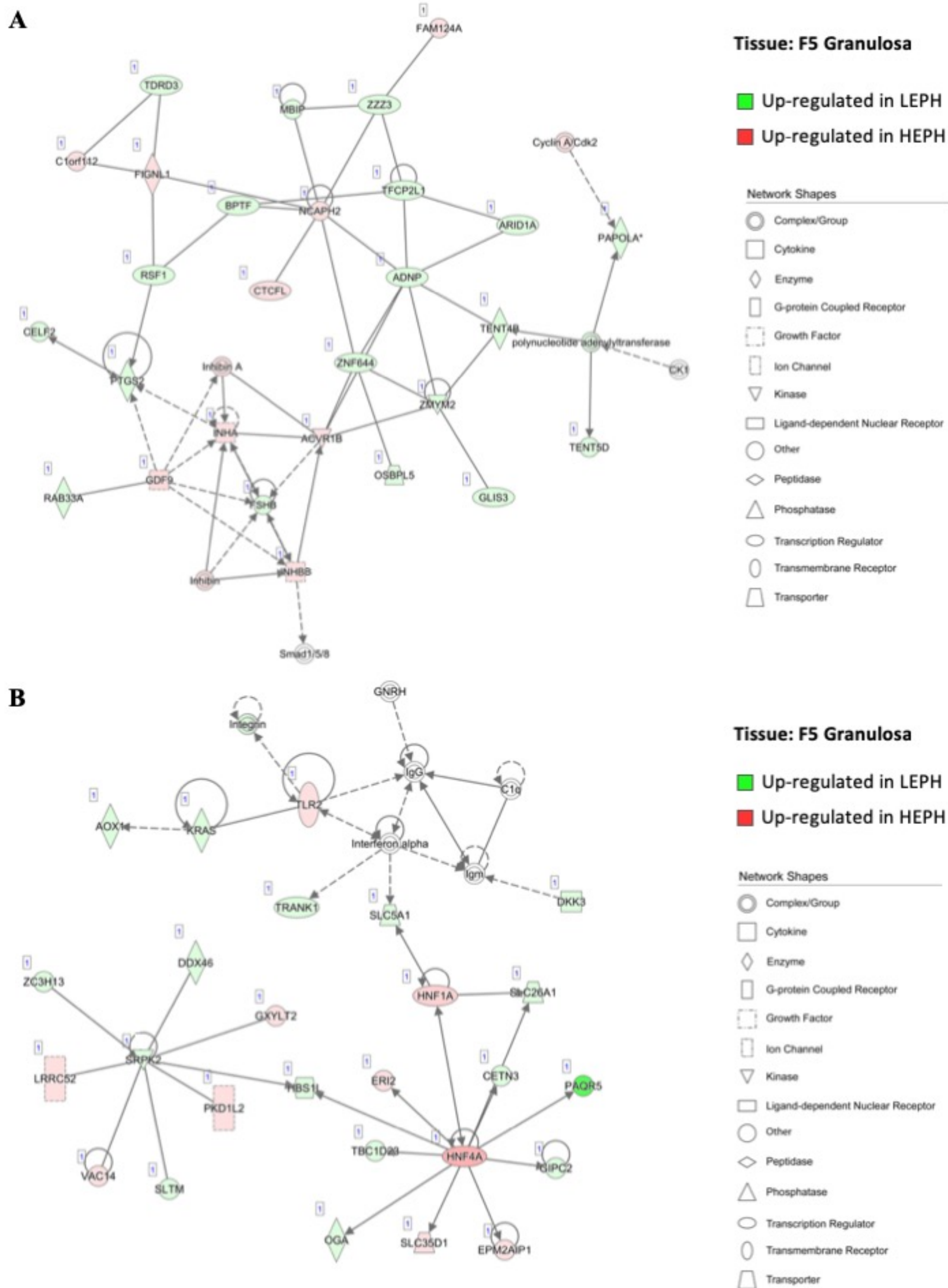


Figure 6.5. IPA network analysis in the F5G comparing LEPH and HEPH gene expression (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.

F5I network analysis

In the F5I, LEPH exhibited increased expression of 17β-hydroxysteroid dehydrogenase 11 (***HSD17B11***), which is involved in AD synthesis (**Figure 6.6A**). This is consistent with previous studies that found genes related to AD production to be up-regulated in the F5I from LEPH compared to HEPH (Chapter 3). In addition, LEPH showed higher expression of DENN domain containing 2D (***DENND2D***), which was previously shown to be up-regulated in bovine atretic follicles (Hatzirodos et al., 2014a). HEPH, on the other hand, showed increased expression of *ESR1* and Rho GTPase activating protein 18 (***ARHGAP18***) when compared to LEPH (**Figure 6.6B**). Upon estradiol binding, *ESR1* binds estrogen response elements in the promotor region of target genes to regulate transcription (Hammes and Davis, 2015). Up-regulation of *ESR1* could allow HEPH to be more responsive to E2 acting in a paracrine fashion. *ARHGAP18* modulates cell signaling and previous studies demonstrated that in chicken and bovine follicles, *ARHGAP18* expression increased significantly during follicle development (Hatzirodos et al., 2014b). Up-regulation of *ARHGAP18* in HEPH, once again, supports the hypothesis that follicles from HEPH are moving through the follicular hierarchy quicker than follicles from LEPH.

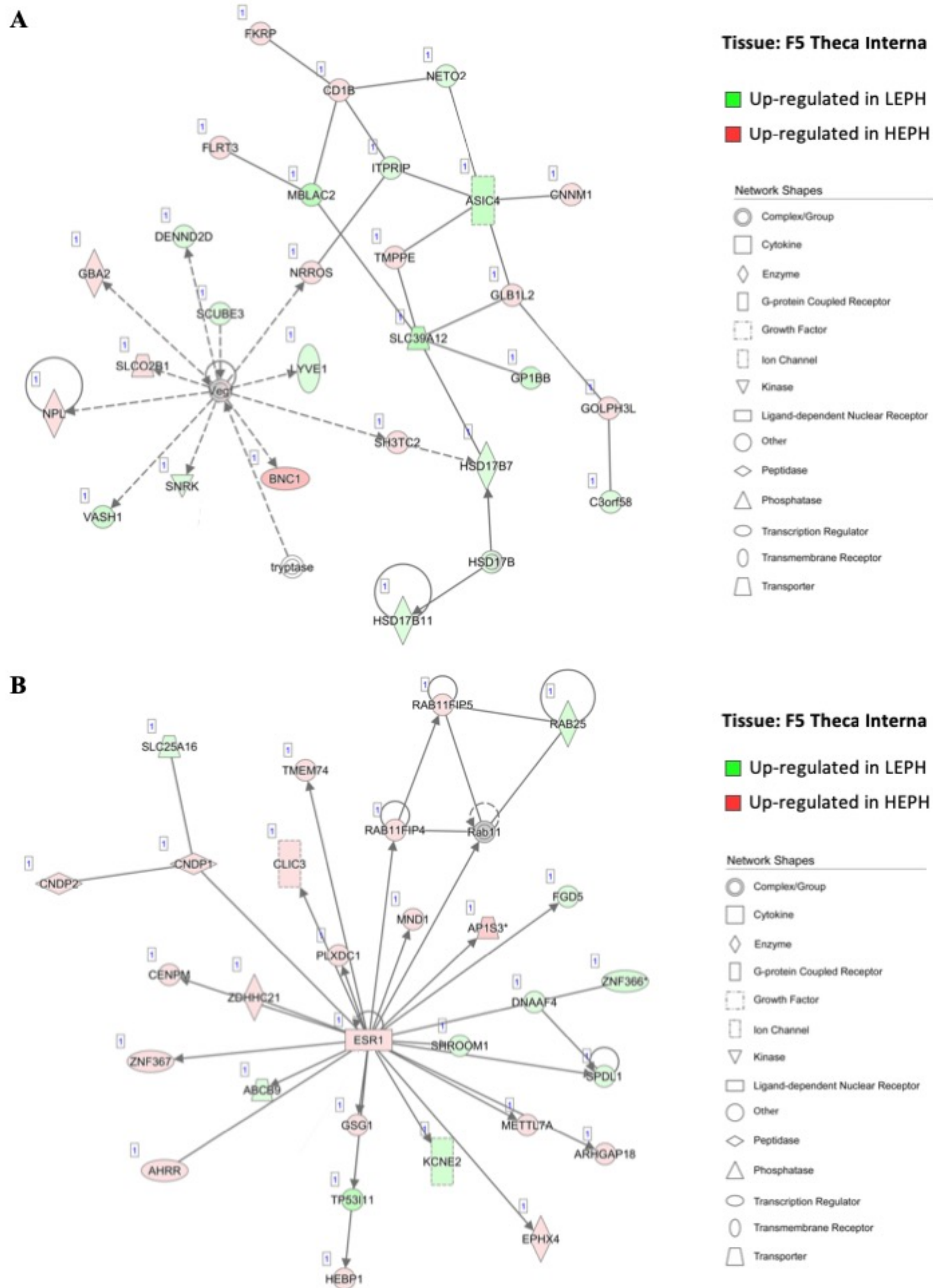


Figure 6.6 IPA network analysis in the F5I comparing LEPH and HEPH gene expression (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.

SWF network analysis

In the SWF, HEPH exhibited up-regulation of *CYP17A1*, which is involved in AD and E2 synthesis (**Figure 6.7A**). This supports results from previous studies that found that genes related to estradiol production were up-regulated in SWF in LEPH compared to HEPH (Chapter 3). HEPH also showed up-regulation of the integrin receptor in SWF relative to levels in SWF from LEPH (**Figure 6.7B**). This is in stark contrast to expression pattern of the integrin receptor in the F5G, which showed up-regulation in LEPH compared to HEPH. Previous studies in laying hens have shown that the subunits of integrin receptor display higher expression in SWF compared to more mature follicle types (Sechman, 2012). Up-regulation of the integrin receptor in SWF from HEPH may increase the sensitivity of HEPH to the non-genomic impact of thyroid hormone on steroidogenesis.

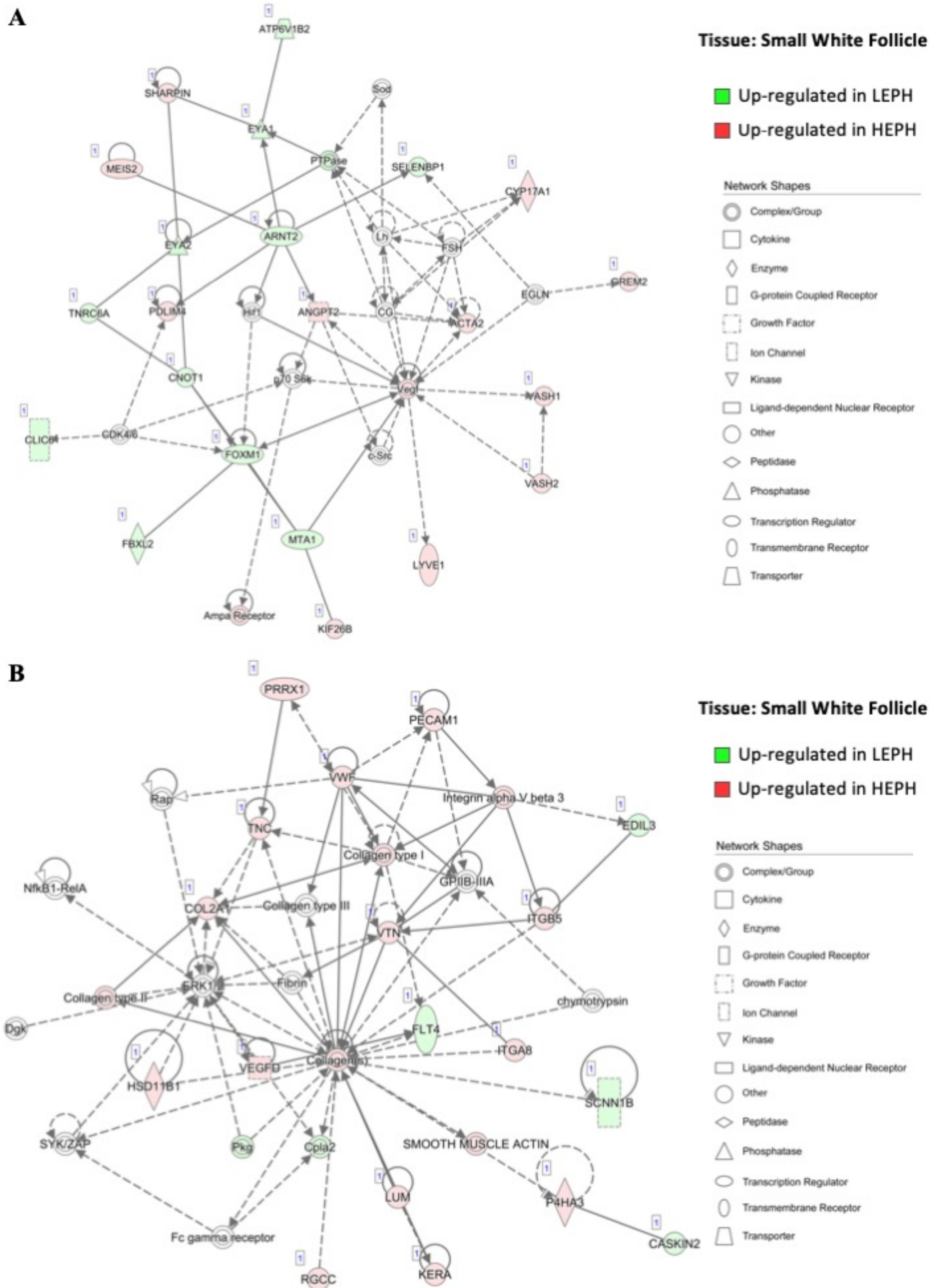


Figure 6.7 IPA network analysis in the SWF comparing LEPH and HEPH gene expression (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH.

Upstream analysis

Analysis of the predicted upstream regulators for the follicle cell types examined showed a general activation by beta-estradiol. While the calculated z-score varied for the comparisons examined, beta-estradiol was the only upstream regulator common to all of the comparisons. Additionally, beta-estradiol was among the top five upstream regulators in the F1G, F5G, and F5I (**Table 6.1**). Beta-estradiol feedback on the hypothalamus and pituitary to impact the ovulatory process but also exerts local regulation in the ovary (Christian and Moenter, 2010; Nelson and Bulun, 2001). For the comparisons between LEPH and HEPH, beta-estradiol was significantly more active in HEPH in the F5I (z-score = 2.268) and SWF (z-score = 2.588). While beta-estradiol was a top upstream regulator in the F1G and F5G, the activation z-score was less than 2 in these tissues. Differentially expressed target genes of beta-estradiol in the F5I and SWF include *CYP17A1*, hydroxysteroid 17 beta dehydrogenase 2 (***HSD17B2***), and *CYP19A1*, all of which are involved in AD and E2 production. Upstream analysis of the hypothalamus and pituitary of LEPH and HEPH also predicted beta-estradiol as significant upstream regulator generally activated in HEPH (Chapter 5).

Table 6.1. Top five significant upstream regulators between LEPH and HEPH for each follicle cell type (RPKM>0.2, P<0.05, |fold change|>1.5).

F1 Granulosa

Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
TP53	transcription regulator	0.447	6.35E-18	287
beta-estradiol	chemical - endogenous	0.661	6.77E-18	300
dexamethasone	chemical drug	0.768	1.69E-17	311
ERBB2	kinase	-1.763	1.15E-14	140
GATA1	transcription regulator	2.387	5.65E-14	63

F5 Granulosa

Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
ESR1	ligand-dependent nuclear receptor	-0.993	8.77E-20	204
beta-estradiol	chemical - endogenous	0.028	2.65E-19	271
E2F4	transcription regulator	0.900	2.21E-11	51
CDKN1A	kinase	-2.092	8.89E-11	49
MITF	transcription regulator	1.889	4.35E-10	50

F5 Theca Interna

Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
beta-estradiol	chemical - endogenous	2.268	1.41E-20	460
TGFB1	growth factor	-0.979	1.4E-15	408
TNF	cytokine	2.607	7.8E-15	403
IFNG	cytokine	2.984	6.56E-12	309
dexamethasone	chemical drug	0.319	1.04E-11	437

Small White Follicles

Upstream Regulator	Molecule Type	Z-Score	P-Value	Target Genes
TGFB1	growth factor	4.26	7.97E-10	84
SRF	transcription regulator	3.198	8.54E-10	30
TNF	cytokine	0.506	1.57E-08	80
SP1	transcription regulator	0.427	7.8E-08	36
DLL4	other	1.782	1.48E-07	9

Effect of T3 on SWF E2 production

Based on IPA analysis of the follicle cell types, the HPT axis was identified as a possible regulator of the differences in steroidogenesis seen in LEPH and HEPH. THRs and thyroid hormone transporters appeared to be up-regulated LEPH in more mature follicles and up-regulated in HEPH in less mature follicles. Previously, HEPH SWF cells were shown to be more responsive to FSH stimulated E2 production than LEPH (Chapter 4). To examine if thyroid hormone impacted the elevated estradiol production levels seen previously in HEPH, isolated SWF cells from LEPH and HEPH were subjected to pretreatment with either NPT or 1.5 ng/mL of T3 for 12 hours, followed by FSH treatment at 0, 10, and 100 ng/mL for 5 hours (**Figure 6.8**).

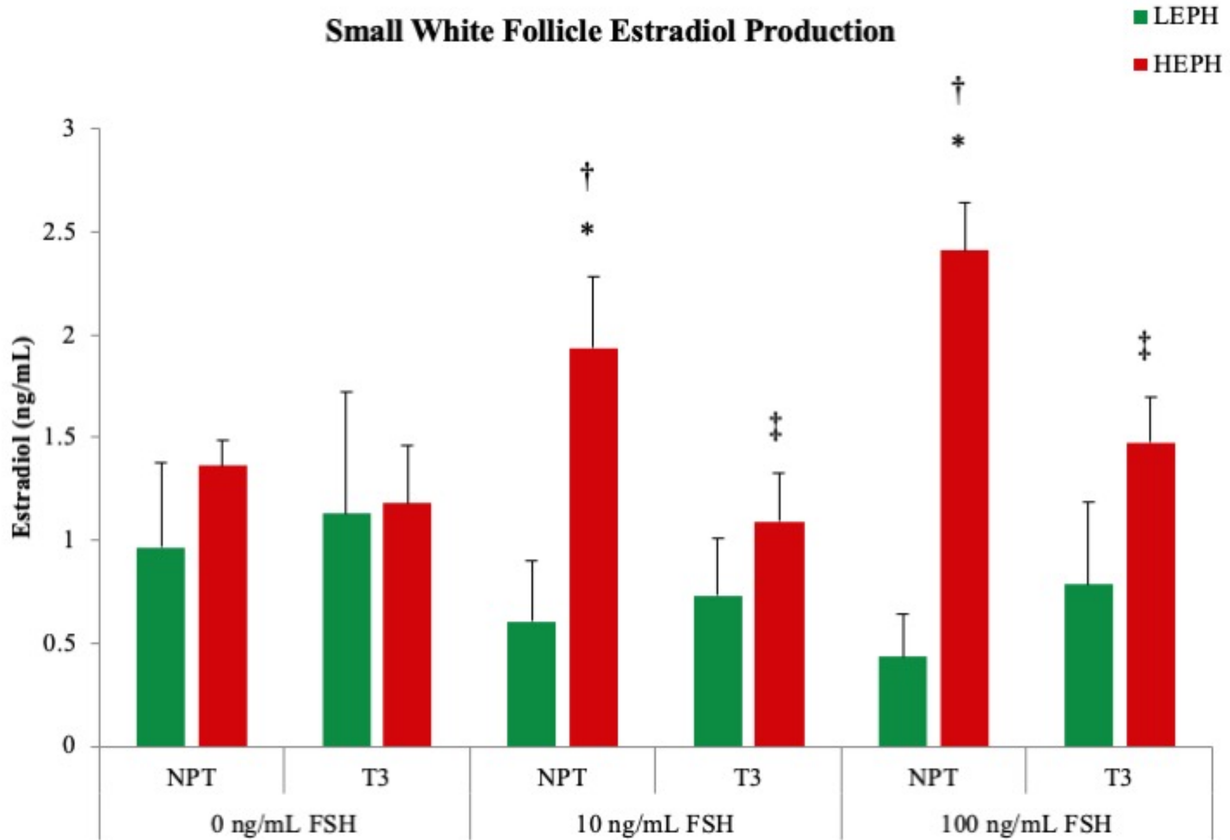


Figure 6.8 E2 production in small white follicle cells from LEPH and HEPH after pretreatment with either NPT or T3 followed by treatment with FSH. Significant differences in E2 production between LEPH and HEPH for a given condition are denoted with an asterisk (*). Significant differences between FSH treatments for a given egg production group are denoted with a dagger (†). Significant differences between pretreatments for a given egg production group are denoted with a double dagger (‡).

Basal E2 production *in vitro* did not differ between SWF cells from LEPH and HEPH, regardless of pretreatment. However, at 10 and 100 ng/mL treatment of FSH, HEPH SWF cells showed significantly higher E2 production when compared to LEPH. SWF cells from LEPH did not respond to either FSH treatment in terms of E2 production. E2 production levels in LEPH and HEPH cells subjected to NPT are consistent with previous results (Chapter 4). Pretreatment with T3, both at 10 and 100 ng/mL of FSH, decreased E2 production in SWF cells from HEPH, reducing E2 production to levels seen in LEPH. The depression of gonadotropin stimulated E2 production following T3 treatment seen in HEPH is consistent with the results previously reported in laying chicken hens (Sechman et al., 2009).

Overall*, in vitro*, SWF cells from HEPH were more responsive to T3 treatment, with T3 treatment significantly decreasing FSH-induced E2 production. Furthermore, the addition of T3 pretreatment caused SWF cells from HEPH to respond to FSH in a similar manner to that seen in cells from LEPH. These results suggest that the HPT axis may play a role in differentially regulating E2 production in the SWF of LEPH and HEPH. Taken together with the up-regulation of integrin subunits in HEPH in SWF network analysis, it could be hypothesized that the impact of T3 on E2 production may be elicited through non-genomic mechanisms. Further studies will be necessary to clarify the role of the thyroid hormone in the regulation of SWF E2 production as well as in the regulation of P4 and AD production from the F1G and F5I, respectively. Additional studies examining the effect of circulating thyroid hormones on steroid hormone feedback on the hypothalamus and pituitary may explain the impact of thyroid hormone regulation of steroidogenesis on HPG axis function, and ultimately egg production rates.

Conclusions

Transcriptome analysis the follicle cell types responsible for P4, AD, and E2 production in LEPH and HEPH provided insights the differential regulation of steroidogenesis and follicle development between the two groups of hens. Several trends in gene expression were seen through the comparison of LEPH and HEPH. The first was up-regulation of genes involved in P4 and E2 production in HEPH while LEPH displayed up-regulation of genes involved in AD production. These results are consistent with previous studies examining LEPH and HEPH (Chapters 3 and 4). The second was the up-regulation of HPT axis related genes in LEPH in more mature follicle, yet the up-regulation of HPT axis related genes in HEPH in less mature follicles. These results are consistent with the hypothesis that the thyroid axis impacts follicular development and steroidogenesis differently in LEPH and HEPH. This hypothesis was further supported *in vitro*, with T3 treatment decreasing FSH-stimulated E2 production in SWF cells from HEPH but did not impact SWF cells from LEPH. Up-regulation of the HPT axis later in follicle development, as is seen in LEPH, is inconsistent with the marked decreased expression of THRs during follicle development established in previous studies and indicates that the HPT axis may play a role in slowing the progress of developing follicles through the follicular hierarchy in LEPH (Sechman et al., 2009). The third was the up-regulation of E2 signaling in HEPH. Significant activation of beta-estradiol upstream regulation in HEPH was predicted in less mature follicle types.

Generally, across the follicle types examined, LEPH showed gene expression consistent with a longer progression through the follicular hierarchy. LEPH displayed up-regulation of genes previously associated with atretic follicles, though macroscopically, atretic follicles were not found in LEPH (Hatzirodos et al., 2014a). These genes may be up-regulated in atretic

follicles due to the decrease in follicle cell proliferation and development seen in atretic follicles. Up-regulation of these genes in LEPH throughout follicle development, may imply that LEPH follicle cells are not developing as fast as those from HEPH. On the other hand, HEPH displayed up-regulation of genes previously associated with healthy follicles, which further supports the hypothesis that follicle development is more rapid in HEPH compared to LEPH. Lastly, prior to ovulation, LEPH up-regulate *FSHR,* which is normally up-regulated earlier in follicle development, whereas HEPH up-regulate genes that have been shown to positively regulate the LH receptor gene, which is normally up-regulated at the end of follicle development.

Collectively, based on the transcriptome analysis of the primary steroid hormone producing follicle cell types in LEPH and HEPH, follicle development appears to be more rapid in HEPH compared to LEPH, and may be responsible for the differential steroidogenesis capabilities between LEPH and HEPH. Follicle development differences in LEPH and HEPH may include regulation by thyroid hormone or E2. Further studies will be necessary to clarify the possible role of thyroid hormones and estradiol in follicle development rates in hens with differential egg production.

Materials and Methods

Hen Selection and Tissue Collection

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual wire cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards. Hens were sampled at 37 weeks of age. Daily egg records were used to calculate each hen's number of EPD by dividing the total number of eggs produced by the number of days in production. Hens were classified as LEPH when EPD<0.6 and as HEPH when EPD>0.8. EPD

cutoffs for LEPH and HEPH were based on previous studies examining average flock egg production and egg production distribution (Chapter 2). The F1, F5, and SWF were isolated from three LEPH and HEPH outside of the PS. Additionally, three LEPH and HEPH were sampled outside of the PS for SWF isolation and culture. The timing of the PS was predicted using hourly egg records as previously described (Chapter 2). All hens were sampled on the second day of the hen's sequence. Blood samples were taken from the wing vein immediately before sampling and fractionated by centrifugation.

Plasma samples were stored at -20°C prior to assessment through RIAs as described below. Plasma P4 levels were examined to confirm correct sampling outside of the PS. F1 and F5 follicles were subjected to isolation of the three cell types from the follicle wall prior to RNA extraction, as described below. Whole SWF samples from LEPH and HEPH were snap frozen in liquid nitrogen and stored at -20°C prior to assessment through RNAseq and RT-qPCR, as described below. SWF samples from LEPH and HEPH exclusively sampled for cell culture were dispersed prior to culture as described below. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland.

Follicle Wall Cell Isolation

The granulosa and theca interna were isolated from the F1 and F5 follicles using an adapted published method, as previously described (Porter et al., 1989; Chapter 2). Briefly, the yolk was drained from each follicle and the follicle was inverted to peel off the granulosa layer. The theca interna layer was scraped from the inverted follicle. All follicle layers were subjected to trypsin dispersion (1 mg/mL) followed by layering onto a Percoll suspension (50%) to remove debris and red blood cells. Only the F1G, F5G, and F5I of the F1 and F5 follicles were used for

this study. Isolated cells were snap frozen and stored at -20°C prior to assessment through RNAseq and RT-qPCR, as described below.

RNA Isolation, cDNA Library Construction, and Sequencing

Total RNA was extracted from isolated F1G, F5G, and F5I cells and from whole SWF using RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA was performed as previously described (Chapter 2). Amplified cDNA was generated using a SMART-Seq Ultra Low Input RNA kit (Takara Bio, Kusatsu, Japan) following the manufacturer's procedure and long distance, as previously described (Chapter 5). Amplified cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) and quantified using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Santa Clara, CA), as previously described (Chapter 5). To generate sequencing libraries, a Nextera XT DNA library kit (Illumina, San Diego, CA), following the manufacturer's procedure, and 150pg of amplified cDNA per library was used, as previously described (Chapter 5). For each sample two libraries were produced (from the same amplified cDNA), each library having a unique index pairing, and libraries were purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), as previously described (Chapter 5). The libraries were quantified using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit (Agilent, Santa Clara, CA). For sequencing 24 libraries (4 tissues) were pooled (10nM). Libraries were pooled so that set 1 for each tissue was sequenced in a different pool than set 2. Pools were submitted to North Carolina State University's GSL facility for paired-end sequencing (75 bp reads) on an Illumina NextSeq 500.

Bioinformatic Analysis of Sequencing Data

Processing and analysis of sequencing data was performed using CLC genomics workbench (Qiagen, Valencia, CA). Adapter sequences and low quality sequences (Phred < 20) were removed from FASTQ files using the NGS trim tool. Trimmed reads were mapped to the *Meleagris gallopavo* reference genome (Turkey_2.01). The RNA-seq analysis suite was used to analyze mRNA libraries. DEGs were determined using the "Differential Expression for RNA-Seq" tool. Pairwise comparisons were made between LEPH and HEPH for each timepoint in the ovulatory cycle as well as between timepoints in the ovulatory for each egg production group. Due to poor annotation of the turkey genome, the protein sequences for DEGs that were unannotated in the turkey were subjected to orthologous comparisons in human, mouse, and chicken protein sequences using BIOMART (Ensembl). Unannotated DEGs were assumed orthologous if greater than 50% identity to the human, mouse, and chicken was seen at the protein level.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, Valencia, CA) was performed on the differential expression data. IPA was used to construct gene networks as well as to predict upstream biological regulators based on DEGs between LEPH and HEPH for each tissue. Only DEGs with RMPK>0.2 were used for IPA analysis. The RPKM threshold was selected based on the distribution of $\log_2$ transformed RPKM values across all of the comparisons examined. The threshold of DEGs was set at $P < 0.05$ and absolute fold change ≥1.5. Pathways and predicted upstream regulators with P-value < 0.05 (Fischer's exact test) were considered to be statistically significant. For upstream regulators, published findings in the Ingenuity knowledge database were used to calculate the activation z-score to infer activation or inhibition of transcriptional

regulators. Upstream regulators with a z-score greater than 2 or less than -2 and $P<0.05$ were considered to be significantly activated or inhibited.

Culture of SWF

SWF were dispersed as previously described (Chapter 4). SWF cells were diluted to a density of 10,000 cells/mL for culture. SWF cells were cultured in SMEM supplemented with 0.1% bovine serum albumen, 100-U/mL penicillin G, and 100-µg/mL streptomycin sulfate in 12x75 mm polypropylene tubes ($1x10^5$ cells per tube). Cells were pretreated with either no NPT (10 µl SMEM added) or 1.5 ng/mL of T3 for 12 hours, followed by treatment with porcine FSH (National Hormone & Peptide Program, Torrance, CA) at 0, 10, or 100 ng/mL for 5 hours. Cells were maintained in a 37.5°C, 5% CO_2 atmosphere during incubation. After incubation, the media from the SWF cell cultures was recovered and stored at -20 °C prior to assessment through an E2 RIA.

RIAs

The RIAs used for P4 and E2 were coated tube kits (MP Biomedicals, Solon, OH). All protocols were performed as directed by the supplier. All samples were assayed in duplicate and were measured in a single RIA for each hormone. Plasma samples were ether extracted and analyzed for P4 to determine that hens were sampled outside of the PS. Culture media from the SWF cell cultures were assayed for E2 content. The standard curve was assessed for linearity as well as dilutional parallelism using serial plasma or culture media dilutions. The intraassay coefficients of variation determined by pools run every 30 samples were 2.89% for P4 and 4.36% for E2.

RNAseq confirmation

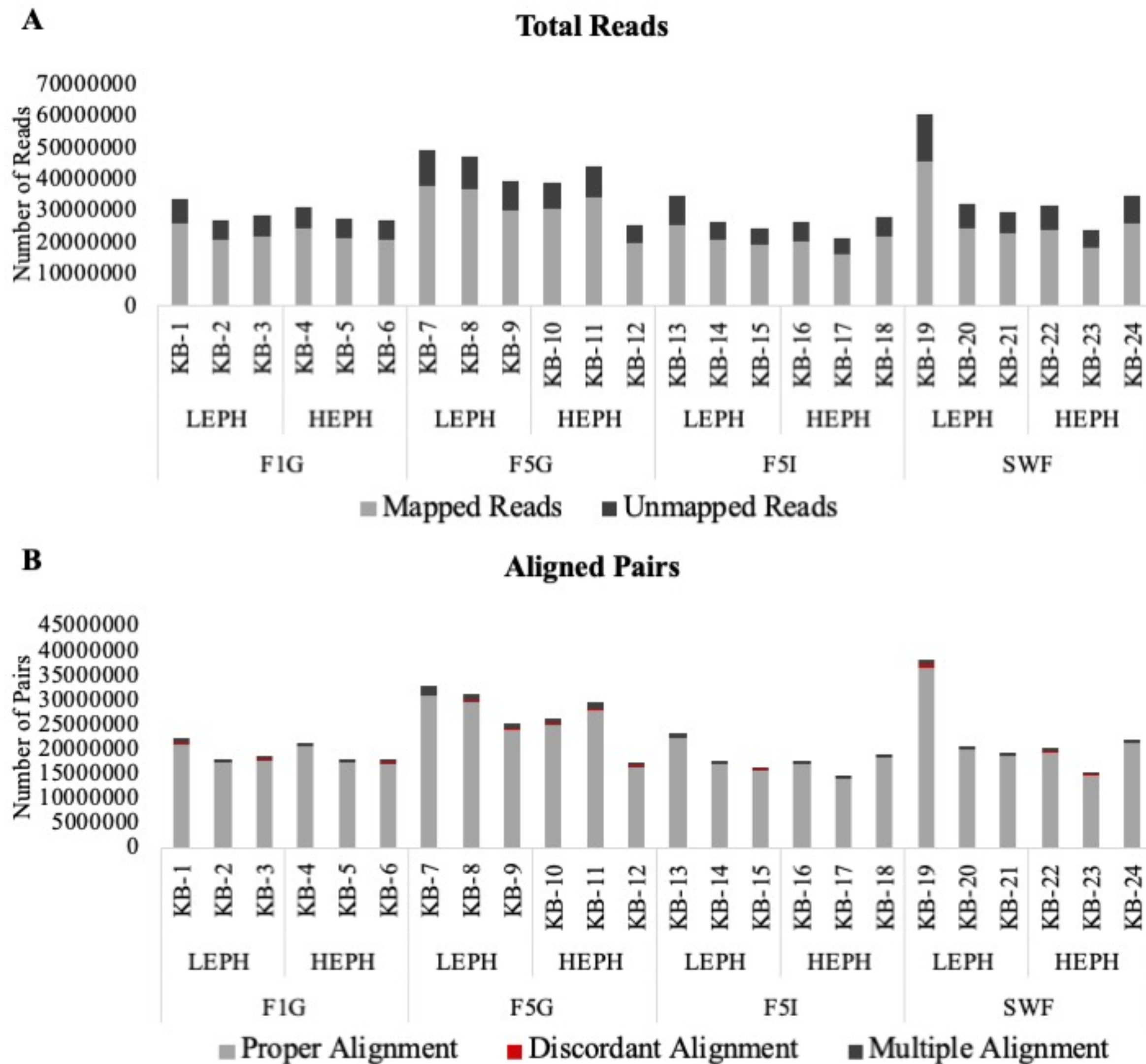
Extracted and quantified RNA from F1G, F5G, F5I, and SWF used for cDNA library construction was reverse transcribed for PCR analysis, as previously described (Chapter 2). A pool of total RNA was made and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. PCR reactions (15 μL) were carried out as previously described using a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA) (Chapter 2). Data from all follicle cell types were normalized to *GAPDH* and were analyzed by the $2^{-\Delta\Delta Ct}$ method. All PCR reactions for each gene in a given cell type were analyzed within a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates. For each tissue, mRNA levels for 6 confirmation genes was determined. DEGs selected for RNAseq confirmation fit the following parameters: $P<0.05$, absolute fold change greater or equal to 1.5, annotated in the turkey genome, encoded by a single transcript, and highly expressed in all follicle cell types examined. Primers were designed as described above. Data are presented as fold increase over mRNA levels for LEPH outside of the PS for each gene.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data for RNA sequencing confirmation genes were $\log_2$ transformed before statistical analysis. A two-way ANOVA using the mixed models procedure was used to compare the $\log_2$ transformed gene expression between LEPH and HEPH from hypothalamus and pituitary samples used for RNAseq confirmation. A three-way ANOVA using the mixed models procedure was conducted to compare the $\log_2$ transformed gene expression between LEPH and HEPH pituitary cell culture samples subjected to different pretreatment and treatment

combinations. The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$.

Supplemental Figures



Supplemental Figure 6.1 (A) The number of reads obtained for each sample. The portion of mapped reads for each sample is represented in blue, whereas the portion of unmapped reads for each sample is represented in orange. (B) The number of aligned pairs obtained for each sample. The portion of aligned pairs with proper alignment is represented in light gray, with discordant alignment in red, and with multiple alignments in dark gray.

CHAPTER 7

Characterization of hypothalamo-pituitary-thyroid axis in the hypothalamus, pituitary gland, and ovarian follicles of turkey hens during the preovulatory surge and in hens with different levels of egg production

Abstract

The HPT axis has been shown to influence plasma P4 levels of the PS that triggers follicle ovulation. Dysregulation of the PS leads to lowered egg production. The presence of THRs in the reproductive axis indicates possible effects of thyroid hormone. Characterization of HPT axis plasma hormone concentrations and gene expression surrounding the PS was performed in AEPH, LEPH, and HEPH (n=3 hens/group). Data were analyzed using the mixed models procedure of SAS, with significance indicated at $P<0.05$. AEPH and HEPH displayed lower levels of T3 and higher levels of T4 inside of the PS, whereas LEPH showed inverse T3 and T4 levels relative to the PS. HPT axis expression of mRNA for hypothalamic *TRH*, pituitary *TSHB*, and the main thyroid hormone metabolism enzyme, *DIO2*, were downregulated during the PS in AEPH and HEPH. LEPH displayed higher expression of mRNA for hypothalamic *TRH* as well as pituitary *TSHB* and *DIO2* compared to HEPH. AEPH expression of THR mRNAs was upregulated during the PS in the hypothalamus but downregulated in the pituitary. HEPH showed decreased expression of THR mRNAs in both the hypothalamus and pituitary when compared to LEPH. In ovarian follicles, THR mRNAs were more prevalent in the thecal layer of the follicle wall compared to the granulosa layer, and expression tended to decrease with follicle maturity. Minimal differences in follicular THR expression were seen between LEPH and HEPH, indicating that THR expression is unlikely to be responsible for steroid hormone production differences seen in LEPH and HEPH. Generally, down-regulation of the HPT axis was seen during the PS, and up-regulation of the HPT axis was seen in LEPH compared to HEPH. Further studies will be required to clarify the role of the HPT axis in the regulation of ovulation and egg production rates in turkey hens.

Introduction

Two neuroendocrine axes have been shown to regulate reproductive activity in avian species, the HPG axis and the HPT axis (Follett and Nicholls, 1985). The HPT axis is comprised of the hypothalamus, the anterior pituitary gland, and the thyroid gland, and predominantly regulates metabolism (Paster, 1991). However, proper HPT axis function is necessary for egg production to occur (Lien and Siopes, 1989a). Previous studies in avian species have defined a role for the HPT axis in the initiation and cessation of egg production as well as in the regulation of seasonal rhythms (Proudman and Siopes, 2006; Nakayama and Yoshimura, 2017). The HPT axis has not been characterized in commercial lines of turkey hens during peak egg production, and the influences of hen egg production level and of the reproductive PS on HPT axis function have yet to be investigated.

Within the HPT axis, hypothalamic *TRH* is released and binds to *TRHR* on pituitary thyrotrophs, leading to release of TSH and upregulation of both TSH subunits (*CGA* and *TSHB*) (Fekete and Lechan, 2014). TSH acts on the thyroid to induce production of thyroid hormones, T3 and T4. T3 and T4 production occurs by the iodination of the tyrosine residues on thyroglobulin followed by protease digestion to release T3 and T4 (McNabb and Darras, 2014). Circulating T4 is inactive but can be converted to active T3 by deiodinases (Decuypere et al., 2005). *DIO2* is responsible for the conversion of T4 to T3 in target tissues (Nakane and Yoshimura, 2014).

Thyroid hormones elicit their actions through the binding of THRs. THRs are present in the each tissue of the HPG axis in avian species, namely the hypothalamus, pituitary, and ovary (Sechman, 2012). Two types of receptors are capable of thyroid hormone binding, nuclear THRα and THRβ and the integrin cell membrane receptor. Nuclear thyroid hormone receptors elicit

genomic effects by recruiting coactivators to TREs in target genes to regulate transcription (Cheng et al., 2010). The integrin receptor regulates non-genomic effects such as protein translocation and phosphorylation events (Davis et al., 2008). Both types of THRs have been shown to be present in the hypothalamus, the pituitary, and the ovarian follicles, indicating that thyroid hormone is capable of eliciting both genomic and non-genomic effects in the tissues of the reproductive axis. (McNabb, 2007b).

Commercial line turkey hens show a large variation in egg production levels, that we have classified as groups of LEPH, AEPH, and HEPH. Hypothalamic, pituitary, and ovarian transcriptome analysis in LEPH and HEPH inferred upregulation of thyroid hormone production and metabolism in LEPH when compared to HEPH (Chapters 5 and 6). This study sought to define normal HPT axis function in AEPH as well as to characterize how HPT axis function might be perturbed in LEPH and HEPH. In addition, the impact of fluctuations in reproductive hormones during ovulatory cycle on HPT function was assessed in the three egg production level groups. This study examined AEPH, LEPH, and HEPH turkey hens sampled outside and inside of the PS to (1) define the plasma concentrations of T3 and T4 and (2) characterize the expression of key HPT axis genes in the reproductive tissues, namely the hypothalamus, pituitary, and the preovulatory follicle layers responsible for P4 and E2 production, the F1 granulosa and the F5 theca externa.

Materials and Methods

Hen Selection and Cell Isolation

Females from a commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual cages. Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed *ad libitum* to NRC standards.

Hens were sampled at 37 weeks of age. Hens were selected and the timing of the PS was predicted using daily egg records as previously described, with the following exceptions (Chapter 2). AEPH, LEPH, and HEPH were used for sampling and were classified by EPD by dividing the total number of eggs produced by the number of days in production. Hens were classified as AEPH when 0.68<EPD<0.72, as LEPH when EPD<0.6, and as HEPH when EPD>0.8. All hens were sampled on the second day of the hen's sequence. The hypothalamus, pituitary, F1 follicle, F5 follicle, and the SWF were isolated from six AEPH, LEPH, and HEPH, half sampled outside of the PS and half during the PS. Hens were confirmed to be outside or during the PS by plasma P4 levels. Blood samples were taken from the wing vein immediately before tissue sampling and fractionated by centrifugation. Plasma samples were stored at -20°C prior to assessment through RIAs as described below. The hypothalamus and pituitary were snap frozen for RNA extraction, while the F1 and F5 follicles were subjected isolation of granulosa and theca externa layers from the follicle wall, as previously described (Chapter 2). Isolated tissues and cells were stored at -80°C prior to RNA extraction. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland.

The granulosa and theca externa layers were isolated from the F1 and F5 follicles as previously described (Porter et al., 1989a). Briefly, the yolk was drained from each follicle and the follicle was inverted to peel off the granulosa layer. The theca interna layer was scraped from the inverted follicle and discarded. The remaining theca externa layer was minced. Both follicle layers were subjected to trypsin dispersion (1 mg/mL) followed by layering onto a 50% Percoll suspension to remove debris and red blood cells. Isolated cells were snap frozen for RNA extraction.

RIAs

The RIAs used for P4, T3, and T4 were coated tube kits (MP Biomedicals, Solon, OH). All protocols were performed as directed by the manufacturer. All samples were assayed in duplicate. The standard curve was assessed for linearity as well as parallelism using serial plasma dilutions. The intraassay coefficients of variation determined by pools run every 30 samples were 4.26% for P4, 2.37% for T3, and 2.06% for T4. All samples were measured in a single RIA for each hormone. The ratio of T3 to T4 plasma concentrations was calculated by dividing the T3 plasma concentration by the T4 plasma concentration for each hen.

RT-qPCR

Total RNA was isolated from the hypothalamus, pituitary, and ovarian granulosa and theca externa cell from the F1 and F5 follicles, respectively, with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA, reverse transcription reactions, and RT-qPCR were performed as previously described (Chapter 2). A pool of total RNA was made, and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. Reactions were diluted by tissue type as previously described prior to PCR analysis (Chapter 2). Primers (IDT, Skokie, IL) were designed and used with cycling parameters described previously (Chapter 2).

Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reaction and was absent from the genomic DNA and water controls. Data were normalized to housekeeping genes and analyzed by the $2^{-\Delta\Delta Ct}$ method. For the hypothalamus, *GAPDH* was used for normalization. For the pituitary, *PGK1* was used for normalization. For all of the follicle cell types, *GAPDH* was used for normalization. All PCR reactions for each gene in a given tissue were analyzed in a single 96-

well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates.

Statistics

All data were analyzed using SAS software (SAS Institute, Cary, NC). Normalized RT-qPCR data were $\log_2$ transformed before statistical analysis. A one-way ANOVA using the mixed models procedure was used to compare plasma hormone concentrations and $\log_2$ transformed gene expression data from AEPH outside and inside of the PS. A two-way ANOVA using the mixed models procedure was conducted to compare plasma hormone concentrations and $\log_2$ transformed gene expression data from LEPH and HEPH outside and inside of the PS. The least squares means for each group were compared using the test of least significant difference, with overall significance level of $P < 0.05$. AEPH data are presented relative to basal steroid hormone concentration or gene expression, whereas, data from LEPH and HEPH are presented relative to LEPH basal steroid hormone concentration or gene expression.

Results

Plasma Hormone Levels

In AEPH and HEPH, plasma T3 concentrations were significantly higher outside of the PS, whereas, LEPH showed no change in plasma T3 concentrations during the ovulatory cycle (**Figure 7.1A and 7.1B**). Additionally, HEPH displayed increased plasma T3 levels outside of the PS when compared to LEPH. No differences in plasma T3 levels were seen between LEPH and HEPH during the PS. Plasma T4 concentrations were significantly higher during the PS in AEPH and HEPH and significantly lower during the PS in LEPH (**Figure 7.1C and 7.1D**). While T4 plasma levels did not differ between LEPH and HEPH outside of the PS, T4 plasma levels during the PS were higher in HEPH when compared to LEPH.

The T3:T4 ratio in AEPH decreased significantly during the PS, dropping from 0.31 to 0.16 (**Figure 7.2A**). This same trend was seen in the T3:T4 ratio of HEPH, whereas LEPH showed a significant increase in the T3:T4 ratio during the PS (**Figure 7.2B**). In addition, HEPH displayed a higher T3:T4 ratio outside of the PS and a lower T3:T4 ratio during the PS when compared to LEPH. Combined T3 and T4 plasma concentrations were higher during the PS in AEPH and HEPH but higher outside of the PS in LEPH (**Figure 7.2C and 7.2D**). Moreover, LEPH displayed a higher combined T3 and T4 plasma concentration outside of the PS but a lower combined T3 and T4 plasma concentration during the PS when compared with HEPH.

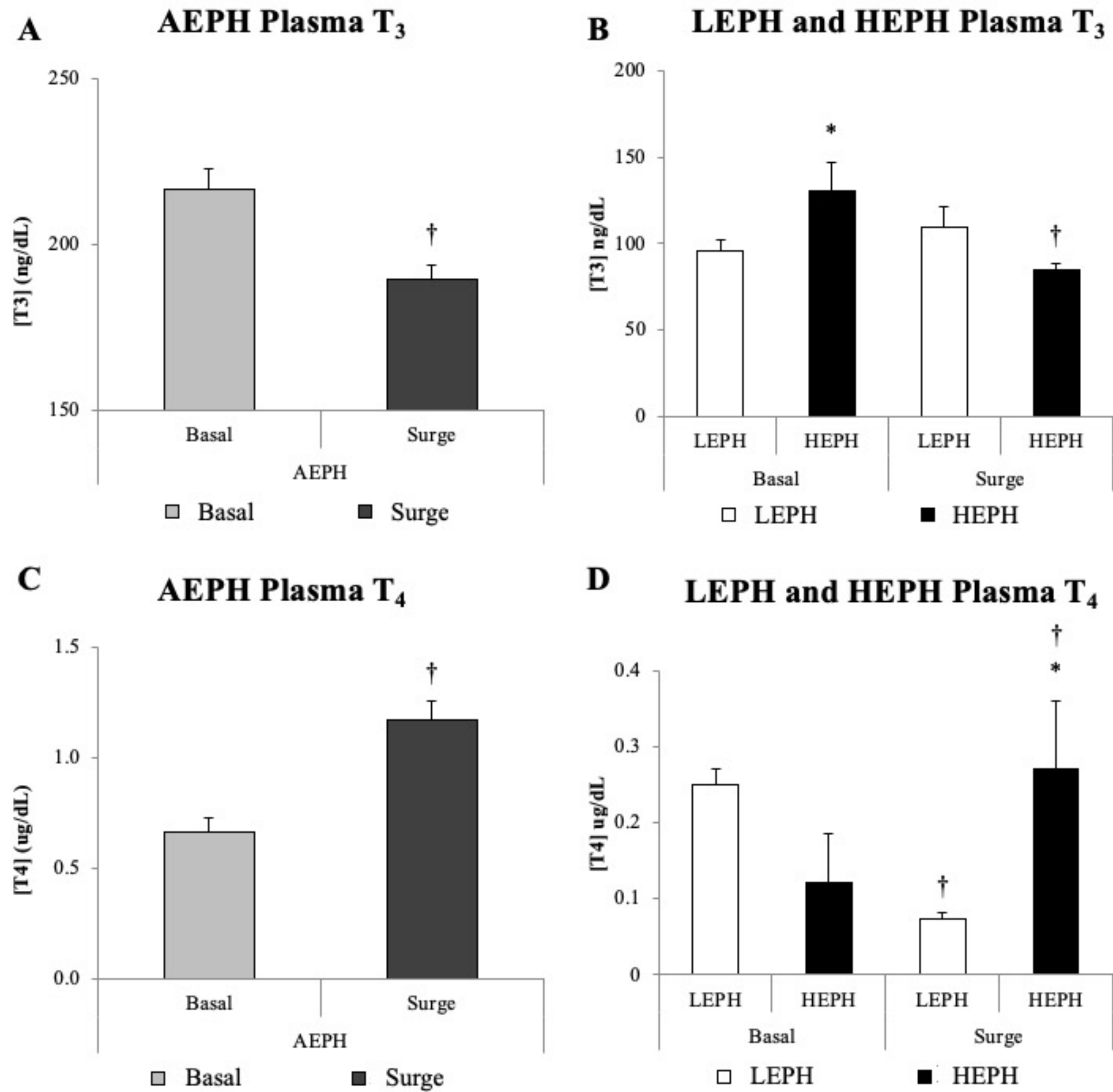


Figure 7.1 Plasma T3 and T4 hormone profiles in AEPH (Figure 1A and 1C) as well as LEPH and HEPH (Figure 1B and 1D). All three groups of hens were sampled outside (basal) and inside (surge) of the PS. Significant plasma thyroid hormone concentration differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge plasma thyroid hormone concentrations for a given egg production group are denoted with a dagger.

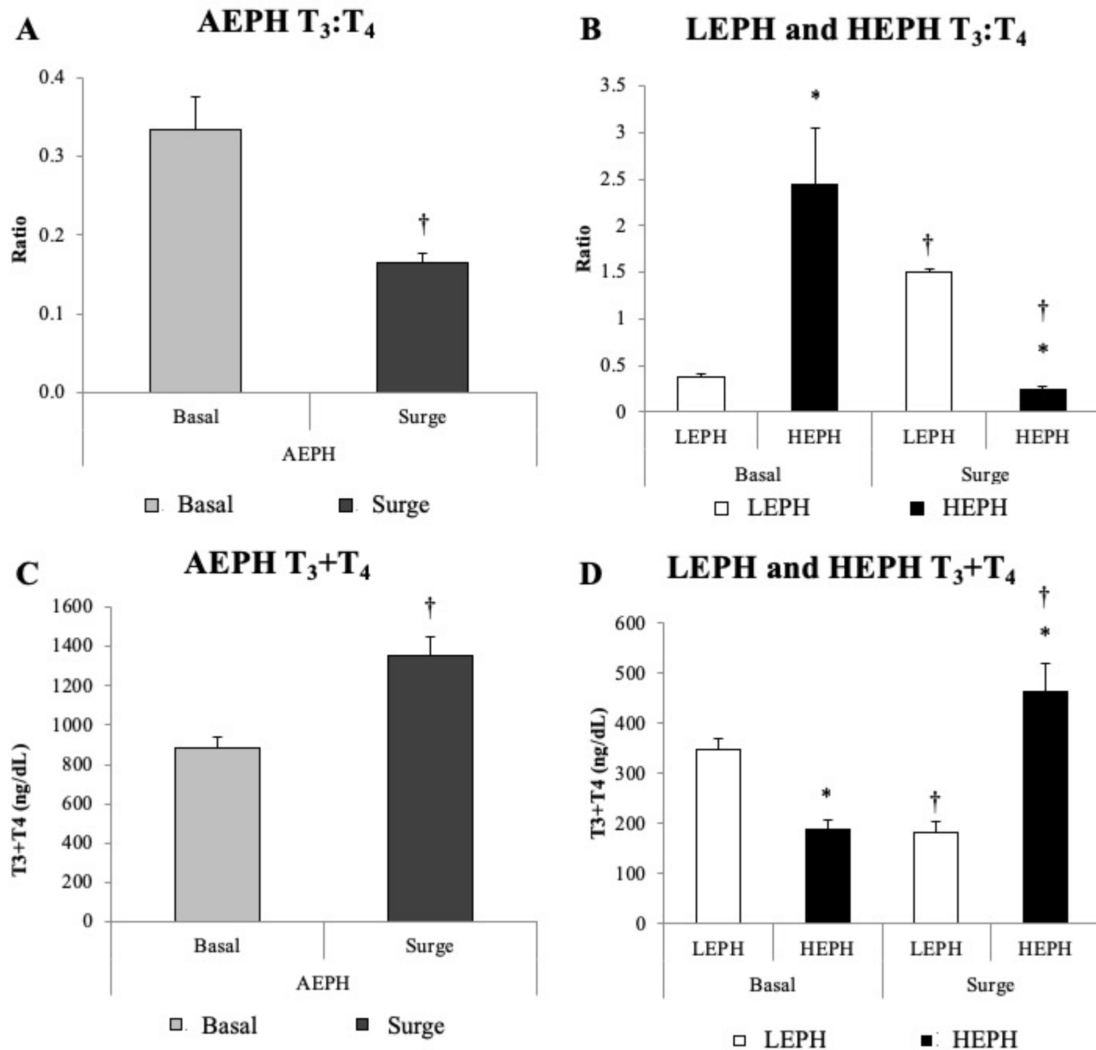


Figure 7.2 T3 to T4 ratio in AEPH (Figure 2A) as well as LEPH and HEPH (Figure 2B). All three groups of hens were sampled outside (basal) and inside (surge) of the PS. Combined T3 and T4 plasma concentrations of AEPH under basal and surge conditions as well as of LEPH and HEPH, taking the PS into account, are shown in Figure 2C and 2D, respectively. Significant thyroid hormone ratio or combined plasma concentration differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge plasma thyroid hormone concentrations for a given egg production group are denoted with a dagger.

Hypothalamic Gene Expression

Hypothalamic expression of the main stimulatory releasing factor as well as two thyroid hormone receptors differed significantly in AEPH outside and during the PS (**Figure 7.3A**). Expression of *TRH*, which is the main stimulatory releasing factor of the HPT axis, was decreased in AEPH during the PS, in contrast to levels outside of the PS. Expression of two thyroid hormone receptors, *THRB* and *ITGB3*, which encode THRs present in the nucleus and plasma membrane, respectively, exhibited upregulation in AEPH during the PS. In LEPH and HEPH, significant hypothalamic gene expression differences were seen in *TRH* and in thyroid hormone receptors *THRA*, *THRB* and *ITGB3* (**Figure 7.3B**). Similar to AEPH, HEPH showed decreased mRNA levels for *TRH* during the PS, whereas LEPH showed increased mRNA levels for *TRH* during the PS. HEPH also exhibited increased mRNA levels for *THRB* during the PS as was seen in HEPH, whereas LEPH showed decreased *THRB* mRNA levels during the PS. On the other hand, LEPH showed increased mRNA levels for *ITGB3* during the PS as was also seen in AEPH, whereas HEPH showed decreased *ITGB3* mRNA levels during the PS. Additionally, LEPH showed higher mRNA levels for *TRH* and for three of the four thyroid hormone receptors than in HEPH. LEPH displayed upregulation of *THRB* and *ITGAV* outside of the PS as well as *ITGB3* during the PS when compared to HEPH.

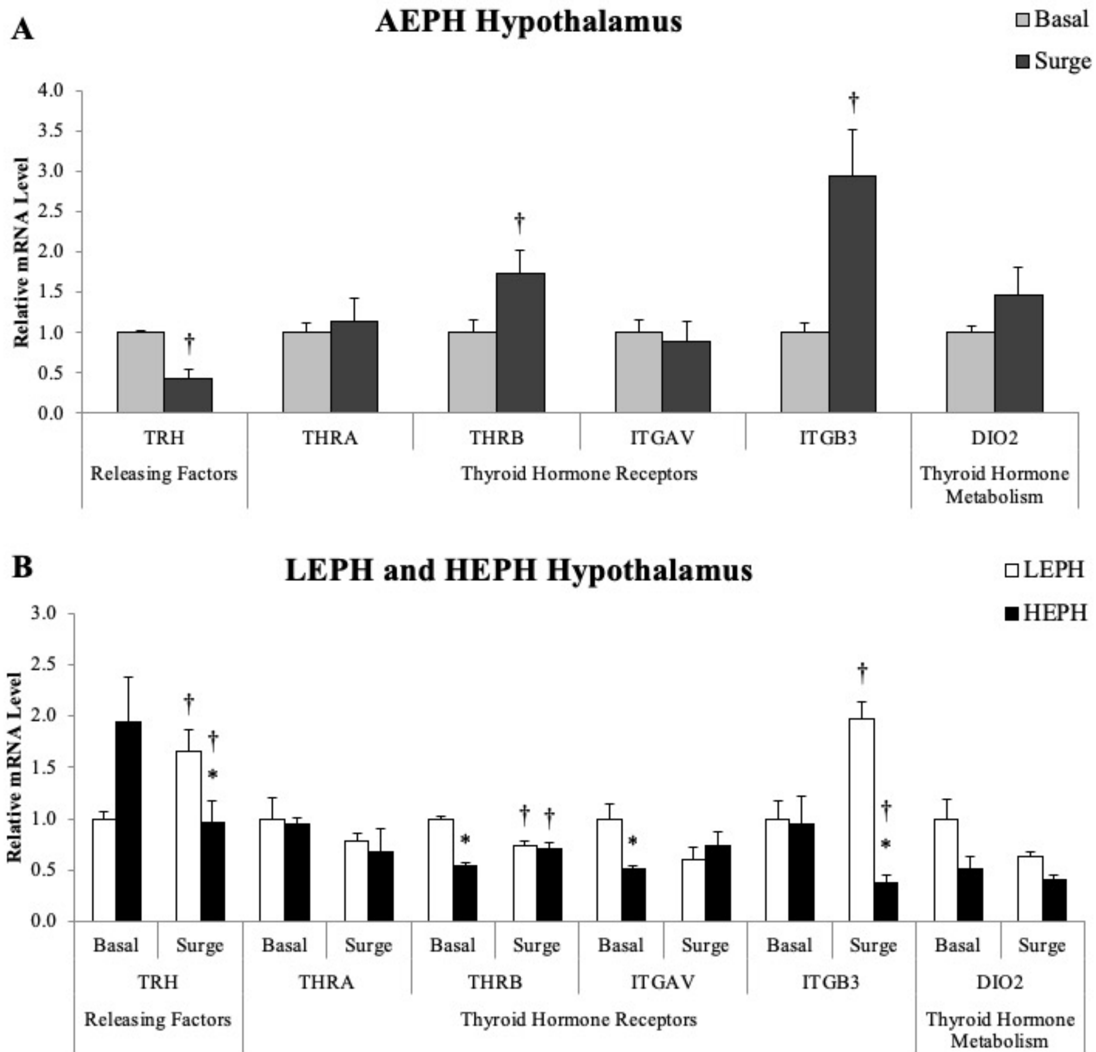


Figure 7.3 Hypothalamic gene expression of HPT axis releasing factors, THRs and thyroid hormone metabolism enzymes in AEPH, LEPH, and HEPH sampled outside (basal) and inside (surge) of the PS. AEPH expression under basal and surge conditions is presented in Figure 7.3A, while LEPH and HEPH expression, taking the PS into account, is presented in Figure 7.3B. Normalized data are presented relative to basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

Pituitary Gene Expression

Pituitary expression of two thyroid hormone receptors, of the main thyrotropin subunit, and of the deiodinase involved in T3 metabolism to T3 differed in AEPH outside and during the PS (**Figure 7.4A**). THRs, *THRB* and *ITGB3*, which encode THRs that elicit genomic and non-genomic actions of thyroid hormone, respectively, both showed reduced mRNA levels in AEPH during the PS. Additionally, reduced expression of *TSHB* and *DIO2* was also seen in AEPH during the PS when compared to mRNA levels outside of the PS. Significant expression differences between LEPH and HEPH were seen for THR genes, *THRB* and *ITGB3*, as well as for *TSHB* and *DIO2* (**Figure 7.4B**). HEPH showed decreased mRNA levels for *THRB*, *TSHB*, and *DIO2* during the PS similar to expression trends seen in AEPH. LEPH also showed decreased mRNA levels for *THRB* and *TSHB* during the PS but did not show decreased expression of *DIO2* during the PS. LEPH also showed increased expression of *ITGB3* under basal and surge conditions, of *TSHB* outside of the PS, and of *THRB* during the PS, when compared to HEPH. HEPH displayed increased mRNA levels for *DIO2* outside of the PS but decreased mRNA levels for *DIO2* during the PS in relation to LEPH expression.

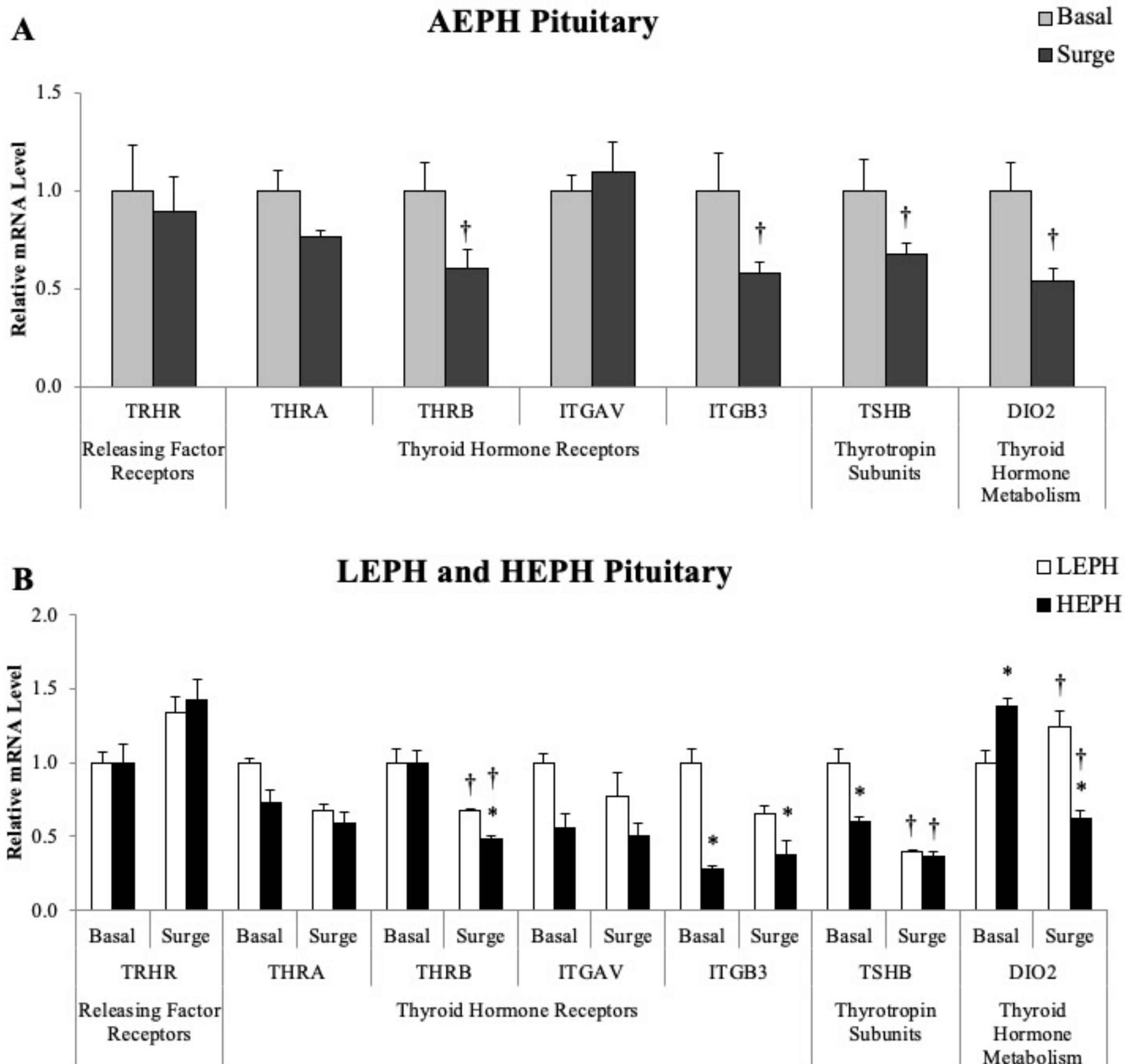


Figure 7.4 Pituitary gene expression for HPT axis releasing factor receptors, thyrotropin subunits, THRs, and thyroid hormone metabolism enzymes in AEPH, LEPH, and HEPH sampled outside (basal) and inside (surge) of the PS. AEPH expression under basal and surge conditions is presented in Figure 7.4A, while LEPH and HEPH expression, taking the PS into account, is presented in Figure 7.4B. Normalized data are presented relative to basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

F1G Gene Expression

THR expression in the F1G layer was not impacted by the PS or by egg production level (**Figure 7.5A and 7.5B**).

F5E Gene Expression

In the F5 theca externa, expression of three of the four THR genes was significantly different between AEPH outside and during the PS (**Figure 7.6A**). In AEPH, *THRA,* which encodes a nuclear thyroid hormone receptor, was downregulated during the PS, while expression of integrin subunits, *ITGAV* and *ITGB3,* which are capable of thyroid hormone binding in the plasma membrane, was upregulated during the PS. LEPH and HEPH only showed differential mRNA levels for *ITGAV* (**Figure 7.6B**). Diverging from AEPH, LEPH showed decreased expression of *ITGAV* during the PS, whereas, HEPH showed no change in *ITGAV* mRNA levels during the PS. Additionally, LEPH showed decreased mRNA levels for *ITGAV* both outside and during the PS when compared to HEPH.

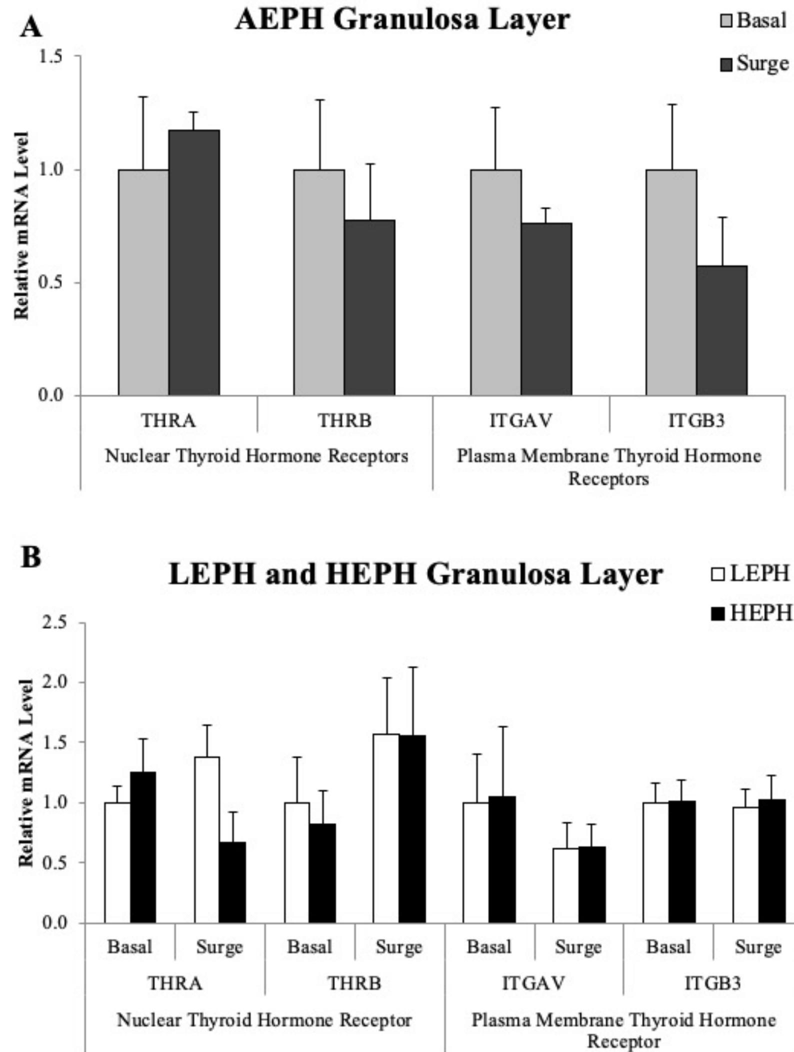


Figure 7.5 F1G layer gene expression of HPT axis THRs in AEPH, LEPH, and HEPH sampled outside (basal) and inside (surge) of the PS. AEPH expression under basal and surge conditions is presented in Figure 7.5A, while LEPH and HEPH expression, taking the PS into account, is presented in Figure 7.5B. Normalized data are presented relative to basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

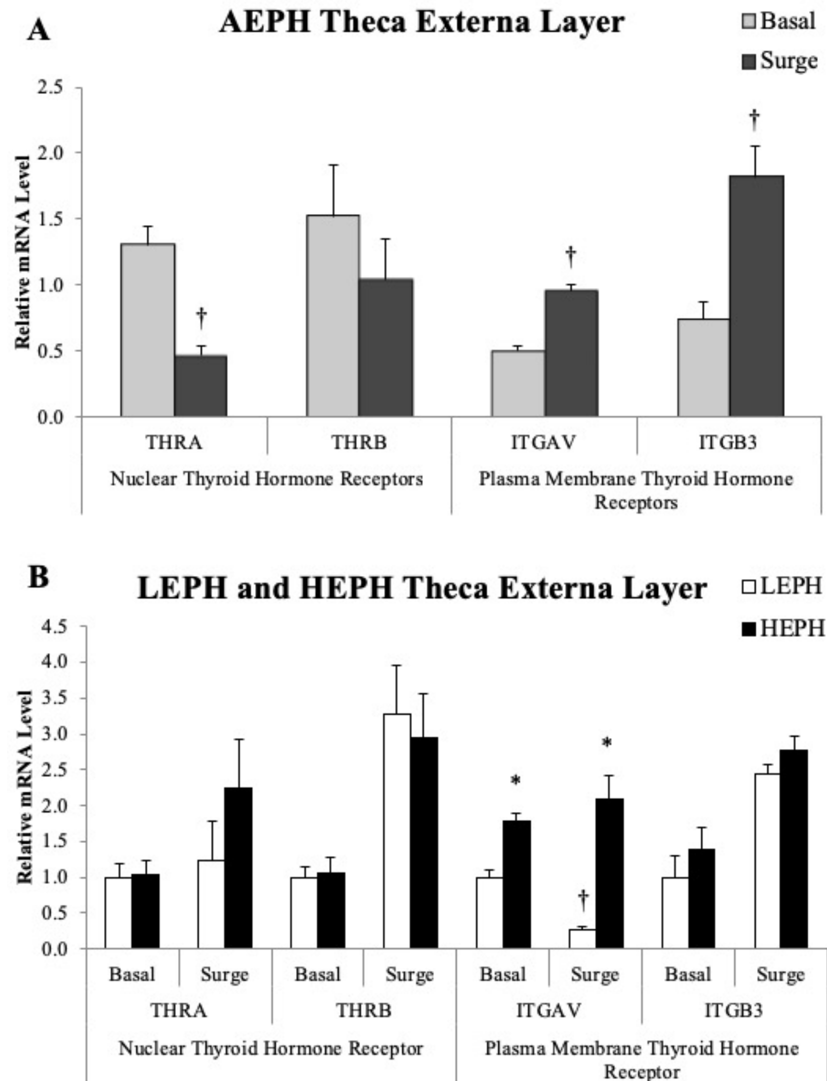


Figure 7.6 F5 follicle theca externa layer gene expression of HPT axis THRs in AEPH, LEPH, and HEPH sampled outside (basal) and inside (surge) of the PS. AEPH expression under basal and surge conditions is presented in Figure 7.6A, while LEPH and HEPH expression, taking the PS into account, is presented in Figure 7.6B. Normalized data are presented relative to basal expression. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk, whereas significant differences between basal and surge expression for a given egg production group are denoted with a dagger.

Discussion

HPT axis circulating thyroid hormone levels and expression of genes related to thyroid hormone production and metabolism differed during the ovulatory cycle and differed with egg production level. In general, HEPH displayed thyroid axis hormone profiles and gene expression consistent with those seen in AEPH, while LEPH tended to display opposite trends. In addition, LEPH tended to exhibit higher basal thyroid axis expression when compared to HEPH. This investigation is the first study to quantify T3 and T4 plasma concentrations surrounding the PS in commercial turkey hens with varied egg production levels. Furthermore, this study provides novel insights into the mRNA expression of key HPT axis genes, also taking the expression changes in regard to egg production levels and position in the ovulatory cycle into account. Based on the results from this study, HPT axis function is not consistent during the hen ovulatory cycle or in hens with differential egg production, reinforcing the intertwining roles of the thyroid and reproductive axes in the regulation of ovulation.

Thyroid hormone concentrations, in regard to the ovulatory cycle or to egg production level, had not been previously examined in avian species. Prior studies focused on plasma concentrations of the thyroid hormones during initiation of egg lay and during periods of ovarian regression, mainly focusing on photoresponsiveness and photorefractoriness (Siopes et al., 2010; Lien and Siopes, 1989b). Earlier studies found that suppression of T3 plasma levels is necessary for initiation of egg lay (Decuypere et al., 2005). In the current study, AEPH and HEPH plasma levels of T3 declined 12.6% and 35.5%, respectively, during the PS, whereas, no changes in plasma T3 levels during the PS in LEPH were exhibited. Decreased circulating T3 outside of the PS may play a role in initiating the next ovulation of a hen's laying sequence. Increased T4 plasma concentrations are associated with molt, or gonadal regression, in both chicken and

turkey hens (Sekimoto et al., 1987; Siopes, 1993). Both AEPH and HEPH, exhibited roughly a two-fold increase in T4 plasma concentrations during the PS, but LEPH T4 plasma concentrations decreased over three-fold during the PS. Increased circulating T4 during the PS may aid in returning the reproductive hormones involved in the PS to basal levels to prepare for the next ovulation. Previous studies show that the T3:T4 ratio in hens does not significantly change throughout the laying cycle (Proudman and Siopes, 2005). However, this is the first study to examine the ratio during the ovulatory cycle. The T3:T4 ratio differed during the ovulatory cycle in each group of hens examined, with AEPH and HEPH showing a similar reduction in the ratio and LEPH showing an increase in the ratio during the PS. These results indicate that not only individual plasma concentrations of T3 and T4, but also the ratio of T3:T4 may play a role in the rate of egg production.

AEPH expression of the main hypothalamic releasing factor as well as pituitary thyrotropin and thyroid hormone metabolism enzymes was down-regulated during the PS. Additionally, AEPH expression of THRs was up-regulated during the PS in the hypothalamus but down-regulated in the pituitary, indicating possible differential regulation in the hypothalamus and pituitary. HPT axis gene expression in the hypothalamus and pituitary of HEPH was more consistent with expression profiles seen in AEPH when compared to LEPH, including similar expression profiles of hypothalamic *TRH* and *THRB* as well as pituitary *THRB*, *TSHB*, and *DIO2*. LEPH, in contrast, only shared similar expression profiles of hypothalamic *ITGB3* and pituitary *THRB* and *TSHB* with AEPH. Earlier studies in mammalian models found that higher T3 and T4 concentrations, both individually and combined, had an inhibitory effect on *TRH* mRNA levels (Kakucska et al., 1992). In the present study, though decreased expression of *TRH* during the PS in AEPH and HEPH coincided with a decrease in plasma T3

concentrations, combined plasma concentrations of T3 and T4 were significantly increased in AEPH and HEPH during the PS. In contrast, LEPH displayed a decrease in combined plasma concentrations of T3 and T4 during the PS, which coincided with increased expression of *TRH*. LEPH displayed increased mRNA levels for three THR genes and for *TRH* in the hypothalamus and for two THR genes and for *TSHB* in the pituitary, when compared to HEPH, suggesting that LEPH may be more capable of HPT axis stimulation and feedback in the hypothalamus and pituitary. HEPH displayed higher mRNA levels for pituitary *DIO2* outside of the PS compared to LEPH. Transcriptome analysis of red-feather Taiwan country chickens revealed that *DIO2* was upregulated in the pituitary of hens with high egg production under basal conditions (Chen et al., 2007).

In regard to ovarian follicle expression, THRs were more prevalent in the thecal layer of the follicle wall than in the granulosa layer, and expression of THRs tended to decrease with follicle maturity. This is consistent with previous studies examining the effects of thyroid hormones on steroidogenesis (Sechman, 2012). However, not previously reported, THRs in the F5 theca externa layer showed expression changes during the PS, further solidifying the potential role of thyroid hormones in the regulation of steroidogenesis. T3 inclusion in F1 granulosa cell cultures in chickens increased P4 synthesis as well as expression of genes related to P4 production (Sechman et al., 2009). Previous work determined that HEPH displayed increased expression of P4 production genes outside of the PS and increased P4 synthesis in response to LH treatment *in vitro* when compared to LEPH (Chapters 3 and 4). Coupled with the increased plasma T3 levels in HEPH outside of the PS in the present study, it is possible that differential levels of circulating thyroid hormones may partially regulate the differences seen in LEPH and HEPH P4 gene expression and *in vitro* production in the F1G layer. *DIO2* was not found to be

expressed in the F1G layer, which may prevent T4 from impacting the F1G layer due to minimal T4 to T3 conversion. Expression of THRs did not change during the ovulatory cycle and did not show differential expression in LEPH and HEPH, indicating that possible thyroid hormone regulation of F1G P4 production is most likely due to differences in circulating thyroid hormones rather than THR expression. Only *ITGAV* in the F5 theca externa layer showed differential expression between LEPH and HEPH. Previous studies have not examined the effects of T3 or T4 treatment on E2 production in the F5 theca externa layer, however T3 treatment did suppress gonadotropin stimulated E2 production in SWF from chicken and turkey hens (Sechman et al., 2009; Chapter 6). Previous work found that HEPH showed increased expression of E2 production genes during the PS in the F5 theca externa, which coincides with decreased plasma T3 concentrations (Chapter 3). Further studies will be necessary to assess the effects of *in vitro* thyroid hormone treatment on E2 production in the F5 theca externa layer. However, due to the minimal THR expression differences between LEPH and HEPH, it is more likely that effects of thyroid hormone on E2 production occurs through differential circulating thyroid hormone levels rather than differential THR expression.

Timing in the ovulatory cycle and egg production level impacted plasma thyroid hormone concentrations and HPT axis gene expression in the hypothalamus and pituitary. While influences of the timing in the ovulatory cycle and egg production level on HPG axis gene expression and ovarian steroid hormone production in the F1 granulosa and F5 theca externa layers have been established, the influence of thyroid hormone on these aspects appears to be due to circulating thyroid hormone levels rather than THR expression. In general, HPT axis expression tended to be down-regulated during the PS and tended to be up-regulated in LEPH. Further studies will be required to elicit the full influence of the thyroid axis on reproductive

function and to determine what impact the thyroid axis has on follicle development and ovulation rates to ultimately impact egg production levels.

CHAPTER 8

Conclusions and Future Directions

Conclusions

The objective of these studies was to define differences in HPG axis function that could ultimately lead to the egg production gap seen between LEPH and HEPH. Differences in egg production between LEPH and HEPH, not only decreases flock uniformity but lowers egg production in LEPH and decreases the profitability of the turkey industry. Additionally, fully understanding the mechanisms governing ovulation rates in turkeys may allow for the selection for higher egg production in breeding hens without compromising meat production traits (Wolc et al., 2016). These are the first studies to examine LEPH and HEPH from commercial line turkey hens and from the same flock (in hens of the same age). Furthermore, these are the first studies in avian species to define HPG axis gene expression and hormone responsiveness in hens with differential egg production. A handful of previous studies have examined global gene expression differences in hens selected for egg or meat production. However, these are the first studies to perform transcriptome analysis on hens with differential egg production from the same line as well as the first study to examine how the transcriptome is impacted by the PS.

Due to the limited knowledge of gene expression changes related to ovulation in the tissues of the HPG axis in the turkey hen, the first objective established baseline function of the HPG axis outside and during the PS (Scanes, 2017). During the PS, down-regulation of ovulation stimulatory genes (e.g. *GNRHR, LHB,* and *PGR*) occurred during the PS, whereas up-regulation of follicle development genes (e.g. *FSHB* and *FSHR*) occurred during the PS (**Figure 8.1**). Additionally, up-regulation of several genes involved in steroid production (e.g. *STAR, HSD17B1*, and *CYP19A1*) took place during the PS. General down-regulation of ovulatory genes, coupled with up-regulation of genes supporting follicle development, could function to terminate the PS and shift focus to the next ovulation (Christian and Moenter, 2010).

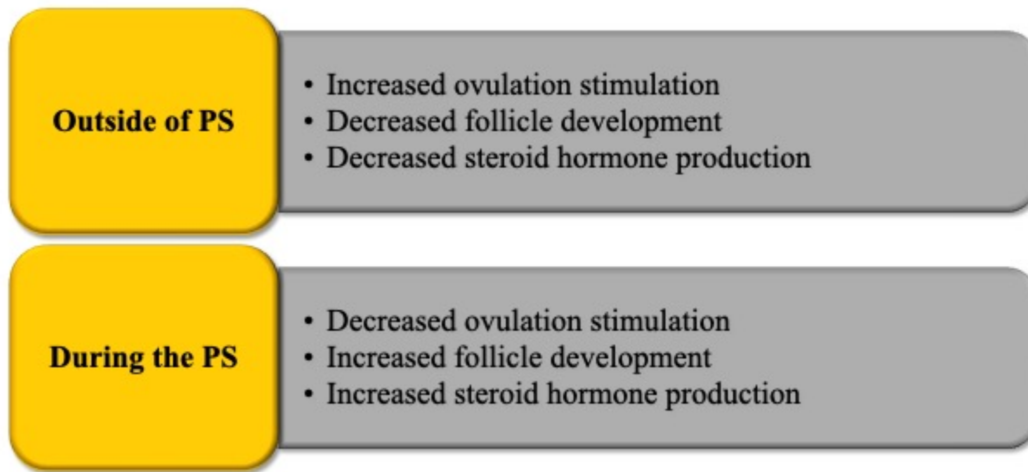


Figure 8.1 Summary of the HPG axis physiological processes associated with the mRNA level differences seen in AEPH outside and during the PS.

Establishment of baseline HPG axis function allowed for identification of perturbations of this baseline function in LEPH and HEPH. Through the second objective, LEPH were found to display increased expression of genes related to ovulation inhibition (e.g. *GNIH* and *GNIHR*), whereas, HEPH were found to display increased expression of genes related to ovulation stimulation and follicle development (e.g. *LHB* and *FSHB*) (**Figure 8.2**). Furthermore, HEPH exhibited increased expression of genes related to P4 production (e.g. *STAR* and *CYP11A1*) and E2 production (e.g. *CYP19A1*) while LEPH exhibited increased expression of genes related to AD production (e.g. *CYP17A1* and *HSD17B1*). Interestingly, HEPH exhibited similar gene expression trends during the PS as were seen in AEPH, with down-regulation and up-regulation of ovulation related and follicle development genes during the PS, respectively. LEPH up-regulation of genes related to ovulation inhibition occurred during the PS, in contrast to the trends seen in AEPH during the PS. A reduction in GnIH expression is proposed to be necessary for the PS to occur (Smith and Clarke, 2010). Based on the results of this objective and results from previous studies, it can be hypothesized that, at the transcript level, LEPH expression of ovulation inhibition genes prevents the PS from occurring, leading to longer ovulation intervals in these hens. Up-regulation of ovulation stimulation genes and of steroidogenic genes in HEPH may lead to a shorter ovulation interval time through a more rapid production of gonadotropin hormones and positive steroid hormone feedback.

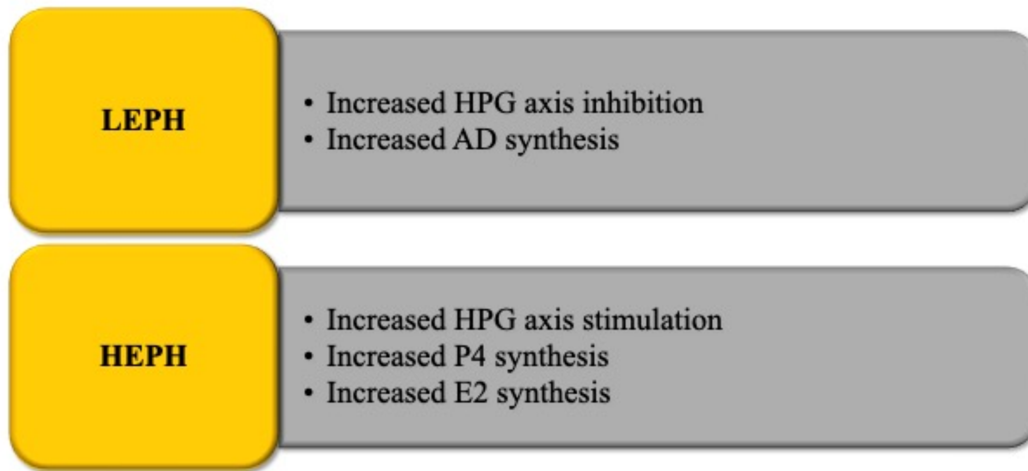


Figure 8.2 Summary of the HPG axis physiological processes associated with the mRNA level differences seen between LEPH and HEPH.

Based on HPG axis gene expression differences seen in LEPH and HEPH, it was hypothesized that LEPH and HEPH may respond differently to HPG axis hypothalamic releasing factors and pituitary gonadotropins in the pituitary and follicle cells, respectively. Results from treatment of pituitary cells *in vitro* with GnRH and GnIH were consistent with LEPH displaying an increased responsiveness to the inhibitory pathways of the HPG axis and with HEPH displaying an increased responsiveness to the stimulatory pathways of the HPG axis (**Figure 8.3**). Furthermore, HEPH displayed an increased *in vitro* response to gonadotropin treatment in F1G cells and SWF cells in terms of steroid hormone production compared to LEPH, which is consistent with the increased mRNA levels of steroidogenic genes previously seen in HEPH. Taken together, the increased the responsiveness to GnRH, FSH, and LH seen in HEPH *in vitro*, may translate to HEPH responding quicker to HPG axis stimulation at the level of the pituitary and the ovary *in vivo*, leading to a shorter time between HPG axis stimulation and ovulation in HEPH (Tsutsumi and Webster, 2009).

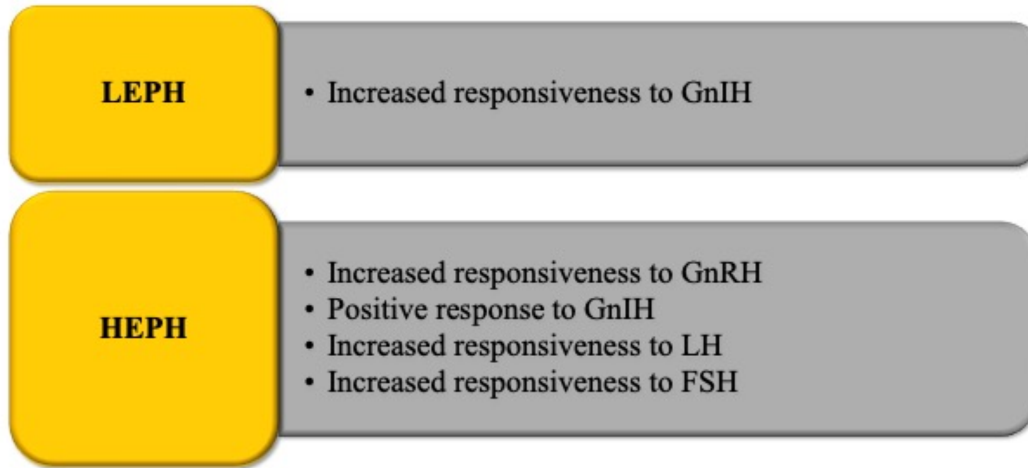


Figure 8.3 Summary of the HPG axis physiological processes associated with the differences in pituitary mRNA levels and follicular steroid hormone production levels seen between LEPH and HEPH.

With the known regulators of reproductive function characterized in LEPH and HEPH, transcriptome analysis of the hypothalamus, pituitary, and follicle cells allowed for a global picture of gene expression changes that could potentially impact egg production rates. Two gene expression trends emerged from the transcriptome analysis: (1) increased HPG axis expression in HEPH, particularly involving beta-estradiol feedback on hypothalamus and pituitary and (2) increased HPT axis expression in LEPH. Moreover, E2 and T3 pretreatment *in vitro* supported the involvement of E2 and thyroid hormone in the regulation of gonadotropin and steroid hormone production, with E2 stimulating production and thyroid hormone inhibiting production (**Figure 8.4**).

LEPH displayed an increased responsiveness in the pituitary to E2 treatment *in vitro* when compared to HEPH. Additionally, increased plasma E2 levels were seen in HEPH and beta-estradiol was predicted to be activated in HEPH as an upstream regulator. Increased circulating E2 levels could be due to the increased responsiveness of HEPH SWF to FSH stimulation, as was seen *in vitro*. Additionally, increased plasma levels of E2 infers increased E2 feedback in HEPH. Increased responsiveness of pituitary cells from LEPH to E2 treatment *in vitro* could be due increased exposure of pituitary cells from HEPH to circulating E2 levels *in vivo* prior to cell isolation and culture. Increased responsiveness of pituitary cells from LEPH to E2 treatment *in vitro* could also be due to E2 receptor desensitization in pituitary cells from HEPH as a result of higher circulating E2 plasma levels in HEPH. E2 receptors influence the transcription of target genes through the binding of estrogen response elements. Several HPG and HPT axis genes were predicted targets of E2 regulation. Increased E2 feedback at the level of the hypothalamus and pituitary may influence ovulation rates through the transcriptional

regulation of gonadotropin production or through the transcriptional regulation of other key genes of the HPG or HPT axis.

HPT axis activity is necessary for egg production to occur but increased circulating thyroid hormone is associated with gonadal regression (Lien and Siopes, 1989; Queen et al., 1997). Therefore, over-expression of the HPT axis in the hypothalamus and pituitary of LEPH may suppress the reproductive axis leading to longer ovulation intervals in these hens (**Figure 8.5**). Additionally, increased circulation of plasma thyroid hormones in LEPH may decrease pituitary gonadotropin production and decrease steroid hormone production in the ovary (McNabb, 2007b). Decreased gonadotropin and steroid hormone production can, in turn, influence the timing of the PS through the regulation of circulating LH and P4 levels. THRs elicit actions on target genes through genomic and non-genomic mechanisms. Target genes of thyroid hormones includes key genes of the HPG and HPT axes. Increased expression of THRs in LEPH may allow for increased down-regulation of the HPG axis and up-regulation of the HPT axis is these hens, which is consistent with mRNA levels of key HPG and HPT axis genes seen in LEPH. Cells from HEPH showed an increased responsiveness to T3 treatment *in vitro* when compared to cells from LEPH. Similar to the *in vitro* responses seen following E2 treatment, cells from HEPH may be more responsive to T3 treatment because cells from LEPH are exhibiting THR desensitization due to higher circulating levels of plasma thyroid hormones. Further exploration of the influence of the HPT axis on the HPG axis and of the differential regulation of the HPT axis seen in LEPH and HEPH will be necessary to determine if up-regulation of the HPT axis in LEPH is connected to lowered ovulation rates in these hens.

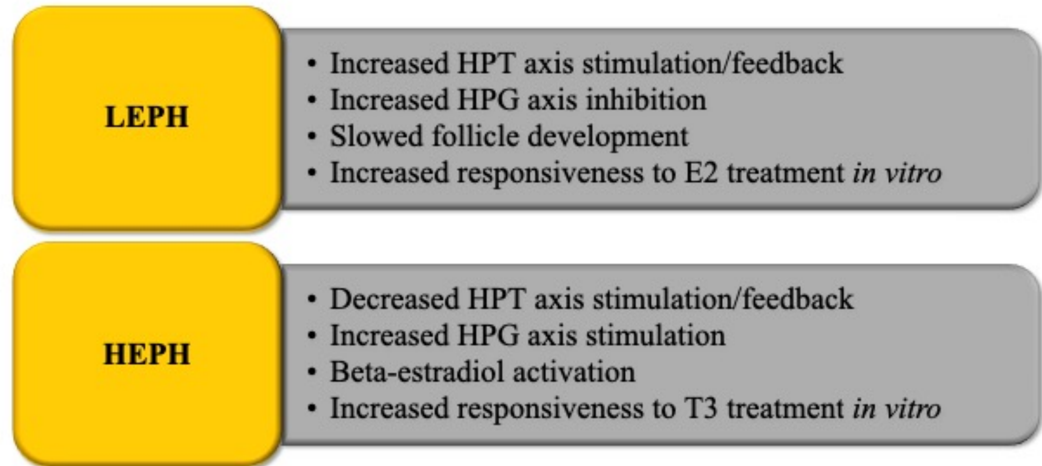


Figure 8.4 Summary of the physiological processes associated with the DEGs and *in vitro* response differences between LEPH and HEPH.

Chapter 7: Summary

AEPH

- Increased T3 outside of the PS and T4 during the PS
- Decreased HPT axis activity during the PS

LEPH

- Decreased T3 outside of the PS and T4 during the PS
- Increased HPT axis activity during the PS
- Increased HPT axis feedback during the PS

HEPH

- Increased T3 outside of the PS and T4 during the PS
- Decreased HPT axis activity during the PS
- Decreased HPT axis feedback during the PS

Figure 8.5 Summary of the thyroid hormone plasma levels and the physiological processes associated with mRNA levels of key HPT axis genes between LEPH and HEPH.

With no apparent differences in ovarian structure between LEPH and HEPH, it was hypothesized that functional differences in the HPG axis may explain differences in ovulation rates between LEPH and HEPH. Through a combination of targeted and global gene expression approaches, key differences between LEPH and HEPH were identified not only in the HPG axis but also in the HPT axis. Furthermore, these differences in key HPG and HPT axis genes between LEPH and HEPH were consistent differences in gonadotropin and steroid hormone production detected between the two groups of hens through *in vitro* methods. Results from these studies, support the role of increased E2 signaling in HEPH and increased HPT axis activity in LEPH as possible contributors regulating ovulation rates in turkey hens. Future studies are necessary to define the full role of E2 and thyroid hormone regulation of follicle development and the PS, and ultimately ovulation rates in turkey hens.

Future Directions

To determine the possible role of E2 and/or thyroid hormone in the regulation of follicle development and the PS, future studies will need to determine the mechanisms regulating levels of circulating E2/thyroid hormone as well as the downstream mechanisms related to reproduction that are activated/inhibited by E2/thyroid hormone. Differences in circulating levels of E2/thyroid hormone between LEPH and HEPH could be due to differential regulation of hormone synthesis. Regulators of genes involved in E2/thyroid hormone synthesis (microRNA, transcription factor binding, etc) should be examined in LEPH and HEPH in future studies to determine how differences in circulating levels of these hormones occur in the two groups of hens (Nelson and Bulun, 2001; Eggo, 2010). Additionally, key HPG or HPT axis genes that are regulated by E2/thyroid hormone in LEPH and HEPH need to be identified (Björnström and Sjöberg, 2005; Hammes and Davis, 2015). Based on the results seen from the current studies, the

rate of follicle development may differ between LEPH and HEPH and may be partially regulated by E2 or thyroid hormones. The rate of progression through the follicular hierarchy can be examined through alternating dyes in the feed each day, and examining the incorporation of the dye into the yolk rings of the follicle (Grau, 1976). This could be examined in LEPH and HEPH to determine if follicle development rate is different between the two groups of hens.

A greater understanding of the differences in regulatory mechanisms of follicle development and ovulation in LEPH and HEPH may allow for the identification of genetic markers that could be used to select for increased egg production. High egg production is usually associated with decreased growth traits (Nestor, 1984). However, body weight differences between LEPH and HEPH were not seen in the present studies. Further studies will be necessary to determine the impact of high egg production on growth traits of progeny. Understanding the crosstalk between the reproductive and growth axes may allow for the identification of genetic markers associated with increased egg production which impart minimal effects on growth (Hocking, 2014).

Furthermore, the mechanisms triggering ovulation are not well understood in avian species and would be an area for future research. Studies determining the regulatory mechanisms (e.g. microRNA regulation, transcription factor binding, etc.) behind the observed changes in gene expression during the PS would be instrumental to further understanding ovulation in avian species. A lack of appropriate available antibodies precluded measurement of HPG axis protein expression in the current study. However, protein studies will be necessary in the future to confirm that these trends are seen beyond the transcriptome.

This project addressed LEPH and HEPH but did not address fertility and hatchability rates in these hens. For HEPH to impact the turkey industry, HEPH must exhibit average or

above average fertility and hatchability compared to industry standards. If fertility and hatchability rates do not differ between LEPH and HEPH, selection for HEPH over LEPH would not only improve egg production levels but also the number of poults produced by a single hen. Additionally, growth parameters in progeny derived from these hens were not examined in these studies. Similar to fertility and hatchability rates, the growth of progeny from HEPH must exhibit average or above average feed efficiency and average daily gain to improve the efficiency of the turkey industry.

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