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The Changes in Hypothalamic mRNA Transcription of Genes Related to Behavior and Feeding in Incubating and Laying Hens: Implications for Laying Period Management

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Hypothalamus integrates environmental and peripheral signals to coordinate and manage growth, reproduction, feeding, sleeping and behavior functions. Transcriptomic profile changes in hypothalamus for transition from laying to incubating state and vice versa in poultry are a good way for bird requirement calculation. Accordingly RNA-Seq data of hypothalamus gene expression were downloaded from NCBI (http://www.ncbi.nlm.- nih.gov/sra/DRA008396). The quality control of sequencing reads was carried out by FastQC software. Filtered reads were evaluated with FastQC. The transcriptional profiling of 4 hypothalamus Sampels including two samples from broodiness and two samples from non-broodiness was assessed using DEseq2 Package . String database was used for the retrieval of interacting genes and Cytoscape software was used to visualize the network. In hypothalamus of laying hens comparing to incubating hens 17 genes showed significant expression change. Eight down-regualtion was related to serotonin receptor 4 (HTR4), prolactin receptor (PRLR), prolactin (PRL), Aguti-related protein (AGRP), Ghrelin (GHRL), neuro-peptide receptor 1 and 2 (NPY1R and NPY2R), vaso-active intestinal peptide (VIP) and nine up-regulation was related to gene expression of melatonin receptor 1 (MTNR1), Arginine vasotocin (AVP), Prolactin Releasing Hormone (PRLH), growth hormone releasing hormone (GHRH), Thyroid releasing hormone (TRH), orexin (HCRT), Pro-Melanin Concentrating Hormone (PMCH), tryptophan hydroxylase 1 and 2 gene expression (TPH1 and TPH2). In conclusion, although pro-lactogenic pathway declined significantly but serotonergic and melatonergic pathways related genes proliferated in hypothalamus of laying hen. AGRP gene and specially ghrelinergic pathway, VIP gene decreased. Accordingly it might to be told more supplementation of tryptophan amino acid and its melatonergic metabolites during laying period is necessary.

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Introduction

The hypothalamus is a center-pivot area located in the central part of the brain. maintaining body homeostasis undertaken by controlling sleep, secretion hormones, body temperature regulation, feeding and behavior. change in the Physiological condition of the body occurred through the

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expression of specific receptor genes and neurogenic pathways in hypothalamic regions. Thus these processes are integrated in the hypothalamus (Hajdarovic *et al.*, 2022). the preoptic area (POA), ventromedial nucleus, and lateral hypothalamus are the leading centers for reproductive behavior regulation in domestic poultry.

Brooding, a common trait of most poultry breeds, is characterized with consistent nesting, egg incubating, and nest defense. These behaviors are initiated by the hypothalamus and illustrated in some endocrine hormones alteration, egg-laying pause, body weight loss, and feed intake reduction (de la Iglesia and Schwartz, 2006). in the incubation period, ovarian regression led to egg-laying reduction and a decline in potential egg production.

One event is an increase in prolactin secretion, a polypeptide hormone secreted from adeno-hypophysis, affected the ovary and then inhibited the ovarian development. High blood prolactin level is associated with high broody behavior and egg production reduction (Yao *et al.*, 2019). Other changes in blood hormones concentration are related to gonadotropins (LH, FSH), VIP, and sex hormones (Progesterone, Estrogen) (Progesterone, Estrogen) (Ye *et al.*, 2019b). furthermore, transcriptomic change with many up and down gene regulations has been observed in the hypothalamus-hypophysis axis (Takeda and Ohkubo, 2019).

On the other hand, indoleamine hormones melatonin, serotonin, and their precursor tryptophan amino acid are identified as essential agents in egg production, calming, and well-being of poultry (Zawilska *et al.*, 2007). Also role of ghrelin hormone in egg production and ovarian folliculogenesis reported (Sirotkin and Grossmann, 2015; Sirotkin *et al.*, 2016).

Thus several genes and cellular pathways have been reported to participate in these processes and understanding the precise molecular mechanisms underlying broodiness and laying behavior is vital for the development of growing and production strategies.

Accordingly based above literature and free-downloadable hypothalamus transcriptom data from NCBI, the purpose was to investigate ghrelinergic, melatonergic, serotonergic , pro-lactonergic related genes differences in hypothalamus of laying and incubating hens.

Material and methods

Data retrieval, quality control and cleaning data

The free-downloadable dataset of hypothalamus gene expression were achieved from NCBI (http://www.ncbi.nlm.-nih.gov/sra/SRP08212) (Takeda and Ohkubo, 2019). The transcriptional profiling of hypothalamus samples from leghorn and local hen in non-broodiness and broodiness condition respectively was carried out at two samples for treated and two samples for control group. The quality control of sequencing reads was carried out by FastQC software(Andrews, 2010) (0.11.8).Trimmomatics software (0.36) was used to remove adaptor sequences and low-quality reads. Reads with an average quality score less than 20 were removed. Filtered reads were evaluated with FastQC.

Idenitification of DEGs

Filtered reads were mapped to the chicken reference genome (GCA_016699485.1) downloaded from the NCBI site using Hisat2 program (2.1.0) (Kim and Chong, 2013). DEGs among the groups (laying or incubating hen) were recognized using DESeq2 package(3.6) (Love *et al.*, 2014). The cutoff criteria were $|\text{LogFC}| \geq 1$ and adjusted p-value < 0.05. Finally, DEGs including upregulated and down-regulated genes were obtained.

Network Analysis

After identifying genes with different expressions, String database was used for the retrieval of interacting genes and Cytoscape software was used to visualize the network. In the network, the nodes represent genes, and the edges represent interactions among them. The three methods including degree centrality, betweenness centrality, and closeness centrality were used to calculate the topology scores of nodes in PPI network using CytoNCA plugin.

Gene ontology (GO) analysis

Gene Ontology resource (http://geneontology.org/) were used for GO enrichment analysis for identification some candidate genes which are involved in incubating and laying behavior (Table 1). The p-values < 0.05 were considered to be significant.

Results

Transcription dataset analysis of hypothalamus in incubating and laying condition showed significant changes in gene expression (Table 2). Ghrelin gene was significantly down regulated in laying hen ($P<0.01$). serotonin receptor (HTR4) showed decreased but TPH 1 and 2, MTNR1A gene expression increased ($P<0.01$). Prolactin releasing hormone gene had significant elevation but PRL and PRLR genes significantly decrease in gene expression of lying hen ($P<0.01$). thyroid releasing hormone (TRH), Growth hormone releasing hormone (GHRH), Arginine vasotocin (AVP), Hypocretin (HCRT), Pro-Melanin Concentrating Hormone (PMCH) significantly showed increase gene expression in hypothalamus of lying hen ($P<0.01$). receptors of neuropeptide (NPY1R and NPY2R), Agouti-related peptide (AGRP), Vasoactive intestinal peptide (VIP) gene expression significantly reduced in laying hen hypothalamus ($P<0.01$).

Based on the data in the STRING database, Gene co-expression network (Figure 1) showed between GHRL, PRL, HCRT with degree 10. These genes were the main hub genes in network with 75% closeness. After that specialized genes in network were TRH, GHRH, AVP with degree 9, 8, 8 respectively. After that VIP, PMCH, TPH1 with degree 7. (Table 3).

To obtain a better understanding of vital differentially expressed genes, the Gene ontology online server was used for GO analysis. In gene ontology, three parts of functional groups including biological process (BP), cellular performance (CC), and molecular function (MF) were analyzed. The p-values < 0.05 were considered to be significant. It was found that the DEGs are enriched in metabilic terms of the biological process which serotonin biosynthetic process, prolactin signaling pathway and Appetite-regulating hormone are the most significant biological process ,About the cellular component analysis, the DEGs mainly were enriched in ghrelin, prolacrin, and serotonin metabolites related genes. The gene ontology molecular function analysis revealed the involvement of DEGs in signaling pathways, protein receptors and feeding behavior (Table 1).

Discussion

Significant differences were observed in dataset analysis of hypothalamus genes expression during incubating and laying period. One of the significant change related to ghrelin gene expression which is mainly synthesized in the stomach and other body organs. ghrelin and its receptors expressed in ovary and hypothalamus of old and young laying hens (Sirotkin *et al.*, 2013). The ghrelin regulated the secretion of growth hormone and other hormones, food intake and energy balance, This hormone inhibited and stimulated food intake in birds and mammalians respectively(Kaiya *et al.*, 2009). Ghrelin addition to In vitro medium of different cell showed proliferating and apoptotic results, ovarian cell culture with ghrelin promoted expression of the apoptotic peptide bax and suppressed the antiapoptotic peptide bcl-2 (Sirotkin and Grossmann, 2008). In our research, hypothalamic ghrelin gene expression was significantly decreased in laying hens .this hormone in the hypophysis is a potent secretagogue for prolactin secretion (Messini *et al.*, 2010). In line with above literature, intra-muscular injection of ghrelin to young- laying leghorn hen reduced egg-laying rate (Sirotkin and Grossmann, 2015). Comparing blood ghrelin concentration during different phases of egg production of four laying hen breeds, showed its reduction during egg production peak (Höhne *et al.*, 2017).Thus reducing hypothalamic ghrelin gene expression in laying hen and its increase in incubating hen suggested ghrelin role on controlling poultry hypothalamus-hypophysis-ovarian function.

Serotonin is made from amino acid tryptophan by the limiting enzyme tryptophan hydroxylase (TH). Two isoforms of tryptophan hydroxylase enzyme, tryptophan hydroxylase 1 (TPH1) and tryptophan hydroxylase 2 (TPH2) have been known until now. TH1 is mainly synthesized in entrochromaphin cells of gut and pineal gland while TPH2 is commonly expressed in central nervous system (Walther and Bader, 2003).

Serotonin modulates many physiological functions including cardiovascular, respiratory and immune function, and energy balance as well as body temperature. Supplying tryptophan in broiler breeder during egg production peak increased intestinal integrity, egg production traits and reduced inflammatory agents (Jiang *et al.*, 2018).

Hypothalamus transcription showed significant increase in TPH1 ,TPH2 and reduction in HTR4 genes expression of laying hen respectively. Broodiness in duck significantly increased hypothalamic serotonergic synapses comparing to laying duck (Ye *et al.*, 2019a). Similarly central injection of serotonin to turkey significantly increased prolactin secretion but 5-HT2C (serotonin receptor) agonist injection acted reversely (Bakken *et al.*, 2014). Accordingly based on which one serotonin receptors involvement in post-receptor signaling, effect of serotonin on prolactin secretion would be changed. Blocking serotonin receptors in rat hypothalamus-hypophysis axis proved 5-HTR4 receptor role in mediating prolactin release (Papageorgiou and Denef, 2007). In our research laying hen showed significant decline in 5-HTR4 gene expression and probably this would led to prolactin secretion decrease. On the other hands melatonin receptor significantly increased during laying period campared to incubating hen. Melatonin produced in pineal gland acts an significant role in the reproductive functions and calcium homeostasis, antioxidant capacity (Wang *et al.*, 2021).

Broodiness behaviors are managed by series of hormones .one of different is related to pro-lactogenic gene expression pathway between laying and incubating hens. prolactin act a critical role in onset and continuing broodiness behaviors (Sarkar, 2022). In our research based on transcription data, prolactin and its receptor gene expression significantly reduced in laying status but prolactin releasing hormone (PRLH) increased. Prolactin ceased egg production and induced aggressive and incubating behavior in hen. There is a negative relationship among blood prolactin level and egg laying rate (Yao *et al.*, 2019). PRLH is involved in PRL secretion and stimulated its production in hypophysis. Its hypothalamic gene expression significantly was higher in high egg production compared to low egg production ones (Yu *et al.*, 2020). This discrepancy may be related to egg laying phase and feed-back mechanisms, so that Yu et al reported and compared low and high egg production of laying hen, as well as according to feed-back mechanism, declining prolactin level induced elevation in its stimulator (namely PRLH). In addition PRLH reported as a non-prolactin releasing agent in lactating and non-lactating rat but decreased food intake, energy balance and body weight in rodent model (Pflimlin *et al.*, 2019). Together RRLH function most likely is as a neuromodulator without hypophysiotrophic role and suppressed food intake by diminishing AGRP signaling in rodent hypothalamus (Cheng *et al.*, 2021). In agreement to Cheng et al, hypothalamic AGRP gene expression reduced significantly in laying hen comparing to incubating hen. This confirm available circuit between AGRP and PRLH neurons in hypothalamus.

Avian vasoactive intestinal polypeptide (VIP) is a 28-amino-acid peptide that originated and produced from the chicken's intestin. This hormone is part of VIP-PRL system in management of reproduction and broodiness behavior (Zhou *et al.*, 2008). VIP effect is stimulation of PRL production and secretion and its production increased during photo-refractory and incubating status (Rozenboim *et al.*, 2022). In our research VIP gene expression reduced in laying hen. Prolactin secretion is positively correlated to VIP production in hypothalamus. Its production has been reported in hypothalamic neurons (Rozenboim *et al.*, 2022).

Maybe the answer to the reason for VIP elevation during broodiness is the ability of this hormone for protecting gastrointestinal integrity, promoting immune system. So, VIP reduced TNF-alpha, IL-6 (pro-inflammatory agents) (Abad and Tan, 2018) and increased tight-junction protein in intestinal cells (Conlin *et al.*, 2009). Poultry broodiness is characterized by incubating in nest, aggressive behavior, nest defensing, food intake reducing and all these behaviors potentiated bird to illness and pathogen attack. Accordingly VIP increase during incubating help to maintain the bird against pathogenic agents.

TRH is one of the most vital hypothalamic signaling agents for modulation the energy balance in poultry as well as a key trigger for regulating the synthesis of triiodothyronine (Singh *et al.*, 2023). In laying hen its gene expression elevated. This probably related to higher metabolic rate in laying compared to incubating hen. In line to recent findings, hypothalamic TRH gene expression in sing-birds increased during breeding season (Verhagen *et al.*, 2019). In addition , fasting reduced TRH gene expression in rat hypothalamus (Campos *et al.*, 2021). Reducing appetite and food intake is characteristic of poultry during broodiness and decreased in hypothalamic TRH

gene might be related to incubating condition.

AVT (Arginine-Vasotocin) is secreted by ovarian of laying hen and its secretion increase during egg laying period (Sirotkin and Grossmann, 2008). Its secretion decreased during food restriction and there is a positive relationship between AVT and progesterone concentration in poultry ovary (Sirotkin and Grossmann, 2015). Also negative relationship demonstrated between ghrelin and AVP production in laying hens ovary (Sirotkin *et al.*, 2016). So both human and poultry ghrelin addition to ovarian cell culture, suppressed AVT production by granulosa cells. In recent findings AVT gene expression has been elevated significantly in laying hen compared to incubating hen. AVT seems to be involved in egg laying, according to above mentioned finding and its hypothalamic gene expression increased during egg laying period specially at time of oviposition (Seth *et al.*, 2004).

Neuropeptide Y (NPY), a neuromodulator /neurotransmitter, is very well known to be involved in regulation of food intake in mammals and birds. But there is information about NPY role in maternal behavior. the number of NPY-immune-reactive neurons within hypothalamus of incubating hen increased specially in PVN nucleus (Kamkrathok *et al.*, 2021). Also density of NPY receptors in broiler compared to layer hypothalamus showed two to three times higher (Merckaert and Vandesande, 1996). These findings implicating that the NPYergic system within hypothalamus has significant effect on maternal behavior and egg production and could be a target for layer breeding.

Orexins and orexine system are G-protein coupled receptors in charge of numerous cell signaling functions and pathways. These are known as hypocretins. regulating feeding behavior, energy homeostasis are roles of orexine in mammalian but these functions have not been reported in chickens (Furuse *et al.*, 1999). Orexine although had elevated arousal but their food intake was not changed in layer chickens (Katayama *et al.*, 2010). in avian species orexine has been shown to have no central effect on feed-intake, however it was declared to have participated in energy metabolism of muscle and liver lipogenesis (Ramser and Dridi, 2022). In recent experiment analysis, laying compared to incubating hen significantly increased orexine gene expression in hypothalamus. Collectively it may be told laying hens for higher metabolic requirement probably had more activated orexigenic system in hypothalamus.

An indolic hormone in body, Melatonin (N-acetyl-5-methoxytryptamine), is synthesized from serotonin in the pineal gland and other extra-brain tissues and manage variety of biological actions through three different receptor subtypes MTNR1A (MTR1), MTNR1B (MTR2), and MTNR1C in avian (Zhao *et al.*, 2011). In recent experiment, laying hens showed higher MTNR1A gene expression significantly compared to incubating hens. In geese this gene introduced as a candidate gene for egg production and MTNR1A polymorphism has near relationship to egg laying capacity and a molecular marker in goose selection specifically for egg laying percentage (Alsiddig *et al.*, 2017). In laying compared to incubating hens, fold change of MTNR1A gene expression was 26. This probably indicates importance of melatonin and its receptors for egg production and egg formation process especially in laying period. In agreement to our results, intraperitoneally injected melatonin to aging laying hen significantly elevated estrogen and luteinizing hormone, also egg production rate of melatonin-treated ones were significantly higher than control hens (Hao *et al.*, 2020).

Conclusion

Hypothalamic nuclei are the main centers for reproduction regulation and its transcription is a good guidance for studying laying and incubating status. In this study lower ghrelin and pro-lactogenic genes, higher gene expression of melatonergic system and tryptophan hydroxylase enzymes of laying hens was observed. Conclusively promoting and supplementing these systems in laying hens Should be recommended for nutrition and breeder specialists.

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Table 2. Transcription data of laying hen hypothalamus compared to local incubating hen.

Gene Code	P-value	Fold change	FDR p-value correction
TPH1	1.48E-04	2.47913	4.42E-03
TPH2	0.035776	1.366844	0.232266
HTR4	0.018516	-1.66812	0.155636
MTNR1A	0.031265	26.93334	0.212352
PRLR	2.13E-08	-1.81432	2.27E-06
prl	9.42E-04	-6.05678	0.018846
PRLH	1.60E-57	10.03064	2.71E-54
GHRH	2.23E-03	1.367323	0.035979
TRH	2.57E-03	1.366786	0.039124
AVP	0.027619	1.366907	0.201471
AGRP	3.94E-17	-8.6375	1.45E-14
GHRL	2.53E-10	-18.2443	3.59E-08
NPY1R	0.031696	-1.3382	0.214157
NPY2R	0.038345	-1.40111	0.243093
VIP	0.029371	-1.49024	0.207242
HCRT	0.021364	1.242721	0.170082
PMCH	4.85E-03	1.500149	0.061844

Table 1. Metabolic pathway of Laying hens hypothalamus compared to own of local incubating hens. Based on investigation from geneontology.org

GO term	P value	FDR	Name of genes
serotonin biosynthetic process	2.39E-08	2.23E-05	TPH1, TPH2
prolactin signaling pathway	8.27E-06	2.44E-03	PRLR
feeding behavior	2.47E-11	2.77E-07	AGRP, GHRH, HCRT, PRLH, TRH
response to nutrient levels	1.67E-04	2.75E-02	HCRT, PRL, HTR4
negative regulation of gonadotropin secretion	5.61E-03	4.66E-01	PRL
neuropeptide Y receptor activity	1.12E-04	1.86E-02	NPY1R, NPY2R
Appetite-regulating hormone	1.73E-06	5.42E-03	GHRL

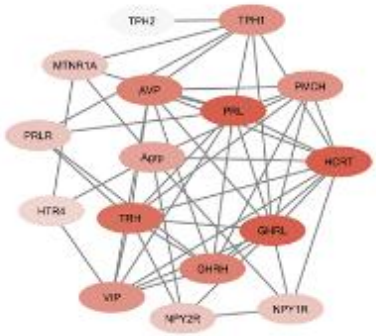


Figure 1. genes co-expression network involved in incubating and laying behavior.

Table 3. The Gene-Gene network showing interactions scores between different genes related to incubating and laying behavior of hen.

Gene symbol	Gene interaction number	Betweenness score	Closeness score
PRL	10	18.29	0.75
HCRT	10	19.78	0.75
GHRL	10	21.38	0.75
TRH	9	12	0.68
GHRH	8	4.41	0.65
AVP	8	3.94	0.68
VIP	7	11.2	0.62
PMCH	7	4.43	0.62
TPH1	7	34.41	0.65
AgRP	6	14.5	0.58
PRLR	4	1.6	0.52
NPY1R	4	1	0.54
NPY2R	4	2.25	0.55
MTNR1A	4	8.86	0.58
HTR4	3	2	0.52
TPH2	1	0	0.40