

PHYSIOLOGY AND REPRODUCTION

Targeted differential monochromatic lighting improves broiler breeder reproductive performance

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ABSTRACT Light perception in birds is composed of the retina and extraretinal sites, located in the brain. Previous studies indicate that selective photostimulation of the eye decreased reproductive performance, whereas extraretinal photostimulation increases it. Differential photostimulation of the retina and extraretinal sites is based on the retina's sensitivity to green wavelengths and on the red wavelengths' ability to penetrate body tissues.

We previously found that short-day exposure to green light within a long-day exposure to red light increases reproductive activity in female turkeys and broiler breeder hens. Furthermore, in a study conducted recently in our laboratory, we found that blue light repressed expression of green light receptor in the retina, which can further enhance reproduction activity in broiler breeders. Here, we examined the "brain activate/eye deactivate" hypothesis on gonadal axis activity and reproductive performance in a broiler breeder flock.

Broiler breeder hens and roosters (ROSS 308) were divided into 5 light-treatment groups (controlled rooms

with light-emitting diodes [LED] lamps): warm white (control), long-day (14 h) red (630 nm) and short-day (6 h) green (514 nm) (red-green), long-day green and short-day red (green-red), long-day red and short-day blue (456 nm) (red-blue), and long-day blue and short-day red (blue-red). Birds were reared from 20 to 55 wk of age. Eggs were collected daily. Weekly egg production calculated. All eggs were incubated for fertility and hatchability examination. Blood was drawn monthly for plasma analysis. At 35 wk of age (after peak production) and 55 wk of age (end of the experiment), 10 hens from each treatment group were euthanized, and selected tissues and glands were taken for gene expression trials.

Providing long-day red light to extraretinal photoreceptors while maintaining retinal photoreceptors on short day with blue or green light significantly improved reproductive activities, manifested by elevated egg production and gonadal axis activity compared with Controls and primary breeder recommendations. Long-day green light reduced reproductive performances. We suggest that targeted photostimulation enhances reproductive and gonadal axis activities in broiler breeders.

Key words: photostimulation, extraretinal photoreceptors, broiler breeder, reproduction, gonadotropin-releasing hormone, gonadotropin-inhibitory hormone

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INTRODUCTION

The only light source for chickens in environmentally controlled houses is an artificial one (Rozenboim et al., 2013). Taking into consideration the fundamental role

of light in the reproduction of domestic fowl (*Gallus gallus domesticus*), light quality (photoperiod, intensity, and spectrum) is an important environmental factor.

In birds, light perception occurs at 2 sites: in the retina of the eye and in different places in the brain (Menaker and Underwood, 1976), including the pineal gland, pituitary, and hypothalamus (Scanes, 2014). Photoreceptors found at those sites are known as extraretinal photoreceptors (ERPR). In mammals which lack ERPR, circadian rhythm is mediated by the retinal photoreceptors (Tosini et al., 2016). Bird eyes, however, are not required for the circadian rhythm and the circannual cycle, as

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shown by the response to photostimulation seen in enucleated ducks (Benoit and Assenmacher, 1954). Covering the head so that light does not penetrate the skull, on the other hand, results in photorefractoriness (Benoit, 1964).

All photoreceptors contain opsins—protein complexes that binds to vitamin-A—that isomerizes in response to light (Bownds, 1967; Hart, 2001). Isomerization allows the opsin molecule to bind a protein involved in signal transfer to the brain—resulting in a biochemical cascade that changes neurotransmitter release from the photoreceptor (Applebury and Hargrave, 1986).

The neuroendocrine response to photostimulation is expressed by release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. Subsequent secretion of gonadotropins from the pituitary gland into blood will initiate gonadal recrudescence (Chaiseha and El Halawani, 2005).

Differential photostimulation, that is, stimulating the ERPR while maintaining the retina under nonphotostimulatory conditions alters reproductive behavior and egg production in domestic fowl (Mobarkey et al., 2010) and turkey hens (unpublished data). It has been shown that the longer wavelengths of the red band of the light spectrum have the ability to penetrate the skull and tissues and stimulate the ERPR—responsible for the activation of the reproductive system (Oishi and Lauber, 1973; Benoit, 1978; Mobarkey et al., 2009)—whereas the shorter wavelengths, such as the green and yellow, are mainly perceived by the retina—stimulating the secretion of gonadotropin-inhibitory hormone (GnIH)—and thereby inhibiting reproduction (Benoit and Assenmacher, 1966; Mobarkey et al., 2009; Bédécarrats, 2015). Selective photostimulation of different photoreceptor sites can be used as an environmental tool for acceleration of reproductive activities in domestic birds.

Two connected endocrine axes that are involved in avian reproduction are associated with photostimulation: the gonadotropic axis and the lactotropic/serotonergic axis. Photostimulation of the gonadotropic axis have been well characterized (Sharp et al., 1998; Saldanha et al., 2001), whereas the exact mechanisms through which photic cues are transduced to neuroendocrine effector neurons remain unknown. There is still much to discover about the connection between the brain ERPR and the reproductive axis. Previous studies have shown that brain photoreceptors communicate directly with the GnRH neurons that stimulate the activation of reproduction (Saldanha et al., 2001; Scanes, 2014). Another possible connection is vasoactive intestinal peptide (VIP) cells, which colocalize with all opsin-expressing cells in birds. Within the opsin system, VIP could potentially regulate reproduction through synaptic interactions all along the trajectory of its axons through the lateral septum and hypothalamus (Hof et al., 1991; Saldanha et al., 2001).

The objective of this study was to examine the effect of retinal photostimulation by green or blue light (for 14 h) while maintaining the ERPR in a nonphotostimulatory

condition by red light (for 6 h), and the effect of ERPR photostimulation by red light (for 14 h) while maintaining the retinal photoreceptors in a nonphotostimulatory condition by green or blue light (for 6 h) on reproductive factors in a model broiler breeder flock. Egg production, egg fertility and hatchability, and other relevant factors are described.

MATERIALS AND METHODS

All procedures were approved by the Animal Care and Welfare Committee of The Hebrew University of Jerusalem on 13 September 2016. Research number AG-16-14904-1.

Experimental Animals

One hundred fifty Ross 308 broiler breeder hens and 15 broiler breeder roosters at 20 wk of age were divided into 5 equal weight groups ($n_F = 1.9 \pm 0.1$, $n_M = 3.39 \pm 0.1$) and housed in 5 light-controlled rooms ($n_F = 30$, $n_M = 3$). Rearing and management were in accordance with the Ross Parent Stock Management guide. In brief, birds were subjected to a restricted feeding program with daily administration of feed in increasing quantities. Water was provided *ad libitum*. The experimental facility was not temperature controlled; consequently, temperature depended on ambient conditions. The experiment conducted during the summer and temperatures were high and usually not lower than 30°C during the day. Each room was of 25 m² and was equipped with separate feeders for males and females, automatic water trough, and 2 manual nest boxes. Rooms were light proof, and all light was provided by light-emitting diodes (LED) lamps.

Light Treatments

The control group was illuminated with a full-light spectrum using warm white LED lamps (intensity of 0.1 W/m²; 20 lux). W/m² units are used to measure light intensity and are therefore more suitable for examining the system that provides skull-penetrating light (white and red). Lux units on the other hand measure brightness, thus used for the eye stimulating system (green and blue). Two rooms had 2 parallel lighting systems: red light (wavelength of 630 nm, intensity of 0.1 W/m²) and green light (514 nm, 20 lux). Two other rooms had also 2 parallel lighting systems: red light (630 nm, W/m²) and blue light (456 nm, 20 lux).

Light intensities were determined using a LI-COR light meter (Lincoln, NE). Light spectra were determined using a spectrometer (MK350S Handheld Spectrometer; UPRtek, Taiwan).

Before photostimulation, birds of all groups were kept under nonphotostimulatory conditions (8L:16D) similar to pullet farm light condition for 2 wk with only white light in the control group and both lighting systems in the other light-treatment groups. Photostimulation was initiated at 22 wk of age, in the control group by gradually increasing the white light to long day (LD,

14h) and in all other groups by gradually increasing the LD lighting system—red, blue, or green, in accordance with the treatment group—while maintaining the other lighting system in a short-day (SD, 8h) condition. For more details, please see [Table 1](#).

We split the lighting treatment into LD and SD to differentiate between retinal and ERPR. Because the retina is most sensitive to short wavelengths such as green and blue ([Benoit and Assenmacher, 1966](#); [Mobarkey et al., 2010, 2013](#)), we used green/blue SD at the beginning of the red LD to reduce retinal photostimulation. Conversely, in the Blue-Red and Green-Red groups, red SD was used to prevent photostimulation of brain ERPR.

Management and Physiological Measures

Eggs were collected daily and weekly, and the egg production rate was recorded. Once a week, all eggs were incubated. On day 10, incubated eggs were candled to determine percent fertility. Hatchability was recorded at hatch.

Blood Sampling

Heparinized blood samples were drawn from the brachial vein of 10 hens in each room once a month for determination of plasma steroids and prolactin (PRL) levels. Plasma samples obtained from each blood sample were stored at -20°C until assay. Steroids and PRL were assayed by competitive ELISA with the corresponding biotinylated tracer.

Hormone Analysis

Prolactin was assayed by competitive ELISA. Plates (MaxiSorp, Nunc-Immuno MicroWell; Sigma-Aldrich, Israel Ltd., Park Rabin, Rehovot, Israel) were coated with 0.1 mL per well of Goat anti-Rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted to a ratio of 1:2,000 in 0.05 mol potassium phosphate buffer pH 7.4 and incubated overnight at 4°C . Surfaces were blocked by addition of 0.1 mL of 0.4% casein in 0.15 mol PBS pH 7.2 containing 1 mmol EDTA and 0.02% thimerosal per well. After overnight incubation at 4°C , plates were washed 3 times in 0.03 mol PBS containing 0.05% Tween 20. The assay buffer was 0.15 mol PBS pH 7.2 containing 0.1% casein, 1 mmol EDTA, and 0.02% thimerosal. Volumes of 50 μL samples (10 μL plasma diluted in 40 of assay buffer) or standards containing 0.039–20 ng PRL (Dr. A. F. Parlow, National Hormone and Peptide Program; Harbor-UCLA R.E.I,

Torrance, CA) were pipetted into the wells. Biotinylated PRL was diluted to a ratio of 1:250,000, and 25 μL was added to each well. Rabbit anti-chicken PRL was diluted to a ratio of 1:250,000 (shows broad phylogenetic recognition of avian PRL, [Dr. John Proudman, USDA, Beltsville, MD]), and 25 μL was added to each well. The antibody used has been validated for chicken, with parallel curves (starting at 5 μL) achieved for chicken (I.R., unpublished data). The plates were incubated at 4°C overnight. Then plates were washed, and 0.1 mL of streptavidin-horseradish peroxidase (1:5000) was added. The plates were incubated 2 h at room temperature, and then, plates were washed. A volume of 0.1 mL ABTS reagent (0.04% 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and 0.015% H_2O_2 in 0.1 mol citrate phosphate buffer, pH 4) was added. After 30 min of incubation at room temperature, the color reaction was measured at 405 nm using a Tecan Sunrise ELISA reader (Tecan Group Ltd., Männedorf, Switzerland). The assay was conducted in duplicates; intra-assay coefficient of variation with pooled chicken plasma was 7% and inter-assay was not computed as all samples were analyzed in a single assay.

Plasma progesterone, estrogen, and testosterone were measured in a single ELISA as per a previously described method ([Nash et al., 2000](#)) using primary antibody and tracer dilutions of 1:5,000 and 1:50 for progesterone, 1:160,000 and 1:160 for estrogen, and 1:20,000 and 1:20 for testosterone, respectively. All samples were analyzed in duplicate, and for every other plate, a separate standard curve was determined. The range of the assay was 0.78 to 400 pg/mL, and the intra-assay CV was 5% for progesterone, estrogen, and testosterone.

Tissue Sampling

At 35 and 55 wk of age, 10 birds from each room were euthanized using CO_2 gas. Each bird was weighed, and the hypothalamus, pituitary, F1 follicle, and retinae were sampled. The hypothalamus was sampled after extracting the brain from the skull. The pituitary was sampled from its case, and 1 eye was removed from its socket for retina sampling. Ovaries were excised from the abdominal cavity and weighed, and the F1 follicle was taken. All samples were placed in liquid nitrogen and stored at -80°C until analysis of mRNA levels.

RNA Extraction and Real-Time PCR

Frozen tissue samples were homogenized using an HG-300 homogenizer with a 7-mm diameter saw tooth. Total RNA was extracted from the different tissues by using

Table 1. Daily light treatment.

	White light	Blue light	Red light	Green light	Total h
Control group	0700–2100 h	—	—	—	14
Blue-Red group	—	0700–2100 h	0700–1300 h	—	14
Red-Blue group	—	0700–1300 h	0700–2100 h	—	14
Green-Red group	—	—	0700–1300 h	0700–2100 h	14
Red-Green group	—	—	0700–2100 h	0700–1300 h	14

Table 2. Primers used for the real-time PCR analysis.

Gene	Primers	Product length	GenBank accession no.
GAPDH	R:CCTGCATCTGCCCATTT F:GGCACGCCATCACTATC	61	NM 204305.1
Actin	R:AAAGCCATGCCAATCTCGTC F:CCGCAAATGCTTCTAAACCG	101	NM 205518.1
Red Opsin	R:GCAGTAGGCCACGATCATCA F:TCGCCATCATCATCCTCTGC	140	XM_025146211.1
Green Opsin	R:ATCCGCGTCACCTCCTTCTCT F:CACTTCATCATCCCAGGTCGT	128	NM_205490.1
GnRH	R:GATCAGGCTTGCCATGGTTT F:TGGTCTTATGGCCTGCAACC	174	NM_001080877.1
LH	R:CCCCATAAGTGCACGCCG F:GAATGCCCCCAATGTATGGCT	118	S_70834
FSH	R:ATTCAGGATGGTCAACCGCAG F:CTGCTTCAACAAGGGATCCAGTA	122	NM_204257.1
VIP	R:CAGCATGCCTTGCATTTCTATCA F:CGCCATGGGTCCTTAAAGT	113	NM_205366.2
PRL	R:AGTCCTCATCAGCGAGTTGC F:TAGTTGGGCGGGTTCATTCT	90	NM_205466.2
GnIH	R:TCAGTCGACAGGAAGTCACAC F:AGGCAATGATAACTGTAGTGAATGA	92	AB193126.1

Abbreviations: FSH, follicle-stimulating hormone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PRL, prolactin; VIP, vasoactive intestinal peptide.

RNAzol RT reagent (100 mg of tissue/1 mL RNAzol RT) as per the manufacturer's protocol (Genecopoeia, Rockville, MD). The concentration of total RNA obtained from the tissues was measured using NanoDrop ND-1000 UV-Vis Spectrophotometer (Wilmington, DE). Reverse transcription reaction to obtain complementary DNA was performed. One microgram of total RNA was reverse transcribed into cDNA in a total volume of 20 μ L using 200 U reverse transcriptase, 250 pg random hexamer primers, and 62.5 ng oligodeoxynucleotides. Real-time PCR was performed using a LightCycler 96 Real-Time PCR system (Roche Diagnostics GmbH; Sandhofer strabe 116 68,305 Mannheim, Germany), using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific, Life Technologies Ltd., Invitrogen™, Paisley, UK). The geometric means of β -actin and glyceraldehyde 3-phosphate dehydrogenase were used as standards. The reaction was incubated under a preplanned thermal cycle, starting with preincubation for 10 min, followed by 45 cycles of amplification steps (each for 50 s), and ending in melting for 2 minutes. Dissociation curves after each real-time run confirmed the presence of only one product and the absence of the formation of primer dimers. The quantification cycle (C_q) for each tested gene X was used to quantify the relative abundance of the gene; arbitrary units were calculated as $2^{-\{\Delta\} C_q} = 2^{-[Ct \text{ gene X} - Ct (\text{geometric mean of } \beta\text{-actin and glyceraldehyde 3-phosphate dehydrogenase})]} \times 1,000$. Primer sequences used in the PCR are shown in Table 2. The results of gene expression were presented in arbitrary units.

Statistical Analysis

Experiment format meant to resemble field condition as closely as possible. Hence, all layers were housed together and laid together. Moreover, low number of birds made every single death statistically significant.

Therefore, production, fertility, hatchability, and plasma levels of steroids and PRL data could not be analyzed in repeated measurements model. Data were analyzed by two-way ANOVA model with age (wk), treatment (light treatment), and their interaction as fix effect.

Gene expression and cumulative egg production data were analyzed by using one-way ANOVA with treatment (light treatment) as fix effect.

The Tukey–Kramer honestly significant difference (HSD) test was used for post hoc testing of the differences between means least square means. Differences between means were considered significant at $P \leq 0.05$. Data are presented as least square mean \pm SEM.1

Data was analyzed using JMP 14 software (SAS Institute, Cary, NC, 2,000).

RESULTS

Reproductive Factors

No interaction was found between hen age and treatment ($P = 0.765$).

The Red-Blue group's laying percentage was significantly higher than that of the other groups (average of 84.7%) and the Green-Red group's was the lowest (67.5%) ($P < 0.0001$) (Figure 1). Although the Blue-Red group started the production period quite well, it soon exhibited poor performances compared with the control group and to the primary breeder's data. Photostimulation by blue SD during a red LD affected cumulative egg production (Figure 2) bringing about an increase of 21 eggs per layer—a 15% increase compared with the primary breeder's recommendations for a similar production period.

The Blue-Red group showed a significant decrease in fertility from wk 42 until the end of the experiment and, overall, had the lowest fertility than those in the other groups ($P < 0.001$) (Figure 3). Hatchability levels (Figure 4) were lower than the primary breeder's data

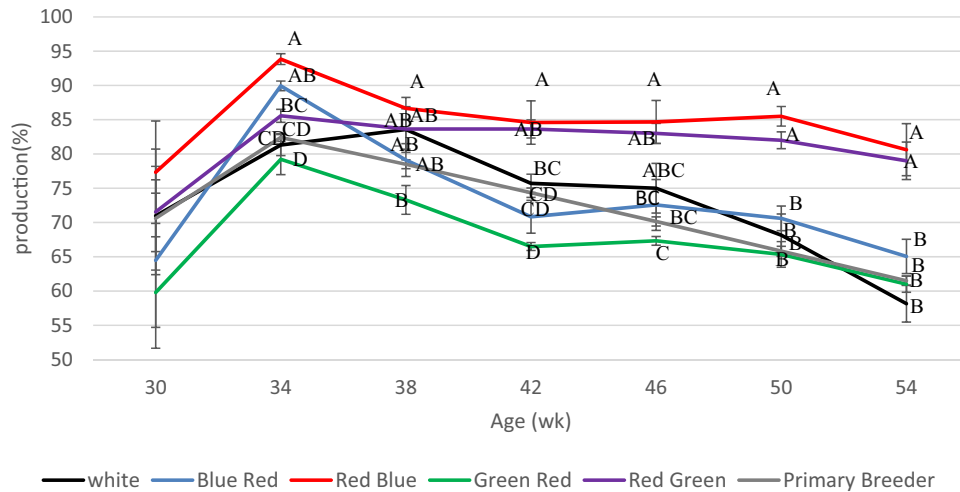


Figure 1. Monthly egg production during the 7 mo of the experiment of broiler breeder hens exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), red light for 14 h with 6 h green light (Red-Green), and primary breeder's recommendations are also presented. Points present monthly laying average \pm SE. Levels with different letters are significantly different ($P \leq 0.05$).

for all treatments for most of the experiment owing to the severe environmental conditions. A change was seen at 50 wk when the Red-Blue, Red-Green, and Green-Red groups exceeded the primary breeder's recommended hatchability rate.

Gonadotrophic Axis Gene Expression

No significant difference was found in hypothalamic GnRH mRNA levels among the different groups at 35 wk. However, at 55 wk, the GnRH mRNA level was significantly higher in hens from the Red-Blue group than in those from the control, Blue-Red and Green-Red groups (Figure 5). Similarly, pituitary luteinizing hormone (LH) mRNA level showed no significant differences among groups at 35 wk but was significantly higher for the Red-Blue group at 55 wk (Figure 6). At 35 wk, pituitary follicle-stimulating hormone (FSH) mRNA levels were significantly higher in the Red-Blue group than in the control, but at 55 wk, no significant difference was seen (Figure 7).

Lactotropic Axis Gene Expression

A significant increase in the hypothalamic VIP mRNA level was observed in the Red-Blue group at 55 wk

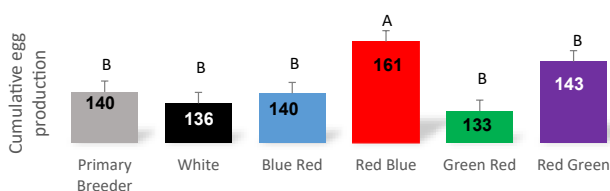


Figure 2. Cumulative egg production of broiler breeder hens exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), red light for 14 h with 6 h green light (Red-Green), and primary breeder's recommendations are also presented. Data are presented as average \pm SE. Levels with different letters are significantly different ($P \leq 0.05$).

compared with all other groups and to the previous age of sampling (35 wk)—where no difference was detected among the groups (Figure 8). The elevation in the hypothalamic VIP mRNA level in the Red-Blue group was followed by an increase in PRL mRNA level at 55 wk (Figure 9). An increase in PRL mRNA level was also seen in the Blue-Red group. Neither of these groups differed significantly from the control group.

Gonadotropin-Inhibitory Hormone Gene Expression

No significant difference was found for hypothalamic GnIH mRNA levels among the different treatments at 35 wk (Figure 10). At 55 wk, on the other hand, photostimulation of the retinae with LD green light while the ERPR were kept under nonstimulatory conditions by the use of SD red light caused an increase in GnIH mRNA level compared with all other groups, except the Red-Blue group.

Opsin Gene Expression

A significant increase in the hypothalamic red opsin mRNA level (Figure 11) was observed in the Green-Red group 13 wk after initiation of photostimulation compared with all other groups, and compared with the colored-light-treatment groups, but not the control group, at 55 wk. Retinal red opsin mRNA levels (Figure 12) were significantly higher in the Red-Blue group than those in all other groups at 55 wk and significantly lower in the Blue-Red group.

A high level of green opsin mRNA was found in the hypothalamus collected from Red-Blue hens at 35 wk. By 55 wk, green opsin mRNA gene expression was higher in the Green-Red group (Figure 13). Similar to the retinal red opsin mRNA levels, the retinal green opsin mRNA levels were also significantly low in the retinae of the Blue-Red group hens at 55 wk (Figure 14).

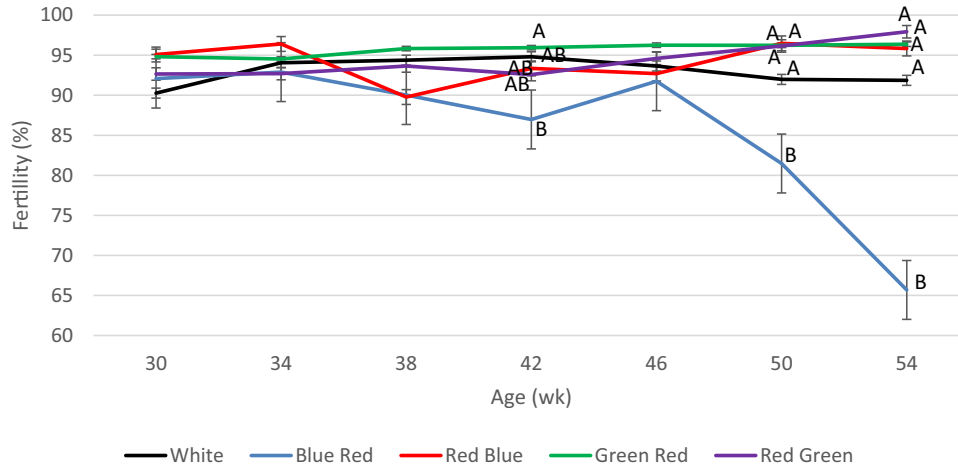


Figure 3. Percentage of fertile eggs of all eggs laid by broiler breeder hens exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), red light for 14 h with 6 h green light (Red-Green), and primary breeder's recommendations are also presented. Data are presented as average \pm SE. Levels with different letters are significantly different ($P \leq 0.05$).

Plasma Steroid Levels

No interaction was found between treatment and hen age. Changes in plasma steroid levels changed significantly with age ($P < 0.001$) as previously reported (Onagbesan et al., 2006); however, no differences were detected among treatments.

DISCUSSION

Relative selective activation of the ERPR using red light (14 h) combined with nonphotostimulatory conditions for the retina (6 h of blue or green light, turned on during the 14 h of red light) improved reproductive performance. A comparison of the 5 treatments indicated that red illumination improves reproduction,

whereas green and blue illuminations suppress it. The conditions in all treatments were the same except for wavelength and lighting duration; therefore, the differential influence on reproduction necessarily derived from those parameters. The improvement in reproductive performance was reflected in higher egg yield and higher expression at the mRNA level of genes involved in reproductive processes. Activation of the retina with green or blue light resulted in decreased reproductive performance, expressed as low egg production and an increase in reproduction-inhibiting gene expression. In addition, we found an increase in the green opsin mRNA level in the retinae of the Green-Red group hens.

Fertility and hatchability are very important parameters of reproductive performance. Egg fertility

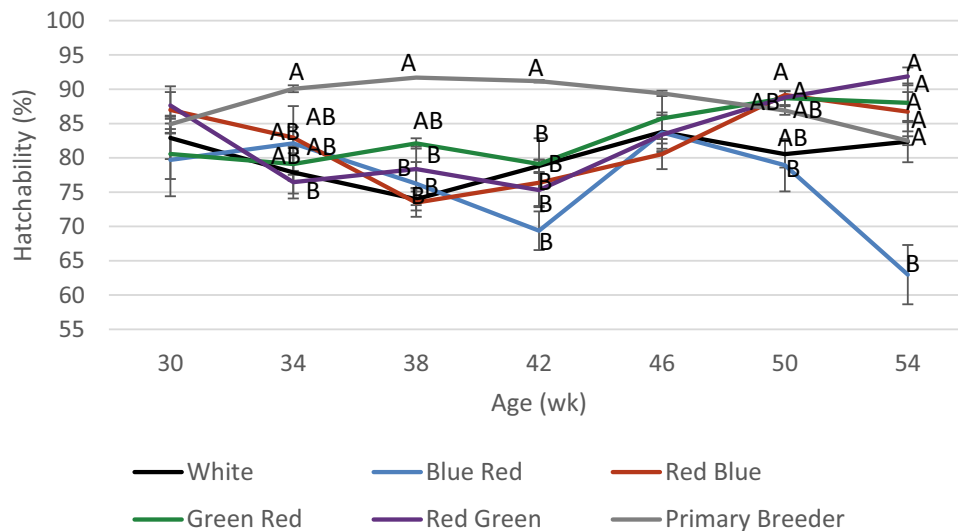


Figure 4. Percentage of hatched eggs of all eggs laid by broiler breeder hens exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), red light for 14 h with 6 h green light (Red-Green), and primary breeder's recommendations are also presented. Data are presented as average \pm SE. Levels with different letters are significantly different ($P \leq 0.05$).

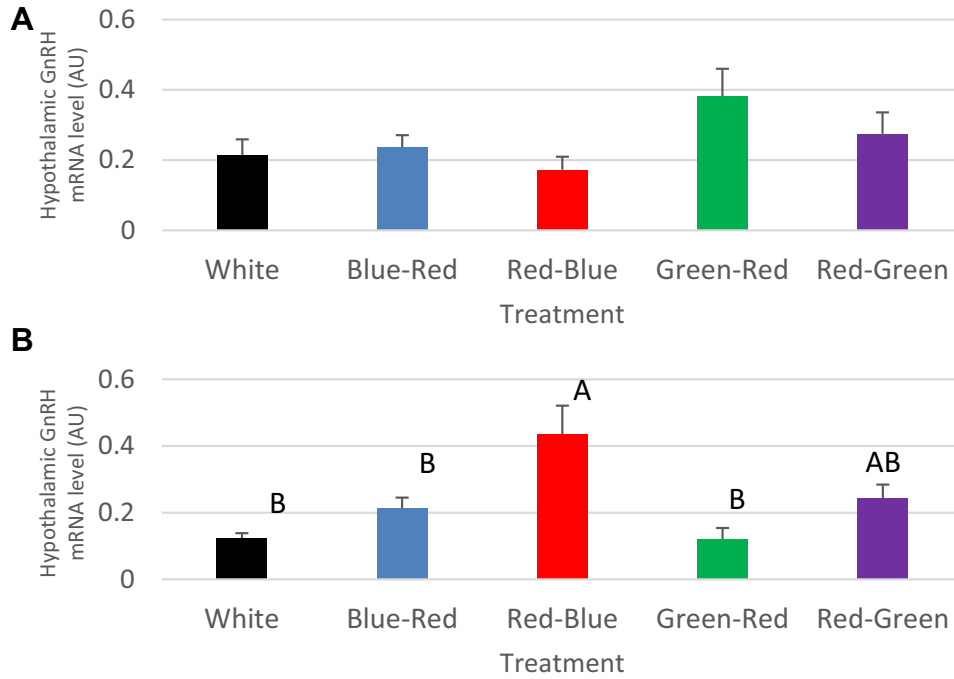


Figure 5. Hypothalamic GnRH mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, n = 10. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: GnRH, gonadotropin-releasing hormone.

levels were high in all treatments throughout the experiment. Nevertheless, the Blue-Red group demonstrated slightly lower fertility levels than the other groups, with a sharp decrease in fertility in the last 2 mo of the experiment. Hatchability percentage depends on many variables, including layer health, genetics, and environmental conditions such as temperature, humidity, and so forth (Stromberg,

1975). Heywang (1944) found a correlation between high temperatures in a chicken coop during the laying period and low hatchability of the resultant eggs (Heywang, 1944). In our study, hatchability percentage declined from wk 30 to wk 46 and was consistently lower than the primary breeder's recommendation. A possible reason for this is the high temperatures prevailing in the chicken house

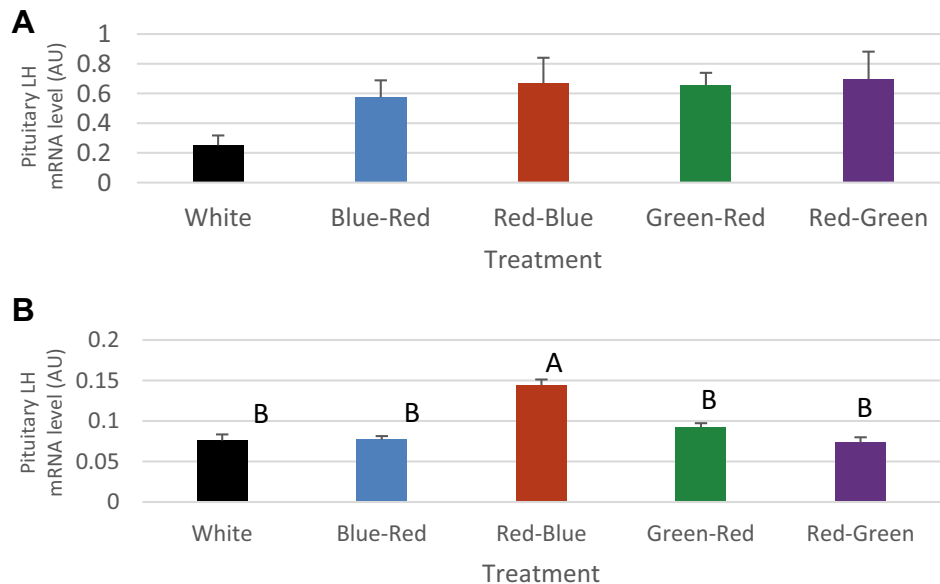


Figure 6. Pituitary LH mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, n = 10. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: LH, luteinizing hormone.

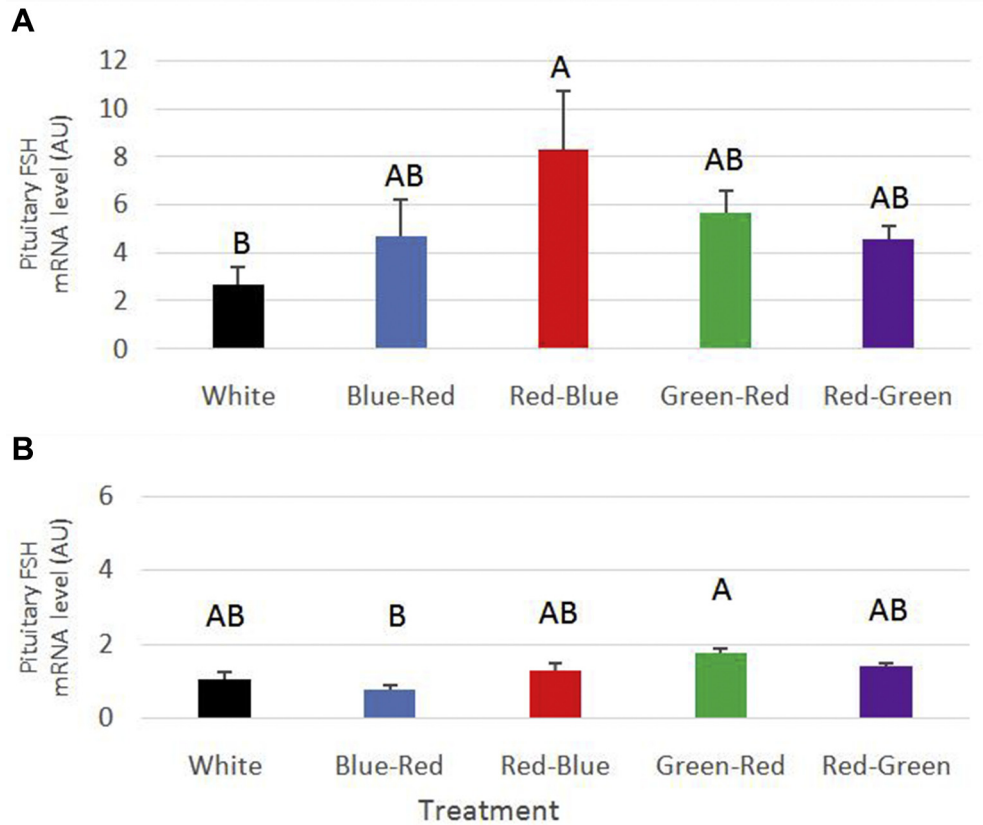


Figure 7. Pituitary FSH mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h and Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, $n = 10$. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: FSH, follicle-stimulating hormone.

during the summer months, which reached 38°C. Indeed, when the temperatures dropped, from wk 47 on, hatchability levels increased and in some groups,

even exceeded breeder recommendations. The control group's results ultimately met the primary breeder's recommendations, whereas in the Blue-Red group,

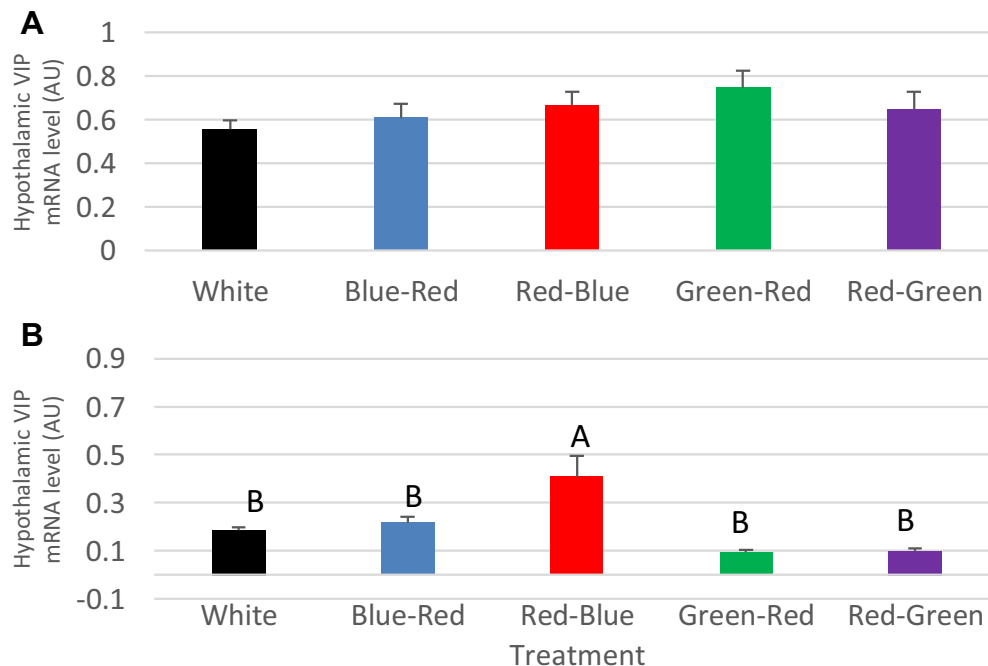


Figure 8. Hypothalamic VIP mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, $n = 10$. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: VIP, vasoactive intestinal peptide.

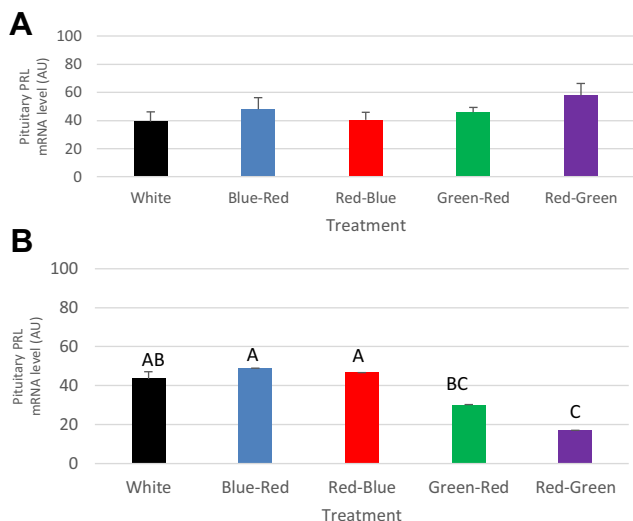


Figure 9. Hypophyseal PRL mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, n = 10. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: PRL, prolactin.

hatchability levels never recovered, reaching only 65% hatchability.

We found an increase in gonadal axis genes' expression in the Red-Blue and Red-Green groups, manifested by an increase in the GnRH mRNA level. This is in line with the excellent reproductive performance of these groups, as among its other functions, GnRH is responsible for sexual behavior, gonadotropin release, and regulation of peripheral tissues of the reproductive system (Schnieder et al., 2006). In general, gonadotropin levels were lower at 55 wk than at 35 wk. Nevertheless, some differences in gene expression were visible among the

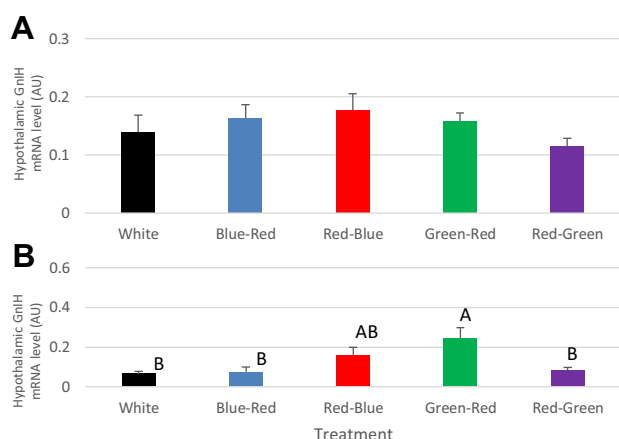


Figure 10. Hypothalamic GnIH mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, n = 10. Levels with different letters are significantly different ($P \leq 0.05$). Abbreviation: GnIH, gonadotropin-inhibitory hormone.

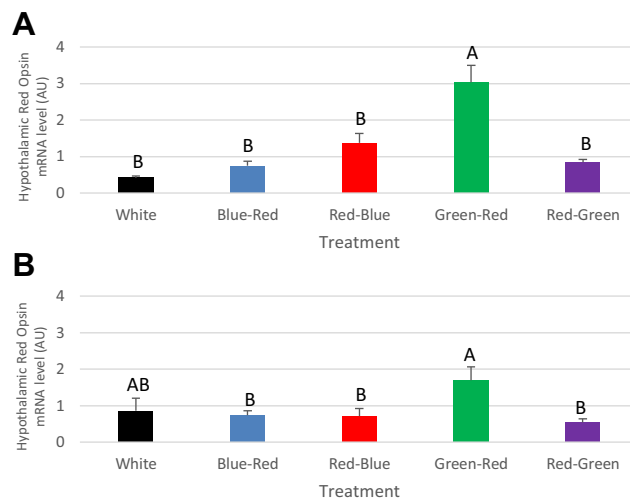


Figure 11. Hypothalamic red opsin mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, n = 10. Levels with different letters are significantly different ($P \leq 0.05$).

groups, with Red-Blue LH mRNA levels being significantly higher than in the other groups. Because LH is responsible for ovulation, high LH levels indicate a rise in egg production, in line with the previously reported positive correlation between plasma LH concentration and egg production (Tanabe et al., 1981). These findings are especially noteworthy considering the decrease in LH responsiveness to GnRH as hens mature (Sharp et al., 1992).

Red opsin mRNA levels increased after relative selective photostimulation of the brain by red light. As already noted, the increase in reproductive activity in the Red-Blue and Red-Green groups was characterized by a significant increase in the GnRH mRNA level. The photoreceptors in the brain are known to communicate directly with GnRH neurons (Saldanha et al., 2001). Therefore, the increase in red opsin mRNA level in the hypothalamus may be responsible for the increase in GnRH expression.

The green opsin mRNA level was lowest in the retinae of chickens from the Blue-Red group. There is evidence of blue light being responsible for apoptosis in the retinae of mature rats (Wu et al., 1999) and cattle (Pautler et al., 1990) and also of short wavelengths, such as blue light, damaging mammalian retinae (Ham et al., 1978, 1980). Moreover, blue light has been found to damage the mouse retina, particularly in the retinal pigmented epithelium area where the photoreceptors are found (Wu et al., 1999), resulting in extensive photoreceptor death (Wu et al., 1999). Because the retina's structure and its cell components are similar in mammals and in poultry, it can be assumed that the avian retina is influenced by blue light in the same manner (Dowling, 1970). Indeed, in the retinae of the Blue-Red group birds (14 h of blue light), we observed significantly lower levels of the green and red opsin mRNA than in the other

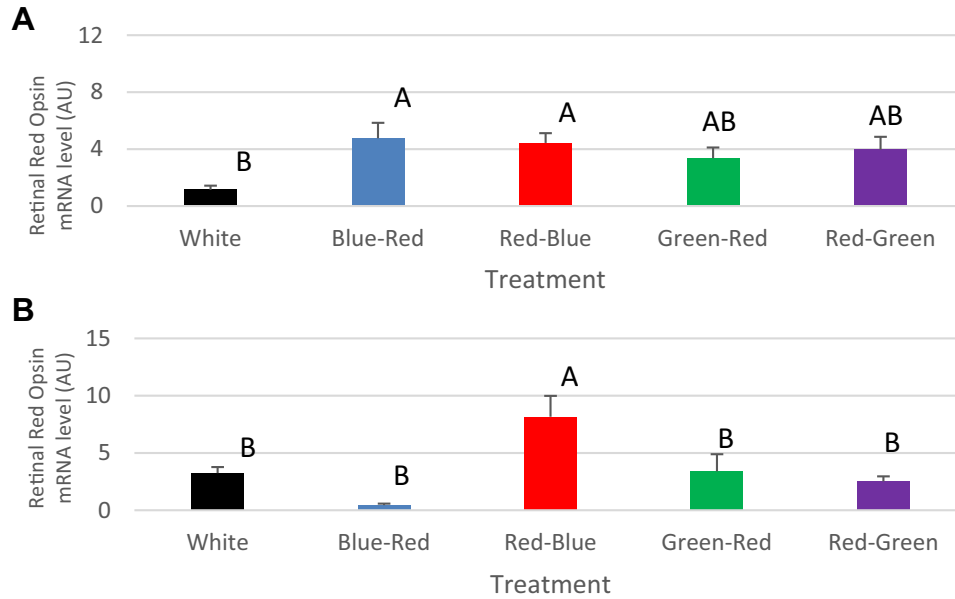


Figure 12. Retinal red opsin mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, $n = 10$. Levels with different letters are significantly different ($P \leq 0.05$).

groups. A similar phenomenon has been previously documented in our laboratory (unpublished data).

Owing to the low tissue penetration by short wavelengths (Benoit, 1964), it is commonly assumed that there is no green opsin gene expression in the hypothalamus. However, the hypothalamus has been reported to contain opsins that react to green light (Foster et al., 1985). In addition, direct illumination of the hypothalamus with short waves, such as blue light, has been found to result in gonadal recrudescence, similar to that with red-light illumination (Benoit, 1964). In this work, we

found obvious expression of the green opsin gene in the hypothalamus (figure 13).

The retinal reproductive inhibition mechanism activated by green light, which includes GnIH, has been well characterized (Bédécarrats, 2015). Gonadotropin-inhibitory hormone inhibits synthesis and secretion of the gonadotropins LH and FSH in domestic fowl (Ciccone et al., 2004) and disrupts gonadal development and activity in quail (Ubuka et al., 2006). We found a significant increase in the GnIH mRNA level in the Green-Red group (figure 10). It should be noted that a

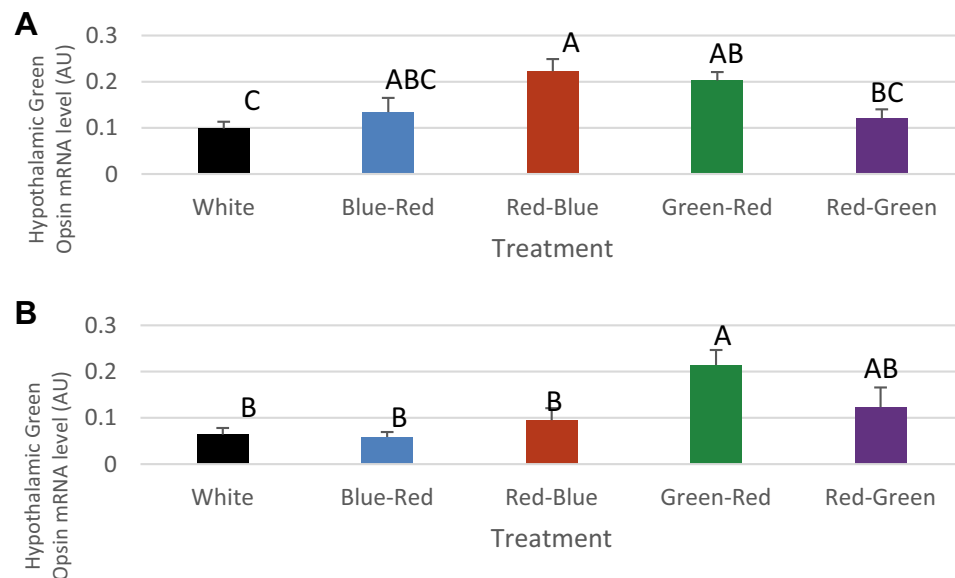


Figure 13. Hypothalamic green opsin mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, $n = 10$. Levels with different letters are significantly different ($P \leq 0.05$).

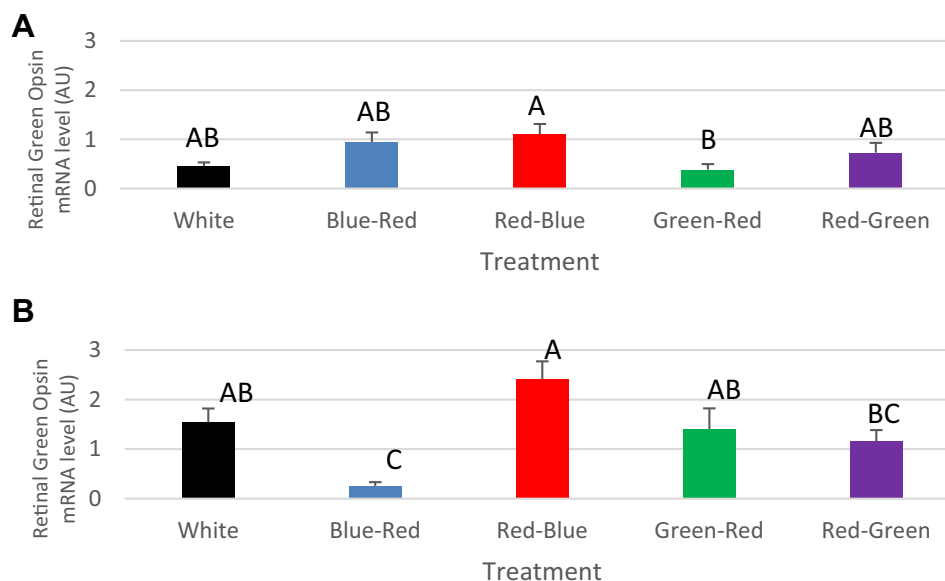


Figure 14. Retinal green opsin mRNA levels in broiler breeder hens (aged 35 wk [A] and 55 wk [B]) exposed to 14 h white light (Control), blue light for 14 h combined with 6 h red light (Blue-Red), red light for 14 h with 6 h blue light (Red-Blue), green light for 14 h with 6 h Red light (Green-Red), and red light for 14 h with 6 h green light (Red-Green). Data are presented as average \pm SE, $n = 10$. Levels with different letters are significantly different ($P \leq 0.05$).

similarly high GnIH mRNA level was also observed in the Red-Blue group, with the best layers. This increase is probably related to the way red light affects melatonin. It turns out that unlike the other wavelengths that inhibit melatonin synthesis, red light does not affect it (Zatz et al., 1988; Zatz and Mullen, 1988; Zawilska et al., 1995). Melatonin, in turn, increases GnIH synthesis and gene expression (Ubuka et al., 2005). There is an apparent contradiction here because a rise in the GnIH levels is expected to suppress LH and FSH gene expression (Ciccone et al., 2004). A possible explanation for this seeming contradiction is that the sensitivity of the pituitary gland to GnRH is higher than its sensitivity to GnIH, so even though GnIH gene expression was high in the Red-Blue group, influence of GnRH was stronger. Ciccone et al. (2005) found that during the breeding season, there is no difference between the expression levels of GnRH and GnIH genes, and there is no influence on the expression of LH and FSH genes. Further studies are needed to verify this hypothesis.

Vasoactive intestinal peptide mRNA levels were significantly higher in the Red-Blue group than in the other groups (Figure 8) and PRL mRNA levels followed a similar trend (Figure 9). High VIP levels inhibit LH synthesis and secretion (Weick and Stobie, 1995) and increase PRL levels, which in turn suppress hypothalamic GnRH secretion (Rozenboim et al., 1993) and hypophyseal LH synthesis (You et al., 1995). We therefore expected to see higher levels of VIP and PRL in the Green-Red and Blue-Red groups. On the other hand, photostimulation increases VIP level in turkey hen hypothalamus (Rozenboim et al., 1993; You et al., 1995) followed by hypophyseal PRL secretion (Nicholls et al., 1988; El Halawani et al., 1990). Hence, low levels of VIP are required for proper release of GnRH and LH

(Samson et al., 1981; Ohtsuka et al., 1988). More importantly, we previously demonstrated that complementary treatment with PRL in old breeder roosters vaccinated against VIP restores their reproductive axis and improves sperm quality (Avital-Cohen et al., 2012). Thus, the decline in VIP and PRL gene expression in the Green-Red group might explain the damage caused to LH gene expression and reproductive performance.

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