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

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Abstract

Background: High egg producing hens (**HEPH**) show increased hypothalamic and pituitary gene expression related to hypothalamo-pituitary-gonadal (**HPG**) axis stimulation as well as increased *in vitro* responsiveness to gonadotropin releasing hormone (**GnRH**) stimulation in the pituitary when compared to low egg producing hens (**LEPH**). Transcriptome analysis was performed on hypothalamus and pituitary samples from LEPH and HEPH to identify novel regulators of HPG axis function.

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

Conclusions: Up-regulation of the HPT axis in LEPH and upstream beta-estradiol activation in HEPH may play a role in regulating HPG axis function, and ultimately ovulation rates. Furthermore, thyroid hormone and estradiol pretreatment impacted gonadotropin mRNA levels following GnRH stimulation, with the inhibitory effects of thyroid hormone being more detrimental in HEPH and estradiol stimulatory effects being more prominent in LEPH. Differential responsiveness to thyroid hormone and estradiol pretreatment may be due to desensitization of target genes to thyroid hormone and estradiol in LEPH and HEPH, respectively, in response general up-regulation of the HPT axis in LEPH and of the HPG axis in HEPH. Further studies will be necessary to identify possible target gene desensitization mechanisms and elicit the full role that the HPT axis and beta-estradiol upstream regulation play in egg production rates in turkey hens.

Background

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

Within the HPG axis, feedback mechanisms of progesterone and estradiol are instrumental for follicle ovulation to occur. Progesterone feedback on the hypothalamus and pituitary triggers a preovulatory surge (PS) of luteinizing hormone (LH) and progesterone, resulting in follicle ovulation, but the role of estradiol feedback during the PS is not well characterized in the turkey hen. In the chicken, estradiol reduces gonadotropin inhibitory hormone (GnIH) production and exerts positive and negative feedback on gonadotropin releasing hormone (GnRH) production in the hypothalamus, indicating that estradiol feedback may play a role in ovulation timing [1, 2].

In addition to the HPG axis, proper function of the HPT axis is necessary for egg production to occur. The full impact of the HPT axis on reproductive function is not well understood, but studies have shown that increased activity of the HPT axis is associated with gonadal regression [3]. On the other hand, studies have shown that HPT axis activity is necessary for the initiation of egg production [4]. Additional studies examined the role of the HPT axis in the regulation of reproductive cycles in seasonally reproductive species, however, the HPT axis has not been characterized in commercial chicken or turkeys during peak egg production and has not been examined in regard to the regulation of the PS [5].

LEPH and HEPH exhibited differential expression of genes within the HPG axis, with LEPH showing higher mRNA levels for genes involved in ovulation inhibition and HEPH showing higher mRNA levels for genes involved in ovulation stimulation [6]. Furthermore, during in vitro culture of isolated pituitary cells, LEPH displayed an increased responsiveness to GnIH treatment, whereas HEPH displayed an increased responsiveness to GnRH treatment [7]. To further understand the mechanisms regulating the differential gene expression and in vitro responses seen in these two groups of hens, transcriptome analysis was performed in the hypothalamus and pituitary of LEPH and HEPH, both under basal conditions (outside of the PS) and during HPG axis stimulation (during the PS).

Results And Discussion

Transcriptome Alignment and Mapping

A total of 852,343,043 sequence reads were obtained from the hypothalamus and pituitary, with an average of 35,514,293 reads per sample (**Supplemental Figure 1A**). A significantly higher number of reads were obtained from the pituitary samples when compared to the hypothalamus samples, but no differences in the percentage of reads mapped to the turkey genome were seen. On average, 79.9% of reads mapped to the turkey reference genome (Ensembl Turkey_2.01). For each sample, read pairs were aligned with minimal discordant pairs or pairs with multiple alignments (average of 0.58% and 2.29% respectively) (**Supplemental Figure 1B**). The number of reads per sample, the number of mapped reads per sample, and the number of properly aligned pairs per sample did not differ significantly between egg production or ovulatory cycle groups in either of the tissue examined.

Overview of DEGs

A total of 1641 and 2778 DEGs were identified in the hypothalamus and pituitary, respectively. Analysis of the genes differentially expressed between LEPH and HEPH revealed a significantly higher number of DEGs in the hypothalamus during the PS and in the pituitary outside of the PS. In the hypothalamus, 248 DEGs were identified outside of the PS, whereas 1393 DEGs were identified during the PS (**Figure 1A**). The pituitary showed the opposite trend, with 2155 DEGs outside of the PS and 623 DEGs during the PS (**Figure 1B**). In the hypothalamus, equal numbers of genes were seen up-regulated in LEPH and HEPH outside of the PS, though a higher number of genes were up-regulated in LEPH during the PS. In the pituitary, both outside and during the PS, a higher number of genes were up-regulated in HEPH compared to LEPH. In the hypothalamus and pituitary, under both ovulatory cycle conditions, unannotated genes accounted for roughly 20-30% of the DEGs, indicating that further progress annotating the turkey genome may reveal additional genes involved in egg production rates or in triggering ovulation.

When comparing each hen group during the ovulatory cycle, LEPH displayed twice as many DEGs in the hypothalamus and pituitary between basal and PS conditions when compared to HEPH (**Figure 1C and 1D**). Of the genes differentially expressed in the hypothalamus during the ovulatory cycle, unannotated genes accounted for 26% of the DEGs unique to LEPH and 47% of the DEGs unique to HEPH. Lower fractions of unannotated DEGs were seen in the pituitary during the ovulatory cycle, with unannotated genes accounting for 21% of the DEGs unique to LEPH and 27% of the DEGs unique to HEPH. In total, LEPH and HEPH shared 64 genes in the hypothalamus and 210 genes in the pituitary that were differentially expressed during the ovulatory cycle. Roughly one-fourth of the common DEGs in the hypothalamus and pituitary were unannotated as well.

Of the DEGs common to both groups of hens during the ovulatory cycle, a majority showed similar expression patterns in LEPH and HEPH (73% of common DEGs in the hypothalamus and 93% of common DEGs in the pituitary) (**Figure 1E and 1F**). A larger percentage of the common DEGs showed down-regulation in both groups of hens in the hypothalamus and pituitary compared to the percentage of DEGs that showed up-regulation in both groups of hens. Among the genes in the hypothalamus showing similar expression patterns during the PS in both groups of hens was fatty acid 2-hydroxylase (***FA2H***) and somatostatin (***SST***). *FA2H*, which was up-regulated in both groups of hens during the PS, is involved in myelin production, which is essential for proper nerve conduction [8]. *SST,* which was also up-regulated in both groups of hens during the PS, is the main inhibitory hormone of the somatotropic axis but has been shown to inhibit GnRH neuron activity in mice [9]. Among the genes in the pituitary showing similar expression patterns during the PS in both groups of hens was Pre-MRNA processing factor 19 (***PRPF19***). *PRPF19*, which was down-regulated in both groups of hens during the PS, has been shown in mouse models to impact the splicing of gonadotropin subunits [10]. Common DEGs with similar expression patterns during the ovulatory cycle in both LEPH and HEPH could indicate a potential role for these genes in the regulation of ovulation.

A small percentage of the common DEGs showed inverse expression patterns in LEPH and HEPH (27% of common DEGs in the hypothalamus and 7% of common DEGs in the pituitary). Of the hypothalamic common DEGs showing inverse expression patterns between LEPH and HEPH, proteasome 26S subunit,

non-ATPase 2 (***PSMD2***) displayed up-regulation in HEPH and down-regulation in LEPH during the PS. In mice, mutations in *PSMD2* have been associated with decreased thyroid hormone production [11]. Of the pituitary common DEGs showing inverse expression patterns between LEPH and HEPH, NADH dehydrogenase 4 (***ND4***) and cyclooxygenase-2 (***COX2***) have been previously associated with reproductive functions [12, 13]. Both *ND4* and *COX2* showed up-regulation in HEPH and down-regulation in LEPH during the PS. Swine selected for high ovulation rates displayed higher pituitary *ND4* gene expression when compared to control lines [12]. *COX2* encodes the rate limiting enzyme in prostaglandin production, and deletion of *COX2* in mice results in decreased ovulation [13]. Common DEGs during the ovulatory cycle with inverse expression patterns in LEPH and HEPH could signify a possible role in the regulation of egg production rates.

RNA sequencing confirmation

A total of 8 genes per tissue were confirmed through RT-qPCR. Confirmation genes were equally distributed to have one of four expression profiles: genes showing up-regulation in HEPH compared to LEPH (both outside and during the PS), genes showing up-regulation in LEPH compared to HEPH (both outside and during the PS), genes showing up-regulation in one hen group outside of the PS and up-regulation in the other hen group during the PS, and genes showing no changes in expression between hen groups (both outside and during the PS). Each of the confirmation genes examined in the hypothalamus (**Figure 2A**) and pituitary (**Figure 2B**) showed expression profiles similar to those obtained through RNA sequencing.

Overview of network analysis

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

The HPG axis

In the hypothalamus during the PS, LEPH displayed up-regulation of genes associated with ovulation inhibition as well as an abnormal up-regulation of ovulation stimulation genes when compared to HEPH (**Figure 3A**). LEPH exhibited up-regulation of neuropeptide VF precursor (***NPVF),*** which encodes avian gonadotropin inhibitory hormone (***GNIH***) and of gonadotropin releasing hormone 1 (***GNRH1***). GnIH negatively regulates the HPG axis to decrease gonadotropin production in the pituitary [14]. Up-regulation of *NPVF* may play a role in reduced ovulation rates seen in LEPH. *GNRH1* mRNA levels were previously shown to decrease during the PS in hens with average egg production, whereas in the present study, LEPH showed increased expression relative to HEPH [15]. In the same study, no expression changes in

NPVF were seen during the PS in average egg producing hens, whereas in the present study, LEPH showed up-regulation of *NPVF* [15]. Up-regulation of *GNRH1* during the PS may prevent hormone levels from returning to basal levels, prolonging the interval between ovulations.

When comparing HEPH outside and during the PS, HEPH showed up-regulation of estrogen related receptor beta (***ESRRB)*** and down-regulation of follicle stimulating hormone (**FSH**) and LH during the PS (**Figure 3B)**. Estrogen related receptors are ligand-dependent transcription factors capable of estradiol binding. Though the function of estrogen related receptors in avian reproduction have not been characterized, function analysis of estrogen related receptors in knock-out mice and zebrafish models indicate that estrogen related receptors are essential for female reproduction [16]. Decreased LH during the PS is consistent with decreased mRNA levels for the beta-subunit of LH (***LHB***) seen in average egg producing hens during the PS [7]. Additionally, in this network, casein kinase 2 alpha 2 (***CSNK2A2)*** is down-regulated in the pituitary of HEPH during the PS. *CSNK2A2* encodes an uncharacterized protein in avian species but this protein was shown to be decreased in laying geese pituitaries when compared to non-laying geese, indicating a possible role in egg production or ovulation [17].

Examination of the expression changes of DEGs related to the HPG axis revealed differential regulation of the HPG axis during the ovulatory cycle in LEPH and HEPH (**Table 1**). Outside of the PS, LEPH showed up-regulation of genes involved in prolactin signaling and androgen signaling. During the PS, LEPH showed up-regulation of genes involved in stimulatory and inhibitory HPG axis signaling, whereas HEPH showed up-regulation of estradiol and prolactin signaling. When LEPH and HEPH were compared individually outside and during the PS, LEPH displayed further increased expression of HPG axis inhibition and prolactin signaling (**Table S1**). HEPH displayed decreased expression of HPG axis stimulatory genes and increased expression of androgen and prolactin signaling. Prolactin signaling showed inverse trends in LEPH and HEPH and was impacted by the PS. Prolactin signaling has been shown to impact LH release in mammals and was up-regulated in HEPH during the PS, indicating a possible role in the shortened ovulation intervals seen in HEPH [18]. Both LEPH and HEPH showed down-regulation of gonadotropin releasing hormone receptor (***GNRHR***) during the PS, which was also seen in average egg producing turkey hens during the PS [7]. Generally, HEPH displayed down-regulation of the HPG axis during the PS, whereas LEPH displayed up-regulation of both genes that stimulate and inhibit the HPG axis during the PS, presumably leading to a longer ovulation interval in LEPH.

The HPT axis

DEGs up-regulated in LEPH compared to HEPH were associated with HPT axis expression in each tissue and condition examined (**Figure 4, S3, and S4**). In the hypothalamus during the PS, LEPH displayed increased expression of thyroid stimulating hormone receptor (***TSHR***) and solute carrier organic anion transporter family member 1C1 (***SLCO1C1***) relative to HEPH (**Figure 4A**). In the pituitary during the PS, LEPH displayed increased expression of the beta-subunit of thyroid stimulating hormone (***TSHB***) in contrast to HEPH (**Figure 4B**). *TSHR* expression in the hypothalamus is related to short loop feedback control on thyrotropin releasing hormone signaling [19]. Retrograde regulation of *TSHB* on the

hypothalamus has also been implicated in increased GnRH production in response to a changing photoperiod in seasonally reproductive birds [20]. It is plausible that retrograde *TSHB* feedback on the hypothalamus could also be involved in the timing of ovulation, due to the role of *TSHB* in GnRH signaling initiation coupled with the finding that molecular clockwork components impact *TSHB* pituitary expression in several mammalian species [21]. *SLCO1C1* is a thyroid hormone transporter that participates in transporting thyroid hormone across the blood-brain barrier [22]. Up-regulation of *SLCO1C1* in LEPH during the PS would allow greater thyroid hormone concentrations in the hypothalamus, which could ultimately have genomic effects on ovulation rates [23].

Additionally, in the hypothalamus during the PS, LEPH showed up-regulation of solute carrier family 16 member 12 (***SLC16A12***) and integrin (encoded by ***ITGAV*** and ***ITGB3***) relative to HEPH (**Figure S3A and S3B**). *SLC16A12* encodes a thyroid hormone transporter similar to *SLCO1C1,* allowing greater transport of thyroid hormone past the blood brain barrier in LEPH rather than HEPH [22]. Integrin is a plasma membrane receptor capable of binding thyroid hormones to elicit non-genomic actions of thyroid hormone, such as protein translocation and phosphorylation [23]. Up-regulation of integrin in the hypothalamus of LEPH relative to HEPH during the PS, infers possible non-genomic implications of thyroid hormone in the hypothalamus of LEPH [24].

In the pituitary during the PS, HEPH showed up-regulation of iodothyronine deiodinase 1 (***DIO1***) relative to LEPH (**Figure S4A**). *DIO1* is capable of converting thyroid hormone to the biologically active form but is also capable for thyroid hormone deactivation [25]. Increased thyroid hormone deactivation could mitigate the effect of thyroid hormone on the tissues of the HPG axis in HEPH. When comparing HEPH outside and during the PS, HEPH showed down-regulation of *TSHB* in the pituitary during the PS (**Figure S4B**). Thyroid stimulating hormone (**TSH**) acts on the thyroid gland to promote the synthesis of thyroid hormones [26]. Down-regulation of *TSHB* during the PS in HEPH could indicate lower circulating levels of TSH, ultimately impacting circulating thyroid hormones.

Examination of the expression changes of DEGs related to HPT axis revealed that LEPH exhibited up-regulation of a majority of the key genes of the HPT axis when compared to HEPH (**Table 2**). Outside and during the PS, LEPH displayed higher expression of genes related to HPT axis signaling, thyroid hormone receptors, thyroid hormone transporters, thyroid hormone metabolism, and thyroid hormone synthesis when compared to HEPH. During of the PS, LEPH displayed further increased expression thyroid related genes. When LEPH and HEPH were compared individually outside and during the PS, LEPH displayed increased expression of HPT axis genes during the PS, whereas HEPH displayed decreased expression of HPT axis genes during the PS (**Table S2**). HEPH during the PS showed down regulation of thyroid hormone transporters and genes involved in HPT axis signaling. Generally, LEPH displayed higher expression of HPT axis genes both outside and during the PS compared to HEPH and displayed further up-regulation of the HPT axis during the PS when compared to levels outside of the PS. HEPH, on the other hand, displayed down-regulation of the HPT axis during the PS and lowered HPT axis expression both outside and during the PS when compared to LEPH.

Upstream analysis

Analysis of the predicted upstream regulators for each comparison made also showed a common theme: the involvement of beta-estradiol. While the calculated Z-score varied for the comparisons examined, beta-estradiol was the only upstream regulator common to all of the comparisons (**Figure 5**). Additionally, beta-estradiol was among the top five upstream regulators in the pituitary both outside and during the PS (**Table 3**). The predicted involvement of beta-estradiol across all conditions examined with target genes involved in the HPG and HPT axes supports the hypothesis that beta-estradiol feedback on the hypothalamus and pituitary impacts the ovulatory process and possibly egg production rates.

For the comparisons between LEPH and HEPH, beta-estradiol was significantly more active in HEPH in the hypothalamus (z-score = 2.011) and pituitary (z-score = 2.079) outside of the PS. Differentially expressed target genes of beta-estradiol in the hypothalamus outside of the PS included thyroid releasing hormone receptor (***TRHR***), *TSHB*, transthyretin (***TTR***), prolactin (***PRL***), hydroxysteroid 17 beta dehydrogenase 2 (***HSD17B2***), and aromatase (***CYP19A1***), while differentially expressed target genes of beta-estradiol in the pituitary outside of the PS included the androgen receptor (***AR***), glycoprotein hormones alpha subunit (***CGA***), steroidogenic acute regulatory protein (***STAR***), and solute carrier family 7 member 5 (***SLC7A5***) (**Table S3**). Beta-estradiol tended to be more active in HEPH in the pituitary during the PS (z-score = 1.75), though not significantly.

For the comparisons during the ovulatory cycle for each hen group, in the pituitary beta-estradiol was significantly more active during the PS for LEPH (z-score = 2.014) and significantly more active outside of the PS for HEPH (z-score = -2.079). Differentially expressed target genes of beta-estradiol in the pituitary of LEPH included albumin (***ALB***), prolactin receptor (***PRLR***), *STAR*, and *TTR,* whereas differentially expressed target genes of beta-estradiol in the pituitary of HEPH included *CGA* and *TSHB* (**Table S4**).

Effect of thyroid hormone and estradiol on pituitary gonadotropin production

To further examine the impact of thyroid hormone and estradiol on HPG axis function, gonadotropin subunit mRNA levels were measured in pituitary cells from LEPH and HEPH after thyroid hormone pretreatment (**T_3**) or estradiol pretreatment (**E_2**) combined with GnRH treatment. Pituitary cells from LEPH and HEPH responded differently to each pretreatment in terms of gonadotropin subunit mRNA levels, indicating functional differences in the response of the HPG axis to thyroid hormone and estradiol that could be related to differences seen in egg production levels between the two groups of hens (**Figure 6**). The *in vitro* effects of T_3 and E_2 were seen both with and without GnRH treatment, indicating that both hormones could be capable of pituitary gonadotropin regulation outside and during the PS.

T_3 decreased *LHB*, follicle stimulating hormone beta subunit (***FSHB***), and *CGA* mRNA levels compared to no pretreatment in HEPH pituitary cells, regardless of GnRH treatment concentration. T_3 also decreased *LHB*, *FSHB*, and *CGA* mRNA levels in LEPH pituitary cells, but only at 10^{-9} M GnRH for *LHB*, 0 M and 10^{-9} M GnRH for *FSHB*, and 0 M and 10^{-8} M GnRH for *CGA*. T_3 negatively impacted *LHB, FSHB,* and *CGA*

mRNA levels in cells from LEPH and HEPH, however the effect was more prominent in HEPH cells. Negative regulation of *LHB*, *FSHB*, and *CGA* by thyroid hormone treatment was also reported in male rats [27, 28]. One possible mechanism for response differences to T_3 between LEPH and HEPH is desensitization or down-regulation of thyroid hormone receptors in LEPH due to the general up-regulation of the HPT axis seen in the hypothalamus and pituitary of LEPH. Thyroid hormone receptor desensitization in the liver has been documented after thyroid hormone injections in mice and *in vitro* thyroid hormone treatment decreased thyroid hormone receptor expression in rat pituitary cells [29, 30]. Generally, T_3 negatively regulated gonadotropin production, independent of GnRH treatment concentration, with a higher negative response from HEPH. These findings suggest that HEPH are more sensitive to the effect of T_3 on gonadotropin production, whereas LEPH are more resistant to the effects of T_3.

E_2 decreased *LHB* mRNA levels in HEPH pituitary cells compared to no pretreatment at 10^{-8} M GnRH. E_2 also decreased *FSHB* mRNA levels in HEPH pituitary cells relative to no pretreatment at 0 M GnRH and increased *FSHB* mRNA levels in LEPH pituitary cells at 10^{-9} M GnRH. E_2 in HEPH pituitary cells decreased *FSHB* mRNA levels at lower GnRH treatment concentrations but decreased *LHB* mRNA levels at higher GnRH treatment concentrations. Previous studies in chickens have shown estradiol to inhibit pituitary LH production [31]. In contrast, E_2 upregulated *FSHB* in pituitary cells from LEPH at 10^{-9} M GnRH. The effect of estradiol on *FSHB* mRNA levels has not been examined in avian species but estradiol injections in quail did not impact FSH plasma levels, which is consistent with the mRNA levels seen in HEPH [32]. Overall, E_2 had varied impacts on gonadotropin production, depending on the rate of egg production of the hens.

Conclusions

Hypothalamic and pituitary transcriptome analysis of LEPH and HEPH provided insights into the involvement of the HPT axis and estradiol signaling on egg production rates. LEPH displayed higher expression of genes related to the HPT axis in both the hypothalamus and pituitary when compared to HEPH, regardless of timing in the ovulatory cycle. During the PS, further up-regulation of the HPT axis was seen in LEPH, whereas the opposite was seen in HEPH. Beta-estradiol was activated as an upstream regulator in the hypothalamus and pituitary of HEPH compared to LEPH under basal conditions. Additionally, beta-estradiol was activated in LEPH and inhibited in HEPH in the pituitary during the PS. These observations support the hypothesis of beta-estradiol playing a role in the regulation of the PS and possibly in HEPH exhibiting decreased ovulation intervals. Furthermore, T_3 and E_2 in vitro impacted gonadotropin production to different degrees in LEPH and HEPH, inferring that LEPH and HEPH respond differently to thyroid hormone and estradiol feedback on the pituitary gland.

Materials And Methods

Hen selection and tissue collection

All animal procedures were approved by the Institutional Animal Care and Use Committees at USDA Beltsville Agricultural Research Center (BARC) and at the University of Maryland, College Park (reference numbers 16 – 002 and XR-16-09, respectively). Two flocks of turkey hens from the same commercial line (Hybrid Turkey, Kitchener, Ontario) were housed at BARC in individual wire cages during two separate time periods six months apart (200 hens at a time). Turkey hens were maintained under standard poultry management practices with artificial lighting (14L:10D) and were provided feed ad libitum to NRC standards. Daily egg records were kept from the onset of lay (around 28 weeks of age) until sampling occurred (at 37 weeks of age). Daily egg records were used to calculate each hen's number of eggs per day (EPD) by dividing the total number of eggs produced by the number of days in production. Based on the distribution of flock egg production rates, the bottom and top 15% of egg production were classified as LEPH and HEPH, respectively. Hens were classified as LEPH when EPD < 0.6 and as HEPH when EPD > 0.8. Egg production distribution and average EPD for LEPH and HEPH groups did not differ between flocks. Daily egg records were also used to predict the timing of the PS prior to ovulation, as described previously [7].

A total of 12 hens were sampled from the first flock and 6 hens were sampled from the second flock. All hens were euthanized by cervical dislocation prior to tissue isolation. Additionally, all hens were sampled on the second day of the hen's sequence. The first flock was used to perform transcriptome analysis of the hypothalamus and pituitary, with six LEPH and six HEPH sampled outside and during the PS (n = 3 per group). From each hen, the hypothalamus and pituitary were isolated, snap frozen as whole tissues in liquid nitrogen, and stored at -80 °C prior to assessment through RNA sequencing and confirmation of RNA sequencing results as described below. The second flock was used to perform follow up in vitro pituitary cultures, with three LEPH and three HEPH sampled exclusively outside of the PS (n = 3 per group). From each hen, the pituitary was isolated and placed in ice cold Dulbecco's Modified Eagle Medium (DMEM) until dispersion, cell culture, and RT-qPCR as described below.

To confirm correct sampling outside or during the PS, blood samples were taken from the wing vein immediately before sampling to measure plasma progesterone levels. Blood was collected in heparinized tubes and fractionated by centrifugation (2000xg for 10 minutes at 4 °C) to isolate plasma. Plasma samples were stored at -20 °C prior to assessment through radioimmunoassays as described below.

Radioimmunoassay

The radioimmunoassay (RIA) used to measure plasma progesterone levels was a coated tube kit (MP Biomedicals, Solon, OH). All protocols were performed as directed by the supplier. All samples were assayed in duplicate in a single RIA. Plasma samples were ether extracted and analyzed for progesterone to determine that hens were sampled at the correct time during the ovulatory cycle, based on experimental group assigned. Hypothalamus and pituitary samples taken from a hen with plasma progesterone levels less than 1 ng/dL were considered to be sampled outside of the PS, while hypothalamus and pituitary samples taken from a hen with plasma progesterone levels greater than 4 ng/dL were considered to be sampled during of the PS. The plasma progesterone cutoffs for outside

and during the PS were based on previous studies determining average plasma progesterone levels during the ovulatory cycle [7].

RNA isolation, cDNA library construction, and sequencing

Total RNA was isolated from whole tissue hypothalamus and pituitary samples with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA was performed as previously described [15]. Amplified cDNA was generated using a SMART-Seq Ultra Low Input RNA kit (Takara Bio) following the manufacturer's procedure. Briefly, for each sample 1 µl of 10X Reaction Buffer was added to 10 ng of total RNA (in 9.5 µl nuclease-free water). 10X Reaction Buffer: 19 µl of 10X Lysis Buffer and 1 µl of RNase Inhibitor. Then, 2 µl of 3' SMART-Seq CDS Primer II A (12 µM) was added and gently vortexed, samples were incubated at 72 °C for 3 min and then placed on ice for 2 min. Then, 7.5 µl of RT Master Mix was added to each sample (Total reaction volume is 20 µl) and incubated at 42 °C for 90 min, followed by 72 °C for 10 min then placed on ice. Master Mix (per reaction) consisted of 4 µl 5X Ultra Low First-Strand Buffer; 1 µl SMART-Seq v4 Oligonucleotide (48 µM); 0.5 µl RNase Inhibitor (40 U/µl); 2 µl of SMARTScribe Reverse Transcriptase (100U/µl). Next, cDNA was amplified by long distance PCR (LD PCR) as follows.

To each 20 µl cDNA sample, 30 µl of PCR Master Mix was added and amplification was carried out using the following conditions: 95 °C for 1 minute, followed by 8 cycles of: 98 °C for 10 sec, 65 °C for 30 sec and 68 °C 3 min, 72 °C for 10 min. PCR Master Mix (per reaction) consisted of 25 µl 2X SeqAmp CB PCR Buffer; 1 µl PCR Primer II A (12 µM); 1 µl SeqAmp DNA Polymerase; 3 µl nuclease-free water. Amplified cDNA was then purified using Agencourt AMPure XP beads as follows. To each PCR reaction 50 µl of AMPure XP beads was added, vortexed and incubated at room temperature for 8 min, briefly centrifuged, and placed on a MagWell Magnetic Separator 96 (EdgeBio) for 5 min. Supernatant was discarded and the beads were washed twice with 200 µl of 80% ethanol. Beads air dried (~ 2 min) and 17 µl of Elution Buffer was added to each sample, vortexed and incubated at room temperature for 2 min. The amplified cDNA was quantified using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit.

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

dried for 15 min. To each library 52.5 µl of Resuspension Buffer was added and shook at 1800 rpm for 2 min then incubated at room temperature for 2 min. Tubes were then placed on a MagWell Magnetic Separator 96 (EdgeBio) until liquid was clear (~ 5 min), then 50 µl of each library was transferred to a new tube. The libraries were quantified using an Agilent 2100 Bioanalyzer and High Sensitivity DNA Kit. For sequencing 24 libraries (2 tissues) were pooled (10 nM). Libraries were pooled so that set 1 for each tissue was sequenced in a different pool than set 2. Pools were submitted to NC State's GSL facility for paired-end sequencing (75 bp reads) on an Illumina NextSeq 500.

Bioinformatic analysis of sequencing data

All FASTQ sequencing files have been deposited to the NIH Short Read Archive (accession numbers SAMN11624488-SAMN11624511). Processing and analysis of sequencing data was performed using the Galaxy (https://usegalaxy.org/) suite. Adapter sequences and low-quality sequences (Phred < 20) were removed from FASTQ files using the TrimGalore tool. Trimmed reads were mapped to the Meleagris gallopavo reference genome (Turkey_2.01). TopHat was used to analyze mRNA libraries. DEGs were determined using the Cuffdiff tool. Pairwise comparisons were made between LEPH and HEPH for each timepoint in the ovulatory cycle as well as between timepoints in the ovulatory for each egg production group. Due to poor annotation of the turkey genome, the protein sequences for DEGs that were unannotated in the turkey were subjected to orthologous comparisons in human, mouse, and chicken protein sequences using Ensembl BIOMART (https://useast.ensembl.org/info/data/biomart/index.html). Unannotated DEGs were assumed orthologous if greater than 50% identity to the human, mouse, and chicken was seen at the protein level.

Ingenuity pathway analysis

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

Culture of pituitary cells

All cell isolation procedures were performed using Minimum Essential Medium, Spinner modification (SMEM) or DMEM as noted below. Media was supplemented with 0.1% bovine serum albumen, 100-U/mL penicillin G, and 100-µg/mL streptomycin sulfate (0.1% BSA and P/S). Dispersion pituitary cells was performed as previously described [7]. Briefly, isolated pituitaries were dispersed in SMEM (0.1% BSA and P/S) using trypsin and collagenase (1 mg/mL of each) and filtered through 70 µm nylon mesh.

Cells were diluted to a concentration of 200,000 cells/mL and plated in serum free medium (DMEM/F12) supplemented with 0.1% bovine serum albumen, 5-µg/mL human insulin, 100-U/mL penicillin G, and 100-µg/mL streptomycin sulfate. Cells were plated in 24 well poly-L lysine coated plates (Corning Life Sciences, Lowell, MA) at 100,000 cells/well and were allowed to attach for 2 hours before pretreatment. Pituitary cells were pretreated with either no pretreatment (NPT) (10 ∝l DMEM/F12 added), T_3 (1.5 ng/mL of thyroid hormone), or E_2 (1.5 ng/mL of estradiol) for 12 hours, followed by treatment with chicken GNRH (Phoenix Pharmaceuticals, Burlingame, CA) at 0, 10^{-9}, or 10^{-8} M for 6 hours. Cells were maintained in a 37.5 °C, 5% CO_2 atmosphere during incubation. Pituitary cells were harvested at the end of incubation by retrypsinization, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

RT-qPCR

Total RNA was isolated from pituitary cell cultures with RNeasy Mini kits (Qiagen, Valencia, CA), including on-column deoxyribonuclease digestion. Quantification of RNA, RT, and RT-qPCR were performed as previously described [7]. A pool of total RNA was made and the reaction conducted without reverse transcriptase as a control for genomic DNA contamination. PCR reactions (15 µL) were carried out as previously described using a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA) [15]. Pituitary data were normalized to phosphoglycerate kinase 1 (PGK1) and were analyzed by the $2^{-\Delta\Delta Ct}$ method. All PCR reactions for each gene in a given tissue were analyzed in a single 96-well plate, allowing accurate performance of relative quantification without the need to include a reference control sample in multiple plates. Primers (Integrated DNA Technologies, Skokie, IL) for turkey PGK1, LHB, FSHB, and CGA mRNA were designed and used with cycling parameters described previously [15]. Data are presented as fold increase over levels in LEPH basal cells for each pretreatment/treatment combination and time point.

RNAseq confirmation

RNA extracted and quantified from whole tissue hypothalamus and pituitary samples for RNAseq was reverse transcribed as described previously [15], with controls for genomic DNA contamination. PCR reactions were carried out as described above. Hypothalamic data were normalized to GAPDH, whereas pituitary data were normalized to PGK1. Data were analyzed as described above. For each tissue, mRNA levels for 12 genes total were determined. DEGs selected for RNAseq confirmation fit the following parameters: $P < 0.05$, absolute fold change greater or equal to 1.5, annotated in the turkey genome, and encoded by a single transcript. DEGs fitting these parameters were selected with the following RNAseq expression profiles: 3 DEGs up-regulated in LEPH both outside and during the PS, 3 DEGs up-regulated in HEPH both outside and during the PS, 3 DEGs which showed up-regulation in one egg production group outside of the PS and up-regulation in the other egg production group during the PS, and 3 control genes which did not show expression changes between egg production levels or during the ovulatory cycle. Primers were designed as described above. Data are presented as fold increase over mRNA levels for LEPH outside of the PS for each gene.

Statistics

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

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committees at USDA Beltsville Agricultural Research Center and at University of Maryland, College Park (reference numbers 16-002 and XR-16-09, respectively).

Consent for publication

Not applicable.

Availability of data and materials

The FASTQ sequencing file datasets supporting the results of this article are available in the NCBI Short Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra), accession numbers, SAMN11624488-SAMN11624511. Differential expression output generated or analyzed during this study are included in this published article (Additional files 4-5)

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KB performed the RNA-seq data analyses, carried out the RT-qPCR confirmation, performed the *in vitro* cell culture experiments, and drafted the manuscript. JAL provided the animals used in the study, participated in the study design and coordination, and helped draft the manuscript. JAH performed the library construction and helped draft the manuscript. HCL participated in the study design and coordination and helped draft the manuscript. TEP conceived the study, obtained the funding, participated in the study

design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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Abbreviations

ACTA2: Alpha-actin-2

ALB: Albumin

AR: Androgen receptor

BARC: Beltsville Agricultural Research Center

BLOC1S4: Biogenesis of lysosomal organelles complex-1, subunit 4

CGA: Glycoprotein hormones alpha subunit

COX2: Cyclooxygenase-2

CRYBB1: Crystallin beta B1

CSNK2A2*:* Casein kinase 2 alpha 2

CYP19A1: Aromatase

DEGs: Differentially expressed genes

DIO1: Iodothyronine deiodinase 1

DMEM: Dulbecco's modified eagle medium

E_2: Estradiol pretreatment

EPD: Eggs per day

ESRRB*:* Estrogen related receptor beta

ESYT3: Extended synaptotagmin 3

FA2H: Fatty acid 2-hydroxylase

FABP6: Fatty acid binding protein 6

FSH: Follicle stimulating hormone

FSHB: Follicle stimulating hormone beta subunit

GnIH: Gonadotropin inhibitory hormone

GNIH: Gonadotropin inhibitory hormone

GnRH: Gonadotropin releasing hormone

GNRH1: Gonadotropin releasing hormone 1

GNRHR: Gonadotropin releasing hormone receptor

HEPH: High egg producing turkey hen

HPG: Hypothalamo-pituitary-gonadal

HPT: Hypothalamo-pituitary-thyroid

HSD17B2: Hydroxysteroid 17 beta dehydrogenase 2

IGF1: Insulin like growth factor 1

IPA: Ingenuity® Pathway Analysis

ITG2A: Integral membrane protein 2A

ITGAV: Integrin, alpha V subuit

ITGB3: Integrin, beta 3 subuit

LEPH: Low egg producing turkey hen

LH: Luteinizing hormone

LHB: Luteinizing hormone beta-subunit

LYVE1: Lymphatic vessel endothelial hyaluronan receptor 1

MANEA: Mannosidase endo-alpha

ND4: NADH dehydrogenase 4

NPT: No pretreatment

NPVF: Neuropeptide VF precursor

PAPPA2: Pappalysin 2

PGK1: Phosphoglycerate kinase 1

PRL: Prolactin

PRLR: Prolactin receptor

PRPF19: Pre-MRNA processing factor 19

PS: Preovulatory surge

PSMA2: Proteasome subunit alpha 2

PSMD2: Proteasome 26S subunit, non-ATPase 2

RIA: Radioimmunoassay

RSAD2: Radical s-adenosyl methionine domain containing 2

SLC16A12: Solute carrier family 16 member 12

SLC7A5: Solute carrier family 7 member 5

SLCO1C1: Solute carrier organic anion transporter family member 1C1

SMEM: Minimum essential medium, spinner modification

SST: Somatostatin

STAR: Steroidogenic acute regulatory protein

T_3: Thyroid hormone pretreatment

TGFBR1: Transforming growth factor beta receptor 1

TRHR: Thyroid releasing hormone receptor

TSH: Thyroid stimulating hormone

TSHB: Thyroid stimulating hormone beta-subunit

TSHR: Thyroid stimulating hormone receptor

TTR: Transthyretin

ZFPM1: Zinc finger protein, FOG family member 1

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Tables

Table 1. Significant gene expression changes in the hypothalamo-pituitary-gonadal (HPG) axis between egg production levels. Fold change and significance are presented for key HPG axis genes outside and during the preovulatory surge (RPKM>0.2, P<0.05). Negative fold change values represent increased expression in low egg producing hens (**LEPH**) and positive fold change values represent increased expression in high egg producing hens (**HEPH**).

Outside Preovulatory Surge

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

During Preovulatory Surge

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

Table 2. Significant gene expression changes in the hypothalamo-pituitary-thyroid (HPT) axis between egg production levels. Fold change and significance are presented for key HPT axis genes outside and during the preovulatory surge (RPKM>0.2, P<0.05). Negative fold change values represent increased expression in low egg producing hens (**LEPH**) and positive fold change values represent increased expression in high egg producing hens (**HEPH**).

Outside Preovulatory Surge

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

During Preovulatory Surge

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

Table 3. Upstream regulators. Significant upstream regulators between low egg producing hens (**LEPH**) and high egg producing hens (**HEPH**), outside and during the preovulatory surge (RPKM>0.2, P<0.05, |fold change|>1.5).

Outside Prevoulatory Surge

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

During Prevoulatory Surge

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

Figures

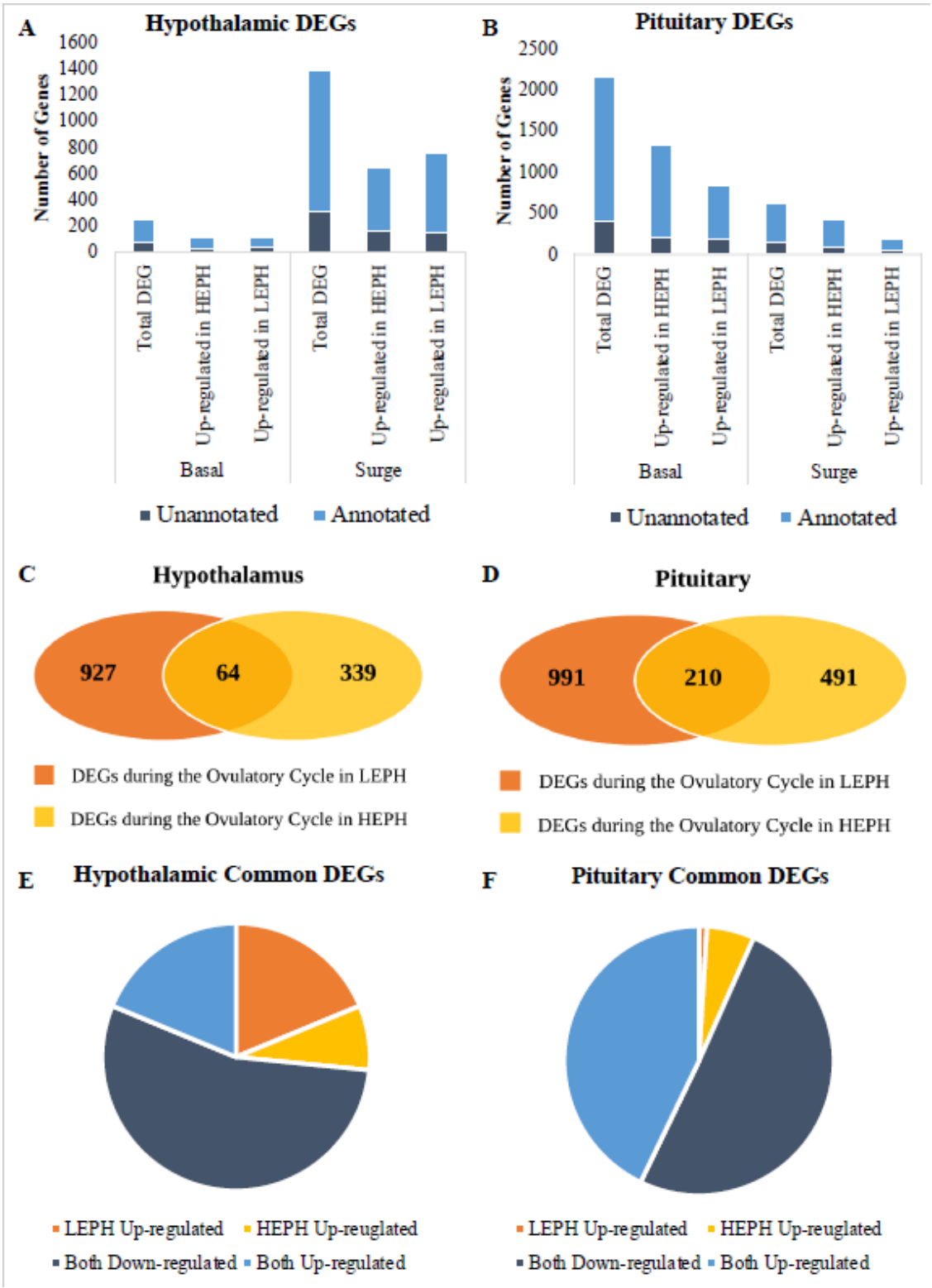

Figure 1

Overview of differentially expressed genes. (A) Numbers of total, up-regulated in high egg producing hens (HEPH), and up-regulated in low egg producing hens (LEPH) differentially expressed genes (DEGs) in the hypothalamus of LEPH and HEPH sampled outside (basal) and during (surge) the preovulatory surge (PS) (RPKM>0.2, P<0.05). The portion of genes that are unannotated in the turkey genome are represented in dark blue and the portion of gene that are annotated in the turkey genome are represented

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

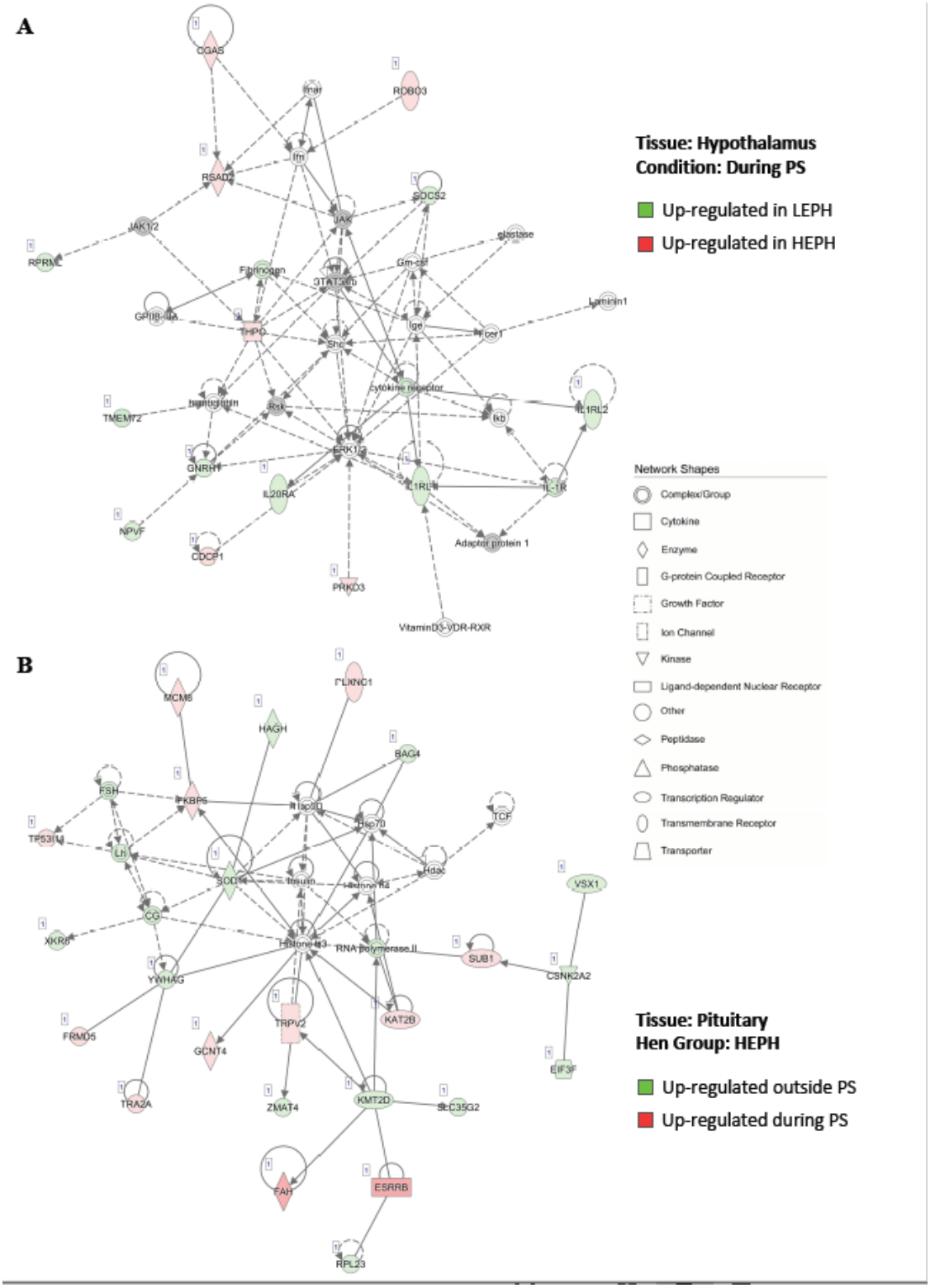

Figure 2

Hypothalamo-pituitary-gonadal axis networks. (A) IPA network analysis in the hypothalamus comparing low egg producing hens (LEPH) and high egg producing hens (HEPH) gene expression during the preovulatory surge (PS) (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in LEPH, whereas red represents genes up-regulated in HEPH. (B) IPA network analysis in the pituitary comparing HEPH gene expression outside and during the PS (RPKM>0.2, P<0.05, |fold change|>1.5).

Green represents genes up-regulated outside of the PS, whereas red represents genes up-regulated during the PS.

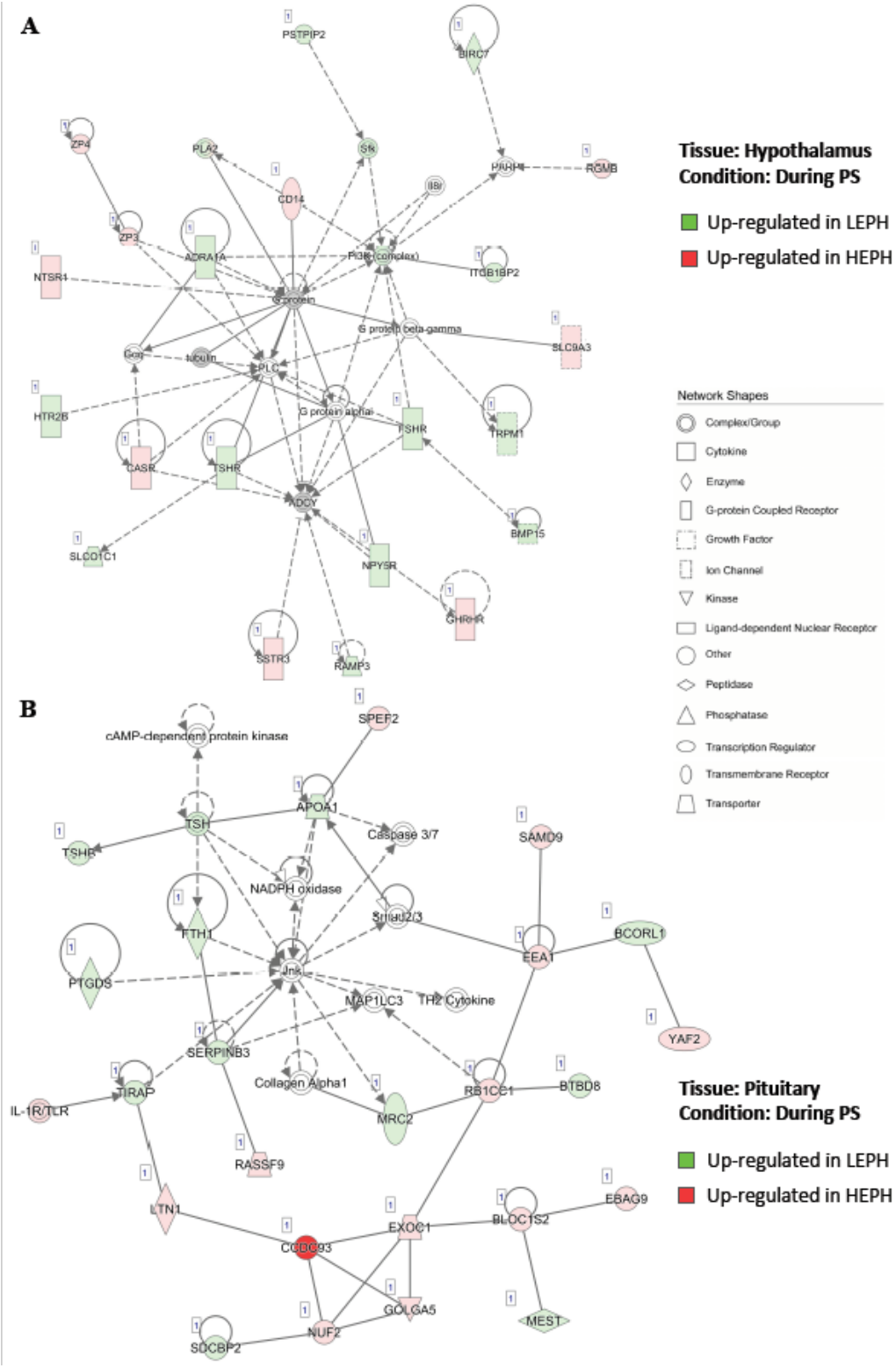

Figure 3

Hypothalamo-pituitary-thyroid axis networks. (A) IPA network analysis in the hypothalamus comparing low egg producing hens (LEPH) and high egg producing hens (HEPH) gene expression during the preovulatory surge (PS) (RPKM>0.2, P<0.05, |fold change|>1.5). Green represents genes up-regulated in

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

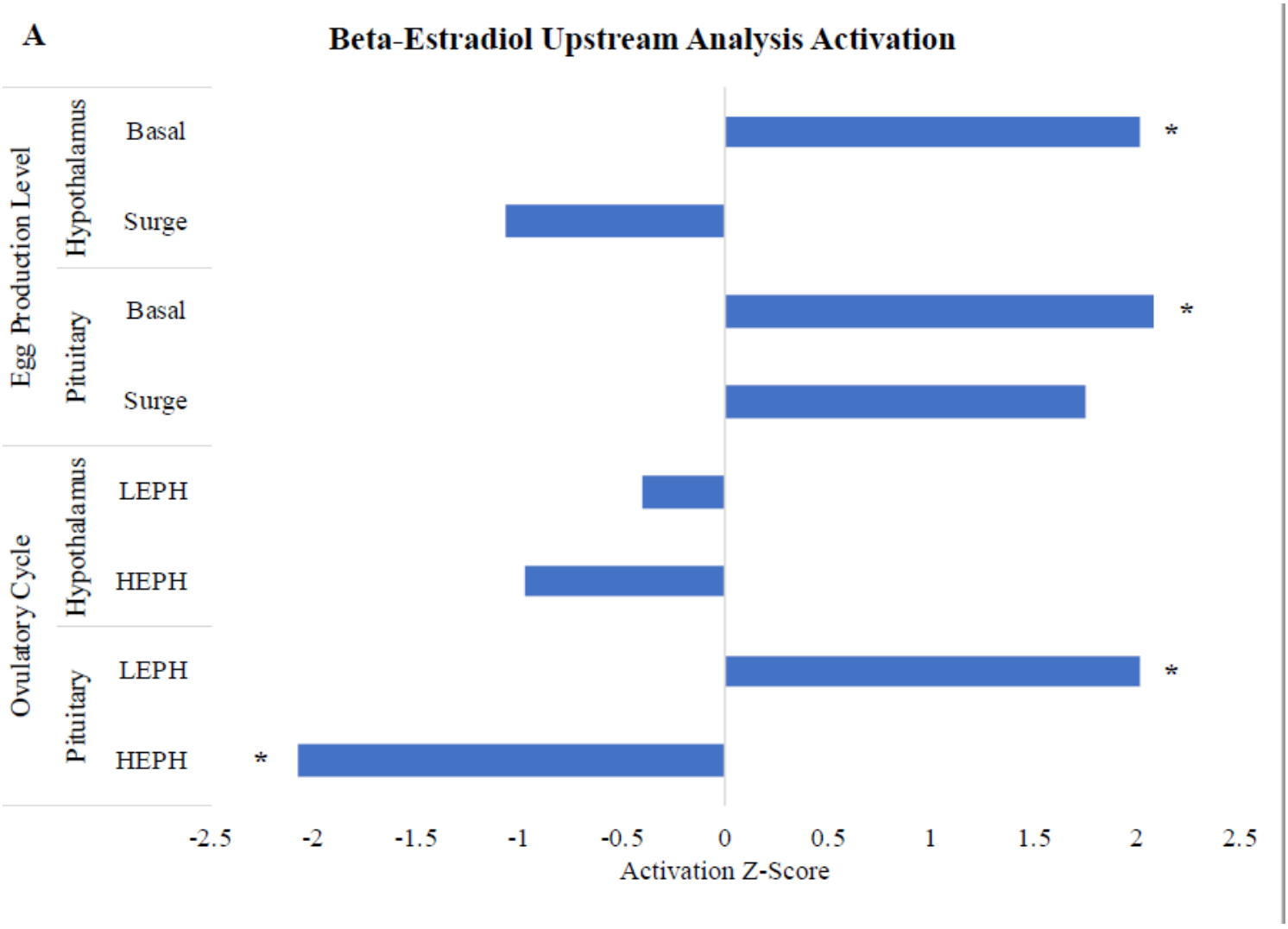

Figure 4

Beta-estradiol as an upstream regulator. Activation z-score calculated for beta-estradiol based on differentially expressed genes (DEGs) (RPKM>0.2, P<0.05, |fold change|>1.5). The top panel shows the calculated z-score for beta-estradiol based on DEGs between low egg producing hens (LEPH) and high egg producing hens (HEPH), both outside (basal) and during (surge) the preovulatory surge (PS). The bottom panel shows the calculated z-score for beta-estradiol based on DEGs between outside and during the PS in LEPH and HEPH individually. Significant predicted activation (z-score ≥ 2) or inhibition (z-score ≤ -2) of beta-estradiol is denoted with an asterisk (*).

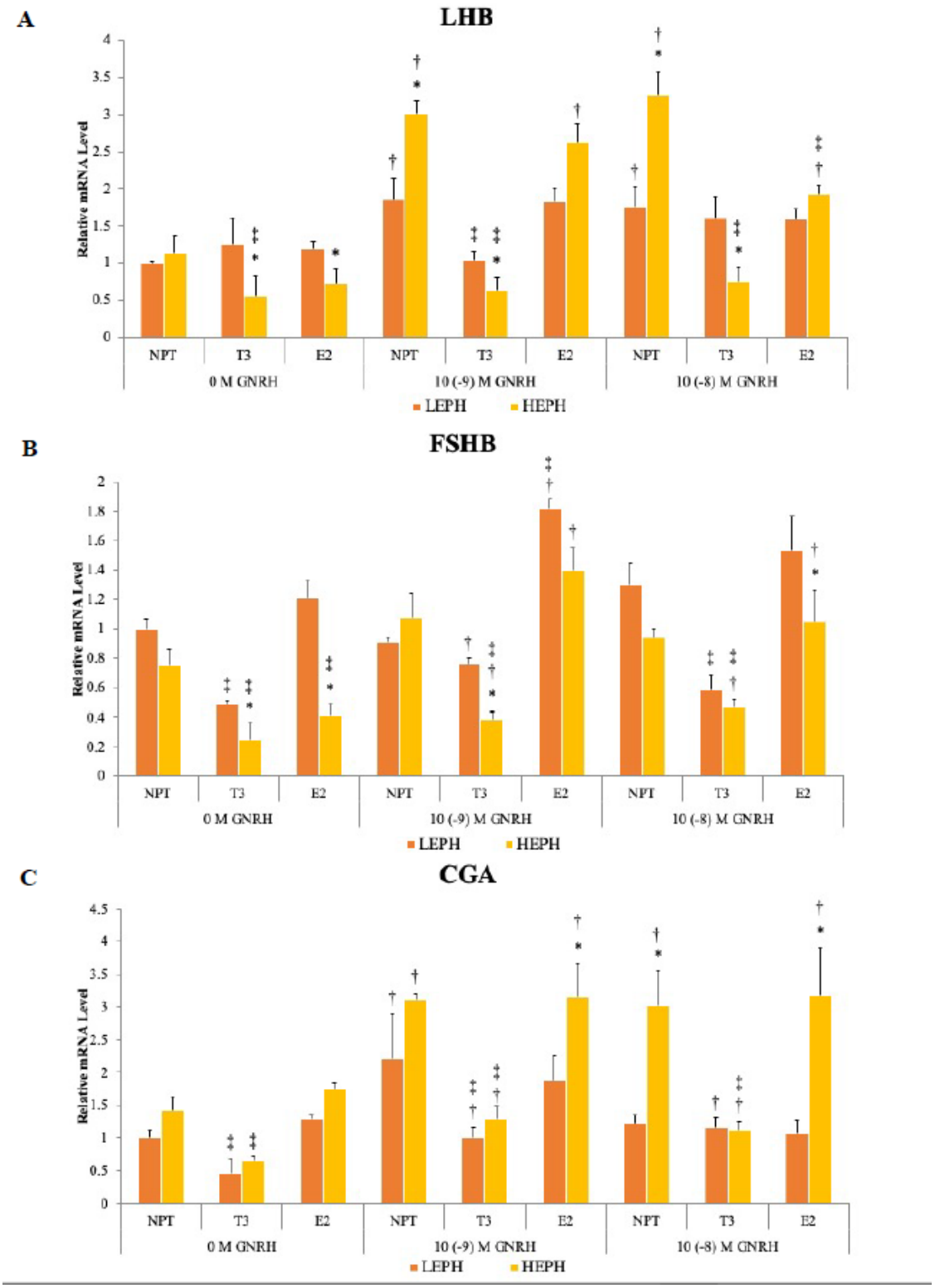

Figure 5

Impact of thyroid hormone and estradiol on gonadotropin production. Relative pituitary expression of the beta-subunit of luteinizing hormone (LHB), the beta-subunit of follicle stimulating hormone (FSHB), and the glycoprotein hormones alpha-subunit (CGA) after pretreatment with no pretreatment (NPT), thyroid hormone (T3), or estradiol (E2) followed by gonadotropin-releasing hormone (GNRH) treatment in low egg producing hens (LEPH) and high egg producing hens (HEPH). Normalized data are presented relative to

LEPH basal expression for each gene. Significant expression differences between LEPH and HEPH for a given condition are denoted with an asterisk (*). Significant differences between GNRH treatments for a given egg production group are denoted with a dagger (†). Significant differences between pretreatments for a given egg production group are denoted with a double dagger (‡).

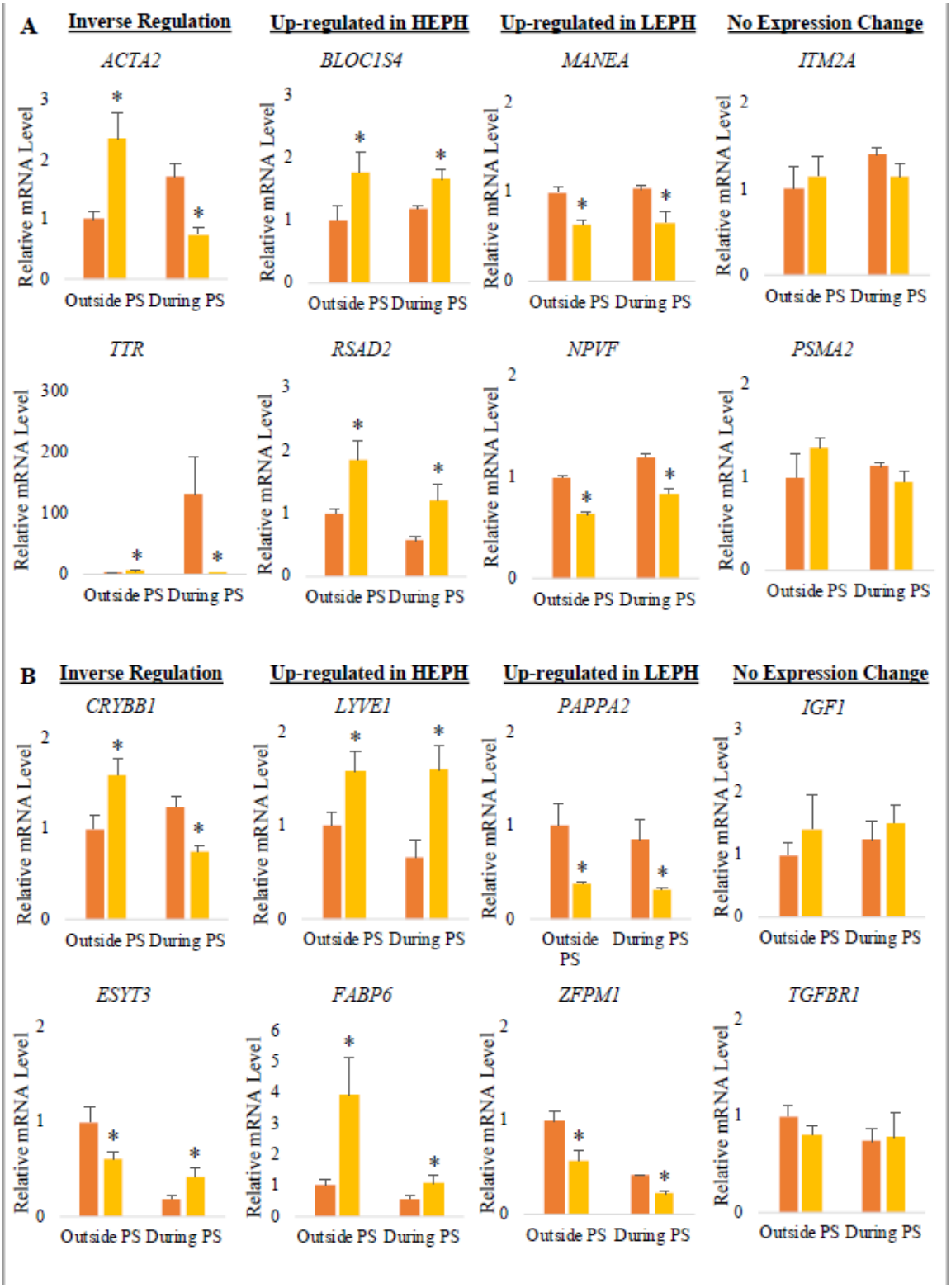

Figure 6

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PituitaryCuffdiffgenedifferentialexpressiontesting.xlsx
- SupplementalTables14.docx
- SupplementaryFigure1.pdf
- SupplementaryFigure2.pdf
- SupplementaryFigure3.pdf
- HypothalamusCuffdiffgenedifferentialexpression.xlsx