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Research Article

GnRH-1, GnIH mRNA and Luteinizing Hormone in Domestic hens (*Gallus gallus domesticus*) Exposed to Different Wavelengths of light

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Abstract: The objective of this study was to establish the effects of red spectrum of light (650nm, treated n=12) and normal spectrum of light (450nm control=12) on GnRH-I and GnIH mRNA expression, amplitude and frequency of luteinizing hormone (LH) and egg production from 42 to 52 weeks of age in white leghorn hens**.** Blood samples were collected at weekly interval from both the groups. At the 47th week of age blood samples from both the groups were collected at every 3 h for 36h to study the pulsatile secretion of LH surges. GnRH and GnIH mRNA expression pattern was studied between control and treated birds. Egg production and pause days were calculated between the two groups. LH concentration in the plasma was increased significantly (P<0.01) in hens exposed to red spectrum of light. Plasma LH concentration was higher (P<0.01) in treated birds with more number of LH surges. The amplitude and frequencies of LH were advanced in birds exposed to red spectrum of light during 36 h of sampling at 3h intervals. GnRH-I mRNA concentration was significantly (P<0.01) higher, whereas GnIH mRNA was significantly (P<0.01) lower in birds exposed to red spectrum of light compared to controls. It is hypothesized that exposure of birds to red spectrum of light enhanced (P<0.01) GnRH-I mRNA, along with LH required for ovulation and egg lay. During 77 days (42-52 weeks of age) of the experimental period, egg production was increased (p<0.01) with lower incidence of pause days in the treated group. It is concluded that low GnIH mRNA and higher levels of GnRH-I mRNA, LH, lower number of pause days enabled the birds to lay more eggs by stimulating GnRH through red spectrum of light.

Keywords: Different wavelengths of light, GnRH-ImRNA, LH surges, GnIH, egg production, hen.

Introduction

It is known that nonmammalian vertebrates detect light by deep brain photoreceptors that lie outside the retina and pineal organ to regulate the seasonal cycle of reproduction (Nakane *et al*., 2010). Birds sense light through the retinal photoreceptors (Lewis and Morris, 2000), and the extra retinal photosensitive (Nakane *et al*., 2010; Reddy *et al*., 2012) cells in the brain play a role for egg lay. Experiments indicate that the photoreception by hypothalamic photosensitive cells located deep in the brain responds to the light passed through the skull instead of light passing through the retina of the eye. Dim light less than 5lux may not be able to penetrate the skull and thus light of that intensity is unable or less likely excite receptors to release GnRH. These receptors upon stimulation rereleases gonadotrophic hormone releasing hormones (GnRH) into the hypothalamus (Reddy *et al.,* 2012; Reddy *et al.,* 2016). GnRH receptors as photoreceptor responds to different wave lengths of light 460–675nm (Bedecarrettes *et al*., 2015). Light perceived by photoreceptors within the hypothalamus is transuded into nervous impulses that initiate the synthesis and release of gonadotropin-releasing hormone (Nakane *et al.,* 2010), thereby triggering the events of the hypothalamo-pituitary-gonadal (HPG) axis resulting in the development of gonads (Sharp, 2005). Our research has shown that the color of light plays a key role in the behavior, growth, and reproduction in domestic hens. However, as observed in mammals, the relative sensitivity of the avian pituitary to GnRH stimulation and the hypothalamic GnRH content is dependent on the physiological age, sex, and stage (Reddy *et al.,* 2012; Reddy *et al.,* 2016) of the birds. Light perceived by the retina, increases serotonin in the retina causing more synthesis of melatonin. Increased melatonin stimulates gonadotropin inhibitory hormone (GnIH) release into the hypothalamus ultimately causing the low secretion of FSH, LH and other steroid hormones for ovulation, egg formation and egg lay in hens (Bedecarrettes *et al.,* 2015). Hence the reproductive system in avians is controlled by two antagonistic neuropeptides: the inhibitory neuropeptide, GnIH and the stimulatory neuropeptide, GnRH-I. Hypothalamic neuropeptide GnRH-I is responsible for stimulating the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the anterior pituitary gland. LH is involved in

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stimulating the production of estradiol and progesterone and causes ovulation in females (Robinson and Etches, 1986), while FSH stimulates follicular maturation and differentiation (Mans and Taylor, 2008). Estradiol, produced by the small follicles, stimulates the development of the reproductive tract, secondary sex characteristics, and behavior and has been speculated to be involved in ovulation (Robinson and Etches, 1986). In this study, we evaluated the effects two different wavelengths of light at 450nm (normal) and 650 nm (red) of wavelengths of light on GnRH, GnIH, LH, LH pulsatile secretion and egg lay during the active period of egg in hens. Results obtained in this study may serve as a managemental tool to optimize the lighting schedule in commercial poultry settings.

Materials and Methods

Experimental Design

Twenty-four white leg horn birds at 42 weeks of divided as control and treated groups consisting of 12 birds in each. Birds were housed in individual cages (one bird per cage) under two-tier battery system. Birds in both the groups were fed as per the standard specifications (Ranjhan, 1993) and were provided 16 h light and 8 h dark. Feed intakes in both the groups were not altered due to treatment. Water was made available round the clock throughout the experimental period. At 42-52 weeks of age the birds in the treatment group were exposed to 650nm of wavelengths of light (red spectrum of light- treatment group). Controls were exposed to normal spectrum of light i.e., 450nm of wavelength of light. LED lights (white and red) 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 a 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 energy level penetrating through the skull (Reddy *et al.,* 2012). The control group was housed in a separate partisan with normal LED light bulbs at 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 for both the groups. Total number of pause days during the 11-week period (77 days) was recorded. The ovipositions patterns in both the groups were observed. The ovipositons were regular with long log sequences with less pause days in birds exposed to red spectrum of light compared to controls. (Fig. 4)

Collection of blood samples

Blood samples were collected from each bird by superficial veinepuncture of the brachial vein starting from 42 weeks of age onwards at weekly intervals and continued until the end of the experimental period at 52 weeks of age. At 47th weeks of age, blood samples were collected with cannulater 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 (Reddy *et al.,* 2012). Plasma was separated and stored at -20^0 C for hormone assay.

Studies *In vitro*

Hypothalami were dissected to include GnRH-I and GnIH (Lal *et al.,* 1990) cell bodies. Hypothami and pituitary glands were weighed and snap frozen in liquid nitrogen and stored at -80^{0}c until RNA extraction. Hypothalamic cell bodies containing GnRH-I and GnIH were cultured separately from treated and control group of birds in Modified eagle Eagle Media (DMEM) with phenol red (invtrogen), 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 1ul Trizol (invitrogen) for hypothalami. The tissues were disrupted using the 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 dH_2O 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 the 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.,* (1996). The sequences for chicken GnIH were made using primer design

software from gene bank 189: FAGAAATGAAAGACTGGGGATCA, 409 R: ATCTCCCAAGCCTGTACGATAA. 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°C, 20s; 62°C, 20S; 72°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 5.48% and 9.22% respectively, with sensitivity of the hormone 0.01 ng/ml per tube as per the method described previously (Sharp *et al.,* 1987).

Statistical analysis

The statistical analyses were carried out following the standard method. Measurements were given as mean±SE. The significance of differences between means was analyzed by F test. The data on egg production, LH, were subjected to correlation coefficient analysis to study the influence of the hormones on egg production. Differences were considered significant at a value of $p<0.01$ (Snedecor and Cochran, 1994).

Results

Hormonal parameters:

GnRH-I mRNA:

There were differences in hypothalamic GnRH-I mRNAs and GnIH mRNA 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.1). GnIH mRNA expression was significantly higher in hens exposed to normal spectrum of light relative to the treated birds.

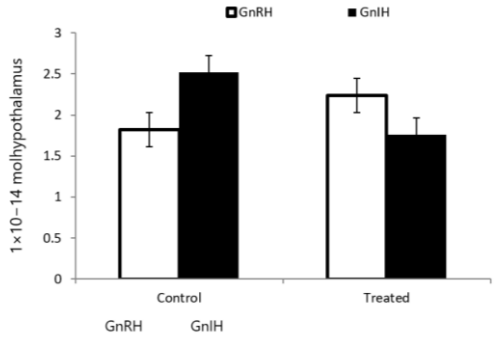

Fig. 1. GnRH and GnIH between control and treated birds.

LH

Plasma LH concentration in control group varied between 2.61 ± 0.01 ng/ml to 5.39 ± 0.1 ng/ml during 42 to 52 weeks of age (Fig 2). In treatment group plasma LH increased from 2.71 ± 0.21 ng /ml to 5.42± 0.3 ng /ml during 42 to 52 weeks of age. LH levels fluctuated between the two groups and birds exposed to red spectrum showed significantly ($P<0.01$) higher levels relative to the controls. Treated group, showed higher levels of LH with greater magnitude for eleven weeks. Three hourly secretion of LH surges in treated birds occurred mostly before the noon with an highest concentration of LH 7.84± 0.19 ng/ml, and in controls LH surges occurred early in the evening in the controls with highest concentration of 6.01±0.15 ng/ml (Fig. 3). However, intermittent fluctuations in LH surges were observed in both control and treated groups.

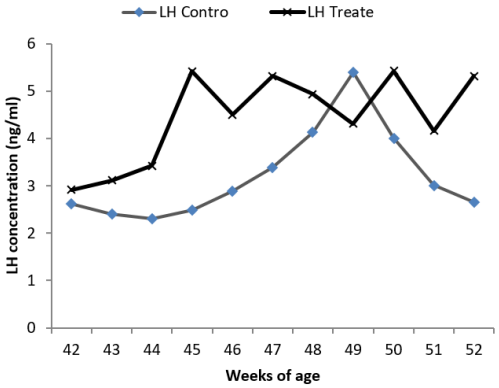

Fig. 2. Mean plasma LH concentration between control and treated birds

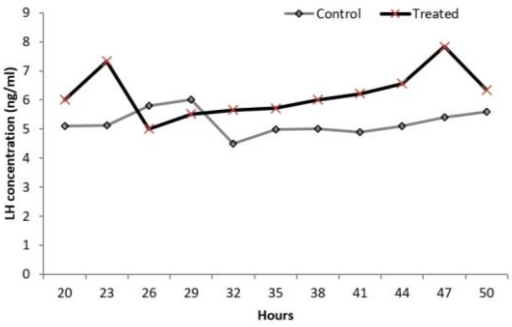

Fig. 3. Hourly LH profile in control and treated birds

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 42 to 52 weeks of age was significantly ($p<0.01$) higher than the controls (Fig. 4). It fluctuated between 80.92% to 85.68% and 88.06% to 93.23% in birds exposed to normal spectrum of light and red spectrum of light respectively. The difference in egg production/ bird/week was significant between

two groups from 44 weeks of age to 52 weeks of age. The percentage of pause days during the 11-week period (77 days) was significantly lower in the treatment group as against the control group. Egg production in treatment group increased by 11.731% between 42 and 52 weeks of age, compared to the control group. Egg production in birds exposed to red spectrum of light, was positively correlated with LH profile (r= 0.69), whereas GnRH I mRNA level was positively correlated with LH as (r = 0.73) and positively correlated with egg lay (0.77). However, GnIH was negatively correlated with LH (r = -0.64) and egg production (r =-0.69) and positively correlated with pause days (r = -0.68).

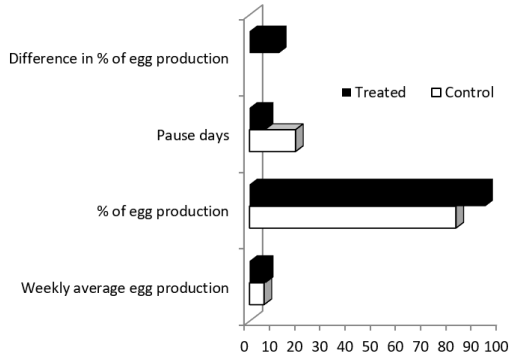

Fig. 4. Egg production, pause days and weekly egg lay in control and treated birds**.**

Discussion

Earlier studies proposed that photoperiod controls the synthesis and release of both hypothalamic neuropeptides which in turn interact at the level of the anterior pituitary gland to regulate the synthesis and release of gonadotropins LH and FSH. Furthermore, it was also pro-posed that beyond controlling hypothalamic outputs, changes in the photoperiod are associated with a switch in pituitary sensitivity from GnIH to GnRH. Our results demonstrate for the first time on the expression pattern of GnRH and GnIH mRNA levels in birds exposed to longer wavelengths of light (675nm i.e., red spectrum of light) and normal/visible wavelength of light (450nm) along with LH profile and egg production during 42-52 weeks of age in hens. In our study, birds exposed to long (red) spectrum of light (treated group) showed significantly (P<0.01) higher levels of GnRH mRNA (Fig.1) as compared to the birds exposed to normal/short wavelengths (white) of light (control group). We found that exposure of birds to longer wavelengths of light resulted in increased GnRH expression, whereas exposure of birds to normal/short wavelengths of light increased the GnIH expression. Stimulation of the hypothalamus with longer wavelengths of light (675nm) increases the secretion of GnRH mRNA (Bédécarrats *et al.,* 2015; Reddy *et al.,* 2012) into the hypothalamus. Monochromatic longer wavelengths of light spectrum stimulate receptors to secrete GnRH into the hypothalamus and these receptors are suggested to be sensitive to the light passed through the skull instead of light passing through the retina of the eye. Longer wavelengths of light of light penetrate the skull and possibility stimulated the receptors to release more GnRH secretion into the hypothalamus (Bédécarrats *et al.,* 2015) as observed in the treated birds. We observed that there were differences in GnRH mRNA s in birds exposed to red spectrum of light and to visible light suggest that changes in the synthesis of GnRH are the immediate cause of increased LH secretion in treated hens. This increase could be attributed to the photons of longer wavelengths (red spectrum of light 650nm) to penetrate the hypothalamus more effectively than photons of short wavelengths (normal spectrum of light 450nm; Lewis and Morris 2000; Bédécarrats *et al.,* 2006) and acts directly on hypothalamic extra-retinal photoreceptors to stimulate HPG-axis (Prescott and Wathes 1999; Chaiseha and Halawani 2005). The decrease in hypothalamic GnRH mRNA found in control hens is in accordance with earlier findings suggest that hypothalamic GnRH peptide content decrease with decreased egg laying in ageing broilers (Sharp *et al.,* 2005; Mobarkey *et al.,* 2010).

Shorter wavelengths of light elicit its responses through retina of the eye because shorter wavelengths of light (450nm) may not be able penetrate the skull and thus light at that intensity is unable or less likely to excite receptors responsible for release of GnRH into the hypothalamus. Decreased testicular size and low FSH concentration in pullets also supports this hypothesis (Bédécarrats *et al.,* 2006). In this experiment, control birds exposed to normal wavelength of light showed low levels of GnRH and higher levels of GnIH expression. It was pro-posed that beyond controlling hypothalamic outputs, changes in GnIH are more associated with photoperiod, stimulation of melatonin and GnIH pathway. Thus, GnIH in the control group was significantly (P>0.01) higher compared to treated group (Fig. 1). In turn, this integration at both the hypothalamic and pituitary levels regulate the maturation and function of the gonads and pituitary sensitivity from GnRH to GnIH (Saldanha *et al.,* 2001; Bédécarrats *et al.,* 2009). Increase in GnIH mRNA in controls exposed to normal wavelengths of light remains to be elucidated. It was proposed that, shorter wavelengths of light stimulate melatonin synthesis in response to photoperiod. Melatonin in turn stimulate GnIH pathway and there by enhances the GnIH secretion in birds (Bédécarrats *et al.,* 2009). It is tempting to speculate that shorter wavelengths of light enhance GnIH thereby affecting the gonadotrophic hormone (LH) as observed in the control group (Fig.3). In addition, the increase in GnIH

expression as observed in control hens photo stimulated with normal wavelengths of light may increase in GnIH, potentially resulting in the inhibition of GnRH thus blocking preovulatory LH surges. However, to date this hypothesis still needs to be experimentally verified (Chowdhury *et al.*, 2010).

Longer wavelengths of light triggers release of gonadotropins by the pituitary gland, which initiates folliculogenesis and estradiol (E2) production. In turn, E2 down regulates the synthesis of GnIH-R at the pituitary level. As maturation progresses, GnRH release dominates GnIH, the pituitary GnIH-R/GnRH-R ratio switches toward GnRH and the axis becomes fully functional (Bédécarrats *et al.*, 2009). In mature hens, high levels of E2 and progesterone (P4) maintain the inhibition of the GnIH. E2 may also contribute to maintain high levels of GnRH (Bédécarrats *et al.,* 2009). Changes in LH profile may be attributed to longer wavelengths are transmitted through neural tissue more readily than shorter wavelengths (Lewis *et al.,* 2005) and might have up regulated the GnRH mRNA and down regulated the GnIH in this study. However, a decrease in GnRH mRNA and an increase in GnIH mRNA found in control hens imply that changes in GnRH release are responsible for decreased LH secretion. This is supported by an observation on a mammalian GnRH cell line (GT1), where steady state levels of GnRH mRNA correlate with GnRH release (Pitts *et al.*, 2001). Low GnRH mRNA in turn decreases the synthesis of pituitary LH and FSH which in turn decreases the production of E2 and P4. Low E2 up regulates the synthesis of GnIH-R at the pituitary level and decreases the production of LH profile which is reflected in control hens. In ageing female rats and women, GnRH pulse frequency is reduced (Rossmanith *et al.*, 1991) and a similar decrease in GnRH /increase in GnIH with LH pulse frequency may explain the changes in plasma LH profile in hens of the control group (Fig.2; Fig.3). In addition, as GnIH neurons project to the median eminence, it was also shown to inhibit the synthesis and release of pituitary gonadotropins. This implies that within the hypothalamus, integration of external and internal signals must converge to allow or prevent egg lay in hens (Bédécarrats *et al.*, 2006) and hence the increase in GnIH mRNA expression and decrease in plasma LH in controls also agrees with a previous study in quails. The results obtained in this study also support this hypothesis. The observation that plasma LH frequency (Fig.3) occurs earlier in birds exposed to red spectrum of light is similar to the finding that in mammals, an increase in GnRH pulse frequency preferentially stimulates LH release whereas a decrease in GnRH pulse frequency decreases in LH release (Bédécarrats *et al.*, 2006). 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 (Reddy *et al.*, 2012) resulting in LH secretion being more sensitive to the decreased stimulatory action of estrogen in hens than in mammals. The observation that during 42 to 52 weeks of age following the exposure of birds to red spectrum of wavelength, the reproductive system of domestic hen in treated group changed from low functional sate to a high functional state as compared to controls exposed to normal spectrum of wavelengths of light and this reflected less pause days between the sequences of egg lay and more egg lay in birds exposed to longer wavelengths of light as observed in treated birds during 42 to 52 weeks of age (Fig. 4).

Conclusion

We observed that red spectrum of light on GnRH mRNA was stronger than the normal incandescent light. The data also indicated that the predominance of higher wave lengths of light from the red band increased GnRH mRNA, compared to the normal light. Further, the birds exposed to normal spectrum of light are associated with an increase in GnIH mRNA and reduced plasma LH. The sensitivity of the bird to long-wave radiation (630–780 nm) is a result of deep tissue penetration (hypothalamic extra retinal photoreceptors) stimulating the reproductive axis. The more numbers of eggs were produced when exposed to longer wavelengths of light owing to its deeper penetrating power to stimulate the extra retinal photoreceptors. 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. Following this model, a substantial body of experimental evidence has emerged further enhancing our understanding of the molecular mechanisms controlling the hypothalamo-pituitary–gonadal axis (HPG) in chickens.

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