Effects of incubation lighting with green or white light on brown layers: hatching performance, feather pecking and hypothalamic expressions of genes related with photoreception, serotonin, and stress systems

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ABSTRACT The aim of this study was to evaluate the effect of 16L:8D photoperiod with green (**GREEN**) or white (**WHITE**) lights during incubation on hatching performance, blood melatonin, corticosterone, and serotonin levels, hypothalamic expressions of genes related to photoreception, serotonin, and stress systems in layers in relation with feather pecking behavior. Dark incubation (**DARK**) was the control. Eggs (n = 1,176) from Brown Nick breeders in 2 batches (n = 588/batch) were incubated in the experiment. A total of 396 female chicks and 261 hens were used at rearing and laying periods until 40 wk. Incubation lighting did not affect hatchability, day-old chick weight, and length, but resulted in a more synchronized hatch as compared with the DARK. The effect of incubation lighting on blood hormones was not significant except for reduced serotonin in the GREEN group at the end of the experiment. There was no effect of incubation lighting on gentle, severe, and aggressive pecking of birds during the early rearing period. From 16 wk, GREEN hens showed increased gentle pecking with increasing age. WHITE hens had the highest gentle pecking frequency at 16 wk while they performed less gentle but higher severe and aggressive pecks at 24 and 32 wk. At hatching, the hypothalamic expression of CRH, 5-HTR1A, and 5-HTR1B was higher for the WHITE group compared with both GREEN and DARK, however, 5-HTT expression was higher in GREEN than WHITE which was similar to DARK. Except for the highest VA opsin expression obtained for WHITE hens at 40 wk of age, there was no change in hypothalamic expression levels of rhodopsin, VA opsin, red, and green opsins at any age. Although blood hormone levels were not consistent, results provide preliminary evidence that incubation lighting modulates the pecking tendencies of laying hens, probably through the observed changes in hypothalamic expression of genes related to the serotonin system and stress. Significant correlations among the hypothalamic gene expression levels supplied further evidence for the associations among photoreception, serotonin, and stress systems.

Key words: incubation lighting, layer, feather pecking, serotonin and stress system, opsin

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INTRODUCTION

Avian embryos are able to sense and respond to their environment (Reed and Clarks, 2011). Therefore, interactions between embryos and their incubation environment are receiving increasing attention. Light is an important environmental factor affecting embryonic development and post hatch behavior and physiology of chickens. It has been well documented that photoreceptors of chicken embryos detect light through photosensitive opsin molecules located in the retina, the pineal gland and the hypothalamus (Lewis and Morris, 2006; Surbhi and Kumar, 2015). Expression of opsins from the OPN1, OPN4, and OPN5 families in the hypothalamus suggest they play a role in regulation of the major endocrine axes that is, the hypothalamus pituitary adrenal (**HPA**), the hypothalamus pituitary gonadal (**HPG**), and the hypothalamus pituitary thyroidal (**HPT**) axis. Recent evidence also showed that light might affect neural development of the embryo from the very beginning of the incubation period, from as early as d 3 of the embryonic development (Chiandetti et al. 2013; Chiandetti and Vallortigara, 2019).

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There are contradictory results regarding the effect of light during incubation on hatching performance in broilers. Compared to dark incubation, 16 light (L):8 dark (D) green LED lighted incubation positively affected hatchability and chick quality and incubation duration of broiler embryos (Tong et al., 2018; Yu et al., 2018). Archer (2017) reported that 12L:12D red or white LED lighting during incubation improved hatchability and chick quality as compared to green LED and dark incubation conditions. However, a 16L:8D schedule using white fluorescent light had no effect on hatching performance as compared with the dark incubation condition (Özkan et al., 2012).

Lighted incubation may have also a positive effect on stress susceptibility of broiler chicks post hatch. It was shown that retinal and extra retinal photoreceptors of chicken embryos can detect and respond to environmental light/dark cycles from embryonic day (**ED**) 18 through melatonin (Zeman et al. 1992; Zeman and Herichova, 2011), which is able to suppress corticosterone (**CORT**) production (Saito et al., 2005). A 16L:8D white fluorescent lighting during the incubation period (ED 0−21) as compared with dark incubation resulted in a lower CORT response to holding stress by 8 h in transportation boxes on the day of hatching (Özkan et al., 2012). The authors concluded that these positive effects of lighted incubation on post-hatch stress responses of chickens might be related with early entrainment of pineal rhythms by secreting its hormone melatonin, which has an important physiological role in the ontogeny of the chicken embryo (Cooper et al., 2011) and may help chicks to cope with environmental changes (Zeman et al. 1999). Archer (2017) found that lighted incubation with white, red or green LED significantly reduced plasma CORT, but increased serotonin levels, as a further evidence for improved stress status of broilers on d 45 as compared to broilers from dark incubation.

However, the studies on the effect of lighted incubation are mostly on broiler embryos and information regarding the effect of lighting incubation on laying hen embryos is scarce. Huth and Archer (2015) reported that lighted incubation improved chick quality of white layer embryos. A recent study from Hannah et al. (2020) indicated that commercial brown and white layer strain embryos had a more synchronized hatching window when incubated under a 12L: 12D photoperiod with no significant effect on hatchability as compared with the dark incubation.

Light during incubation may also affect feather pecking behavior. Feather pecking is a multifactorial problem and one of the important welfare issues of laying hens (Rodenburg et al., 2013). Van Hierden et al. (2004) reported that feather pecking is exacerbated by low serotonin (5-hydroxytryptamine, 5-HT) neurotransmission. In an earlier study, higher frequencies of gentle feather pecking directed to familiar pen mates were reported in layer chicks exposed to 2-h incandescent light pulses with a high intensity (750−100 lux) at ED 18 and 19 (Riedstra and Groothuis, 2004). The authors concluded that light exposure during the last stage of incubation, which commonly occurs under natural incubation and in industry, could be risky for commercial layers as the gentle feather pecking observed in the study may turn into severe feather pecking. In contrast, Rogers et al. (2007) incubating layer eggs under incandescent light with a lower light intensity of 150 to 200 Lux between ED 18 to 21 showed that lighting positively affected the development of normal pecking behavior and the ability of chicks to discriminate edible objects from nonedible objects. It has been suggested that lighted incubation may affect development of damaging feather pecking by the improvement in the birds' ability to discriminate food and thus reduce risk of damaging feather pecking (de Haas et al., 2021).

There is a large accumulation of information regarding the effect of light during incubation on growth, response to environmental stressors, health, and welfare of broilers. However, scarce information is available on the effect of lighted incubation on molecular changes in relation to photoreception, serotonin and stress systems in laying chicks at hatch and laying period. To the best of our knowledge, there has been only one earlier report regarding the effect of lighting during the embryo stage on opsin expressions in hypothalamus of the broiler chickens (Rozenboim et al., 2013). The authors of this report referred to their unpublished data in which they found that photostimulation of eggs with green light reduced retinal gene expression of the green and red opsin during late embryogenesis and at hatching. However, they did not detect hypothalamic red and green opsins during late embryogenesis and concluded that enhancement of muscle growth might be governed by retinal photostimulation. They further reported that the downregulation of the retinal green and red opsins in response to incubation lighting persisted until 9 d post hatch, suggesting a possible epigenetic effect. Moreover, information on functional connections of extra retinal photoreceptors to the endocrine system, behavior, and metabolic processes are limited (Perez et al., 2019) and any information regarding to the role of extraretinal photopigments in birds' physiology and behavior would help to uncover photoperiodic control of these functions by extraretinal photoreceptors.

Considering the suggested possible role of light on morphological, physiological, behavioral development of chicken embryos we hypothesized that a photoperiodic lighting schedule using different color of LED lights (Green and White) during incubation could affect hatching performance, blood melatonin, corticosterone and serotonin levels, feather pecking and hypothalamic expressions of genes related with photoreception, serotonin, and stress systems in layer chicks and hens.

MATERIALS AND METHODS

Animal experimental procedures were approved by the Ege University Animal Research Ethics Committee,

protocol no. 2014-063 and 2016-012 for rearing and laying periods, respectively.

Incubation Conditions

The experiment was carried out in 2 batches and was conducted with 3 incubators/batch that combined both the setter and hatcher (VGS, Istanbul, Turkey) with a capacity of 196 eggs and 4 egg trays/incubator. Therefore, each batch consisted of total of 588 hatching eggs from the same brown layer (Brown Nick, H&N International, Öztavuk, Bursa, Turkey) breeders, aged 36 and 40 wk, respectively.

The eggs stored for 3 d prior to incubation were numbered, weighed, randomly divided into 3 groups and incubated under 16L:8D lighting schedule throughout incubation (E0 to 21) using 1) full spectrum white (6,500 K) light emitting diode (**LED**) lamps (WHITE), 2) green (8,000 K) LED lamps (GREEN), or 3) darkness (DARK). Center wavelength of WHITE and GREEN LED lamps was 442 and 518 nm, color rendering indexes were 69.67 and 17.28, respectively. LED light intensity was measured at 4 different points of each one of the egg trays at egg levels using a Testoterm luxmeter (model 0500, Testo, Germany) and ranged between 150 and 250 Lux with an average of 200 Lux. Average egg weight was 57.4, 57.8, and 57.2 g, for WHITE and GREEN lighted, and DARK groups, respectively.

Incubators were set at 37.7 ± 0.1°C and 60% relative humidity. Temperature and humidity was also monitored with data loggers. During the second half of the incubation, eggshell temperature was measured by an infrared thermometer (Testo 845, Testo, Germany) on 4 eggs per incubator twice per day to maintain a constant egg temperature of 37.7 ± 0.1°C and incubator temperature was adjusted if necessary. Eggshell temperatures did not vary with the treatments and ranged between 37.54 and 37.80°C. At d 18, eggs were candled to remove non-fertile eggs and the remaining fertile eggs were transferred to hatcher trays. At the end of the 504 h, the hatching period was terminated.

Rearing and Laying Period Conditions

A total of 198 non-beak trimmed female chicks from each batch (66 chicks from each incubation lighting/batch) were transferred to the poultry house and assigned to one of 9 floor pens/batch covered with wood shaving and reared for 14 weeks. There were 22 chicks per pen (1.4 × 1.2 m) per batch, and three replications per incubation lighting treatment per batch. Pens were provided with one bell drinker and one feeder. The temperature was kept constant at 33°C during the first 3 d and lowered until 22°C was reached on d 42. Thereafter, temperature and humidity ranged between 20 and 22°C and humidity was 55 to 60% throughout the rearing period. Lighting started with 23-h light and 1 h darkness (23L:1D) and gradually decreased to 16L:8D on d 5, 14L:10D on wk 2, 12L:12D on wk 5, 10L:14D on wk 9 and was kept constant until moving to the laying house in week 14. Average light intensity at chicks' level was 20 to 25 Lux during the first week and kept at 8 to 10 Lux during the rearing period. Birds were fed with commercial diets that meet or exceed the nutrient recommendations of laying hens specified by the NRC (1994). The chicks were fed a starter mash diet (2900 kcal/kg ME and 21% protein) from 0 to 3 wk, grower-1 diet (2750 kcal/kg ME and 19% protein) from 4 to 8 wk, a grower-2 diet (2,700 kcal/kg ME and 18% protein) from 9 to 14 wk, respectively. Feed and water were provided ad libitum throughout the experiment.

At the age of 14 wk pullets were moved to a laying house with windows, having 2 separate units each containing 6 floor pens (2.3 × 2.1 m). Leg bands were attached to the pullets from each one of the batches for individual identification, and birds were randomly distributed onto floor pens (21−22 birds/pen) with 2 replicate pens for each treatment/batch (a total of 4 pens per treatment) and reared until 40 wk of age. Each pen had one hopper type feeder and four nipple drinkers. Perch length per bird was 20 cm and perch height was 50 cm. Nest boxes (6 per pen) were provided from wk 18 onward. Pullets received commercial grower diet in mash form (2,700 kcal/kg ME and 18% protein) between 14 and 18 wk of age and a layer mash diet (2800 kcal ME and 17.0% protein, 3.75% Ca, 0.69% P) during the laying period. The light schedule was kept at 10L:14D between 14 and 17 wk and a 13L:11D light schedule was started at the age of 18 wk. By gradual increases, the light schedule reached 16L:8D at 24 wk of age and was constant until the end of the experiment. Lighting was provided by compact white fluorescent lamps (2,700 K) during the rearing and laying periods. The average light intensity ranged between 7 and 12 Lux among the pens at birds' level. To protect birds from direct sun light, windows were shaded by black curtains to limit daylight during the laying period.

Measurements During Incubation Period and Day of the Hatch

Hatching Performance After 472 h of incubation, all eggs were examined every 8 h to determine the moment of hatching. At 496 h of incubation 30 hatched chicks per incubation light treatment and per batch were sampled, weighed, and chick length was measured.

Unhatched eggs were broken out to determine the number of infertile, early dead (0−7 d of incubation), mid dead (8−14 d of incubation), late dead (15−21 d of incubation), and pipped but not hatched eggs. Hatchability and mortalities were presented as a percentage of fertile eggs.

Blood Hormones At 484 h of incubation, when the eggs were in the mid of the 8-h dark period in lighted incubation treatments, 8 hatched chicks/treatment/batch were randomly sampled to obtain blood to measure night-time (darkness) blood melatonin and CORT concentrations. The blood was collected into heparinized

Table 1. Primer sequences and probes for Real-time PCR.

Genes	Accession no	Pimer sequences (5'-3')	Probes catalog no
Green opsin	NM_205490	F: cacttcatcatcccggtcgt R: atccgcgtcacctccttctct	04694295001- UPL# 143
Red opsin	NM_205440	F: caagtcggccaccatctac R: gagacggaggagttggaga	04694503001- UPL# 163
VA opsin	NM_001105318	F: tcaatcatcaccaggcattg R: cagatcacaatgtaccgctca	04688511001-UPL#54
RHO	NM_001030606	F: ggtgaaattgctctctggtca R: ggcttacagaccaccacgtatc	04694490001- UPL#162
5-HTT	NM_213572	F: tggagatttccctacatatgctatc R: ggccataattgtgtaaggaatga	04688627001-UPL#63
5-HTR1A	NM_0011170528	F: gaccccatcgactatgtcaac R: cgggatggatatcaagaagc	04694201001- UPL#136
5-HTR1B	NM_001172781	F: ccaggtgaaggtgaaggtgt R: cctaaagtctttgtcgctttcc	04688660001 - UPL#67
CRH	NM_001123031	F: catctccctggacctgactt R: tcagtttcctgttgctgtgg	04688619001-UPL#62
ACTB	NM_205518	F: tggcaatgagaggttcagg R: cacaggactccatacccaaga	04685105001-UPL#11

Abbreviations: ACTB, actin beta; CRH, Corticotropin Releasing Hormone; RHO, Rhodopsin; VA opsin, vertebrate ancient opsin; 5-HTT, serotonin transporter;5-HTR1A, 5-hydroxytryptamine receptor 1A; 5-HTR1B, 5-hydroxytryptamine receptor 1B, .

tubes via immediate neck cut. A similar procedure was applied at 496 h of incubation to measure day-time (lightness) melatonin and CORT concentrations of chicks at hatch. Blood samples were centrifuged at 4,500 × *g* and 4°C for 15 min and immediately frozen at −80°C for hormone analyses. Plasma melatonin and CORT levels were measured using commercial ELISA kits (Cusabio, www.cusabio.com) according to the manufacturer's instructions. Several dilution rates were tested before analyses and dilution rates of 1/4 and 1/5 were used for CORT and melatonin hormones, respectively.

Sampling of the Hypothalamus and Real-Time PCR Analysis On the day of hatching a total of 5 chicks per incubation/batch were used for brain tissue collection. After decapitation, the brain was immediately removed from the skull and the whole brain was dipped into liquid nitrogen for approximately 7 to 8 s before dissection to get firmness of the tissue to make precise cuts for hypothalamus sampling. Identification of the hypothalamus was performed according to A7.6 of the brain atlas of the chick (Kuenzel and Masson, 1988) and the hypothalamus was dissected by making an approximately 1.5 to 2 mm cut parallel to the midline on both sides and a 1 mm depth from starting septopallio-mesencephalic tract to the third oculomotor nerve (Piekarski et al., 2016). Hypothalamus samples were immediately frozen in liquid nitrogen and stored at −80°C until PCR analyses.

Total RNA was extracted from the hypothalamus using TriPure Isolation Reagent (Roche, Mannheim, Germany). The RNA concentration and purity were estimated by measuring the absorbance values at 260 and 280 nm. iScriptTM cDNA synthesis kit (BIO-RAD, CA) was used to transcribe total RNA samples, according to the manufacturer's instructions. Primers and probes for Vertebrate ancient opsin (**VA opsin**), Green opsin, Red opsin, Rhodopsin, Serotonin transporter (**5-HTT**), Serotonin-1A receptor (**5-HTR1A**), serotonin -1B receptor (**5-HTR1B**), corticotropin releasing hormone (**CRH**), and actin beta (**ACTB**) were designed using the NCBI (https://www.ncbi.nlm.nih.gov/genbank/) and ENSEMBLE (https://www.ensembl.org/index.html) GenBank Gallus gallus-specific (Table 1). ACTB (actin, beta) was used as the housekeeping gene. Gene expressions were determined by mixing 2.5 μL cDNA, 5 μL 2x LightCycler 480 Probes Master (Roche, Mannheim, Germany), primer pairs and probes (**UPL**, Universal Probe Library) at 10 μM concentrations in a final volume of 10 μL on a Roche LightCycler 480 II. Gene expressions of VA opsin, Green opsin, Red opsin, Rhodopsin, 5-HTT, 5-HTR1A, 5-HTR1B, and CRH were calculated using the ΔΔCt method.

Measurements During Laying Period

Blood Hormones At 14, 24, and 40 wk of age, blood samples of 6 birds/incubation/batch were collected from the wing vein to measure whole-blood 5-HT level. The blood was collected into EDTA coated tubes for 5-HT concentration analysis. Whole blood 5-HT concentration was determined using a fluorescence assay as described in Bolhuis et al. (2009). At 14 and 40 wk, blood was also collected into heparin tubes to measure CORT levels.

Sampling of Hypothalamus and Real-Time PCR Analysis At 16 and 40 wk of age, the hypothalamus of 4 and 3 laying hens per incubation/batch, respectively, was obtained using the same procedure applied on the day of the hatch.

Feather Pecking Behavior Observations in the Home Pen During the rearing, feather pecking was recorded at pen level at 1, 5, and 8 wk of age. All birds in the 3 replicated pens of each incubation group for each batch (in total 6 pens per incubation group) were observed for pecking behavior directed to pen mates as gentle, severe, and aggressive pecking according to the

definitions by Bilcík and Keeling (1999). Each peck was recorded from video observations during 10 min of 3 sessions in the morning (between 8:00 and 9:00 h), noon (12:00−13:00), and evening (17:00−18:00 h) on 2 consecutive days. Thus, each pen was observed for 60 min at each age. The number of pecks was averaged across all birds in a pen thus was presented as pecks/bird. In the laying house, the total number of replicate pens of each was reduced to 4 pens per incubation treatment and direct observations were made by the same observer at the age of 16, 24, and 32 wk. However, at 24 and 32 wk of ages, noon observations were between 13:00 and 14:00 h and evening observations were between 21:00 and 22:00 h, according to the lighting schedule as lights went off at 22:00 h.

Scoring for Plumage Damage, Comb, and Vent Pecking Wound Each bird was scored for feather condition, pecking wound at comb, given on a 3-point scale (Welfare Quality, 2009). Feather scoring was performed on 3 body parts (head and neck, back and ramp, and abdominal area). These 3-point scaling (0: no damage or a few feathers lacking, 1: moderate feather damage and one or more featherless areas less than 5 cm in diameter, 2: one or more body parts have featherless area larger than 5 cm in diameter) was combined to get a single score for each hen at the age of 40 wk. Pecking wounds at the comb were also assessed by 3 scale scoring (0: no pecking wound, 1: less than 3 wounds, and 2: ≥ 3 wounds). Apart from feather damage and loss, pecking wounds at the vent area were also assessed using 1 −0 scaling to get more information regarding vent pecking.

STATISTICAL ANALYSIS

The hatching performance, chick length, hatch body weight, gene expressions at different ages, and hormone levels were analyzed by ANOVA with the GLM procedure of the JMP 5.1. of the SAS statistical package. The statistical model to analyze hatching performance included lighted incubation treatment (I), batch (B), interaction between I and B, and egg tray effect nested within the I. The data on chick length, hatch body weight and gene expressions were analyzed with a model including I, B, and interaction between them. For melatonin and CORT hormones at hatch, sampling time (day/night) was also included in the model with all possible interactions. However, insignificant interaction effects were removed from the model. In the analysis of serotonin and CORT hormones data from pullets and layers, the statistical model included I and pen effects nested within the I and B. When significant differences were observed, means for the effect were subjected to post hoc test for pairwise comparisons with Tukey. Significance was based on $P \leq 0.05$. Associations between expression levels of genes were analyzed via Pearson-s'correlation coefficient. Plumage damage, comb and vent pecking wounds data were subjected to chi-square analysis. Behavior data from observations at the home pens were analyzed with a Mixed model using the MIXED procedure of the SAS statistic package version 8.2 including I, age (week), batch, session and interactions among them as fixed effects. The batch effect was not significant for any of the behaviors and it was therefore, removed from the model. Pen within I and B was added as a random effect. For significant effects, lsmeans were separated with pre planned t tests using pdiff option of SAS with a significance level at $P \leq 0.05$. Prior to the analyses, data were checked for normality by the Shapiro-Wilk test and a logarithmic transformation was used for the gene expression data except for red and rhodopsin, where arcsin square root transformation was used. For the other traits, arcsin square root transformation for severe and aggressive pecking and logarithmic transformation for gentle pecking and hormones data were used. Actual values are presented in the tables and graphs.

RESULTS

At the Day of the Hatch

Lighted incubation did not affect hatchability, embryo mortality rates, chick weight, and length at hatching day (Table 2). The significant effect of batch showed that chick weight and length ($P \leq 0.05$) were higher in first batch. There was no significant interaction between treatment and batch on any of the traits measured at the day of the hatch. Figure 1 presents the distribution of accumulated hatching rates of the incubation groups. Significantly lower hatching rates were observed in the GREEN group as compared to the DARK at different time points from 472 h to 488 h of incubation, namely 32 to 16 h before hatching was completed (Figure 1). The WHITE group was intermediate and had significantly lower hatching rates than DARK incubation at 480 h but similar hatching rates as the other 2 groups at 472 and 488 h of the incubation period. The GREEN group caught up with the others at 496 h of incubation.

Neither incubation nor batch effect was significant for blood melatonin and CORT hormone levels of newly hatched chicks (Table 2). However, sampling time significantly affected blood CORT levels at hatch being higher at night time observations as compared with the day time observations.

Relative expression of photoreceptor genes, namely green opsin, red opsin, rhodopsin, and VA opsin, was not influenced by treatments at the day of the hatching (Figures 2A−2D). Lighted incubation affected 5-HTT, 5-HTR1A, 5-HTR1B, and CRH expression levels (Figures 3A−3D) in the hypothalamus of day-old chicks ($P \leq 0.05$). Light color had a significant impact on the expression levels on the day of hatch and chicks exposed to GREEN incubation treatment had higher 5-HTT expression than those exposed to WHITE incubation treatment ($P \leq 0.05$) while the DARK group had an intermediate expression level and was similar to both lighted groups (Figure 3A). WHITE incubation lighting significantly increased 5-HTR1A, 5-HTR1B, and CRH

Table 2. Effect of incubation treatment (white light, green light or dark incubation), batch and sampling time on hatchability, embryonic mortalities as a percentage of fertile eggs, chick weight and length and blood corticosterone (CORT), and melatonin concentration at hatch.

Treatments	Hatchability %	Embryonic mortalities %				Chick			
		Early	Mid	Late	Pipped	Weight g	Length cm	CORT ng/ml	Melatonin pg/ml
Incubation (I)									
White	88.64	5.27	0.79	1.58	3.70	41.74	16.82	6.96	65.36
Green	92.56	2.65	1.41	0.71	2.66	41.24	16.82	6.46	66.36
Dark	90.67	3.59	1.08	2.51	2.15	40.39	16.81	7.93	48.01
SEM	0.91	0.82	0.50	0.64	0.56	0.28	0.04	0.76	11.29
Batch (B)									
1	90.92	3.95	0.79	1.13	3.19	41.20[a]	16.98[a]	6.74	59.51
2	89.33	3.72	1.39	2.06	2.47	40.38[b]	16.66[b]	7.49	60.32
SEM	0.77	0.69	0.42	0.54	0.56	0.23	0.03	0.63	9.26
Sampling time (ST)									
Night	-	-	-	-	-	-	-	8.07[a]	67.94
Day	-	-	-	-	-	-	-	6.17[b]	51.89
SEM	-	-	-	-	-	-	-	0.63	9.24
Statistical analysis					*P* values (Significance)				
I	0.076	0.175	0.712	0.222	0.242	0.101	0.956	0.310	0.327
B	0.620	0.831	0.377	0.287	0.344	0.012	<0.001	0.705	0.730
I × B	0.066	0.376	0.220	0.383	0.106	0.781	0.087		-
ST	-	-	-	-	-	-	-	0.008	0.178

[a,b]Means in the same column within the same variable with different superscript differ significantly ($P \leq 0.05$).

expression in hypothalamus of day-old chicks (Figures 3B–3D) as compared to GREEN and DARK incubation treatments ($P \leq 0.05$).

Prelay and Laying Period

Whole blood 5-HT hormone levels measured at 14 and 24 wk did not differ with the lighted incubation (Table 3). However, a significant I × B interaction indicated reduced 5-HT in the GREEN group (66.73 ± 3.55 nmol/mL) as compared to both WHITE and DARK (87.82 ± 3.55 and 79.34 ± 3.55 nmol/mL, respectively) in the second batch while there was no difference among the incubation groups in the first batch (data not presented in the tables). Incubation treatment significantly affected 5-HT levels of hens at 40 wk of age ($P \leq 0.05$). Hens from the GREEN group had lower whole blood 5-HT concentrations than those from the DARK ($P \leq 0.05$). Hens from WHITE incubation had similar serotonin levels to both DARK and GREEN incubation groups. No interaction effect was observed. Basal CORT levels of hens were not affected by treatment, batch and their interaction at 14 and 40 wk of ages (Table 3).

Except at wk 40, expression of photoreceptor genes in the hypothalamus did not vary with the treatments (Figure 2). At 40 wk of age, VA opsin expression differed with incubation treatment being lower in hypothalamus of hens from the WHITE group ($P \leq 0.05$). The batch effect was significant only for red opsin expression at the

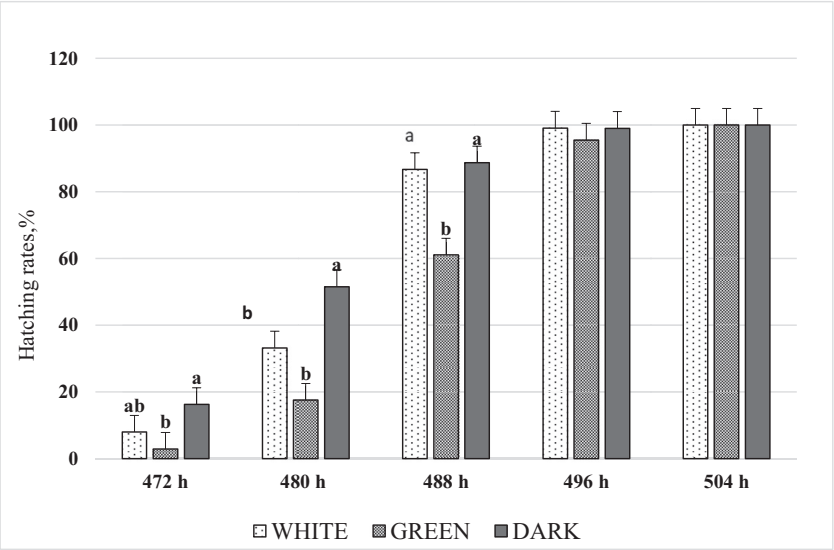

Figure 1. Distribution of accumulated hatching rates for incubation treatments at different time points. [a,b] Means in the same hour with different superscript differ significantly ($P \leq 0.05$).

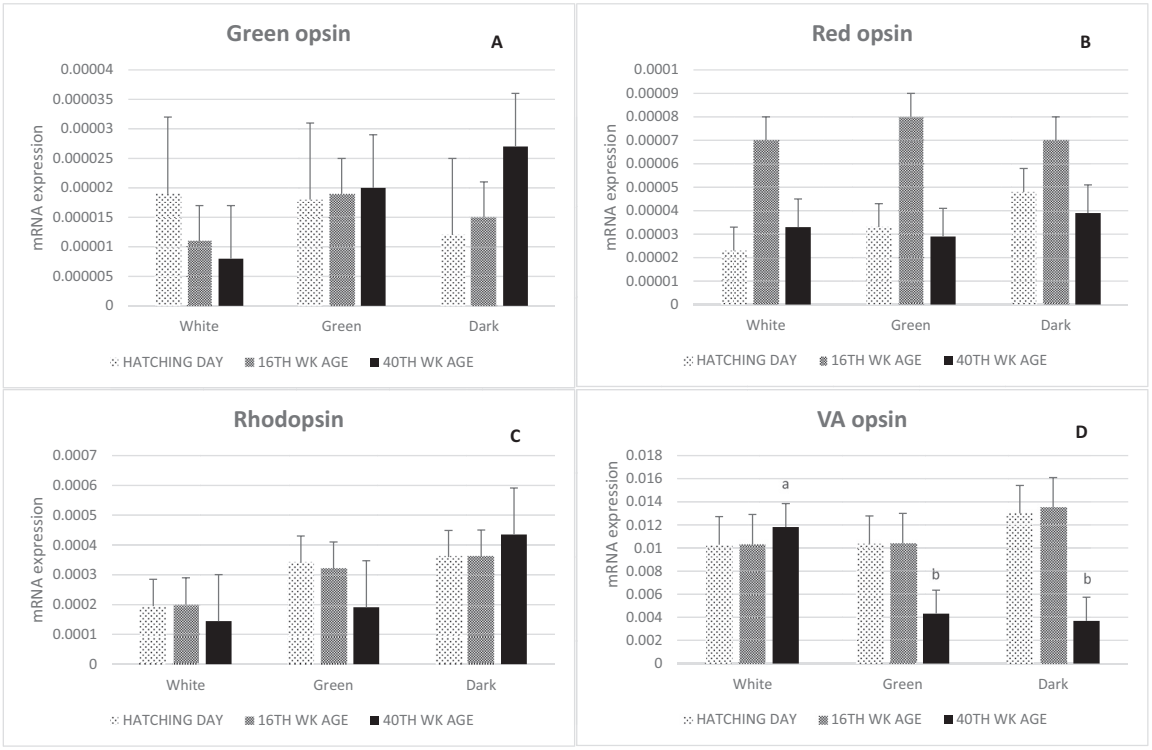

Figure 2. Effects of WHITE, GREEN, or DARK incubation on mRNA expression of Green opsin (A), Red opsin (B), Rhodopsin (C), and VA opsin (D) at the age of hatching, 16 wk and 40 wk. [a,b]: Different superscripts indicate significant differences between incubation groups at the age of 40 wk.

age of 16 wk and was higher in the second batch. No interaction effect was observed for photo pigments expression at any of the ages.

No significant difference between treatments was observed in the expression levels of serotonin and stress system-related genes at 16 wk; however, the batch effect was significant for CRH expression being lower

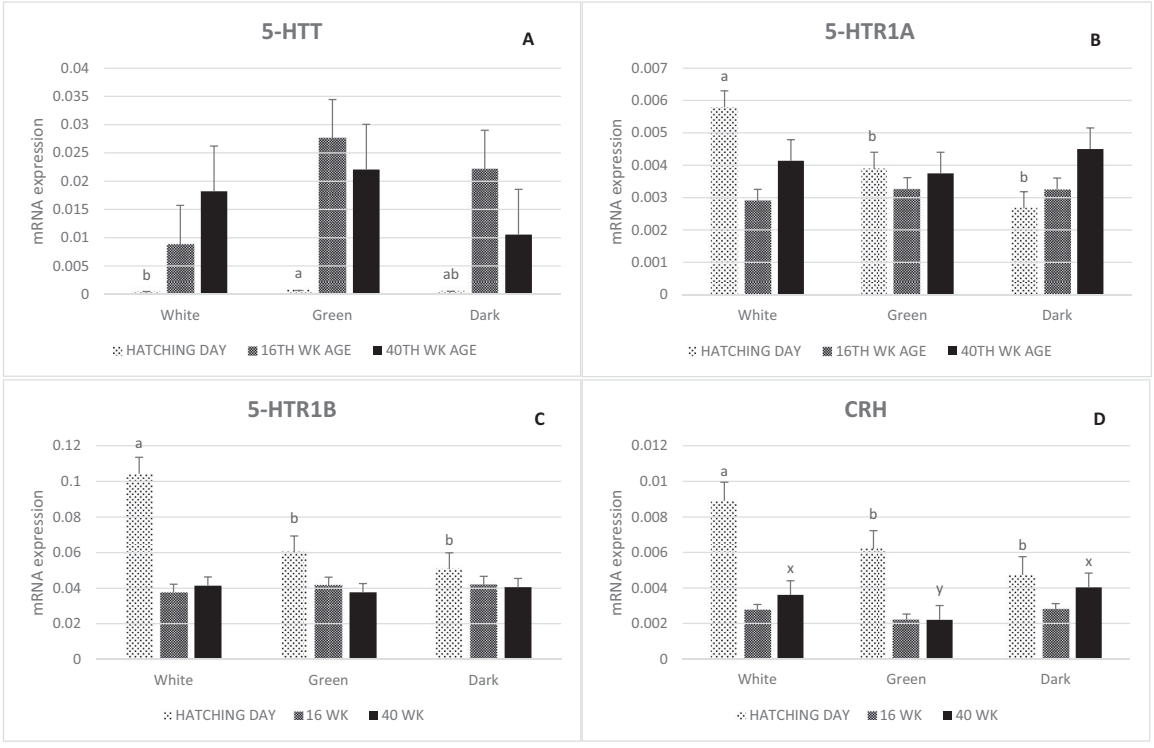

Figure 3. Effects of WHITE, GREEN, or DARK incubation on mRNA expression of 5-HTT (A), 5-HTR1A (B), 5-HTR1B (C), and CRH (D) at the ages of the hatching, 16 wk and 40 wk. [a,b]: Different superscripts indicate significant differences between incubation groups at the age of hatching; [x, y]: different superscripts indicate significant differences between incubation groups at the age of 40 wk.

Table 3. Effect of incubation treatment (white light, green light or dark incubation), and batch on corticosterone (CORT) and Serotonin hormone levels of layer chickens at different ages.

Treatments	Serotonin, nmol/ml			CORT, ng/mL	
	Wk 14	Wk 24	Wk 40	Wk 14	Wk 40
Incubation (I)					
White	65.91	79.29	58.92[ab]	4.50	6.35
Green	64.47	74.60	46.05[b]	5.32	7.95
Dark	70.62	74.70	67.04[a]	4.75	10.80
SEM	2.06	2.51	4.30	0.94	0.99
Batch (B)					
1	65.48	74.43	56.93	5.02	8.72
2	69.52	77.96	55.70	4.69	8.01
SEM	1.68	2.05	3.49	0.77	0.81
Statistical analysis	*P* values (significance)				
I	0.113	0.441	0.016	0.864	0.194
BB	0.038	0.309	0. 965	0.6	0.376
I × B	0.689	0.001	0.67	0.955	0.062
Pen	0.045	0.001	0.518	0.949	0.142

[a,b]Means in the same column within the same variable with different superscript differ significantly ($P < 0.05$)

in the second batch. CRH was the only gene which was influenced by incubation treatment at 40 wk of age. Hens from GREEN incubation had lower expression levels (Figure 3D) than those from WHITE and DARK incubation ($P \leq 0.05$). Significantly lower expression levels of CRH in the hypothalamus of hens from the GREEN group was observed in the second batch (0.000740±) as compared to the first batch (0.003682 ± 0.001100), as indicated by a significant I × B interaction effect (P ≤ 0.05) at 40 wk of age (data not tabulated).

Observations from the rearing period (1, 5, 8 wk) did not show any significant effect of incubation lighting on gentle, severe and aggressive pecking of birds directed to pen mates (Table 4). The age effect was significant for all types of pecking behavior ($P \leq 0.05$) and pecking frequency increased with the increase in age. The session effect was significant only for gentle feather pecking being highest at the noon observation which was different from the evening. However, morning session was intermediate and similar to both sessions. The session effect was not significant for severe pecking, but a

Table 4. Effect of incubation treatment (white light, green light, or dark incubation), week (age), and session on pecking behaviors directed to pen mates (peck/bird/10 min) during the rearing period.

Treatments	Gentle peck	Severe peck	Aggressive peck
Incubation			
White	0.376	0.050	0.012
Green	0.369	0.034	0.021
Control	0.279	0.038	0.031
SEM	0.085	0.016	0.010
Week (W)			
1	0.566[a]	0.073[a]	0.046[a]
5	0.134[b]	0.007[b]	0.000[b]
8	0.324[b]	0.042[b]	0.018[b]
SEM	0.085	0.016	0.010
Session (S)			
Morning	0.382[ab]	0.052	0.023
Noon	0.428[a]	0.035	0.018
Evening	0.214[b]	0.036	0.023
SEM	0.085	0.016	0.010
Statistical analysis	*P*-value (Significance)		
I	0.517	0.714	0.484
W	<0.001	<0.001	<.001
S	0.046	0.434	0.464
W × I	0.376	0.210	0.196
W × S	0.068	0.048	0.344
I × S	0.784	0.358	0.133
W × I × S	0.581	0.601	0.556

[a,b]Means in the same column within the same variable with different superscript differ significantly ($P < 0.05$).

significant week × session interaction was found, with significantly higher severe feather pecking frequencies in the morning session as compared to the noon and evening sessions at the 8th wk ($P \leq 0.05$). At the first and 5th wk, severe feather pecking did not differ between the sessions (Figure 4).

Observations from 16 wk onward revealed that gentle feather pecking, severe feather pecking, and aggressive pecking were all affected by incubation treatment ($P \leq 0.05$) and the incubation × week interaction ($P \leq 0.05$). Severe feather pecking and aggressive pecking were affected by week ($P \leq 0.05$) while session effect was significant for gentle and severe pecking ($P \leq 0.05$, Table 5). Gentle, severe, and aggressive pecking frequencies in different weeks for the different treatments are presented in Figure 5.

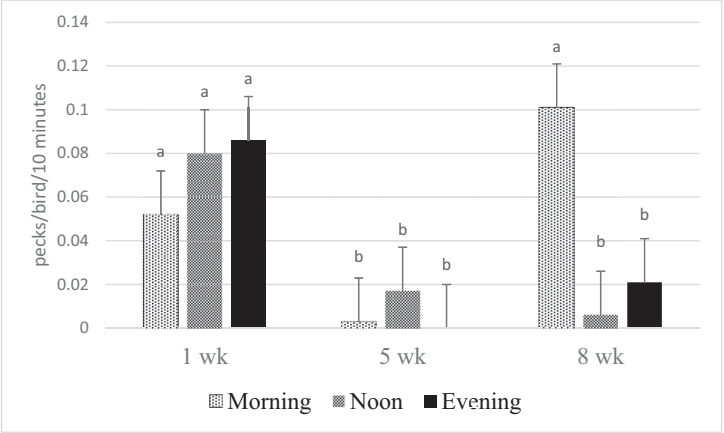

Figure 4. Severe feather pecking in the morning, noon, and evening sessions at different ages. [a,b]: Means with different superscript differ significantly ($P \leq 0.05$).

Table 5. Effect of incubation treatment (white light, green light, or dark incubation), week (age), and session on pecking behaviors directed to pen mates (peck/bird/10 min) during the laying period.

Treatments	Gentle peck	Sever peck	Aggressive peck
Incubation (I)			
White	0.236[ab]	0.424[a]	0.340[a]
Green	0.323[a]	0.224[b]	0.147[b]
Control	0.169[b]	0.359[a]	0.273[a]
SEM	0.025	0.045	0.045
Week (W)			
16	0.251[a]	0.110[b]	0.025[c]
24	0.266[a]	0.445[a]	0.303[b]
32	0.210[b]	0.452[a]	0.432[a]
SEM	0.025	0.033	0.033
Session (S)			
Morning	0.204[b]	0.341[a]	0.270
Noon	0.415[a]	0.394[a]	0.250
Evening	0.108[c]	0.272[b]	0.239
SEM	0.025	0.033	0.033
Statistical analysis		*P*-value (Significance)	
I	0.004	0.005	0.005
W	0.155	<0.001	<0.001
Session (S)	<0.001	<0.001	0.190
W × I	<0.001	0.003	0.005
W × S	0.313	0.011	0.196
I × S	0.759	0.578	0.228
W × I × S	0.034	0.405	0.907

[a,b,c]Means in the same column within the same variable with different superscript differ significantly ($P < 0.05$).

A significant effect of W × I × S interaction was also observed for gentle pecking (Table 5) and frequencies of gentle pecking in different weeks and sessions for the different treatments are presented in Figure 6 ($P \leq 0.05$). At 16 wk of age, the WHITE group had significantly higher numbers of gentle pecking than the GREEN and DARK groups at noon. However, hens from GREEN incubation had the highest gentle pecking numbers at noon session as compared to both WHITE and DARK at 24 and 32 wk. Evening observations did not show any significant effect on incubation groups at any age.

As it can be seen in Figure 5, for severe and aggressive pecking no difference was observed between the incubation groups at the age of 16 wk. However, GREEN hens had significantly lower severe and aggressive pecking frequencies than both WHITE and DARK groups at 24 wk of age. At the age of 32 wk, the number of severe pecks was higher in the WHITE group than in the DARK group, with the GREEN group intermediate. Aggressive pecking frequency at 32 wk of age did not differ between the groups (Figure 5).

Severe feather pecking in the morning and noon sessions was higher than the evening observation. A significant effect of W × S interaction was observed for severe pecking ($P \leq 0.05$) and presented in Figure 7. There was no difference among the sessions at 16 wk. However, significantly higher severe pecking was observed at the noon session as compared with the morning and evening sessions at 24 wk. At 32 wk, highest severe pecking frequencies were observed in the morning session which was significantly different from the evening but similar to the noon session (Figure 7).

Lighted incubation had a significant effect on feather, comb and vent scoring of hens at 40 wK of age (Table 6). There was no hen with a perfect feather score of "0" in any group. A hundred percent of hens in the DARK group had score 2 (high feather damage) while the GREEN group had a lower (94.52%) percentage of score 2 ($P \leq 0.05$). The number of hens with score 2 (≥3 pecking wounds) was the lowest in the GREEN group while the WHITE and DARK groups had similar percentages. For vent pecking wounds chi square analyses revealed that a lower number of birds in WHITE group had pecking wounds at vent area compared to GREEN and DARK groups ($P \leq 0.05$).

Correlations Between Gene Expression Levels

Table 7 presents the correlations among the expression levels of genes investigated in the study. Significant positive correlations were observed between 5-HTR1A and 5HTR1B, between 5-HTR1B and red opsin, and between CRH and red opsin, consistently across the

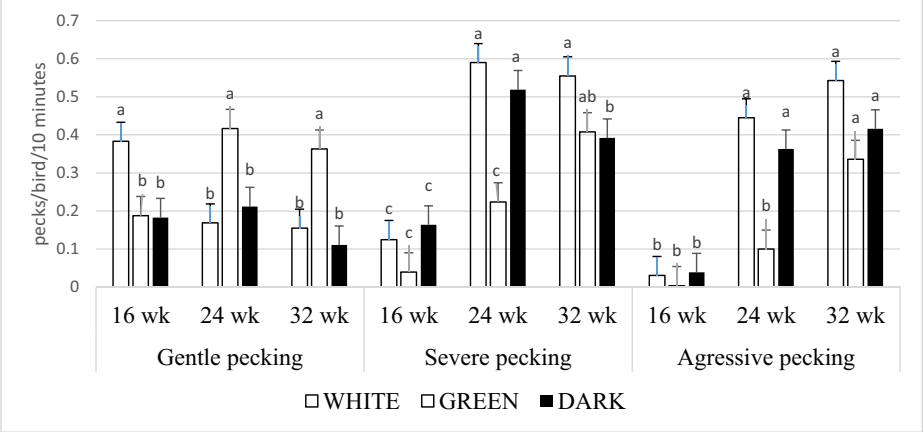

Figure 5. Gentle, severe and aggressive pecking (peck/bird/10 min) in hens exposed to different incubation treatment with white light (WHITE), green light (GREEN), or dark incubation (DARK) at different ages (week × incubation lighting interaction). [a,b, c]: Within each pecking type, means for incubation groups with different superscript differ significantly ($P \leq 0.05$).

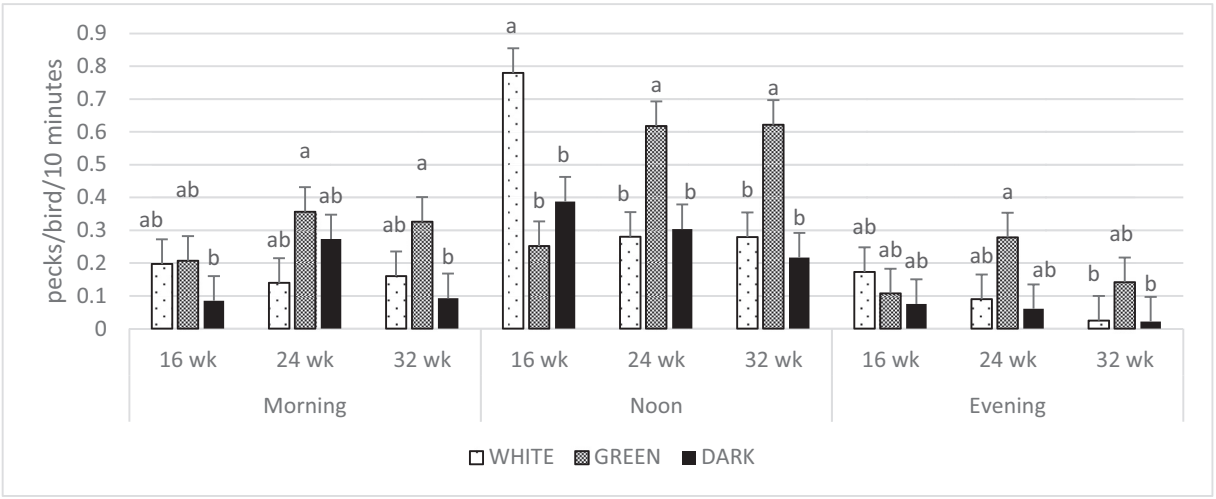

Figure 6. Gentle pecking (peck/bird/10 min) in hens exposed to different incubation treatments with white light (WHITE), green light (GREEN), or dark incubation (DARK) at different sessions and ages (week × incubation lighting × session interaction). [a,b]: Means for incubation groups with different superscript within the same session differ significantly ($P \leq 0.05$).

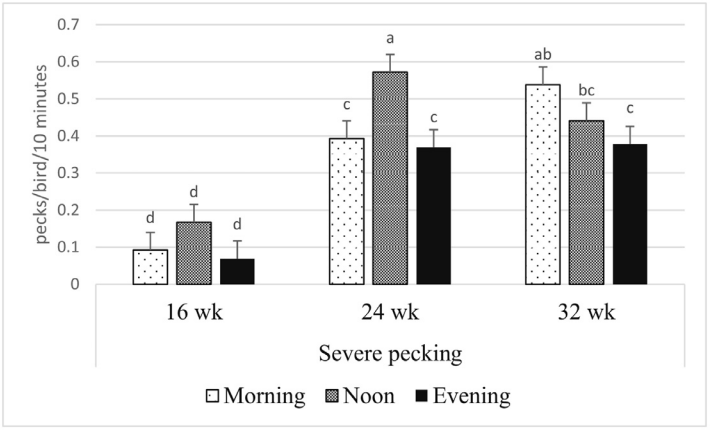

Figure 7. The effect of week × session interaction on severe pecking during the laying period. [a,b, c]: Means for sessions with different superscript at different ages differ significantly ($P \leq 0.05$).

Table 6. Effect of incubation lighting on feather and comb scores of 40 weeks old laying hens (% of hens in each score).

	Feather score[1]		Comb score[1]			Vent score[2]	
			%				
Score	1	2	0	1	2	0	1
Treatment							
White	1.37	98.63	12.33	21.92	65.75	80.82	19.18
Green	5.48	94.52	31.51	28.77	39.73	65.75	34.15
Dark	0.00	100.00	17.86	15.48	66.67	60.71	39.29
Chi square	6.600		16.460			8.143	
Significance (P value)	0.037		0.003			0.017	

[1]Three scales of scoring were used for both feather (0: no damage or a few feathers lacking, 1: moderate feather damage and one or more featherless area less than 5 cm in diameter, 2: one or more body parts have featherless area larger than 5 cm in diameter) and comb (0: no pecking wound, 1: less than 3 wounds, and 2: ≥ 3 wounds). However, for feather score, there was not any hen scored with 0 (no feather damage).

[2]Apart from feather loss, one-zero scaling was used for vent wounds.

Table 7. Correlations between hypothalamic expression levels of different genes in treatment groups (white light, green light or dark incubation).

	Correlation coefficient, r		
Related genes	White	Green	Dark
5-HTR1A - 5HTR1B	0.83**	0.39*	0.40*
5-HTR1A - Red opsin	0.67**	0.19	0.06
5-HTR1B - Red opsin	0.69**	0.37*	0.44*
5-HTT - 5-HTR1B	−0.67**	−0.50**	0.23
5-HTT - 5-HTR1A	−0.52**	−0.20	0.46*
5-HTT - Red opsin	−0.77**	−0.31	−0.55**
CRH - Red opsin	0.44*	0.45*	0.45*
CRH - Green opsin	0.53**	0.31	0.15
CRH - 5-HTR1A	0.49*	0.30	0.31
CRH - 5-HTR1B	0.42*	0.40*	0.35
CRH - 5-HTT	−0.52**	−0.43*	−0.34
Rhodopsin -Green opsin	0.14	0.31	0.42*

*$P \leq 0.05$.
**$P \leq 0.01$.

incubation groups ($P \leq 0.05$). However, there was a significant negative correlation between the CRH and 5-HTT expression within each of the incubation groups. Correlations between 5-HTR1A and red opsin; CRH and green opsin; CRH and 5-HTR1A were found to be significant only in WHITE ($P \leq 0.05$).

In both of the lighted incubation groups, significant negative correlations between 5-HTT and 5-HTR1B, and positive correlations between CRH and 5-HTR1B were observed ($P \leq 0.05$). A significant and negative correlation was observed between 5-HTT and red opsin in the WHITE and DARK groups ($P \leq 0.05$). The correlation between 5-HTT and 5-HTR1A was significant in the WHITE and DARK groups ($P \leq 0.05$). However, direction of the relation was negative in the WHITE while it was positive in the DARK group. The only significant correlation between rhodopsin and green opsin was observed in the DARK incubation group ($P \leq 0.05$).

DISCUSSION

This study aimed to investigate the effect of a 16L:8D lighting schedule using either GREEN or WHITE light during incubation on hypothalamic expressions of genes related with photoreception (VAopsin, rhodopsin, green opsin, red opsin), serotonin (5-HTT, 5-HTR1A, 5-HTR1B), and stress (CRH) systems in layer chickens and possible links to blood hormone levels (melatonin, CORT, and serotonin), feather pecking behavior, and hatching performance.

At the Day of the Hatch

There have been contradictory reports in the literature regarding the effect of lighted incubation on hatching performance. Some of the studies reported no effect of lighted incubation on hatching performance of layer eggs (Huth and Archer, 2015; Hannah et al. 2020) but improved chick quality (Huth and Archer, 2015) and synchronized hatching time (Hannah et al. 2020). Wang et al. (2020) observed no change in hatchability from fertile eggs, but shorter hatching time in different layer strains with green lighted incubation as compared with the dark incubation. In our study, hatching performance was not affected significantly by incubation lighting even though a numerical improvement in hatchability was observed in the GREEN group. The only statistically significant effect of GREEN and WHITE incubation in this study was lower rates of hatching at the earlier hours of the hatch window as compared with the DARK incubation. Although the total hatch time was not shortened in lighted groups, these lower accumulated hatching rates at the beginning of the hatch window, namely from 472 to 488 h, may have high value for commercial settings. The differences obtained for chick weight and length between batches could be due to the difference of 4 wk in age of breeder stock.

We found significant alterations in the hypothalamic expression of serotonergic genes, namely 5-HTR1A, 5-HTR1B, and 5-HTT, at the hatching day, due to the lighting and wavelength of light applied to embryos during incubation. Neural 5-HT has key roles in stress sensitivity (Jiang et al., 2009) locomotor activity, anxiety-related behaviors and emotional states. One of the most pronounced results in our study, the GREEN group had higher expression of 5-HTT on the day of hatch than the WHITE group, with levels of the DARK group in between. It has been reported that increased expression of 5-HTT in chickens was associated with lower fear levels in adults (Krause et al., 2017) and also in newly hatched chicks (Phi van et al., 2018). Along with the 5-HTT, 5-HTR1A, and 5-HTR1B receptors are suggested to modulate the individual differences in aggressive behavior (Popova, 2006, 2008). Dennis et al. (2013) reported that modification of the serotonergic system during embryonic life had long lasting effects on birds' physiology and behavior. They found that embryonic 5−HT injection significantly reduced aggressive behaviors at 9 and 18 wk of ages as compared to 5-HTR1A agonist (8-OH-DPAT) injection or saline control. They further concluded that lower aggression at 9 and 18 wk in birds exposed to a high level of 5-HT during the embryonal stage was associated with reduced dopamine and increased serotonin concentrations in the brain and increased developmental instability as a measure of stress along with the increase in fearfulness. Our results showed that GREEN and DARK incubation groups had significantly lower 5-HTR1A and 5-HTR1B expression as compared with the WHITE group. Moreover, these findings can be accounted for more beneficial effects of GREEN incubation light on feather pecking. We found that lighted incubation affected stress and serotonin system-related hypothalamic gene expression in newly hatched chicks, which may have long lasting consequences for physiology and behavior of chickens including aggressiveness and feather pecking. Increased CRH expression in the hypothalamus of the WHITE group at hatch might be related with the increased stimulation of HPA axis of newly hatched chicks. Stimulation of HPA axis by a stressor results in CRH secretion from the hypothalamus along with arginine vasotocin (**AVT**), followed by secretion of ACTH in the pituitary which ultimately results in the release of CORT from adrenals (Blas, 2015). Indeed, intra-cerebroventicular injection of CRH increased plasma CORT levels and had significant impact on behavior of day-old chicks which was indicated by increased spontaneous activity and distress vocalization (Zhang et al., 2003). Injection of 5-HT together with CRH has been found to attenuate CRH-induced behavioral changes in day old chicks (Zhang et al., 2004). We did not detect any difference among the incubation groups for day-old chick's blood CORT levels, which agrees with our previous study (Özkan et al., 2012) and with the report of van der Pol et al. (2019a). There was not a clear day/night rhythm of melatonin on the hatching day, contrary to the rhythmic changes in melatonin hormone reported in earlier lighted incubation studies (Zeman et al., 1992, 1999; Özkan et al., 2012; Archer and Mench, 2014). However, Van

der Pol et al. (2019b) also reported no significant effect of lighted incubation on blood melatonin of embryos at the last stage of incubation (age of E19-E20). Differences among the reports might be related with the difference in lines used, layers vs. broilers, and also differences in eggshell pigmentation of these lines which affect penetration of light into the embryo level (Huth and Archer, 2015).

At the day of the hatch, we did not find any significant change in hypothalamic expression levels of photopigments. Rozenboim et al. (2013) found changes in retinal expression levels of red and green opsins in lighted incubation groups as compared with dark incubation. Differences between expression levels of opsins in retina and brain would be expected because the light has to penetrate into brain passing through the skin and skull. Therefore, the same light intensity and wavelength may differentially affect photopigments in hypothalamus and in retina.

Prelay and Laying Period

With an exception of VA opsin at 40 wk of age, in ovo lighting did not result in a significant alteration in expression of opsin photopigments (green, red, rhodopsin, VA opsin) in the hypothalamus of pullets and layers. We found significantly higher expression level of VA opsin in WHITE hens at 40 wk as compared to GREEN and DARK and VA opsin is suggested to have a prime role in controlling reproduction in birds (Garcia-Fernadez et al., 2015; Perez et al., 2019). In a recent study, Hanlon et al. (2021) investigated the changes in VA opsin expression in hypothalamus of different layer genotypes and reported that expression level was stabile within genotypes through the ages from 12 to 100 wk of age. The author's conclusion was that this stabile expression of VA opsin might be necessary to sustain photosensitivity, thus egg production and to prevent photorefractoriness. Egg production did not differ with the incubation lighting in this experiment and hen day egg production was 93.04, 90.89, and 92.71% for WHITE, GREEN, and DARK groups, respectively (Dayıoğlu, 2018). However, we terminated the study at 40 wk of age and it is not possible to conclude whether a difference in VA opsin expression might have accounted for further differences in egg production.

Accumulated information suggests the importance of neural 5-HT in modulation of locomotor activity, anxiety related behaviors, emotional states and stress related disorders in vertebrates (Baxter, 2001; Bouwknecht et al., 2001). Indeed, Wysocki et al. (2013) found that 5-HTR1B gene expression was significantly higher in the brain of chickens selected for high feather pecking. Several studies show that the serotonergic system is associated with feather pecking behavior in laying hens. Increased feather pecking in laying hens has been reported due to experimentally induced low serotonin turnover using a somatodentritic 5-HTR1A autoreceptor agonist (5-15535) which acts as postsynaptic 5-HTR1B receptor antagonist (Van Hierden et al., 2004). According to their data, the authors' conclusion was that control of feather pecking involves different postsynaptic 5-HT receptors other than those controlling aggressive behavior. Dennis et al. (2008) further reported that 5-HTR1A and 5-HTR1B antagonist injection modulated gentle, severe, and aggressive pecking behaviors in laying hens. However, responses of hens to the injections varied with the genetic background. A line selected for low group productivity and survivability increased all types of pecking with 5-HTR1A antagonist injection while in commercial line increased aggressive pecking only. Both of these lines did not respond to 5-HTR1B antagonist injection, but the line selected for high group productivity and survivability increased gentle, severe, and aggressive pecking.

In our study WHITE chicks had higher hypothalamic expression of CRH, 5-HTR1A, and 5-HTR1B compared with both GREEN and DARK at hatch. Ahmed et al. (2014) reported that there was a significant increase in aggressive behaviors, namely grabbing and pecking directed to other birds' head and neck, induced by high dose in ovo CORT injection. They found that these behavioral changes were also associated with the increased expression of serotonergic genes, namely 5-HTR1A and monoamine oxidase (**MAO**). This may partly explain the overall highest frequencies of severe and aggressive pecking in WHITE hens, which also had increased expression levels of 5-HTR1A at the day of the hatch in our study. In line with our findings, increased gentle feather pecking in layer chicks (Riedstra and Groothuis, 2004) and in broiler chicks (Dayıoğlu and Özkan, 2012) exposed to white light during the incubation have been reported in other studies. However, on the day of hatch, chicks from GREEN incubation had higher 5-HTT expression than WHITE, which was similar to DARK. This increased expression of 5-HTT in day-old chicks might be associated with the lower fear levels (Krause et al., 2017; Phi van et al., 2018) and support the suggested link to reduced feather pecking in the GREEN group. Furthermore, GREEN hens performed lower number of severe and aggressive pecking, but more gentle pecking as compared to WHITE and DARK after egg production started. This reduced number of severe feather pecking and aggressive pecking (to head and neck) was accompanied by lower percentages of feather and comb damage scores in GREEN hens at 40 wk of age.

Reduced expression levels of CRH in hypothalamus of birds from GREEN incubation at hatch and at 40 wk of age were not accompanied by a significant change in blood CORT levels in this group. This possibly was due to negative feedback control of HPA axis. Indeed, Ahmed et al. (2014) reported that high dose in ovo CORT injection to the embryos resulted in downregulated hypothalamic CRH and AVT expressions as compared to a control group, indicating negative feedback control.

Archer (2017) reported a higher 5-HT but lower CORT concentration in the blood of 42-day-old broiler

chickens from lighted incubation regardless of the light color including white, green and red. However, there is a lack of literature on blood hormone levels of layers subjected to light during the incubation. In this study, blood CORT levels measured at pullet and laying stages were not different between the groups. However, whole blood serotonin level of hens from GREEN group was significantly lower than the others at 40 wk of age.

It seems that although hens from GREEN incubation had a lower tendency to display severe and aggressive pecking than WHITE and DARK birds at the laying period; they also tended to perform more vent pecking. Unfortunately during the feather pecking observations, we did not record the body part where the peck was directed to. Therefore, we cannot show data, but we may assume that a higher number of pecking wounds at the vent area is associated with a higher pecking tendency toward this area. Vent pecking has a different motivation than other pecks (Savory, 1995) and more related to egg production and environmental conditions which may predispose hens to vent pecking (Lambton et al., 2015). Therefore, GREEN incubation light reduces severe feather pecking and aggressive pecking but not vent pecking.

Correlations

There has been no research investigating correlations among the hypothalamic opsins consisted by photoreceptor cells and serotonin and stress system related gene expressions. Our results are the first data reporting correlations among the expression levels of genes in relation to serotonin, CRH, and photoreceptor pigments in the hypothalamus of chickens those subjected to either photoperiodic (16L:8D) lighting schedule (WHITE and GREEN) or darkness (DARK) during the incubation period. Regardless of the incubation background, there were positive correlations between 5-HTR1A and 5-HTR1B, 5-HTR1B and red opsin, CRH and red opsins; while a negative correlation between CRH and 5-HTT was common for all groups indicating a clear relationship among the mentioned systems. Red opsin was positively correlated with both 5-HTR1B and CRH. These findings provide further evidence for the relationship among the light, serotonin and stress systems. Photostimulation of broiler breeders with red light resulted in increased expression of red opsin in the hypothalamus and was associated with improved reproduction (Mobarkey et al., 2010). However, we used white and green light during the incubation, but only the white light in the post-hatch period, and could not find a significant increase in red hypothalamic opsin expression at any age. Negative correlations between CRH and 5-HTT also gave indication of relations between stress and serotonin systems (Summers and Winberg, 2006). If decreased CRH expression in the hypothalamus may account for lowered activity of HPA axis, an increase in 5-HTT in the hypothalamus may also account for reduced extracellular 5-HT. We observed significantly higher 5-HTT expression in GREEN chicks at hatch with a significantly lower expression of CRH gene. However, the only observed effect of incubation treatment on blood 5-HT was at 40 wk of age, with lower levels for the GREEN group than for the other 2 groups. There was no effect of incubation on 5-HT at earlier ages in this experiment namely, 14 and 24 wk. Reduced 5-HT turnover in the brain has been reported in high feather pecking lines at early ages by 56 d (van Hierden et al., 2002, 2004).

Our results provide preliminary evidence that lighted incubation modulates the pecking tendencies of laying hens which was indicated by reduced severe feather pecking and aggressive behavior in birds exposed to GREEN incubation light, probably through the observed changes in hypothalamic expression of genes related to 5-HT and stress systems in this study.

In our study, WHITE chicks had higher hypothalamic expression of CRH, 5-HTR1A, and 5-HTR1B compared with both GREEN and DARK incubation at hatching day. However, 5-HTT expression in GREEN was higher than WHITE which was similar to DARK. This may partly explain lower frequencies for severe and aggressive pecks in GREEN hens. GREEN incubation light reduces severe feather pecking and aggressive pecking but not vent pecking. Regarding photoreceptor genes, lighted incubation only affected hypothalamic VA opsin expression with no evidence for alteration in egg production between groups by 40 wk of age. However, because limited information is available in the area (Perez et al., 2019) any data regarding the role of extraretinal photopigments in birds' physiology and behavior would help to improve our understanding on photoperiodic control of these functions by extraretinal photoreceptors.

Future studies regarding the effect of lighted incubation on modulation of feather pecking in laying hens would incorporate pharmacological and molecular research to uncover the underlying mechanisms regarding serotonin and stress systems together with dopamine (de Haas and van der Eijk, 2018; de Haas et al., 2021).

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DISCLOSURES

The authors declare no conflicts of interest.

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