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Different wavelengths of light on GnRH mRNA, LH, steroid hormones and egg lay in the ageing hens (*Gallus gallus domesticus).*

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Abstract: The aim of this study was to investigate the effects of red spectrum of light (650nm, treated n=12) and normal spectrum of light (450nm control=12) on circulating concentrations of luteinizing hormone (LH), progesterone (P_4), estradiol ($E_2\beta$), GnRH mRNA, pause days and egg production in birds later in the reproductive period from 92-102weeks of age. Twenty-four White Leghorn birds of same age group were divided into two groups of 12 in each as control and treated. Birds in the control group were exposed to normal spectrum of light (450nm of length) and birds in the treated group were exposed to red spectrum of light (650nm, treated n=12). Egg production and inter sequence pauses were recorded daily from both the groups. Plasma LH, $E_2\beta$ and P_4 concentrations were estimated in blood samples collected at weekly intervals. At 97th weeks of age, blood samples from treated and control birds were obtained every 3 h for 36 h to study the surges of LH. It was found that plasma GnRH was higher ($p < 0.01$) in treated birds with high concentrations of LH, its 3 h LH surges, $E_2\beta$ and P_4 in plasma. Higher egg production, less pause days in treated birds may be the result of high GnRH associated with positively correlated responses of high concentrations of LH (with regular interval and duration of LH surges), $E_2\beta$ and P_4 concentration required for completion of egg formation and oviposition. In conclusion, red spectrum of light enhanced GnRH mRNA ($p < 0.01$), increased ($p < 0.01$) steroid hormones and LH surges, for egg formation and oviposition and enabled the birds to lay more eggs even later in the productive period with the available resources under normal husbandry practices.

Key Words: Red light; GnRH; egg lay in aged hens.

Introduction

In poultry farming, the number of eggs laid per hen, the duration of the laying period and male fertility are all critical factors. Layers routinely produce over 300 eggs per year and a laying cycle can last over 52 weeks. As for all vertebrates, reproduction in poultry is controlled by the hypothalamo-pituitary-gonadal axis (1,2). Sexual maturation can be induced by increasing the photoperiod (photostimulation) and results in an increase in gonadotropin releasing hormone (GnRH) secretion from the hypothalamus followed by the stimulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion by the pituitary gland. In turn, increasing levels of circulating LH and FSH stimulate the development and maturation of the reproductive organs resulting in the lay of the first egg and semen production in hens and roosters, respectively (3,4,5). At this stage, hens enter an active laying period and gonadal steroids act both negatively and positively at the hypothalamic and pituitary level to control successive ovulations (6). After reaching a production peak, laying rates progressively decrease. It is reported that wave length from the red spectrum have the ability to penetrate the skull and directly stimulate the hypothalamic photoreceptors (7,8), while wavelength from the normal spectrum barely penetrate the brain and mainly stimulate the retina. Taken together, the discovery of hypothalamic peptides and their extra retinal photoreceptors has allowed for the elaboration of revised hypothesis on the photoperiodic control of reproduction in poultry (1), and it is now critical to experimentally test these new theories. In industry settings, white incandescent lights (entire spectrum) are generally used to control the photoperiod. Results from this experiment will help determine the optimum lighting regimen to be used in an industry setting, and will help reduce the energy cost associated with incandescent lighting.

Materials and Methods

Experimental Design: At 92 weeks of age, 24 White leg horn birds were housed in individual cages (one bird per cage) under two-tier battery system. Birds were divided into two groups consisting of 12 birds in each. All birds were fed on the same layer ration (16 per cent CP and 11. 72 MJ ME Kg^{-1}) as per the standard recommendations (9) and were provided 16 h light and 8 h dark. Feed intakes were not affected by the treatment in both the groups. Clean water was made available round the clock throughout the experimental period. From 92-102 weeks of age the birds in treatment group were exposed to 650nm of wavelengths of light (red spectrum of light- treatment group). Controls were exposed to normal spectrum of light with white bulbs at 450nm of wavelength of light and treated group were exposed to red spectrum of light at 650nM of wavelengths of light with red bulbs. Bulbs were fixed in the experimental unit so as to provide uniform intensity of light to all the birds within the group without any variation as for as source of light with an intensity of 0. 1 W/m2 at bird-head level. Watts per square meter units were used because extra retinal photoreceptors located in bird's head detect

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energy level penetrating through the skull. The control group was (housed in separate partisan) provided with normal light bulbs 0. 1 W/ m2 at bird-head level, and served as controls. The lighting schedule was 16h light and 8h darkness. Daily egg production was recorded for each hen at the same time for 77 days. Mean weekly egg production was recorded from both the groups. Total number of pause days during 11-week period (77 days) was recorded. Ovipositions were recorded between 9AM to 12 noon in both the groups. Ovipositions in the control group were irregular with more days between the sequences of egg lay compared to treated group. (Figure 6).

Collection of blood samples; Blood samples were collected from each bird by superficial venepuncture of the brachial vein starting from 92 weeks of age onwards at weekly intervals and continued until the end of experimental period at 102 weeks of age. At 97th weeks of age, blood samples were collected with cannulate at every 3 h for 36h starting at 6. 00 h to study the pattern of LH frequency and amplitude from both the groups. . The sampling took about 1 h and the birds were always sampled in the same order to ensure a period of 3h between each sample. During lights were off (between 10PM to 6AM), hand torches were used for the sampling to minimize disruption of the lighting schedule. Plasma was separated and stored at – 20^{0} C for hormone assay. Oviposition times were recorded while collecting blood samples in both the groups.

Studies *In vitro*: To determine the effect of red spectrum of light (treated) and normal spectrum of light (incandescent light) on reproductive ageing on the distribution of yellow-yolky follicles (YYFs) greater than 8 mm in the ovary, birds in both the groups were decapitated at the end of 102 weeks of age. Hypothalami were dissected to include GnRH-I cell bodies (10). Hypothalami and pituitary glands were weighed and snap frozen in liquid nitrogen and stored at -80^{0}c until RNA extraction. The number of yellow yolky follicles >8mm, follicles between 5 and 8 mm, and the residual ovary and oviduct weights were recorded (Fig. No. 7). Hypothalamic cell bodies containing GnRH-I were cultured separately from treated and control group of birds in DMEM with phenol red (Invitrogen), supplemented with 3. 75%fetal calf serum and 65% horse serum and antibiotics:100u/ml streptomycin and 100ug/ml penicillin. The culture plates were placed in air tight containers, and after equilibration with 95% O_2 and 5%CO_2 transferred to an incubator maintained at 37^{o} C. Media changed for every 2 days and maintained for six days. Superficial fluid is removed and the cells which were attached are subjected to RNA extraction.

RNA Extraction: Total RNA was extracted from neuroendocrine tissues in eppendorf tubes containing 1 ul Trizol (Invitrogen) for hypothalami. The tissues were disrupted using homogenizer. Final precipitation of RNA was facilitated by addition of 2ul of glycogen solution. The total RNA pellet was briefly dried under vacuum and reconstituted in 100-150ul of double distilled water. The yield of RNA was quantified by measuring the optical density of a sample diluted to 1:50 at 260 and 280nm, and its quality was confirmed by running a sample out on a formaldehyde gel.

Reverse transcription of total RNA: A sample of (4ul) of total RNA was reverse transcribed using a first strand synthesis kit (Amersham Pharmacia biotech UK Ltd) following the manufacturer's instructions. Reverse transcribed samples were diluted to 40ul in dH_2O. PCR Primers in QC RT-PCR assays for GnRH mRNA were made from gene bank Accession no: X69491. GnRH-I, forward primer as **TGGGTTTGTTGATGGTGTTGT** and reverse primer as **ATTTTCCAGCGGGAAGAGTTG**. Chicken GnRH-I in both control and treated group were measured by quantitative (QC) RT_PCR assays as per Dunn *et al.,* (11). Oligonucleotide primers for the amplification of neuroendocrine gene GnRH were designed using the "primer" software package version 0. 5 and published cDNA sequences. The PCR amplification of was carried out in a Thermo-Fast low profile 96 well plate on a programmable heating block. The PCR conditions were 30 cycles (94^{o}C, 20s;62^{o}C, 20S;72^{o}C,20s) for GnRH-I.

Analysis of hormones: Chicken cLH antisera and pure hormone were obtained from John. A. Proudman, USDA as a gift from USA. The intra and inter coefficient variation for cLH was 4. 45%and 8. 12% respectively with sensitivity of the hormone 0. 01 ng/ml per tube as per the method described by previously Sharp, *et al.,* (4). E_2ß and P4 samples were analyzed with RIA kits obtained from ImmunoTech, France.

Statistical analysis

Measurements were given as mean ± SE. The significance of differences between means was analysed by F test. The data on egg production, LH, E_2ß and P_4 were subjected to correlation coefficient analysis to study the influence of the hormones on egg production. Differences were considered significant at a value of $p<0.01$. The statistical analyses were carried out following the standard method (12). Log transformed data was performed for GnRH-I mRNA data by one-way ANOVA analysis.

Results

Plasma LH concentration in control group varied between 2. 29 ± 0. 06 ng/ml to 3. 11 ± 0. 6 ng/ml during 92 to 102 week of age [Fig 3]. In treatment group plasma LH increased from 2. 54 ± 0. 18 ng /ml to 4. 91± 0. 05 ng /ml during 72 to 82 week of

age. LH levels fluctuated between the two groups and treated birds showed significantly (P<0. 01) higher levels over the controls. In treatment group, the increase in LH level was of greater magnitude due to the exposure of red spectrum of light (treatment) for eleven weeks. Three hourly secretion of preovulatory LH surges in treated birds occurred mostly between the 9AM to 12 Noon with a highest concentration of LH 3. 11± 0. 60 ng/ml, whereas these surges in controls occurred around 3PM to 5PM in the controls with highest concentration of 4. 91± 0. 05 ng/ml (Fig 4). The plasma $E_2ß$ level in birds of control group varied between 230. 77 ± 1. 06 pg/ml to 239. 11 ± 1. 09 pg / ml during 92 to 102 weeks of age (Fig 1). In treatment group plasma $E_2ß$ increased from 230. 45 ± 1. 65 pg /ml to 275. 11± 1. 11 pg/ml during 92 to 102 weeks of age. The P_4 secretion in the two groups also followed a similar pattern as estradiol and is presented in Figure 2. However, intermittent hormonal fluctuations were observed in both control and treated groups. Egg production in birds exposed to red spectrum of light, was positively correlated with $E_2ß$ (r= 0. 69), P4 (r =0. 79) LH (r =0. 84) whereas GnRH I mRNA level was positively correlated with $E_2ß$ (r = 0. 73), P_4 (r = 0. 81) and LH (r =-0. 69) and negatively correlated with pause days (r = - 0. 68).

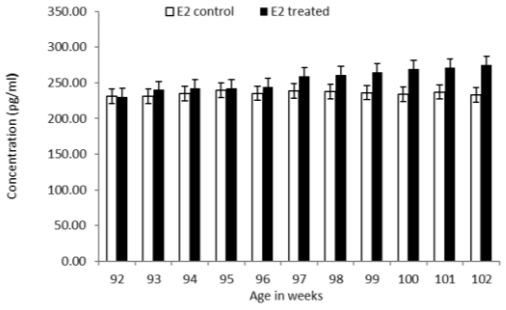

Fig.1: Plsma Estradiol (pg/ml) beteween control and treated birds.

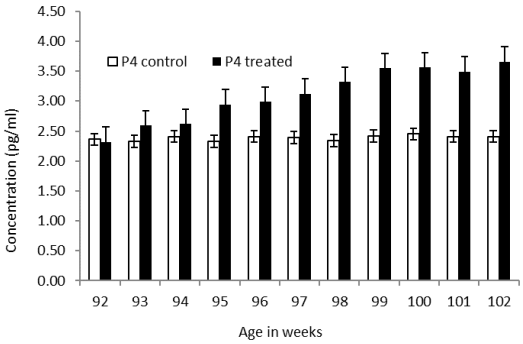

Fig.2: Plsma progesterone (ng/ml) beteween control and treated birds.

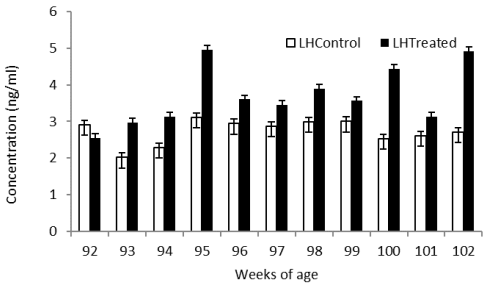

Fig.3: LH concentration between control birds and treated birds

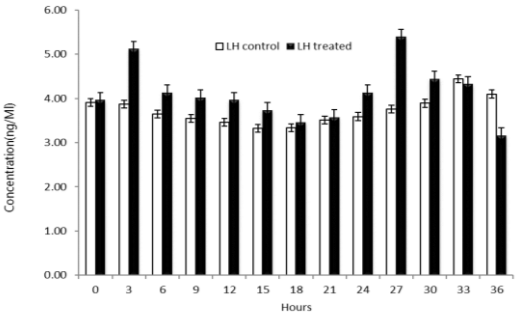

Fig.4:Three hourly LH concentration (ng/ml) in control birds (exposed to normal spectrum of light) and treated birds (exposed to red spectrum of light

GnRH-I mRNA

There were differences in hypothalamic GnRH-I mRNAs expression between treated and control hens. GnRH-I mRNA concentration was significantly higher in birds exposed to red spectrum of light over the control birds. GnRH concentration was expressed in 10^{-14}moles/hypothalamus (Fig. 5).

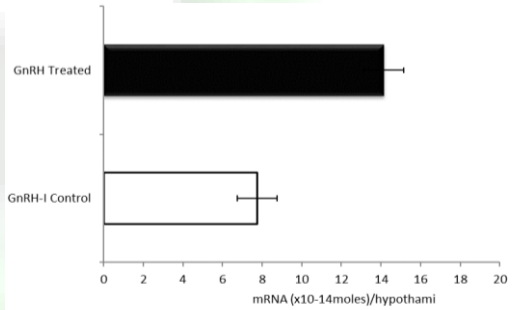

Fig.5: Hypothalamic GnRH-I mRNA in control birds and treated birds.

Influence of different spectrum of light on egg production parameters: The average egg production (bird/week) exposed to red spectrum of light (treatment group) from 92 to 102 weeks of age was significantly (p<0. 01) higher than the controls (Figure 6). It fluctuated between 78% t0 81. 81% and 69 to 73% in birds exposed to red spectrum of light and incandescent light respectively. The difference in egg production/ bird/week was significant between two groups from 94 weeks of age to 102 weeks of age. The percentage of pause days during the 11-week period (77 days) (Figure 6) were 18. 18± 0. 19 in the treatment group as against 28. 57 ± 0. 21 in control group. Residual ovary is the one after removal of the hierarchal follicles F1 to F5. It

comprises of small yellow and white follicles but not stroma alone. Egg production in treatment group between 92 and 102 weeks of age, improved by 10. 39% over control group (Figure 6).

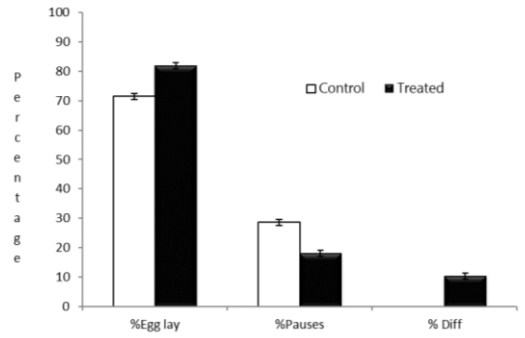

Fig.6: Percentage of egg production, percentage of pause days, difference in percentage of egg production between control and treated birds .

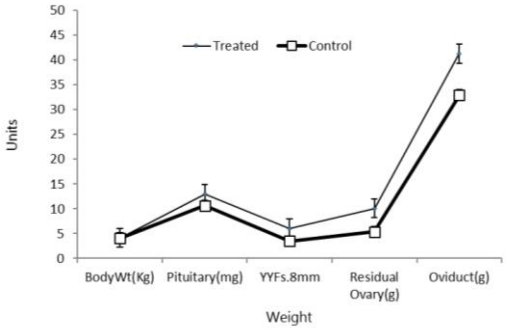

Fig.7:Effect of wavelengh of light on body weight , pituitary weight , residual ovary weight , and oviduct weight and numbers of yellow yolk follicles in birds exposed to 450 and 650 nm wavelengths of light.

Discussion

Wavelength affects the colour of light and is often referred to as the quality of light. Knowledge of the impact of wavelength on poultry productivity is not extensive but there are indications that it can impact bird performance (13). Based on our results, it is evident that wavelengths from the red spectrum are the most potent stimulator of gonadal maturation and egg-lay aged hen. In this study the reproductive system of domestic hen changed from low functional sate to a high functional state following the exposure of birds to red spectrum of light from 92-102 weeks of age and increased egg production by10. 39% in treated group (Figure 6). This may be explained by the fact that wavelengths of higher intensities possess higher power and are thus able to penetrate the skull, brain tissue more easily and stimulate receptors responsible for gonadotropin-releasing hormone (GnRH) release in the hypothalamus and these receptors are suggested to be sensitive to light directly passing through the skull instead of perception of light by retina (14,15,16,17,2). GnRH intern stimulates gonadotrophic and gonad steroid. It is well established that GnRH, LH, FSH and steroid hormones act simultaneously on follicular development folliculogenesis, ovulation, egg formation and egg lay in hen. Amplitude and frequency of LH surges were less prominent (Figure3) in control group either at weekly interval or 3hr interval (Figure 3). This may be attributed to low GnRH in the control group relative to treated group (Figure5). Further, normal wavelengths of light may not be able to penetrate the skull (15,16,18) and thus, light at this intensity may be unable or less likely to excite receptors to release GnRH. Decreased GnRH and low E_2ß and P4 (Figure 1, 2) levels in control birds under normal wave lengths of light also support this hypothesis. In this study, the plasma LH frequency occurs earlier in birds exposed to red spectrum of light is similar to the finding that in mammals, a fast GnRH pulse frequency preferentially stimulates LH release (18) whereas a slow GnRH pulse frequency stimulates FSH release (19). It is suggested that this may be a consequence of low circulating concentrations of plasma estrogen, compared to treated hens which exert a reduced stimulatory effect on LH (9,20) resulting in LH secretion being more sensitive to the decreased stimulatory action of estrogen in hens than in mammals (21). Hens maintained under red light showed high plasma concentrations of E_2ß, P_4, and LH within 4wk after photostimulation than the birds exposed to normal light. Low levels of E2ß, P_4, LH and GnRH in controls is in agreement with various studies and suggests that shorter wavelength of light is ineffective in properly mediating a stimulatory photoperiod. This is further strengthened by the fact that under our experimental conditions normal wave length light. The decreased GnRH in the control group failed to significantly elevate circulating LH, E2B, P4 levels, whereas red light was the most effective. Higher levels of estradiol during the initiation of egg laying have been correlated with the activity of small follicles (15). This effect of light wavelength on ovarian activity was independent of a fully functional retina of the eye and is thus most likely mediated via extra-retinal photoreceptors, most likely hypothalamic. The earlier studies by Bedecarrats, *et al.*, (15) suggests that, ovarian activity in chickens is tightly regulated by a balance of stimulatory HPG axis with inputs from the hypothalamic receptors, coupled with a switch in sensitivity of GnRH at the level of the anterior pituitary. The effect of red spectrum of light on ovarian morphology was stronger than the normal light. This study shows that reduced egg production in birds exposed to normal spectrum of light is associated with a reduction in YYFs and reduced plasma LH (Figure 7). The reduction in numbers of YYFs and in plasma LH agrees with a previous study in broiler breeders of hens (4). It is therefore likely that reduced ovarian function in control hens is caused by a reduction in gonadotropin secretion (6). Changes in pituitary glands may be attributed to longer wavelengths are transmitted through neural tissue more readily than shorter wavelengths Lewis, *et al.*, (17). The feed intake was recorded only during the 92 to 102-week period of the study and no

difference was found in the intake between the groups within the short period of time. The body weights were taken only at slaughter and whatever difference observed may be due to the changes before the initiation of study prior to 92 weeks' age of the birds. Results of this study shows that red light is necessary to adequately initiate the activation of the reproductive axis, increase ovarian activity, maintain high levels of production, and increase total number of eggs in aged birds.

In conclusion the decline in egg production was strongly related to more pause days, a decrease in plasma GnRH-I mRNA decreases LH and steroid hormone concentrations. Similarly, decreased plasma LH in hens with regressed ovaries is not associated with decreased GnRH peptide but is associated with decreased hypothalamic GnRH-I mRNA (11), which suggests that the decrease in ovarian function in old laying hens could be mediated by a reduction in GnRH mRNA transcription and/or stability. Additionally, the duration of one ovulation cycle was prolonged due to aging of the birds and this may explain the increase of pause days. Stimulating the GnRH levels with red spectrum of light enhanced reproductive performance in ageing hens by reducing the number of pause days and shortened the ovulation cycle, same was reflected in hormonal parameters. The longer intervals and more pause days suggest that the synchronization between follicular development and LH peaks during the ovulation cycle (and thus the responsiveness of the F1 follicle to LH) is not 'optimal' with ageing but that this can be improved by stimulating the GnRH concentration. This is in agreement in domestic pullets with the results obtained later. Results from this experiment will help determine the optimum lighting regimen to be used in an industry setting, and will help reduce the energy cost associated with incandescent lighting. Furthermore, this approach and procedure could be extended to other species of economic importance such as ducks, geese and quails in tropics/subtropics in particular.

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