

Studying the Effect of Light Wavelength on Laying Hens (*Gallus gallus*)

By

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A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

In partial fulfilment of requirements

for the degree of

Master of Science

in

Animal & Poultry Science

Guelph, Ontario, Canada

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ABSTRACT

STUDYING THE EFFECT OF LIGHT WAVELENGTH ON LAYING HENS (*Gallus gallus*)

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University of Guelph, 2014

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In poultry, higher wavelengths of light are more effective at stimulating extra-retinal photoreceptors, increasing reproduction. In practice, multiple light sources are used in layer barns, each emitting different light spectra. This study aimed to design a novel LED bulb and evaluate the effect of light wavelength on egg production. Our initial results in non-commercial laying hens, found that red light is critical to stimulate the reproductive response, therefore we designed a LED bulb emitting 60% red light (LED-R). This bulb was able to effectively stimulate the reproductive axis in commercial laying hens without negatively affecting egg production, egg quality or stress in birds maintained either in cages or on floor pens. When exposed to light from the LED-R, feed consumption and body growth was reduced, without affecting cumulative number of eggs. As well, the LED-R bulbs consumed less electricity thus reducing cost of production.

This thesis is dedicated to:

My Parents,

John and Patricia Baxter

Acknowledgements

I would like to thank my parents, for all their support and love and being so understanding while working on my thesis. Supporting my decision to go into a field they were unfamiliar with means a lot and I really appreciate it. I would like to thank my friends, for their support and help with both editing and at the farm. I would also like to thank my partner Jacob, for all his love and patience in the writing of my thesis. I would also like to thank all the undergraduate students and grad students who helped throughout all the trials. The farm staff was also essential with each trial, ensuring the birds were well taken care. I would also to thank Alex Thies from Thies Electrical for his involvement on the design and production of the LED bulb.

I would like to thank my advisory committee, Dr. Jim Squires and Dr. Alexandra Harlander. Your advice and input during the writing and analyzing of my data has been tremendously helpful and I appreciate the time and patience you had with me. The Animal Poultry Science department has been key to my graduate studies for all the administrative staff and support, who took the time to help me, I really appreciate it!

I would especially like to thank my advisor Gregoy Bedecarrats, who has provided me with great advice and encouragements during my graduate studies. He has provided me with amazing opportunities both academically and within the industry. He has been essential to my development as a scientist, which is the foundation of my future career.

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Chapter 1: Review: The Effects of Light Wavelength on Reproduction, Growth, Immune Response and Behaviour in Poultry

1.1 Introduction

Birds have retinal photoreceptors responsible for vision and non-visual photoreceptors (extra-retinal) responsible for detecting photoperiod and synchronizing their physiology to the environment (Kumar and Rani, 1999). Photoreceptors are composed of large proteins called opsins attached to an aldehyde of vitamin A, referred to as a chromophore (Hart, 2001). When a chromophore absorbs light, the photoreceptor goes through a conformational change triggering a biochemical signal affecting the release of neurotransmitters from the photoreceptor cell (Hart, 2001). Photoreceptors are located in three main organs: the retina of the eye, pineal gland and hypothalamus. The retina contains three photoreceptors: rods, cones and double cones (Perry, 2004). Rods are responsible for vision during low illumination and do not detect colour (Hart, 2001). The role of the double cone is unclear, but it does respond to changes in light and may be involved in luminance perception (Prescott and Wathes, 1999; Hart, 2001). Cones are responsible for photic colour vision and are tetrachromatic in fowls, containing four different photoreceptive cone pigments (Prescott and Wathes, 1999). Photoreceptive pigments have a maximum sensitivity to violet (415nm), blue (455nm), green (508nm), or red (571nm) (Yoshizawa, 1992; Perry, 2004). The cone photopigment in the red spectrum of the chicken retina is referred to as iodopsin (Yoshizawa, 1992). Arylalkylamine N-acetyltransferase (AA-NAT) is the first enzyme in the stepwise conversion of serotonin to melatonin. In the chicken, AA-NAT mRNA is highly expressed in the outer nuclear layer of the retina, where photoreceptors nuclei are located (Bernard et al, 1997). This suggests that retinal serotonin is converted into melatonin which exerts an inhibitory effect on reproduction (Bernard et al, 1997).

Pineal photoreceptors contain the pigments pinopsin and melanopsin (Holthues et al., 2005). Photoreceptors receive light signals and transmit them to oscillators which control the bird's circadian rhythm via the synthesis and release of melatonin (Pelham and Ralph, 1972; Pang et al., 1974; Nir et al., 1987; Kumar and Rani, 1999). Pinopsin is responsible for mediating the inhibitory effect of light on melatonin synthesis (Holthues et al., 2005). Although the role of melanopsin is unclear, evidence suggests that it may entrain the effects of light in chicks (Holthues et al., 2005). Melatonin, produced during the dark phase, is responsible for daily and annual circadian rhythms in birds (Trivedi and Kumar, 2014). Melatonin has also been reported to be involved in reactivating immunity in immunosuppressed birds by acting as immune enhancing agent in unchallenged quails (Moore and Siopes, 2002) and can increase plasma growth hormone concentration (Zeman et al., 1999). It is also responsible for the activation of the inhibitory pathway of the reproductive axis (Ubuka et al., 2005). It has yet to be determined if pinealopsin and melanopsin have a maximum sensitivity to a specific wavelengths of light, therefore it is unclear how light spectrum effects pineal photoreceptors.

Encephalic photoreceptors were first discovered by Benoit and Ott (1944) who found that eye enucleated and pinealectomized male ducks retain a photoperiodic gonadal response. Direct illumination of the hypothalamus with a quartz rod that emits various colours of light resulted in an equal gonadal response by each colour, suggesting hypothalamic photoreceptors are not sensitive to specific wavelength (Benoit and Ott, 1944). Foster et al. (1985) determined that deep brain photoreceptors of quail have a peak sensitivity to 492nm, which is similar to the peak sensitivity of rhodopsin, suggesting rhodopsin-like pigments in the hypothalamus are involved in photoperiodic gonadal response in quail (Foster et al., 1985a; Oishi and Ohashi, 1993). However, Davies et al. (2012) found vertebrate ancient (VA) opsin, which is expressed in neurons in the

hypothalamus, also has an absorbance sensitivity of 490nm and could be another potential hypothalamic photoreceptor (Davies et al., 2012). Nakane et al. (2010) also detected the photoreceptor Opsin 5, in neurons contacting the cerebrospinal fluid in the paraventricular organ and neurons extending to the median eminence. They reported a peak sensitivity of Opsin 5 to wavelengths at 420nm, reiterating that encephalic photoreceptors are more sensitive to shorter wavelengths (Nakane et al., 2010). Since longer wavelengths have a higher radiant flux, it is hard to determine if the encephalic photoreceptors have a maximum sensitivity to longer wavelengths or if longer wavelengths simply have more energy and are capable of passing through the brain more effectively to stimulate these photoreceptors (Foster et al., 1985a). As multiple photopigments have been suggested to be involved, it still remains unclear which one mediates the photoperiodic response of the deep brain photoreceptors.

Characteristics of light depends on light quantity (number of photons delivered) and quality (spectral composition). In modern day poultry houses, the light intensity is measured in lux, a unit based on human spectral sensitivity. Humans and birds perceive light differently due to differences in spectral sensitivity and density of photoreceptive pigments between human and avian retinas (Prescott and Wathes, 1999). Prescott and Wathes (1999) determined the retinal spectral sensitivity curve of the chicken retina and found birds are visually most sensitive to wavelengths of 533-577nm. The perceived intensity is relative to the spectral power output of the light and the spectral sensitivity of the fowl retina. For example, incandescent light at 100 lux will be perceived the same as fluorescent light at 77 lux due to the spectral sensitivity of the hen (Prescott and Wathes, 1999). Red light has also been reported to appear

brighter to birds than blue light at lower intensities (Prayitno and Phillips, 1997). The retina can be stimulated with light at low intensities, whereas higher light intensities are required

to stimulate hypothalamic photoreceptors (Harrison and Becker, 1970). Therefore, it has been suggested that lower wavelengths (blue/green light) require higher intensities to stimulate hypothalamic photoreceptors (Pang et al., 1974).

The majority of commercial poultry operations rely on controlled environments in which artificial lighting is used to manage bird's growth, behaviour, and production. Incandescent lights have been the primary light source used by the North American poultry industry. Incandescent light has a broad spectral output ranging from 400-1050 nm with a gradual peak at 925 nm, which mimics sunlight (Siopes and Wilson, 1980b; Chignell et al., 2008; Benson et al., 2013). However, these bulbs are energy inefficient and the global push to reduce greenhouse gases has forced producers to find alternative light sources. Two of the main alternative light sources used by the industry are fluorescent light and light emitting diodes (LED). Fluorescents emit light using electricity to excite mercury vapour (Benson et al., 2013) resulting in a spectral output ranging from 400-750 nm with a sharp peak in the green spectrum (558 nm) (Siopes and Wilson, 1980b; Chignell et al., 2008). Although fluorescent lights are more efficient, they typically flicker at low intensity, they do not dim well, and require specialized disposal due to the mercury (Benson et al., 2013). LEDs emit light using a solid-state semiconductor (Benson et al., 2013). They are among the most efficient light sources, can be manufactured to deliver a defined and stable spectral output (Steranka et al., 2002) and are dimmable (Benson et al., 2013). With new lighting technologies and the advancement in genetic selection of domestic poultry, there is often conflicting evidence as to the effects of light spectrum on poultry. Older studies used older technologies with less control over the spectral output and used birds of a different genetic makeup. As light spectrum has been shown to affect the physiology of birds, this review intends

to summarize the current state of knowledge on its effects on reproduction, growth, immunity and behaviours in avian species.

1.2 Reproduction

1.2.1 Overview

The avian reproductive axis is tightly regulated by two antagonistic neuropeptides: the inhibitory neuropeptide, gonadotropin inhibitory hormone (GnIH) and the stimulatory neuropeptide, gonadotropin releasing hormone (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 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 behaviour and has been speculated to be involved in ovulation (Robinson and Etches, 1986; Etches, 1996; Rangel and Gutierrez, 2014). Estradiol is also involved in stimulating the hepatic synthesis of major yolk components and increases the activity of calcitriol, which increases calcium levels in the blood, making it available for shell synthesis (Etches, 1996). Progesterone, produced by the large follicles, is involved in regulating ovulation (Robinson and Etches, 1986; Ottinger and Bakst, 1995; Rangel and Gutierrez, 2014). GnIH, a hormone responsible for the tonic inhibition of the reproductive axis, acts directly on GnRH neurons in the hypothalamus to decrease GnRH neuronal activity (Bentley et al., 2003; Ubuka et al., 2008; Bedecarrats et al., 2009; Tsutsui et al., 2010) and also acts directly on the pituitary gland, inhibiting synthesis and release of gonadotropins, preventing development of the ovary (Ubuka et al., 2006).

Both stimulatory and inhibitory pathways regulating the reproductive axis are under the influence of photoperiod. The stimulatory pathway is activated under long day photoperiods, as GnRH-I mRNA and peptide levels increase during photostimulation (Dunn and Sharp, 1999; Bedecarrats et al., 2006; Thayananuphat et al., 2007). Also, opsin-positive neurons have been reported to be in direct contact with GnRH-I dendrites, indicating a potential pathway for photoreceptors to regulate the reproductive axis (Saldanha et al., 2001). The inhibitory pathway is activated during short day photoperiods and is regulated by melatonin, a hormone synthesized during the dark phase. Removing melatonin sources, via enucleation and pinealectomy, decreased GnIH mRNA and peptide levels, which were restored when melatonin was administered (Ubuka et al., 2005; Chowdhury et al., 2013). As well, GnIH neurons in the quail express melatonin receptors, allowing melatonin to directly regulate GnIH (Ubuka et al., 2005). Thus, when extrapolating these findings, birds have a higher amount of melatonin under short day photoperiods, stimulating GnIH synthesis and release, which inhibits the stimulatory pathway while the lack of photostimulation of deep brain photoreceptor maintains birds in a sexually immature state (Bedecarrats et al., 2009). Photostimulation directly stimulates the GnRH pathway increasing the amount of gonadotropins which stimulate ovarian development. Simultaneously, an increase in day length also decreases the amount of melatonin, decreasing GnIH, and removing the inhibition on the stimulatory axis (Bedecarrats et al., 2009). As birds remain under long day photoperiods, they continue to sexually mature, stimulating gonadotropin release which increases production and the effects of sex steroids (Bedecarrats et al., 2009).

The role of the avian retina in controlling reproduction remains controversial. Experimental evidence has shown that hypothalamic photostimulation activates the reproductive axis (Dawson et al., 2001; Saldanha et al., 2001), whereas retinal stimulation may decrease

reproductive performance in chickens (Siopes and Wilson, 1980b; Mobarkey et al., 2010; Mobarkey et al., 2013). However, it is still unclear whether photoreceptors are sensitive to specific light wavelengths or if stimulation depends on light intensity. For example, the retina can be stimulated by light at low intensities, where higher intensities are required to stimulate hypothalamic photoreceptors (Harrison and Becker, 1970). As well, higher wavelengths contain more energy and are able to more easily penetrate through the skull and brain tissue and to stimulate the hypothalamus (Pang et al., 1974; Foster et al., 1985; Oishi and Ohashi, 1993; Mobarkey et al., 2010). Therefore, it was suggested that lower wavelengths (blue/green light) require higher intensities to stimulate hypothalamic photoreceptors (Pang et al., 1974). The avian retina has a peak sensitivity to wavelengths in the yellow and green spectrum, but it has been demonstrated that birds exposed to green light have an a delay in rate of sexual maturity, lower egg production, and lower levels of steroids and GnRH-I mRNA expression (Siopes and Wilson, 1980a; Mobarkey et al., 2010; Gongruttananun, 2011; Mobarkey et al., 2013; Baxter et al., 2014). Therefore, it has also been suggested that exposure to green light inhibits reproduction via stimulating only retinal photoreceptors (Siopes and Wilson, 1980a; Mobarkey et al., 2010; Gongruttananun, 2011; Mobarkey et al., 2013). Alternatively, exposure to higher wavelengths increased level of egg production and resulted in higher steroid and gonadotropin concentrations and higher neuropeptide mRNA expression (Foss et al., 1972; Kim et al., 2012; Reddy et al., 2012; Hassan et al., 2013; Huber-Eicher, et al., 2013; Baxter et al., 2014). Generally these results have been consistent over a number of different avian species for both males and females. The potential mechanism of retinal inhibition on reproduction is unknown, however a major contributor is thought to be serotonin as it is synthesized in the retina and the hypothalamus (Mobarkey et al., 2010; Norgren and Silver, 1989). It has been suggested that serotonin may act

directly to inhibit reproduction or maybe a precursor to secondary messengers such as melatonin (Mobarkey et al., 2013).

1.2.2 Sexual Maturity

In commercial poultry systems, the onset of sexual maturity can be stimulated by increasing photoperiod, which can be modified by the spectral output. The first study looking at the effect of light spectrum on sexual maturity was performed by Beniot and Ott (1944), when male ducks were exposed to monochromatic lights, illumination with red light led to the largest testis growth (Benoit and Ott, 1944). To determine hypothalamic photoreceptors sensitivity to light spectrum, the hypothalamus was directly illuminated with monochromatic light (Benoit and Ott, 1944). They discovered that there was no difference in testis size between the birds exposed to different light spectra (Benoit and Ott, 1944). This suggests hypothalamic photoreceptors are not sensitive to wavelength and the higher testis growth under red light was due to higher wavelengths being more efficient at stimulating the reproductive axis (Benoit and Ott, 1944). Red light has also been reported to increase testis weight in cockerels (Foss et al., 1972), Japanese quail (Woodard et al., 1968; Oishi and Lauber, 1973; Oishi and Ohashi, 1993), and black headed buntings (Kumar and Rani, 1996). Red light not only affects testis growth but also has been reported to increase testis activity in starlings (Bissonnette et al., 1931; Burger, 1943) and stimulate higher comb weight in the Japanese quail (Foss et al., 1972). A recent study performed on starlings determined one hour pulses of red light increase testis development regardless of intensity, whereas birds exposed to green light did not show a photoperiodic response (Kumar and Kumar, 2013). Red light has a similar effect on sexual maturity in female avian species. Red light triggers earlier age at first age in laying chickens (Gongruttananun, 2011; Kim et al., 2012; Hassan et al., 2013; Baxter et al., 2014) and Japanese quail (Woodard et al.,

1968). Thus, from the research it is evident that red light enhances the photosexual response in both males and females.

Exposing birds to lower wavelengths often results in lack of stimulation of the reproductive axis. This is evident as there was no difference in testis weight between cockerels exposed to light in the green and blue areas of the spectrum and those in constant darkness, and these treatments had significantly lower testis weight compared to birds treated with red and white light (Foss et al., 1972). Similar results were found in Japanese quail (Woodard et al., 1968; Oishi and Ohashi, 1993). At equated energy levels, blue light resulted in cockerels having significantly lower testis weight than birds under red, green or white light (Oishi and Lauber, 1973). In addition, there was no difference in testis growth under blue light at two different intensities, which was similar to birds exposed to complete darkness (Oishi and Lauber, 1973). The different responses to wavelengths observed under varying energy levels suggest that wavelength is the most important parameter in controlling photo-sexual response; however it is unclear if this is due to absorption qualities or characteristics of the photoreceptor (Oishi and Lauber, 1973). Beyond a lack of stimulatory effect on sexual maturity, recent evidence also suggests that green light may be inhibitory (Mobarkey et al., 2010; Gongruttananun, 2011). Thai native laying hens exposed to day light and supplemented with fluorescent light, had a delay in age at first egg (Gongruttananun, 2011). The delay was attributed to the inhibitory effect of the green light being emitted from the fluorescent lights. There has also been a report of a delay in age at first egg for laying hens exposed to blue light (Hassan et al., 2013) and broiler breeder hens exposed to green light (Mobarkey et al., 2013) compared to birds that remained under the same light source they were raised on. Green light may have an inhibitory effect on germ cell activity in male starlings (Bissonnette et al., 1931). Although most studies indicate that green

light may cause a delay in age at first egg, Harrison et al. (1969) found that both male and females under filtered incandescent green and blue light began laying slightly earlier than those under red and white. Conversely to other data cited, laying hens (Siopes and Wilson, 1980b) and Japanese quail (Siopes and Wilson, 1980a) exposed to incandescent light (peaking at 925nm) had a significant delay in the onset of lay compared to blind birds exposed to incandescent light and sighted and blind birds exposed to fluorescent (peaking at 558nm) light. This indicates that incandescent light may have an inhibitory effect via the eye (Siopes and Wilson, 1980a; Siopes and Wilson, 1980b). However, when birds were photostimulated, there was no difference in age at first egg regardless of sight status or light treatment, suggesting that length of photoperiod had more to do with the delay (Siopes and Wilson, 1980b).

Although evidence strongly suggests an effect of light spectrum on sexual maturation, several studies failed to identify any effect. Lewis et al. (2007) found that illuminating birds with green and white light had no effects on the age at first egg. However in this study, green light was produced from filtered incandescent light and emitted the majority of its light in the red spectrum suggesting that the lack of difference in age at first egg was due to the similarity in spectral composition (Lewis et al., 2007). Another study, found no effect of light spectrum on the age at first egg in laying and turkey hens (Pyrzak et al., 1986; Pyrzak and Siopes, 1986). In this study, light was normalized for each light treatment suggesting enough energy was delivered to hypothalamic photoreceptors to stimulate the reproductive axis regardless of spectrum (Pyrzak and Siopes, 1986). However when birds were kept after molt, red and incandescent light resulted in earlier age at first egg, suggesting that higher wavelengths have a greater penetrating abilities through peripheral tissue to stimulate deep brain photoreceptors (Pyrzak and Siopes, 1986). Lastly, Nakane et al. (2010) exposed birds to white, blue, UVA and UVB light and found no

difference in testis size of pinealectomized and enucleated Japanese quail (Nakane et al., 2010). The lack of difference in testis weight between birds exposed to different light treatments was likely due to all light intensities meeting the threshold to stimulate the hypothalamic photoreceptors, as quails had been enucleated and pinealectomized.

1.2.3 Egg Production

Along with sexual maturation, light spectrum has also been reported to affect egg production in a number of avian species. Studies have found that laying hens maintained under monochromatic red light had higher egg production than birds maintained under white, green (Huber-Eicher, et al., 2013; Baxter et al., 2014) and blue LED light (Kim et al., 2012; Hassan et al., 2013). Similar results were seen with Japanese quail, where birds exposed to red light reached 50% rate of lay two weeks earlier and maintained a higher rate of production than birds exposed to blue and green light until 16 weeks of age (woa) (Woodard et al., 1968). Laying hens exposed to light sources containing higher wavelengths produced significantly more eggs than hens exposed to light sources with lower wavelengths during the first laying cycle (Pyrzak et al., 1987). The effects of higher wavelengths were more prominent during the second laying cycle after birds were molted (Pyrzak et al., 1987). This suggests that as birds age there is a change in transmittance of light through peripheral tissue, making longer wavelengths more efficient (Pyrzak et al., 1987). This was corroborated by another study that found leghorns exposed to red light at 72 woa and onward had significantly higher production than hens under incandescent light (Reddy et al., 2012). Broiler breeder hens exposed to fluorescent light had lower egg production the birds maintained under incandescent light from 58 woa and onward (Ingram et al., 1987). Laying hens under monochromatic green LEDs had a one week delay to peak production and produced significantly fewer eggs (Li et al., 2014). Using the Thai native hen as a model,

there was no significant difference in egg production but hens exposed to daylight supplemented with fluorescent light produced on average ten less eggs than those exposed to red and daylight (Gongruttananun, 2011). However, each light treatment was set at different intensities, suggesting that at these intensities lower wavelengths produced enough energy to maintain sufficient egg production (Gongruttananun, 2011). Harrison et al. (1974) found birds exposed to shorter wavelengths had consistently changing times between consecutive oviposition suggesting shorter wavelengths are less efficient at entraining time of lay.

Not only has red light been reported to be stimulatory, but green light has also been found to have an inhibitory effect on egg production. In a study conducted by Mobarkey et al. (2010), broiler breeder hens were stimulated with combinations of red and green light to activate hypothalamic or retinal photoreceptors. Hens under green light treatment had significantly lower production than hens exposed to red and white light (Mobarkey et al., 2010). These results support the theory that the eye is unnecessary for stimulating the reproductive axis and, the retina may in fact have an inhibitory effect (Mobarkey et al., 2010). This was further confirmed in a follow up study in which broiler breeder hens exposed to green light had a significantly lower cumulative number of eggs and overall production levels than birds exposed to white light (Mobarkey et al., 2013). When blocking the serotonin pathway, a potential reproductive inhibitor, with parachlorophenylalanine, the inhibition on the reproductive axis in hens was alleviated when birds were maintained under green light. This suggests that the retina may inhibit reproduction via serotonin pathway (Mobarkey et al., 2013). Similarly, Baxter et al. (2014) found sighted hens exposed to monochromatic green light dropped out of production significantly earlier than blind birds, further suggesting retinal stimulation inhibits reproduction. With no difference between blind and sighted birds exposed to red and white light, inhibitory

effects may be less prevalent when there is sufficient hypothalamic stimulation (Baxter et al., 2014).

However, as for sexual maturity, not all studies report a stimulatory effect of higher wavelengths on egg production. Jones et al. (1982) found a high intensity of white light negatively effects egg production in turkey breeders, but red light at high intensities and red and white light at low intensities resulted in no difference in egg production. It should be noted each light treatment was not normalized to the number of photons delivered to the bird (Jones et al., 1982). Similar results were found by Carson et al. (1958) where chickens exposed to various spectra via filtered florescent light, had no difference in age to 50% percent production and mean egg production per bird over 400 days. However it should be noted between the two studies that various light sources were used and birds were exposed to different lighting schedules.

1.2.4 Neuropeptides, Gonadotropins and Steroids

Rate of sexual development and higher reproductive performance suggest that light spectrum may act directly at the level of the hypothalamus. This is supported by data on gene expression of reproductive neuropeptides and gonadotropins, organ weights and gonadotropins and steroid hormone concentrations. When broiler breeder hens were exposed to combinations of red and green light, birds exposed to longer periods of red light had a significantly higher levels of hypothalamic GnRH-I mRNA and pituitary LH- β mRNA (Mobarkey et al., 2010). Furthermore, broiler breeder hens maintained under white light had significantly higher GnRH-I, LH- β or FSH- β mRNA than hens under green light (Mobarkey et al., 2013). Similar results were seen in laying hens where birds exposed to red light at the end of lay (72 woa) had higher hypothalamic GnRH-I mRNA levels (Reddy et al., 2012). Exposure to higher wavelengths also

increases steroid and gonadotropin levels in circulation. Broiler breeders exposed to red light had increased levels of estradiol, progesterone and testosterone during the first week post-photostimulation (Mobarkey et al., 2010). Laying hens under monochromatic red light or a combination of red and green light had a higher concentration of estradiol and FSH compared to blue and green monochromatic lights (Hassan et al., et al., 2013). As well, cockerels exposed to red light had higher levels of circulating gonadotropins (Foss et al., 1972). Blind and sighted laying hens under red light had significantly higher levels of estradiol at photostimulation than hens under white and green light regardless of sight status (Baxter et al., 2014). Similar results were seen by Reddy et al., (2012) who reported that hens under red light had higher LH surge and overall levels of LH, estradiol and progesterone. Thai native hens exposed to monochromatic red light had higher serum estradiol levels (Gongruttananun, 2011). This suggests that the higher level of production in birds exposed to higher wavelengths can be attributed to an increase in stimulatory neuropeptides (GnRH-I) which may stimulate gonadotropin gene expression and release, resulting in ovarian stimulation and steroid hormone production. More stimulation of reproductive organs can translate to higher organ weight. It was reported that laying hens exposed to red light during the end of lay had significantly higher pituitary weight, ovary weight, oviduct weight and number of yellow follicles greater than 8 mm in diameter (Reddy et al., 2012). Higher ovarian weight, stromal weight and number of follicles were also seen in hens under red light and a combination of red and green light (Hassan et al., 2013). On the other hand, Mobarkey et al. (2010, 2013) found retinal stimulation with green light inhibited reproduction and reduced gonadotropins and steroid levels. Foss et al. (1972) observed that birds maintained under low wavelengths or wavelengths above 700 nm had the same gonadotropin content as

birds kept under darkness. This suggests that lower wavelengths may not have an inhibitory effect but rather a lack of stimulation on the reproductive axis.

1.2.5 Egg Quality

Egg weight and shell strength are important factors for the sale of table eggs, however, observation made on the effects of light spectrum on these parameters are conflicting. Laying hens under blue light had heavier eggs than birds under red and white light from 41-50 woa (Kim et al., 2012). Similar results were seen by Pyrzak et al. (1987) where shell quality was improved under green light and egg weights were higher under green and blue light during the first and second laying cycle. Conversely, no differences were found in egg weight and eggshell quality under red, green, white and blue light, combined light colour treatments of red, blue and green (Hassan et al., et al., 2013), and daylight supplemented with fluorescent or LED light (Gongruttananun, 2011). Li et al. (2014) found that birds under red and white light had the heaviest egg and hens under blue and green light produced significantly lighter eggs, however, egg shell strength was higher in birds under green light than birds under white and blue light (Li et al., 2014). Fertility and hatchability are important factors for breeding flocks, but few studies have looked at the effects of light spectrum on these parameters. Woodard et al. (1968) found that fertility decreases in Japanese quail kept under blue light but hatchability and egg weights are unaffected by light spectrum. Conversely, Ingram et al. (1987) found that exposing broiler breeder hens to incandescent or fluorescent lights had no effect on fertility, hatchability and egg weights (Ingram et al., 1987). With limited and conflicting results, it is evident that more research is needed to assess the effects of light spectrum on egg quality in birds.

1.2.6 Conclusion

Overall, based on the current literature, higher wavelengths of light are more effective at stimulating the reproductive axis; an overview of results can be seen in Table 1.1. However, whether these effects are the result of a direct stimulation of hypothalamic photoreceptors specific to higher wavelengths or the non-specific effects of wavelengths strong enough to penetrate through the skull and or peripheral tissues more effectively is still unclear. Nonetheless, it is safe to state that red light is the most effective at stimulating the reproductive axis. On the other hand, the possible inhibitory effects of green light on the reproductive axis are less clear. Many studies indicate that the poor reproductive performances of birds maintained under green light is due to a lack of stimulation rather than an inhibition (Woodard et al., 1968; Foss et al., 1972; Oishi, 1993) while it has also been suggested that green light may inhibit reproduction via retinal stimulation (Mobarkey et al., 2010; Mobarkey et al., 2013). Variation between results may be due the use of different light sources, and strains of birds, as well as the differences in light intensity. Therefore, more research is required to determine if and how green light may inhibit the reproductive axis.

Table 1.1 An Overview of the Effect of Higher Wavelengths on the Reproductive Axis in Avian Species.

The Effect of Higher Wavelengths on the Reproductive Axis	Reference
Higher testis weight	Benoit and Ott, 1944; Woodard et al., 1968; Foss et al., 1972; Oishi and Lauber, 1973; Oishi and Ohashi, 1993; Kumar, 1996
Increased testis development	Bissonnette et al., 1931; Burger, 1943; Kumar et al., 2013
Earlier age at first egg	Woodard et al., 1968; Gongruttananun, 2011; Kim et al., 2012; Hassan et al., et al., 2013; Baxter et al., 2014
Higher egg production and cumulative number of eggs	Ingram et al., 1987; Pyrzak et al., 1987; Gongruttananun, 2010; Mobarkey et al., 2010; Kim et al., 2012; Reddy et al., 2012; Huber-Eicher et al., 2013; Mobarkey et al., 2013; Hassan et al., 2013; Baxter et al., 2014; Li et al., 2014
Higher expression of GnRH-I, LH-B and FSH-B genes	Mobarkey et al., 2010; Reddy et al., 2012; Mobarkey et al., 2013
Higher LH surge and circulating level of gonadotropins and steroid hormones	Foss et al., 1972; Mobarkey et al., 2010; Gongruttananun, 2011; Reddy et al., 2012; Baxter et al., 2014
Higher pituitary, oviduct, ovary weights and higher number of small follicle	Reddy et al., 2012

1.3 Growth

Growth can be measured as body weight gain, feed intake, feed conversion rate, muscle cell proliferation and protein and mRNA levels in both *in ovo* and post hatch animals. The current state of knowledge on the effects of light spectrum on growth in poultry is presented below. However it should be noted that the majority of the research has been performed on broiler birds.

1.3.1 *In ovo* Illumination and Post Hatch Growth in Broilers

Extensive research has been performed on how light spectrum effects growth of broilers birds, starting *in ovo* and continuing as chicks hatch and grow. From 5 days of incubation until hatch, eggs were exposed to monochromatic green light in 15 min light increments or complete darkness. They found eggs exposed to green light increased embryonic weight after 14 days of incubation and resulted in higher percentage of body weight (Rozenboim et al., 2004b). Fourteen days incubation corresponds to when retinal photoreceptors become active in chicks (Bruhn and Cepko, 1996), suggesting that retinal photoreceptors may be involved in this increase in body weight. Post hatch, *in ovo* illuminated male chicks had a higher body weight after 7 days and females after 14 days until 42 days of age regardless of rearing illumination (Rozenboim et al., 2004b). It should be noted that stimulating *in ovo*, and rearing birds under green light had no additive effect, but higher growth was achieved during *in ovo* illumination. Similar results were found by Zhang et al. (2014), where continuous *in ovo* stimulation with green light led to significantly higher body weight and pectoral muscle at 7 days post hatch. *In ovo*, stimulation with green light resulted in higher satellite cell mitotic activity 1 and 3 days after hatch and had significantly higher myofibers in the pectoral muscle 5-7 days post hatch (Zhang et al., 2012).

Chicks exposed to green light *in ovo* had higher number of cells per gram of muscles at 1 and 3 days post hatch (Halevy et al., 2006). To determine the effects of *in ovo* illumination at the cellular level, Halevy et al. (2006) measured Pax7 and myogenin protein levels in muscles, as indicators of pre-differentiation and differentiation *in ovo*. *In ovo* stimulation with green light promoted Pax7 on day 3 and 6 and myogenin on day 1 after hatch suggesting that green light enhance the number of cells available for proliferation and differentiation (Halevy et al., 2006). As well, embryos that were exposed to green light *in ovo* had a higher number of adult myoblasts. Therefore, *in ovo* stimulation with green light increased rate of adult myoblast proliferation, resulting in higher body weights (Halevy et al., 2006). Along with protein, expression of transcription factors can be measured as an indicator of growth. MyoD, a marker of satellite cell activation, and myogenin, a marker for cells entering terminal differentiation, was investigated in embryos illuminated with green light during incubation (Zhang et al., 2011). Illuminated embryos had higher MyoD and myogenin mRNA levels during late incubation and early hatch, further indicating that green light enhances satellite muscle proliferation and differentiation (Zhang et al., 2014). Myostatin, a negative regulator of skeletal muscle growth, was also upregulated in illuminated embryos, possibly implementing a mechanism to prevent excessive proliferation and differentiation (Zhang et al., 2014). Melatonin may also play a role in regulating cell proliferation and differentiation, as pineal pacemakers produce melatonin and are light sensitive (Zhang et al., 2014). Therefore, it is evident that green light stimulates growth via satellite muscle cell proliferation *in ovo*, which may be mediated by pineal photoreceptors and melatonin.

1.3.2 Growth in Broilers

Not only does green light affect embryonic development, but it also has been reported to stimulate growth in chicks. Broiler chicks maintained under blue and green light had higher body and breast muscle weight at 35 days of age than birds under red light. Higher muscle weight was due to increase satellite cell proliferation during the first days post hatch (Halevy et al., 1998). Further investigation found birds under green and blue light had a low protein to DNA ratio and higher growth hormone mRNA levels, indicating active satellite muscle cells (Halevy et al., 1998). The role of light spectrum on growth regulators such as growth hormone, thyroxine and prolactin has not been extensively investigated. However unpublished results have shown chicks illuminated with green light post-hatch had higher growth hormone receptors mRNA expression in satellite muscle cells and higher levels of growth hormone and insulin-like growth factor in muscle tissue (Rozenboim et al., 2013). Rozenboim et al. (1999a) found broilers exposed to green and blue light had accelerated growth rates, however, the effects of the spectrum was dependant on age. Broilers under green light had higher body weights than those exposed to red and white lights from 3 to 20 days of age due to higher skeletal muscle satellite cell proliferation (Rozenboim et al., 1999a). Birds under blue light had significantly higher body weight than those exposed to monochromatic red and white from 20 to 34 days of age due to higher levels of androgens, which increases protein synthesis and reduces protein breakdown (Rozenboim et al., 1999a). To determine the optimal spectrum combination to manipulate body weight, combinations of monochromatic green and blue light were use at different ages. Body weight and growth was highest when birds were raised under monochromatic green light until 10 days and then switched to blue light until 46 days (Rozenboim et al., 2004a). Overall there was no difference in feed conversion (Rozenboim et al., 2004a), however this article noted that retinal

stimulation with monochromatic light may cause changes in behavioural activity levels, thus altering energy partitioning and growth rates (Rozenboim et al., 2004a). Similar results were identified by Cao et al. (2008), where chicks reared under green light had a significantly higher body weight and a lower feed conversion rate than those maintained on red and white light until 38 days of age. After 38 days, chicks under blue light had a higher body weight and a lower feed conversion rate, than birds maintained under red, white and green light (Cao et al., 2008). The higher body weight in birds under blue light was attributed to higher carcass, breast muscle, thigh and crus weight (Cao et al., 2008). Myofiber growth in the breast muscle levels was largest under green light at 21 days and blue light at 49 days of age (Cao et al., 2008). Changes in testosterone levels reflected changes in myofiber growth (Cao et al., 2008). Initially birds under green light had higher testosterone, but near the end of production birds under blue light had higher levels of testosterone, identifying a mechanism for the increase in myofiber growth (Cao et al., 2008). Therefore it is evident that green light stimulates growth during early rearing and blue light stimulates growth during the later stages of production via myofiber growth and satellite muscle cell proliferation.

However, both male and female broilers reared under mini fluorescent lights and incandescent light had higher growth rates than those under fluorescent light, which tend to have a higher amount of green light (Rozenboim et al., 1999b). Unfortunately, spectral output of the bulbs was not measured and higher amounts of flickering in the fluorescent bulbs may have affected activity levels, impacting body growth (Rozenboim et al., 1999b). Kim et al. (2013) found that birds reared under yellow light had a higher body weight gain at 5 woa than white, red and blue LED light, however, there was no consistent effect of LED light on body weight gain (Kim et al., 2013). Feed intake was higher under yellow light, which may be attributed to higher

body weights and was also suggested to be a result of wavelength mediated feeding behaviours (Kim et al., 2013). When looking at hematological parameters as an indicator of growth response, Kim et al. (2013) found that birds under yellow light had higher amounts of red blood cells, platelets and hematocrit (%), which correlates with their higher growth. These results do not corroborate previous studies; however, intensity was measured in lux indicating variations may have been due to intensity and not light spectrum.

1.3.3 Growth in Other Breeds and Species

The effect of light spectrum is not as extensively researched in layers. Baxter et al. (2014) found hens under green light had significantly higher body growth than birds under red and white lights, however, feed consumption was not measured and birds under green light laid significantly fewer eggs. Therefore it was speculated that a reduction in egg production resulted in more energy being put towards body growth (Baxter et al., 2014). Cockerels exposed to monochromatic green light displayed higher body growth but there was no difference in feed consumption (Foss et al., 1972). It was suggested that with the same amount of energy being delivered, green light doesn't stimulate growth but rather is lacking wavelengths that inhibits it (Foss et al., 1972). Male turkeys exposed to blue light had significantly higher body weight gain than those exposed to red and white, which is consistent to previous research, however after 16 woa, birds exposed to blue light had a significant decline in body growth (Levenick and Leighton, 1988). Although this study is in contrast to what was seen in broilers (Rozenboim et al., 1999a; Rozenboim et al., 2004a), filtered light was used and spectral output was not measured (Levenick and Leighton, 1988). Layer chicks illuminated with pink light *in ovo* had a lower body weight at hatch and rearing chicks under the same light led to a depression in growth, suggesting a negative additive effect (Tamimie, 1966). However in this experiment, spectral output was not

recorded therefore it is unclear which wavelength is causing a depression in growth (Tamimie, 1966). Interestingly, adult birds under red light have been reported to have higher body weight due to higher weights of reproductive organs. Chickens exposed to red light had higher body weight than those exposed to incandescent later on in lay, however this may be due to higher weight of the ovary and oviduct and more yellow yolk follicles (Reddy et al., 2012). Similar results were seen in the Japanese quail, where hens under red and incandescent lights had higher body weight than those under blue and green lights after a 5 week rearing period, when there was no difference in feed efficiency (Woodard et al., 1968). They speculated this was due to higher weight of reproductive organs; however this was never measured (Woodard et al., 1968).

1.3.4 Conclusion

Overall, it is evident that green and blue light stimulates growth in broilers. This occurs *in ovo* with increased proliferation of myofiber, myoblast, and satellite muscle cells (Halevy et al., 2006; Zhang et al., 2012). The stimulatory effects of green and blue light continue post hatch to promote growth via skeletal muscle satellite cell proliferation and myofiber growth and maybe due to stimulatory effects of protein synthesis (Halevy et al., 1998; Rozenboim et al., 2004a; Cao et al., 2008). However, as for the effect of light spectrum on growth in non-broiler poultry species, there are conflicting results which may be due to the variations in light spectrum output, intensity of light and strain of bird used.

1.4 Immunity and Stress

1.4.1 Introduction

Few studies have been performed on the effect of light wavelengths on birds' immune and stress response. Stress has been found in multiple cases to increase the number of heterophils

due to the rapid response to inflammation, and causes a decrease in the number of lymphocytes, which are slower acting immune cells (Kliger et al., 2000). Using a light dark schedule instead of continuous light has been shown to enhance immune function by lowering the heterophil to lymphocyte ratio, decrease corticosterone concentrations (Abbas et al, 2008), and enhance the spleen's immune function (Kliger et al., 2000). The light dark lighting schedule is thought to enhance immunity indirectly via the action of melatonin (Kliger et al., 2000), where continuous light may stress the bird (Abbas et al, 2008). Thus, any effect of light wavelength on the immune system may involve the effectiveness in stimulating melatonin while reducing stress.

1.4.2 Splenocytes, Antibodies and Growth Factors

Studies performed by Xie et al. (2008a; 2008b) found that exposing broilers to monochromatic light affected immunological organ weight and cell proliferation. Rearing birds under green light until 21 days led to larger spleens due to splenocyte proliferation, and birds under blue light had heavier spleens (Xie et al., 2008a) and higher lymphocyte proliferation (Xie et al., 2008b). This lighting program with monochromatic green and blue light has also been reported to stimulate growth (Rozenboim et al., 2004a). This suggests that exposing broilers to shorter wavelengths leads to enhanced immunity which may allow for better growth. The anti-Newcastle's disease virus antibody titers were also highest for birds under blue and green light throughout the trial. This indicates that birds exposed to green and blue light have longer lasting effective antibodies, a higher antibody production, and humoral immune function (Xie et al., 2008b). Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine playing a key role in the stress response. It stimulates the hypothalamus to release corticotropin-releasing hormone (CRH), which elicits a response from the adrenal cortex, to produce corticosterone. The level of IL-1 β was highest in birds exposed to white light, while exposure to blue light produced the lowest

amount of corticosterone, indicating low stress levels (Xie et al, 2008b). It also explains why birds under green and blue light had a larger spleen, as stress decreases the size of this secondary lymphoid structure (Xie et al, 2008b). Nitric Oxide (NO) is an important modulating factor of the immune system, by suppressing lymphocyte proliferation. Birds exposed to red light had significantly elevated levels of NO which increased as birds' aged, where birds under green and blue light had significantly lower NO at 49 days of age (Xie et al, 2008a). Interleukin-2 (IL-2), an indicator of lymphocyte proliferation, had higher bioactivity under green and blue light compared to red light. This indicates that birds under red light have a lower lymphocyte proliferation and higher amount of lymphocyte suppression (Xie et al, 2008a). Exposing turkey hens to monochromatic blue, green, red light and incandescent light did not significantly change levels of erythrocytes, leukocytes and corticosterone (Scott and Siopes, 1994). However, there was a depression in heterophil counts in birds under red light at 15 and 23 woa and green light at 15 woa. As well, the heterophil/lymphocyte ratio, an indicator of stress response, was lower in both these light treatments (Scott and Siopes, 1994). Nonetheless, this did not result in differences in stress levels in the hens (Scott and Siopes, 1994).

1.4.3 Intestinal Structure

Light sources have also been reported to influence the immunological barrier of the small intestines. Xie et al. (2011) found birds under green and blue light had a better intestinal mucosal structure with longer villus and smaller crypt depth. Concentration of factors that can act as key intestine immunological barriers, such as iIEL, IgA, and GC, were more abundant under green light during early rearing (Xie et al., 2011). Blue light increased number of iIEL, IgA+ cells, and GC during the later growth stage (Xie et al., 2011). The better structural integrity

of the villus and a higher number of gut mucosal immune-associated cells in birds under blue and green light, suggest enhanced immunity compared to birds maintained under red light.

1.4.4 Melatonin as a Potential Mediator between Light and Immunity

Melatonin has an immunomodulatory effects on chickens by enhancing the immune response and reduce factors that cause a depression in immune response such as stress, disease, aging, or drug treatments. Increased melatonin levels have been found to activate T helper-2 responses leading to the production of TH2 cytokines, known to enhance B cell activation and increase antibody production (Kliger et al, 2000; Jin et al, 2011). Due to the spectral sensitivity of retinal photoreceptors, stimulation with green light has been suggested to increase serotonin levels, which can be converted into melatonin (Mobarkey et al., 2013). Therefore, there is increased immune response in birds under green light (Xie et al, 2008a; Xie et al, 2008b), and an increase in retinal serotonin production under green light suggest that melatonin may act as a key messenger in relaying the effect of light wavelength on immune response. However it should be noted that the majority of circulating melatonin is produced by the pineal gland in quail and chickens, as studies have found melatonin levels are significantly decreased after pinealectomy (Pelham et al., 1972; Pang et al., 1974) but not bilateral enucleation (Pang et al., 1974; Nir et al., 1987). Jin et al. (2011) found that after a pinealectomy there was a reduction in plasma melatonin, but without a pineal gland, melatonin still fluctuated depending on exposure to light or darkness, suggesting that there is another source of melatonin in birds, possibly from the retina. As a matter of fact, they also found arylalkylamine N-acetyl-transferase (AANAT) mRNA expression, the rate limiting enzyme involved in melatonin synthesis, to be significantly higher in the retina of birds under green light compared to red, blue and white light at day 7 and 14 (Jin et al., 2011). As well, birds under red and green light had higher AANAT mRNA

expression than birds under blue and white light at day 7 (Jin et al., 2011). This suggests that green light may increase melatonin synthesis in both the retina and pineal gland, which acts to enhance the birds' immunity compared to birds under blue and white light.

1.4.5 Conclusions

With limited research available, to optimize immune and stress response it is best exposing broilers to green light during early rearing and blue light during late rearing (Xie et al, 2008a; Xie et al, 2008b; Xie et al, 2011). The stimulatory effects of green and blue light may be due to higher amounts of melatonin; however this was not specifically measured in these studies. As well, this higher immunity and reduced stress may have led to higher growth. It should be noted current research indicates that no light sources have been found that diminish the immunological response, but rather provide beneficial results to the immune system, or have no impact on the responses built up by the layer or broiler. However most of these few studies were performed on broilers and it is unknown how monochromatic light affects other breeds at older ages.

1.5 Behaviour

Birds rely heavily on visual information when conducting various behaviours. Intensive modern poultry facilities allow for controlled lighting environments, having a large impact on behaviour and welfare of the birds (Mench, 1991). Several studies have assessed the behaviour of poultry under various lighting conditions; however, when assessing the effects of lighting on poultry behaviour, it is important to consider light intensity, light source, and frequency. Currently, the poultry industry is shifting towards the use of LED lights for their energy efficiency. Nonetheless, due to the spectral sensitivity of the fowl and the spectral limitation of

artificial light, lack of specific wavelength may affects bird's behaviour (Prescott and Wathes, 1999). For example, incandescent light peaks in the red but lacks UVA light, which may have an effect of the breeding fitness of breeders (Prescott and Wathes, 1999). Thus, when using a new light source, it is important to fully assess its impact on behaviour.

1.5.1 Aggression, Time Budget and Fear Response

Previous studies have assessed and compared the effect of light colour on behavioural responses of poultry (Prayitno et al. 1997a; Sultana et al. 2013). In a study by Sultana et al. (2013), fear and behaviours was assessed in broiler raised under eight different light treatments, comparing monochromatic and combined LED light. Results showed birds under higher wavelengths had an increase in walking behaviour and birds under shorter wavelengths spent more time sitting and standing (Sultana et al., 2013). The increase in activity may have been due to better visual acuity of birds under red light compared to blue light (Sultana et al., 2013). Fear response was measured using the tonic immobility test, where birds that are more stressed stay immobile longer. Initial testing found that birds under red light had a longer time to recovery, while in a follow up tests birds under yellow light had a longer recovery rate. Although the results were inconsistent, they speculated that the higher activity of birds under longer wavelengths may increase duration birds remain immobile (Sultana et al., 2013). Also, birds under shorter wavelengths did not display a significant difference in duration of immobility suggesting that they have a decreased fear response (Sultana et al., 2013). Age had a significant influence on behaviour, as younger birds were more active than older birds regardless of light spectrum (Sultana et al., 2013). This study clearly indicates that light spectrum and age affect bird's behaviour, and higher wavelengths may increase fear response. When broiler chicks were exposed to blue, red, green, or white filtered incandescent light, chicks under red and white lights

were more active, and birds under red light had a higher incidence of aggressive behaviours, while birds under green and blue light spent more time sitting (Prayitno et al., 1997a). Hassan et al. (2014) found similar results, where layer chicks under red light had a higher incidence of feather pecking, however, this was not statistically significant. Both groups suggested that red light is better able to penetrate hypothalamic photoreceptors, which may activate interactive behaviours (Prayitno et al., 1997a). Broiler chicks exposed to bright red light had an increase in activity, which helped reduce leg disorder at the end of production (Prayitno et al., 1997b). When determining the bird's preference for certain colours of light, birds were reared under one colour and given the preference to choose another (Prayitno et al., 1997a). Preference was determined based on the number of minutes a hen stayed under each light treatment (Prayitno et al., 1997a). They found that all birds showed preference for a novel colour, and the highest preference was towards blue (Prayitno et al., 1997a). The birds that were reared under red light showed the weakest preference to remain under red light. This suggests that there is an aversion to red light, maybe due to the stimulation of the hypothalamus and preference towards blue, which may be due to spectral sensitivity of the avian retina (Prayitno et al., 1997b). However, these studies measured intensity in lux, which does not reflect the spectral sensitivity of the chicken retina. Since chickens perceive red light brighter than humans (Prescott and Wathes, 1999), measuring intensity in lux results in an overly bright output which may have increased aggression. Conversely, Huber-Eicher et al. (2013) exposed laying hens to monochromatic light at equal intensities, and found birds under red light had a reduction in aggressive behaviour. Schumaier et al. (1968) found similar results where birds under red light had lower incidence of feather pecking and cannibalism. However, birds under green light were exposed to higher intensities, which may have influenced the incidence of feather pecking rather than the wavelength. A study

by Kristensen et al. (2007) assessed behaviour of broilers reared under various light sources with intensity adjusted to the spectral sensitivity of the fowl. Birds under Biolux, a fluorescent light with major peaks in the violet, blue and green spectrum, had a higher incidence of feather pecking than birds under warm white fluorescent tubes, which had peaks in the blue, green, orange and red spectrum. This may have been due to UVA light emitted by Biolux (Kristensen et al., 2007). Better time-budget assessments and behaviour recording may be necessary in future studies to accurately assess aggressive behaviour.

1.5.2 Conclusion

Higher wavelengths are better able to stimulate the hypothalamus and have been suggested to increase activity leading to a reduction in leg disorders, increasing birds' health (Prayitno et al., 1997a; Prayitno et al., 1997b; Hassan et al., 2014). However, due to the limited amount of research there is no indication on the effect of monochromatic light on the welfare status of birds, and may not reflect its true effect on behaviours. Variations in light sources, light intensity, age of birds, breeds of birds and methods to record behaviour indicate that more research is required to determine the effect of light spectrum on poultry behaviour.

1.6 Conclusion

Overall, birds are photoperiodic and rely on different photoreceptors to regulate various physiological processes. It is evident that higher wavelengths are able to stimulate hypothalamic photoreceptors more efficiently than short wavelengths, which is necessary to effectively stimulate the reproductive axis in birds but may increase activity and aggression. Lower wavelengths, such as those in the green spectrum, in part via retinal photoreception, may enhance the birds' immune response, increase growth and may trigger more sitting and perching

behaviour. However many of these studies were performed using various light sources, allowing for variation in spectral output and light intensity, important components of light. As well, studies were performed on various breeds of birds spanning over more than 50 years thus compounding possible effects of heavy genetic selection for specific production traits. With different effects of light wavelength on different physiological systems and a continuously evolving genetic makeup, it is imperative to focus research and determine the right light source for the right production type.

Chapter 2: Red Light is Necessary to Activate the Reproductive Axis in Chickens Independently of the Retina of the Eye

Baxter, M., N. Joseph, V. R. Osborne, and G. Y. Bedecarrats. 2014. Poult. Sci. 93:1289-

1297

2.1 Abstract

Photoperiod plays an essential role in manipulating sexual maturity and reproductive performance in avian species. Light can be perceived by photoreceptors in the retina, pineal gland and hypothalamus. Higher wavelengths are able to penetrate through the skull more easily than lower wavelengths to stimulate the hypothalamus. The purpose of this experiment was to test the effects of light wavelengths on reproduction and growth parameters of laying hens in a conventional cage system. Three optically isolated rooms were equipped with LED strips providing pure green, pure red or white light set to 10 lux (hens levels). The involvement of the retina on mediating the effects of light wavelength was assessed by using blind and sighted Smoky Joe hens. No significant difference was observed between blind and sighted birds, suggesting the retina does not participate in controlling of reproduction under this setting. The red and white treatments displayed higher estradiol concentrations after photostimulation indicating higher ovarian activity, which translated to a significantly lower age at first egg when compared to the green treatment. Similarly, hens from the red and white treatments had a longer and higher peak egg production and higher cumulative egg number than the green group. No significant difference in body weight gain was observed during sexual maturation. However, further into the laying cycle, birds under green light showed higher body growth which was likely due to their lower egg production. Although corticosterone levels were higher under the red treatment, concentrations were below the levels that can be considered to be the result of

stress and reproduction was not compromised. In summary, red light is required to stimulate the reproductive axis, independent of the retina while green light resulted in a lack of stimulation or stimulation of an inhibitory pathway which resulted in a decreased reproductive activity.

2.2 Introduction

Avian species detect light through retinal and extra-retinal photoreceptors (Siopes and Wilson, 1980; Saldanha et al., 1994; Dawson et al., 2001). The retina allows birds to see and respond to their environment and mediates the effect of light on growth and behaviour (Wilson and Lindstrom, 2011). The retina contains two types of photoreceptors, cones and rods, with cones having a peak sensitivity to blue (450nm), green (550nm), red (700nm) or violet (415nm) light, allowing chickens to see some ultra violet (UV) light (Lewis et al., 2007). Consequently, poultry perceive light differently than humans, resulting in some light sources appearing brighter (Pyrzak et al., 1987). Extra-retinal photoreceptors are located in the pineal gland and the hypothalamus (Siopes and Wilson, 1980b; Saldanha et al., 1994; Dawson et al., 2001). The pineal gland is responsible for controlling bird's circadian rhythm via the synthesis and release of melatonin (Pelham et al., 1972, Pang et al., 1974, Nir et al., 1987, Kumar et al., 1999). The hypothalamus, located deep within the brain tissue, directly controls or is involved in the control of most homeostatic and physiological processes, including reproduction.

In birds, reproduction is tightly regulated by stimulatory (gonadotropin releasing hormones; GnRHs) and inhibitory (gonadotropin inhibitory hormone; GnIH) hypothalamic neuropeptides. Upon photostimulation, GnRH stimulates the release of gonadotropins (follicle stimulating hormone FSH, and luteinizing hormone LH) from the anterior pituitary gland which in turn triggers gonadal development and the synthesis of steroid hormones (progesterone from the granulosa cells of the large follicles and estradiol from the small follicles) (Robinson et al.,

1986; Ottinger and Bakst, 1995; Bedecarrats et al., 2009; Dunn et al., 2009; Tsutsui et al., 2010). Although hypothalamic photoreceptors remain elusive, GnRH neurons have been shown to be directly innervated by opsin containing “photoreceptors” cells (Saldanha et al., 2001) and exposure to long day photoperiod increases GnRH mRNA (Dunn and Sharp, 1999). On the other hand, GnIH acts on both the hypothalamus and the anterior pituitary to prevent the release of GnRH and gonadotropins, respectively (Tsutsui et al., 2010), and its release is stimulated by melatonin produced by both the retina and pineal gland during dark phases (Chowdhury et al., 2010). These findings have led us to propose a model in which increasing photoperiod reduces melatonin production, which decreases GnIH and indirectly stimulates the release of GnRH. Simultaneously, light stimulation of the hypothalamus also triggers the release of GnRH and as a result, under a short photoperiod sexually immature birds have high levels of GnIH resulting in the tonic inhibition of the reproductive axis while exposure to long photoperiod induces sexual maturity by reducing GnIH and increasing GnRH (Bedecarrats et al., 2009, Tsutsui et al., 2010).

Although the relative contribution of the retina of the eye and hypothalamic photoreceptors on reproduction is still controversial, experimental evidence showed that hypothalamic photostimulation activates the reproductive axis (Dawson et al., 2001; Saldanha et al., 2001) while retinal stimulation may decrease reproductive performance in chickens (Siopes et al., 1980b, Mobarkey et al., 2010, Gongruttananun, 2011, Mobarkey et al., 2013). However, it is still unclear whether light wavelength is a deciding factor. For example, the retina can be stimulated with light at low intensities, whereas the hypothalamus requires higher levels (Harrison et al., 1970). As well, higher wavelengths contain more energy and are able to penetrate through the skull and brain tissue to easily stimulate the hypothalamus (Pang et al., 1974, Foster et al., 1985, Oishi et al., 1993, Mobarkey et al., 2010). Therefore, it was suggested

that lower wavelengths (blue/green light) require higher intensities to stimulate hypothalamic photoreceptors (Pang et al., 1974). In addition to reproduction, light spectrum also plays a role in growth and behaviour. Exposing broilers chicks to green light during initial production and blue light near the end of production caused satellite muscle cells proliferation and a subsequent increase in muscle mass (Rozenboim et al., 2004). Furthermore, behavioural studies performed on broilers found birds reared under green light are less active and spent more time sleeping and relaxing (Prayitno et al., 1997a), whereas birds reared under red and white light had increased walking activity, floor pecking, wing stretching and aggression (Prayitno et al., 1997b).

Since photoperiod is essential to control sexual maturation and reproductive performance in avian species, artificial lighting is commonly used in commercial poultry production.

Incandescent lights have been the primary lighting source used by the North American poultry industry; however they are energy inefficient and the global push to reduce greenhouse gases has forced producers to find alternative sources. Light Emitting Diodes (LED's) are among the most efficient light sources and can be manufactured to deliver a defined and stable spectral output (Steranka et al., 2002). As the spectral characteristics of the light used may impact the reproductive performances, growth, behaviour and health of the birds, it is essential to fully test and validate any new light source before it can be recommended for use by the industry. This study aims at establishing if light spectrum influences egg production, body growth and stress in laying hens maintained in individual cages, and if these effects are mediated in part by the retina of the eye.

2.3 Materials and Methods

2.3.1 Experimental Animals

In order to investigate whether the impact of light wavelength is mediated via retinal and/or extra-retinal photoreceptors, this study was performed using Smokey Joe hens. This strain of Leghorn chickens harbors an autosomal recessive mutation that causes retinal degeneration (Salter et al. 1997; Tran et al., 2013). To obtain both blind and sighted birds, males and females from the parent colony were bred by artificial insemination following a strict pedigree protocol (Perttula and Bedecarrats, 2012). Photoperiod was kept at 24 h for the first 2 weeks of age (woa) then dropped to 8 hours thereafter. From hatch to 14 woa, light was provided by incandescent bulbs set to 10 lux intensity at birds' level. Sight status (blind or sighted) was determined at 14 woa based on their reaction to visual stimulation (pupillary reflex), eye phenotypes (bugling, atrophied eyes) and pedigree. Throughout the trial, birds were fed *ad libitum* commercial diets (starter and layers) meeting or exceeding NRC requirements (1994) and had free access to water. All procedures were approved by the University of Guelph Animal Care Committee.

2.3.2 Lighting Paradigm

A single room at the University of Guelph research station was partitioned into three optically isolated sections each containing identical individual cages. LED light strips (STR2 RGB) were purchased from GVA Lighting Inc. (Mississauga, Ontario, Canada) and mounted on top of cages. Each light strip section (1.2 m long) was assigned an individual internet protocol (IP) address and the intensity of individual diodes (red, green and blue) within each section was remotely adjusted. Light fixtures were then connected to an E:cue Butler interface and light spectrum and intensity was controlled using the Terminal Emulator V5.2 computer software (e:cue Lighting Control, Paderborn, Germany). Each partition of the experimental room was

illuminated with either pure green (464 nm; G), pure red (623 nm; R) or white (equal portions of red, green, and blue; W). Light intensities were adjusted to 10 lux at the hens' level for all treatments and spectral output was verified using a portable spectroradiometer (LI-1800 Portable Spectroradiometer; Li-Cor Inc.). At 14 woa, 26 sighted and 34 blind hens were randomly assigned to the green (9 sighted and 11 blind birds), white (8 sighted and 12 blind birds) and red (9 sighted and 11 blind birds) treatments. To reduce placement variables, blind and sighted birds were staggered. Before LEDs were turned on, birds were given 1 week to adjust to their new environment and were exposed to an 8 h photoperiod under incandescent light (10 lux). At 15 woa, LED lights were turned on with an 8 h photoperiod and, at 20 woa birds were photostimulated by an abrupt transfer to a 14 h photoperiod.

2.3.3 Measurement of Growth, Stress and Reproduction

Body weights were recorded at placement (14 woa) and biweekly from 14 to 23 woa and monthly from 37 to 52 woa. Sexual maturity and ovarian activation were determined by recording the age at first egg and measuring plasma levels of estradiol, respectively. Individual egg production was recorded daily throughout the trial (until 67 woa) and reproductive performances were expressed as weekly percent production (egg/hen/day). The effect of light treatment on stress was estimated by measuring plasma corticosterone concentrations.

2.3.4 Hormone Analysis and Enzyme immunoassays (EIA)

Blood samples were taken from each bird by venipuncture of the brachial vein at 14, 15, 20 and 23 woa, 2 to 4 h after lights were turned on. Approximately 2 ml of blood was collected and placed in a sodium heparin blood vacutainer. Immediately after collection, blood plasma was recovered by centrifugation at 900 g for 15 min at 4 °C and stored at -20 °C until hormone extraction and assay. Prior to immuno-assays, corticosterone and estradiol were extracted from

plasma using ethanol. In brief, thawed samples were diluted with cold ethanol at a 5:1 (ethanol:plasma) ratio. Samples were then vortex, centrifuged for 5 min at 20 °C at 1800 g and frozen in a -80 °C freezer. The organic (ethanol) phase was recovered, decanted into new tubes and dried under nitrogen flow. Samples were reconstituted in half the original volume with Trizma assay buffer (20 mM Trizma, 0.3 M NaCl, 0.1 % BSA; pH 7.5) and stored at -20°C until assayed. Plasma corticosterone was quantified using a corticosterone EIA developed by C.J. Munro (University of California, Davis, CA) and modified by Graham et al. (2001). Briefly, microtitre plates were coated with affinity purified goat anti-rabbit gamma globulin (25 µg/plate; Sigma Chemicals, St. Louis, MI) dissolved in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃; pH 9.6) and incubated overnight at room temperature. Coating buffer was removed, wells were refilled with Trizma assay buffer and assay plates were stored at room temperature for at least 30 minutes. Coated plates were washed with 0.04 % Tween 20 and samples (between 80 µl -300 µl) or standards (3.9 – 500 pg/well) were added into the appropriate wells. Horse-radish peroxidase-labeled corticosterone was then added to wells followed by anti-corticosterone antibody (Reference number: CJM006) provided by C.J. Munro (University of California, Davis, CA). Following incubation overnight at room temperature, plates were washed with 0.02 % Tween 20 and incubated with substrate solution (0.5 ml of 16 mM tetramethylbenzidine and 0.1 ml of 0.175M H₂O₂ diluted in 22 ml of 0.01M C₂H₃O₂Na; pH 5.0). After incubation (45 min, room temperature) the reaction was terminated with 50 µl of 3 M H₂SO₄ and the optical density was measured with a Microplate reader (Bio Rad, model: 550) at 450 nm (reference 595 nm). The standard curve and samples were then plotted and analyzed using Microsoft Excel. The intra and interassay coefficients of variation were < 15 %.

Plasma estradiol was quantified using the same general procedure as described above for corticosterone except that an anti-estradiol antibody raised in rabbits and estradiol conjugated to horse-radish peroxidase provided by C.J. Munro (University of California, Davis, CA) were used. The standard curve for estradiol ranged from 3.9 to 500 pg/well and the intra and interassay coefficients of variation were also < 15 %.

2.3.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 software (Graphpad Software, La Jolla, CA). For each parameter, a two-way ANOVA was used to determine the overall effect and possible interaction between light treatment and sight status (blind or sighted), and between light treatment and age/time. When significant differences were detected, further analyses were performed using Bonferroni post-hoc tests. Significance was based on $P < 0.05$. To normalize for differences in initial body weight between animals, cumulative body growth was measured by subtracting individual initial body weight at each time point.

2.4 Results

2.4.1 Sexual Maturity and Egg Production

The onset of sexual maturity, calculated based on the age at first egg (Table 2.1), was significantly delayed ($p < 0.0001$) for hens maintained under green light (189.6 ± 2.4 d) compared to hens under white (166.8 ± 1.1 d) and red (165.9 ± 1.3 d) lights. No significant difference was observed between blind and sighted hens for each light treatment and no significant interaction was detected between light and sight status (Table 2.2A).

Table 2.1: Effect of Light Wavelength on Age at First Egg

The average number of days to the age at first egg, total and post-stimulation, for each overall light treatment and for each sight status within each light treatment. ^{A,B} Values with different superscript letters within columns correspond to significantly different mean age at first egg ($p < 0.001$). Capitalized superscripts indicate comparison between overall lights treatments (combining blind and sighted hens). Lowercase superscripts indicate comparison between blind and sighted birds within each light treatment. Data are presented as mean \pm S.E.M.

Experimental group / Status	Age at first egg (days) (mean \pm S.E.M.)	Days post-stimulation (mean \pm S.E.M.)	Number of observations per mean (n)
Green	189.6 \pm 2.4 ^A	48.6 \pm 2.4 ^A	19
Blind Green	191.0 \pm 3.5 ^a	50.0 \pm 3.5 ^A	10
Sighted Green	188.3 \pm 3.3 ^a	47.3 \pm 3.3 ^A	9
White	166.8 \pm 1.1 ^B	25.8 \pm 1.1 ^B	19
Blind White	166.8 \pm 1.7 ^b	25.8 \pm 1.7 ^B	11
Sighted White	166.8 \pm 1.2 ^b	25.8 \pm 1.2 ^B	8
Red	165.9 \pm 1.3 ^B	24.9 \pm 1.3 ^B	20
Blind Red	164.4 \pm 1.0 ^b	23.4 \pm 1.1 ^B	11
Sighted Red	167.8 \pm 2.6 ^b	26.8 \pm 2.6 ^B	9

Table 2.2A: Statistical analysis (2-way ANOVA) of age at first egg, estradiol concentrations and total number of eggs produced¹

	Sight effect			Light treatment effect			Interaction		
Age at first egg	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
	0.305	1	0.583	58.2	2	<0.0001	0.383	2	0.684
Estradiol Concentration	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
15 woa	1.436	1	0.237	0.097	2	0.907	0.034	2	0.966
20 woa	1.248	1	0.269	21.160	2	<0.0001	1.154	2	0.323
23 woa	0.047	1	0.829	0.567	2	0.571	1.057	2	0.355
	Time/age effect			Light treatment effect			Interaction		
Overall Estradiol Concentrations	92.180	2	<0.0001	16.810	2	<0.0001	15.610	4	<0.0001
	Sight effect			Light treatment effect			Interaction		
Total egg production	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
	0.007	1	0.932	11.010	2	<0.0001	0.830	2	0.442

¹ Bolded P values are significant

Estradiol concentrations in plasma were measured at different time points around sexual maturation to reflect activation of small follicles (Figure 2.1). At 15 woa, estradiol levels were not significantly different between light treatments (G: 0.84 ± 0.10 ng/ml; W: 0.82 ± 0.09 ng/ml; R: 0.87 ± 0.08 ng/ml), nor were there any significant differences between blind and sighted hens or interaction between sight status and light treatment (Table 2.2A). At 20 woa, levels of estradiol were significantly higher in birds maintained under red light when compared to green light, regardless of sight status (G: 1.67 ± 0.23 ng/ml; W: 3.33 ± 0.39 ng/ml; R: 5.04 ± 0.44 ng/ml). At 23 woa, no significant difference was observed between light treatments or sight status nor were there any interactions (G: 1.19 ± 0.22 ng/ml; W: 1.07 ± 0.094 ng/ml; R: 1.30 ± 0.13 ng/ml). As no significant difference were observed between blind and sighted animals for each light treatment, blind and sighted data were combined and changes in estradiol concentration were analysed overtime (Figure 2.1D). Both age and light treatment had a significant effect with a significant interaction (Table 2.2A). The red and white treatments resulted in significantly higher levels of estradiol at 20 woa compared to 15 and 23 woa. Conversely, no significant change in estradiol levels was observed in hens maintained under green light between 15 and 23 woa.

The effect of light treatment on egg production over the course of the trial is presented in Figure 2. There was a significant interaction between sight status and the light treatment on weeks 25, 52, 58-61 (Table 2.2B). When the effects of sight and light treatment on egg production were analyzed on a weekly basis (Table 2.2B), the effect of sight was significant for only 5 separate weeks (29, 36, 39, 45 and 64 woa), whereas the effect of light treatment was significant for 16 out of 45 weeks (from 23-28 woa and at 30, 33, 36, 40, 44, 45, 47, 50, 52 and 58 woa) with hens under red or red and white lights producing significantly more eggs than hens under green light.

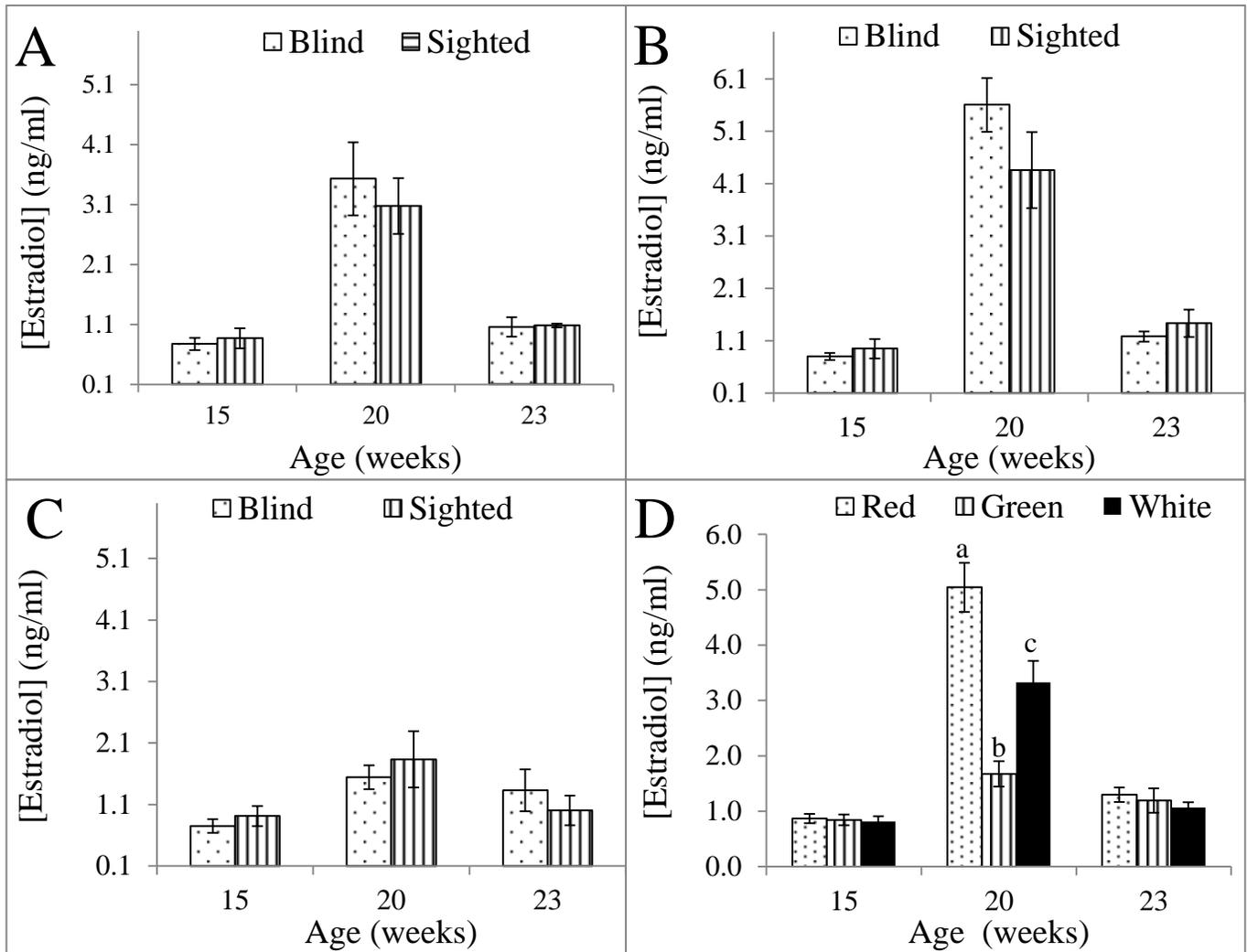


Figure 2.1: Effect of light treatment on plasma estradiol concentrations.

Estradiol concentrations in blind and sighted birds for the green, white, and red treatments are displayed in panels A, B, and C, respectively. Panel D corresponds to the combined values from blind and sighted hens for each light treatment at each time point. Different letters (a–c) indicate significant differences at ($p < 0.0001$). All birds in each light treatments were sampled, data is presented as mean and error bars represent the S.E.M.

Table 2.2B: Statistical analysis (2-way ANOVA) of weekly egg production²

Age (weeks)	Sight effect			Light treatment effect			Interaction		
	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
23	0.516	1	0.476	8.049	2	<0.001	0.215	2	0.807
24	0.745	1	0.392	16.020	2	<0.0001	0.529	2	0.592
25	0.113	1	0.738	75.200	2	<0.001	6.289	2	0.003
26	0.343	1	0.560	38.600	2	<0.001	0.273	2	0.762
27	0.098	1	0.756	29.560	2	<0.0001	0.582	2	0.562
28	2.014	1	0.162	14.170	2	<0.0001	2.884	2	0.065
29	4.063	1	0.049	2.068	2	0.137	0.723	2	0.490
30	0.784	1	0.380	4.345	2	0.018	0.173	2	0.842
31	2.905	1	0.094	0.836	2	0.439	0.408	2	0.667
32	0.472	1	0.495	2.241	2	0.116	1.590	2	0.214
33	2.092	1	0.094	3.360	2	0.042	0.771	2	0.468
34	3.697	1	0.060	0.642	2	0.530	1.383	2	0.260
35	0.226	1	0.636	2.098	2	0.133	0.170	2	0.844
36	6.669	1	0.012	4.195	2	0.020	2.372	2	0.103
37	0.394	1	0.533	0.839	2	0.438	0.237	2	0.790
38	0.330	1	0.568	1.439	2	0.246	0.143	2	0.867
39	4.086	1	0.048	2.599	2	0.084	0.972	2	0.385
40	0.217	1	0.644	3.244	2	0.047	2.793	2	0.070
41	0.012	1	0.913	0.298	2	0.743	0.040	2	0.961
42	0.091	1	0.764	2.220	2	0.119	0.883	2	0.419
43	0.010	1	0.920	1.962	2	0.151	0.920	2	0.561

² Bolded P values are significant

44	0.784	1	0.380	3.807	2	0.028	1.804	2	0.175
45	4.574	1	0.037	6.817	2	0.002	0.564	2	0.577
46	0.953	1	0.333	1.565	2	0.219	1.204	2	0.308
47	0.312	1	0.572	3.466	2	0.038	0.651	2	0.525
48	1.695	1	0.198	0.487	2	0.617	0.229	2	0.796
49	0.811	1	0.372	1.814	2	0.173	2.609	2	0.083
50	0.005	1	0.945	4.394	2	0.017	1.180	2	0.315
51	2.648	1	0.110	1.985	2	0.147	1.246	2	0.296
52	1.492	1	0.227	3.406	2	0.041	3.994	2	0.024
53	1.197	1	0.279	2.194	2	0.121	1.782	2	0.178
54	0.953	1	0.333	1.263	2	0.291	2.032	2	0.141
55	1.709	1	0.197	0.967	2	0.387	2.910	2	0.063
56	3.214	1	0.079	0.747	2	0.479	1.893	2	0.161
57	3.524	1	0.066	2.351	2	0.105	1.634	2	0.205
58	0.338	1	0.563	4.539	2	0.015	3.722	2	0.031
59	0.301	1	0.585	1.512	2	0.230	4.215	2	0.020
60	0.223	1	0.638	2.805	2	0.069	4.595	2	0.014
61	1.313	1	0.252	2.013	2	0.144	5.610	2	0.006
62	0.917	1	0.342	0.917	2	0.290	3.090	2	0.054
63	0.007	1	0.935	1.973	2	0.149	1.349	2	0.268
64	4.972	1	0.030	0.969	2	0.386	4.569	2	0.015
65	0.093	1	0.763	1.045	2	0.313	2.975	2	0.093
66	0.543	1	0.464	0.572	2	0.568	0.824	2	0.444
Overall	Time/age effect			Light treatment effect			Interaction		
	26.300	43	<0.0001	114.700	2	<0.0001	2.869	86	<0.0001

When egg production from blind and sighted hens was combined for each light treatment, it appeared that overall, light treatment had a significant effect on egg production throughout the course of the trial with hens under green light having the lowest production (Table 2.2B, Figure 2.2D). Interestingly when analyzing the effect of sight for each light treatment individually, overall, sighted hens under green light had significantly lower production than blind animals ($p < 0.0001$; Figure 2.2A). However, Boniferroni post hoc test shows that the difference between sighted and blind hens was significant only at 61 and 64 woa. Under white light, age had a significant effect on egg production, yet there was no difference between blind and sighted birds ($p = 0.5807$), nor was there any interaction (Figure 2.2B). Under red light, sighted birds had a slightly higher level of production than blind birds ($p = 0.0455$; Figure 2.2C). However when a Boniferroni post hoc was performed for each week no significant difference between blind and sighted birds was observed. Similar to the white treatment, age had a significant effect on production with no interaction between sight status and age.

The average cumulative number of eggs produced is displayed in Table 2.3. Hens from the red and white treatments had significantly higher cumulative egg production than those in the green treatment. There was no significant difference between blind and sighted hens for each treatment nor was there any significant interaction between light treatment and sight status (Table 2.2A).

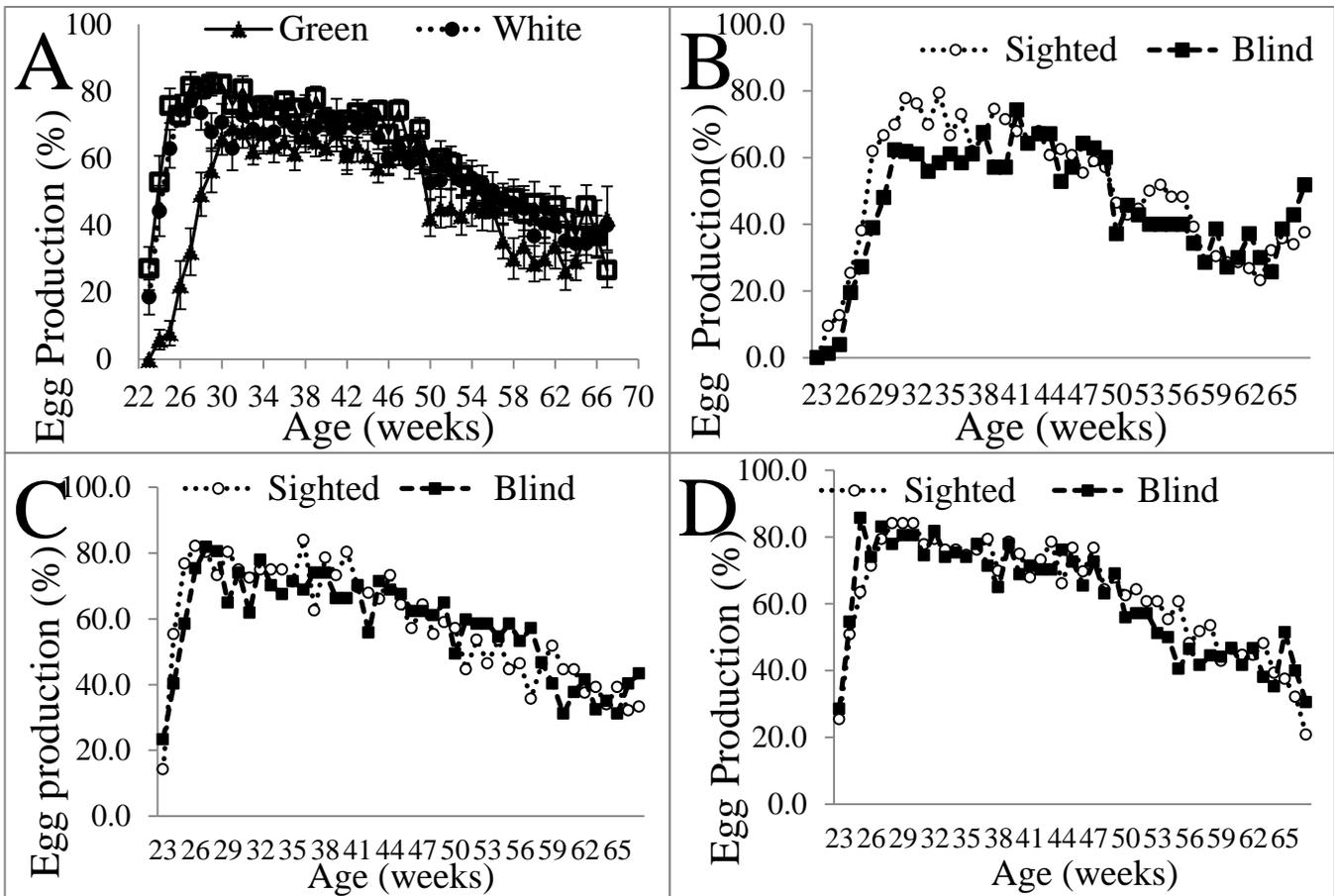


Figure 2.2: The effect of light treatment on weekly egg production.

There was a significant interaction between sight status and the light treatment on 25, 52, 58-61 woa. Light treatment had a significant effect of egg production during 23-28, 30, 33, 36, 40, 45, 47, 50 and 52 woa, where red or red and white had significantly higher egg production than the green treatment. There was only a significant difference between blind and sighted birds on 29, 36, 39, 45, and 64woa therefore blind and sighted values were average for each treatment and compared at each time point (A). The difference in egg production between blind and sighted bird for the green, white and red treatment are displayed in Figure B, C, and D.

Table 2.3: Effect of light wavelength on cumulative egg production.³

Experimental group	Cumulative egg number / bird (mean \pm S.E.M.)
Green	140.5 \pm 0.3 ^A
Blind Green	151.4 \pm 0.4 ^a
Sighted Green	135.0 \pm 0.5 ^a
White	180.2 \pm 0.3 ^B
Blind White	178.6 \pm 0.3 ^{a,b}
Sighted White	182.3 \pm 0.4 ^B
Red	190.3 \pm 0.3 ^B
Blind Red	185.4 \pm 0.4 ^b
Sighted Red	196.1 \pm 0.4 ^b

³ Different superscript letters indicate significantly different values. Capitalized superscripts indicate comparison between overall lights treatments (combining blind and sighted hens; ^{A,B} $p < 0.001$).

Lowercase superscripts indicate comparison between blind and sighted birds within each light treatment (^{a,b} $p < 0.05$).

2.4.2 Body Growth

The percent body growth is shown in Figure 2.3. At 14 and 15 woa, blind birds had significantly lower body weights than sighted, regardless of light treatment. However from 16 woa onward, there was no significant difference in body growth between blind and sighted birds. Until 23 woa, there was no significant difference in body weight change between light treatments. However, from 23 to 52 woa, with the exception of 41 woa, hens from the green treatment had significantly higher body weight gain than those from the red and white treatments. Overall, there was a significant effect of light treatment on body growth, regardless of age (Table 2.2C). There was no significant interaction between light treatment and sight status for body growth (Table 2.2C).

2.4.4 Stress

Stress was evaluated as per corticosterone levels in plasma (Figure 2.4; Table 2.2D). At 14 woa, before birds were exposed to the various lights, no significant difference between light treatments (G: 0.35 ± 0.08 ng/ml; W: 0.29 ± 0.05 ng/ml; R: 0.30 ± 0.09 ng/ml) or between sight status was observed. Similarly, after one week exposure to the light treatment (15 woa) no difference in corticosterone levels was observed (G: 0.30 ± 0.04 ng/ml; W: 0.27 ± 0.04 ng/ml; R: 0.24 ± 0.03 ng/ml). Yet at that age, there was a significant difference between blind and sighted hens with sighted birds having significantly higher levels of corticosterone than blind birds, regardless of light treatment. At 20 woa (after 5 weeks exposure to light treatment), birds under red light had significantly higher levels of corticosterone (0.72 ± 0.12 ng/ml) than hens under green (0.30 ± 0.02 ng/ml) or white (0.37 ± 0.03 ng/ml) lights. As there was only a significant difference in corticosterone levels between the blind and sighted birds at 15woa data were combined and analysed overtime (Figure 2.4D; Table 2.2D). A two-way ANOVA revealed that

the age had a significant effect on corticosterone levels while light treatment did not have a significant effect on its own. However, the interaction between the age of hens and light treatment was significant (Table 2.2D).

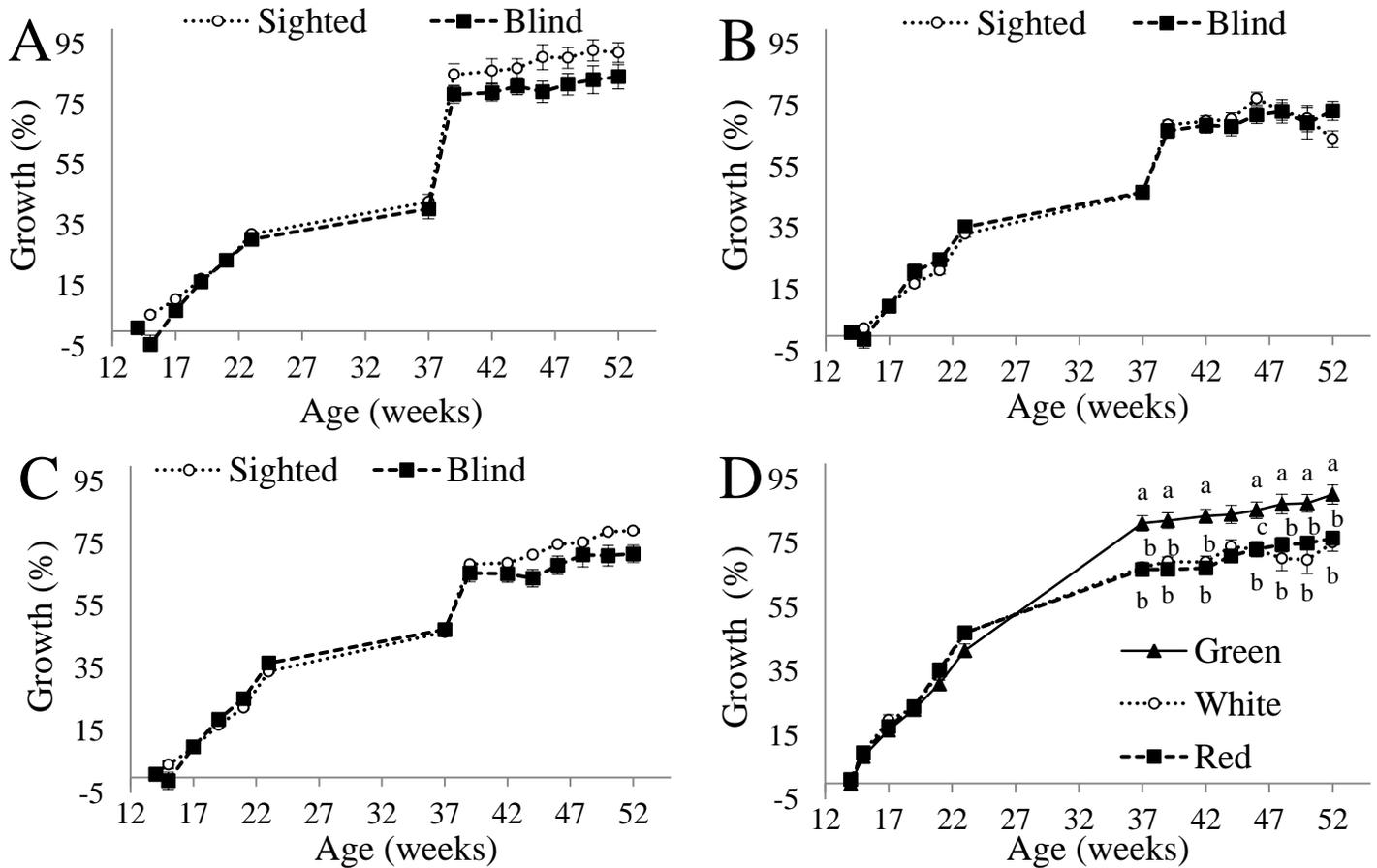


Figure 2.3: Effect of light treatment on percent body growth.

Body growth between blind and sighted birds for the green, white, and red treatments is displayed in panels A, B, and C, respectively. Panel D corresponds to the combined values from blind and sighted hens for each light treatment at each time point. Body growth was measured by subtracting individual initial body weight at each time point. Letters (a,b) correspond to significant differences ($p < 0.001$) between light treatments in panel D, whereas c indicates significant differences at $p < 0.01$.

Table 2.2C: Statistical analysis for Body Growth

Results from the two-way ANOVA									
Body Growth	Sighted effect (blind or sighted)			Light treatment			Interaction		
	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
Weeks of Age									
14	9.815	1	0.0028	0.1147	2	0.8919	0.9316	2	0.4003
15	9.815	1	0.0028	0.1147	2	0.8919	0.9316	2	0.4003
17	1.971	1	0.1662	1.255	2	0.2934	1.407	2	0.2538
19	4.009	1	0.0504	0.1139	2	0.8926	0.7059	2	0.4982
21	0.6453	1	0.4254	3.058	2	0.0554	1.024	2	0.3662
23	0.01718	1	0.8962	3.875	2	0.0269	0.3149	2	0.7312
37	2.89	1	0.095	18.24	1	<0.0001	0.3659	2	0.6953
39	3.099	1	0.0841	18.76	2	<0.0001	0.6129	2	0.5456
42	5.641	1	0.0213	21.81	2	<0.0001	0.4779	2	0.6228
44	1.335	1	0.2532	1.531	2	0.226	0.2003	2	0.8191
46	2.364	1	0.1302	9.41	2	0.0003	0.8015	2	0.4541
48	2.797	1	0.1004	8.303	2	0.0007	0.5239	2	0.5953
50	0.2694	1	0.6059	9.716	2	0.0003	2.655	2	0.0798
52	2.784	1	0.1012	11.14	2	<0.0001	1.029	2	0.3646
	<i>row factor: time</i>			<i>column factor= light treatment</i>			<i>Interaction</i>		
Overall	940.3	13	<0.0001	6.641	2	0.0026	8.817	26	<0.0001

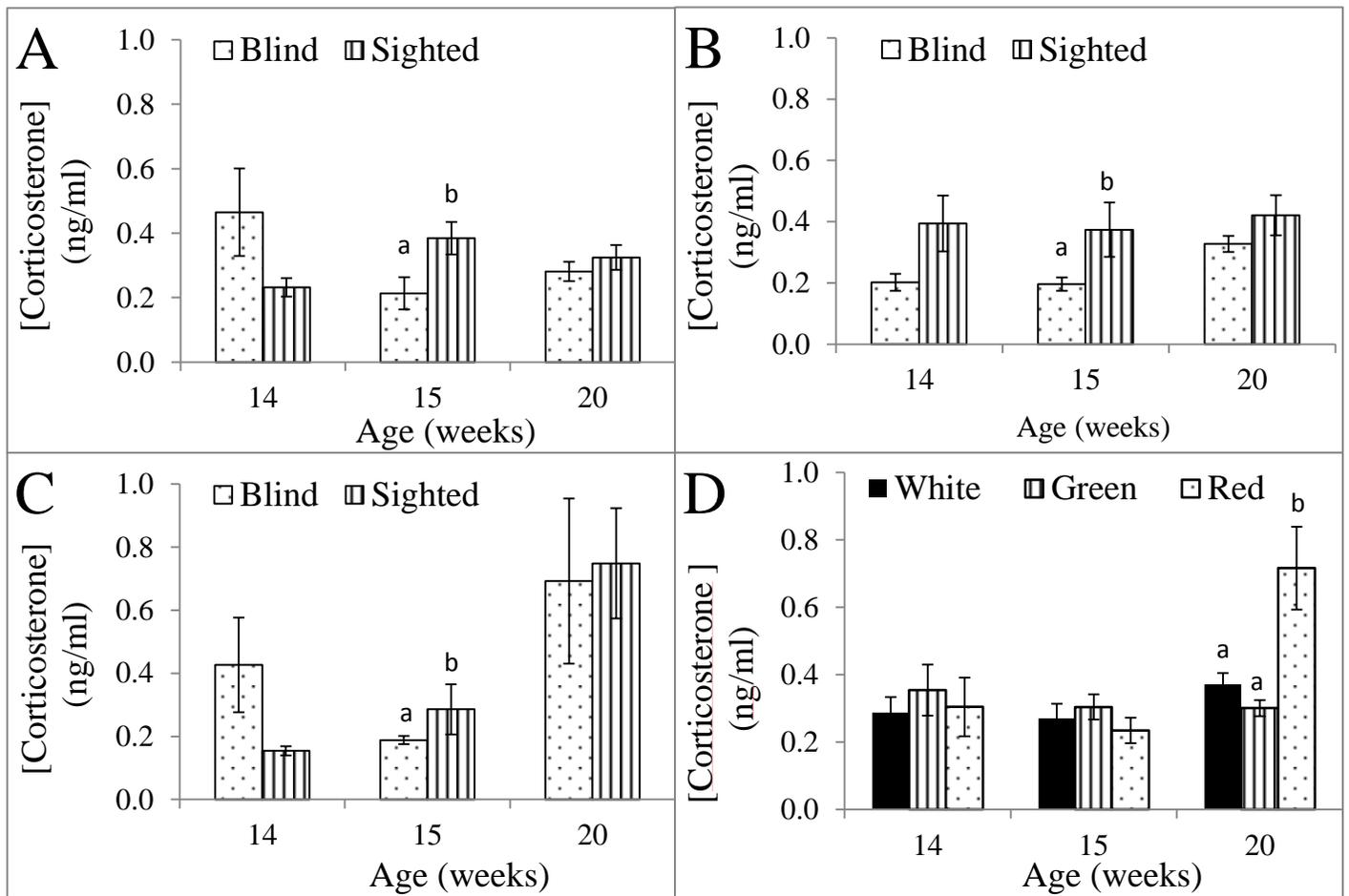


Figure 2.4: Effect of light treatment on plasma corticosterone concentrations. Corticosterone concentrations between blind and sighted birds for the green, white, and red treatments are displayed in panels A, B, and C, respectively. Panel D corresponds to the combined values from blind and sighted hens for each light treatment at each time point. Different letters (a,b) indicate significant differences at ($p < 0.001$).

Table 2.2D: Statistical Analysis for Corticosterone Concentrations

Results from the two-way ANOVA									
Significance bolded	Sighted effect			Light treatment			Interaction		
	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
C1: 14woa	2.234	1	0.141	0.9423	2	0.3963	2.421	2	0.0987
C2: 15woa	1.31	1	0.2577	33.96	2	<0.0001	0.1175	2	0.8894
C3: 20woa	0.3215	1	0.5732	6.992	2	0.0022	0.7726	2	0.4671
	Sighted effect			Light treatment			Interaction		
	F-value	DF	P-value	F-value	DF	P-value	F-value	DF	P-value
<i>Overall</i>	<i>3.174</i>	<i>2</i>	<i>0.0444</i>	<i>1.151</i>	<i>2</i>	<i>0.3188</i>	<i>3.666</i>	<i>4</i>	<i>0.0069</i>

2.5 Discussion

2.5.1 Effect on Reproductive Performance

Based on these results, it is evident that wavelengths from the red spectrum are the most potent stimulator of sexual maturation and egg-laying in both blind and sighted Smokey Joe hens. This may be explained by the fact that wavelengths of lower frequencies are able to penetrate the skull and brain tissue more easily, as demonstrated by Oishi and Ohashi (1993). Hens maintained under red and white light reached sexual maturity within 3 weeks after photostimulation (23 woa), while birds exposed to green light displayed a 23.7 days (over 3 weeks) delay. Interestingly, 27 weeks is the age at which Smoky Joe hens mature spontaneously in the breeding colony when left under an 8 h photoperiod (unpublished data). This observed delay in egg production under green light is in agreement with previous studies (Harrison et al. 1970, Kumar et al. 1996, Mobarkey et al. 2010, Gongruttananun 2011) and suggests that it is ineffective in properly mediating a stimulatory photoperiod. This is further strengthened by the fact that under the experimental conditions, green light failed to significantly elevate circulating estradiol levels while 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 (Robinson et al., 1986). This effect of light wavelength on ovarian activity was independent of a functional retina of the eye and is thus mediated via extra-retinal photoreceptors, most likely hypothalamic. To the best of the authors' knowledge, this is the first study linking circulating levels of estradiol to light wavelength and subsequent sexual maturation.

As we and others previously proposed (Bedecarrats et al., 2009; Tsutsui et al., 2010), sexual maturation in chickens is tightly regulated by a balance of stimulatory (GnRH) and inhibitory (GnIH) inputs from the hypothalamus, coupled with a switch in sensitivity to these

peptides at the level of the anterior pituitary. In that model, it was postulated that the increase in GnRH observed upon photostimulation is under the direct influence of hypothalamic photoreceptors while the synthesis and release of GnIH is under the control of melatonin produced during the dark phases. This is supported by evidence that melatonin from both the pineal gland and the retina of the eye contribute to the production of GnIH and is in part responsible for inhibiting LH release in both quails and chickens (Chowdhury et al., 2013). Moreover, stimulation of the retina of the eye with green light in broiler breeders results in decreased reproductive performances (Mobarkey et al., 2010) and, supplementation with the serotonin inhibitor parachlorophenylalanine partially restored egg production (Mobarkey et al., 2013). It is possible that stimulation of the retina with green light promotes the synthesis of serotonin which is further converted into melatonin during the dark phases, inhibiting the reproductive axis via GnIH. Interestingly, in this study no significant difference was observed between blind and sighted birds under red and white lights suggesting that the inhibition from the retina of the eye may not be prevalent when sufficient stimulation of the hypothalamus is provided. On the other hand, when the stimulatory effect of red light is removed and hens are maintained under pure green light, sighted hens dropped out of production significantly earlier than blinds suggesting that without proper extra-retinal stimulation, an inhibitory effect via the retina can be observed. However, whether the degenerative retina in the blind Smoky Joe line is associated with a decreased melatonin synthesis remains to be determined. Interestingly, in a previous study performed on Smokey Joe roosters (Perttula and Bedecarrats, 2012), blindness resulted in advanced sexual maturity in both unphotostimulated and photostimulated birds. In this study, incandescent light bulbs that emit a broader spectrum were used and it is possible that the ratio of green versus red is the determining factor to observe any antagonistic effect of

stimulatory extra-retinal and inhibitory retinal photoreceptors on reproduction. However, although sighted birds began dropping out of production sooner under green light, no significant difference in cumulative egg number between blind and sighted birds was observed. This is most likely due to the fact the significance was detected when both blind and sighted were below 60 % production. Nonetheless, significance may have been reached if the trial had continued for a full 52 week production cycle.

Although red light was the most effective wavelength to stimulate the production of estradiol, no significant difference in age at first egg or overall production were observed between hens under red and white lights. Thus, a white light containing 33% red may be sufficient to adequately stimulate and maintain reproduction. However, although not statistically significant, hens under red light did have slightly better reproductive performances than hens under white light, as they began laying earlier, and had a slightly higher level of production. This may be of importance for large commercial operations where slight increases in production translate into higher profit, and more than 33% of red spectrum may be beneficial to increase reproductive performances.

2.5.2 Effect on Body Weight

The initial differences in body growth observed between blind and sighted hens could be explained by the fact that at placement, blind hens had to first “map” their new environment to locate feeders and water dishes. In a previous study, when Smoky Joe birds were monitored from a younger age and kept in the same environment no such difference between blind and sighted was observed (Perttula and Bedecarrats 2012). However in the present study, the initial difference was no longer visible after 2 weeks.

Prayitno et al. (1997b) previously reported that birds exposed to red light had higher feed intake and increased initial body growth, although higher activity levels also resulted in decreased final body weight. Rozenboim et al. (1998) suggested that feed intake of caged laying hens was mainly affected by light intensity and not wavelength. On the other hand in broiler chicks, initial exposure to green and blue lights does increase growth later in development, and this effect is mainly due to proliferation of satellite muscle cells rather than changes in feed consumption (Rozenboim et al. 2004). In this experiment, light intensity was the same across all treatments and all birds were housed in individual cages thus limiting physical activity. Although feed consumption was not monitored, hens under green light grew at a significantly faster pace than those under white and red light from 23-52 woa. Since birds were exposed to their respective light treatment at 15 woa, this difference cannot be attributed to an effect of green light on satellite cells proliferation neither can it be attributed to differences in physical activity. Instead, this may have resulted from changes in energy expenditure associated with the delayed entry in lay and lower overall egg production observed in the green treatment.

2.5.3 Effect on Stress

Corticosterone produced by the adrenal glands is the main mediator of the stress axis in birds and daily fluctuation in laying hens ranges from 7-11 ng/ml (Beuving and Vonder 1977). Corticosterone levels in the study were lower than these values suggesting that birds under the three light treatments were not physiologically stressed. However, significantly higher levels of corticosterone were observed in the red treatment at 20 woa. At a behavioural level, prior studies have shown that birds reared under red and white light display increased walking activity, floor pecking, wing stretching and aggression (Prayitno et al., 1997b), while birds reared under green light are less active with increased sleeping patterns which may decrease the hens' corticosterone

levels (Prayitno et al., 1997a). Under the experimental conditions, birds were confined to individual cages and these behaviours could not be assessed. Thus, it cannot be determined whether the observed increase was linked to behavioural changes and additional research should be conducted to investigate the effect of light spectrum. Nonetheless, the higher levels observed under red light at 20 woa were below what is deemed physiological stressed (Beuving and Vonder, 1978) and had no negative impact on reproduction.

2.6 Conclusion

In conclusion, 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. Although birds in the red treatment had higher levels of corticosterone, these levels were below what can be considered a physiological stress and reproduction was not compromised. As no significant difference between blind and sighted birds was observed, it appears that under the experimental conditions, retinal stimulation or lack of does not impact initiation of reproduction.

Chapter 3: Does Light Spectrum Impact Production, Growth, Stress and Behaviour in Laying Hens Maintained in Floor Pens

3.1 Abstract

Light emitting diodes (LED) are an efficient lighting source which can emit a pre-defined and stable spectrum. In poultry, longer wavelengths of light contain more energy and are more efficient in activating the reproductive axis. Using a strain of blind Leghorns (Smoky Joe), we previously reported that light from the red spectrum is instrumental in triggering and maintaining egg-laying, independently of the retina. Thus, we designed an LED bulb that emits 60% red light (LED-R), specifically for laying hens. The purpose of this experiment was to evaluate the effect of green, red and modified white light on behaviour, stress, reproduction and growth in birds maintained in floor pens. In this study, we used 3 identical rooms each containing 6 floor pens, equipped with a perch, 3 nest-boxes and populated with 8 Lohmann LSL-lite hens per pen. Rooms were illuminated with either pure red (662nm), pure green (524nm) or LED-R light, set to 10 lux at the hen's level. There was no significant effect of light treatment on egg production and cumulative egg numbers. However, birds exposed to red light had higher level of estradiol before birds began laying, suggesting red light is more effective at stimulating the reproductive axis. Birds under green light laid significantly more eggs during the dark phase, suggesting that green light may not entrain oviposition. Birds under green light had a higher body growth rate although no difference in feed consumption was observed. There was no significant difference in stress or activity levels between treatments. However, light spectrum may have an effect on bird behaviours as birds under green light used the nestbox more often. This is likely a learned behaviour and further investigation is required to determine how light spectrum affects nesting. There were a higher number of aggressive pecks in birds under LED-R, however no other

adverse behaviours were different and the number of pecks varied significantly between pens. In conclusion, in commercial layers maintained in collective floor pens, light spectrum may not have a significant effect on reproductive performance although hormonal patterns indicate that red light does stimulate the reproductive axis more efficiently. Furthermore, green light does promote body growth, and appears to fail to synchronize oviposition/ovulation with photoperiod.

3.2 Introduction

Artificial lighting is required in commercial poultry barns to control photoperiod, which includes light quantity (number of photons delivered) and light quality (spectral composition). Birds can detect light through photoreceptors in the retina, pineal gland and hypothalamus. Retinal photoreceptors are most sensitive to 533-577nm and have a low sensitivity to the colours at the extreme ends of the visible spectrum (Prescott and Wathes, 1999). Pineal photoreceptors receive light signals and transmit it to oscillators which control circadian rhythms via the synthesis and release of melatonin (Pelham and Ralph, 1972; Pang et al., 1974; Nir et al., 1987; Kumar and Rani, 1999). Hypothalamic photoreceptors act as an intermediate to relay photic information to regulate homeostatic and physiological processes such as reproduction and stress in birds. In modern day poultry houses, light intensity is measured in lux, a unit based on human spectral sensitivity however, this is perceived differently by chickens (Prescott and Wathes, 1999). The perceived intensity therefore is relative to the spectral power output of the light and the spectral sensitivity of the fowl (Prescott and Wathes, 1999). The retina can be stimulated with light at low intensities, where higher levels are required to stimulate hypothalamic photoreceptors (Harrison and Becker, 1970). Therefore, it was suggested that shorter wavelengths (blue/green light) require higher intensities to stimulate hypothalamic photoreceptors (Pang et al., 1974).

Light spectrum can effect reproduction, growth, and behaviour in birds. Longer wavelengths contain more energy and are able to penetrate through the skull and brain tissue to stimulate the hypothalamic photoreceptors which activate the reproductive axis in poultry (Woodard et al., 1968; Ingram et al., 1987; Oishi and Ohashi, 1993; Mobarkey et al., 2010; Kim et al., 2012; Hassan et al., 2013; Huber-Eicher, et al., 2013; Mobarkey et al., 2013; Baxter et al., 2014). This effect has been reported to be more pronounced after a molt or later in production (Pyrzak et al., 1987; Reddy et al., 2012). Previous reports indicate that the avian retina may act to inhibit reproduction, with retinal photoreceptors being more sensitive to wavelengths in the green spectrum, stimulation with green light maybe responsible for this inhibition (Foss et al., 1972; Oishi and Ohashi, 1993; Siopes and Wilson, 1980b; Mobarkey et al., 2010; Gongruttananun, 2011; Mobarkey et al., 2013). There is however, conflicting evidence regarding light spectrum effects on the onset of lay and egg production (Carson et al., 1958; Jones et al., 1982), suggesting there may be other controls such as body growth and metabolic status that may also regulate the onset of lay (Zelenka et al., 1984; Dunnington and Siegel, 1984).

The effects of light spectrum on the stress response, immune system and growth have been well documented in broilers, but not as well researched in commercial laying hens. Often, the birds immune status is used as an indicator of physiological stress and exposing broilers to green light during early rearing and blue light during late rearing resulted in heavier spleen weights (Xie et al., 2008a) and higher lymphocyte proliferation (Xie et al., 2008b). As well, birds exposed to green and blue light had longer lasting effective antibodies, a higher antibody production and humoral immune function. Exposure to white light resulted in higher Interleukin-1 β (IL-1 β) a pro-inflammatory cytokine playing a key role in stress (Xie et al, 2008a; Xie et al, 2008b). Similar results have been observed for body growth, where exposure to shorter

wavelengths optimizes growth in broiler chicks. Exposure to green light during initial production and blue light near the end of production caused satellite muscle cells proliferation and subsequent increases in muscle mass (Halevy et al., 1998; Rozenboim et al., 1999a; Rozenboim et al., 2004; Cao et al., 2008). Most trials looking at the effects of light spectrum on growth are conducted on broilers, and did not look at the effect of light treatment past 6 woa. Therefore, it is unclear if the effects of light spectrum on growth vary as birds' age, suggesting further research is required.

Intensive modern poultry facilities allow for controlled lighting environments, which has a large impact on behaviour and welfare of the birds (Mench, 1991). Behavioral studies performed on broilers found birds reared under green light are less active and spent more time sleeping and relaxing, whereas birds reared under red and white light had increased walking activity, floor pecking, wing stretching, and aggression (Prayitno et al., 1997a; Prayitno et al., 1997b; Sultana et al., 2013). Some reports show that layers reared under red light have increased feather pecking (Hassan et al., 2014), however, a reduction in aggression and feather pecking have also been reported (Schumaier et al., 1968; Huber-Eicher et al., 2013). Therefore, how light spectrum affects layers' behavior and aggression during their production period it still unclear.

Light emitting diodes (LED) are an efficient lighting source which can emit a pre-defined and stable spectrum. In the previous chapter using a strain of blind Leghorn (Smoky Joes) we reported that light from the red spectrum is instrumental in triggering and maintaining egg-laying, independently of the retina. Thus, an LED bulb that emits 60% red light (LED-R) was designed specifically for laying hens. The purpose of this experiment was to evaluate the effect of green, red and modified white light on behaviour, stress, reproduction and growth in birds maintained in floor pens.

3.3 Materials and Methods

3.3.1 Experimental Animals

The effects of monochromatic light on a commercial strain of laying hens, was performed using Lohmann LSL-Lites. The birds were housed at the Arkell Poultry research station in Guelph, Ontario. Chicks arrived at the research station on day of hatch and were maintained in brooding cage systems under incandescent light. From 1 to 3 days of age, birds were maintained on 23 hours of light at 20 lux. From 4 to 12 woa, birds were maintained on a 10 hours photoperiod at 10 lux. At 13 woa, 144 birds were randomly assigned to one of the three treatment rooms. Six pens were used within each room and 8 hens were randomly placed into each pen (1.83 X 2.36 M), at a stocking density of 0.540 m² per bird. Birds were fed *ad libitum* commercial diets meeting NRC requirements (1994). From 0-6 woa, chicks were fed standard starter diet (crumbles), from 7 to 16 woa pullets were fed a standard poultry grower (crumbles), and from 17 woa to the end of the trial, birds were fed a standard layer-breeder ration (crumbles). At the hatchery (Archers Hatchery, Brighton, ON) birds were injected with Mareks Disease Vaccine (live virus), sprayed with Bronchitis Vaccine (live vaccine) and administered infectious Vaccitec. From 3-5 woa, birds were sprayed with Newcastle-bronchitis vaccine (live virus), and ILT vectormune FP-LT-AE (live virus) was administered via the wing web. From 10-12 woa Newcastle-Bronchitis vaccine was administered via spray. At 18 woa, birds received intramuscular injection of Newcastle Bronchitis Vaccine (killed Virus). All animal procedures were reviewed and approved by the University of Guelph Animal Care Committee and at the end of the trial; all the birds were euthanized humanely.

3.3.2 Lighting Protocol and Treatment

After transfer to treatment rooms at 13 woa, hens were kept on a 9 hours photoperiod under incandescent light for 1 week to acclimatize them to their new environment. At 14 woa, rooms were equipped with either pure red (632nm), pure green (526nm) or 60% red LED spectrum (LED-R) lights (20% green (518nm), 20% blue (465nm) and 60%red (662nm)) bulbs supplied by Thies Electrical Distributing Company, located in Cambridge, Ontario, Canada (See Figure 3.1). Intensity in all rooms was adjusted to 10 lux at the hens' eye level. From 14 woa to 18 woa, birds were maintained under a 9 hour photoperiod. At 18 woa, birds were photostimulated with an abrupt change to a 16 h photoperiod. Spectral output was measured at the end of the trial by placing a spectrophotometer light probe approximately 3 feet directly under each light source.

3.3.3 Growth and Feed Consumption

At 14 woa, birds were weighed to determine their initial body weight, which was used as a reference point to measure growth. Body weights were measured every 2 weeks from 13-25 woa and every 4 weeks from 29-49 woa. A final weighing was also performed at 66 woa. Feed consumption was measured weekly by subtracting the initial weight of the feeder from total weigh of feeder when new feed was added. However, in this case feed consumption also included feed that may have been spilled by hens within the pen.

3.3.4 Egg Production and Time of Lay

Egg production as well as egg location (nest box vs floor) was recorded daily. Time of lay was estimated for four consecutive days at 28 and 33 woa by collecting eggs every 2 hours from the time lights were turned on (7 am) until 2 pm when most of the eggs should have been

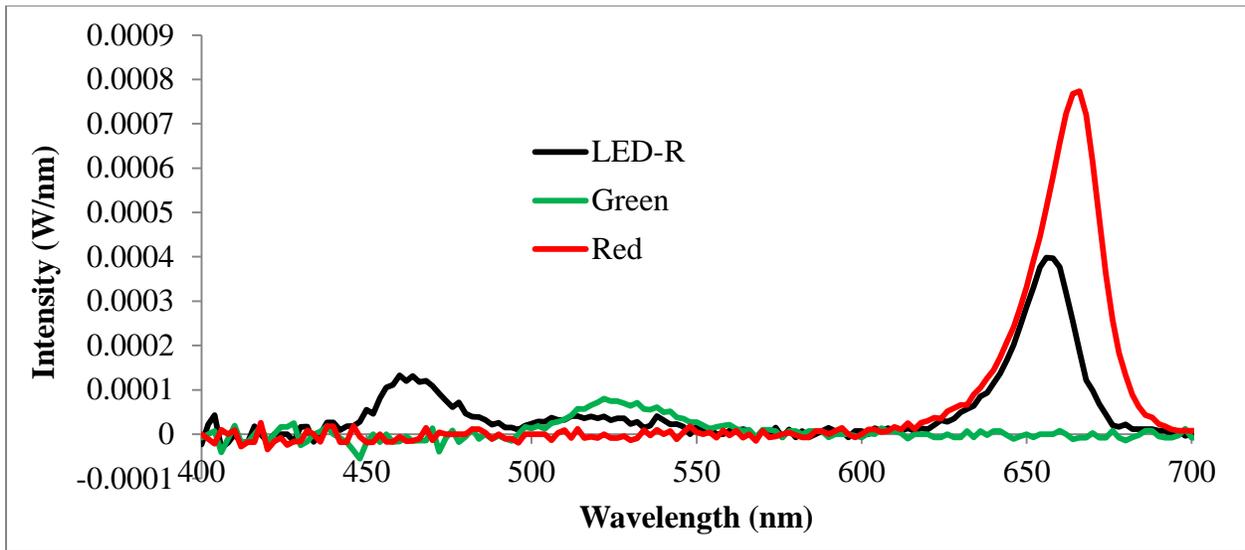


Figure 3.1: Spectral Output of each Light Treatment

Spectral output recorded by a spectrophotometer, with each light source set to 10 lux.

laid. Pens were also checked for eggs before lights were turned off at 9 pm, to determine if eggs were being laid during late afternoon and during the dark phase.

3.3.5 Behaviour and Stress

The ethogram of behaviours recorded is presented in in Table 3.1. Live observations were used for time budget and included scan sampling, where the entire pen was viewed from one end to the other, recording what each bird was doing at the first instant it was observed. Live observations were conducted by four different observers biweekly from 14 to 18 woa and weekly from 19 to 22 woa. For each time point, observation was performed twice a day beginning at 8:00 am, 30 minutes after lights were turned on and then again at 1:30 pm. To measure aggressive behaviour, two video cameras were placed in each pen. One was directed at the nest-box, and the other placed on the ceiling to cover the rest of the pen. Videos of each pen were recorded from light-on at 7:00 am for approximately 6 h from 25-29 woa, during peak production. To determine the most appropriate time of day to record aggressive behaviours, a pilot analysis was conducted to assess differences between the morning and afternoon, and to verify that the quality of video recording was sufficient to ensure proper assessment of behaviour. For this pilot analysis, two different observations methods were performed; the first included watching four 15 min observations from both sets of cameras at random time points throughout the day during three different days, and the second observation method involved 1 hour observations in the morning and in the afternoon. As no significant difference between the two methods was detected, the 1 hour observation in the mornings and afternoons paradigm was selected to allow observation of hens during nesting (when lights were turned on). All videos were viewed by one observer to reduce potential observer error.

Table 3.1: Ethogram for Time Budget and Aggressive Behaviours

3.1A Time Budget Behaviours	Operational Definition
Standing	Bird is immobile with its abdomen held above the ground.
Sitting	Bird is resting with its abdomen resting on the ground
Drinking	Bird is at the water dispenser actively drinking
Eating	Bird is at the feeder actively eating.
Walking	Moving forward
Perching	Bird is standing on the perch in the pen or perching on the nestbox
Preening	Bird is either grooming itself or another bird.
Nest Box	Bird is inside nest box
Dustbathing	Birds sitting or standing in substrate, may involve head or side rubbing, vertical wing shaking, pecking in plumage, scratching and wing and leg stretching
Foraging	Birds scratching and pecking while walking or standing
3.1B Aggressive Behaviours	Operational Definition
Aggressive Pecking	Frontal pecking directed at head or neck region with downward motion toward another hen (Estevez et al., 2002). May be associated with neck raising or slight chase/charge before attack. If a subsequent aggressive peck was done immediately following the initial peck – this counted as one count.
Position supplementation	A hen supplants its cage mate; can be done by force, or movement towards other hen causing the other to move out of the way. May occur at feeder, nest box, or water dispenser. Majority counts occurring at water dispenser and feeder.
Threat	A hen places itself in front of another bird with its neck stretched in attempt to gain height, may ruffles its feathers, stretch both wings, and views target bird downwards (associated with pre-aggressive behaviour). No pecks exchanged, may lead to fight. Maybe a minor threat in which hen stretches neck toward other hen, neck feathers may be ruffled. No aggressive peck involved.

3.3.6 Hormone Analyses: Corticosterone and Estradiol

Blood samples were taken from the wing vein of the same 12 birds (2 birds per pen) in each room at approximately 8:30 am on each sample day. Samples were taken biweekly from 13-25 woa and at 29, 33, 37, 41, 45, and 49 woa. Approximately 2 mL of blood were collected and placed in a sodium heparin vacutainer. Immediately after collection, blood plasma was recovered by centrifugation at $900 \times g$ for 15 min at 4°C and stored at -20°C until hormone extraction and assay. Corticosterone and estradiol assays were previously validated for chicken plasma (Baxter et al., 2014) and procedures were identical to the ones described in Chapter 2. The intra- and inter-assay coefficients of variation were $< 15\%$.

3.3.7 Statistical Analysis

Data was analyzed using Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Using the average value per pen as a replicate for each time point, a two-way ANOVA was performed on egg production, estradiol concentrations, feed consumption, body weight gain (%), and corticosterone concentrations, where age was the independent variable (X treatment) and light was the treatment (Y treatment). To account for a potential room effect on egg production, hens and bulbs were rotated between each room during the peak of lay. During phase 1, from 26 -31 woa, birds were kept in their initial rooms. At 31 woa, birds and bulbs were rotated between rooms for 4 weeks (phase 2). At 35 woa, birds and bulbs were rotated a third time between rooms to ensure all birds were exposed to every room and thus eliminating the potential room effect. For aggressive behaviours, a two-way ANOVA was performed, where the number of occurrences per pen was the replicate, behaviour was the dependent variable (Y treatments) and light treatment was the independent variable (X treatment). To analyze the time

at which birds laid their egg, a two-way ANOVA was performed where pens were the replicate, time of lay was the independent variable (X treatment) and light treatment was the dependent variable (Y treatment). There was no difference between each of the collections dates or pens. To determine if there was a difference between the days eggs were collected, a two-way ANOVA was performed for each light treatment, where pen was the replicate, time of lay was the independent variable (X treatment) and the collection date was the dependent variable (Y treatment).

Where eggs were laid was analyzed with a two way ANOVA for each location, on the nestbox or on the floor, where pen was the replicate, age was the independent variable (X treatment) and light treatment was the dependent variable (Y treatment). As well, a one-way ANOVA was performed to determine if there was a difference between pens within each light treatment where age was the independent variable (X treatment) and pen was the dependent variable (Y treatment).

As time budget data was collected in both the morning and afternoon, a one-way ANOVA was performed in each light treatment to determine if there was a difference in behaviour between collection times, where behaviour was the independent variable and morning or afternoon collections were in the dependent variables. Since there was no difference in behaviour between morning and afternoon, a two-way ANOVA was performed, where collection date was the replicate, behaviours were the independent variable (X treatment), and light treatment was the dependent variable (Y treatment). To ensure there was no difference in time budgets between pens within each treatment, a one-way ANOVA was performed for each treatment, where behaviours were the independent variable (X treatment) and light treatment was the dependent variable (Y treatment).

To ensure there was no difference in body weight between the pens within each light treatment, a two-way ANOVA was performed for each light treatment where age was the independent (X treatment) and pen was the dependent (Y treatment). Significance was based on $p < 0.05$. Body growth was measured by subtracting initial body weight from each subsequent body weight measurement. To determine if body weight correlated to when birds began laying, correlation was calculated where age at first egg was compared to the birds body weight, regardless of light treatment, from 14-22 woa.

3.4 Results

3.4.1 Egg Production

Reproductive performance was determined as egg production and is shown in Figure 3.2. Interestingly, birds were already at over 20 % production before photostimulation at 18 woa, suggesting other internal factors may affect the onset of lay. There was no significant difference in overall egg production between each light treatment ($p = 0.5638$). However, a Tukeys multiple comparison test determined significant differences between light treatments during four individual weeks. At 18 woa, birds maintained under red light ($31.1 \% \pm 6.82$) had a higher level of production than birds under green light ($p < 0.05$; $22.3 + 1.77 \%$). At 20 and 21 woa birds maintained under green light (20 woa: $90.2 \pm 2.55 \%$; 21 woa: $96.4 \pm 1.3 \%$) had higher production ($p < 0.01$) than birds under LED-R light (20 woa: $77.7 \pm 3.15 \%$; 21 woa: $85.1 \pm 2.67 \%$). At 63 woa, birds maintained under green light ($93.5 + 1.9 \%$) had a higher level of

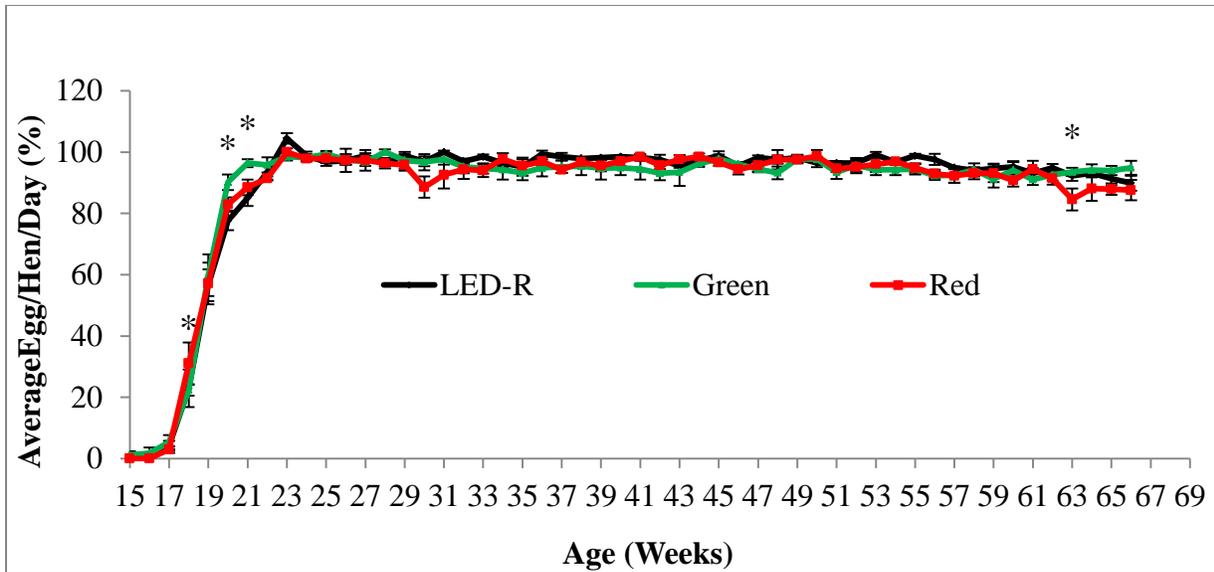


Figure 3.2: Egg production of birds maintained in collective floor pens.

Egg production was recorded weekly for each pen within each treatment. At 18 woa, birds maintained under red light had a higher level of production than birds under green light ($p < 0.05$). At 20 and 21 woa birds maintained under green light had a higher level of production than birds under LED-R light ($p < 0.01$). At 63 woa, birds maintained under green light had a higher level of production than birds under red light ($p < 0.05$). * indicates significance of $p \leq 0.05$. Overall, there was no significant difference between the three light treatments ($p = 0.5638$).

production than birds under red light ($p < 0.05$; $84.6 \pm 3.6 \%$). There was no difference in production at any other time point.

3.4.2 Estradiol Concentrations

Estradiol was measured throughout the trial to determine the activation of the ovary and indirectly measures recruitment of small follicles (Figure 3.3). Overall, light treatment had a significant effect on the level of estradiol in plasma ($p = 0.0214$). A Tukey's multiple comparison test determined there was a significant difference between light treatments at 15 and 17 woa. Birds under green light had significantly lower levels of estradiol at 15 woa, than birds under red ($p < 0.0001$) and LED-R ($p < 0.05$) ($W = 2.5 \pm 0.11$ ng/ml; $G = 1.49 \pm 0.05$ ng/ml; $R = 3.22 \pm 0.15$ ng/ml). As well, estradiol peaked at 17 woa, where birds under red light had a significantly higher level of estradiol than green ($p < 0.001$) and LED-R ($p < 0.0001$). ($W = 2.63 \pm 0.12$ ng/ml, $G = 3.07 \pm 0.16$ ng/ml; $R = 4.55 \pm 0.20$ ng/ml). There was no significant difference between light treatments for the remainder of the trial. When looking at changes in estradiol for each individual treatment overtime, levels are expected to decrease following initial follicular recruitment ($p < 0.0001$). However we did notice a slight increase in estradiol in all light treatments, occurring at around 45 woa.

Table 3.2: Cumulative egg number from 15-66 woa.

There was no significant difference in cumulative egg number between each light treatment.

Light Treatment	Avg. number of Eggs per Bird from 15-66woa.
LED-R	320.4 \pm 0.74
Green	320.1 \pm 2.4
Red	316.8 \pm 3.8

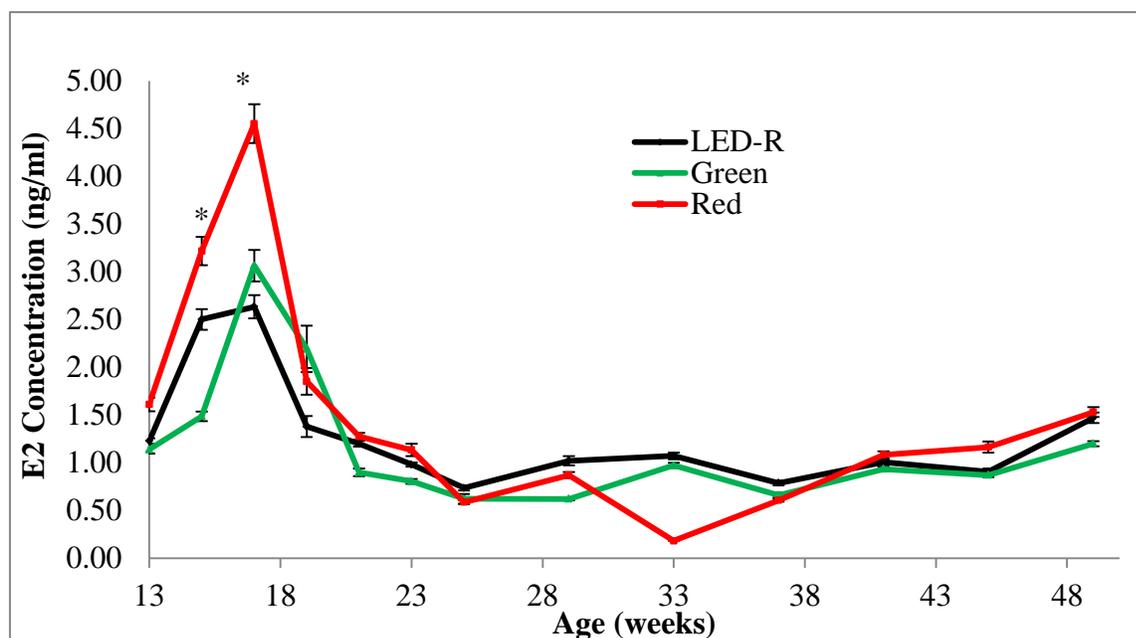


Figure 3.3: Estradiol concentrations in the plasma under each light treatment

Overall, light treatment had a significant effect on the level of estradiol in the plasma ($p = 0.0024$; ng/ml \pm standard error of the mean (SEM)), this was significant at 15 and 17 woa during peak estradiol production. Birds under monochromatic red light had significantly higher levels of estradiol at 15 woa than birds under green light ($p < 0.01$). The peak of estradiol at occurred at 17 woa, birds under red light had a significantly higher level of estradiol than birds under LED-R ($p < 0.01$) and green ($p < 0.05$).

3.4.3 Time and Location of Lay

Time of lay was recorded to determine if light spectrum influenced the entrainment of oviposition (Figure 3.4). At 28 woa, birds maintained under green light laid significantly more eggs in the dark than birds under red and LED-R light ($p < 0.0001$). Birds under red and LED-R light laid significantly more eggs between 9-11 am than birds under green light ($p < 0.05$). There was no significant difference between each consecutive collection date, nor was there any significant difference between pens. To determine if birds under green light continued to lay in the dark and if there was a room effect, another egg collection was performed at 33 woa after birds' rotation (Figure 3.4B). At that time, birds under green light still laid significantly more eggs than birds under red and LED-R light before lights were turned on ($p < 0.01$). Birds under red and LED-R light laid significantly more eggs between 9-11 am than birds under green light ($p < 0.05$). Again there was no difference between the collection dates, nor was there any significant difference between pens.

Throughout the trial, egg location was recorded to determine if the different light spectrum influenced nesting and is displayed in Figure 3.5. On average, birds under green light laid 39.3 % of their eggs on the floor, and 59.7 % in the nestboxes. Birds under LED-R light laid 86.4% of their eggs on the floor and 17.6% in the nestboxes. Birds under red light laid 75.26% of their eggs on the floor and 22.3% in the nestboxes. Overall, birds

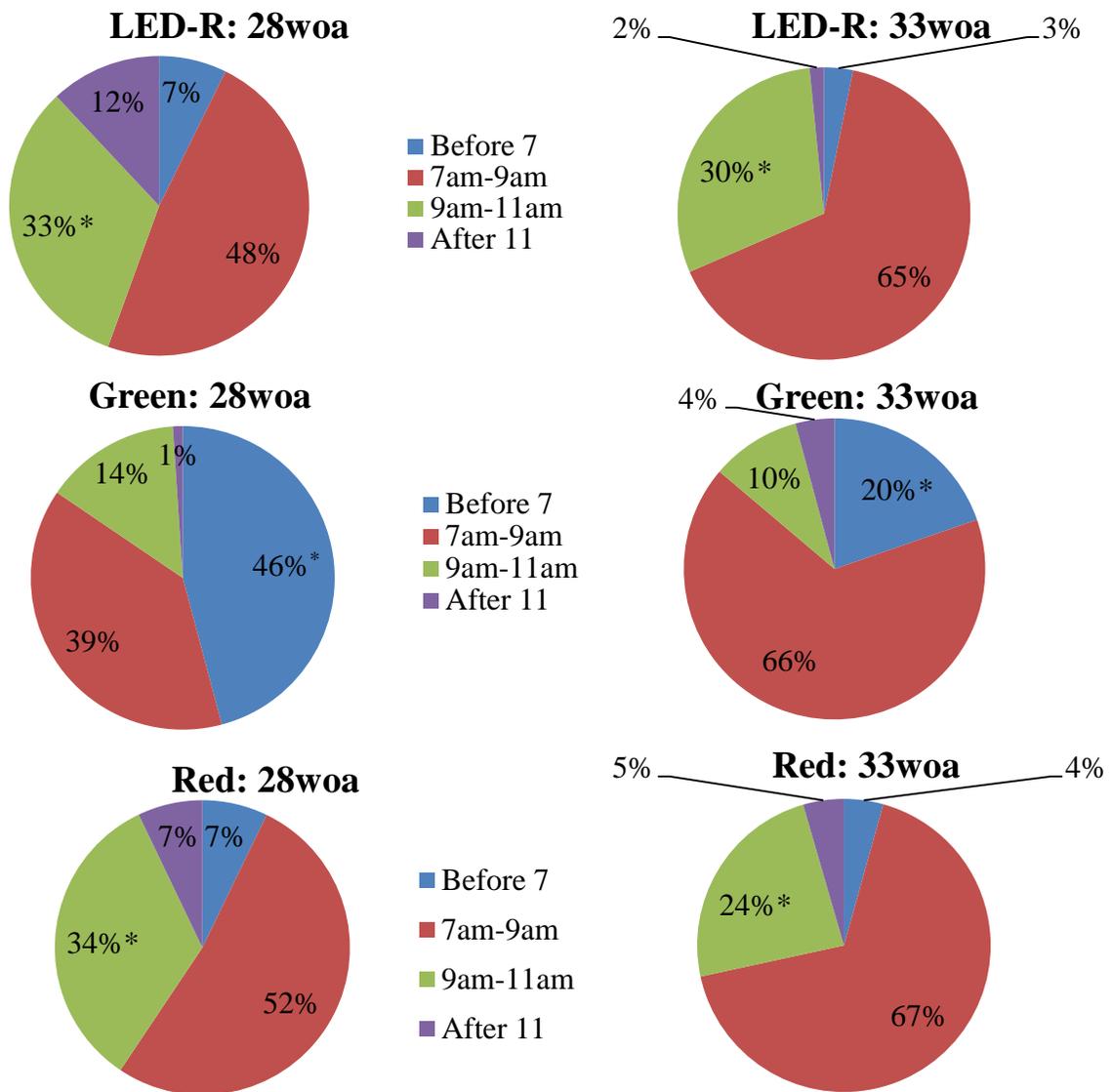


Figure 3.4: Time of lay for each treatment observed at 28 and 33 woa.

Time of lay was recorded to determine if light spectrum influenced the entrainment of oviposition. At 28 woa, birds maintained under green light laid significantly more eggs in the dark than birds under red and LED-R light ($p < 0.0001$). Birds under red ($p < 0.05$) and LED-R ($p < 0.05$) light laid significantly more eggs between 9-11 am than birds under green light. At 33 woa, birds under green light still laid significantly more eggs before lights were on than birds under red ($p < 0.05$) and LED-R light ($p < 0.01$). Birds under red ($p < 0.05$) and LED-R ($p < 0.001$) light laid significantly more eggs between 9-11 am than birds under green light.* indicates significant of $p < 0.05$ different between light treatments.

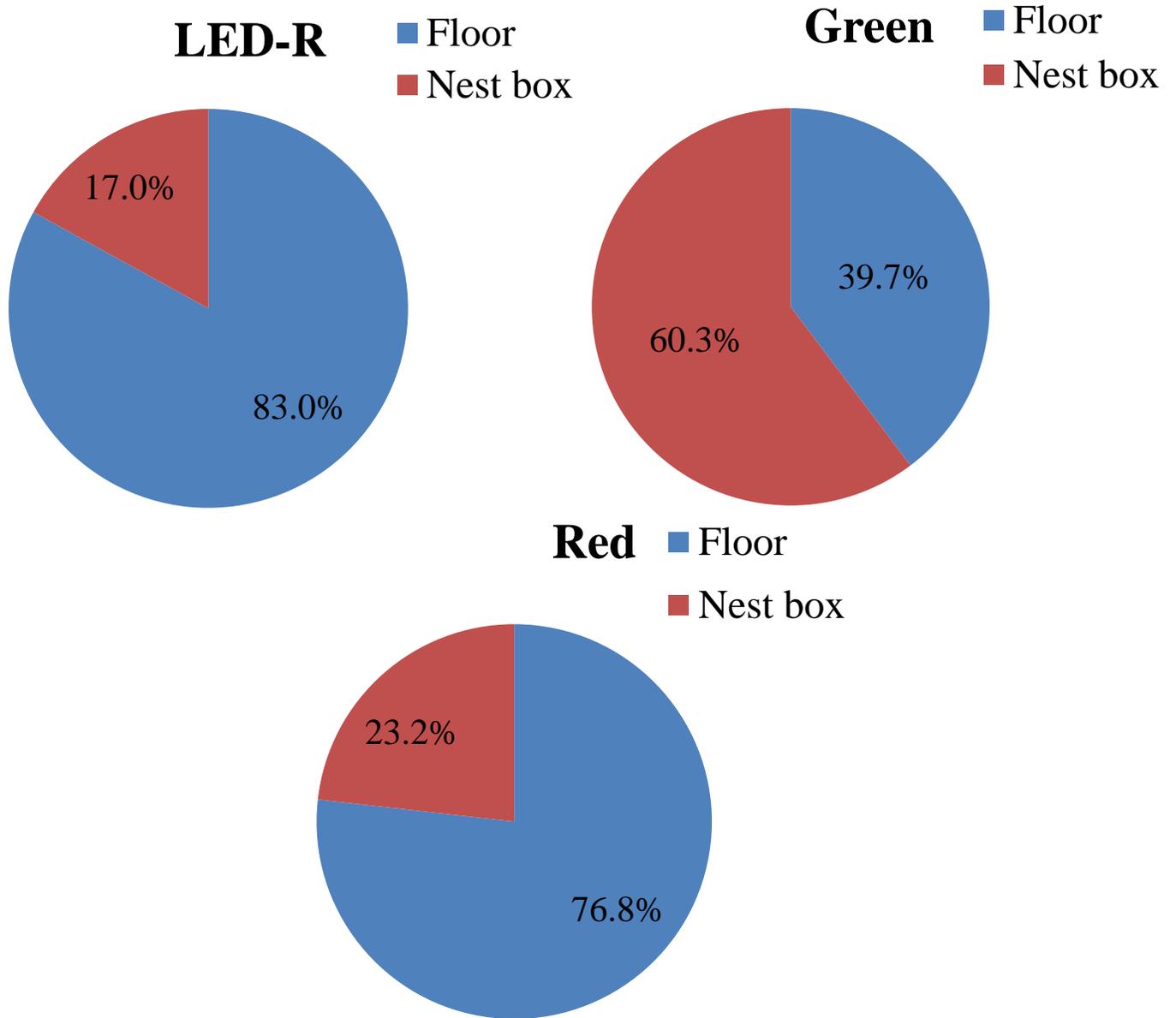


Figure 3.5: Average location of where eggs were laid for each treatment

Egg location was recorded daily and averaged for the entire trial. Birds under the green light treatment laid significantly more eggs in the nest box than those birds under LED-R and red light treatments ($p < 0.0001$).

under green light laid significantly more eggs in the nest box than birds under red and LED-R ($p < 0.0001$). There was a significant pen effect for egg location (floor: $p < 0.0001$; nestbox: $p < 0.0001$), where hens in certain pens, regardless of light treatment, laid significantly more eggs on the floor. It appears that the age of the hens also had a significant effect as to where birds lay their eggs (floor: $p < 0.0001$; nestbox: $p < 0.0001$), birds in all light treatments had a higher number of floor eggs as the trial progressed.

3.4.4 Stress

Corticosterone was measured throughout the trial as an indicator of physiological stress (Figure 3.6). There was no significant difference between any of the light treatments for samples taken from 13 to 25 woa ($p = 0.6016$). There was no significant difference between pens within each light treatment (W: $p = 0.3906$; G: $p = 0.9022$; R: $p = 0.2340$). Corticosterone levels did fluctuate over time ($p < 0.0001$); however there were no consistent trends between each collection date.

3.4.5 Body Weight Gain and Feed Consumption

Body weight and feed consumption were measured to assess the effects of various light spectrums on growth (Figure 3.7). Overall, light treatment had a significant effect on body weight gain, where birds under LED-R and green light had a significantly higher body weight gain than birds under red light throughout the trial ($p = 0.0039$). LED-R light had higher growth than birds maintained under red and green at 17, 19, 33, 41 and 66 woa. Birds under LED-R had significantly higher growth than under red light at 37 and 49 woa. Hens under green light had a significantly higher body weight gain than red and LED-R at 21 and 23 woa. And no pen effect was detected for each individual treatment. The birds' age had a significant effect on

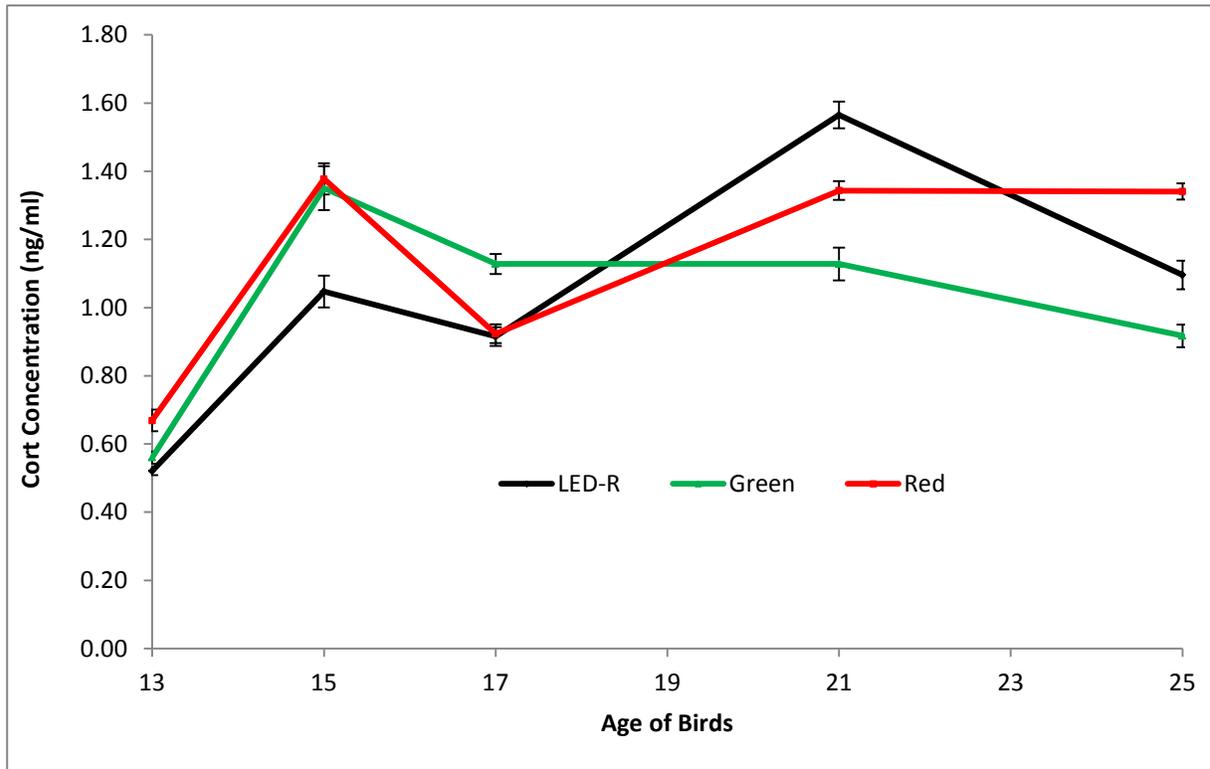


Figure 3.6 Corticosterone levels in the plasma under each light treatment

Corticosterone concentrations (ng/ml \pm SEM) in the plasma were measured throughout the trial. Overall, there was no significant difference between light treatments ($p = 0.6016$) however, corticosterone levels did fluctuate over time ($p < 0.0001$).

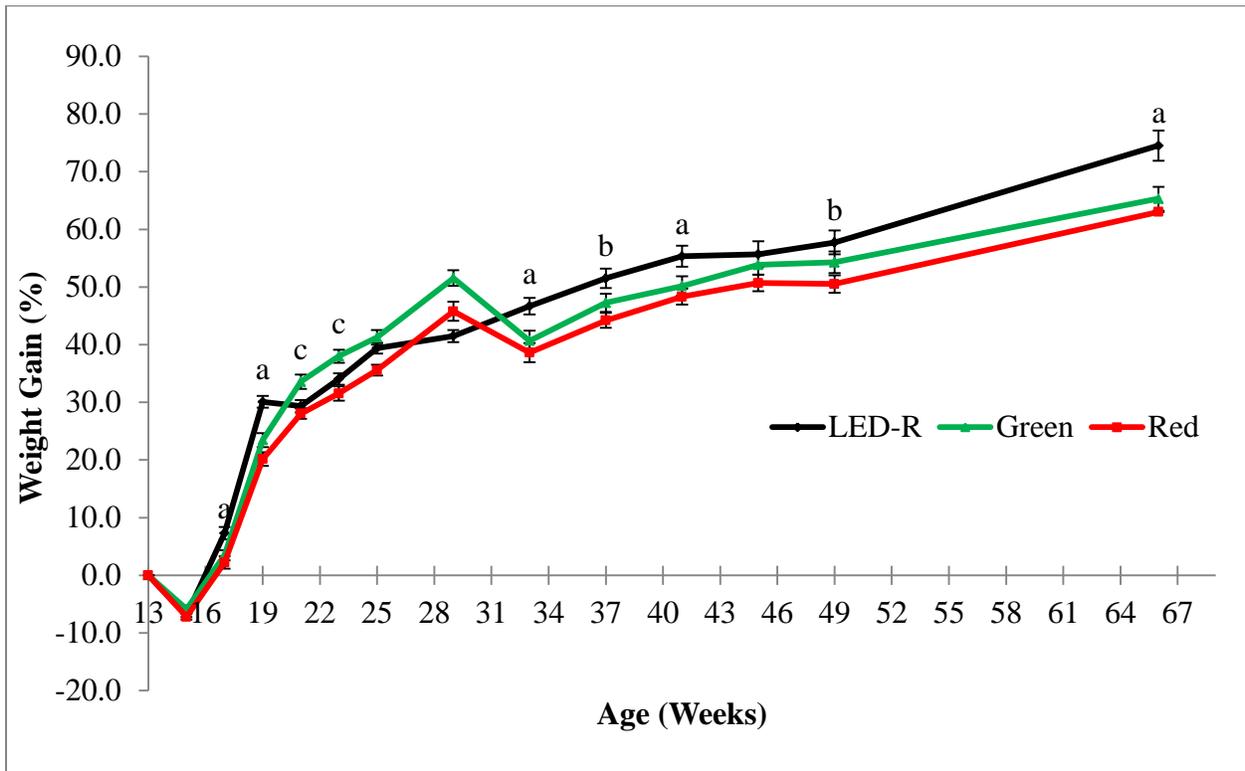


Figure 3.7: Body weight gain of birds maintained on collective floor pens exposed to various light treatments

Initial weights were used as a reference point to measure for treatment during collection date. Overall, light treatment had a significant effect on body growth, where birds under LED-R and green light had a significantly higher level body weight gain than birds under red light throughout the trial ($p = 0.0039$). Superscript indicates significant difference of $p \leq 0.05$ between the light treatments. Superscript ^a indicates LED-R had significantly higher body weight gain than birds under red and green light; ^b indicates LED-R had significantly higher body weight gain than birds under red light; ^c indicates birds under green light had significantly higher body weight gain than birds under red light; ^d indicates birds under green light had significantly higher body weight gain than birds under LED-R and red lights.

body weight, where birds gained significantly more weight as they aged ($p < 0.0001$), which caused significant interaction between light treatment and time ($p < 0.0001$).

Feed consumption was measured weekly to determine any effect of light (Figure 3.8). Overall there was no significant difference in feed consumption between light treatments ($p = 0.9649$). There were significant differences within pens between each treatment. Regardless of light treatment, time had a significant effect on feed consumption ($p < 0.0001$).

3.4.6 Time Budget and Aggressive Behaviours

A time budget was recorded from live observations to determine if light treatments affect behaviour (Figure 3.9). There was no significant difference between behaviours performed during morning or afternoon therefore each recording was averaged. As well, within each treatment there was no difference between pens, therefore all pens were included within the analysis. Overall, there was difference in behaviour between treatments, when performing a Tukey multiple comparison test, some differences between treatments were detected. Birds under red light spent less time standing than birds under LED-R ($p < 0.01$) and more time eating than birds under green ($p < 0.01$) and LED-R lights ($p < 0.001$). Birds under red light spent less time traveling than birds under LED-R ($p < 0.05$). Birds under red light spent more time preening and drinking than birds under green ($p < 0.05$). Birds under LED-R light spent more time foraging than birds under red ($p < 0.001$) and green ($p < 0.001$) light. Aggression, displayed in Figure 3.10, was measured to determine if light had a negative impact on behaviour. Overall, when aggressive behaviours were combined (aggressive pecks, threats and displacements) there was no difference in behaviours between light treatments. However, when looking at the occurrences of each individual behaviour, birds under LED-R light had a significantly higher number of

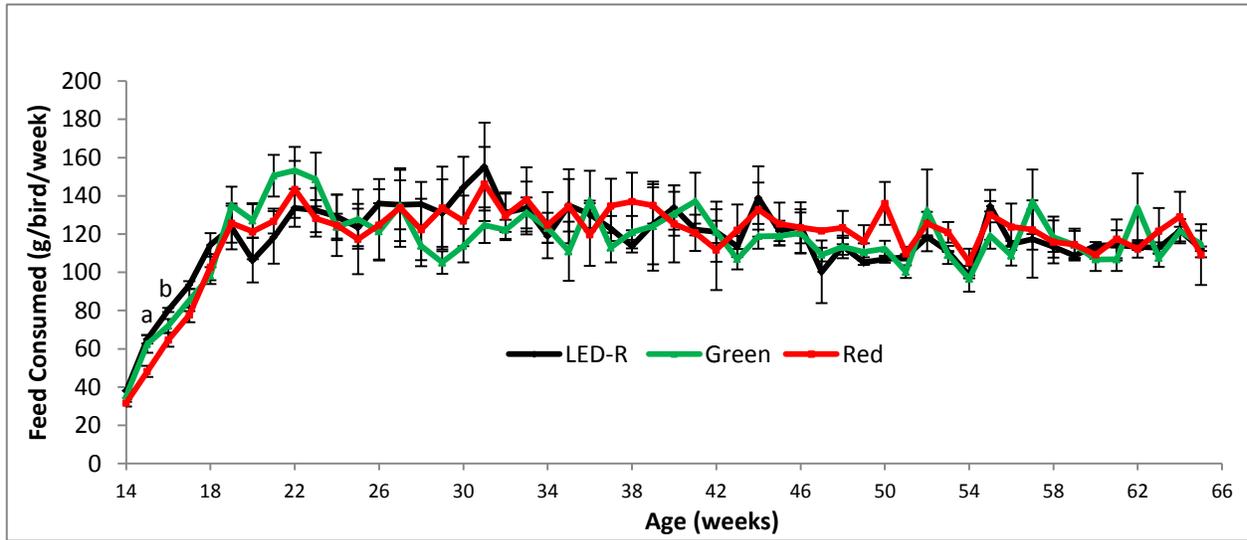


Figure 3.8 Feed consumption of birds maintained on collective floor pens under various light treatments

Feed consumption was measured weekly to determine if there was a difference between light treatments. Overall there was no significant difference in feed consumption between light treatments ($p = 0.9649$).

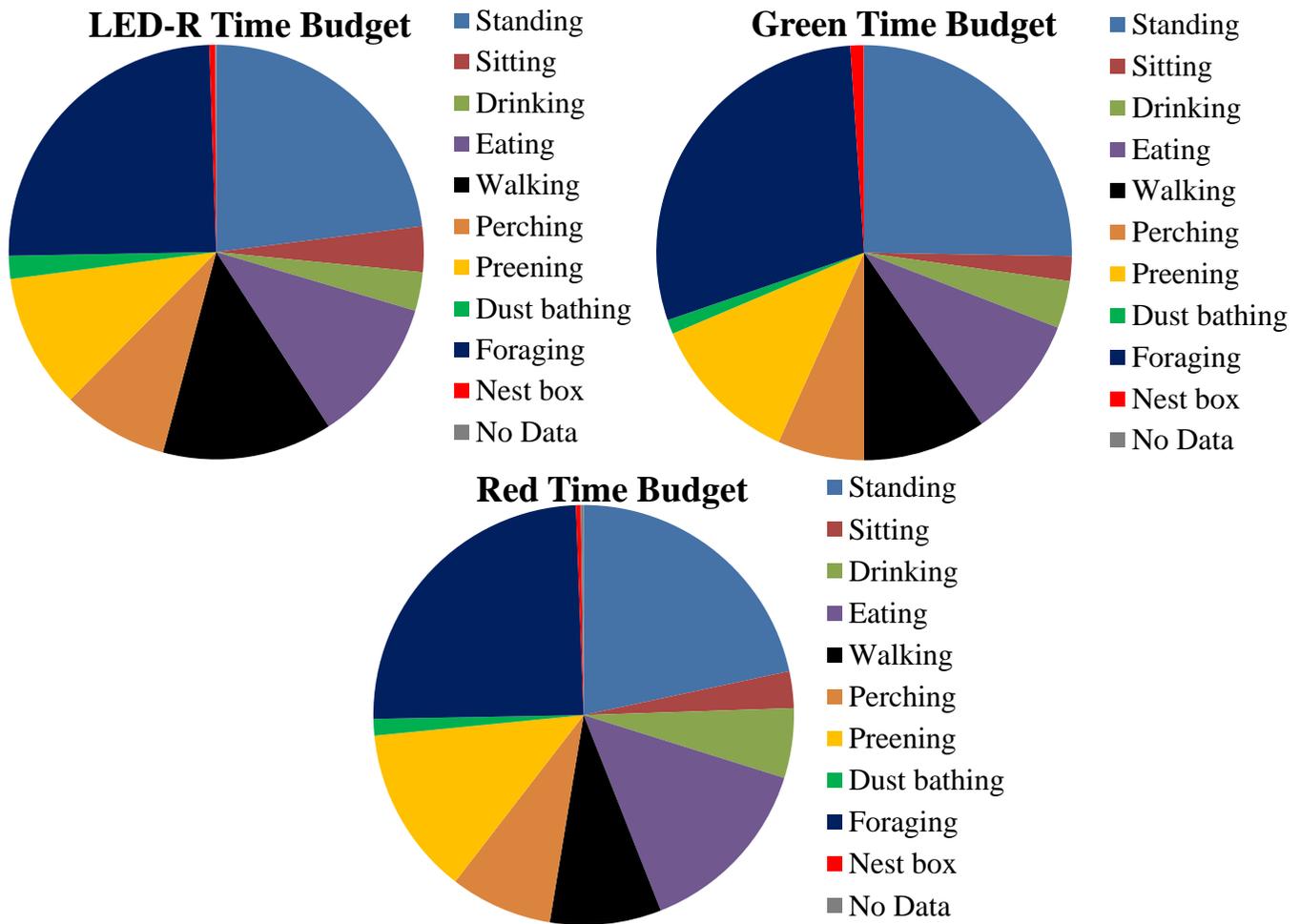


Figure 3.9: Time budget behaviours recorded via live observation under each light treatment

A time budget was recorded via live observation to determine if light treatments affect birds' behaviour. Overall, there was no major difference in behaviour between treatments, but when performing the Tukey multiple comparison test, some differences between treatments were detected. Birds under red light spent less time standing than birds under LED-R ($p < 0.01$) and more time eating than birds under green ($p < 0.01$) and LED-R light ($p < 0.001$). Birds under red light spent less time traveling than birds under LED-R ($p < 0.05$). Birds under red light spent more time preening and drinking than birds under green light ($p < 0.05$). Birds under LED-R light spent more time foraging than birds under red ($p < 0.001$) and green light ($p < 0.001$).

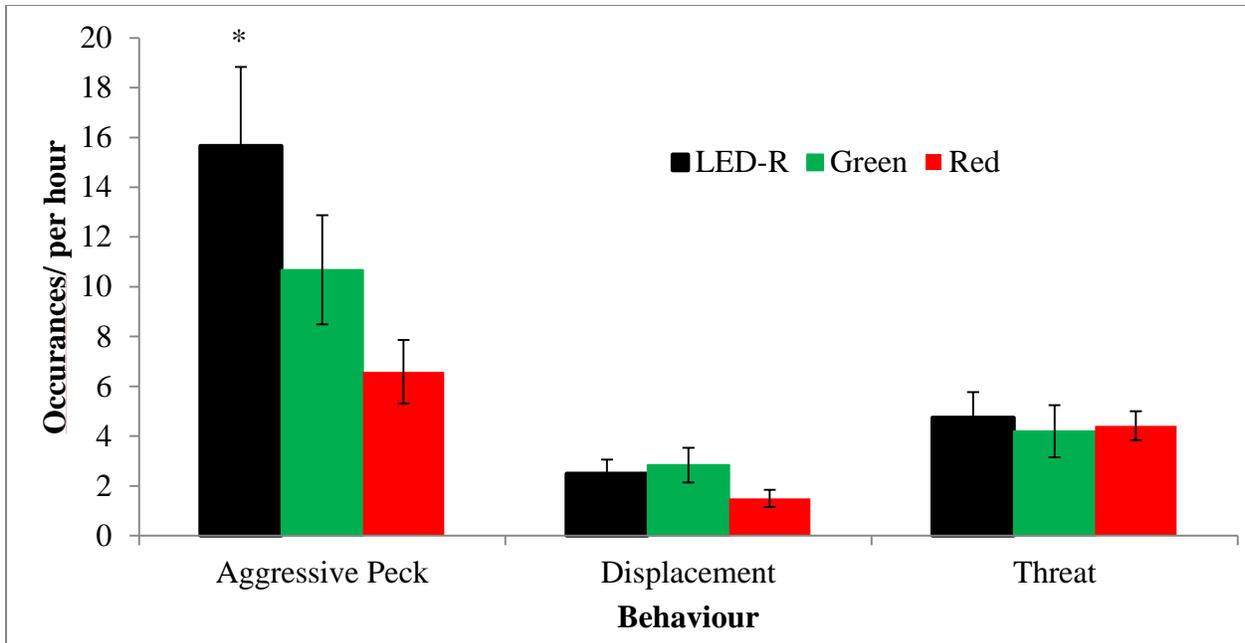


Figure 3.10: Aggressive behaviours recorded via video observation under each light treatment

Overall, when aggressive behaviours were grouped together (aggressive pecks, threats and displacements) there was no difference between light treatments (W: $p = 0.3343$; G: $p = 0.1230$; R: $p = 0.3542$). However, when looking at the occurrences of each individual behaviour, birds under LED-R light had a significantly higher number of aggressive pecks than birds maintained under red and green light ($p < 0.0001$), indicated by the superscript *. There was no difference in the number of threats and displacements.

aggressive pecks than birds maintained under red and green light ($p < 0.0001$), but there was no difference in the number of threats and displacements.

3.5 Discussion

3.5.1 Reproductive Parameters

Within each light treatment, birds began laying before photostimulation, suggesting that this commercial strain of layers does not only rely on photoperiodic cues to initiate the onset of lay but may rely on other internal factors. A study performed on Japanese quail found that quail matured earlier when they reached a critical body lipid level (Zelenka et al., 1984). It has also been reported that early maturing leghorn hens began laying at a similar body weight to those hens that matured later, indicating that there is a correlation between body weight of birds and initiation of lay (Dunnington and Siegel, 1984). The strain recommended average body weight of birds at 20 woa is between 1.3-1.4 kg (Lohmann Tierzucht, 2013). At 21 woa, the average body weight of birds in the present study, was higher than the recommended weight of 1.42 kg (LED-R= 1.437 kg; Green=1.476 kg; Red=1.453 kg), which may account for why birds reached peak production before expected. Interestingly, at 21 woa birds under green light had higher egg production and body weight, suggesting that they may have reached their mature body weight, allowing them to reach peak production sooner. For reference, this strain is expected to reach 50 % production between 21-22 woa in a non-cage system (Lohmann Tierzucht, 2013). In our study, regardless of light treatment, birds reached over 50% production by 19 woa (W = 56.5 %; G = 59.8 %; R = 57.1 %). Thus, conversely to what we reported in Chapter 2 using a non-commercial strain of layers maintained in cages (Baxter et al., 2014), light spectrum did not affect the initiation and maintenance of lay in commercial birds maintained in floor pens. Over the last 60 years, higher egg production has largely been achieved through improvements in

genetic selection programs (Hocking, 2010) and, data from older literature may not apply to newer strains of commercial birds.

There was no difference in overall egg production or number of cumulative eggs among treatments. This is similar to Jones et al. (1982) who found no difference in egg production for turkey hens exposed to incandescent and red light; however it should be noted that the number of photons delivered to the bird was not normalized between filtered and non-filtered incandescent light (Jones et al., 1982). Similar results were found by Carson et al. (1958) where chickens exposed to various spectra using filtered fluorescent light, found no difference between treatments at 50 % production and after 400 days of production. However it should be noted between the two studies that various light sources were used and birds were exposed to different lighting schedules. In a recent study using the Thai native hen as a model, there was no difference in egg production but hens exposed to daylight and fluorescent light produced on average ten less eggs than those exposed to red and daylight (Gongruttananun, 2011). Light intensity under each treatment may have reached the intensity threshold which resulted in similar levels of egg production between the light treatments. It should also be noted that, even though each light sources was set to 10 lux, when measuring the light spectrum there was variation in the true intensity, measured in spectral radiant flux (W/nm), between the light treatments (Figure 3.1).

The results from the present study are contrary to those reporting that birds under monochromatic red light had significantly higher egg production than birds maintained on green and blue light (Woodard et al., 1968; Ingram et al., 1987; Kim et al., 2012; Huber-Eicher et al., 2013; Hassan et al., 2013; Baxter et al., 2014). In this study we used the Lohmann LSL-Lite laying hens, a strain of white leghorn heavily selected for egg production (Lohmann Tierzucht, 2013). Birds used in previous trials assessing the effect of red light on egg production were

performed on quail (Woodard et al., 1968); chickens unselected for egg production (Baxter M 2014); broiler breeders (Ingram et al., 1987; Mobarkey et al., 2010; Mobarkey et al., 2013) or other strains of commercial laying hens (Kim et al., 2012; Huber-Eicher et al., 2013; Hassan et al., 2013). There have been reports that the effects of higher wavelengths were more prominent during the second laying cycle after birds were molted (Pyrzak et al., 1987) or later on in lay from 73-82 woa (Reddy et al., 2012). An inhibitory effect or lack of stimulation of monochromatic green light was not observed in this trial as there was no difference in production between treatments. This is in contrast with studies that showed birds maintained a poor reproductive response under green light due to lack of stimulation (Woodard et al., 1968; Foss et al., 1972; Oishi and Ohashi, 1993), or may even be delayed due to possible inhibitory effects of green light via the retina of the eye (Mobarkey et al., 2010; Mobarkey et al., 2013; Baxter et al., 2014). Variation between results may be due to the use of different light sources, species/strain of birds and differences in light intensity. Therefore it is evident that more research is required to determine how light wavelength affects the reproductive axis in commercial hens.

Estradiol is produced from the small follicles of the ovary and peaks three to five weeks prior to the initiation of lay (Etches, 1996). Birds under red light had a significantly higher level of estradiol at 15 and 17 woa. This suggests that monochromatic red light is more efficient at stimulating the reproductive axis to increase ovarian activity. Similar results were seen in broiler breeders where birds under red light had an increase in estradiol, progesterone and testosterone during the first week of photostimulation (Mobarkey et al., 2010). Additionally, hens under monochromatic red light and combined red and green light had a higher level of estradiol than hens under blue and green monochromatic lights (Gongruttananun, 2011; Hassan et al., 2013; Baxter et al., 2014). Similar results were seen by Reddy et al. (2012) observing that hens under

red light had higher LH surge and overall levels of LH, estradiol and progesterone. In this trial, higher levels of estradiol did not appear to effect production as seen in other studies (Mobarkey et al., 2010; Baxter et al., 2014). Regardless of light spectrum, birds were producing almost an egg a day therefore it may be physiologically impossible for higher levels of estradiol to translate to higher egg production. Nonetheless, higher estradiol may affect other aspects of the birds' physiology such as calcium deposition or time of ovulation (Beck and Hansen, 2004).

3.5.2 Time of Lay and Location

Time of lay was recorded to determine if light spectrum effected timing of oviposition and thus ovulation. Birds maintained under green light laid significantly more eggs before the lights were turned on than those under red and LED-R light. This suggests that birds under green light had a free-running oviposition rhythm, which may have been due to lack of stimulation or longer mean intervals between consecutive daily oviposition (Harrison, 1974; Etches, 1996). Typically, the light-dark photoschedule regulates when ovulation occurs, and under the 14 h photoperiod as used in this trial, the majority of eggs are laid within the first couple hours after dawn (Etches, 1996). This result was observed in birds maintained under red and LED-R; who laid the majority of their eggs less than 4 hours after light was turned on. The lack of synchronicity in birds under green light was also seen by Harrison et al. (1974) when birds exposed to shorter wavelengths had consistently changing times between consecutive ovipositions. This may suggest shorter wavelengths are less efficient at stimulating the bird's circadian rhythm, preventing light from entraining time of lay. However due to limited data collection further studies need to be conducted to determine if birds under green light are on free-running circadian rhythms due to the lack of stimulation.

Birds under green light laid significantly more eggs in the nestboxes than birds under red and LED-R light. A study by Huber- Eicher et al. (2004) found that birds had a preference to nestboxes painted yellow. They also found that there was a lot of individual variability in nest choice between laying hens (Huber-Eicher et al., 2004; Zupan et al., 2005) and many factors can affect nesting site, such as nest stimuli and social affects (Huber-Eicher et al., 2004; Zupan et al., 2005). Although the light intensity for each treatment was set to 10 lux, avian species have a different spectral sensitivity and certain light may appear brighter. Prescott and Wathes, (1999a) reported that the avian retina has a higher sensitivity to green light, and may appear brighter to birds. Therefore due to the perceived brightness of green light, the nestbox may have been interpreted as the darkest, most secluded nesting site for hens to lay their eggs (Appleby et al., 2004). It should be noted that floor eggs were often found in certain areas of the pen, which may have been perceived as nesting sites, especially shaded areas under the nest box and corners. Nesting behaviours vary between hens; where some hens lay in the same nesting site every time, others will always lay in the most isolated area (Riber and Birte, 2013). It should also be noted that there was a miscommunication with the farm staff and shavings were placed in the nestboxes in the green treatment, potentially encouraging hens to use the nestbox. There was no significant difference between nesting location in hens over time suggesting than nesting location was conserved. However, due to grouped housing conditions within each pen, it could not be determined if this behaviour was truly conserved.

3.5.3 Stress

The inconsistent variation in corticosterone levels as birds aged and the lack of difference indicate that light treatment did not affect stress levels in laying hens maintained on collective floor pens. Similar results were seen in turkey hens exposed to monochromatic blue, green, and

red light and incandescent light where there were no significant differences in erythrocyte, leukocyte and corticosterone concentrations (Scott and Siopes, 1994). Fear response is often measured using the tonic immobility test, where birds that are more stressed stay immobile longer. In birds maintained under various light wavelengths, initial testing found that birds under red light had a longer time to recovery, and the second set of tests found birds under yellow light had a longer recovery rate (Sultana et al., 2013). Although the results were inconsistent, the authors speculated that the higher activity of birds under longer wavelengths may increase duration birds remain immobile (Sultana et al., 2013). As interleukin-1 β (IL-1 β), a pro-inflammatory cytokine, can elicit the release of corticosterone, it can be used as an indicator of physiological stress (Xie et al., 2008b). The level of IL-1 β was shown to be highest in birds exposed to white light, while exposure to blue light produced the lowest amount of corticosterone, indicating low stress levels (Xie et al., 2008b). The lack of consistent results within the current study may be due to bird handling before sampling or difference in perceived intensity. It should be noted that the level of corticosterone is below what is considered physiologically stressed, where daily fluctuations in laying hens ranges from 7 to 11 ng/mL (Beuving and Vonder, 1978).

3.5.4 Feed Consumption and Body Weight Gain

There was no difference in feed consumption between treatments, yet birds under red light had significantly lower body weight gain than birds under LED-R and green light. It is evident from the literature that broilers under green light and or a combination of green and blue lights results in significantly higher body growth (Halevy et al., 1998; Rozenboim et al., 1999a; Rozenboim et al., 2004a; Rozenboim et al., 2004b; Cao et al., 2008). However, these studies were performed on broilers which have a completely different genetic make-up and growth

curves compared to layers, and often reach market weight before 10 woa while still at a juvenile stage. Nonetheless, it is possible that the higher weight gain observed under LED-R light compared to pure red is due to the blue and green component of the light. Broilers under green and blue light had higher body weights than those exposed to monochromatic red and white. This was due to higher skeletal muscle satellite cell proliferation and higher levels of androgens which increase protein synthesis and reduces protein breakdown (Rozenboim et al., 1999a). It has also been reported by Praynito et al. (1997a) that broilers under red light had higher activity levels, which may be a potential reason for the reduced body growth. In the present trial, there was no correlation between time budget behaviours and light treatment suggesting activity level didn't differ and thus did not affect growth. The effect of light spectrum on growth has not been as extensively researched in layers; however Baxter et al. (2014) found hens under green light had significantly higher body weight gain than birds under red and white, however feed consumption was not measured and birds under green light laid significantly fewer eggs. Therefore it was speculated that a reduction in eggs resulted in more energy being put towards body growth than egg production (Baxter et al., 2014). Cockerels exposed to monochromatic green light displayed higher body growth but there was no difference in feed consumption (Foss et al., 1972). It was suggested that, each bulb was emitting the same amount of energy; therefore higher growth in birds under green light was due to this component of the spectrum lacking a wavelength that inhibits growth (Foss et al., 1972). Thus, various light spectrums may affect birds differently depending on ages, as well as strains. In our results we observed that birds under green and red light had a drop in body growth from 19 to 33 woa. It should be noted that this was the time in which birds and bulbs were rotated between the rooms which may have caused handling stress or exposure to diseases that led to this drop in growth.

3.5.5 Time Budget and Aggressive Behaviour

Due to the spectral sensitivity of the avian retina and the limitation of spectral output from artificial light, lack of certain light wavelengths may affect behaviour (Prescott and Wathes, 1999). Our results indicate that there is a slight variation in standing, preening, eating, traveling and foraging behaviour between birds in each of the treatments. However there was no correlation between behaviours performed and light spectrum; therefore no definitive conclusions could be made from our observations. Prayitno et al. (1999a & 1999b) observed a higher activity of birds under red light. In a study by Sultana et al. (2013), broilers exposed to higher wavelengths had an increase in walking behaviour and birds under shorter wavelengths spent more time sitting and standing. Hassan et al. (2014) found similar results, where layer chicks under red light had a higher incidence of feather pecking, however this was not statistically significant. Previous research suggests that red light is better able to penetrate hypothalamic photoreceptors, which may activate interactive behaviours (Prayitno and Phillips, 1997). However, most studies were conducted on broiler birds and therefore the contrast in results may be due to variations between strains. Furthermore, age has a significant influence on behaviour, as younger birds are more active than older birds regardless of light spectrum (Sultana et al., 2013). Under our experimental conditions, behaviour was observed between 14 and 22 weeks, and differences may have been more definite at a younger or older age.

Birds under LED-R light had a higher incidence of aggressive pecks than birds under red or green light. LED-R bulbs in the present study emitted a wider range of the visible light spectrum. This may have increased the bird's visual acuity leading to higher amounts of aggression. When Huber-Eicher et al. (2013) exposed laying hens to monochromatic light at equal intensities; it was found that birds under red light had a reduction in aggressive behaviour.

Schumaier et al. (1968) found similar results where birds under red light had lower incidence of feather pecking and cannibalism. This is in contrast to Prayitno et al. (1997a, 1997b) who observed a higher incidence of aggression in birds maintained under red light. In the current trial, the higher incidence of aggressive pecks in birds under LED-R light, maybe due to the relatively low stocking density, 0.540 m² when the code of practice requires 0.17m² (Canadian Agri-Food Research Council, 2003) as birds have formed social hierarchies (Nicol et al., 1999). Another study observed that group size preferences depended on both sufficient space and density but also depends on the number of dominant hens (Lindberg and Nicol., 1996). This suggests that even if each pen had the same amount of space, there were more dominant hens under the LED-R treatment, which is most likely the reason for the higher number of aggressive pecks. Due to limitations in equipment there was only one observation time for each pen, suggesting the aggression may have been due to a social hierarchy or a potential room effect. Better time budget assessments and aggressive behaviour recording may be necessary to accurately determine the effects of light spectrum on laying hen behaviour.

3.6 Conclusion

In conclusion, light did not affect the onset of lay and spectrum did not impact egg production for this commercial strain of birds under the experimental conditions, as birds began laying before photostimulation and had over a 90 % production at the end of the trial. However, the higher concentrations of estradiol under monochromatic red light suggest that higher wavelengths are more efficient at stimulating the reproductive axis to increase ovarian activity. It also appeared that shorter wavelengths are less efficient at entraining oviposition and ovulation, as hens under green light exhibited a lack of synchronicity between light schedule and time of lay. Birds under LED-R and green lights had a higher body growth compared to birds exposed to

red light. Although there was no difference in feed consumption or time budgeting, previous reports suggest that birds under red light may have higher activities levels due to deep brain stimulation. Light spectrum may have an effect on birds' behaviours as birds under green light used the nestboxes more often. This is likely a learned behaviour and further investigation is required to determine how light spectrum affects nesting. There was also a slightly higher amount of aggression observed in birds under LED-R light, which is likely due to social hierarchy within each pen suggesting a dominant hen within the pens may have influenced the data, however limited number of samples were taken. Therefore more research is required to determine if these effects are consistent. It is evident from this experiment that commercial strains of layers are programmed to produce eggs regardless of light spectrum or photoperiod. Previous results suggested red light was necessary to initiate the onset of lay and maintain persistent egg production however this may not be the case in commercial laying hens maintained in group floor pens. It is unclear what internal factors are responsible for the onset of lay, or how hens are able to maintain such a high level of production but metabolic cues such as mature body weight may be a primary trigger. Nonetheless, higher amount of red light within a LED-R bulb did not negatively affect production, stress and growth in birds maintained on collective floor pens suggesting that it is an appropriate light source to be used for the layers in alternative housing system.

Chapter 4: Evaluation and Comparison of the Effect of Various Light Sources on Reproductive and Growth Parameters in Laying Hens Maintained in Cages

4.1 Abstract

In poultry, higher wavelengths have been shown to more effectively stimulate extra-retinal photoreceptors increasing reproduction. In practice, multiple light sources are used in barns, each with a different specific spectrum and level of energy consumption. We developed an LED bulb with 60% red light (LED-R) to promote egg production. To test the effects of this LED bulb on production performances and electricity consumption, 3 rooms with individual cages were equipped with LED-R (10 Watt), incandescent (100 Watt) or compact fluorescent (CFL, 15 Watt) light bulbs. Each room was populated with 96 layers (Lohmann LSL-Lite) divided among 4 rows of cages. Egg production, egg quality, feed consumption, body weight and bulb energy consumption was monitored for each room from 14 to 69 weeks of age. Birds were maintained on a 10h photoperiod until photostimulation starting at 18woa (1 h increase every 2 weeks until 14h). Data were compared among treatments by ANOVA, since only one room was used per treatment; possible room effect was not assessed. Birds exposed to CFL and incandescent lights began laying 3 days earlier than birds under the LED-R light treatment. However, all birds peaked above 95% at 22woa, and there were no difference ($p < 0.2939$) in overall egg production among treatments, or in cumulative egg number. Birds under LED-R light treatments averaged a higher level of estradiol than incandescent and CFL. At 52 woa, a second increase in estradiol was observed in all treatment groups. Light source did not impact gene expression of GnRH-I, GnIH, LH- β , FSH- β , α -subunit, and GnRH-RIII. Overall egg weight

increased ($p < 0.0001$) and shell strength decreased ($p < 0.0001$) over time; with no difference in egg quality among treatments. Birds under CFL and incandescent light had significantly higher body weight gain and feed consumption than those under LED-R. Cumulative energy consumption of ten LED-R (10W) was 306kW, less than the incandescent (100W; 2514kW) and CFL (15W; 422kW) bulbs. In summary, light treatment did not significantly impact egg production; however with sustained peak production over 95%, it is possible that this commercial line is not particularly responsive to light quality and photoperiod. Interestingly, the reduced feed consumption and body weight gain observed in the LED-R treatment indicates that their energy was utilized mainly by egg production. This could result in significant reduction in feed cost, which combined with the significant savings in electricity, could reduce the cost of production.

4.2 Introduction

The avian reproductive axis is tightly regulated by two antagonistic neuropeptides; the inhibitory neuropeptide being Gonadotropin inhibitory hormone (GnIH) and the stimulatory neuropeptide being Gonadotropin releasing hormone-I (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. LH is involved in stimulating the production of estradiol and progesterone and is the trigger for 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 secondary sex characteristics and behaviour and has been suggested to be involved in ovulation (Robinson and Etches, 1986; Rangel and Gutierrez, 2014). Estradiol is also responsible for negative feedback on the hypothalamus and pituitary to reduce

mRNA expression of LH- β and α -subunit (Terada et al., 1997). Furthermore, estradiol is involved in stimulating the hepatic synthesis of major yolk components and increases the activity of calcitriol, which increases calcium levels in the blood making it available for shell synthesis (Etches, 1996). On the other hand, progesterone, produced by the large follicles, is involved in regulating ovulation (Robinson and Etches, 1986; Ottinger and Bakst, 1995; Rangel and Gutierrez, 2014). GnIH, a hormone responsible for the tonic inhibition of the reproductive axis, acts directly on GnRH neurons in the hypothalamus to decrease activity (Bentley et al., 2003; Ubuka et al., 2008). GnIH also acts directly on the pituitary inhibiting synthesis and release of gonadotropins, preventing the activation of the ovary (Ubuka et al., 2006).

The regulation of the reproductive axis relies heavily on photoperiod. The ovarian stimulatory pathway is activated under long day photoperiods, as GnRH-I mRNA and peptide levels increase when photoperiod increases (Dunn and Sharp, 1999; Bedecarrats et al., 2006; Thayananuphat et al., 2007). Also, opsin-positive neurons have been reported to directly synapse with GnRH dendrites, allowing for a pathway for photoreceptors to regulate the reproductive axis (Saldanha et al., 2001). The inhibitory pathway is activated during short day photoperiods and is regulated by melatonin, a hormone synthesized during the dark phase. Removing melatonin sources, via eye-enucleation and pinealectomy, decreased GnIH mRNA and peptide levels, which was restored when exogenous melatonin was administered (Ubuka et al., 2005). In quail GnIH neurons express melatonin receptors, allowing melatonin to directly regulate GnIH (Ubuka et al., 2005). Under short day photoperiods, birds have a higher amount of melatonin, stimulating GnIH synthesis and release, which inhibits the stimulatory pathway while the lack of photostimulation maintains birds in a sexually immature state (Bedecarrats et al., 2009). Upon photostimulation, the activation of the GnRH pathway results in increased

amount of gonadotropins, thus stimulating ovarian development (Dunn et al., 1999). This increases the amount of gonadal steroids, estradiol and progesterone, which feedback on the pituitary to decrease GnIH receptor mRNA expression (Maddineni et al., 2008). In addition, an increase in day length decreases the amount of melatonin, decreasing GnIH, thus removing the inhibition of the stimulatory branch (Bedecarrats et al., 2009).

Characteristics of light depends on light quantity (number of photons delivered) and quality (spectral composition). In modern day poultry house, the light intensity is measured in lux, a unit based on human spectral sensitivity. Due to the difference in spectral sensitivity of photoreceptive pigments between humans and birds, light is perceived differently (Prescott and Wathes, 1999). For example, incandescent light set to 100 lux will be perceived the same as fluorescent light set to 77 lux (Prescott and Wathes, 1999). The general consensus of the effect of light spectrum on birds is that higher wavelengths contain more energy and are able to penetrate through the skull and brain tissue to easily stimulate the hypothalamic photoreceptors (Pang et al., 1974; Foster et al., 1985; Oishi and Ohashi, 1993; Mobarkey et al., 2010), while lower wavelengths (blue/green light) require higher intensities to stimulate hypothalamic photoreceptors (Pang et al., 1974). Moreover, the avian retina has peak sensitivity to green light, and experimental evidence suggests that green light inhibits reproduction in birds via retinal photoreceptors (Siopes and Wilson, 1980b; Mobarkey et al., 2010; Gongruttananun, 2011; Mobarkey et al., 2013). However, over the years there have been some conflicting results as to whether higher and lower wavelengths are stimulatory and inhibitory, respectively.

Light spectrum also affects growth and stress in poultry. Previous trials in broilers indicate that green and blue light stimulate growth via skeletal muscle satellite cell proliferation and myofiber growth and may have stimulatory effects on protein synthesis (Halevy et al., 1998;

Rozenboim et al., 2004a; Cao et al., 2008). Although the effects of green light on growth in layers is not as well documented, similar results have been observed in hens and cockerels, where exposure to green light led to higher body growth (Foss et al., 1972; Baxter et al., 2014) with no difference in feed consumption (Foss et al., 1972). The effect of light spectrum on stress has not been extensively documented. Some have speculated that higher activity of birds under longer wavelengths may increase the stress levels (Sultana et al., 2013). In addition, when looking at immunity as an indicator of physiological stress, Interleukin-1 β (IL-1 β) can be used as it increases the release of corticosterone (Xie et al., 2008b). The level of IL-1 β was highest in birds exposed to white light, while exposure to blue light produced the lowest amount of corticosterone, indicating low stress levels (Xie et al., 2008b). However, further research is required to determine whether light spectrum is directly implicated in stress in birds.

The majority of commercial poultry operations rely on controlled environments in which artificial lighting is used to manage bird's growth, and production. On a practical side, light sources commonly used by the poultry industry emit various complex combinations of spectrums and it is unclear how this affects the reproductive axis of the commercial laying hens.

Incandescent bulbs have been the primary light source used by the North American poultry industry. Incandescent bulbs produce a broad spectral output ranging from 400-1050 nm with a gradual peak at 925 nm mimicking sunlight (Siopes and Wilson, 1980b; Chignell et al., 2008). However, these bulbs are energy inefficient and the global push to reduce greenhouse gases has forced producers to find alternative sources. The two main alternative light sources used by the industry are fluorescent light and light emitting diodes (LED). Fluorescent emit light using electricity to excite mercury vapour (Benson et al., 2013) resulting in a spectral output ranging from 400-750 nm with sharp peaks in the green spectrum (558 nm) (Siopes and Wilson, 1980b;

Chignell et al., 2008). Although fluorescent lights are more efficient, they typically flicker, do not dim well and involve specialized disposal of the mercury (Benson et al., 2013). LED's emit light using a solid-state semiconductor (Benson et al., 2013). They are among the most efficient light sources, can be manufactured to deliver a defined and stable spectral output (Steranka et al., 2002), and they are dimmable (Benson et al., 2013). A previous report on a strain of blind Leghorn called Smoky Joes determined that the red spectrum is instrumental in triggering and maintaining egg-laying, independently of the retina (Baxter et al., 2014). Thus, we designed an LED bulb that emits 60 % red light, specifically for laying hens. With new lighting technologies and the advancement in genetic selection of domestic poultry, there is often conflicting evidence as to the effects of light spectrum on poultry. Older studies used older technologies with less control over the spectral output, and the genetic makeup of birds significantly evolved through stringent selection processes. As light spectrum has been shown to affect the physiology of birds, the purpose of this experiment was to evaluate the effect of three different light sources, 100 W incandescent, 15 W Compact fluorescent (CFL) and 10 W LED bulbs with 60 % red light (LED-R), on reproduction stress, and growth and feed consumption, in birds maintained in individual cages. In addition, energy consumption between light sources was recorded over an entire production cycle.

4.3 Materials and Methods

4.3.1 Experimental Birds and Housing Conditions

Day old Lohmann LSL-Lite chicks were purchased from **Archer's Poultry Farm** (Brighton, ON) and raised at Arkell research station. From day one to three, chicks were maintained on 23 hours of light at 20 lux under incandescent lighting. The photoperiod was then

reduced to 16 hours at 4 days of age and at 1 woa, a step down protocol was implemented (14 h at 1 woa, 13 h at 3 woa, 12 h at 4 woa, 11 h at 5 woa, 10 h at 6 woa and 9 h from 7 to 13 woa). At 14 woa, 288 birds were randomly transferred to 3 separate rooms (n = 96 per room) equipped with individual cages and photoperiod was set to 10 hours. Birds were fed ad libitum commercial diets meeting or exceeding NRC requirements (1994) with a standard poultry chick starter (crumbles) from 0-6 woa, a standard poultry grower (crumbles) and from 7-16 woa and a standard layer-breeder ration (crumbles) from 17 woa to the end of the trial. At hatch, chicks were vaccinated against Marek's Disease (live virus), Infectious Bronchitis (live vaccine) and administered infectious Vaxxitek HVT+ IBD (live virus for Bursal and Marek's Disease). After placements, birds were further vaccinated against Newcastle and infectious-bronchitis (live virus at 3 woa), and against fowl pox, laryngotracheitis and avian encephalomyelitis (live virus) at 8 woa. Follow up boosters against Newcastle-Bronchitis were administered at 10 and 18 woa. All animal procedures were reviewed and approved by the University of Guelph Animal Care Committee and strictly adhered to the guidelines of the Canadian Council on Animal Care (CCAC). At 18 woa, birds were photostimulated using a step up lighting program, increasing photoperiod by one hour every two weeks until 14 hours of light.

4.3.2 Lighting Paradigm

The trial was conducted in three identical separate rooms at the University of Guelph Arkell poultry research station. Each room was equipped with 10 bulbs of either 100 W incandescent bulbs (Power Surge, Philips, The Netherlands), 15 W compact fluorescent (CFL, TCP, TrueDim Lamps; Aurora, OH) or 10 W LED with 60 % red bulbs (LED-R, supplied by Thies Electrical Distributing Company, Cambridge, Ontario, Canada). Each light source was adjusted to 10 lux at hen level and spectral output was measured using a spectrophotometer at

placement (14 woa) and at 39, 44, 46, 50, 57, 67 and 70 woa. Figure 4.1 displays the average spectral output of the same bulb throughout the trial. To measure electrical consumption of the different light sources, a residential electrical meter was installed on the line after the light controller for each room (GE Digital Energy; I-210+ Smart Grid Meter).

4.3.3 Measurement of Body Weight Gain, Stress and Reproduction

At 14 woa, birds were weighed to determine their initial body weight, which was used as a reference point to measure body weight gain. Body weights were recorded weekly from 14-31 woa and throughout the trial at 41, 46, 52, 60 and 69 woa. To measure feed consumption, containers were assigned to groups of 8 hens (12 containers per room) and were weighed weekly. Individual egg production was recorded daily throughout the trial and reproductive performance was expressed as the average percent weekly production (egg/hen/day over a week). Sexual maturity was measured by recording age at first egg for each hen. Egg quality was assessed by measuring egg weight (in grams) and shell strength using the Egg Forces Reader (Orka Food Technology Ltd, Bountiful, UT) which measures the number of kg of force required to crack the shell. Eggs were collected for 5 consecutive days and egg weights were recorded at 24, 35, 50 and 65 woa while shell strength was recorded at 35, 50 and 65 woa.

4.3.4 Hormone Analysis: Corticosterone and Estradiol

Blood samples were taken from the wing vein of the same 16 birds (4 birds per row of cages) in each room at approximately 8:30am on each sample day. Serial blood samples were taken biweekly from 13 to 25 woa and at 29, 33, 37, 41, 45, and 49 woa. Approximately 2 mL of blood was collected and placed in a sodium heparin vacutainer. Immediately after collection, blood plasma was recovered by centrifugation at $900 \times g$ for 15 min at 4°C and stored at -20°C

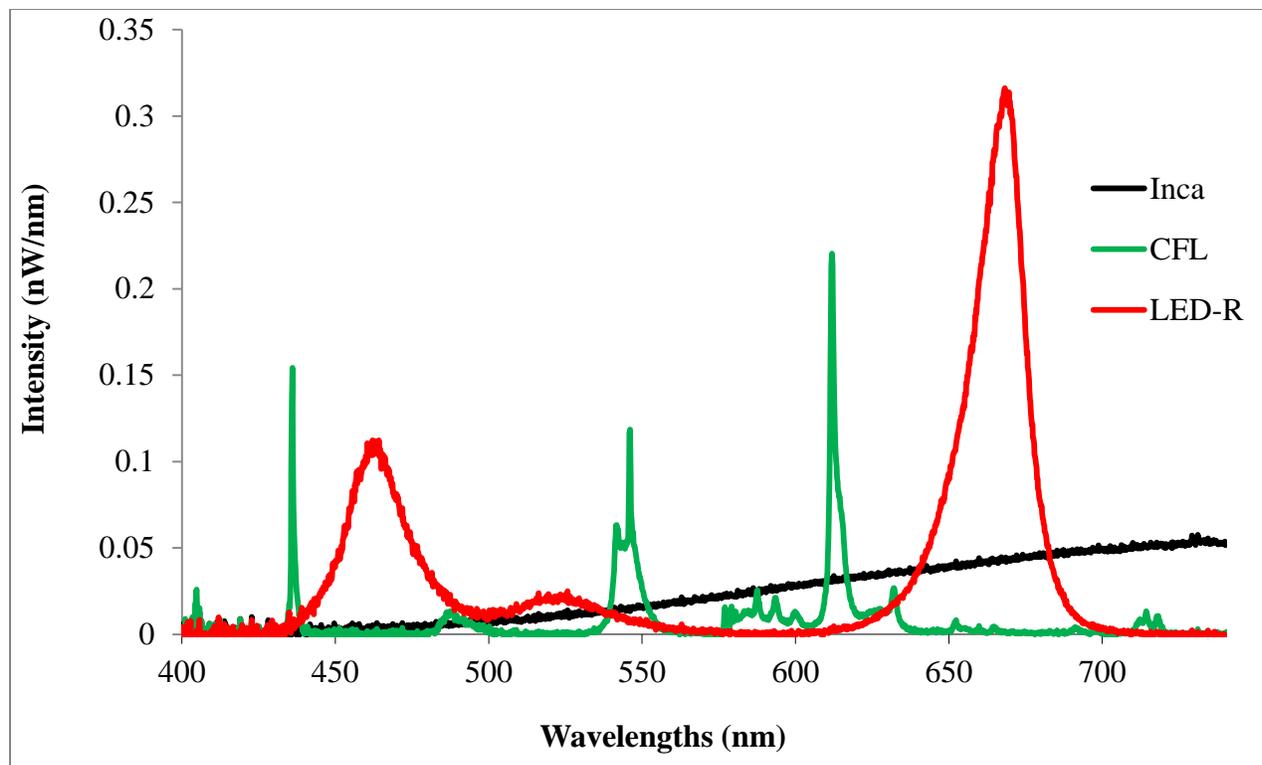


Figure 4.1 Spectral output and wavelength peaks from each light source

Spectral output recorded by a spectrophotometer, with each light source set to 10 lux.

until hormone extraction and assay. Corticosterone and estradiol assays were previously validated for chicken plasma (Baxter et al., 2014). The intra- and inter-assay coefficients of variation were <15%. Assay procedures were identical to the ones described in Chapter 2.

4.3.5 RNA Extraction and cDNA Synthesis

To determine at which level of the reproductive axis light may elicit its effect, tissue samples were collected at 15 woa (baseline before experimental lights were turned on; 5 birds exposed to incandescent light); 29 woa (4 birds from each treatment, one from each row were randomly chosen,) and 70 woa (4 birds from each treatment, one from each row were randomly chosen). To remove tissues, birds were euthanized by cervical dislocation and the diencephalon (containing the hypothalamus), pituitary and the ovary were removed. Tissues were weighed and snap frozen in liquid nitrogen then stored at -80°C until further extraction.

RNA was extracted from the diencephalon and ovary with Trizol Reagent (Invitrogen, Life Technologies, Burlington, ON, Canada). Briefly, 1 mL Trizol Reagent was added per 100 mg of tissue sample and samples were then homogenized via sonication. For phase separation, 200 uL chloroform was added to the samples, vortexed, incubated on ice for 15 mins, and vortexed again. They were then centrifuged for 15 mins at 17000 g to precipitate proteins, DNA and other cellular components. The RNA present in the aqueous phase was pipetted into a sterile Eppendorf tube and precipitated with 500 uL of isopropanol. Samples were then stored at 4°C overnight (approximately 12 hours) and RNA pellets were recovered by centrifugation at 17000 g for 15 mins. Supernatant was removed and the pellet was washed twice with 70% chilled ethanol. Sample were then re-suspended in 100 uL dH₂O and stored at -80°C. Due to the small size of the pituitary gland, the RNA extraction procedure used the same steps as described above, with the following alterations: 600uL of Trizol reagent was used during homogenization,

followed by 100uL of chloroform for phase separation. After centrifugation and recovery of the aqueous phase, 2 uL of molecular biology grade glycogen (Fisher Scientific, Pittsburgh, PA) was added to aid in pelleting of the RNA precipitate and 600 uL isopropanol was added. Samples were then processed as described above. Pituitary RNA samples were resuspended in 30 uL sterile dH₂O and stored at -80°C. Concentration and purity of RNA were analyzed using a NanoDrop and all samples were treated with DNase to remove potential genomic DNA contamination. Briefly, 1 unit (1 unit/μl) of DNase (Promega Madison, WI) and 2 μl 10X DNase Buffer (Promega, Madison, WI) were added to 10 μg of RNA, bringing the total volume up to 20 ul. The DNase treated samples were then heated to 65 °C for at least 10 mins to deactivate the DNase.

Subsequent cDNA synthesis reactions were performed on 2 μg RNA from all samples. RNA was mixed with 1 uL oligo (dT) primer, 1 uL 10mM dNTP, and sterile water was added to 10 uL. The sample was incubated for 5 min at 65°C and then incubated on ice for approximately 1 min. Reaction mix was prepared (2 μL 10X RT Buffer, 4 μL 25mM MgCl₂, 2 μL 0.1M DTT, 1 μL sterile water) and 8 μl of the reaction mix was added to each sample, vortexed, and then incubated for 2 min at 42°C. Samples were then placed on ice and 1μL Superscript II reverse transcriptase (Invitrogen, Life Technologies, Burlington, ON) was added. Samples were incubated at 42°C for 50 min for reverse transcription and the enzyme was deactivated by incubating for 15 min at 70°C. Samples were returned on ice for 5 min and 1 μL RNase H (Invitrogen, Life Technologies, Burlington, ON) was added. Samples were incubated at 37°C for 20 min, and then stored at -20°C until further analysis. A general pool of cDNA was prepared by mixing equal amounts from each individual sample to validate the amplification for quantification (see below).

4.3.6. Semi-quantitative Polymerase Chain Reaction (qPCR)

Semi-quantitative Polymerase Chain Reaction (qPCR) was used to assay the mRNA levels of GnRH-I, and GnIH in the hypothalamus and LH- β , FSH- β , α -subunit and GnRH-RIII in the pituitary. Primers were designed for each gene based on the literature and were then subjected to a BLAST search to ensure specificity (Table 4.1). qPCR was performed using a Rotor-Gene (RG-3000; Corbett Research, Corbett Robotics Inc, San Francisco, CA) with iTaq Universal SYBR green Supermix (Bio-Rad Laboratories Ltd. Mississauga, ON). For each reaction, 10 μ L of SYBR green Supermix, 0.152 pmol of each of the forward and reverse primers and 2 μ l of 1/8 dilution of cDNA were mixed. Sterile H₂O was used as a non-template control instead of the cDNA. Samples were quantified in duplicates, to ensure accuracy. Before qPCR was performed on samples, the amplification parameters were optimized with the general pool to ensure PCR efficiencies between 85-115%. Optimization also included ensuring proper dissociation curves to confirm the presence of a single amplicon and to adjust the temperature at which data acquisition was done. Final cycling temperatures are displayed in Table 4.2. LinRegPCR (Dr. J.M. Ruijter, Academic Medical Centre, Amsterdam, Netherlands) was used to determine Ct values. Expression was quantified relative to the average of the first samples taken at 15 woa (used as calibrator). Two housekeeping genes, β -Actin and GAPDH, were used to calculate the geometric mean to normalize the range of data. The Δ Ct was calculated subtracting the average geometric mean from the average mean of the gene of interest for each sample, and the $\Delta\Delta$ Ct was calculated subtracting the Δ Ct of the calibrator (samples from initial sampling at 15woa) from the average delta Ct described above. The relative expression (%) was calculated using the following equation for each sample $[2^{-1*\Delta\Delta Ct} * 100]$. To determine the relative expression of each gene under each light treatment, samples were averaged at each time point.

Table 4.1: List of Primers sequences used in the qPCR

Name	Sequence Direction (F-Forward; R-Reverse)	Sequence (5'-3')	Reference
GnRH-RIII	F	GCTCGCTGTGCCGCAGCTGT	(Joseph et al., 2009)
	R	ACGTCCCTGGAGGAGAAGAG	
FSH- β	F	CCACGTGGTGCTCAGGATACT	NM_204257 (Avital-Cohen et al., 2013)
	R	AGGTACATATTTGCTGAACAGATGAGA	
GnIH	F	CGAGTGCTTATTTGCCTTTG	AB120325.1 (Ahmed et al., 2014)
	R	CTCCTGGACACCTTGAGATACA	
LH- β	F	GTGTTGGTGCTGATGACCCT	
	R	ACCGCCACCGTTACGTTTAT	
α -subunit	F	ATTGAACAAGGGAGAAAGATC	S70833 (Ciccone et al., 2004)
	R	AACAGCCATCCATCTCATTC	
GnRH-I	F	TGGGTTTGTGATGGTGTGTTGT	X69491 (Ciccone et al., 2004)
	R	ATTTCCAGCGGGAAGAGTTG	
GAPDH	F	AATGGGCACGCCATCACTAT	NM_204305.1
	R	AGCTGAGGGAGCTGAGAGA	
B-ACTIN	F	GTATGGAGTCCTGTGGTAT	NM_205518.1
	R	CACATCTGCTGGAAGGTGG	

Table 4.2: qPCR cycling conditions of each gene to determine relative expression

House Keeping Genes		B-Actin		GAPDH					
		Temp (°C)	Time (mins)	Temp (°C)	Time (mins)				
Initial Denaturation		95	10mins	95	10mins				
Cycling	Denaturation	94	10sec	95	10sec				
	Annealing	60	40sec	59	20sec				
	Amplification/ Acquiring	72	15sec	72	15sec				
Melt		60-95		59-95					
Hypothalamus		GnRH-I		GnIH					
		Temp (°C)	Time (mins)	Temp (°C)	Time (mins)				
Initial Denaturation		95	10min	95	10min				
Cycling	Denaturation	95	20sec	95	20sec				
	Annealing	62	20sec	60	30sec				
	Amplification	72	20sec	72	20sec				
	Acquiring	80	15sec	79	20sec				
Melt		62-95		60-95					
Pituitary		GnRH-RIII		LH-β		FSH-β		Alpha	
		Temp (°C)	Time (mins)						
Initial Denaturation		95	10mins	95	10mins	95	10min	94	10mins
Cycling	Denaturation	95	15sec	95	10sec	95	15sec	94	20sec
	Annealing	60	30sec	61	20sec	60	20sec	59	20sec
	Amplification	72	20sec	72	20sec	72	20sec	71	20sec
	Acquiring	86	15sec	86	15sec	76	15sec	78	15sec
Melt		60-95		61-95		60-95		59-94	

4.3.7 Statistical Analysis

Data was analyzed using Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Using the average value per row of cages as a replicate for each time point, a two-way ANOVA was performed on age at first egg, egg production, egg weight, shell strength, feed consumption, body weight (kg), body weight gain (%), corticosterone and gene expression, where age was the constant (X treatment) and light was the treatment (Y treatment). For age at first egg, a two-way ANOVA was performed, where the row was the constant (X treatment) and light was the treatment (Y treatment). To ensure there was no difference between the rows of cages within each light treatment, a two-way ANOVA was performed where age was the constant (X Treatment) and row was the treatments (Y treatment). A Tukey's multiple comparison test was performed both between the different collection ages and then between the different light treatments to determine significance at different ages. Electrical consumption and radiant flux was analyzed using a one-way ANOVA, where time was the constant independent variable (X treatment) and the light treatment was the dependant variable (Y treatment). Significance was based on $p < 0.05$. Body weight gain was measured by subtracting initial body weight from each time body weight was recorded. To determine if body weight correlated to when birds began laying, correlation was calculated where age at first egg was compared to the birds' body weight, regardless of light treatment, from 14-22 woa.

4.4 Results

4.4.1 Egg Production

Age at first egg and average number of eggs per hen is displayed in Table 4.3. Birds under LED-R light had a significant delay in age at first egg compared to birds under CFL and

incandescent ($p < 0.0001$). However the average age at first egg in each light treatment occurred between 19 and 20 woa when birds were under 11-12 hours of light, indicating that regardless of light treatment, birds began maturing before reaching a stimulatory photoperiod. There was no significant difference between rows within each light treatment (I: $p = 0.5911$; C: $p = 0.1461$; L: $p = 0.0569$).

Egg production was measured over a 52 weeks period (14-66 woa) and is presented in Figure 4.2. Overall there was no significant difference between light treatments ($p = 0.2939$) and there was no significant difference in total cumulative number of eggs per hen ($p = 0.8256$; Table 4.3B). However, production levels did fluctuate over time and, at 19 woa birds under LED-R had significantly lower egg production than both incandescent and CFL light (I = 34.8 ± 3.73 %; C = 38.4 ± 3.95 %; L = 19.0 ± 3.04 %),); at 20 woa, birds under LED-R had significantly lower production than birds maintained under CFL light (I = 66.82 ± 3.91 %; C = 75 ± 3.21 %; L = 58.3 ± 3.90 %), and at 24 woa, birds under LED-R had significantly higher level of production than both incandescent and CFL (I = 95.8 ± 1.55 %; C = 96.1 ± 1.00 %; L = 101.6 ± 1.42 %).

4.4.2 Estradiol

As shown in Figure 4.3, throughout the entire trial birds under LED-R light treatments averaged a higher level of estradiol than incandescent and CFL ($p = 0.0069$). In all treatment groups estradiol levels peaked at 16 woa, however, although differences were not significant, birds under LED-R light had the highest numerical values (I = 2.22 ± 0.38 ng/ml; C = 2.61 ± 0.30 ng/ml; L = 3.19 ± 0.22 ng/ml). At 52 woa, a second increase in estradiol was observed in all treatment groups. At that time, birds under LED-R light had a significantly higher level of estradiol than incandescent and CFL ($p = 0.0117$; I = 1.22 ± 0.18 ng/ml; C = 1.27 ± 0.16 ng/ml;

L = 2.05 ± 0.27 ng/ml). There were no other significant differences in estradiol levels between light treatments for any other time points recorded. As expected, age had a significant effect on overall estradiol levels regardless of light treatment ($p < 0.0001$).

4.3.3 Egg Weight and Shell Strength

Egg weight was measured at four time points (24woa, 35woa, 50woa and 65woa) and results are displayed in Figure 4A. Overall, light treatment had no significant effect on egg weight ($p = 0.8832$) at 24woa (I = 53.9 ± 0.21 ; C = 54.5 ± 0.26 ; L = 53.7 ± 0.15), 35woa (I = 61.1 ± 0.51 ; C = 61.4 ± 0.21 ; L = 61.0 ± 0.12), 50woa (I = 63.4 ± 0.20 ; C = 64.07 ± 1.21 ; L = 62.9 ± 0.31) and 65woa (I = 65.1 ± 0.29 ; C = 64.3 ± 0.28 ; L = 65.2 ± 0.24). Egg weight increased as age increased regardless of placement within the room and light treatment ($p < 0.0001$). Average egg weight increased significantly with each collection date in birds under LED-R and incandescent light. Birds under CFL light also had a significant increase in egg weight between each collection date except between 50woa and 65woa. Shell strength, measured in kg-force required to crack the egg, was measured at three time points (35woa, 50woa and 65woa) and is displayed in Figure 4B. Overall, there was no difference in shell strength between light treatments ($p = 0.4567$) at 35woa (I = 4.81 ± 0.04 ; C = 4.79 ± 0.01 ; L = 4.84 ± 0.03), 50woa (I = 4.33 ± 0.03 ; C = 4.39 ± 0.01 ; L = 4.34 ± 0.03) and 65woa (I = 4.05 ± 0.22 ; C = 4.02 ± 0.12 ; L = 3.88 ± 0.025). Again, time had a significant effect over time where shell strength decreased as birds aged in all treatments ($p < 0.0001$).

Table 4.3: Average age at first egg and total number of eggs per hen

The age at first egg of each bird was recorded and averaged per light treatment (Mean \pm S.E.M.). Birds under LED-R light had a significant delay in age at first egg compared to birds under CFL and incandescent ($p < 0.0001$). Overall there was no significant difference in total cumulative number of eggs per hen ($p = 0.8737$).

	Age at First Egg (days)	Avg. total Egg #
Inca	137.4 \pm 0.57 ^a	314.2 \pm 2.32
CFL	137.4 \pm 0.65 ^a	320.5 \pm 1.11
LED-R	140.8 \pm 0.67 ^b	315.4 \pm 1.69

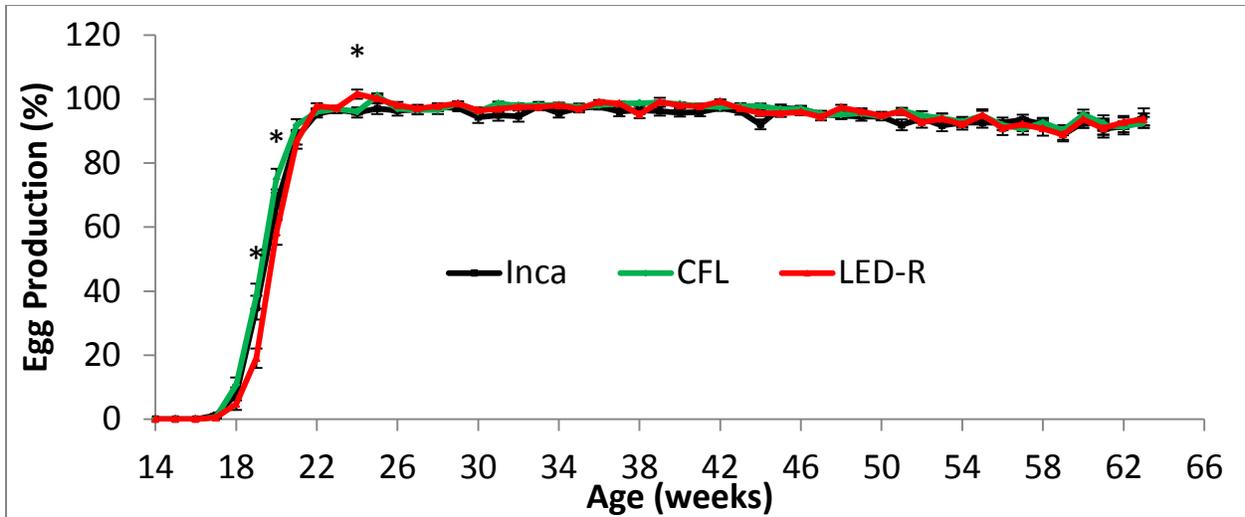


Figure 4.2: Egg production of birds maintained in individual cages maintained under various light sources.

Egg production was measured daily for each individual hen and averaged per room per week. Overall there was no significant difference between light treatments ($p = 0.2939$). At 19 woa birds under LED-R had significantly lower egg production than both incandescent ($p < 0.001$) and CFL light ($p < 0.0001$); at 20 woa birds under LED-R had significantly lower production than birds maintained under CFL light ($p < 0.05$), and at 24 woa, birds under LED-R had significantly higher level of production than CFL ($p < 0.05$). * indicates significance difference between light treatments on the graph.

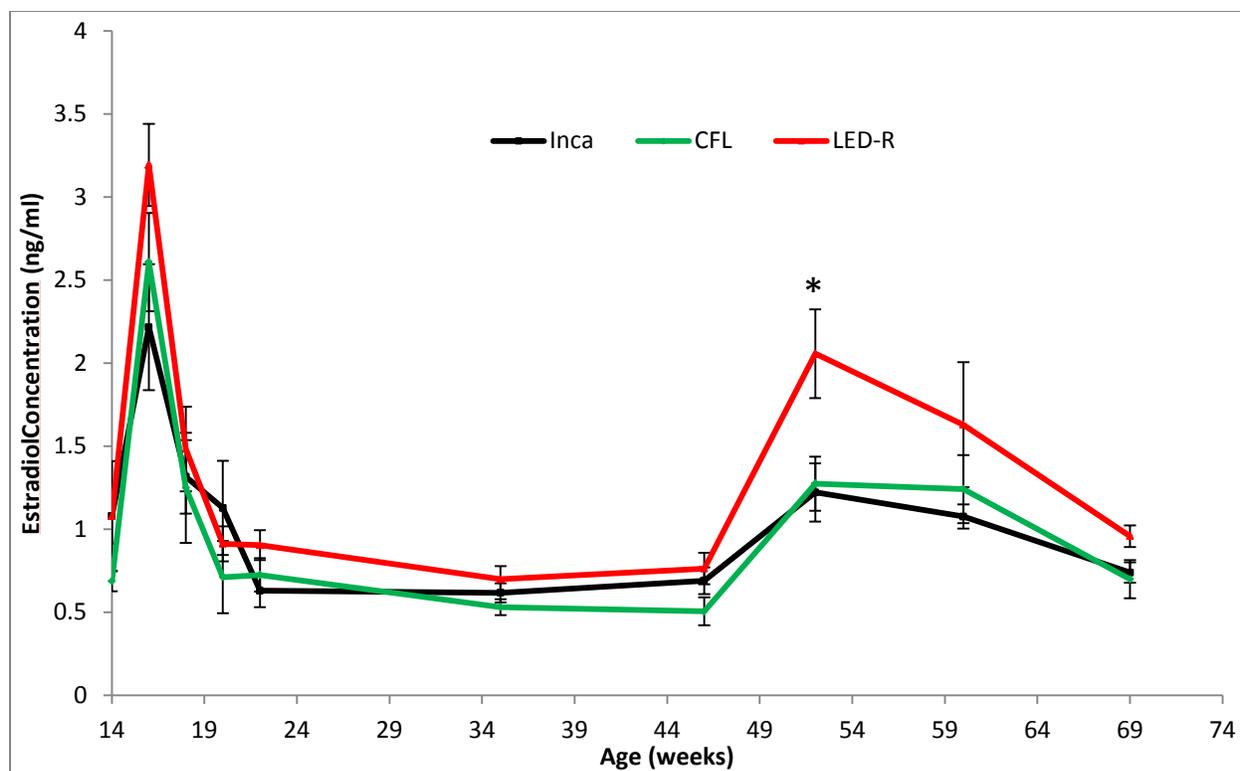


Figure 4.3: Plasma estradiol concentration of birds maintained in individual cages under various light sources.

Overall, light treatment had a significant effect on the level of estradiol in the plasma (Mean \pm S.E.M.), where birds under LED-R light treatments averaged a higher level of estradiol than incandescent and CFL ($p = 0.0069$). Levels in hens from each light treatment peaked at 16 woa. Birds under LED-R numerically had higher levels of estradiol than the other light sources at 16 woa, however this was not significant. At 52 woa, a second increase in estradiol was observed in all treatment groups. * an asterisk indicate LED-R had significantly higher level of estradiol than incandescent and CFL ($p = 0.0117$).

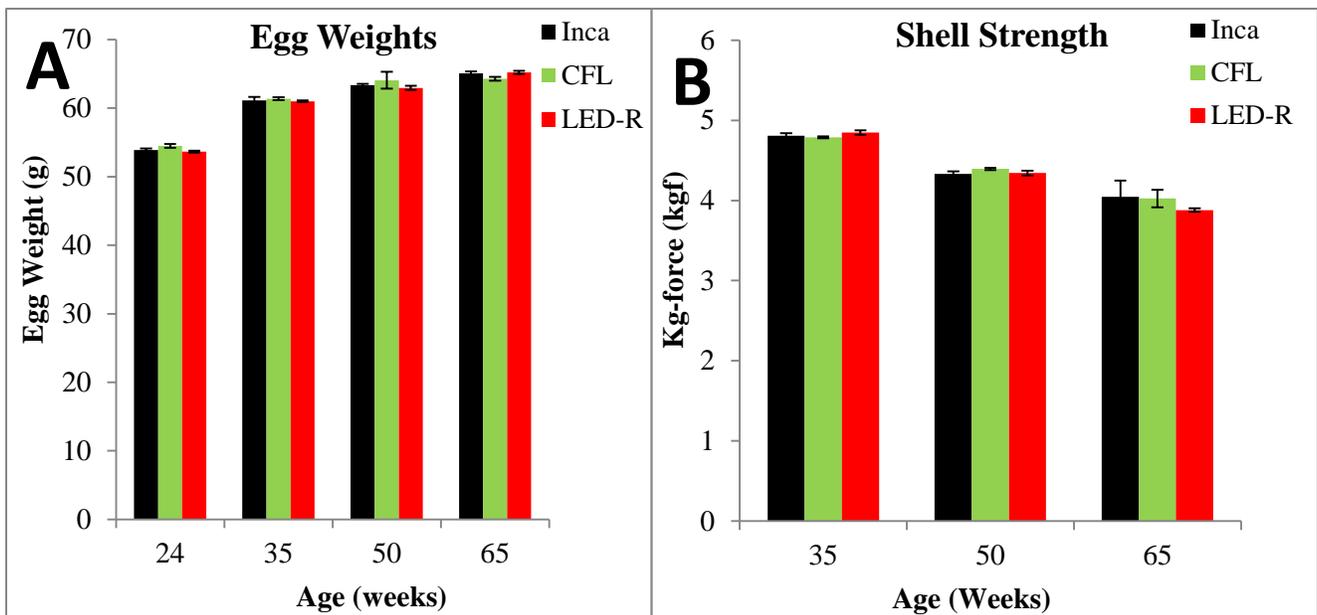


Figure 4.4 Egg weight and shell strength of birds under light treatments

(A) Eggs were weighed for individual birds over four consecutive days at 24, 35, 50 and 65 woa. There was a significant increase in egg weight as birds aged ($p < 0.0001$), however there was no difference between light treatments ($p = 0.8832$). (B) Egg Shell strength was measured by the Egg Force Reader as Kg of force required to crack the egg for each individual birds over four consecutive days at 35, 50 and 65 woa. Overall, there was no difference in shell strength between light treatments ($p = 0.4567$) and age had a significant effect where shell strength decreased as birds aged for all treatments ($p < 0.0001$).

4.4.4 Levels of mRNA of Reproductive Hormones and Receptors

Tissues were collected before photostimulation (15 woa), at peak production (29 woa) and in late lay (70 woa) in order to capture important time points at which mRNA levels of hypothalamic neuropeptides and pituitary gonadotropin subunits were expected to change (Figure 4.5). Levels of GnRH-I mRNA in the hypothalamus did not change over time ($p = 0.1161$) nor did they differ between light treatments ($p = 0.7216$) (Figure 4.5A). Similarly, levels of GnIH mRNA did not change over time ($p = 0.9528$) (Figure 4.5B). However, at 29 woa birds under incandescent light had significantly higher GnIH mRNA levels than both CFL and LED-R ($p < 0.0293$).

Levels of GnRH-RIII mRNA significantly differed over time ($p < 0.0009$). For birds under incandescent light, levels were significantly higher at 29 and 70 woa compared to 15 woa (Figure 4.5C) while birds under CFL, had levels that were significantly higher at 29 woa than 15 woa. Conversely, there were no changes in gene expression for birds kept under LED-R light. Although there was no overall significant difference in gene expression between light treatments, at 29 woa, a Tukey's multiple comparison test determined birds under CFL had a higher level of GnRH-RIII mRNA than under incandescent. At 70 woa, birds under incandescent light had significantly higher levels of GnRH-RIII mRNA expression than under those treated with LED-R. Furthermore, a significant interaction between light treatment and age of the birds ($p < 0.0204$) was detected suggesting that the effect of light treatment on GnRH-III mRNA expression may depend on the birds' age.

For LH- β mRNA, the effect of age was significant ($p < 0.0026$) with birds under incandescent light displaying significantly higher level of LH- β at 70 woa than at 15woa and 29 woa ($p < 0.0001$; Figure 4.5D). In addition, light treatment also had a significant effect on LH- β

mRNA expression ($p < 0.0002$), following Tukey's multiple comparison test, at 29 woa birds under CFL light had significantly higher levels of LH- β mRNA than birds under LED-R and incandescent ($p < 0.05$). At 70 woa, birds under incandescent light had significantly higher LH- β mRNA expression than birds under LED-R ($p < 0.005$) and CFL ($p < 0.001$). Overall, there was a significant interaction between light treatment and the age of the birds ($p < 0.0204$), again suggesting that the effect of light treatment on LH- β mRNA expression may depend on the birds' age. FSH- β mRNA levels did not change between treatments ($p < 0.8680$; Figure 4.5E) however, there was an increase in FSH- β within each treatment over time ($p < 0.0061$), although this significance was not detected following multiple comparison test. Alpha mRNA levels did not fluctuate as the birds aged ($p < 0.5022$), nor was any difference in gene expression between treatments ($p < 0.9751$; Figure 4.5F).

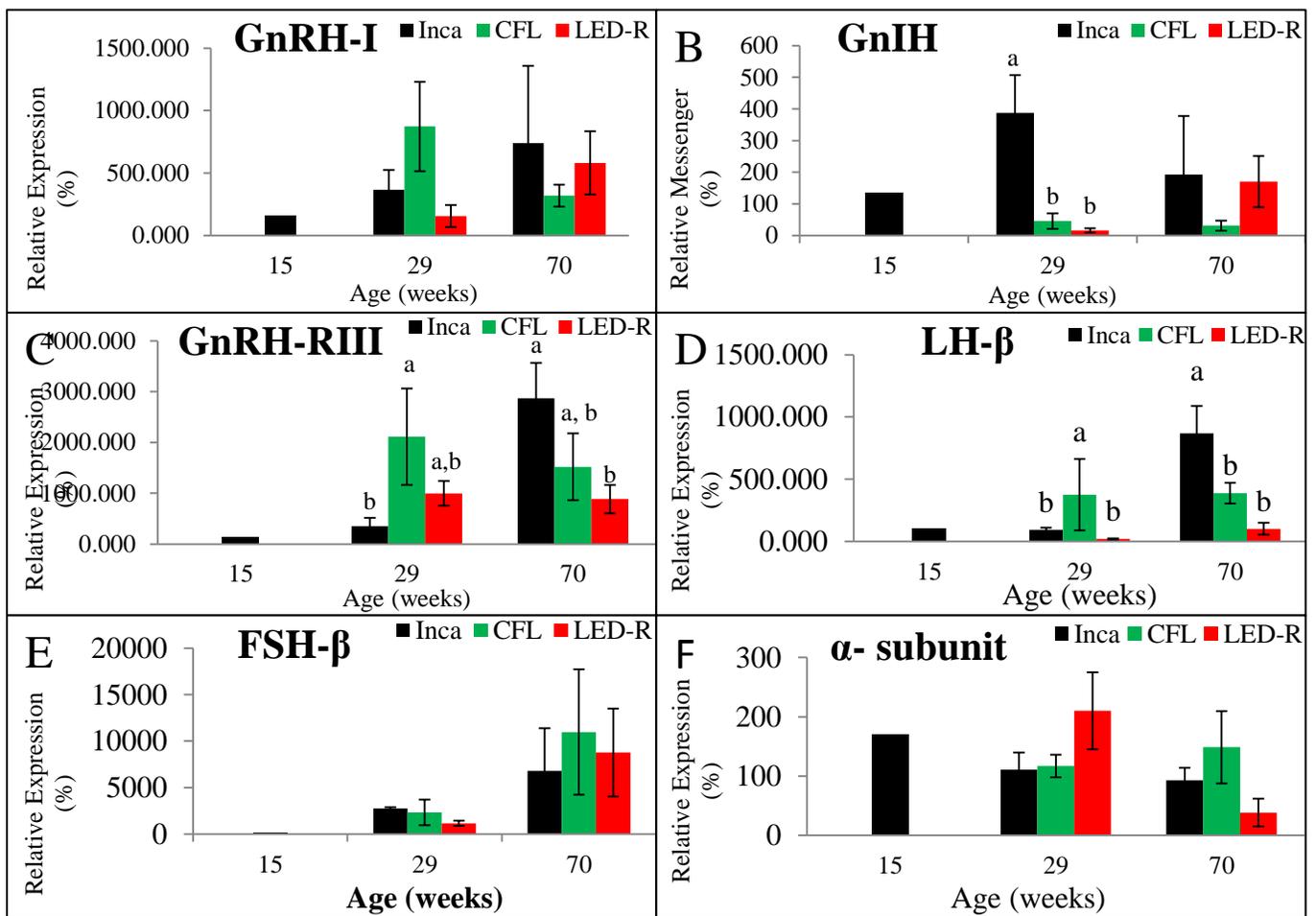


Figure 4.5: Gene expression of reproductive neuropeptides in the hypothalamus and, neuropeptide receptors and gonadotropins subunits in pituitary.

Panel A displays GnRH-I mRNA expression in the hypothalamus, which did not change over time ($p = 0.1161$) nor did they differ between light treatments ($p = 0.7216$). Panel B displays expression levels of GnIH mRNA, which did not change over time ($p < 0.9528$), however, at 29 woa birds under incandescent light had significantly higher GnIH mRNA levels than both CFL and LED-R ($p < 0.0293$). Panel C displays levels of GnRH-RIII mRNA which significantly differed over time ($p < 0.0009$). Although there was no overall significant difference between light treatments, birds under CFL had a higher level of GnRH-RIII mRNA than incandescent ($p < 0.05$) at 29 woa. At 70 woa, levels in hens under incandescent light had significantly higher levels of GnRH-RIII mRNA expression than under LED-R ($p < 0.05$). Panel D displays LH-β mRNA expression, light treatment had a significant effect, at 29woa birds under CFL light had significantly higher levels of LH-β mRNA expression than birds under LED-R and incandescent ($p < 0.05$). At 70 woa, birds under incandescent light had significantly higher expression than birds under LED-R ($p < 0.005$) and CFL ($p < 0.001$). Panel E displays FSH-β mRNA levels, where no different between treatments was observed ($p < 0.8680$) however, there was an increase in FSH-β within each treatment over time ($p < 0.0061$), although this significance was not detected following multiple comparison test. Panel F displays common alpha subunit mRNA levels, which did not fluctuate as the birds aged ($p < 0.5022$), nor was any difference in gene expression between treatments ($p < 0.9751$). ^{a,b} subscripts indicates significant difference of at least $p < 0.05$ between the light treatments.

4.4.5 Feed Consumption

Overall birds under LED-R light ate significantly less than birds under CFL and incandescent ($p = 0.0016$; $I = 0.1026$ kg/bird/day; $C = 0.1032$ kg/bird/day; $L = 0.099$ kg/bird/day; Figure 4.6). Multiple comparison post hoc tests indicate that birds under LED-R light consumed significantly less feed than birds under CFL and incandescent at 17, 18, 20, 28, 56, 57, and 58 woa (Figure 4.6). Exposure to incandescent light resulted in higher feed consumption than LED-R at 35, 37 and 45 woa. Birds under CFL consumed more kg per bird per day than those under LED-R at 16, 19, 21, 22, 38, 44, 60 and 63 woa. Birds under CFL consumed more kg per day per bird than those under incandescent at 21 and 53 woa. Age had a significant effect on feed consumption, where birds ate more as they aged ($p < 0.0001$).

4.4.6 Body Weight Gain

Overall light treatment had a significant effect on percent body weight gain (Figure 4.7). Hens from LED-R groups had a significantly lower body weight gain compared to birds under CFL and incandescent lights ($p = 0.0054$; $I = 38.48$ %; $C = 38.6$ %; $L = 35.73$ %). Multiple comparison post hoc tests indicate that birds under LED-R had significantly lower weight gain than birds under both CFL and incandescent at 19, 24, 26 and 28 woa. Hens under LED-R had significantly lower body weight gain than under incandescent at 27, 29 and 60 woa. Birds exposed to LED-R had significantly lower weight gain than birds under CFL light at 22, 33, 34 and 41 woa. As birds aged, there was an increase in body weight gain within each treatment ($p < 0.0001$). When looking at the body weight of birds under each treatment in kilograms (Figure 4.8), light treatment had a significant effect on body weight ($p = 0.00141$). After multiple comparison test, birds under LED-R weighed significantly less than birds under incandescent

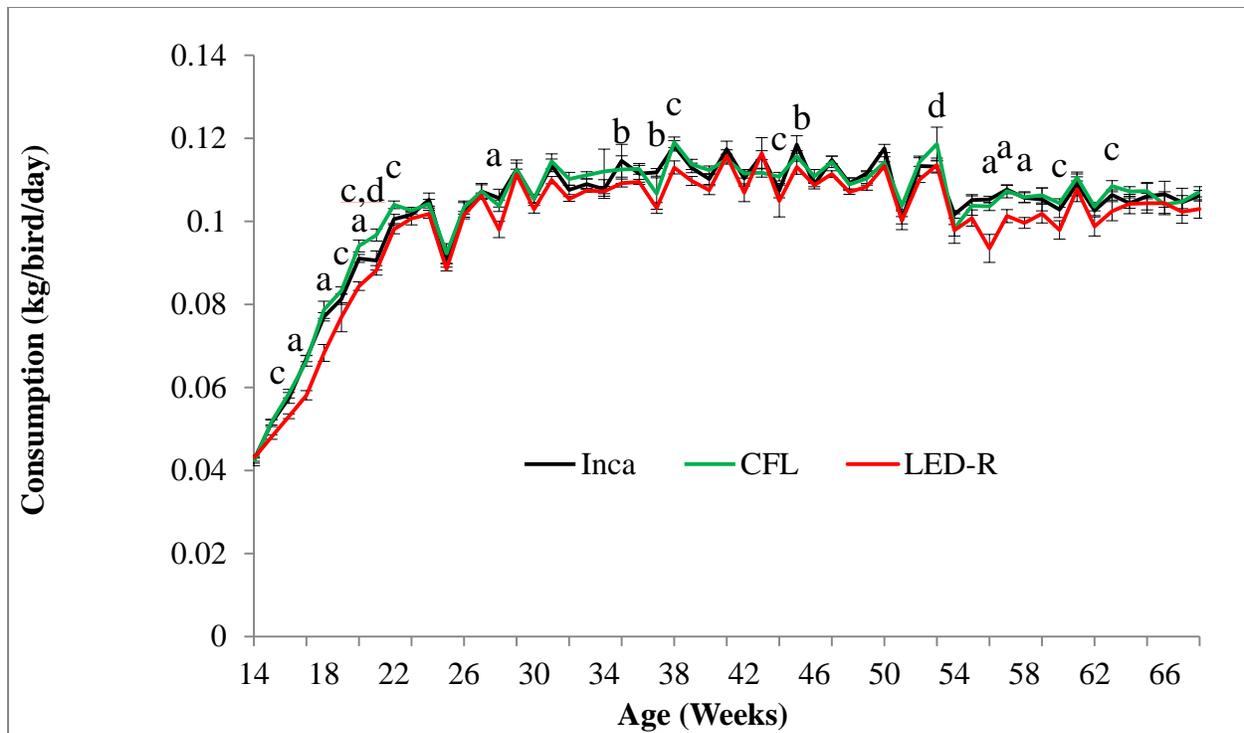


Figure 4.6: Feed consumption of birds maintained in individual cages maintained under various light treatments

Feed consumption was measured weekly to determine any effect of light. Overall birds under LED-R light ate significantly less than birds under CFL and incandescent ($p = 0.0016$).

Superscript indicate significant difference of $p \leq 0.05$ between the light treatments. Superscript ^a indicates feed consumption was significantly lower under LED-R than both Incandescent and CFL; ^b indicates feed consumption was significantly lower under LED-R than incandescent; ^c indicates feed consumption was significantly lower under LED-R than CFL. ^d indicates that feed consumption under CFL was significantly higher than incandescent.

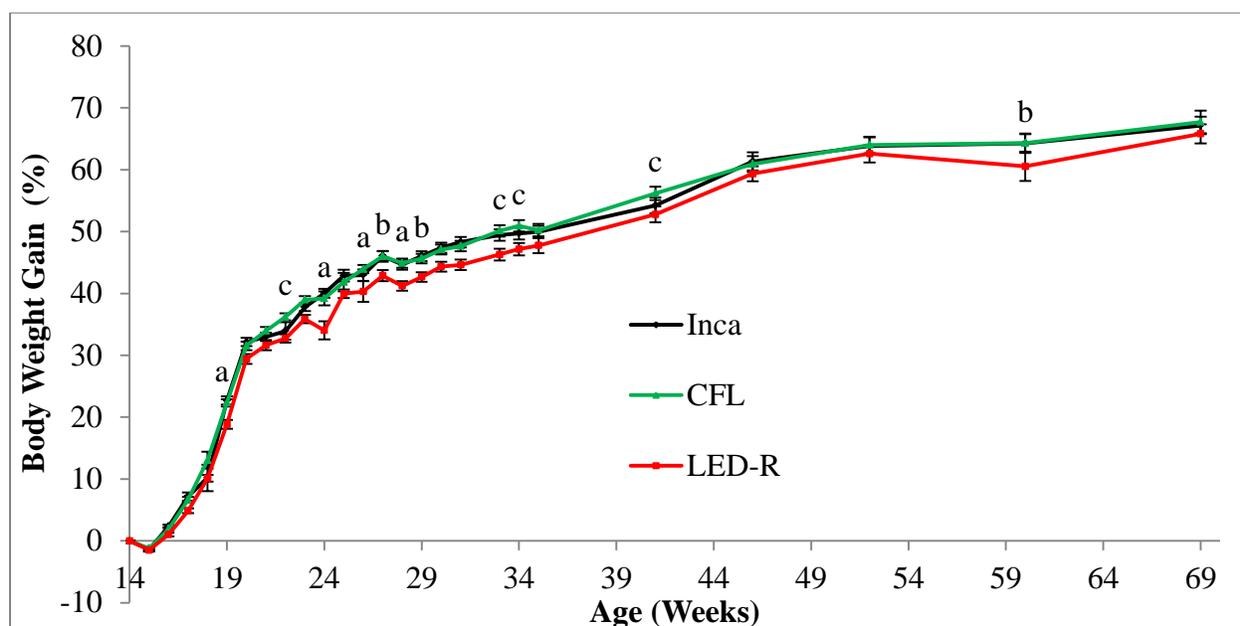


Figure 4.7: Body weight gain of birds under different light sources in individual cages

Body weight gain was measured by subtracting initial body weight from the growth of each time body weight throughout the trial. Overall, birds under LED-R had a significant lower body weight gain compared to birds under CFL and incandescent lights ($p = 0.0054$). Superscript indicates significant difference of $p \leq 0.05$ between the light treatments. Superscript ^a indicates body weight gain of hens under LED-R was significantly lower than both incandescent and CFL; ^b indicates weight gain growth of hens under LED-R was significantly lower than incandescent; ^c indicates that hens under LED-R had a significantly lower body weight gain than CFL.

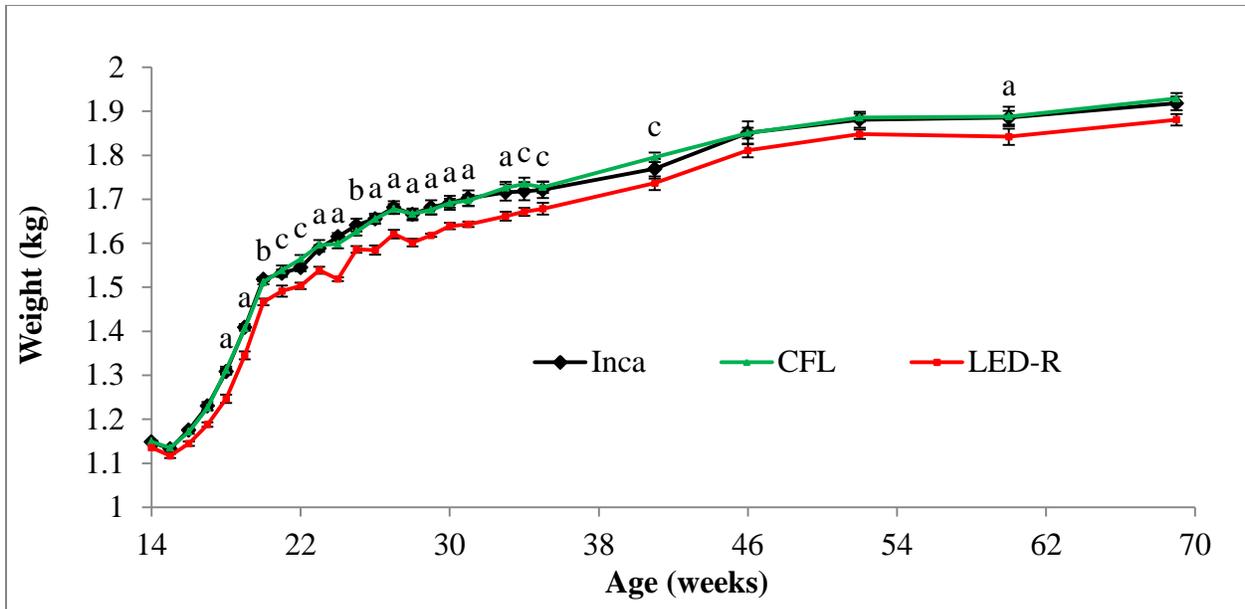


Figure 4.8: Average body weight of birds in each light treatment

Body weight was measured throughout the trial. Overall, light treatment had a significant effect on body weight ($p = 0.00141$). Superscripts indicate significant difference of $p \leq 0.05$ between the light treatments. Superscript ^a indicates LED-R was significantly lower body growth than both Incandescent and CFL; ^b indicates LED-R had significantly lower body growth than incandescent; ^c indicates LED-R had a significantly lower body growth than CFL.

and CFL at 18, 19, 23, 24, 26, 27, 28, 29, 30, 31, 33, 34, 35, 41, and 60 woa. Exposure to LED-R resulted in a significantly lower body weight than birds under incandescent at 20 and 25 woa. Exposure to LED-R resulted in a significantly lower body weight than birds under CFL at 21, 22, 34, 35, 41, and 69 woa. Overall, time had a significant effect on body weight, where birds became heavier as they aged ($p < 0.0001$).

The correlation between age at first egg and bodyweight (kg) was calculated and it was determined that there was a negative correlation between age at first egg and body weight in birds under LED-R at 18 woa ($p < 0.0001$ $r = -0.2774$); 19 woa ($p = 0.0006$; $r = -0.2009$). There was a negative correlation between age at first egg and body weight of birds at 18 and 19woa. Therefore age at first egg decreases as body weight increases in birds regardless of light treatment.

4.4.7 Corticosterone

Changes in corticosterone concentrations over time are displayed in Figure 4.9. Overall, there was no significant difference between treatments ($p = 0.5785$). However, at 22 woa there was a slightly lower level of corticosterone in birds under CFL light than for birds maintained under LED-R and incandescent light ($p = 0.05$; I = 0.556 ± 0.17 ng/ml; C = 0.24 ± 0.07 ng/ml; L = 0.71 ± 0.14 ng/ml). Age of birds had a significant effect on the cortocosterone concentrations ($p < 0.0001$).

4.4.8 Electrical Consumption and Radiant Flux of the Various Light Bulbs

To determine any changes in light output, the spectrum and energy was measured using a spectrophotometer throughout the trial (Figure 4.10; Table 4.4). The electrical consumption (kW per hour) of the lighting system was also measured for each room (Figure 4.10). Overall the

LED-R bulbs used significantly less energy than CFL and incandescent, ($p < 0.0001$; I = 1210 kW/hr; C = 202.8 kW/hr; L = 152.7 kW/hr). As well, CFL bulbs used significantly less energy than incandescent ($p < 0.0001$). Although light intensity was set to 10 lux in each room, there was a significant difference between the amounts of energy emitted per bulb. LED-R bulbs produced significantly higher energy than incandescent and CFL lights. As well, incandescent light emitted significantly more energy than CFL bulbs (I = 7.37 ± 0.84 nW; C = 2.25 ± 0.34 nW; L = 15.47 ± 1.94 nW). It should be noted that there was a significant difference between collection time points ($p = 0.0043$), however, it is unclear whether this was a result from changes in bulb output (wear and/or dust accumulation) could not be precisely determined as the light probe was very sensitive to light angle and placement under the bulb.

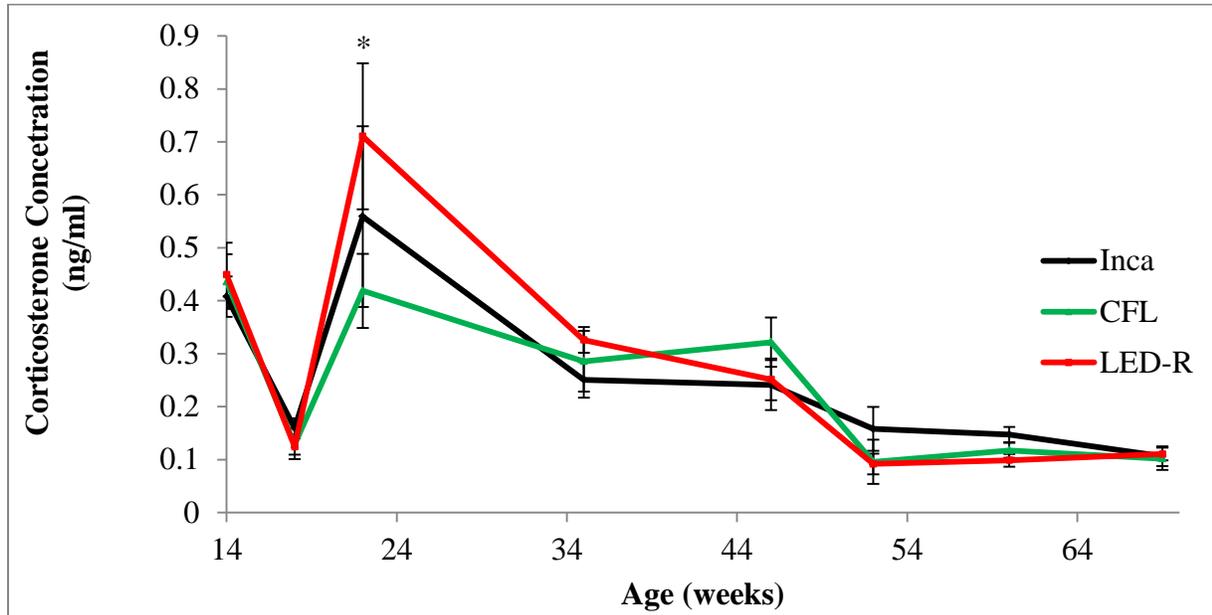


Figure 4.9: Average corticosterone levels in plasma of hens maintained in cages under various lights

Age of birds had a significant effect on the corticosterone concentrations ($p < 0.0001$). Overall, there was no significant difference between treatments ($p = 0.5785$; $\text{ng/ml} \pm$ standard error of the mean (SEM)), however at 22 woa, LED-R had significantly higher level of corticosterone than CFL ($p = 0.05$), indicated by * an asterisk.

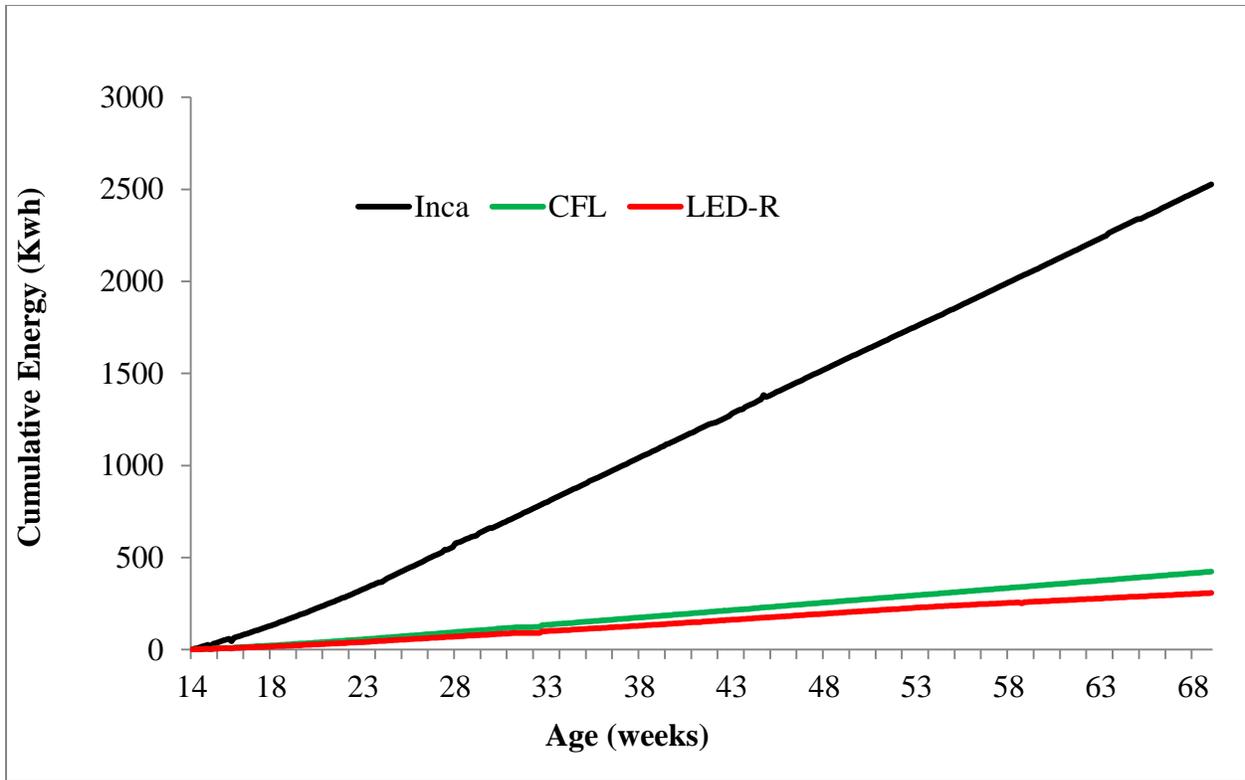


Figure 4.10: Electrical consumption of each light source over 51 week

Hydro meters were placed outside each room, containing 10 bulbs per room. Overall the LED-R bulbs used significantly less energy than CFL and incandescent, ($p < 0.0001$; $I = 1210$ kW/hr; $C = 202.8$ kW/hr; $L = 152.7$ kW/hr). As well, CFL bulbs used significantly less energy than incandescent ($p < 0.0001$).

Table 4.4 Cumulative energy consumption for each light source and the average energy emitting per bulb

Although light sources were set to 10 lux, LED-R bulbs produced significantly higher energy and utilized less energy than incandescent and CFL lights ($p < 0.0001$). Incandescent bulb emitted significantly more energy, yet utilized significantly more electricity than CFL bulbs ($p < 0.0001$). ^{a,b,c} subscripts indicate significant difference between parameters of at least ($p < 0.05$).

	Cumulative Energy Consumption (Kwh)	Energy emitted/bulb (nW)
Inca	2528 ^a	7.31 \pm 0.93 ^b
CFL	424 ^b	2.24 \pm 1.6 ^c
LED-R	308 ^c	15.21 \pm 2.3 ^a

4.5 Discussion

4.5.1 Reproduction

With vast advancements in genetic selection in laying hens, optimizing environmental conditions is key (Lewis, 2009). The onset of sexual maturity is often measured by the age at which hens lay their first egg, which can be initiated by increasing photoperiod. In the present study, the LED-R bulb with a higher amount of red light did not advance maturation and in fact resulted in a significant delay in the average age at first egg. This was unexpected, as previous studies determined higher wavelengths are better able to stimulate the reproductive axis, resulting in earlier age at first age in laying chickens (Gongruttananun, 2011; Kim et al., 2012; Hassan et al., 2013 ; Baxter et al., 2014; Li et al., 2014) and Japanese quail (Woodard et al., 1968). While other studies found birds exposed to green light have a delay in rate of sexual maturity suggesting that exposure to green light inhibits reproduction via retinal photoreceptors (Mobarkey et al., 2010; Gongruttananun, 2011; Hassan et al., 2013; Mobarkey et al., 2013). In the present study, the spectral output of incandescent and LED-R bulbs peak in the red spectrum and CFL bulbs peak in the orange and green spectrum, yet birds under incandescent and CFL light began laying earlier than birds under LED-R light. This suggests that light spectrum may not have been the primary drive in the onset of lay under the present conditions. Furthermore, sexual maturation occurred before photostimulation which further indicates sexual maturation is independent of light and photoperiod. Although age at first egg was significantly different, numerically the delay was only by three days and there was no significant difference between total numbers of cumulative eggs nor was there a difference in egg production. The Lohmann LSL-Lite laying hens used in our experiment is a strain of white leghorn heavily selected for egg production and is expected to reach 50% production between 20 and 21 woa in a cage system (Lohmann Tierzucht, 2013) indicating a “pre-programmed” early initiation of lay, and may rely

on other internal factors, such as body weight. The impact of metabolic status and body weight on maturity was previously shown in Japanese quail where birds matured earlier when they reached a critical body lipid level (Zelenka et al., 1984). It also has been reported that early maturing leghorn hens began laying at a similar body weight to those hens that matured later, indicating that there is a correlation between body weight of birds and initiation of lay (Dunnington and Siegel, 1984). Eitan et al (1998) found that restricted fed layers had a slight delay in the onset of lay, although not significant, suggesting that bird had met their threshold body weight to initiate lay. As well, laying hens had larger combs before 22 woa, which could be attributed to higher body weight (Eitan et al., 1998). In our study, correlation between body weight and age at first egg was calculated and there was a negative correlation between these two factors in birds at 18-19 woa, where an increase in age at first egg (in days) correlated to a lower body weight (kg). The breeders recommended average body weight for this strain at 20 woa is between 1.3-1.4 kg (Lohmann Tierzucht, 2013). The average body weight of birds in the present study was higher than the recommendation at 20 woa (I = 1.52 ± 0.01 kg; C = 1.51 ± 0.01 kg; L = 1.48 ± 0.02 kg) for hens under CFL and incandescent lights, respectively.

In Chapter three, we reported a potential increase in estradiol occurring around 45 woa; however in that trial, samples were not taken often enough to determine the exact timing of this second peak. In the current trial, a significant well defined second peak in estradiol occurring at 52 woa was observed. This occurred in all birds irrelevant of treatment; however, birds under LED-R had significantly higher amplitude. This confirms our previous findings that higher amounts of red light in the LED-R are more efficient at stimulating the production of estradiol (Baxter et al., 2014), possibly by better stimulating the hypothalamic photoreceptors later in the production cycle. The effects of higher wavelengths have been reported to be prominent during

the second laying cycle, after birds were molted, as peripheral tissues are harder to penetrate as birds' age (Pyrzak et al., 1987). This is in line with results from another study, where leghorns exposed to red light at 72 woa and onward have significantly higher production than birds exposed to incandescent light (Reddy et al., 2012). Therefore, the higher amount of red light within the LED-R light and the higher amount of energy emitted from the bulb was more efficient at stimulating the reproductive axis than CFL and incandescent light, although the increased estradiol levels did not translate into higher egg production. Regardless of light source, birds were producing almost an egg a day over the course of the trial making it physiologically impossible for higher levels of estradiol to increase production. Nonetheless, higher estradiol may affect other aspects of the bird's physiology such as calcium deposition or time of ovulation (Beck and Hansen, 2004). The second peak of estradiol suggests that there is a second spontaneous wave of follicular recruitment. In a previous study performed by Johnson et al (1986), there was no difference between serum estradiol concentrations in old (53-63 woa) and young hens (28-38 woa). However as birds aged, there was a decrease in estradiol in preovulatory follicles, leading to longer intervals between ovulation, decreasing egg production (Johnson et al., 1986). In the present study, all hens were still above 90 % production by the end of the trial, it is possible that this second peak in estradiol accounts for the extended laying persistency observed in commercial hens. This is a very interesting finding which will require further research to pinpoint the exact source and regulators.

As there was no difference between treatments in egg production and cumulative number of eggs and, incandescent and LED-R bulbs peak in the red spectrum and CFL peak in the orange spectrum, it is possible that each light source has delivered sufficient amount of red light to stimulate hypothalamic photoreceptors. Previous studies performed on non-commercial strains

of laying hens and Japanese quail indicate that exposing birds to light with higher wavelengths results in higher egg production than birds maintained under white, green (Pyrzak et al., 1987; Huber-Eicher et al., 2013; Baxter et al., 2014; Li et al., 2014) and blue LED light (Kim et al., 2012; Hassan et al., 2013). Similarly, Japanese quail exposed to red light maintained a higher rate of production than birds exposed to blue and green until 16 woa (Woodard et al., 1968). As well, broiler breeder hens exposed to fluorescent light had a lower level of egg production from 58 woa and onward (Ingram et al., 1987). Contrary to the studies above, Er et al. (2007) found that there was no effect of light treatment on egg production in a commercial strain of laying hens, when birds were exposed to either red, green or blue light or complex white light. However, birds under red light had higher production than birds under green and blue light from 38-52 woa (Er et al., 2007). This coincides with another study that found leghorns exposed to red light at 72 woa and onward have significantly higher production than those exposed to incandescent from 73 to 82 woa (Reddy et al., 2012), suggesting that higher wavelengths are better able to simulate the reproductive axis as birds age. This also suggests that if birds in our trial were monitored beyond 69 woa, a difference between light treatments may have been observed. Therefore, differences in strains of birds used, the age of birds, light sources and lighting programs used could have been the cause of the contrasting results. In our trial, pullets reached sexual maturity before photostimulation and thus initiated the activation of the HPG prior to being exposed to the light treatments. Thus, in retrospect better management of pullets which were left under incandescent light and fed ad libitum should be taken into consideration prior to exposing hens to spectrum lighting. Nonetheless, it is clear that the higher egg production of current commercial laying hens is largely due to significant improvements in

genetic techniques (Hocking, 2010) and therefore, data from older literature may not apply or compare to newer strains of birds.

In order to evaluate whether the effect of light spectrum is integrated at the higher level of the reproductive axis, we measured mRNA levels of hypothalamic peptides and their receptors and mRNA levels of pituitary gonadotropin sub-units. Surprisingly, there was no change in GnRH-I or GnIH mRNA levels over time. It has previously been established that GnRH-I gene expression increases upon photostimulation (Dunn and Sharp, 1999) while GnIH release increases during the dark phase (Chowdhury et al., 2010). Furthermore, Reddy et al (2012) reported that birds under red light had a higher hypothalamic GnRH-I mRNA expression than those exposed to green light from 72 woa onwards. Similarly, it was also reported that in broiler breeders, exposure to longer wavelengths results in higher hypothalamic GnRH-I mRNA expression at 65 woa (Mobarkey et al., 2010; Mobarkey et al., 2013). In our study, samples were collected at 15, 29 and 70 woa, where hens began laying before photostimulation and displayed a peak in estradiol at 16 woa, it is thus likely that we missed the initial activation of the reproductive axis. Furthermore, at 70 woa birds were still at a 90 % production level, regardless of treatment, again suggesting that hens were still at peak production and the reproductive axis was still fully activated. As well, spectrums from each light source did include a component of the red and or orange light, it is possible that sufficient activation of hypothalamic photoreceptors led to equal GnRH-I gene expression. Nonetheless, the photoperiodic induced GnRH-I mRNA expression (Dunn and Sharp, 1999) is transitory and our collection time points may not have captured this event. We did observe an increase in GnIH mRNA level in birds under incandescent light at 29 woa, which did not appear to impact egg production and, as reported by Ciccone et al (2005), changes in GnRH-I and GnIH mRNA levels may not reflect

peptide release and reproductive status of the bird. However, a decrease in gene expression has been found to reduce peptide release as reported by Dunn et al (1996) who found that during incubation, bantam hens had a decrease in GnRH peptide which was associated with a decrease in hypothalamic GnRH-I mRNA. Interestingly, we decided to collect tissue sample at 70 woa as this is normally associated with a drop in production (end-of lay), however, due to the high level of production of this commercial strain, all birds were still at levels close to peak production.

At 29 and 70 woa, there were higher levels of GnRH-RIII mRNA than at 15 woa regardless of light treatment. This is in agreement with previous results that show higher amounts of GnRH-RIII in sexually mature birds compared to immature or juvenile birds (Shimizu and Bedecarrats, 2006; Joseph et al., 2009). However, Shimizu et al (2006) also recorded a decrease in GnRH-RIII mRNA expression at 52 woa, time at which hens were considered to be closer to the end of the laying period. We did not observe such a decrease but in our case, none of the hens were displaying a drop in production, thus results cannot be compared solely based on age. It is also worth mentioning that due to the relatively small number of samples taken at each time point, inter-individual variability exacerbated changes in mRNA levels as represented by the large standard error.

Overall, LH- β mRNA expression significantly increased as birds aged ($p < 0.0002$). There was also a significant difference between treatments, where at 70 woa birds under incandescent light had a higher level of LH- β mRNA expression than LED-R. A previous study reported that ovariectomized hens had a reduction in plasma estradiol which led to an increase in pituitary LH- β and alpha-subunit mRNA levels, suggesting that ovarian estradiol negatively feeds back onto the pituitary to inhibit LH- β and α -subunit gene expression (Terada et al., 1997). Therefore, the constant level or lack of increase in LH- β gene expression in birds under LED-R

light may be due to higher amounts of estradiol negatively feeding back onto the anterior pituitary. Interestingly, the observed second peak in estradiol decreased in all treatments at 69 woa suggesting that the increase in LH- β mRNA levels may be due to a decrease in negative feedback. However, Ciccone et al (2005) did observe an increase in LH- β and FSH- β mRNA expression in out of lay hens (60 woa) compared to young hens (30 woa) and old laying hens (60 woa). This again suggests that feedback from estradiol may be a key regulator of LH subunit gene expression, especially in older hens (Ciccone et al., 2005). As stated above, in our experiment at 70 woa birds were still at over 90% production which may explain the difference observed with results reported by Ciccone et al. (2005). Alternatively, the strain of chicken used may also account for the difference, as broiler breeders (Ciccone et al., 2005) aren't genetically selected for egg production like commercial laying hens.

There was no difference in FSH- β mRNA levels between light treatments however there was an increase in expression as birds aged. Such inconsistency has previously been reported as no difference in FSH- β and LH- β mRNA levels were observed regardless if birds were kept under short day and long day photoperiods, yet expression increased with age (Ni et al., 2013). Regarding the α -subunit, higher levels of mRNA were observed in younger hens compared to older laying hens or out-of-lay hens (Ciccone et al., 2005). In our study, all hens were laying and remained at peak for each tissue collection; the lack of change in alpha-subunit mRNA as birds aged or between treatments could have been expected.

4.5.2 Egg Quality

There was no difference in egg weight and shell strength between light treatments. These results are similar to previous studies that found no difference in egg weight and eggshell quality

under various monochromatic lights (Hassan et al., 2013) and under daylight supplemented with fluorescent or LED light (Gongruttananun, 2011). Conversely, Er et al. (2007) found that egg weight was heaviest under white light, and lightest under red light. They also determined that birds under monochromatic green light had better shell strength than those under white and blue lights. Others have reported that laying hens under blue light had heavier eggs than birds under red and white from 41-50 woa (Kim et al., 2012). Shell quality was also reported to be superior when hens were maintained under green light and egg weights were higher in hens under green and blue light during the first and second laying cycle (Pyrzak et al, 1987). Li et al (2014) found that birds under red and white light had the heaviest eggs and hens under blue and green light produced significantly lighter eggs, however egg shell strength was better in birds under green light than birds under white and blue light (Li et al., 2014). Thus, there is no consensus regarding the effect of light wavelength on egg quality, and strain, nutrition, and metabolic status may have stronger influence on egg quality than light. Interestingly, although in our experiment birds under LED-R ate significantly less and had lower body weights; this did not impact egg weight or quality. It has also been observed that reduced body weight did not result in smaller egg weight (Lewis, 2009).

4.5.3 Body Weight Gain and Feed Consumption

The effect of light wavelength on feed consumption, feed efficiency and body growth has mainly been studied in broilers. Rozenboim et al. (1999a) found broilers under green light had higher body weights from 3 to 20 days and exposure to blue light had significantly increased body weight from 20 to 34 days (Rozenboim et al., 1999a). To determine the optimal spectrum to manipulate body weight, combinations of monochromatic green and blue light were used at different ages. Body weight and growth was highest when birds were raised under

monochromatic green light until 10 days and then switched to blue light until 46 days (Rozenboim et al., 2004a). These effects appear to be partly mediated by direct stimulation of muscle growth, but higher feed intake and better feed conversion were also reported in broilers under white (colour peak at 500nm) and yellow (colour peak at 635nm) LED compared to birds under CFL light, (spectral output not measured) (Mendes et al., 2013). However, broilers and layers are two completely different birds and one cannot extrapolate results from one breed to another. Furthermore, broiler birds reach market weight by 6 woa and are thus considered juvenile. Nonetheless, Hassan et al. (2014) also found that laying hens under red light had better feed conversion, which is similar to results seen in our trial as birds under LED-R are producing the same number of eggs while consuming less feed. When comparing feed conversion in layers, it is important to consider environmental housing, as energy expenditure directly relates to the level of activity or thermoregulatory activities. In our experiment, all birds were placed in individual cages so that their energy expenditure would be minimal and remain the same between treatments. Still, a higher amount of red light appears to reduce feed intake and growth. We previously reported similar results on growth as Baxter et al. (2014) found hens under green light had significantly higher body growth than birds under red and white, however feed consumption was not measured and birds under green laid significantly fewer eggs. Therefore it was speculated that a reduction in eggs resulted in more energy being put towards body growth, as birds were also placed in individual cages (Baxter et al., 2014). It has been previously reported that UV light may accelerate growth (Barott and Pringle, 1951). Incandescent light had a broad spectral output and may emit wavelengths in the UV spectrum potentially contributing to the higher growth under incandescent light. However UV light could not be detected by our spectrophotometer probe.

4.5.4 Stress

As we observed in Chapter 3 for laying hens maintained on floor pens, there was no definite trend regarding plasma corticosterone concentrations as birds aged, nor was there any difference between light treatments. This indicates that different light sources may not affect physiological stress levels in laying hens maintained in individual cages. It should be noted that the level of corticosterone was below what is considered physiological stress, where daily fluctuations in laying hens ranges from 7 to 11 ng/mL (Beuving and Vonder, 1978). Similar results were seen in turkey hens under monochromatic blue, green, and red light or incandescent light where no significant differences in erythrocyte, leukocyte and corticosterone concentrations were observed (Scott and Siopes, 1994). Beyond corticosterone, fear response was previously measured using the tonic immobility test, where birds that are more stressed stay immobile longer (Sultana et al., 2013). Initial testing found that birds under red light had a longer time to recovery on the first test, while during a second set of tests birds under yellow light had a longer recovery rate. Although the results were inconsistent, they speculated that the higher activity of birds under longer wavelengths may increase the duration birds remain immobile (Sultana et al., 2013). In addition, birds under shorter wavelengths did not display a significant difference in duration of immobility suggesting that they did not have a decreased fear response (Sultana et al., 2013). When looking at immunity as an indicator of physiological stress, Interleukin-1 β (IL-1 β), a pro-inflammatory cytokine, can elicit the release of corticosterone (Xie et al., 2008b). The level of IL-1 β was highest in birds exposed to white light, while exposure to blue light produced the lowest amount of corticosterone, indicating low stress levels (Xie et al., 2008b). The lack of consistent results within this study may be due to bird handling before sampling, similarities in light spectrum between light sources or difference in perceived intensity. As well, corticosterone

levels may not reflect the physiological stress levels in birds, and other factors may need to be investigated to find the true effect of light source on stress levels.

4.5.5 Bulb Characteristics

Throughout the trial, spectral output was measured with a spectrophotometer, and the spectral integrity for each bulb remained constant indicating a stable output. Electrical consumption (kW/hr) between bulbs did vary, where the room with the LED-R lights used significantly less energy than both the CFL and incandescent. The large difference in energy consumption between incandescent and LED-R is a testament of energy efficiency with the majority of energy wasted as thermal output for incandescent bulbs due to the energy required to heat the tungsten filament (Andrews and Zimmermann, 1990). The LED bulb has high photoelectric conversion efficiency therefore a low thermal output allowing for long lasting bulbs that use less energy (Yeh and Chung, 2009). Energy emitted per bulb was measured as the radiant flux which is the number of watts emitted per second from the light sources (nW) (Prescott et al., 2003). Although maintained at the same intensity (10 lux), the LED-R bulbs emitted significantly higher radiant flux than both the CFL and incandescent. Incandescent and CFL bulbs have a relatively well distributed isotropic intensity distribution (Shaw and Goodman, 2008). An LED bulb with 75 LED elements had the highest intensities of light below the light source, indicating that the LED light intensity is anisotropic (Shaw and Goodman, 2008). Although different bulbs were used between our experiment and those used by Shaw and Goodman (2008), the higher energy emitted from the LED bulb may have been due to large cluster of light being detected by the light probe. Therefore based on the previous research performed by Shaw and Goodman (2008), the difference in radiant flux between the bulbs may have been due to the distribution of light emitted from each of the bulbs.

4.6 Conclusion

Overall, red light was more efficient at stimulating the reproductive axis as seen with the higher concentrations of estradiol; however, this didn't translate to higher egg production. Similarly, light source did not impact gene expression of GnRH-I, GnIH, LH- β , FSH- β , α -subunit, and GnRH-RIII. However, we did not anticipate that hens would sexually mature prior to photostimulation (at time of placement) and display such a high level of laying persistency. This is likely due to the heavy genetic selection for egg production in commercial birds over the last decades, especially during the last 10 years. Interestingly, we did observe a defined second peak of estradiol around 52woa, which may be why hens maintained high levels of production when they are supposed to progressively go out-of-lay. These observations suggest that the commercial strain of layers from 2013-2014 may not fit the avian reproductive model developed over the years (Etches, 1996, Bedecarrats et al., 2009). As there was a significant correlation between age at first egg and body weight, other internal factors such as metabolic status may be important cues to trigger sexual maturation beyond photoperiod. . It also appeared that shorter wavelengths are less efficient at entraining oviposition and ovulation, as hens under green light exhibited a lack of synchronicity between light schedule and time of lay. Light sources did not affect egg quality, nor was there any effect on corticosterone levels in the plasma. However, birds under CFL and incandescent lights had significantly higher body growth and consumed more feed than birds under LED-R light, yet they produced the same number of eggs. This suggests that caged birds under LED-R have better feed efficiency, although lighting is only a component of proper management. As both incandescent and LED-R lights peak in the red spectrum, it was expected they would have similar effects on body weight and feed consumption; however, this was not the case. It is possible that beyond the spectrum, energy output, and the

relative intensity of each wavelength of the bulb are important. Overall, the LED-R bulb used significantly less electricity and emitted a higher radiant flux which makes it an ideal candidate for retrofit system.

Chapter 5: Conclusion

Exposing a non-commercial strain of blind and sighted laying hens (Smokey Joe hens) to red, green and white light showed red light was the most effective wavelength to stimulate the production of estradiol and thus the reproductive axis. While hens under green light had a delay in age at first egg and lower and shorter egg production suggesting green light was ineffective at stimulating the reproductive axis. Although not significant, hens under red light did have slightly better reproductive performances than hens under white light, as they began laying earlier, and had a slightly higher level of production. Thus, it was concluded that wavelengths in the red spectrum are required for stimulating the hypothalamic-pituitary-gonadal (HPG) axis. Furthermore, as no difference was observed between blind and sighted hens, the effect of light wavelength is independent of the retina of the eye. From this preliminary study we speculated that a white light containing 33% red or more is sufficient to adequately stimulate and maintain reproduction and so, our team designed an LED bulb emitting 60% red light. The subsequent work performed as part of this thesis was then aimed at testing and further validating this novel lighting system on commercial laying hens under various housing conditions. Surprisingly, when using a commercial strain of laying hens (Lohmann LSL-Lite) both in cages and on collective floor pens, we observed that light spectrum did not impact the onset of lay, nor the persistency of production. However, beyond production, a higher amount of red spectrum was still more efficient at stimulating the reproductive axis as higher estradiol levels were seen under both environmental conditions. Nonetheless, regardless of light source or spectrum, in each trial with these commercial hens, birds began laying before photostimulation and production peaked sooner and was maintained longer than expected. Correlating age at first egg with body weight revealed that higher body weight did lead to earlier onset of lay and peak production. Thus, we

speculate that internal metabolic triggers may be responsible for initiating the onset of lay in modern layers and further studies will be required to confirm that hypothesis. Nonetheless, regardless of light treatment, light spectrum did not affect egg quality but spectrum did affect feed consumption and body weight gain, as Smoky Joes under green light gained more weight, and commercial birds in cages under the LED-R had a lower feed consumption, leading to lower body weight, while producing the same number of eggs. This suggests that the LED-R bulb along with proper management techniques can allow for better feed conversion in birds maintained in cages. It also appeared that shorter wavelengths are less efficient at entraining oviposition and ovulation, as hens under green light exhibited a lack of synchronicity between light schedule and time of lay. It is unclear as to how light source affected growth and feed consumption, but we can speculate that spectrum, energy output, and the relative intensity of each wavelength of the bulb are all involved.

Unexpectedly, in birds maintained in individual cages, a second peak in estradiol was observed, occurring around 45-50 woa. The trigger for this second peak in estradiol is unclear as it has never been documented in the literature. However, it is most likely linked to the heavy genetic selection for egg production in commercial strains over the last couple of decades, especially in the last 10 years. As such, it may contribute to the high level of persistency seen in modern commercial layers. This is very intriguing and further investigation is required to determine the cause of this increase in estradiol and how it affects the bird's physiology and behaviour.

In an attempt to elucidate at what level light from the red spectrum does activate the HPG, levels of mRNA from hypothalamic neuropeptides and their receptors and reproductive hormones were measured. However, mRNA levels did not reflect the reproductive status of the

bird which may have been due to the sampling times not representing the expected reproductive status of commercial layers (immature versus peak and late lay). Beyond production, it appeared that light spectrum did not negatively affect behaviour, although a higher number of aggressive pecks in birds under LED-R were observed in birds maintained in floor pens. However, variation between pens within each treatment suggests that aggressive pecks may have more to do with social hierarchy than light treatment. On a practical level, the LED-R bulb used significantly less electricity and emitted a higher radiant flux than comparable incandescent or compact fluorescent lights. In addition, our prototype was fully dimmable (thus suitable for all type of lighting program).

In conclusion, this thesis shows that an LED bulb with 60% red light has no adverse effects on production, stress, behaviour and growth. With a reduction in feed costs for birds maintained in cages and a significant reduction in electrical consumption, the potential financial savings/gains for producers stand to be significant which make our bulb an ideal candidate for layer barn retrofit.

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