# ABSTRACT

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| Title of Thesis: | CHARACTERIZING THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN TURKEYS GENETICALLY SELECTED FOR INCREASED EGG PRODUCTION |
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Within the United States, the turkey industry has historically focused on increased meat production. Studies have shown that there is a negative correlation between meat and egg production. Previous research in our laboratory compared high egg producing hens (HEPH) to low egg producing hens (LEPH). It was found that HEPH express increased levels of mRNA for genes associated with stimulating the hypothalamic-pituitary-gonadal (HPG) axis. We also demonstrated that the hypothalamic-pituitary-thyroid (HPT) axis exerts some effect on egg production. In order to increase our understanding of differences in egg production, we focused on a genetically selected line with increased egg production (E line) and a random bred control line (RBC1). Related to the HPG axis, differences were found related to steroidogenesis and feedback mechanisms. Within the HPT axis, the RBC1 line

tended to have increased mRNA levels of genes associated with stimulation of the axis compared to E line.

# CHARACTERIZING THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN TURKEYS GENETICALLY SELECTED FOR INCREASED EGG PRODUCTION

by

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# Dedication

For my parents. Thank you for everything.

# Acknowledgements

I would like to thank my graduate committee for their help and guidance throughout this process. Especially Dr. Porter for always being available to answer my many questions and to remind me that science is not always the results we expect. Thank you also to my fellow Porter lab mates. Your willingness to help and listen to any problem large or small was incredibly helpful and appreciated. And finally thank you to my family who have always supported and encouraged me to achieve my goals.

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# List of Abbreviations

| Abbreviation | Definition |
|---|---|
| AEPH | average egg producing hens |
| AVT | arginine vasotocin |
| BARC | Beltsville Agricultural Research Center |
| BSA | bovine serum albumin |
| $Ca^{2+}$ | calcium |
| CAMK | calmodulin kinase |
| cAMP | cyclic adenosine monophosphate |
| CGA | common alpha subunit |
| CYP11A1 | cholesterol side chain cleavage enzyme |
| CYP17A1 | 17, 20 lyase |
| CYP19A1 | aromatase |
| DA | dopamine |
| DAG | diacylglycerol |
| DIO2 | iodothyronine deiodinase 2 |
| $E_2$ | estradiol |
| EPD | eggs per day |
| ESR1 | $E_2$ receptor alpha |
| ESR2 | $E_2$ receptor beta |
| F1 | largest preovulatory follicle |
| F1G | F1 granulosa |
| F5 | fifth largest preovulatory follicle |
| FSH | follicle stimulating hormone |
| FSHB | FSH beta |
| FSHR | FSH receptors |
| GnIH | Gonadotropin-releasing hormone |
| GnIHR | GnIH receptors |
| GnRH | gonadotropin-releasing hormone |
| GnRHR | GnRH receptor |
| HEPH | high egg producing hens |
| HGP | hypothalamo-pituitary-gonadal |
| HPT | hypothalamo-pituitary-thyroid |
| HSD17B1 | hydroxysteroid dehydrogenase 17β1 |
| HSD3B1 | 3β-hydroxysteroid dehydrogenase |
| $IP_3$ | 1,4,5-trisphosphate |
| ITGAV | integrin alpha-V |
| ITGB3 | integrin beta-3 |

| | |
|---|---|
| LEPH | low egg producing hens |
| LH | luteinizing hormone |
| LHB | LH beta |
| LHCGR | LH receptor |
| mRNA | messenger RNA |
| $P_4$ | progesterone |
| PGR | progesterone receptor |
| PGR | $P_4$ receptor |
| POA | preoptic area |
| PRL | prolactin |
| PVN | paraventricular nucleus |
| RBC1 | random bred control |
| RIA | radioimmunoassay |
| RT-qPCR | reverse transcription quantitative PCR |
| SMEM | minimum essential medium, spinner modification |
| SNP | single nucleotide polymorphisms |
| STAR | steroidogenic acute regulatory protein |
| SWF | small white follicles |
| T | testosterone |
| $T_3$ | triiodothyronine |
| $T_4$ | thyroxine |
| THRA | thyroid hormone receptor alpha |
| THRB | thyroid hormone receptor beta |
| TRH | thyrotropin-releasing hormone |
| TRHR | TRH receptor |
| TSH | thyroid stimulating hormone |
| TSHB | TSH beta |
| VIP | vasoactive intestinal peptide |

# Chapter 1: Literature Review

## Turkey Industry

In 2019, according to the USDA National Agricultural Statistics Service, 2.8 million turkey poults were hatched in the United States (United States Department of Agriculture, 2019). Of the poults hatched and raised, the majority will be harvested for meat consumption and sold to the US consumer in a variety of turkey-based products. Due to the lack of turkey egg consumption, the industry has placed focus on growth and size of individual turkeys to increase the value per carcass. In order to achieve this growth, producers must select for increased body weight in egg laying hens (Nestor, et al., 2006). These hens will produce offspring with increased body weight in comparison to offspring from smaller hens.

In multiple studies performed by Nestor et al., it has been shown that a producer can easily increase body weight in a hen line with repeated crossing to a male line that also has prominent body weight (Nestor, et al., 1997). This will yield offspring with higher body weights over time. Over generations, they also observed increased breast muscle size in comparison to the offspring of the original hen line. Body weight and breast muscle size are traits often selected for due to the greater yield in consumable (and profitable) meat. Unfortunately, in these studies it was evident that there was a negative correlation between body weight and efficient egg laying traits such as decreased number of eggs throughout the production period and decreased hatch of fertilized eggs (Nestor, et al., 1997).

## HPG Axis

Reproduction in turkey hens is governed by the hypothalamo-pituitary-gonadal (HPG) axis. The HPG axis is responsible for stimulation of ovarian steroidogenesis, folliculogenesis, and ultimately ovulation. The axis is comprised of

the hypothalamus, pituitary, and ovary and is regulated by the secretion of stimulatory and inhibitory hormones. Gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibiting hormone (GnIH) are neuropeptides produced by neurons in the hypothalamus portion of the brain (Ubuka, et al., 2013). GnRH is synthesized and released by neurons originating in the preoptic area (POA) of the hypothalamus. These neurons extend down into the median eminence where they terminate and release GnRH (Saldanha, et al., 2001). The hormone is then transported to the anterior pituitary by long portal veins within the hypothalamo-hypophyseal portal system. Stimulation of the anterior pituitary by GnRH binding to GnRH receptors (GnRHR) results in the release of gonadotropin hormones. The two main gonadotropins are follicle stimulating hormone (FSH) and luteinizing hormone (LH).

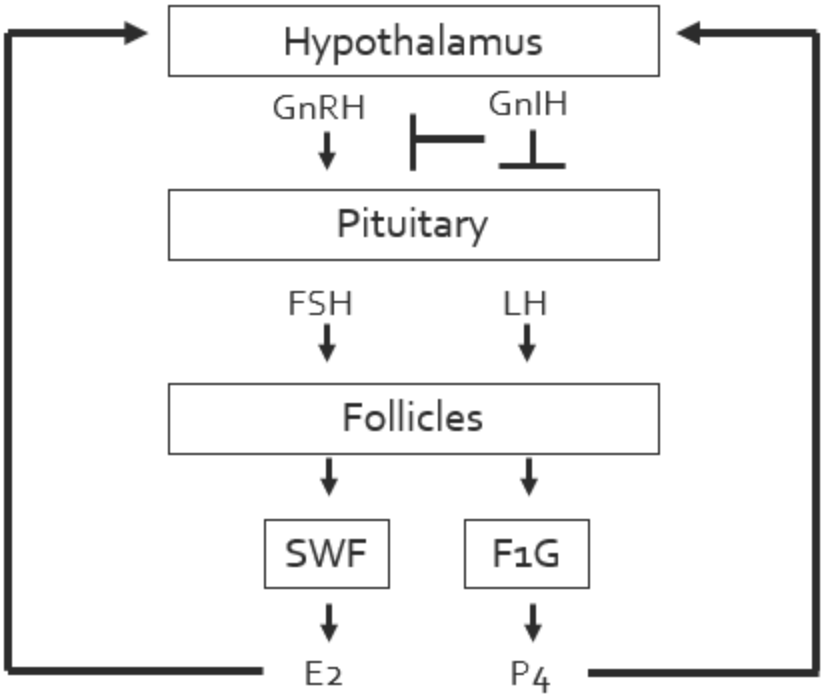


**Figure 1.1**: HPG axis hormone cascade.

GnRHR is a seven-transmembrane domain, G protein-coupled receptor, meaning the receptor is found within the plasma membrane extending out into the extracellular fluid (Millar, et al., 2004). When GnRH binds to GnRHR on the membrane of gonadotrophs in the anterior pituitary, $G_{\alpha q}$ dissociates from the protein and stimulates the phospholipase C pathway. This results in activation of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$), which results in increased intracellular calcium ($Ca^{2+}$). $Ca^{2+}$ binds calmodulin which activates calmodulin kinase (CAMK) leading to phosphorylation of proteins and the ultimate release of synthesized gonadotropins (Stojokovic, et al., 1994). DAG activates protein kinase C which is an enzyme that catalyzes phosphorylation of proteins to elicit a response at the cellular level (Hammes & Mendelson, 2012).

Gonadotropin release from the anterior pituitary can be inhibited by GnIH. GnIH releasing neurons are also found within the hypothalamus, but originate from the paraventricular nucleus (PVN) instead of the POA (Tsutsui, et al., 2009). Neurons from the PVN terminate in the median eminence, and the hormone travels through the hypothalamo-hypophyseal portal system to the anterior pituitary gland, where it binds specific GnIH receptors (GnIHR). GnIHR is also a G-coupled protein receptor. It has been proposed in chickens that GnIHR works by inhibiting intracellular cyclic adenosine monophosphate (cAMP) through the adenylyl cyclase pathway (Shimizu & Bedecarrats, 2010). Binding of the receptor stimulates dissociation of $G_{\alpha i}$, which inhibits adenylyl cyclase.

GnIH inhibits the secretion of gonadotropins by the anterior pituitary in a rapid action. (Bentley, et al., 2006). In addition to this rapid inhibition of

gonadotropin release, there is evidence to support that GnIH neurons from the PVN extend to the POA and terminate in close proximity to GnRH neuron bodies within the POA (Tsutsui, et al., 2009). This suggests a possible inhibition of GnRH release through inhibition of the GnRH neurons by GnIH. Regardless of its mode of action, GnIH works to inhibit or reduce the release of key gonadotropin hormones from the anterior pituitary.

As previously stated, the key gonadotropins involved in the HPG axis of turkey hens are FSH and LH. Both hormones are synthesized and released from gonadotrophs within the anterior pituitary and are glycoproteins that share a common alpha subunit (CGA) (Pierce & Parsons, 1981). The difference in the beta subunit (LHB or FSHB) determines the specific structure and function of the glycoprotein, but the presence of the alpha subunit is still required to ensure proper physiological activity (Burke, et al., 1979). Studies in chickens show that gonadotroph cells of avian species within the anterior pituitary synthesize either LH or FSH and that there is a higher proportion of LH-synthesizing gonadotrophs (Proudman, et al., 1999).

FSH is responsible for the stimulation of the ovary and development of small white follicles (SWF) (Calvo & Bahr, 1983). LH is responsible for follicle maturation of the preovulatory follicles. Both gonadotropins are involved in the process of steroidogenesis. Within avian species, the process of steroidogenesis occurs in a three cell model of steroidogenesis (Porter, et al., 1989), with cholesterol being the common derivative for all steroid hormones produced within the model. The binding of LH to the LH receptor (LHCGR) on granulosa cells of the preovulatory follicles activates a G protein-coupled pathway through the activation of adenylate cyclase and

an increase in cAMP (Park, et al., 2019). Increased cAMP activates steroidogenic acute regulatory protein (STAR) to transport intracellular cholesterol to the inner mitochondrial membrane. Following this step, LH-induced cholesterol side chain cleavage enzyme (CYP11A1) converts cholesterol into pregnenolone. These two steps are recognized as the rate limiting steps for steroidogenesis (Park, et al., 2019). Pregnenolone is then converted to progesterone ($P_4$) by the enzyme 3β-hydroxysteroid dehydrogenase (HSD3B1) (Huang & Nalbandov, 1979), which is regulated by LH-induced cAMP (Park, et al., 2019). At this point, $P_4$ can be secreted by the granulosa cell and bind progesterone receptors (PGR) in the hypothalamus or pituitary, or it can move to the theca interna cell layer of the follicle. Within the theca interna cells, $P_4$ is metabolized to androstenedione (androgen intermediate) by 17, 20 lyase (CYP17A1) and then converted to testosterone (T) by hydroxysteroid dehydrogenase 17β1 (HSD17B1) (Park, et al., 2019). These steps are also regulated by LH. T can then be secreted by the theca interna cells or move to the theca externa cells where it is catalyzed to estradiol ($E_2$) by aromatase (CYP19A1) (Kato, et al., 1995). Conversion of T to $E_2$ by CYP19A1 is regulated by FSH binding to FSH receptors (FSHR) on theca externa cells. FSHR induces an adenylate cyclase pathway similar to LHCGR (Li, et al., 2019). $E_2$ is secreted by the theca externa cells and

enters the circulation where it can bind estrogen receptors (ESR1 or ESR2) in the hypothalamus or pituitary.

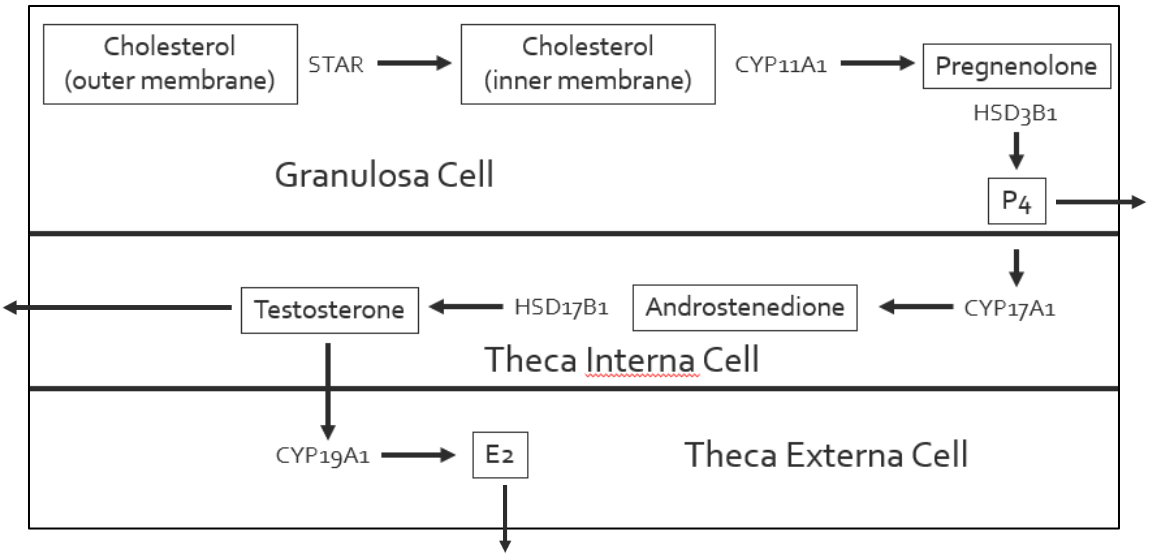


**Figure 1.2**: Three-cell model of steroidogenesis by the follicle cell layers.

Specific secretion of steroids from the three cell layers of the follicle is also dependent on of the maturity of the follicle. This will be explored more in the section below focusing on folliculogenesis, but briefly, the next follicle to ovulate (F1 follicle) will be responsible for most of the $P_4$ production in response to LH stimulation (Porter, et al., 1989). FSH on the other hand, is the major gonadotropin stimulating smaller follicles such as the fifth largest preovulatory follicle (F5) and the small white follicles. The theca cell layers of these follicles are responsible for the majority of production of androgens and $E_2$ respectively (Porter, et al., 1989; Robinson & Etches, 1986).

Ovarian steroids produced by the follicles provide feedback on the hypothalamus and anterior pituitary to regulate the release of gonadotropins. Steroids

bind nuclear hormone receptors that are found within the cell. Specific hormones will bind specific receptors to illicit a response from the cell. The receptor and hormone complex will bind to response elements on the DNA as either homodimers or heterodimers (Thompson & Kumar, 2003). Response elements are unique sequences of DNA that are upstream of the transcription site that is the target sequence of alteration. Alteration of transcription factors will influence protein production (O'Malley & Schrader, 1976) and, ultimately, influence cell action. For example, it has been shown in chickens that $E_2$ will bind either estrogen receptor alpha (ESR1) or estrogen receptor beta (ESR2) located within the cytosol of target tissues (Smith et al, 1979). When $E_2$ binds its receptor, the complex moves into the nucleus, and the DNA binding portion of the receptor will bind the $E_2$ response element sequence of the strand as a homodimer (Loven et al., 2001). This binding will attract co-activator proteins that will bind to the complex and initiate transcription at the target gene sequence downstream of the response element (Thompson & Kumar, 2003).

Increased production of $P_4$ by the granulosa cell layer of the F1 follicle acts in a positive feedback mechanism to stimulate LH production within gonadotrophs in the anterior pituitary (Johnson, et al., 1985). $P_4$ will bind progesterone receptors (PGR) found in the hypothalamus to stimulate the continuous release of GnRH from the POA and LH from the anterior pituitary in order to stimulate $P_4$ secretion. LH continues to stimulate $P_4$ production by the granulosa cells of the F1 follicle until a surge in $P_4$ results in the preovulatory surge of LH and subsequent ovulation of the F1 follicle. This is demonstrated by the documented increase in $P_4$ production by the F1

follicle and reduced production of other hormones prior to ovulation (Kato, et al 1995).

$E_2$, on the other hand, exerts negative feedback on gonadotropin secretion. A study conducted in chickens found a decrease in messenger RNA (mRNA) that encodes for LHB when $E_2$ increased and a subsequent increase in LHB when $E_2$ decreased as a result of ovariectomy (Terada, et al., 1997). This decrease in the beta subunit will result in decreased LH synthesis by gonadotrophs in the anterior pituitary and subsequent decrease in steroidogenesis within the follicle cell layers.

**Folliculogenesis**

Unlike mammals, only one ovary is active within the turkey hen, and at any given point within the reproductive period, the ovary can have four different types of follicles present at various stages of maturation. Primordial and primary follicles begin to develop in the late embryonic stage and early post hatch time period. As the hen grows, small primary follicles are recruited to become larger prehierarchical follicles. These small white follicles (SWF) are responsible for most of the production of $E_2$ found within the reproductive axis (Robinson & Etches, 1986). As prehierarchical follicles continue to develop, a select number will differentiate into larger, preovulatory follicles (Johnson, 2015a). There are typically eight to ten large preovulatory follicles found on the turkey ovary during the production period. The largest, F1 follicle will be the next follicle to ovulate and is responsible for most of the $P_4$ secretion due to stimulation of its granulosa cell layer by LH (Porter, et al., 1989).

Initial recruitment of primordial follicles to primary follicles occurs as a result of the formation of the theca cell layer (Johnson, 2015b). Granulosa cells are initially arranged in a single layer around the primordial germ cell with a thin layer of perivitelline membrane between the granulosa cells and oocyte (Johnson, 2015b). During initial recruitment, the theca cells develop and are separated from the granulosa cells by the basal lamina. As these primary follicles continue to grow, the theca layer differentiates into the theca interna and theca externa layers, and the follicles accumulate a protein-rich yolk (Johnson, 2015a) that will continue to serve as the nutrient source for the growing oocyte. Eventually, a cohort of primary follicles will be recruited to become prehierarchical follicles that are characterized as SWF containing a yolk rich in lipids that have a focused production of $E_2$ by the theca externa cells (Porter, et al., 1991) in response to FSH.

Selection of the largest SWF to become a preovulatory follicle occurs next. At this point, the follicle will go through rapid growth in size and yolk accumulation in comparison to previous developmental stages (Ghanem & Johnson, 2018). It has also been suggested that during this transition to a preovulatory follicle, the follicle's granulosa cells become responsive to gonadotropins through activation of the cAMP pathway by FSHR (Tilly, et al., 1997; Johnson, 2015b). This allows steroidogenesis to occur within the granulosa cells and is also characterized by a transition from FSH regulation to LH regulation via LHCGR. An increase in *LHCGR* mRNA expression has been suggested as evidence that preovulatory follicles are more responsive to LH during the final stage of maturation (Johnson, 2015a). So as the follicle goes through

the various stages of development and the cell layers differentiate, the cells that are responsible for steroid synthesis change.

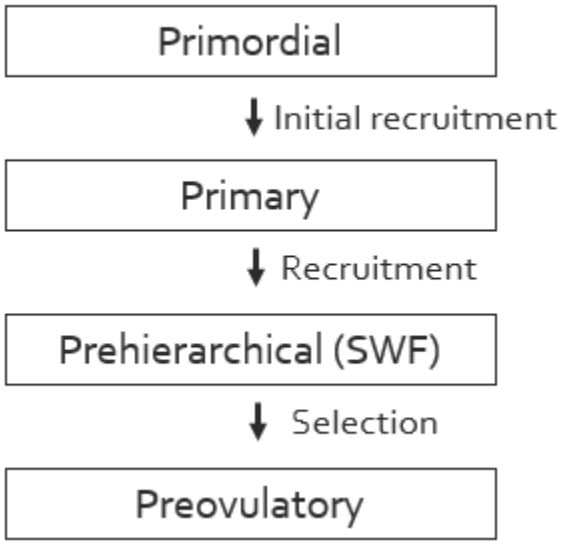


**Figure 1.3**: Process of folliculogenesis in the turkey hen.

**LH surge and Ovulation**

As the preovulatory follicle continues to grow through the accumulation of yolk, steroidogenesis continues to occur in response to LH stimulation from the anterior pituitary. At this point, the granulosa cells are synthesizing and secreting increasing concentrations of $P_4$ that have a positive feedback for continuous production of LH (Johnson, Johnson, & van Tienhoven, 1985). Johnson et al. were able to demonstrate a correlation between increased $P_4$ and a subsequent increase in LH in chickens. Later, they added to this relationship by demonstrating an increase in STAR in granulosa cells of preovulatory follicles especially within the cells of the F1 follicle (Johnson & Bridgham, 2001). This upregulation of STAR results in increased

steroid production, with the primary product being $P_4$. This positive feedback loop of LH and $P_4$ eventually leads to the preovulatory surge in both hormones.

In turkey hens, the preovulatory surges of LH and $P_4$ have been documented as occurring six to ten hours before ovulation (Yang, et al., 1997), with the LH surge following the surge in $P_4$. It has also been shown that it is necessary for the increased concentration in $P_4$ to subside in order to have continual surges of LH resulting in subsequent ovulations throughout the reproductive period (Bacon & Liu, 2004).

The physical process of ovulation involves the breakdown of the follicle wall along the stigma that results in the expulsion of the oocyte. In response to LH, enzymes along the stigma of the follicle wall begin to break down the wall and, in concert with smooth muscle contractions and localized apoptosis, leads to the rupture of the follicle (Johnson, 2015b). The postovulatory follicle still retains the cell layers of the follicle, but exhibits a drastic decrease in steroid synthesis due to decreased LH stimulation of the cAMP pathway. Studies in the chicken have shown a steady decrease in expression of the enzymes involved in steroidogenesis over time in the postovulatory follicle (Armstrong, et al., 1977).

**Egg Production and Movement**

The released ovum will enter the oviduct through the infundibulum, which is the cranial portion of the female reproductive tract. In avian species, the ovum will remain in the infundibulum between fifteen and thirty minutes depending on the species (Johnson, 2015b). Next, the ovum (fertilized or unfertilized) moves to the magnum where it begins to accumulate albumin over two to three hours. Following this, the ovum will move through the isthmus portion of the tract where the inner and

outer shell membrane will form around the ovum. The ovum will spend the greatest amount of time in the shell gland portion of the oviduct. Over the course of eighteen to twenty-six hours, calcification will occur and the egg shell will form. The fully formed egg will pass into the vagina for oviposition (Johnson, 2015b). In the turkey hen, the time between ovulation and oviposition is roughly twenty-six hours. The egg laying rate of an individual hen is correlated with the individual's ability to successfully achieve ovulation (Bacon & Liu, 2004).

Oviposition regulation in avian species has been shown to be regulated by the neurohypophysis hormone arginine vasotocin (AVT). Increased plasma concentrations of AVT have been associated with oviposition in the chicken (Shimada, et al., 1986). AVT neurons are located in the POA and supraoptic regions of the brain (Jurkevich & Grossman, 2003), and the axons terminate in the posterior lobe of the pituitary. AVT receptors in the shell gland of the chicken initiate contraction of the gland in response to increased levels of AVT (Koike, et al., 1988). AVT has also been shown to be synthesized by the ovary and follicle cell layers in varying amounts throughout folliculogenesis and the oviposition cycle (Saito, et al., 1990), but those levels are much lower than the amount of AVT released from the hypothalamus (Saito & Grossman, 1999). This suggests that hypothalamic AVT plays a more important role in oviposition than locally synthesized AVT.

**High vs Low Egg Laying Turkey Hens**

Previous research within our laboratory has demonstrated the correlation between ovulation frequency and egg production within a commercial turkey breeding flock. Initial experiments showed the relative distribution of egg laying rates

within a flock. In a given flock, most hens will lay about between 0.6 and 0.8 eggs per day (EPD) throughout the production period (Brady, et al., 2019). These hens were designated as average egg producing hens (AEPH). It was also demonstrated that deviation from this average will occur in a bell-shaped curve distribution, with individuals on the extreme ends of EPD. In the commercial flock analyzed (n=200 hens), it was found that the top fifteen percent of the flock produced greater than 0.8 EPD. These individuals were classified as high egg producing hens (HEPH). On the other end of the curve, the bottom fifteen percent of the flock produced less than 0.6 EPD and were classified as low egg producing hens (LEPH). This distribution was consistent across multiple commercial breeding flocks and lead to investigation into the factors causing these differences in egg production between the top and bottom portions of the flocks.

First, a study was conducted to determine the effect that the preovulatory surge has on the hormone profile and expression of genes related to the HPG axis in AEPH. Tissue and blood samples were collected from AEPH either during or outside the preovulatory surge of $P_4$. A hen was determined to be in or out of the surge based on plasma $P_4$ concentration, which has been demonstrated to be a good indicator of the LH surge in turkey hens (Yang, et al., 1997). The hormone profile analysis demonstrated the large increase of plasma $P_4$ concentration in hens timed to be within the preovulatory surge compared to outside of the surge. The average $P_4$ concentration of the preovulatory surge hens demonstrated an eight-fold increase compared to outside surge hens (Brady, et al., 2019). There was, however, no difference in $E_2$ concentration between hens inside the surge and hens outside of the

surge. Tissue samples from relevant HPG structures were also collected, and relative mRNA expression was compared for genes related to the axis between surge and outside of the surge. In the hypothalamus, significantly increased mRNA expression of the $E_2$ hormone receptors (*ESR1* and *ESR2*) was observed in the surge hens compared to outside the surge hens. There was also a significant decrease in relative expression of the *GNRH* gene in the surge hens. This suggests suppression of gonadotropin synthesis and release is occurring while a hen is within the surge. In the pituitary, surge hens demonstrated a significant increase in mRNA expression of two receptors associated with the axis (*GNIHR1* and *ESR1*) and a significant increase in expression of the *FSHB* gene. Surge hens also showed a significant decrease in several genes including *GNRHR*, *PGR*, and *LHB* (Brady, et al., 2019). Again, these results support the hypothesis that hens within the surge are experiencing suppression of gonadotropin release (specifically LH) through disruption of the positive feedback loop of $P_4$ and LH that stimulates ovulation. Increased expression of *GNIHR1* also indicates direct inhibition of LH release via PVN neurons. The increased expression of *ESR1* and *FSHB* could be due to increased focus on growth and recruitment of primary to prehierarchical follicles after a designated F1 follicle has reached maximum maturation. Expression of important genes were also analyzed in the three cell layers of the F1 and F5 (fifth largest preovulatory follicle) follicles. In the granulosa layer, it was observed that there was only a significant increase in *STAR* mRNA in the F1 follicle of the surge hens compared to outside the surge hens. There was, however, a significant decrease in *LHCGR* and *CYP11A1* in F1 granulosa cells of surge hens. This indicates cholesterol is still being transported into the

mitochondria, but *CYP11A1* is converting less of it into pregnenolone due to reduced activation of the enzyme by *LHCGR*. In the F5 granulosa, there was a significant increase in *LHCGR* mRNA in the surge hens suggesting these follicles are still responsive to LH and producing $P_4$. In the theca interna layer, there was a significant increase in *FSHR*, *LHCGR*, and *HSD3B1* in the F1 follicle and *HSD17B1* in the F5 follicle. In the theca externa layer, there was a significant increase of *FSHR* and *CYP17A1* in the F1 follicle and *CYP19A1* in the F5 follicle (Brady, et al., 2019). The theca interna and externa layers were still expressing genes related to androgen and estradiol production in the surge. Together, these data demonstrate the effect the preovulatory surge of LH has on the various cascades involved in maturation, and eventual ovulation, of preovulatory follicles.

The next study focused on differences between HEPH and LEPH. The study collected samples from hens both inside and outside the preovulatory surge. Hormone assays were performed, and again it was shown that there was a significant increase in plasma $P_4$ concentration in surge versus outside surge hens (Brady, et al., 2020). This occurred regardless of group (HEPH or LEPH). There was also a significant difference in $E_2$ concentration, but this was group dependent, not surge dependent. $E_2$ concentration was increased in HEPH in both non-surge and surge conditions relative to LEPH. In the hypothalamus, *GNIH* mRNA expression was significantly increased in LEPH under both conditions, which could suggest overall inhibition of gonadotropin release. *PGR* and *ESR2* mRNA levels were decreased in HEPH during the surge compared to their non-surge levels, while *ESR1* was increased in HEPH compared to LEPH in the surge. LEPH decreased expression of *ESR1* during the

surge compared to outside the surge. This indicates differences in responsiveness to steroid feedback between HEPH and LEPH. In the pituitary related to releasing factors, there was a significant decrease in *GNRHR*, *GNIHR1*, and *GNIHR2* mRNA expression in HEPH compared to LEPH during the surge. *GNRHR* and *GNIHR2* were decreased in HEPH when comparing the surge versus non-surge levels. The gonadotropin subunits *LHB* and *FSHB* mRNA expression also showed some variation. *LHB* was increased in HEPH under non-surge conditions compared to non-surge LEPH. It was also increased in non-surge HEPH compared to surge HEPH. *FSHB* was only significantly increased in HEPH compared to LEHP during the surge. *PGR* was decreased for both groups during the surge. *ESR1* was increased in HEPH outside the surge, but was decreased and similar to LEPH during the surge. These data show there was a difference in expression when comparing the groups and this difference can be more pronounced due to the surge depending on the gene. In the F1 granulosa layer, *STAR* mRNA expression was increased in non-surge HEPH and significantly increased during the surge. LEPH also demonstrated an increase during the surge compared to no surge. *CYP11A1* mRNA was increased in HEPH outside the surge, but LEPH showed an increase during the surge compared to non-surge. Both of these genes are vital to $P_4$ production. In F1 theca interna, only *CYP17A1*expression was significantly different. It was increased in HEPH compared to LEPH in non-surge hens and decreased in HEPH during the surge both in comparison to LEPH in the surge and HEPH non-surge levels. On the other hand, *CYP17A1* significantly increased in LEPH during the surge compared to outside the surge. In F1 theca externa, *FSHR* expression was significantly decreased in HEPH under non surge

conditions. However, during the surge it was increased in HEPH compared to both LEPH and non-surge levels. *LHCGR* was also decreased in HEPH compared to LEPH in non-surge hens, but increased during the surge compared to outside the surge. *CYP19A* was significantly increased in HEPH during the surge compared to non-surge HEPH and surge LEPH (Brady, et al., 2020). In the SWF, *LHCGR* mRNA expression was increased in HEPH in both non-surge and surge conditions compared to LEPH. *LHCGR* decreased during the surge compared to non-surge for LEPH. Enzymes involved in $E_2$ production were altered both by group and condition. *CYP17A1* expression was increased in HEPH versus LEPH, but both groups saw a decrease during the surge compared to outside the surge. For *HSD17B1*, surge LEPH were decreased compared to non-surge LEPH, and HEPH were increased compared to LEPH in the surge. *CYP19A1* decreased in surge LEPH compared to non-surge, while surge HEPH increased compared to non-surge and surge LEPH (Brady, et al., 2020). This study concluded that the difference in egg production between the extreme ends of the flocks is due to differences in gene expression related to the HPG axis. Both groups saw an expected and appropriate increase in plasma $P_4$ concentrations at the time of the preovulatory surge, indicating their ability to achieve the surge and ovulate. However, the frequency of ovulation and subsequent egg laying is being influenced by the alterations in expression of genes related to steroidogenesis and their various feedback mechanisms. There were also some differences in stimulation and inhibition of the axis as demonstrated by the increase in *GNIH* and *GNIHR* in LEPH. It seems HEPH are more sensitive to stimulation of the HPG axis and are more sensitive to feedback from the products of steroidogenesis.

HEPH also demonstrate increased expression of the enzymes involved in steroidogenesis, allowing them to be more efficient throughout folliculogenesis and ovulation**.**

**E line and RBC1 line**

The relationship between growth and reproduction in the turkey has been observed and tested over the last seven decades. Utilizing this selection process, researchers have been able to maintain genetically selected lines of turkeys that have valued traits such as increased body weight, increased egg production, or a cross of various commercial lines (Nestor, 1971). These experiments have allowed researchers to determine how these traits interact over time. One such study involved the use of a line selected for increased body weight over time (F line) and a line selected for increased egg production (E line). F line generational studies have shown that the line has increased body weight and size, but egg production throughout production period remains negatively correlated (Nestor, et al., 2008). Crossing of the two lines (FE line) resulted in a line tandemly selected for both increased body weight and increased egg production (Nestor, 1985). At various generation intervals, either growth or egg production was preferentially selected for. They found that during these selection periods there was a negative correlation between the selected trait and the unselected trait. They also found an additive effect over generations. Body weight decreased in every subsequent generation that was selected for egg production (Nestor, 1985). These results were also replicated when comparing the E line to its original line (random-bred control; RBC1). The RBC1 line is a random bred control population of turkeys that were derived from crossing four commercial lines that

boasted one or more economically important traits (growth, conformation, or reproduction). The line was initiated in 1957 and closed in 1958 (McCartney, 1964). The purpose of the line was to maintain a genetic control for other genetically selected lines for research purposes. One line derived from RBC1 was the E line. Initial selection was based on eighty-four-day egg production for the first three generations from RBC1 line. Final selections were based and maintained according to one hundred and eighty day egg production (McCartney, et al., 1968). Body weight and egg laying characteristics were compared between the two lines. E line birds had increased eggs laid throughout the production period as well as increased sensitivity to photostimulation (Emmerson, et al., 2002). Again, E line birds had decreased body weight when compared to the RBC1 line, but were more efficient layers. Interestingly, a study focused on the genetic interaction between breast muscle morphology and egg production found there was no significant difference between the E and RBC1 lines at various ages (Velleman, et al., 2007). While there was a slight decrease in scoring in E line birds, it was not enough to conclude that increased egg production negatively impacts breast muscle morphology. In order to determine where these genetic differences in egg production are coming from, a line comparison between the E line and RBC1 line would be beneficial.

The selection of the E line from the RBC1 line has resulted in historical differences between the two lines. RBC1 hens have increased body weight compared to E line hens, while RBC1 line hens are less efficient egg layers (Emmerson, et al., 2002). Both of these lines have been maintained and still manifest these key characteristic differences related to body weight and egg production. By determining

what factors of the HPG axis are affecting the difference in egg production between the lines, it might be possible to determine the relationship between meat and egg production and implementing that knowledge into practice in commercial flocks.

**HPT Axis**

While the HPG axis is considered the most important factor affecting egg production in avian species, evidence suggests hormones and genes more associated with the hypothalamo-pituitary-thyroid (HPT) axis may exert some influence on the HPG axis that could be contributing to egg production rates. For example, a study in induced hypothyroid chickens found that there was a reduction in the number of eggs laid as a result of the treatment (van Herck, et al., 2013). While there was a reduction in eggs laid, there was no change in quality of the eggs, suggesting that thyroid hormone is playing a part in follicle maturation or ovulation. A study conducted in turkey hens established a correlation between the thyroid, thyroid hormones, and egg production. Following thyroidectomy at various ages, hens experienced either no initiation of egg laying or reduction in egg laying if already within the production period (Lien & Siopes, 1989).

Thyrotropin-releasing hormone (TRH) is released by the hypothalamus to stimulate the anterior pituitary. Specifically, neurons in the PVN region of the hypothalamus release TRH into the median eminence where it is transported to the anterior pituitary (Geris, et al., 1999). TRH binds to the TRH receptor (TRHR) on thyrotrophs in the anterior pituitary. TRHR is a seven transmembrane G protein coupled receptor that binds $G_{\alpha q}$ to initiate the phospholipase C pathway (Sun, et al., 1998). In response to activation of TRHR, thyrotrophs will synthesize and release

thyroid stimulating hormone (TSH) (Groef, et al., 2005). TSH shares the same common alpha subunit as the other glycoproteins FSH and LH, but differs in the beta subunit (TSHB) (McNabb & Darras, 2015). TSH stimulates the thyroid gland to synthesize and secrete triiodothyronine ($T_3$) and thyroxine ($T_4$). In avian species, there is a greater concentration of $T_4$ in the blood than $T_3$ (McNabb & Darras, 2015).

Thyroid hormones exert their effects throughout the body via various thyroid hormone receptors (THR). THRA and THRB are both nuclear hormone receptors that act through genomic pathways to alter DNA transcription in response to thyroid hormone binding (Cheng, 2000). There are also plasma membrane integrin receptors (ITGAV and ITGB3) that thyroid hormones can bind to and elicit a response from a cell using a non-genomic pathway (Cheng, et al.,2010). These receptors are found throughout the body including in the hypothalamus and pituitary. In the chicken, $T_3$ has been shown to downregulate the expression of *TSHB* (Gregory & Porter, 1997). Thyroid hormone acts in a negative feedback mechanism to inhibit TRH and TSH release. The presence of thyroid hormone receptors in cells associated with follicle maturation and steroidogenesis suggests that thyroid hormones could affect those pathways (Sechman, 2013).

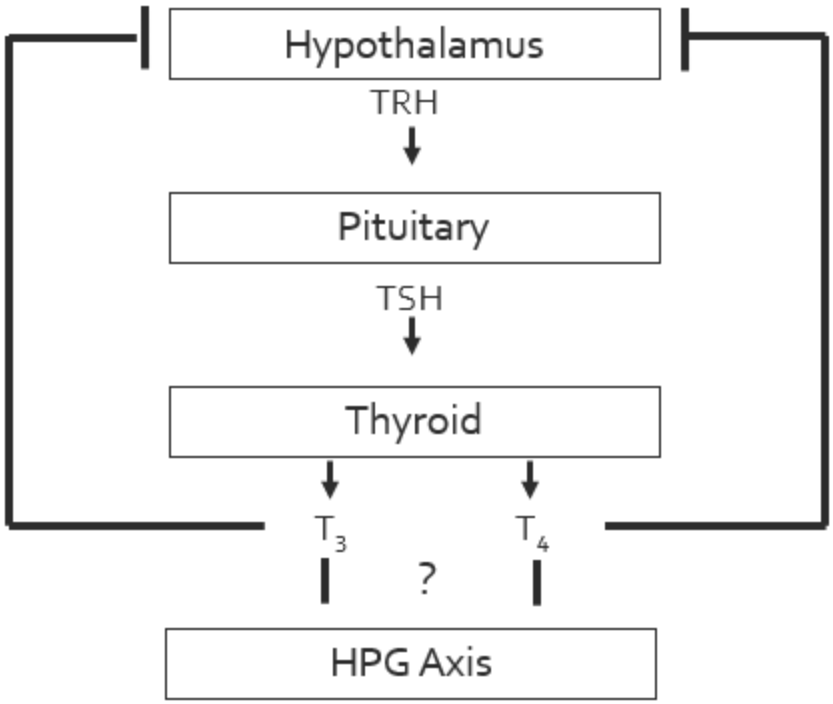


**Figure 1.4**: HPT axis hormone cascade.

Previous work within our laboratory has suggested there is possible regulation of egg production occurring due to differences in the HPT axis between HEPH and LEPH. Briefly, one experiment involving transcriptome analysis found that variations in thyroid hormone transporters and thyroid hormone receptors exist between HEPH and LEPH. The HPT axis was upregulated in F5 follicles in HEPH, while the axis was upregulated in F1 follicles in LEPH (Brady, 2019). Investigation into how this regulation might be affecting the HPG axis lead to *in vitro* experiments involving SWF samples taken from HEPH and LEPH. It was found that treatment with $T_3$ affected $E_2$ production in response to FSH. HEPH showed increased $E_2$ production in response to FSH when no $T_3$ was present compared to LEPH. However, when pre-treated with $T_3$, HEPH produced $E_2$ in response to FSH at levels similar to LEPH

(Brady, 2019). This suggests that $T_3$ is disrupting or suppressing steroidogenesis even when FSH is present and that the difference of when upregulation of the HPT axis is occurring during follicle maturation could indicate a potential cause for differences in ovulation frequency between HEPH and LEPH.

Hormone profiles and gene expression analysis were performed on hormones and genes related to the HPT axis in a commercial laying flock to determine how the axis was affected in different groups (AEPH, HEPH, and LEPH) and under different conditions (outside or within the preovulatory surge). AEPH had significantly increased plasma $T_4$ concentrations during the surge, but decreased $T_3$ concentration also during the surge. Non-surge HEPH had increased $T_3$ compared to LEPH, but that concentration was significantly decreased in HEPH during the surge. $T_4$ in HEPH during the preovulatory $P_4$ surge was increased both compared to surge LEPH and non-surge HEPH. $T_4$ decreased in surge LEPH compared to non-surge LEPH (Brady, 2019). These data showed that the preovulatory $P_4$/ LH surge was associated with changes in $T_3$ and $T_4$, that these changes differ between HEPH and LEPH, and that the hormone profile of HEPH is similar to AEPH.

Analysis of HPT axis related mRNA levels in HPG axis tissues also showed some differences between HEPH and LEPH. In the hypothalamus, *TRH* mRNA expression was significantly decreased in HEPH during the surge both in comparison to non-surge HEPH and surge LEPH. On the other hand, there was an increase in *TRH* in surge LEPH compared to non-surge LEPH. There were also differences in expression of mRNA levels related to thyroid hormone receptors. There was a decrease in expression of *THR* in HEPH versus LEPH regardless of inside or outside

of the preovulatory surge (Brady, 2019). This suggests reduction in the HPT axis pathway in HEPH, especially during the preovulatory $P_4$/LH surge.

In the pituitary, *THR* expression was again decreased in HEPH compared to LEPH. *TSHB* was also decreased in non-surge HEPH compared to LEPH and was decreased during the surge in both groups compared to non-surge conditions (Brady, 2019). There were no differences in gene expression in the F1 granulosa cell layer. These differences in gene expression, along with the effects of $T_3$ *in vitro* treatment of SWF cells, point to some effect by the HPT axis on egg production worth investigating further.

**Objectives**

Based on the past research conducted in our laboratory and by others, the objectives of the current project were to determine whether differences exist in expression of genes in the HPG and HPT axes between the E line and RBC1 line hens and how these differences might be influencing egg production. We have shown that differences exist between HEPH and LEPH of commercial flocks within the HPG axis concerning mRNA expression of key genes and enzymes. By comparing these genes between the E line and the RBC1 line we can determine whether the genes governing egg production in a commercial flock are similarly different between the two genetically selected lines. Comparing the E line and RBC1 line will also allow us to better understand how egg production and meat production may be interacting. The E line has historically been selected for increased egg production which has resulted in decreased body weight. The RBC1 line has decreased body egg production and inconsistent lay rates in comparison to the E line, but has still maintained an increased

body weight compared to the E line. By including genes related to the HPT axis in our investigation, we might be able to broaden our understanding of how the HPG axis and HPT axis work together (or against one another) during the egg laying cycle. We hypothesize that E line hens will have increased expression of genes related to upregulating the reproductive axis and decreased expression of genes related to upregulating the thyroid axis.

# Chapter 2: HPG and HPT gene expression differences in E line and RBC1 line turkey hens

**Abstract**

Previous studies involving commercial flocks of turkeys have demonstrated that mRNA levels for genes associated with the hypothalamo-pituitary-gonadal (HPG) axis and the hypothalamo-pituitary-thyroid (HPT) axis of high egg producing hens (HEPH) differ from those of low egg producing (LEPH) hens. HEPH express higher levels of mRNA for genes associated with stimulation of the HPG axis, while expressing lower levels of mRNA for genes associated with stimulating the HPT axis than LEPH. The objective of this study was to determine if those differences would be in similar in a line of turkeys genetically selected for high egg production (E line) when compared to a random bred control line (RBC1). The average eggs per day for E line and RBC1 hens in the study was 0.84±0.03 and 0.54±0.03 eggs/day, respectively. RNA was extracted from the hypothalamus, pituitary, F1 granulosa, and small white follicle tissue samples and analyzed for mRNA levels using reverse transcription quantitative PCR. Expression levels of the genes in E line samples compared to RBC1 samples were analyzed using the GLIMMix protocol in SAS. Within the hypothalamus, *ESR2* was significantly downregulated ($P<0.05$) in the E line while all genes associated with the HPT axis tended to be downregulated in the E line compared to RBC1 line. In the pituitary, significant upregulation ($P<0.05$) was observed in mRNA levels for *PRL* and *GNIHR2* in the E line, while *FSHB* was significantly downregulated ($P<0.05$). In the small white follicles, multiple genes associated with estradiol production tended to be upregulated in the E line. There was a significant downregulation ($P<0.05$) of a thyroid plasma membrane integrin receptor (*ITGB3*) in the E line, and the other HPT genes also tended to be downregulated in the E line. The results indicate that differences exist in the HPG and

HPT axes between the two genetic lines that are not the same as the differences observed previously between HEPH and LEPH within modern commercial flocks.

**Introduction**

In the United States turkey industry, the majority of poults hatched and raised will be harvested for meat consumption. Due to this demand for edible turkey meat, the industry historically has placed an emphasis on growth and size of individuals birds to increase the value per carcass a producer can earn. Increased selection for body weight in egg laying hens will ensure increased body weight in their offspring and will shift the overall body weight of a flock to produce larger and more valuable birds (Nestor, et al., 2006). However, it has been observed that as body weight increases, egg production decreases (Nestor, et al., 1997). It is important to find this balance between meat production and egg production to ensure that producers continue to keep up with the demand for turkey meat while also being able to maintain viable and self-sustaining flocks that will continue to produce poults year after year. In order to accomplish this goal, an improved understanding of the underlying basis for the difference in egg production among turkey hens is needed.

Reproduction in turkey hens is governed by the hypothalamo-pituitary-gonadal (HPG) axis. Interactions between the hypothalamus, pituitary, and ovary are responsible for stimulation of ovarian steroidogenesis, folliculogenesis, and ovulation. Release of gonadotropin-releasing hormone (GnRH) from neurons in the preoptic area of the hypothalamus (Saldanha, et al., 2001) stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH stimulates the ovary and development of small white follicles (SWF) (Calvo &

Bahr, 1983). LH stimulates the follicles to rapidly mature and ovulate following a preovulatory surge of LH and progesterone ($P_4$) (Johnson, et al., 1985). Both LH and FSH stimulate the three follicle cell layers to synthesize steroid hormones (Porter, et al., 1989). The largest pre-ovulatory follicle (F1) will be the next follicle to ovulate, and the granulosa layer of this follicle (F1G) is responsible for the majority of $P_4$ production within the axis (Porter, et al., 1989). Previous research in our laboratory has demonstrated differences among individual hens in mRNA expression of genes associated with stimulation and inhibition of the HPG axis within a commercial breeding flock (Hybrid Turkey, Kitchener, Ontario). It was shown that high egg producing hens (HEPH) tended to express increased mRNA levels of genes associated with stimulating the axis (ex: *GNRH*, *LHB*, *STAR*), while expressing decreased levels of genes associated with inhibiting the axis (ex: *GNIH*, *GNIHR1*) compared to low egg producing hens (LEPH) (Brady, et al., 2020).

Another possible factor affecting egg production in turkey hens is the interaction and/or interference of the hypothalamo-pituitary-thyroid (HPT) axis with tissues of the HPG axis. A study in chickens found a reduction in eggs laid as result of induced hypothyroidism (van Herck, et al., 2013). Another study performed in turkeys found similar disruption of egg laying as a result of thyroidectomy. They found that, depending on the age of the hen when the procedure was performed, the birds either did not start laying eggs or there was a reduction in eggs laid (Lien & Siopes, 1989). Experiments in our laboratory have shown interactions occurring between the HPG axis and HPT axis. Reduction in SWF FSH-induced $E_2$ synthesis occurred in response to treatment with $T_3$ *in vitro* (Brady, 2019). Transcriptome

analysis also showed variation in thyroid hormone transporters and receptors between HEPH and LEPH (Brady, 2019). The genes found to be upregulated in the LEPH where associated with stimulating the HPT axis suggesting increased expression of the HPT axis is associated with decreased egg production in the turkey hen.

Based on this previous research, the current study focused on two historical turkey lines to determine what differences in expression of genes related to the HPG and HPT axes may exist that could contribute to the variation in egg production between the two lines. The E line is a closed line initiated in 1957 that was selected for increased egg production over time and is derived from the RBC1 line, which is a random bred control population of turkeys that were produced by crossing four commercial lines that boasted one or more economically important traits (growth, conformation, or reproduction) (McCartney, 1964). Over time, both lines have maintained key differences in that the RBC1 line has increased body weight but decreased egg production compared to the E line (Emmerson, et al., 2002). It was hypothesized that E line hens will have increased expression of genes related to stimulating the reproductive axis and decreased expression of genes related to stimulating the thyroid axis compared to the RBC1 line.

## Methods and Materials

### *Hen Selection*

Twelve turkey hens each from the E line and RBC1 line, housed at the Beltsville Agricultural Research Center (BARC), were selected based on eggs laid per day (EPD) for a total of twenty-four hens. Hens were maintained following standard management practices with *ad libitum* access to food and an artificial light schedule

of fourteen hours light and ten hours dark. All animal procedures were approved by the Institutional Animal Care and Use Committee at BARC and at the University of Maryland. Year 1 flock hens were sampled six weeks into their production period over the course of three days. Year 2 flock hens were sampled thirteen weeks into their production period on the same day. Hen selection was based on daily egg records and EPD. EPD was determined by dividing the total number of eggs laid per individual hen by the days since that hen came into production. All birds were selected with the intention that they were outside of the preovulatory surge of $P_4$ and LH. In order to ensure this parameter, a radioimmunoassay (RIA) from MP Biomedical was performed on ether-extracted plasma from blood samples taken at the time of sample collection to determine $P_4$ concentration following an established protocol. Any hen with a plasma $P_4$ level greater than 2ng/ml was defined to be within the preovulatory surge and was excluded from further analysis. One E line hen from the year 1 flock had a $P_4$ concentration of 3.94 ng/mL and was subsequently removed from further analysis. The corresponding RBC1 line hen sampled at the same time was also excluded from analysis to maintain balanced group replicate numbers for statistical comparison.

*Sample Collection*

At the time of sampling, approximately one milliliter of blood was collected from the brachial vein. Hens were then euthanized via cervical dislocation. Body weight, ovary weight, and oviduct weight were recorded. The presence or absence of an egg within the reproductive tract was recorded. All preovulatory follicles were removed from the body cavity and the number recorded. The largest follicle (F1) and

fifth largest (F5) were also weighed. The hypothalamus, pituitary, and small white follicles (SWF) were dissected from the hen and immediately snap frozen in liquid nitrogen.

*Cell Layer Isolation*

The F1 was placed in cold SMEM culture media (0.1% bovine serum albumin (BSA) and 100-U/mL penicillin G and 100-µg/mL streptomycin sulfate added to media) and transported on ice to the University of Maryland. Hypothalamus, pituitary, and SWF were stored at -80ºC until RNA extraction was performed. F1 follicle layers were isolated following an established procedure (Porter, et al., 1988). The granulosa layer was isolated by first cutting a small incision in stigma of the follicle and allowing the yolk to drain. The cell layer was then removed by inverting the follicle and carefully removing the granulosa layer. The layer was then placed in a 1mg/mL trypsin and SMEM solution in a 37ºC shaking water bath for fifteen minutes. Samples were triturated every five minutes using a Pasteur pipette. After fifteen minutes, the sample was filtered using a 70uM nylon mesh and centrifuged at 50% speed for ten minutes. Media was then removed and the cell pellet resuspended in 10mL SMEM. Sample was then pipetted onto 50% Percoll and centrifuged for ten minutes. The interface between the SMEM and Percoll was removed and added to 10mL SMEM before being centrifuged and resuspended again two more times. Following the final centrifugation, cells were resuspended in 3mL of SMEM and centrifuged at 4ºC for ten minutes. The media was then removed, cell pellet snap frozen, and stored at -80ºC until RNA extraction.

*RNA Extraction and Purification*

All samples were homogenized in Trizol, and chloroform was used to separate the aqueous layer. RNA purification was performed on all samples using QIAGEN RNeasy kits including on-column DNAse digestion. Following extraction, RNA was quantified using Quant-iT Ribogreen Reagent. The calculated RNA concentration was used to determine amount of the RNA needed for a 1μg reverse transcription reaction. RNA from each sample was pooled for a "no RT" sample for each tissue type. This sample had all components of the reaction except for the reverse transcriptase enzyme and served as a control for DNA contamination. RNA was combined with 200 U/μL M-MLV Reverse Transcriptase (Invotrogen), an oligo-Dt primer, DNTPS, RNAse Out, and ultra-pure water for total reaction volume of 20 μL. Following cDNA synthesis, all samples were diluted with 80uL of ultra-pure water to yield 100uL of cDNA for each sample.

*RT-qPCR*

Reverse transcription quantitative PCR (RT-qPCR) was performed on samples to determine mRNA levels of relevant genes associated with each tissue type. Each sample was run in duplicate, as well as duplicate of "no RT" and water controls for each gene. Following RT-qPCR, the raw data Ct values and melt curves were analyzed for any abnormalities in duplicates and/or samples for each gene. Samples were excluded from analysis if duplicates were not within an acceptable range of each other or were consistently cycling at ranges suggestive that the PCR product was not the gene of interest. mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method by subtracting the average Ct value of the normalization gene for a sample from the average Ct of the gene of interest for that same sample. The normalization gene was

β-actin for all tissue types. This was determined following analysis of multiple normalization genes with ultimate selection based on increased reduction of variance in the genes of interest. The potential normalization genes evaluated in each tissue included the following: *GAPDH*, *β-tublin*, *PGK1*, *Cyclophilin*, *ITM2A*, *PSMA4*, *ESYT3*, *RPL4*, and *BLGC154*. *β-actin* was selected for normalization of all results for all tissues.

Fold change was based on average mRNA levels found in RBC1 line samples. Average fold change between RBC1 line and E line for each gene was statistically analyzed using the GLIMMix protocol in SAS. Data were analyzed for normality and homogeneity of variance and statistical significance was set to $P < 0.05$. Where normality was a concern, data were log transformed. In the hypothalamus, this was performed for *GNRH* and *PGR*. In the pituitary, this was performed for *β-actin*, *FSHB*, *PGR*, *PRL*, and *ITGB3*. In the F1 granulosa, this was performed for *β-actin*, *STAR*, and *CYP11A1*. In the SWF, this was performed for *FSHR*, *LHCGR*, *CYP19A1*, *TSHR*, and *ITGAV*. Regardless of transformation, untransformed means are presented.

Samples were collected from hens from two flocks of E line and RBC1 line turkeys. Hens one to twelve were collected in fall 2018 (year 1 flock). Hens thirteen to twenty-four were collected in fall 2019 (year 2 flock). Samples collected from the year 1 flock included hypothalamus, pituitary, SWF, F1 granulosa cells, and plasma. Samples collected from the year 2 flock included hypothalamus, pituitary, SWF, and plasma. F1 granulosa cells were not collected from the year 2 flock, because no differences in mRNA levels for the genes studied were found from the year 1 flock. Body weights were recorded for all hens. Hens from the year 1 flock had the

following information recorded: F1 follicle weight, F5 follicle weight, ovary weight, oviduct weight, and number of preovulatory follicles. Hens from the year 2 flock had number of preovulatory follicles recorded. F1 follicle weight, F5 follicle weight, ovary weight, and oviduct weight were not recorded for hens from the year 2 flock.

RT-qPCR analysis of HPG genes in the hypothalamus, pituitary, and SWF was performed on all samples regardless of year with the exclusion of the one E line hen from the year 1 flock found to be within the surge and the corresponding RBC1 line hen sampled at the same time. Four exceptions are *GNIHR2, GNRHR*, and *ESR1* in the pituitary and *HSD3B1* in the SWF which were only analyzed in the year 2 flock samples. RT-qPCR analysis of HPT genes were performed on year 2 flock samples only.

**Table 2.1**: Primer sequences for RT-qPCR.

| Symbol | Forward Primer | Reverse Primer |
|---|---|---|
| *β-Actin* | ATATTGCTGCGCTCGTTGTTG | TCTGGCCCATACCAACCATC |
| *GNRH1* | TGGCAATCTGCTTGGCTCA | CCAGGGCATTCAGCCTTC |
| *GNIH* | CAGTGGCGTTTCTAACACC | ACTCCTCTGCTTTTCCTCC |
| *GNRHR* | TCCCAGGAGGGAACTTCAC | TTCATGCGTGCCTTGGAG |
| *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 |
| *TRH* | TGGGAGCCACATGCTTCAAT | GGTCCCACAGTGACCTTCTG |
| *DIO2* | TGAAGCACATGGTGCTGTTTC | TTGCCCTTGGCTATGTGGAT |
| *THRA* | CATCTTCGACCTCGGCAAGT | GGTACGTCTCCTGGCACTTC |
| *ITGAV* | TCAGTGTGCACCAGCAATCT | TCTCCTTGGGCTGCCAATTT |
| *ITGB3* | CTCATCACCATCCACGACCG | GGAAGCACATCCCTGCAGTA |
| *TSHR* | GCCCAAAACACATGGACGTT | ACTGCCAGTCTGGTTACACA |
| *TRHR* | GGCTCAACAAAACAAGACTGTGAA | TCGATAGGGCATCCACAGAAA |
| *PRL* | TGCTGCGTTCCTGGAATGAT | GTCTGGAGTCCTCATCAGCG |

**Results**

*General Characteristics*

A significant difference (P=0.0004) was found when comparing the weight of the F1 follicle. The RBC1 line had an increased average weight compared to the average weight of the E line (Table 2.2). There were no other significant differences

observed for F5 weight, ovary weight, oviduct weight, or number of preovulatory follicles. There was also a significant difference (P=0.0037) when comparing the overall body weight of both lines. There was an increased average body weight in the RBC1 line compared to the E line (Table 2.2). There was a significant difference (P=<.0001) when comparing EPD between the two lines. Average EPD was significantly lower in the RBC1 line compared to the E line (Table 2.2). Average $P_4$ concentration of blood samples taken at the time of sampling were not significantly different between the two lines.

**Table 2.2**: Variables of interest collected at time of tissue sampling. n value equals hens from each line.

| Variable | RBC1 (mean ± SEM) | E (mean ± SEM) | Flock | n | p-value |
|---|---|---|---|---|---|
| F1 weight (g) | 25.32 ± 0.71 | 20.09 ± 0.65 | 1 | 6 | 0.0004 |
| F5 weight (g) | 8.65 ± 1.23 | 8.33 ± 1.23 | 1 | 6 | 0.8601 |
| Ovary weight (g) | 107.63 ± 9.11 | 91.25 ± 9.11 | 1 | 6 | 0.2324 |
| Oviduct weight (g) | 91.00 ± 8.02 | 87.00 ± 8.02 | 1 | 6 | 0.7313 |
| Body weight (kg) | 15.63 ± 1.069 | 13.89 ± 1.069 | 1,2 | 12 | 0.0037 |
| Number of Preovulatory follicles | 7.67 ± 0.33 | 7.50 ± 0.31 | 1,2 | 12 | 0.7195 |
| EPD | 0.54 ± 0.03 | 0.84 ± 0.03 | 1,2 | 12 | <.0001 |
| $P_4$ (ng/mL) | 0.60 ± 0.16 | 0.86 ± 0.30 | 1,2 | 12 | 0.4481 |

*Hypothalamus*

In the hypothalamus, average mRNA expression for E line hens was determined as a fold change relative to RBC1 line hens. For genes associated with the HPG axis, *ESR2* was significantly downregulated (P<0.05) in the E line compared to RBC1 line (Figure 2.1). While no other genes were significantly different, *ESR1* tended to be downregulated in E line compared to RBC1 line (Figure 2.1). For genes associated with the HPT axis, no significant differences were found. However, *THRA* and *ITGB3* were both approaching significance (P=.0865 and P=.0838 respectively)

(Figure 2.1). Both genes tended toward downregulation in the E line compared to the RBC1 line. All other HPT axis genes also tended to be downregulated in the E line compared to RCB1 line (Figure 2.1). However, these differences were not significant.

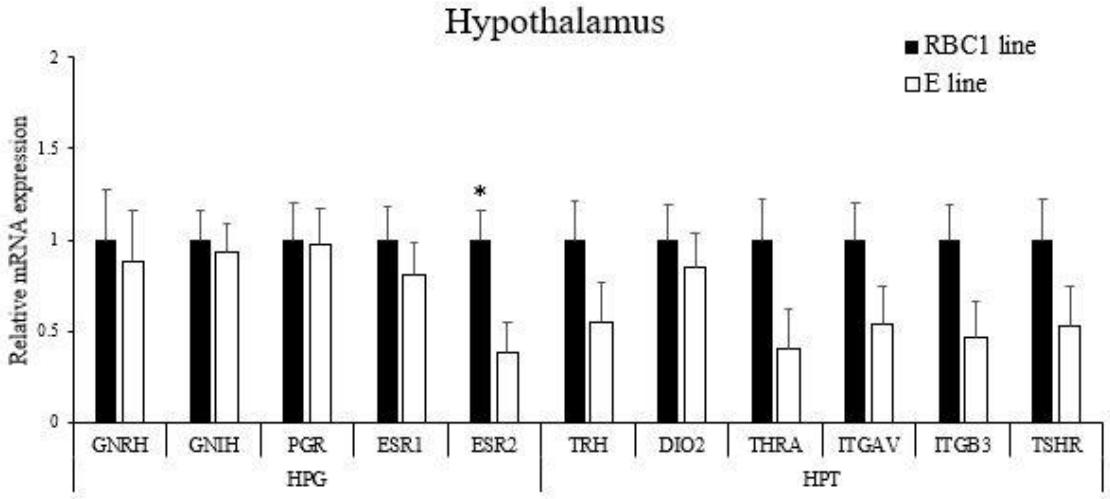


**Figure 2.1**: Relative mRNA expression of genes in the Hypothalamus associated with the HPG and HPT axis. Data are normalized to β-actin and presented relative to RBC1 line for each gene. Significant differences ($p<0.05$) in expression for a gene is indicated with an asterisk. (For HPG genes, n= 11 per line; for HPT genes, n=6 per line)

*Pituitary*

In the pituitary, average mRNA expression for E line hens was determined as a fold change relative to RBC1 line hens. For genes associated with the HPG axis, *FSHB*, *PRL*, and *GNIHR2* were significantly different (Figure 2.2). *FSHB* was significantly downregulated ($P<0.05$) in the E line compared to the RBC1 line. *PRL* was significantly upregulated ($P<0.05$) in the E line compared to the RBC1 line. *GNIHR2* was also significantly upregulated ($P<0.05$) in the E line compare to the RBC1 line. *GNRHR* and *ESR1* tended to be upregulated in the E line, though neither

were statistically significant compared to the RBC1 line (Figure 2.2). For genes associated with the HPT axis, no significant differences were observed. *ITGB3* was approaching significance (P=0.0862). It tended to be downregulated in the E line compared to the RBC1 line (Figure 2.2). There was a slight upregulation of mRNA expression of *ITGAV* and *TRHR* in the E line, but neither were statistically significant compared to the RBC1 line (Figure 2.2).

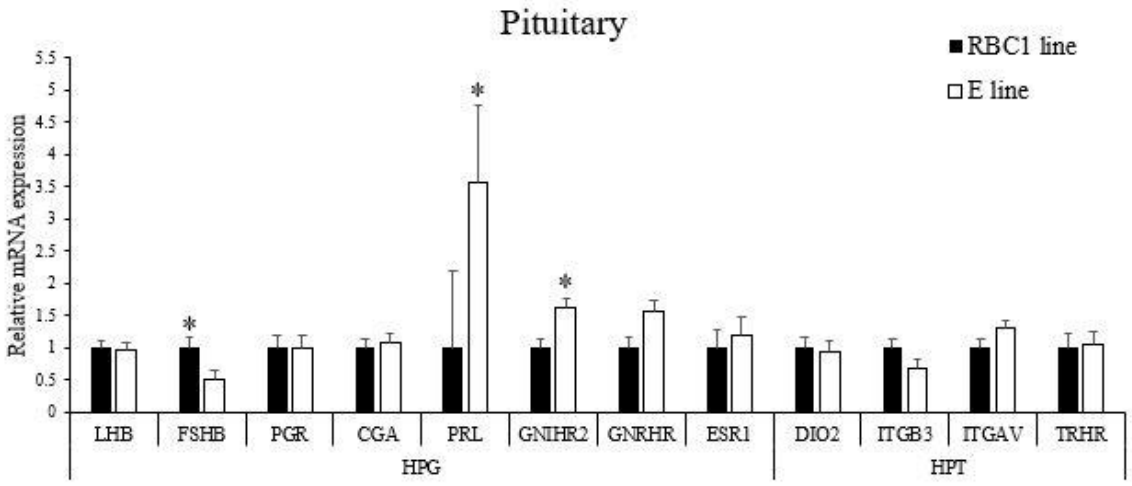


**Figure 2.2**: Relative mRNA expression of genes in the Pituitary associated with the HPG and HPT axis. Data are normalized to β-actin and presented relative to RBC1 line for each gene. Significant differences ($p<0.05$) in expression for a gene is indicated with an asterisk. (For genes LHB, FSHB, PGR, CGA, and PRL, n=10 for each line; for genes GNRHR and ESR1, n=6 per line; for GNIHR2 n=5 per line; for HPT genes, n=6 per line)

*F1 Granulosa*

In the F1 granulosa cell layer, average mRNA expression for E line hens was determined as a fold change relative to RBC1 line hens. No statistically significant

differences were observed in expression of these genes associated with the HPG axis. Both *FSHR* and *LHCGR* appeared to be upregulated in the E line, but neither were statistically significant compared to the RBC1 line (Figure 2.3).

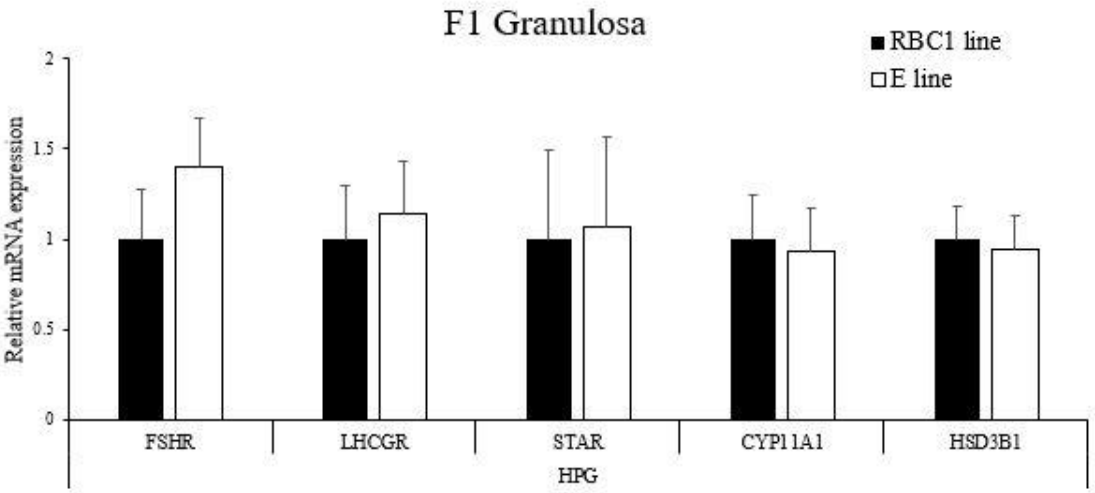


**Figure 2.3**: Relative mRNA expression of genes in the F1 Granulosa associated with the HPG axis. Data are normalized to β-actin and presented relative to RBC1 line for each gene. Significant differences ($p<0.05$) in expression for a gene is indicated with an asterisk. (n=4 for each line)

*Small White Follicles*

In the small white follicles, average mRNA expression for E line hens was determined as a fold change relative to RBC1 line hens. For genes associated with the HPG axis, no statistically significant differences were observed. However, *FSHR*, *LHCGR*, and *CYP19A1* tended to be upregulated in the E line compared to the RBC1 line (Figure 2.4). *HSD17B1* also appeared to be slightly upregulated in the E line compared to the RBC1 line (Figure 2.4). On the other hand, *HSD3B1* tended to be downregulated in the E line compared to the RBC1 line (Figure 2.4). For genes

associated with the HPT axis, *ITGB3* was significantly downregulated (P<0.05) in the E line compared to the RBC1 line (Figure 2.4). *DIO2* approached significance (P=0.057). It was downregulated in the E line compared to the RBC1 line (Figure 2.4). All other genes in the HPT axis tended to be downregulated in the E line compared to the RBC1 line (Figure 2.4).

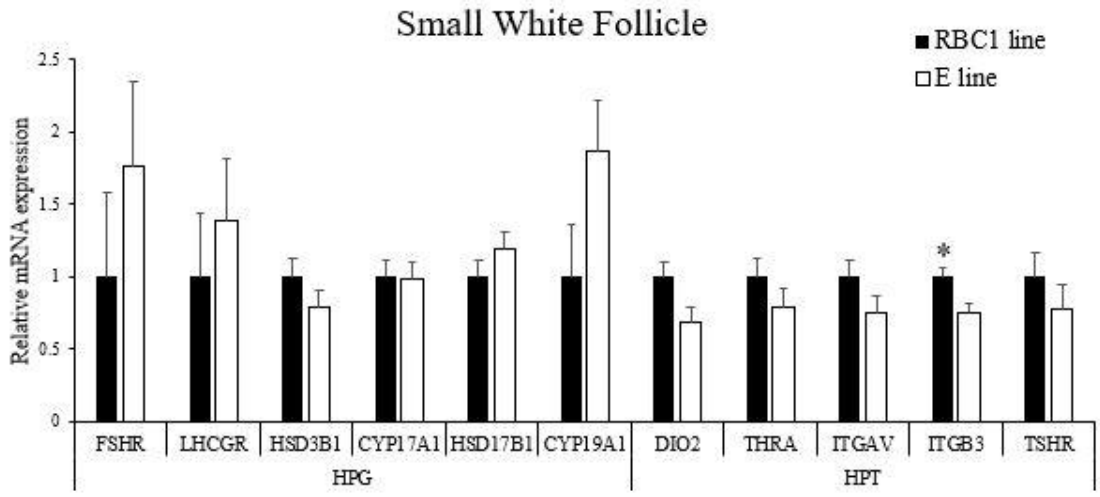


**Figure 2.4**: Relative mRNA expression of genes in the Small White Follicle associated with the HPG and HPT axis. Data are normalized to β-actin and presented relative to RBC1 line for each gene. Significant differences (p<0.05) in expression for a gene is indicated with an asterisk. (For HPG genes except HSD3B1, n=10 for each line; for HSD3B1, n=6 for each line; for HPT genes, n=6 for each line)

**Discussion**

In the current study, differences were observed in the mRNA expression of genes related to the HPG axis and HPT axis between the E line and RBC1 line of turkey hens. Previous studies focused on body characteristics of these genetically selected lines have found an apparent negative correlation between body weight and egg laying (Nestor, et al., 1997). When comparing general characteristics between the

two lines in the present study, there was a significant difference between average overall body weight and EPD between the two lines, with the RBC1 line having increased body weight but decreased EPD compared to the E line. The relationship between these data are similar to other studies conducted that focused on the heritability relationship between body weight and egg laying (Nestor, et al., 1996; Kranis, et al., 2007). Both of these previous studies found that as selection for body weight increased, egg laying throughout the production period decreased. This suggests that the relationship between body weight and egg laying characteristics could have an effect on EPD at the molecular level. Previous research in our laboratory demonstrated differences in expression of genes related to the HPG axis within a commercial flock of laying turkey hens when comparing HEPH versus LEPH (Brady, et al., 2020). It was found that HEPH tended to demonstrate upregulation of genes associated with stimulation of the HPG axis, while demonstrating downregulation of genes that suppress the HPG axis compared to LEPH. Our laboratory has also shown using transcriptome analysis and *in vitro* experiments that hormones and genes associated with the HPT appear to affect cells of the HPG axis (Brady, 2019). The present study is the first to focus on what could be contributing to the difference in egg laying at the mRNA level of the E line and RBC1 line.

Related to the HPG axis in the hypothalamus, the apparent downregulation of *ESR2* in the E line could mean that, outside the preovulatory surge, the hypothalamus is less receptive to feedback from the $E_2$ pathway. In the Chinese alligator, it was observed that transcription levels of *ESR2* were higher when individuals where

outside of the reproductive period (Zhang, et al., 2017). During the reproductive period, *ESR2* expression decreased. While not significant, *ESR1* tended to be downregulated in the E line compared to the RBC1 line. *ESR1* was similarly observed to be downregulated in HEPH (Brady, et al., 2020). These differences in expression could be due to genetic differences between the commercial flock and E line/RBC1 line flocks. In the pituitary, *PRL* expression was significantly increased in the E line. PRL secretion in response to increased $P_4$ results in broodiness or incubation behavior (El Halawani & Rozenboim, 1993). It would not be expected that the E line would have increased PRL while still in the production period, but this could indicate diminished negative feedback on the hypothalamus in response to PRL resulting in no reduction of egg laying. The increase in PRL could also be attributed to the age of the hens at the time of sampling. PRL secretion tends to increase later in the production period (El Halawani, et al., 1980). *GNIHR2* was also significantly upregulated in the E line. Increased expression of the receptor for GnIH could indicate less binding of GnIH occurring in the E line due to less overall secretion. *GNRHR* also tended to be upregulated in the E line, which was not see in HEPH (Brady, et al., 2020). This could indicate that the E line is more responsive to stimulation of the HPG axis regardless of mRNA expression of the hormones, since there was no tendency for difference of expression of *GNRH* and *GNIH* in the hypothalamus. *FSHB* was significantly downregulated in the E line. FSH stimulates follicle growth and recruitment of the largest SWF to become the smallest preovulatory follicle (Ghanem & Johnson, 2018). This suggests decreased focus on follicle growth and recruitment and more focus on follicle maturation in the E line. This may also explain the

significant difference between the F1 follicle weight. A study performed by Ghanem and Johnson (2019) found that when hens were injected with equine chorionic gonadotropin as an FSH substitute, ovulation was blocked but the F1 follicle continued to grow. The transition from FSH to LH responsiveness in the preovulatory follicles could be more efficient in the E line. No differences were noted in F1G, indicating both lines were capable of sufficient $P_4$ production, which is supported by no significant difference in plasma $P_4$ levels. This was similarly seen in the commercial flock. Outside of the preovulatory surge, there was no significant difference in $P_4$ concentration between the HEPH and LEPH (Brady, et al., 2020). In the SWF, both *FSHR* and *LHCGR* tended to be upregulated in the E line. LHCGR was similarly upregulated in HEPH (Brady, et al., 2020). The upregulation of these receptors, plus the tendency of upregulation of *HSD17B1* and *CYP19A1,* suggests increased $E_2$ synthesis in the E line. However, decreased expression of *ESR1* and *ESR2* in the hypothalamus could mean that the E line is overall less sensitive to $E_2$ feedback despite increased synthesis by SWF.

This study also compared the mRNA expression of genes related to the HPT axis to determine if genetic selection for egg production affected the HPT axis. Previous studies in chickens and turkeys have shown that the thyroid and thyroid hormones are necessary for initiation and maintenance of egg laying (van Herch, et al., 2013; Lien & Siopes, 1989). However, research in our laboratory has shown that the HPT axis might affect the number of eggs a hen lays throughout the production period. Transcriptome analysis showed an upregulation of genes related to the HPT axis in LEHP compared to HEPH (Brady, 2019). *In vitro* experiments also showed

decreased steroid production following thyroid hormone treatment (Brady, 2019). In the hypothalamus, all HPT genes tended to be downregulated in the E line compared to the RBC1 line. This suggests increased activity of the HPT axis in the RBC1 line which could be affecting the HPG axis. In the pituitary, the trend was not as obvious. While there were no significant differences, *ITGAV* tended to be upregulated while *ITGB3* tended to be downregulated in the E line. Both genes encode for integrin plasma receptors that bind thyroid hormone (Cheng, et al., 2010). The variability in expression of the HPT axis genes could indicate no difference in responsiveness to thyroid hormone and TRH in the pituitary between the two lines. However, there could be a difference in the synthesis and release of thyroid hormone, but in the present study we did not perform analysis on thyroid tissue only on potential target tissues. In the SWF, all HPT genes tended to be downregulated in the E line with *ITGB3* being significant. Again, this suggests increased activity of the HPT axis in the RBC1 line which could be affecting the HPG axis. As shown by Brady (2019), SWF are susceptible to thyroid hormone actions that could be affecting steroidogenesis and feedback to the hypothalamus. More research is needed to determine if thyroid hormone is only exerting a negative effect on SWF steroid production or if thyroid hormone can also disrupt feedback higher up the HPG cascade. It is important to note that this experiment only measured mRNA levels. While common practice to perform this type of analysis, mRNA levels do not always directly correlate with protein expression, enzyme activity, and hormone or neuropeptide secretion.

In this study, we demonstrated differences between the E line and RBC1 line at the molecular level that could have an effect on overall egg production. While we did not see a clear upregulation of the genes we expected in the E line, the data suggest there are still key variations between the two lines. The apparent overall downregulation of the HPT axis in the E line was expected due to similar results in previous research comparing HEPH and LEPH. Along with the few differences in the HPG axis, this could be an important factor in the differences in egg production between the two lines. Selection of the E line for increased overall egg production has proven to be successful, but the negative correlation with body weight and meat production still leaves questions of why these two important traits cannot co-exist. This study has provided some examples of where differences are present, but continued research into how the HPT axis affects reproduction may prove to be the answer.

| Gene Expression | | | | | |
|---|---|---|---|---|---|
| Tissue | E | RBC1 | Gene | HEPH | LEPH |
| Hypothalamus | | | GNRH | | |
| | | | GNIH | | |
| | | | PGR | | |
| | | | ESR1 | | |
| | | | ESR2 | | |
| | | | TRH | | |
| | | | DIO2 | | |
| | | | THRA | | |
| | | | ITGAV | | |
| | | | ITGB3 | | |
| | | | TSHR | | |
| Pituitary | | | LHB | | |
| | | | FSHB | | |
| | | | PGR | | |
| | | | CGA | | |
| | | | PRL | | |
| | | | GNIHR2 | | |
| | | | GNRHR | | |
| | | | ESR1 | | |
| | | | DIO2 | | |
| | | | ITGB3 | | |
| | | | ITGAV | | |
| | | | TRHR | | |
| F1 Granulosa | | | FSHR | | |
| | | | LHCGR | | |
| | | | STAR | | |
| | | | CYP11A1 | | |
| | | | HSD3B1 | | |
| SWF | | | FSHR | | |
| | | | LHCGR | | |
| | | | HSD3B1 | | |
| | | | CYP17A1 | | |
| | | | HSD17B1 | | |
| | | | CYP19A1 | | |
| | | | DIO2 | | |
| | | | THRA | | |
| | | | ITGAV | | |
| | | | ITGB3 | | |
| | | | TSHR | | |

**Supplemental Figure 2.1**: Summary of gene expression differences between E line vs. RBC1 line and HEPH vs. LEPH. Dark red represents significant increase ($P<0.05$) and light red represents tendency to increase. Dark green represents significant decrease ($P<0.05$) and light green represents tendency to decrease. Yellow represents no difference.

# Chapter 3: Future Directions

While expression of only a few genes was significantly different between the two lines, the results of these experiments offer some possible avenues of exploration for future research. One surprising difference that was found was the upregulation of prolactin in the E line. Prolactin is associated with broodiness and nesting behavior in turkeys and often results in decreased egg production (El Halawani & Rozenboim, 1993). The increased levels of prolactin mRNA could indicate decreased sensitivity of the hypothalamus to prolactin or a breakdown in the prolactin cascade. Prolactin is released from the anterior pituitary in response to vasoactive intestinal peptide (VIP). VIP is a neuroendocrine hormone that is released from axons in the hypothalamus (El Halawani, et al., 1990). Potential differences could exist in the signaling pathways, so it would be interesting to treat isolated pituitary cells *in vitro* with VIP and measure the response of those cells by measuring PRL secretion and/or mRNA levels of genes associated with the cascade. Measuring PRL receptor mRNA could also show a difference between the E line and RBC1 line. Another aspect of the PRL cascade that could be of interest is the effect of dopamine (DA) on anterior pituitary cells. In birds, DA inhibits the secretion of PRL (Hall, et al., 1986). *In vitro* treatment of pituitary cells with DA followed by PRL hormone assays and mRNA analysis could lead to differences between the two lines.

Single nucleotide polymorphisms (SNP) analysis could also reveal where the differences in gene expression arise between the E line and RBC1 line. Analysis of the 5' flanking region of the genes found to be significantly different in the current study may reveal variations in the genetic code of the E line and RBC1 line. The tendency for downregulation of HPT genes in the E line may also prove to be a result

of genetic variation that could be impacting egg production as well as body composition. It was found that the E line had a decreased body weight compared to the RBC1 line and this could be due to decreased stimulation of the HPT axis.

At the time of sampling of the year 1 flock, the F5 follicle was also collected and the cell layers isolated. Performing RT-qPCR analysis on those cell layers as well as the *theca interna* and *theca externa* layer of the F1 follicle could offer more insight to any variation related to steroidogenesis.

It would also be interesting to compare the E line to HEPH of a commercial flock to see if there are differences when comparing genes of the reproductive axis. It could be that the genetic selection over time of the E line has led to increased egg production through a different mechanism or pathway than the mechanism or pathway that results in increased egg production in HEPH.

# Bibliography


Bacon, W. L., & Liu, H. K. (2004). Progesterone injection and egg production in turkey hens. *Biology of Reproduction*, 878-886.

Bentley, G. E., Jensen, J. P., Kaur, G. J., Wacker, D. W., Tsutsui, K., & Wingfield, J. C. (2006). Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH). *Hormones and Behavoir*, 550-555.

Brady, K. (2019). Indentifcation of the Molecular Networks Governing Ovulation Frequency in Low and High Egg Producing Hens. Doctoral Dissertation.

Brady, K., Porter, T. E., Liu, H., & Long, J. A. (2019). Characterization of gene expression in the hypothalamo-pituitary-gonadal axis during the preovulatory surge in the turkey hen. *Poultry Science*, 7041-7049.

Brady, K., Porter, T. E., Liu, H., & Long, J. A. (2020). Characterization of the hypothalamo–pituitary–gonadal axis in low and high egg producing turkey hens. *Poultry Scince*, 1163-1173.

Burke, W. H., Licht, P., Papkoff, H., & Bona Gallo, A. (1979). Isolation and characterization of luteinizing hormone and follicle-stimulating hormone from pituitary glands of the turkey (Meleagris gallopavo). *General and Comparative Endocrinology*, 508-520.

Calvo, F. O., & Bahr, J. M. (1983). Adenylyl cyclase system of the small preovulatory follicles of the domestic hen: responsiveness to follicle-stimulating hormone and luteinizing hormone. *Biology of Reproduction*, 542-547.

Cheng, S. (2000). Multiple Mechanisms for Regulation of the Transcriptional Activity of Thyroid Hormone Receptors. *Reviews in Endocrine and Metabolic Disorders*, 9-18.

Cheng, S., Leonard, J. L., & Davis, P. J. (2010). Molecular Aspects of Thyroid Hormone Actions. *Endocrine Reviews*, 139-170.

El Halawani, M. E., & Rozenboim, I. (1993). The Ontogeny and Control of Incubation Behavior in Turkeys. *Poultry Science*, 906-911.

El Halawani, M. E., Burke, W. H., & Dennison, P. T. (1980). Effect of nest-deprivation on serum prolactin level in nesting female turkeys. *Biology of reproduction*, 118-123.

El Halawani, M. E., Silsby, J. L., & Mauro, L. J. (1990). Vasoactive intestinal peptide is a hypothalamic prolactin-releasing neuropeptide in the turkey (Meleagris gallopavo). *General and Comparative Endocrinology*, 66-73.

Emmerson, D. A., Velleman, S. G., & Nestor, K. E. (2002). Genetics of Growth and Reproduction in the Turkey. 15. Effect of Long-Term Selection for Increased Egg Production on the Genetics of growth and Egg Production Traits. *Poultry Science*, 316-320.

Geris, K. L., D'Hondt, E., Kuhn, E. R., & Darras, V. M. (1999). Thyrotropin-releasing hormone concentrations in different regions of the chicken brain and pituitary: an ontogenetic study. *Brain Research*, 260-266.

Ghanem, K., & Johnson, A. L. (2018). Follicle dynamics and granulosa cell differentiation in the turkey hen ovary. *Poultry Science*, 3755-3761.

Ghanem, K., & Johnson, A. L. (2019). Response of hen pre-recruitment ovarian follicles to follicle stimulating hormone, in vivo. *General and Comparative Endocrinology*, 41-47.

Gomez, Y., Velazquez, P. N., Juarez-Oropeza, M. A., & Padernera, E. (1998). Steroid metabolism in granulosa and theca interna cells from preovulatory follicles of domestic hen (Gallus domesticus). *Animal Reproduction Science*, 81-91.

Gregory, C. C., & Porter, T. E. (1997). Cloning and Sequence Analysis of a cDNA for the β Subunit of Chicken Thyroid-Stimulating Hormone. *General and Comparitive Endocrinology*, 102-190.

Groef, B. D., Vandenborne, K., Van As, P., Darrae, V. M., Kuhn, E. R., Decuypere, E., & Geris, K. (2005). Hypothalamic control of the thyroidal axis in the chicken: Over the boundaries of the classical hormonal axes. *Endocrinology*, 104-110.

Hall, T. R., Harvey, S., & Chadwick, A. (1986). Control of prolactin secretion in birds: A review. *General and Comparative Endocrinology*, 171-184.

Hammes, S., & Mendelson, C. (2012). Mechanisms of Hormone Action. In W. Kovacs, & S. Ojeda, *Textbook of Endocrine Physiology* (pp. 58-98). New York: Oxford Univeristy Press.

Huang, E. S., & Nalbandov, A. V. (1979). Steroidogenesis of chicken granulosa and theca cells: in vitro incubation system. *Biology of Reproduction*, 442-453.

Johnson, A. L. (2015a). Ovarian follicle selection and granulosa cell differentiation. *Poultry Science*, 781-785.

Johnson, A. L. (2015b). Reproduction in the Female. In *Avian Physiology* (pp. 635-641). Elsevier Inc.

Johnson, A. L., & Bridgham, J. T. (2001). Regulation of Steroidogenic Acute Regulatory Protein and Luteinizing Hormone Receptor Messenger Ribonucleic Acid in Hen Granulosa Cells. *Endocrinology*, 3116-3124.

Johnson, P. A., Johnson, A. L., & van Tienhoven, A. (1985). Evidence for a positive feedback interaction between progesterone and luteinizing hormone in the induction of ovulation in the hen, Gallus domesticus. *Endocrinology*, 478-485.

Jurkevich, A., & Grossman, R. (2003). Vasotocin and reprodcutive functions of the domestic chicken. *Domestic Animal Endocrinology*, 93-99.

Kato, M., Shimada, K., Saito, N., Noda, K., & Ohta, M. (1995). Expression of P45017α-hydroxylase and P450aromatase Genes in Isolated Granulosa, Theca Interna, and Theca Externa Layers of Chicken Ovarian Follicles during Follicular Growth. *Biology of Reporudction*, 405-410.

Koike, K., Shimada, K., & Cornett, L. (1988). Plasma levels of immunoreactive mesotocin and vasotocin during oviposition in chickens: relationship to oxytocic action of the peptides in vitro and peptide interaction with myometrial membrane binding sites. *General Compartive Endocrinology*, 119-126.

Kranis, A., Hocking, P. M., Hill, W. G., & Wooliams, J. A. (2007). Genetic parameters for a heavy female turkey line: impact of simultaneous selection for body weight and total egg number. *British Poultry Science*, 685-693.

Li, J., Luo, W., Huang, T., & Gong, Y. (2019). Growth differentiation factor 9 promotes follicle-stimulating hormone-induced progesterone production in chicken follicular granulosa cells. *Endocrinology*, 69-76.

Lien, R. J., & Siopes, T. D. (1989). Effects of Thyroidectomy on Egg Production, Molt, and Plasma Thyroid Hormone Concentrations of Turkey Hens. *Poultry Science*, 1126-1132.

Loven, M. A., Wood, J. R., & Nardulli, A. M. (2001). Interaction of estrogen receptors α and β with estrogen response elements. *Molecular and Cellular Endocrinology*, 151-163.

McCartney, M. (1964). A Randombred Control Population of Turkeys. *Poultry Science*, 739-744.

McCartney, M. G., Nestor, K. E., & Harvey, W. R. (1968). Genetics of Growth and Reproduction in the Turkey: 2. Selection for Increased Body Weight and Egg Production. *Poultry Science*, 981-990.

McNabb, F. A., & Darras, V. M. (2015). Thyroids. In *Avian Physiology.*

Millar, R. P., Lu, Z., Pawson, A. J., Flanagan, C. A., Morgan, K., & Maudlsey, S. R. (2004). Gonadotropin-Releasing Hormone Receptors. *Endocrine Reviews*, 235-275.

Nestor, K. E. (1971). Genetics of Growth and Reproduction in the Turkey: 4. Strain Crossing For Improvement of Growth and Reproduction. *Poultry Science* , 1683-1689.

Nestor, K. E. (1985). Genetics of Growth and Reproduction in the Turkey.: 10. Tandem Selection for Increased Body Weight and Egg Production. *Poultry Science*, 2221-2222.

Nestor, K. E., Anderson, J. K., Patterson, R. A., & Velleman, S. G. (2008). Genetics of Growth and Reproduction in the Turkey. 17. Changes in Genetic Parameters Over Forty Generations of Selection for Increased Sixteen-Week Body Weight1. *Poultry Science*, 1971-1979.

Nestor, K. E., Anderson, J. W., Patterson, R. A., & Velleman, S. G. (2006). Genetics of Growth and Reproduction in the Turkey. 16. Effect of Repeated Backcrossing of an Egg Line to a Commercial Sire Line. *Poultry Science* , 1550-1554.

Nestor, K. E., Noble, D. O., & Emmerson, D. A. (1997). Genetics of growth and reproduction in the turkey. 13. Effects of repeated backcrossing of an egg line to two sire lines. *Poultry Science*, 227-235.

Nestor, K. E., Noble, D. O., Zhu, J., & Moritsu, Y. (1996). Direct and Correlated Responses to Long-Term Selection for Increased Body Weight and Egg Production in Turkeys. *Poultry Science*, 1180-1191.

O'Malley, B., & Schrader, W. (1976). THE RECEPTORS OF STEROID HORMONES. *Scientific American*, 32-43.

Park, J., Kim, Y., Lee, S., Kim, J. Y., Chung, J., Jeong, S., . . . Kim, J. (2019). Drp1 phoshporylation is indespensible for steroidgenesis in Leydig cells. *Endocrinology*, 729-743.

Pierce, J. G., & Parsons, T. F. (1981). Glycoprotein Hormones: Structure and Finction. *Annual Review of Biochemistry*, 465-495.

Porter, T. E., Hargis, B. M., Silsby, J. L., & el Halawani, M. E. (1989). Differential steroid production between theca interna and theca externa cells: a three-cell model for follicular steroidogenesis in avian species. *Endocrinology*, 109-116.

Porter, T. E., Hargis, B. M., Silsby, J. L., & El Halawani, M. E. (1991). Characterization of Dissimilar Steroid Productions by Granulosa, Theca Intern and Theca Externa Cells during Follicular Maturation in the Turkey (Melagris gallopavo). *General and Comparative Endocrinology*, 1-8.

Proudman, J. A., Vandesande, F., & Berghman, L. R. (1999). Immunohistochemical Evidence That Follicle-Stimulating Hormone and Luteinizing Hormone

Reside in Separate Cells in the Chicken Pituitary. *Biology of Reproduction*, 1324-1328.

Robinson, F. E., & Etches, R. J. (1986). Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus domesticus). *Biology of Reproduction*, 1096-1105.

Saito, N., & Grossman, R. (1999). Gene expression of arginine vasotocin in ovarian and uterine tissues of the chicken. *Asian-Australian Journal of Animal Science*, 695-701.

Saito, N., Kinzier, S., & Koike, T. (1990). Arginine vasotocin and mesotocin levels in theca and granulosa layers of the ovary during the oviposition cycle in hens (Gallus domesticus). *General and Comparative Endocrinology*, 54-63.

Saldanha, C. J., Silverman, A.-J., & Silver, R. (2001). Direct Innervation of GnRH Neurons by Encephalic Photoreceptors in Birds. *Journal of Bilogical Rythms*, 39-49.

Sechman, A. (2013). The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. *General and Comparative Endocrinology*, 68-75.

Shimada, K., Neldon, H. L., & Koike, T. I. (1986). Arginine vasotocin (AVT) release in relation to uterine contractility in the hen. *General and Comparative Endocrinology*, 362-367.

Shimizu, M., & Bedecarrats, G. Y. (2010). Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. *General and Comparative Endocrinology*, 331-337.

Smith, R. G., Clarke, S. G., Zalta, E., & Taylor, R. N. (1979). Two estrogen receptors in reproductive tissue. *Journal of Steroid Biochemistry*, 31-35.

Stojokovic, S. S., Reinhart, J., & Catt, K. J. (1994). Gonadotropin-Releasing Hormone Receptors: Structure and Signal Transduction Pathways. *Endocrine Reviews*, 462-499.

Sun, Y., Millar, R. P., Ho, H., Gershengorn, M. C., & Illing, N. (1998). Cloning and Characterization of the Chicken Thyrotropin-Releasing Hormone Receptor. *Endocrinology*, 3390-3398.

Terada, O., Shimada, K., & Saito, N. (1997). Effect of oestradiol replacement in ovariectomized chickens on pituitary LH concentrations and concentrations of mRNAs encoding LH beta and alpha subunits. *Journal of Reproduction and Fertility*, 59-64.

Thompson, E. B., & Kumar, R. (2003). DNA binding of nuclear hormone receptors influences their structure and function. *Biochemical and Biophysical Research Communications*, 1-4.

Tilly, J. L., Kowalski, K. I., & Johnson, A. L. (1997). tage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. *Biology of Reproduction*, 304-314.

Tsutsui, K., Saigoh , E., Yin, H., Ubuka, T., Chowdhury, V., Osugi, T., . . . Bentley, G. (2009). A New Key Neurohormone Controlling Reproduction, Gonadotrophin-Inhibitory Hormone in Birds: Discovery, Progress and Prospects. *Journal of Endocrinolgy*, 271-275.

United States Department of Agriculture. (2019). *National Agricultural Statistics Service.* Retrieved from nass.usda.gov.

van Herck, S. L., Geysens, S., Bald, E., Chwatko, G., Delezie, E., Dianati, E., . . . Darras, V. M. (2013). Maternal transfer of methimazole and effects on thyroid hormone availability in embryonic tissues. *Journal of Endocrinology*, 105-115.

Velleman, S. G., Coy, C. S., Anderson, J. W., & Nestor, K. E. (2007). The Effect of Genetic Increases in Egg Production and Age and Sex on Breast Muscle Development of Turkeys. *Poultry Science*, 2134-2138.

Yang, J., Long, D. W., & Bacon, W. L. (1997). Changes in Plasma Concentrations of Luteinizing Hormone, Progesterone, and Testosterone in Turkey Hens during the Ovulatory Cycle. *Endocrinology*, 281-292.

Zhang, R., Yin, Y., Sun, L., Yan, P., Zhou, Y., Wu, R., & Wu, X. (2017). Molecular cloning of ESR2 and gene expression analysis of ESR1 and ESR2 in the pituitary gland of the Chinese alligator (Alligator sinensis) during female reproductive cycle. *Gene*, 15-23.