SHINING A LIGHT TO CHICKEN EMBRYOS: A NEW PERSPECTIVE ON HATCHERY MANAGEMENT

by

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ABSTRACT

The transition towards limiting the use of antibiotics in the production of commercial broiler chickens has challenged the Canadian poultry industry to consider alternate approaches to managing bird health and disease. A focus on optimizing early health and performance has led us to investigate parameters in the incubation environment that encourage superior embryo development and chick quality. Two experiments were conducted to understand how different lighting programs affect embryonic development, hatch traits and production parameters of commercial broilers. Experiment 1 focused on the effects of light wavelength and consisted of four replicate trials. Broiler hatching eggs (Ross 308) were incubated in dark or illuminated with white, red or blue LED light for 12 h per d^{-1} (12L:12D). Our results demonstrated that providing a 12L:12D photoperiod did not affect hatch traits compared to those incubated under darkness. Provision of blue or white illumination resulted in increased feed consumption and body weight, and a reduced variation in cloaca temperature for chicks at a young age. Red light stimulation had a stronger response to vaccination on d 14 of age. The effects of blue LED illumination with two photoperiods (12L:12D or 18L:6D) on two strains of broilers (Ross 308 and Cobb 500) were compared to no illumination in Experiment 2. Both strains incubated with periodic illumination had lower air cell temperatures than those under constant darkness. The response of air cell temperature to periodic illumination differed between two strains. Cobb embryos had lower air cell temperature in 12L:12D than those incubated with 18L:6D from d 16 of incubation onwards, whereas lower air cell temperature was found in Ross embryos when illuminated with 18L:6D photoperiod compared to those under 12L:12D. Broilers hatched under 12L:12D had improved navel condition than those under dark, and higher feed consumption in the first 6 h post-placement compared to 18L:6D. In conclusion, *in ovo* photostimulation with white and blue light under a 12L:12D photoperiod improved early growth of broilers. The depressed production parameters found in 18L:6D suggests that the duration of darkness should be more than 6 h d^{-1} when providing a periodic illumination during incubation.

Key words: broiler, incubation, light, air cell temperature, productivity

LIST OF ABBREVIATIONS USED

12L:12D	12 hours of light : 12 hours of dark
18L:6D	18 hours of light : 6 hours of dark
AA-NAT	Arylalkylamine N-acetyltransfaerase
CAM	Chorioallantoic membrane
CORT	Corticosterone
DBPs	Deep brain photoreceptors
ED	Embryonic day
ELISA	Enzyme-linked immunosorbent assay
EWL	Egg weight loss
FCR	Feed conversion ratio
h d-1	Hours per day
GH	Growth hormone
GHRH	Growth hormone releasing hormone
H/L ratio	Heterophil to lymphocyte ratio
HPA	Hypothalamus-pituitary-adrenal
HPS	Heat shock protein
HPT	Hypothalamus-pituitary-thyroid (HPT)
HW	Hatch window
IGF-1	Insulin-like growth factor 1
IMEI	2-[125I]iodomelatonin
KLH	Keyhole limpet hemocyanin
LED	Light-Emitting Diodes

LHN	Lateral hypothalamic nucleus
MDA	Malondialdehyde
mSCN	Median suprachiasmatic nucleus
NDV	Newcastle disease virus
Opn2	Rhodopsin
Opn3	Pinopsin
Opn4	Melanopsin
Opn5	Neuropsin
PACAP	Pituitary adenylate cyclase-activating polypeptide
PGC-1	Peroxisome proliferator activated receptor-coactivator 1 □
RH	Relative humidity
RHT	Retinohypothalamic tract
SCN	Suprachiasmatic nucleus of the hypothalamus
VA-opsin	Vertebrate ancient opsin
VIP	Vasoactive intestinal peptide
vSCN	Visual suprachiasmatic nucleus
YFBW	Yolk-free body weight

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CHAPTER 1. GENERAL INTRODUCTION

Earth rotates on its own axis approximately every 24 hours and results in cyclic changes in light exposure, temperature, humidity and food availability. Most organisms on our planet, especially those inhabiting the temperate zone, have developed an internal time-keeping system to adapt to those daily periodic changes (*zeitgebers*) in our environment. As early as 1729, Jean-Jacques d'Ortous de Mairan, who found that a self-sustained day-night pattern of movement for branches and leaves continued under an environment of constant darkness in *heliotrope* plants (de Mairan, 1729). This phenomenon was repeatable, and the author suggested the existence of an endogenous biological clock system controlling the leaf expansion during the day and folding up at night. The output measured as leaf movement was caused by environmental inputs (light, temperature or other factors), but the internal components involved in this time-keeping control were unknown. Knowledge of the physiology and properties of circadian timekeeping mechanisms has advanced significantly in both mammals and birds over the past centuries (Kumar, 2015; Reppert and Weaver 2002).

Light, one of the primary *zeitgebers*, is a powerful exogenous signal in synchronizing the biological rhythms and artificial lighting has been widely used as a management tool for poultry production across the world for many years to improve animal welfare, health and production efficiency (Blatchford et al., 2012; Schwean-Lardner et al., 2012a,b; Wei et al., 2020; Oke et al., 2021). As the duration of incubation continues to encompass a larger portion of the life of high-yield broiler chickens, it is becoming increasingly difficult to ignore the needs of an optimal micro-environment (temperature, humidity, ventilation, air composition and maybe lighting) of incubation for developing embryos. Light is a critical

environmental stimulus for both chickens and chicken embryos. Naturally brooded chicken eggs experience varying degrees of light exposure when the hen turns the eggs frequently at the beginning of incubation to maintain the temperature or leaves the nest for foraging (Archer and Mench, 2014a). While most commercial incubation units are not illuminated except for when humans enter the system to set eggs or clean the unit after use, this process excludes the opportunity for embryos to perceive light. Studies have been conducted to evaluate the impacts of providing illumination during incubation for chicken embryos on development, productivity and welfare. The effects of provision of light reported in early studies includes accelerated embryo development, shortened incubation time and enhanced post-hatch production parameters (Siegel et al., 1969; Walter and Voitle, 1973; Lauber, 1975). However, the growth-promoting effect of photostimulation has been considered as a confounding effect of illumination and increased embryo temperature when illuminated with conventional light sources. With the advancement of lighting technology, the effect of generating heat from the light source could be excluded by using light-emitting diodes (LED). LED is a great source of illumination with low energy consumption, extended lifespan and can provide both full spectrum and monochromatic colors with minimum heat production.

A closer look at the literature on the effects of LED light illumination on embryo development, chick quality and post-hatch performance suggests that the optimal lighting regimes (intensity, the composition of the spectrum and photoperiod) for chicken embryos are still debatable. For instance, a number of authors reported that photostimulation during incubation accelerated embryo development (Halevy et al., 2006; Özkan et al., 2012a; Wang et al., 2017), increased hatchability (Archer et al., 2017;

Yameen et al., 2020), enhanced production efficiency (Özkan et al., 2012b; Yameen et al., 2020), improved bone development (van der Pol et al., 2019) and reduced susceptibility to stress post-hatch (Özkan et al., 2012b; Archer and Mench, 2014a; Archer et al., 2017; Archer, 2018). However, other studies have reported that light stimulation has no significant effects on embryonic development or hatching traits (Rozenboim et al., 2003, 2004a; Khalil, 2009). Shafey et al. (2005) found that providing continuous green light at high light intensity even resulted in a higher embryo mortality. These inconsistent effects of photostimulation may be due to variability in lighting treatments including the source of light, light intensity, wavelength emitted, photoperiod or a combination of these factors.

In commercial practice, newly hatched chicks are exposed to a near-constant illumination (20-23 h d^{-1}) during the first week post-hatch. Few studies have been conducted to examine the effects of photostimulaiton with a photoperiod longer than 12 h d^{-1} during incubation. Little is known about the impacts of extended photoperiods during incubation on developing embryos and their effects on post-hatch performance in broiler chickens. Many studies involving the light spectrum have demonstrated that green light enhanced post-hatch muscle growth and production parameters (Halevy et al., 2006; Rozenboim et al., 2004; Wang et al., 2014; Zhang et al., 2012, 2016), and red and white light improved hatchability and chick quality (Khalil, 2009; Archer, 2017; Archer et al., 2017). However, studies involving light emitted in other parts of the spectrum such as blue or yellow are still insufficient.

Although incubators traditionally do not come equipped to provide illumination during the incubation progress, in recent years, early feeding programs have been developed to

provide feed and water in the hatcher for newly hatched chicks and have resulted in the installation of lights within these units. The lights are typically left on continuously to facilitate consumption of nutrients for the last three days of incubation. The need to illuminate incubation equipment to provide light for chicks to eat and drink may be one aspect of evolving incubation technologies that will drive the need to know more about the impact of light on developing embryos. But few conclusions can be drawn from the recent studies regarding the use of illumination during incubation as a potential environmental tool to improve newly hatched chicks' quality and their adaptation to the post-hatch environment. The implementation of a lighting program during incubation deserves to be explored further.

Aim and outline of the dissertation

The aim of this dissertation is to provide information for a better understanding on the mechanism of how light characteristics (wavelength, photoperiod and their combination) impacts embryogenesis and productivity of broiler chicks.

The literature review, Chapter 2, focused on the state of knowledge of light perception and circadian time measurement in birds, including recently discovered molecular and physiological mechanisms. The ontogeny of the central clock system in chicken embryos, biological rhythm entrainment and the effects of light stimulation during incubation on development, hatch traits, productivity and health are described.

The magnitude of the effects of providing light of different colors (white, red, blue or dark) during incubation on embryonic development and hatching traits was addressed in Chapter 3. It investigated how embryonic organ development, the distribution of hatch time and chick quality responded to the periodic light stimulation with different

wavelengths of the light spectrum. In Chapter 4, the productivity of Ross broilers hatched with incubation lighting programs was investigated and provided novel information on adaption performance, body temperature and daily chick growth during brooding stage. The immune organ development and humoral immune response to a vaccination program were furthermore evaluated. In Chapter 5, an experiment to evaluate the effects of blue light illumination with different photoperiods on hatching traits, thermoregulation and productivity in two commercial strains of broilers is described.

In the general discussion in Chapter 6, results from experiments are discussed to gain insight into the overall effects of light illumination to broiler embryos on embryonic development, production parameters and health. The potential physiological pathways, which may be able to explain the results found in experiments, are discussed.

CHAPTER 2. LITERATURE REVIEW

2.1 Light perception and circadian time measurement of birds

Light is a critical exogenous environmental factor and affects many aspects of animals' life including development, health and behavior. In a natural setting, the duration of light exposure per day provides an environmental cue to birds and allows them to anticipate the preparation for predictable environmental changes. To be able to regulate the physiological and behavioral responses (outputs) to the changes of external photic signals, the temporal cue (inputs) must be perceived by photoreceptors. The central pacemakers in the brain containing self-sustained circadian oscillators that produce a stable rhythm in clock genes and proteins expression and entrain the oscillators in many peripheral tissues (like the liver, kidneys, skeletal muscle and gonads). The synchronized central and peripheral clocks work together as a functional unit to convey rhythmic information to the downstream cells, tissues and organs and drive the daily rhythms of physiological and behavioral events as the net outputs. The evidence of the presence of the "input-pacemaker-output system" at three structures – retina of the eyes, pineal gland and hypothalamus will be discussed in following sections.

2.1.1 Types of photoreceptors

Retinal photoreceptors

The photoreceptor molecules are opsin-based transmembrane proteins coupled to the retinaldehyde to trap light and thus play a role in the perception of photic information. The photoreceptors in avian species have been intensively studied. Light can be acquired by both ocular photoreceptors and extra-retinal photoreceptors located in pineal gland

and hypothalamus (Kumar et al., 2004). The retina of a bird has at least four main types of photoreceptor cells: rod, cone, double cone cells and retinal ganglion cells. Rod cells are highly light-sensitive but cannot differentiate colours due to the single absorbance spectra of rhodopsin (Opn2) at about 505 nm (Bowmaker, 1977; Hart, 2001). Meanwhile, cone cells are approximately 25-100 times less sensitive for luminance detection than rods and play the main role in determining variations in colour (Hart, 2001). Chicken single cone cells can be further classified into 3 types according to their spectral sensitivity to the long-, middle- and short- wavelength. Several studies confirmed the sole contribution of single cones in color discrimination has a trivial effect on the spectral sensitivity thresholds (Osorio et al., 1999; Jones and Osorio, 2004). The retina of birds contains the fourth type of cone cell, double cone cells, which consist of two electrically coupled cells including principal member and a smaller accessory member, and the outer membranes of both members are connected by gap junctions (Smith et al., 1985). The double cone cells were thought to play a role in luminance-base tasks, like movement and pattern detection but not wavelength discrimination as both members of double cone cells express the same wavelength sensitive opsin, which has a maximum spectral absorbance between 565 and 570 nm in *Galliformes* (Bowmaker et al., 1997). However, the precise functions of the double cone cells in birds are still debated. Additional to the cone and rod opsins (Opn1 and Opn2), the expression of the encephalopsin (Opn3), melanopsin (Opn4) and neuropsin (Opn5), which have the peak spectrum absorbance at violet or ultra-violet spectrum range are detected in the ganglion cells of chickens' retina and provide a boarder photosensitivity spectrum range for birds than mammals (Tomonari et al., 2005; Lima et al., 2006; Tomonari et al., 2008; Kato et al., 2016).

Pineal gland photoreceptors

Initially, retinal photoreceptors were considered as the only photoreceptors in avian species like mammals. The existence of extra-retinal photoreceptor in the brain has been observed in Benoit's study, which found that enucleated (blinding) domestic ducks (*Anas platyrhynchos*) displayed the reproductive responses to changing photoperiod, whereas the gonadal responses to the illumination was inhibited when their heads were covered with a black cap (Benoit, 1964). The presence of rhodopsin-like photosensitive substance in the pinealocytes was first proposed by Deguchi (1981). The action spectrum for photosensitivity of the isolated chicken pineal gland was found to be similar to the peak absorption spectrum of rhodopsin at 500 nm. Immunocytochemical procedure has been applied to further investigate the types and locations of photoreceptors in the pineal gland (Foster and Follett, 1985; Foster et al., 1989). The expression of Melanopsin (Opn4) mRNA in chicken pineal gland persisted a rhythmic pattern when transferred to constant darkness from a light-dark cycle (Chaurasia et al., 2005). In Japanese quail pineal gland, the rod-opsin specific antibodies have been found in pinealocytes at day 2 of age and rod opsin increased from 1.16 pmoles opsin/pineal to 2.2 pmole opsin/pineal then reach a plateau at 2.0 pmole opsin/pineal on day 30 of age (Foster et al., 1989). Another unique photopigment with a maximum absorbance at 470 nm found in chicken pinealocytes is pinopsin (Opn3), which was first cloned by Okano and colleagues (Okano et al., 1994). Like its name, the pinopsin messenger RNA is specifically expressed in the pineal gland of avian species including chicken, Japanese quail and pigeon (Okano et al., 1994; Kawamura and Yokoyama, 1996). The identification of opsin molecules in the pinealocytes of birds suggests that pineal gland can perceive light directly. An *in vivo*

study conducted by Deguchi (1979) also demonstrated that chicken pineal gland contains the photoreceptors for perceiving the photic information. The arylalkylamine N-acetyltransfaerase enzyme (AA-NAT), a key enzyme in melatonin biosynthesis, reached the maximum activity in the subjective night and decreased during the subjective day in the cultured pineal gland cells when illuminated with 12 h d^{-1} (Deguchi, 1979). In addition, the entrained daily rhythm of AA-NAT activity in the pineal gland persisted for up to 4 days when kept under a continuous darkness environment (Kasal et al., 1979). Similarly, the circadian rhythm of pinopsin mRNA level in chicken pineal gland entrained by the light-dark cycle can persist under constant darkness (Holthues et al., 2004). The results from above studies suggested that the avian pineal gland can perceive light and has a self-sustained circadian oscillator to mediate the synthesis of melatonin in a clock-driven manner.

Deep brain photoreceptors in the hypothalamus

Addition to the photoreceptors located in the retina and pineal gland, evidence suggests that the hypothalamus of birds can perceive photic information directly as well. Surgical removal of either the pineal gland or eyes of house sparrows (*Passer domesticus*) did not abolish the impacts of long-day illumination on testicular recrudescence, whereas injecting India ink under the head skin of pinealectomized sparrow resulted in lower testis weight than those underwent pinealectomy only (Menaker et al., 1970). The stimulative effect of long-day photostimulation on testicular growth was also observed in blinded and pinealectomized Japanese quails (*Coturnix japonica*) (Oishi and Ohashi, 1993; Nakane et al., 2010). These results clearly showed that the involvement of extra-retinal and extra-pineal photoreceptors in the brain in transferring photic information to

the hypothalamus-pituitary-gonadal axis. Detecting the presence of antibodies specific to known retinal and pineal gland photopigment is the classic approach to locating the photoreceptors in the brain tissue. To date, several opsin-based photopigments have been characterized as the deep brain photoreceptors (DBPs) (Table 2.1). The expression of rhodopsin (Opn2) with an absorption spectrum between 480-495 nm, Opn4 with an absorption spectrum between 480-495 nm, neuropsin (Opn5) with an absorption spectrum between 480-495 nm and vertebrate ancient opsin (VA-opsin) with an absorption spectrum between 480-495 nm have been detected in the brain tissue and involved in the transduction of photic information to oscillators (Deguchi, 1981; Foster and Follett, 1985; Bailey and Cassone, 2005; Chaurasia et al., 2005; Davies et al., 2012).

Table 2.1 The types and location of the deep brain photoreceptors in avian species

Expression area	Opsins	Species
Lateral septal area	Rhodopsin	Ring doves, Japanese quail, duck (Silver et al., 1988); Pigeon (Wada et al., 1998)
Mediobasal hypothalamus	Vertebrate ancient opsin	Chicken (Tomonari et al., 2007; Halford et al., 2009; Davies et al., 2012)
Premammillary nucleus	Melanopsin	Turkey (Kang et al., 2010)
Paraventricular organ	Neuropsin	Japanese quail (Nakane et al., 2010)

2.1.2 Pacemakers and circadian outputs

There has been much progress in the past decades in establishing the anatomical and physiological basis of circadian time measurement in birds. Birds are equipped with the most complex circadian pacemaker system among all animal species due to the presence of multiple oscillators (Reviewed by Bell-Pedersen et al., 2005). Unlike in mammals, which have photoreceptors and pacemakers localized in different organs or tissues, a complete input-pacemaker-out system has been found at each of retina, pineal gland and hypothalamus in birds (Gwinner et al., 1997). The molecular mechanism governing circadian rhythmicity in the brain of birds is guided by the knowledge of the circadian clock mechanism in fruit fly (Konopka and Benzer, 1971). An autoregulatory intracellular transcriptional-translational feedback loop has been proposed to regulate the expression of core clock genes (*Clock, Bmal,* families of *Period* and *Cryptochrome*) in a rhythmic manner and contributing to generate the circadian rhythmicity for both mammals and birds (Helfer et al., 2006). In the daytime, rhythmic transcription of CLOCK and BMAL1 proteins form heterodimers and activate the transcription of the *Per* and *Cry* genes in the nucleus. The mCRY and mPER proteins are phosphorylated with casein kinase in the cytoplasm and then translocated back into the nucleus and interact with CLOCK-BMAL1 heterodimers to inhibit the transcription of *Per* and *Cry* genes during the subjective night. In the next light-dark cycle, the transcriptional activator, CLOCK-BMAL1, can active a new round of transcription of *Per* and *Cry* genes (Reviewed by Reppert and Weaver, 2002). The rhythmic expression of avian *Per2*, *Per3*, *Bmal1*, *Bmal2*, *Cry1*, *Cry2* and *Clock* genes have been found in retina, pineal gland and hypothalamus in birds (Yoshimura et al., 2000; Okano et al., 2001; Bailey et al., 2002; Fu

et al., 2002; Helfer et al., 2006; Jiang et al., 2016). The rhythmic changes in core clock genes and their protein products are responsible for the circadian time measurement. The circadian rhythm in transcript levels varies among clock genes and proteins and the difference in daily expression profile may suggest the different roles in bird clock oscillator mechanisms.

Retina

It is difficult to investigate the role of the retina on circadian time measurement as the photic information can be perceived by extra-retinal photoreceptors in birds. The most notable circadian outputs from the retina are AA-NAT activity and melatonin synthesis. Pinealectomy alone or blinding alone cannot abolish the circadian rhythm of blood melatonin and locomotion completely in pigeons (Ebihara et al., 1984; Underwood et al., 1984). It has been found that complete removal of the eye resulted in arrhythmicity in body temperature of Japanese quail (Underwood, 1994). Those observations offer some support for role of retina in circadian rhythm entrainment. A further study reported that the rhythmic melatonin synthesis in the eye of Japanese quail could be directly entrained by the light-dark cycle. Providing an opposite light-dark cycle stimulation (the timings of light on and off were opposite) directly to the eye or pineal gland by covering the eyes or the top of head (where the pineal gland located at) with black-painted patches for 7 days resulted in the rhythmic secretion of melatonin being retained in both structures (Barret and Underwood, 1991). In migratory songbird, pinealectomy decayed the circadian rhythm of clock genes expression in the hypothalamus and peripheral tissues. However, the gene expression profile of *Bmal1*, *Clock*, *Per*, *Cry* and *Rev-erbα* in retina still showed significant circadian patterns (Trivedi et al., 2016). A recent work of Bian et al. (2019)

also showed the relatively independent role of retina as a circadian oscillator in broiler chickens. The retina exhibited the circadian rhythm of *Bmal1*, *Cry1*, *Per2*, *Per3* expression and melatonin synthesis when reared under a 12L:12D photoperiod in the pinealectomized chicks. Taking together these results suggest that the chicken retina contains an autonomous circadian oscillator that can response to the inputs of periodic light.

Pineal gland

A pineal gland transplantation study demonstrated that pineal gland contains the oscillators in regulating daily behavioral pattern. Zimmerman and Menaker (1979) transplanted the pineal gland, which isolated from donors entrained under a 12L:12D photoperiod, into the anterior chamber of the eye of pinealectomy house sparrows. The circadian rhythm of locomotion was restored on the first day post transplantation under constant darkness condition and the activity onset of hosts were similar to that of donors. The re-established circadian rhythm with pineal transplantation indicated that the pineal gland contains a self-sustained oscillator and it is possible the behavior rhythmicity is driven by the hormone output (melatonin) oscillation from the pineal glands of donors. The inhibition effects of α_2-adrenergic receptors in the pineal gland on melatonin production have been found in chick pineal cell cultures (Pratt and Takahashi, 1988). In addition, the expression of *Alfa2A*, a gene encoding the α_2-adrenergic receptor, exhibited daily patterns with higher expression observed during the subjective day (Adamska et al., 2019). Clock genes in pineal gland in both chicken and house sparrow showed a significant 24-h rhythm. The expression of *clock*, *Bmal1* and *Bmal2* reached the peak during the day-night transition, and *Cry* and *Per* genes expression has been shown to be

high during the beginning of the day (Helfer et al., 2006; Turkowska et al., 2014; Jiang et al., 2016). The activity of AA-NAT in chicken pineal gland cells exhibited a circadian rhythm to the environmental light-dark cycles and the rhythm could be rapidly inverted when using a reversed illumination schedule (Deguchi, 1979). The rhythmic expression of AA-NAT mRNA in chicken pineal gland was also observed under both light-dark cycle and constant illumination conditions *in vivo* (Bernard et al., 1997; Zatz et al., 2000). The persisted pineal AA-NAT activity rhythm under constant illumination suggests that AA-NAT activity is driven by an endogenous circadian oscillator in the pineal gland. The circadian rhythm in melatonin synthesis is one of the oldest hormonal outputs of the circadian timing system. High production of pineal melatonin is found during the scotophase of the light-dark cycle and the level of melatonin is affected by the duration of the darkness. Melatonin synthesis can be regulated by the release of neurotransmitters (norepinephrine, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP)) from the sympathetic nerve fibers (Adamska et al., 2019). In avian species, norepinephrine turnover was higher during the subjective day and binds to α_2-adrenergic receptors in the pineal gland to inhibit the AA-NAT activity, which results in a low melatonin production during the photophase (Cassone et al., 1990; Voisin et al., 1990). Even though the pineal gland is not the only organ producing melatonin in birds, some studies reported that the circadian rhythm in plasma melatonin level was abolished in pinealectomized broilers, house sparrows and European starlings *in vitro* (Janik et al., 1992; Jin et al., 2011). It has been found that a repeated 4-h illumination during the subjective night shifted the rhythmic phase on melatonin secretion within two 24-h cycles in chicken pineal gland (Csernus et al., 2007). The daily

pattern of clock genes expression, melatonin biosynthesis and AA-NAT activity from both *in vivo* and *in vitro* studies demonstrated that the pineal gland contains photoreceptors and circadian oscillators, which are capable to regulate the rhythmic melatonin synthesis as the reliable circadian output. However, the role of the pineal gland as the pacemaker in controlling circadian rhythm entrainment varied among avian species. For example, pinealectomy ablated the circadian locomotion rhythm in constant conditions in the house sparrow (Gaston and Menaker, 1968), while the circadian rhythms of body temperature and locomotion persisted in pinealectomized Japanese quail in both light-dark cycle and constant darkness (Underwood, 1994).

Hypothalamus

In contrast to mammals, which have the central pacemaker only located in the SCN, the master clock in avian hypothalamus includes two anatomically distinct cell groups, median suprachiasmatic nucleus (mSCN) and visual suprachiasmatic nucleus (vSCN). Two nuclei of the mSCN are located bilaterally on either side of the third ventricle above the rostral portion of the optic chiasm (Takahashi and Menaker, 1982) and the vSCN or also called lateral hypothalamic nucleus (LHN) are located at the lateral hypothalamic area and caudal to the mSCN (Cassone and Moore, 1987; Norgren and Silver, 1989). Over the past 50 years, the structures in avian hypothalamus equivalent to the mammalian SCN is still controversial regarding the circadian oscillator function of mSCN and LHN. The discovery of circadian clock genes in the hypothalamus provided molecular evidence in locating the pacemakers in the SCN of hypothalamus. The lesions aimed at both mSCN and LHN coupled with the investigation of the expression of core clock genes and

metabolic biomarker were employed to identify the relative roles of mSCN and LHN in the circadian oscillation function in birds.

Yoshimura and his colleague proposed that mSCN to be the avian SCN homologue of the mammalian SCN. The evidence was: (1) the mSCN is anatomically similar to the mammalian SCN in terms of its location and cell population (Cantwell and Cassone, 2006) and exhibits immunoreactive responses to light stimulation in several species of birds (Norgren and Silver, 1989); (2) the clock genes (*Clock*, *Per2* and *Per3*) were expressed in the mSCN, but not in the LHN of the hypothalamus of chicken, Japanese quail, Java sparrow and pigeon in their studies (Yoshimura et al., 2001; Yasuo et al., 2002). However, studies on other species gave different results. The expression of *Per2* and *Cry2* were detected in the LHN of the house sparrow (Brandstätter et al., 2001) and chickens (Bailey et al., 2002); (3) The temporal pattern of *Per2* expression with a high level during the light phase and undetectable at the middle of subjective night was only observed in the mSCN in Japanese quail and chicken embryos (Yoshimura et al., 2001; Yasuo et al., 2002; Okabayashi et al., 2003).

In contrast, studies conducted by Cassone and his colleague have implicated the LHN is the cell group representing the equivalent to the mammalian SCN. This assumption was based on (1) the presence of the photic information transmission in the LHN via the retinohypothalamic tract (RHT). A stronger retinohypothalamic projection was observed in the LHN than in the mSCN in house sparrow (Cassone and Moore, 1987); (2) The circadian uptake of the circadian metabolic marker, 2-deoxy[^{14}C]glucose (2DG) showed a rhythmic pattern in the LHN, but not in the mSCN in house sparrow (Cassone, 1988; Lu and Cassone, 1993); (3) A higher degree of presence of the 2-[^{125}I]iodomelatonin

(IMEI) binding sites was found in the LHN than in the mSCN of young chicks. When transferred to a constant dark condition, a higher binding density was observed during the daytime with a peak in the late afternoon in both LHN and mSCN. The overall amplitude of the binding rhythm in the LHN was higher than it in the mSCN. However, the circadian rhythm of IMEI binding was damped in the mSCN when chicks were kept under a light-dark cycle (Brooks and Cassone, 1992); (4) The involvement of the LHN in circadian rhythm entrainment was also supported by measuring the daily pattern of pineal norepinephrine efflux in a lesion study performed on chickens. No rhythmicity in norepinephrine turnover were found in chickens with ablated LHN. While birds with precise mSCN lesion or sham surgeries in LHN did not affect the rhythmicity of norepinephrine efflux (Cassone et al., 1990). Moreover, photic induction of the *c-fos*, a member of the *Fos* family of immediate early genes involves in photic entrainment, expression was only detected in the LHN, but not in the mSCN of chickens, Japanese quail and European starlings (Wallman et al., 1994; King and Follett, 1997).

The available information regarding the circadian oscillator in the SCN of hypothalamus is inconclusive to make a statement of either the mSCN or LHN as the avian SCN homology to the mammalian SCN. But the results from lesioning studies clearly demonstrated the presence of circadian pacemaker in the SCN of hypothalamus. Java sparrows with either pinealectomy or ocular enucleation were still able to entrain the locomotor rhythms under light-dark cycles (Ebihara and Kawamura, 1981). Similarly, Oishi and Ohashi (1993) observed the photoperiodic gonadal response in blinded-pinealectomized Japanese quails and suggested the existence of a pacemaker other than the eye and pineal gland. House sparrows with large lesions in the general area of the

suprachiasmatic nucleus of hypothalamus (SCN) demonstrated a loss of circadian rhythm of locomotion under a constant light condition (Ebihara and Kawamura, 1981). However, sparrows with incomplete lesion still retained the locomotion rhythm but with a reduced precision of the running onset (Takahashi and Menaker, 1982). This observation suggests that the effectiveness of circadian rhythm entrainment depends on the lesion degree of the hypothalamus.

2.2 Ontogeny of circadian system and circadian rhythm entrainment in embryos

The entrainment of circadian rhythm in chicken has been found during embryogenesis (Zeman et al., 1993). To establish an early circadian rhythm, avian embryos need to possess mature photoreceptors to perceive light and functional pacemakers to transform the photic information into neuroendocrine signals through phototransduction pathways (Kumar, 2015). Chicken embryonic development can be divided into two stages: the differentiation period and the growth period. Around 90% of the organ development occurs in the differentiation stage and a fully formed chick can be recognizable by day 12 of incubation (Deeming, 2002). There are several critical time points involving the circadian rhythm entrainment during embryogenesis.

The development of the retina can be classified into four stages: (1) The appearance of the ganglion cells, the output neurons of the retina, was observed by day 4 of incubation and the number of neurons in the ganglion cell layer reaches the peak around day 10-12 of incubation (Straznicky and Chehade, 1987; Rogers, 1995); (2) The outer nuclear layer, which is composed of the nuclei and cell bodies of the photoreceptor cells was observed being clearly separated from the inner nuclear layer by day 10 of incubation (Wu et al., 2003). (3) The first indication of the appearance of immature photoreceptor segments was

around day 10 of incubation and the rod, single cone and double cone cells were identified by the size and shape of their inner segments. Subsequently, the size of the photoreceptor inner segment further increased until day 14 of incubation (Wai and Yew, 2002; Wai et al., 2006). (4) The photoreceptors became mature with similar cell size in the adult birds and functional by the 18th day of incubation (Rogers, 1995; Wai and Yew, 2002).

The biosynthesis of melatonin has been detected in the retina during embryogenesis. The melatonin synthesizing enzyme, AA-NAT, can be detected as early as day 7 of incubation (Espinar et al., 1994). However, no difference in AA-NAT activity was observed between constant dark and lighted environments suggesting that the retinae melatonin enzyme activity is not controlled by illumination during the early stage of embryo development. The light exposure inhibited AA-NAT activity and melatonin secretion in chicken retina on the last two days of incubation when illuminated with constant light (Espinar et al., 1994). This observation provides the first evidence of the melatonin synthesis in chicken embryos' retinae can be regulated by light illumination in the final stage of embryo development.

Besides the development of the retina, the formation of chicken pineal gland and hypothalamus is also important for the ontogeny of the circadian system. Chicken pineal gland formed from day 3 to 6 of incubation and became functionally mature until later in development (Romanoff, 1960). The rhythmic expression of pinopsin in pineal gland and melanopsin in the lateral septal area of chicken have been observed under a light-dark condition (Holthues et al., 2004). The pineal gland of a bird is the main secretory organ for melatonin and can perceive light directly. Within the pineal gland, serotonin is

initially N-acetylated to N-acetylserotonin, then converted to melatonin by hydroxyindole-O-methyltransferase (Moore, 1996). The release of melatonin can provide the internal temporal cue to peripheral tissues including the liver, heart, skeletal muscle and adipose tissue, which express melatonin receptors in a circadian manner (Jha et al., 2015). The development of the circadian rhythmicity of melatonin is a prenatal event in chickens. There was a significant increase in the number and size of pineal intracytoplasmic lipid droplets in the light exposed embryos after 18 days of exposure (Aige-Gil and Murillo-Ferrol, 1992). Research has shown that circadian rhythms can be entrained in chicken embryos by exposing embryos to periodic illumination. Providing periodic illumination during incubation increasing melatonin secretion during the dark period was documented in several studies (Zeman et al., 1992; Espinar et al., 1994; Lamosova et al., 1995). Pineal melatonin secretion was detected as early as day 10 of incubation (Möller and Möller, 1990). An irregular pattern of melatonin level during the mid-embryonic age (day 13 of incubation) has been observed in chicken pineal gland. In later developmental stage (day 17-20 of incubation), the daily rhythm of melatonin secretion has been clearly observed under the same condition (Csernus et al., 2007). Akasaka et al. (1995) also observed a significant light-dark difference in melatonin levels when the pineal cell was isolated from the 14-day embryos and cultured under a light-dark regimen. The rhythmicity of melatonin cannot persist under constant darkness. However, when the same procedure was applied on the pineal cell from the 18th day embryos and the circadian melatonin rhythm was able to persist for 2 days after transfer to a constant darkness condition. These data clearly demonstrated that not only the presence of the circadian oscillator during embryogenesis, but the pineal gland can utilize

the light-dark cycle information to entrain the rhythmicity of melatonin to the environmental changes in the final stage of embryonic life. The effects of light spectra on melatonin biosynthesis are inconsistent. Chicken embryos illuminated with a periodic red and white light had a higher level of pineal melatonin synthesis during the scotophase phase than those under green and blue light on the 20th day of incubation (Drozdova et al., 2019). On the contrary, broilers under blue and green light had a higher melatonin secretion than those under red and white light (Abdel-Azeem and Borham, 2018). Similarly, a lower serum melatonin level was found in geese when illuminated with white light than those kept under blue and red light (Chang et al., 2016). The effects of light spectra on the establishment of rhythmic melatonin secretion deserve to be further investigated.

Research is still ongoing to find out the mechanism of how periodic illumination affects the ontogeny of the avian circadian system. The avian circadian clock genes have been successfully cloned and are used as a marker for analyzing the ontogeny of the circadian clock directly. The presence of key clock genes, *Cry1*, *Cry2*, *Clock* and *Bmal1*, was detected in the pineal gland as early as day 15 of incubation (Csernus et al., 2007). The daily rhythmicity of *Cry1* and *Per2* expression was first detected on day 16 and 18 of incubation in the SCN of the hypothalamus and the pineal gland of chicken embryos when illuminated with light-dark cycles (Okabayashi et al., 2003; Csernus et al., 2007). Wang et al. (2014) explored the effect of light illumination during incubation on the expression of melatonin receptors and found that continuous illumination with short wavelengths (green and blue) upregulated the expression of *Mel 1c* in chicken embryo liver and increased plasma melatonin on day 21 of incubation. Broiler hatching eggs

illuminated with a 12L:12D light regime also exhibited the establishment of the circadian rhythm in plasma melatonin on day 19 of incubation (Archer and Mench, 2014b). However, either melatonin level or behavior rhythms at 5-week of age were affected by incubation lighting programs. The subsequent environmental lighting conditions during the rearing period may have override effects on the entrained circadian rhythm of melatonin during incubation. But the incubation light exposure may prime the light-dark cycles to the embryos and enhance the sensitivity of young chicks to diurnal changes. Early development of the circadian controlling system suggests the importance of circadian organization for predicting the environmental changes for neonatal chicks.

2.3 Impact of *in ovo* photostimulation on embryonic development and hatching traits

Hatchability is an important indicator for determining the success of incubation management. Hatchability can be calculated as the percentage of hatched chicks to the total eggs or fertile eggs set. The goal of hatcheries is not only to maximize hatchability but also produce chicks with good quality. A solution must be found to improve the health and quality of newly hatched chicks. The effects of *in ovo* photostimulation on embryonic development and hatching traits have been evaluated by several studies. As described in section 2.1.1, chicken embryos can differentiate the light colors and utilize the light-dark cycle information, the following sections review how *in ovo* photostimulation affecting the embryonic development and hatching traits with respect to the light color and photoperiod.

2.3.1 Embryonic development and hatchability

Light color (wavelength)

Various studies involving colored light illumination during incubation demonstrated that white and green light accelerated embryo development and muscle growth (Rozenboim et al., 2004b; Zhang et al., 2012, 2016; Wang et al., 2017). Khalil (2009) found that continuous white light illumination improved embryonic growth and hatchability in Japanese quails compared to those incubated under darkness. The same study also found that light stimulation increased the metabolic rate of developing embryos with a higher level of plasma biochemical parameters including total protein, albumen, globulin, glucose, total lipids and triglycerides. Continuous illumination with green fluorescent light for the first 18 days of incubation resulted in a higher embryo weight at day 11, 13 and 15 of incubation than those kept under dark (Shafey and Al-mohsen, 2002). Wang et al. (2014) compared continuous illumination with different colors (blue, green, red and white) throughout the incubation and observed that both blue and green lights accelerated the pectoral muscle growth (weight and myofiber area) of broiler embryos in the final stage of embryo development when compared with red light and dark. The mechanisms regarding the accelerated muscle growth by light with short wavelength are still not clear. Studies conducted by Dishon et al. (2017, 2018, 2021) suggested that green light exposure during the last 3 days of incubation would effectively activate the somatotropic axis and stimulate the growth of broiler embryos. The incubation of broiler eggs under a 15 min L : 15 min D photoperiod using green LED light upregulated the expression of hypothalamic growth hormone releasing hormone, liver growth hormone receptor, insulin-like growth factor-1 and increased plasma growth hormone and prolactin levels from day 14 to 16 of incubation onwards. However, green light stimulation did not affect embryo body weight, liver or breast muscle weight (Dishon et al., 2017; 2018). The effect

of green light on upregulating somatotropic axis genes' expression have also been found in some studies when using continuous illumination (Bai et al., 2016; Wang et al., 2017). The molecular study revealed that green light illumination upregulated mRNA expression of MyoD, myogenin and myostatin in chicken embryos and newly hatched chicks (Zhang et al., 2014). Wang et al. (2017) found that continuous illumination with green and blue light during incubation promoted the myofiber growth of skeletal muscle and plasma insulin-like growth factor 1 level of broiler embryos. It is important to note however that these studies provided continuous illumination, so the stimulated effects on embryo growth can be achieved even without the presence of the rhythms of light and darkness. Red light has been documented to have a positive effect on hatchability when compared with monochromatic blue or green light. The mechanism for the improvement in hatchability by photostimulation with long wavelength is still unknown and required future research. It is possible that red light is more effective in regulating the mitochondrial activity and increase the rate of cellular division. Broiler embryos incubated under red light for 12 h per day had a lower mortality at the early stage of embryogenesis compared to those incubated under dark condition (Archer et al., 2017; 2018). However, Sabuncuoğlu et al. (2018) observed that Japanese quail eggs illuminated with continuous green or blue light had no negative impact on total embryo mortality or hatch weight when compared to a dark environment, but has potential benefits in terms of reducing the early and late embryonic mortality by green and blue LED light, respectively. The conflicting results noted in previous literature on the impact of wavelength on hatchability may be related to confounding factors, such as variance in light sources, genetics, and number of animals used in the experiments.

Lighting schedule (photoperiod)

The photoperiod of illumination has also been the subject of interest. Providing a 12 h of illumination per day (12L:12D) is the most common photoperiod used in the previous studies. The use of green light under a 12L:12D photoperiod increased the heart weight, beak length and third toe length at different stages of embryogenesis (Tong et al., 2018). Illuminated with red LED light (12L:12D) reduced the early embryonic mortality and increased the hatchability of fertile eggs (Archer et al., 2017). In addition, Yameen et al. (2020) and Riaz et al., (2021) compared white light with different photoperiods and observed a higher hatchability in the 12L:12D group than the constant illumination conditions. However, the positive effects of *in ovo* photostimulation with white light (12L:12D) on embryonic development, embryo mortality or hatchability of fertile were not detected in other studies (Archer et al., 2009; Huth and Archer, 2015). Similarly, no differences in embryo mortality or hatchability were observed in illuminated layer embryos with green light under a 12L:12D photoperiod (Wang et al., 2020). The effect of a longer photoperiod than the 12L:12D with cool white fluorescent light on embryo growth and hatching traits were evaluated by Özkan et al. (2012a) and Cooper et al. (2011). Exposuring house sparrow eggs to a 18L:6D photoperiod throughout incubation increased metabolic rate during the photophase and shortened the incubation time (Cooper et al., 2011). Broiler embryos illuminated with a 16L:8D photoperiod in different stages of incubation either the entire (0-21 day) or the last stage (14-21 day) of incubation period supplied promising information regarding the growth-promoting effects of long-day illumination on breast muscle and embryo weight (Özkan et al. 2012a). It is not known if the response on embryo development was solely affected by the

illumination or a confounding effect of heat production from the conventional light source. The only information regarding the effect of LED light with different photoperiods on the hatching performance was from Geng et al. (2021), who reported that white light illumination with a 12L:12D and 16L:8D resulted in a higher proportion of chicks without leg problem at hatch without affecting hatchability or hatch weight of dual-purpose native chickens than those under 8L:16D or 24D:0L photoperiods. To our knowledge, no study investigated the effect of monochromatic light with different photoperiod on embryonic development and hatching traits and further investigation is needed.

2.3.2 Time of hatch and the spread of hatch

The normal incubation time for chicken embryos is around 21 days. An earlier study found that embryos incubated under fluorescent light required less time to hatch than those incubated in the dark (Shafey and Al-mohsen, 2002). Furthermore, it was found that longer light exposure resulted in less incubation time for broilers (Walter and Voitle, 1972). The shortened incubation time from light exposure may be simply due to an overheating effect by conventional light source or related to an accelerated embryonic growth rate or higher embryo activity. Egg weight loss was used as an indirect indicator of embryo temperature. Zhang et al. (2016) noted that broiler hatching eggs continuously illuminated with green and white LED light did not increase the egg weight loss during incubation as compared to the dark condition.

The spread of hatch, the duration from the first chick hatched to the last chick, is another parameter to evaluate hatchery management. Broiler chicks usually hatch after 461 h to 510 h of incubation. A large hatch window (HW) has been reported to be associated with

poor day-old chick quality, which negatively affects the welfare and growth performance after placement on rearing farms. Chicks hatched 30 h before removal from incubator can lose up to 10% of their body weight, while chicks that hatch late (2 h before HW closed) lose only 1 to 3% of their body weight in the incubator (Careghi et al., 2005). As the HW narrows, the uniformity of the flock of chicks increases.

Light color (wavelength)

Effects of the monochromatic lighting programs at different wavelengths applied during incubation on time of hatch and the spread of hatch have been investigated by several studies but the results are inconsistent. White and yellow light illumination during incubation reduced the incubation time have been found in both broilers (Hluchý et al., 2012) and layers (Shutze et al., 1962). However, some studies demonstrated that the incubation time or the spread of hatch were not affected by light exposure. Rozenboim et al. (2004a) reported no difference in incubation time for eggs incubated in the dark compared to eggs illuminated with monochromatic green light (15 min L:15 min D) for broilers. Similarly, Zhang et al. (2016) found that continuous green light illumination did not affect hatch time or HW for broiler embryos. Sabuncuoğlu et al. (2018) found that the total duration of incubation did not differ among continuous green, blue and dark conditions. But the blue light illumination had a slower start of hatching process with 17.56% of chicks hatched at 410 h of incubation compared to a dark condition (81.78%). The actual timing depends on both pre-incubation factors and incubation factors and might be regulated by the synthesis of thyroid hormones and CORT (Almeida et al., 2008; Romanini et al., 2013). To date, limited information on the effects of *in ovo* photostimulation with different colors on altering thyroid hormones and CORT

production is available. Further study is required to understand how periodic light affects the hormone profiles and the hatching process.

Lighting schedule (photoperiod)

Periodic illumination during incubation may shorten the time required for incubation (Siegel et al., 1969; Fairchild and Christensen, 2000). It is found that broilers required less time to hatch when illuminated with conventional light sources for a longer duration of photophase (Walter and Voitle, 1972). Studies were conducted using LED lights to minimize the heating effect from incandescent light and fluorescent light. Wang et al. (2020) reported that layer embryos stimulated with green LED light under a 12L:12D photoperiod shorten the incubation time without affecting the duration of the hatch window. Providing green LED light with a 12L:12D regime did not affect the initial time of hatching process and the duration of HW, but the lighted groups resulted in a shorten average incubation time (3.4 h earlier) than those under dark (Tong et al., 2018). The acceleration in development and reduced incubation time might be related to the increased metabolism or stimulated the cell proliferation that gives formation to body structure (Halevy et al., 2006; Cooper et al., 2011). However, no differences in incubation time for eggs incubated in the dark compared to those illuminated with light have been reported in some studies (Rozenboim et al., 2004b; Özkan et al., 2012a; Zhang et al., 2016). In general, most studies counted the number of chicks at different time points during the last three days of incubation and calculated the average incubation time. However, this method cannot provide the detailed information on the distribution of hatch. Hannah et al. (2020) analyzed the spread of hatch of three strains of layers by using a nonlinear regression model and found that Lohmann Brown embryos took less

time to reach 50% of total chicks hatched and reduced the time between 50% to 75% of total chicks hatched when 12 h d^{-1} of full spectrum light was provided for the entire incubation period. However, no difference in the spread of hatch was found in the other two strains. Differences in the spread of hatch among strains might be related to genetic selection for growth and production. No information regarding the effect of periodic illumination on the spread of hatch for broilers was found in the literature.

2.3.3 Chick quality

Good quality chicks can be defined as clean, dry, without dirt, contamination and deformities, with bright and clear eyes, clean and a completely sealed navel area (Deeming, 2000). Chick quality can be determined through a variety of qualitative and quantitative parameters including chick length, chick yolk-free body weight, navel condition, beak and joint conditions and deformities. Chicks with good quality have a better start in life with lower mortality during the early stages of the production period.

Light color (wavelength)

The use of light with different wavelength has shown positive effects on chick quality, specifically, the improvement in the percentage of chicks with better navel closure condition and chick weight. A series of studies by Archer and his colleagues found that exposing broiler eggs to white or red light for the first 18 days of incubation improved navel closure condition and reduced the proportion of chicks with defects (Archer, 2015; Huth and Archer, 2015; Archer, 2018). However, the results on navel closure condition are inconsistent. Some previous studies reported that no effects on chick weight or chick quality at hatch were found when providing light illumination during incubation either (Zhang et al., 2012; Tong et al., 2018). The improved navel maturation could be related

to light stimulation accelerating embryo development or regulating the hypothalamus-pituitary-adrenal axis activity. Most of the literature reported that *in ovo* photostimulaiton does not affect chick weight or length at hatch with the exception of a few contrasting results showing an increased body weight at the terminal of incubation process when illuminated with white or green light. For example, Bai et al. (2016) compared continuous illumination with different colors (blue, green, red and white) throughout the incubation and observed a higher body weight at hatch under green lights than those illuminated with other colors. A recent study reported that red light illumination improved hatchability and hatch weight of turkeys when compared to blue light or dark (Abd El Naby et al., 2021). The mechanism behind the impact of *in ovo* photostimulation on the hatch weight is not clear. The increased body weight might be attributed to the improved growth of myofiber and myoblast cells by green light stimulation.

Lighting schedule (photoperiod)

Different durations of light illumination demonstrated distinct effects on navel closure conditions. Using a light regime with 12L:12D increased the percentage of chicks with perfect navel condition in comparison to the hatchlings incubated under darkness (Archer et al., 2017). It has also been shown that white LED light stimulation with a photoperiod of 12L:12D improved the physical asymmetry and heterophil to lymphocyte ratio on newly hatched broilers (Riaz et al., 2021). Providing white light with a 12L:12D photoperiod resulted in a heavier chick at the end of incubation than those incubated in darkness (Yameen et al., 2020). Rozenboim et al. (2004b) showed that broilers exposed to green light stimulation with a intermittent lighting schedule (15 min L:15 min D) had a higher pectoralis muscle weight from day 9 of incubation and the growth-promoting

effect was carried through the first week post-hatch. The positive effect of intermittent green light on body weight and hatching rate was also reported by Halevy et al. (2006). The mechanisms regarding the acceleration of embryonic development and increased body weight at hatched are still not clear. In contrast, a study by Özkan et al. (2012a) found that lighted incubation with a 16L:8D photoperiod increased breast muscle growth with no noticeable changes in chick quality parameters including body weight, relative heart weight or relative liver weight at hatch in comparison to the 0L:24D photoperiod. Similarly, illumination with a 16L:8D photoperiod (green LEDs) did not affect residual yolk weight, yolk-free body weight or organ weight of female broilers (Güz et al., 2021). Results from the literature showed that neither 12L:12D nor intermittent lighting schedule (15 min L:15 min D) affected organ development in poultry species (Fairchild and Christensen, 2000; Dishon et al., 2017). Limited research is available regarding the effects of periodic illumination on bone development. An improvement in leg health of broilers was found when embryos were illuminated with a 12L:12D or 16L:8D photoperiod (van der Pol et al., 2017). The longer femur was also observed in day-old chicks when incubated with a 12L:12D photoperiod than those incubated under darkness (Geng et al., 2021). However, no difference on tibia morphological parameters and mineral density were found between 16L:8D green light illumination and under dark (Güz et al., 2021). These findings deserve to be investigated further to establish a clear conclusion regarding the effects of photostimulation on bone development of chickens.

2.4 Impact of *in ovo* photostimulation on productivity and health

2.4.1 Post-hatch productivity

Light color (wavelength)

The genetic potential of commercial strain chickens can be fully expressed with optimal management through both incubation and rearing periods. Therefore, providing an excellent incubation condition can achieve high economic efficiency in poultry production. After transferring to the grow-out facility, the changes in nutritional resources and environmental factors (temperature, humidity, light exposure and handling) may be challenging for young chicks. It has been reported that broiler chicks hatched under continuous green LED light illumination had higher body weight gain and improved growth of breast muscle by d 6 of age compared to those incubated under dark condition, whereas the response of productivity under white light group was intermittent (Zhang et al., 2016). Also, *in ovo* intermittent green light (15 min L:15 min D) during incubation increased the number of skeletal muscle cells and enhanced satellite cell proliferation in broilers on day 1 and 3 post-hatch (Halevy et al., 2006). Their results suggested that green light modulates the production of hormones involved in growth and stimulates myogenic activity by modulating energy metabolism. Furthermore, the application of light during incubation has been reported to have positive impacts on brain lateralization. A series of studies conducted by Chiandetti and her colleagues have shown that chicken embryos can respond to light stimulation even before the visual system being fully functional and an improved ability to filter out distractors in young chicks was found in their studies (Chiandetti et al., 2005, 2013; Chiandetti and Vallortigara, 2019). Sabuncuoğlu et al. (2018) noted that chicks hatched under green light had a higher number of peeps and a longer defecation time when placed into the rearing environment, whereas chicks hatched under blue light were more active with a higher number of

jumping. Archer et al. (2009) noted that providing white light illumination increased the feeding behavior during the first 2-h period after the lights turned on. These results suggest that *in ovo* photostimulation has an impact on the epigenetic adaption response to a new environment. The mechanism underlying this effect could be related to the reduction in fear, accelerated brain lateralization, entrainment of the circadian rhythm of melatonin or a combination of those.

The effects of light wavelength during incubation on overall productivity are contradictory in the literature. Some studies showed that broilers hatched under continuous green light illumination during incubation had a higher body weight and breast muscle yield of male broilers through the rearing period than those incubated with blue light or under darkness (Zhang et al., 2012). The use of green light during incubation also resulted in accelerated muscle growth with the intermittent green light and the growth-promoting effect was observed throughout the whole growth period (Rozenboim et al., 2004b). Recent evidence also showed that blue light had a similar growth-promoting effect as green light (Bai et al., 2016). A higher body weight and pectoral muscle weight were found in the later stage of development when incubated under green and blue light illumination. No differences in muscle growth or plasma insulin-like growth factor 1 (IGF-1) were found between dark and red light illumination at any point of measurement. The proliferation activity of satellite cells by the same study revealed that both green- and blue-lighted incubation increased the total number of satellite cells in chicks up to d 10 of age. Continuous green and blue light illumination during incubation also increased the plasma IGF-1 from d 1 to 21 of age. However, Drozdova et al. (2021) reported that periodic red light illumination during incubation increased the body weight

by day 21 of age. The improved growth might be affected by a more active behavior when illuminated with blue light during incubation. The blue light group showed more foraging, fighting and wing-flapping behavior than those hatched under red light. In addition, the effect of light wavelength on muscle growth might be gender- and species-specific. Rozenboim et al. (2003) found that female turkey embryos exposed to monochromatic green light enhanced body weight and the growth of breast muscle at a later age, while no difference in body weight was found among green, white and dark condition in males. Japanese quail eggs exposed to continuous blue or green light during incubation did not affect the weekly body weight by 42 days of age (Sabuncuoğlu et al., 2018). Considering the conflicting information regarding the effects of *in ovo* photostimulation on bird growth, it is still unknown what spectra of lighting is best for improving certain production traits.

Lighting schedule (photoperiod)

It has been found that physiological doses of melatonin can regulate some daily and seasonal physiological processes, like growth, development, thermoregulation, immune function and behavior (Rozenboim et al., 1998; Zeman et al., 1999; Blatchford et al., 2009). It has been noted that periodic illumination during incubation resulted in birds that adapted more easily to novel environments than those incubated under complete darkness. Improved feed consumption and body weight during the first week have been observed when providing a photoperiod during incubation (Li et al., 2021b). Providing a 16L:8D white light illumination during incubation increased the body weight gain of broilers during the first week without affecting residual yolk weight, heart weight or liver weight. By d 6 of age, broilers in the 16L:8D group had a higher plasma melatonin level

and lower Malondialdehyde (MDA) level in the brain than those incubated under darkness (Özkan et al., 2012b). These findings indicate that photoperiodic light stimulation during incubation may mitigate the oxidative stress for neonatal chicks.

The use of light with different photoperiods during incubation does not appear to affect the overall growth during the growth period. Archer et al. (2019) found that no treatment effects on body weight when illuminated with different photoperiods during incubation. Similarly, no differences in feed consumption, body weight gain or mortality of broilers were detected among *in ovo* photostimualtion with different photoperiods (0L:24D, 1L:23D, 6L:18D, 12L:12D or 24L:0D) (Archer et al., 2009; Archer and Mench, 2014). However, photoperiodic white light illumination improved FCR compared to those under constant lighting conditions (Riaz et al., 2021). They speculated that the periodic lighting program increased melatonin production and further accelerated the development of hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-thyroid (HPT) axes. The melatonin and CORT may have a confounding effect on the synthesis of downstream hormones including GH, GHRH, IGF-1 and the expression of their receptors in the liver.

2.4.2 Health and Welfare

Light color (wavelength)

The impact of light wavelength on chicken welfare is still limited. Composition asymmetry is commonly used as the primary indicator of the effects of developmental stressors. Market-age broilers hatched under periodic white and red lights showed a lower composite asymmetry score and the number of vocalizations, an indicator of more fearfulness, during an isolation fear test than those hatched under darkness (Archer, 2017). The author also observed broilers incubated under periodic red and white light had

a lower plasma CORT at the end of production. In addition, continuous green light illumination during incubation reduced the fearful behavior (jumping and defecation) after being placed to the grow-out environment on Japanese quail without a noticeable change in tonic immobility between light colors (Sabuncuoğlu et al., 201). The reduction of response to stress can facilitate the growth and development of chickens. A recent study found that *in ovo* photostimulation also affected post-hatch behavior. Broilers hatched under red light had a higher percentage of resting behavior, whereas blue light treatment resulted in a higher foraging behavior (Drozdova et al., 2021).

Lighting schedule (photoperiod)

The application of illumination with different wavelengths during incubation has been shown to impact several aspects of animal welfare. The *in ovo* photostimulaiton does not seem to affect the mortality during the production period. No difference in mortality was found between 12L:12D and constant light conditions (Archer et al., 2009). The duration of illumination has been shown to affect eye health in chickens during the rearing period. Prolonged photoperiod could induce ocular abnormalities in broiler (Lewis and Gous, 2009) and turkey (Leis et al., 2017). The heavier eye weight associated with the characteristic lesion of light-induced avian glaucoma has been found when chicks were exposed to a duration of photophase longer than 16 h d^{-1} (Oishi and Murakami, 1985). Archer et al. (2009) also found heavier eye weight in constant lighting conditions (0L:24L & 24L:0D) than those incubated under 12L:12D. But no lesions in the epithelial cells of the retina were observed in either lighting treatment. The result suggests that periodic illumination during incubation may have beneficial effects on the development of the eye in broiler chickens. On the other hand, Zhang et al. (2016) reported that

providing continuous light illumination during incubation has no negative effect on the development of eyes and other primary organs by d 6 of age.

Evidence of an improvement in stress resilience by periodic light illumination has been reported by Archer and Mench (2013). These authors observed broilers incubated under a 12L:12D photoperiod had a lower CORT response when introducing to a stress condition at market compared to those incubated under darkness. Heterophil to lymphocyte ratio (H/L) is also used as an index to evaluate levels of stress in poultry. This ratio can increase during stressful situations, including environmental changes such as social stress (Gross and Siegel, 1983) or even lighting programs (Huth and Archer, 2015). A higher H/L ratio in blood was found when birds were raised under constant light compared to the 12L:12D light treatment (Zulkifli et al., 1998). Riaz et al. (2021) compared the effect of white light illumination with different photoperiods on stress susceptibility in broiler chicks. Chicks hatched under 12L:12D had less stress with a lower H/L ratio and physical asymmetry than those hatched under darkness. The positive impacts on stress response might be due to the periodic light illumination entraining the circadian rhythm of melatonin and reducing the secretion of CORT.

The effect of established circadian rhythm in melatonin secretion by incubation illumination has been found to carry through the production period and affect the development and behavior of chickens. Chicken embryos exposed to 12 h d^{-1} from day 13 to 18 of incubation resulted in a body temperature change from morning to evening in chicks and the daily rhythm of body temperature persisted up to 5 days post-hatch under a constant illumination condition (Hill et al., 2004). The application of photostimulation during incubation may accelerate the development of HPA and HPT axes and regulate

the production of hormones involved in the thermoregulation of chicken embryos during the final stage of incubation. Thyroid hormones and CORT are also important for the hatching process and the established circadian rhythm may synchronize the time of hatch to improve chick quality. The effect of the circadian rhythm entrainment by lighting programs on regulating body temperature of developing embryos requires further investigation.

Since the administration of melatonin enhanced the cellular and humoral immune responses during the post-hatch period (Moore and Siopes, 2000), the effect of periodic illumination on broiler immunity has also been studied. Yammen et al. (2020) reported that both continuous and periodic white light illumination increased the relative bursa of Fabricius weight on day 35 of age. In addition, a higher level of NDV titer was found in birds incubated with a 12L:12D photoperiod than those under dark condition. A study comparing different photoperiods revealed that periodic white light had a stronger humoral immune response to a non-pathogenic protein antigen, keyhole limpet hemocyanin (KLH). The anti-KLH IgG level was significantly higher in the 12L:12D groups than the dark and 1L:23D groups on 4 days after the second injection (Archer and Mench, 2013). Thus, *in ovo* photostimulation, especially the photoperiod may have a considerable influence on poultry immunity.

2.5 Conclusion

In recent years, there has been a dramatic growth in the number of studies exploring the optimal incubation lighting program to improve chicken health and productivity. Previous studies have demonstrated that green light enhances muscle growth and proposed the possible mechanisms involved in accelerating muscle cell proliferation and

regulating growth factors. However, the effect of wavelengths on embryo development and hatching traits is highly controversial and the exact mechanisms of these responses are not completely understood. Limited information on periodic blue light is available in the literature and should be of greater interest to researchers in the future. Many previous studies often compared two photoperiods only, assessing the monochromatic light illumination with graded photoperiods can help to understand the inconsistent results between studies. To conclude, when considering the use of illumination during incubation, it is essential to further understand the impact of light wavelength, photoperiod and their combinations on embryonic development, hatching traits and productivity, and examine the possible mechanisms for these changes to occur.

2.6 Hypotheses

LEDs illumination during incubation can serve as a potent circadian stimulator and positively affect the hatching traits, production performance and health of broiler chickens.

Specific hypothesis

1. Embryonic development, time of hatch and chick quality will be impacted by incubating broiler hatching eggs in different light wavelength treatments (white, red, or blue). Light with short wavelength will enhance muscle growth and immune response to challenge.
2. The circadian rhythm of embryo temperature will be entrained by periodic blue LEDs illumination and will change with different photoperiods. Early productivity will be improved with a matched photoperiod treatment between incubation and rearing periods.

CHAPTER 3. PROVIDING COLORED PHOTOPERIODIC LIGHT STIMULATION DURING INCUBATION: 1. EFFECTS ON EMBRYO DEVELOPMENT AND HATCHING PERFORMANCE IN BROILER HATCHING EGGS*

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3.1 Abstract

Providing lighting schedule during incubation has been shown to improve chick quality and reduce stress posthatch. This study was conducted to evaluate the effects of providing light of different colors during incubation on embryo development, air cell temperature, the spread of hatch, and hatching performance. Four batches of eggs (n = 2,176, 1,664, 1,696 and 1,600) from Ross 308 broiler breeders were used in the experiment. In each trial, eggs were randomly distributed into four lighting treatments. The incubation lighting treatments included: incubated under dark as control, illuminated with white, red or blue lights for 12 h daily. There were no incubation lighting treatment differences in embryo development, the spread of hatch, hatchability, embryo mortality, hatch weight, chick length, navel closure quality, yolk-free body weight or relative spleen weight. However, embryos incubated under red light had lower average air cell temperature than those in dark, white or blue light treatments. This finding may suggest higher melatonin secretion during the scotophase when illuminated with red light. Male chicks incubated under dark had a higher bursa of Fabricius weight than males illuminated with blue light. In conclusion, these results suggest that the red, white and blue light stimulation during incubation had no negative effects on hatchability, embryo mortality, spread of hatch or day-old chick quality, but may have potential impacts on immunity and energy metabolism in broiler embryos.

Key words: photostimulation, light color, air cell temperature, hatchability, chick quality

3.2 Introduction

Avian embryogenesis is a perfect platform to examine the effects of exogenous factors on embryonic development due to the physiological independence from the hen (Hill et al.,

2004). During incubation, the management of cabinet temperature, humidity, egg turning and air composition are critical to achieving successful artificial incubation. Light is an important exogenous factor for controlling many physiological and behavioral processes in animals but the use of light in artificial incubation is not commonly practiced. In nature, hens are off the nest for drinking and eating, especially during the last week of incubation (Archer and Mench, 2014). The developing chicken embryos would receive light stimulation when hens were off-nest. Normally, commercial incubation units are not illuminated except for when humans enter to deliver eggs or to clean the unit after use. We know, from *in vitro* studies, that the pineal gland of avian embryos responds to light exposure. Aige-Gil and Murillo-Ferrol (1992) reported a significant increase in the number and size of pineal intracytoplasmic lipid droplets in embryos exposed to light for 18 days of incubation. Light sensing opsins can be detected in an embryo on d 14 of incubation (Bruhn and Cepko, 1996).

Light intensity, the composition of the spectrum and photoperiod (daily pattern of light and dark exposure) are three main parameters of light when used as a tool to manage poultry production. There has been interest in determining the impact of providing light to the incubation environment on hatching performance and the quality of newly hatched chicks. Implementing a light regime during incubation has been studied for over 50 years (Peters et al., 1958). Incandescent and fluorescent lamps were used in early studies and had a positive effect on accelerated embryo development and a decrease in incubation time to hatch (Erwin et al., 1971; Andrews and Zimmerman, 1990; Shafey and Al-mohsen, 2002; Archer et al., 2009; Cooper et al., 2011). But the additional heat emitted from the light source may be confounded with the effects of light (Rozenboim et al.,

2003). Recently, the light-emitting diode (LED) lamp has become commercially available and produces far less heat than conventional lamps. Since then, numerous studies have been conducted to examine the effects of application of LED lights during incubation on embryo development and hatching performance parameters (Rozenboim et al., 2004a; Özkan et al., 2012; Archer and Mench, 2013; Archer, 2017; Archer et al., 2017; Sabuncuoğlu et al., 2018; Hannah et al., 2020).

It has been found that light stimulation during incubation could potentially affect embryonic cell proliferation measured by changes in total embryo and breast muscle weight (Rozenboim et al., 2004a; Halevy et al., 2006). Archer and Mench (2014a) found a rhythmic secretion of melatonin on d 19 of incubation when illuminated with photoperiodic light during embryogenesis. Eggs incubated in the presence of light have been shown to have accelerated embryo development (Shafey and Al-mohsen, 2002; Cooper et al., 2011), increased hatchability (Shafey and Al-mohsen, 2002; Archer, 2017; Archer et al., 2017) and improvements in the quality of chicks at hatch (Archer, 2017; Archer et al., 2017). However, the results on embryonic development, hatchability and chick quality are inconsistent. Some studies demonstrated that light stimulation during incubation reduce or do not affect hatchability or body weight at hatch (Archer et al., 2009; Archer and Mench, 2014a; Özkan et al, 2012; Zhang et al., 2016; Sabuncuoglu et al., 2018). These disagreements among studies suggest that there are some factors influencing the response of chicken embryos to light stimulation during incubation.

Melatonin is produced rhythmically when vertebrates are entrained by the day and night cycle, and it is involved in thermoregulation, metabolic functions and immune responses in chickens (Zeman et al., 2001; Gharib et al., 2008). Whether embryonic growth rate and

the development of the skeletal system are affected by rhythmic melatonin production during embryogenesis is not yet clear.

Hatchlings are removed from incubators when the majority of chicks have hatched. The duration from the time of first chick to emerge to late-hatching chicks can be up to 48 h (Decuypere et al., 2001). This time period is commonly referred to as the hatch window (**HW**) (Decuypere et al., 2001). An extended HW has been reported to be associated with poor chick quality at hatch, which negatively affects the welfare and growth performance of a flock after placement at a rearing facility. Improved synchronization of hatch can decrease the number of dehydrated chicks. Hannah et al. (2020) found that Lohmann Brown embryos took less time to reach 50% of total chicks hatched and reduced the time between 50% to 75% of total chicks hatched when 12 h d^{-1} of full spectrum light was provided for the entire incubation period. This indicates that there is potential for improved hatch synchronization with the use of lights. There are few studies regarding the effect of light spectra on hatch time. Rozenboim et al. (2004a) reported no difference in incubation time for eggs incubated in the dark compared to eggs illuminated with monochromatic green light (15 min L : 15 min D) for broiler chicken embryos. Zhang et al. (2016) found that continuous illumination with green light did not affect hatch time or HW for broilers. However, an earlier study found that embryos incubated under 20-watt green fluorescent light required less time to hatch than those incubated in the dark (Shafey and Al-mohsen, 2002). Furthermore, it is found that longer light exposure required less time to hatch in broilers (Walter and Voitle, 1972). The shortened incubation time from light exposure that may be simply due to an overheating effect by conventional light source or related to an accelerated embryonic growth rate or higher

embryo activity. The mechanism involved in this process during incubation is still unknown.

There is evidence from studies with broiler hatching eggs exposed to different wavelengths of light that wavelength is influential in embryo response. Archer (2017) reported an improvement in hatchability and lower susceptibility to stress when broiler eggs were given 12 h d^{-1} of white or red LED lights as compared to green light or a dark environment. It makes sense that embryos may respond differently to different wavelengths of light based on what we know from studies post-hatch. Light wavelengths can affect the growth, development and behavior of chickens during rearing (Rozenboim et al., 2004b; Xie et al., 2008; Sultana et al., 2013). Sensitivity of birds to specific wavelengths of light varies at different developmental stages including during incubation and post hatch. Green light has been reported to accelerate broiler growth at an early age, while blue light had stronger effects on growth of broilers after 10 d of age (Rozenboim et al., 2004b). The color of the eggshell also affects how the light passes through and is received by embryos. This may explain why researchers like Hannah et al. (2020) found differences in distribution of hatch among lines of chickens that lay different colored eggs. However, few conclusions can be drawn regarding the optimal wavelength and its combination with photoperiod on embryo development and hatching performance parameters. Information on effects of entrainment of circadian rhythm by different colors of light for chicken embryo during incubation period on embryonic development, the spread of hatch, and day-old chick quality is limited. The objective of this project was to evaluate the effects of providing white, blue or red LED lights for 12 h daily during incubation period on embryonic development, embryo mortality, hatchability, air cell

temperature, the spread of hatch and chick quality in commercial broiler chickens. We hypothesized that different colors of photoperiodic LED light exposure during incubation would affect the early entrainment of circadian rhythm and result in different responses for embryo growth and hatching traits.

3.3 Materials and methods

The experimental protocol was carried out in accordance with the Canadian Council of Animal Care Guidelines (CCAC, 2009).

3.3.1 Incubation

The experiment consisted of four incubation trials. In the first trial, a total of 2,176 hatching eggs from a 54-wk Ross 308 broiler breeder flock were obtained from a commercial hatchery. Eggs were randomly divided into 4 experimental groups with 2 replicate incubators. A total of eight recently calibrated ChickMaster single-stage incubators (ChickMaster G09, Cresskill, NJ) with a capacity of 1,188 eggs per incubator were used for each trial. Two incubators were operated without light (0L:24L, Dark) as a control group, the remaining 6 incubators were illuminated with white (color temperature 4100K, Canarm, Brockville, ON), red (Once Innovations, Plymouth, MN) or blue (Once Innovations, Plymouth, MN) LED lights from the beginning of incubation. The spectrum of red and blue light is presented in Figure 1. Four LED strips were attached to metal frames on the left side within each incubator and were on 12 h d^{-1} (0700 to 1900 h). Light intensity at egg level was adjusted to 200 lx, as measured with a digital luxmeter (61-686 Digital Light Meter, Ideal, Ajax, Ontario, Canada). The LED lights were low profile and did not restrict airflow within the incubator. Eggs were turned through 90° once every 45 min for the first 444 h (18.5 d) of incubation. Incubation units were maintained at a

temperature of 37.5 °C for the entire incubation period. During the first 476 h (19 d plus 20 h) of incubation, relative humidity (RH) was set at 55%. RH was increased to 64% at 476 h, 72% at 488 h, 82% at 509 h and set back to 55% at 509 h of incubation (3 h before pulling out). The temperature, humidity and light intensity were monitored using data loggers (OMYL-M62, Omega® Engineering, Quebec, Canada) placed in the incubators to ensure that conditions were comparable among incubators. All eggs were candled at 444 h of incubation. Nonviable eggs were removed and broken open to determine fertility status. If fertile, the d of embryonic death was estimated and categorized as early (d 0-7), middle (d 8-14) or late (after d 15) death. The viable eggs were transferred into hatching trays and incubated in the original incubators without turning for the remaining incubation period.

The same experimental design was applied in all trials. The second, third and fourth trials had 1,664, 1,696 and 1,600 eggs from breeders at 31, 55 and 38 wk of age, respectively. The lighting treatments were rotated among incubators for each trial so that each treatment was represented once in each incubator to prevent the systematic effect of the incubation units.

3.3.2 Embryonic development

Embryo development parameters were measured at 1 h after lights came on (0800 h) on embryonic day (**ED**) 10, 14 and 18. An additional 192 eggs within a narrow weight range were weighed and randomly distributed into 8 incubators to evaluate embryonic development. On each of ED 10, 14 and 17 of incubation, 8 numbered eggs per incubator were removed from the incubator and weighed individually. The percentage of egg weight loss was calculated using the initial egg weight. Eggs were opened and the front-

to-back eyeball diameters of both eyes and embryo diameter were measured by digital calipers from the outermost portion of the curled tail to the outermost part of the curved neck with the natural curled position. Then embryos were separated from the residual yolk and euthanized by cervical dislocation. The residual yolk weight (only on ED 14 and 18), embryo length and weight, diameter and weight for both eyes, and heart weight were measured to the nearest 0.1 mm and 0.001 g, respectively.

3.3.3 Air cell temperature

Eighty eggs (10 eggs per incubator) within a narrow weight range were set on the top tray of each incubator to measure the embryo temperature twice per d. Air cell temperature was measured with transponders (implantable programmable temperature transponder, IPTT-300; BMDS Inc., DE, USA) in the trial 1, 3 and 4. All transponders were factory-calibrated for a range of 32 °C to 43 °C with an accuracy of 0.5 °C and a resolution of 0.2 °C before arrival. Each transponder was sterile and contained a battery-free microchip, read with a probe (DAS-6007 Smart probe, BMDS Inc., DE, USA). The probe reader detected a low frequency radio signal from the transponder within a range of 5 cm and received transponder ID and temperature. On ED 7.5, the pre-selected eggs were candled and implanted with transponders in the air cell of eggs with viable embryos. The eggshell surface area surrounding the site of eggshell perforation was disinfected with 70% ethanol. An oblong shape opening (around 3 mm width and 1 cm length) was perforated in the large end of each egg. Subsequently, a sterile transponder was inserted into the air cell without disrupting the inner shell membrane and chorioallantoic membranes. The opening was covered with a piece of clear packing tape and sealed with melted paraffin wax. Egg weight was determined before and after implantation, and on

ED 18. Between ED 8 and ED 18, air cell temperature was measured every 12 h (1 h after lights turned on or off). In the current study, approximately 74% (178 out of 240) of eggs implanted with transponders in the air cell were hatched and mortality did not differ ($P>0.05$) among incubation lighting treatments.

3.3.4 Spread of hatch

The number of hatched chicks per tray was recorded in 3 h intervals from 454 h to 512 h of incubation to calculate the spread of hatch. Chicks were considered as hatched when they were completely emerged and free from the eggshell. The counting process was carried out in the dark by using a head lamp (0.1-0.2 lux) as the only source of light to minimize the effects of external light on the embryos. At each check, the total number of chicks hatched within the 3 h period was moved to the back of each tray (separated by a barrier) and recorded.

3.3.5 Hatchability and chick quality

At the end of incubation (512 h of incubation), all hatched chicks were counted and weighed in a batch for each incubator. Hatchability was calculated based on total fertile eggs. The chicks were feather sexed and chick quality was assessed by navel closure condition and overall appearance. Navel closure condition was scored as 1 (clean and closed navel area), 2 (black button up to 2 mm or black string), or 3 (black button exceeding 2 mm or open navel area) (Molenaar et al., 2010). In addition, the chick length of 40 males and 40 females per incubator/replicate were randomly selected and measured. All unhatched eggs were broken open to determine the approximate d of embryonic death. An additional 8 male and 8 female chicks per replicate were randomly

selected and euthanized by CO for assessing chick development. The intact chick was weighed and dissected. The residual yolk and yolk-free body weight (YFBW) were calculated as percentage of intact chick weight. spleen, bursa of Fabricius and liver were harvested and calculated as the percentage of YFBW.

3.3.6 Statistical analysis

This experiment was a randomized complete block design with a set of 4 incubators as one block (2 blocks per trial). Incubator was used as the experimental unit with 8 blocks for each lighting treatment. One incubator (dark-control) in trial 2 was not functioning as consistently as the other seven incubators and was removed from the analysis. The residuals of error met the assumptions of normal distribution, independently distributed with mean zero and had constant variance before further analysis. Embryonic development data were analyzed by sampling day using the Mixed Procedure of SAS v. 9.4 (SAS Inc., Cary, NC, 2013). The air cell temperature data were analyzed as repeated measures in SAS v. 9.4 (SAS Inc., Cary, NC, 2013). In repeated measures analysis, four covariance structures, first order autoregressive, compound symmetry, toeplitz, and variance components were compared. The covariance structure which provided the lowest absolute values for Akaike Information Criterion and Bayesian Information Criterion was selected for repeated measures analysis. Based on these criteria, covariance structure toeplitz was selected for all parameters. The spread of hatch data was analyzed using nonlinear regression model of SAS v. 9.4 (SAS Inc., Cary, NC, 2013). A 3-parameter logistic growth model (Eq. 1) was found to be the best nonlinear regression model that described the relationship between the cumulative percentage of hatched chicks (Y) and h of incubation (X) for all treatments. To compare the spread of hatch

among lighting treatments, a new equation (Eq. 2) was converted from the predicted model (Eq. 1) for each experimental unit (incubator). The h of incubation reaching 10%, 25%, 50%, 75% and 90% of chicks hatch were calculated and then analyzed using the Mixed Procedure of SAS v. 9.4 (SAS Inc., Cary, NC, 2013).

$$Y = \frac{\theta_1}{1+\exp(-\frac{(X-\theta_2)}{\theta_3})} + \varepsilon \qquad \text{(Eq. 1}^*\text{)}$$

$$X = \theta_2 - \left[\ln\left(\frac{\theta_1}{Y} - 1\right)\theta_3\right] + \varepsilon \quad \text{(Eq. 2}^*\text{)}$$

*The parameters represent the asymptote (θ_1), the time to half the asymptote (θ_2), and the interval from half to until ¾ of the asymptote (θ_3).

Data for hatching performance (initial egg weight, fertility rate, hatchability, egg weight loss percentage, embryo mortality, and hatch weight) were analyzed using the Mixed Procedure of the SAS v.9.4 (SAS Inc., Cary, NC, 2013) to determine the effect of lighting treatments. For the navel score, chick length and chick quality parameter obtained from dissected chicks, the statistical model included the gender effect. In all cases, if significant effects were found, the Tukey-Kramer test was applied to differentiate the means at 5% level of significance.

3.4 Results and discussion

3.4.1 Embryo development

There were no differences (P>0.05) among the four groups regarding percent of weight loss, embryo size and weight, residual yolk weight, eyeball diameter or relative heart weight on either ED 10, 14 or 18 (Table 3.1). Similar results have been reported previously (Zhang et al., 2016; Dishon et al., 2017). Dishon et al. (2017) reported that illuminated broiler eggs with LED light did not affect body, muscle or liver weight. However, the effects of the photostimulation on embryo development regarding the

embryo weight or physiology parameters were not consistent in past studies. Coleman and McDaniel (1976) reported that White Leghorn embryo development was accelerated from the first d of receiving continuous white fluorescent light. Similar results have been found in Northern Bobwhite quail (Walter and Voitle, 1973); House sparrows (Cooper et al., 2011), Hybro broilers (Shafey and Al-mohsen, 2002) and layers (Shafey, 2004) when illuminated with incandescent or fluorescent light during incubation. The additional heat from those conventional lights may have an over-heating effect and have a cofounding effect with light stimulation on the embryo development. Wang et al. (2014) reported advanced embryo development with continuous green LED light exposure during incubation for chicken embryos. However, no differences in embryo development or liver index at ED 15 and 18 among red, blue and dark were found in their study, which indicates that light wavelength may be a critical factor affecting embryo and organ development. Rozenboim et al. (2004a) reported higher embryo weight as percent of egg weight on ED 14, 15, 17 and 20 but not at hatching when illuminating with monochromatic green LED light (15 min ON and 15 min OFF). The mechanism behind acceleration of embryo development may be related to an alteration of somatotropic axis hormones (growth hormone, insulin-like growth factor-I and prolactin) production from light exposure. A significant increase in hypothalamic growth hormone-releasing hormone RNA expression was found from ED 16 to ED 20 when embryos were illuminated with green light (15 min light / 15 min dark). In addition, plasma growth hormone levels were significantly higher on ED 14, 16, 18 and 20 in embryos with green light exposure (Dishon et al., 2017; 2018).

Accelerated embryo development as a result of light exposure was not detected in the current study. Several factors that characterize light that can affect embryo development need to be considered. These factors include the source of the light, the duration of light exposure, the spectra produced and the intensity of light. Acceleration of embryonic development was found in some studies, which used different photoperiods during incubation. For instance, 15 min on and 15 min off (Rozenboim et al., 2004a), 12 h d^{-1} (Walter and Voitle, 1972; 1973) and 16 h d^{-1} of light exposure (Özkan et al., 2012; Yu et al., 2018) had positive impacts on embryo development. Those results suggested that providing photoperiod during incubation may stimulate pineal melatonin secretion and regulate growth hormone synthesis. However, an increased overall embryo weight as well as embryo muscle weight were also found when light exposure was continuous, which excluded the exogenous time Zeitgeber for chicken embryos (Shafey and Al-mohen, 2004; Wang et al., 2014). Those results indicated that muscle growth may depend on light exposure, but was not only associated with circadian rhythms entrained by photoperiod. Light wavelength and intensity can influence the amount of light that can pass through the eggshell and reach the embryo. The light intensity in the current study may be one of the key factors affecting mitosis in neural crest mesoderm during the early stage of embryo development. Yu et al. (2018) found green LED light at a low intensity (50 lux) stimulated embryo growth during incubation. However, no improvement in embryo weight to initial egg weight was detected when green light was set to 150 or 300 lux. The intensity in our study was approximately 200 lux at egg level, which may be too high as reported by Yu et al. (2018). However, an increased embryo weight was found when illuminated with fluorescent light at 200 to 300 lux (Özkan et al., 2012). Whether

light intensity of LED light at 200 lux stresses the broiler embryos should be evaluated in a future study. The difference between findings on embryo weight may relate to the light source, wavelength, intensity, or their combination with eggshell characteristics, such as, pigment intensity (Shafey et al., 2004) and thickness (Maurer et al., 2015). The response to incubation light stimulation may also vary among avian species. Further studies are needed to investigate whether genetic factors are closely related to cell proliferation, hormone (growth hormone and IGF-1) regulation and embryo development when exposed to photostimulation during incubation.

Eyeball diameter and heart weight were not affected by the presence of light during incubation in the current study (Table 3.1). Zhang et al. (2016), who reported similar results, incubated eggs with continuous green or white light and did not find differences in relative weight of heart, liver and eyeball among treatments. The dimensions of the chicken eyeball at market age are not affected by the provision of fluorescent light (550 lux) during incubation (Archer et al., 2009). It has been reported that domestic chickens reared under continuous fluorescent light (1,044 lux) develop abnormal buphthalmic eyes (Whitley et al., 1984). Our results indicated that providing LED light at 200 lux up to 12 h daily did not negatively impact eye development.

3.4.2 Air cell temperature

The air cell temperature was significantly affected by light color treatments ($P<0.01$) as well as the embryo age ($P<0.01$) (Table 3.2). Embryos incubated under red light with 12 h d^{-1} of light exposure had lower air cell temperature than those in the dark, or with white or blue light. There were no difference in air cell temperature among white, blue and dark groups. Archer et al. (2017) reported that eggshell temperature was not affected by

illumination with red LED light. Considering the results of air cell temperature and egg weight loss percentage (Table 3.1 and 3.2), providing LED light at 200 lux for 12 h d^{-1} does not cause an over-heating effect on broiler hatching eggs. Although, we do not have a clear explanation for lower air cell temperature found in eggs illuminated with red LED light for 12 h d^{-1}. One possible explanation may be that red light increases the accumulative melatonin secretion during scotophase. Melatonin can interact with growth hormone production (Zeman et al., 1999) and thermoregulation (Rozenboim et al., 1998). Zeman et al. (2001) reported that reduced heat production in female broilers when providing 150 mg kg^{-1} of melatonin as a feed supplement at 2 and 3 wk of age. To the best of our knowledge, there are no previous studies reporting thermoregulation as an indicator for the establishment of a circadian rhythm for chicken embryos. It is worth monitoring air cell temperature continuously and its correlation to the melatonin production within a 24 h cycle in future research.

3.4.3 Spread of hatch

There were no differences in incubation time to reach 5%, 10%, 25%, 50%, 75%, 90% or 95% of chicks hatched among light color treatments (Table 3.3). In addition, the duration between specific percent of hatch did not differ among treatments (Table 3.4). Our results contrast with some previous studies (Walter and Voitle, 1972; Fairchild and Christensen, 2000; Shafey and Al-mohsen, 2002;) which reported a reduction in hatch time by the presence of light during incubation, but agrees with the several studies, which reported no differences in time of hatch when providing light exposure during incubation for Japanese quail (Sabuncuoglu et al., 2018) and broilers (Rozenboim et al., 2004a; Özkan et al., 2012; Zhang et al., 2016). No differences in internal pipping time and length of

hatch window were found between incubation in the dark and providing photoperiodic green light during the first 18 d of incubation in Ross broilers (Tong et al., 2018). Counting the number of chicks hatched within certain time intervals and calculating the average incubation time is a common method used for monitoring hatch time (Özkan et al., 2012; Zhang et al., 2016; Sabuncuoglu et al., 2018). However, the information on hatch time distribution within hatching process cannot be drawn by simply comparing the average incubation time. An analysis of the spread of hatch using a nonlinear regression model can provide more information on the distribution of hatch time. Hannah et al. (2020) found a synchronized HW for Lohmann Brown embryos when illuminated with white LED light during incubation. The result from the current study indicated that HW of broiler Ross strain was not synchronized when illuminated with 12 h d^{-1} of light regardless of the colors of light. Differences in HW responses to light stimulation between strains might be related to genetic selection for growth and production.

3.4.4 Hatchability and chick quality

No differences in hatchability of fertile eggs, hatch weight and embryo mortality were found among lighting treatments in the current study (Table 3.5). Our results agreed with the results reported by Rozenboim et al. (2003) in turkey, Shafey (2004) in layers and Sabuncuoglu et al. (2018) in Japanese quail. Similarly, no difference in hatchability was found among different photoperiods when illuminated with full spectrum fluorescent light on Cobb broiler hatching eggs (Archer et al., 2009; Archer and Mench, 2014a). Zhang et al. (2012; 2016) reported that providing continuous green light (560 nm) during incubation does not affect hatchability, hatch weight or embryo mortality. However, the effects of incubation lighting illumination on hatchability and embryo mortality were not

consistent among studies. Illuminating lighter brown color broiler hatching eggs with excessive light intensity (1430–2080 lux) had negative effects on hatchability and increased embryo mortality as the amount of light that passed through the eggshell may above the optimal level (Shafey et al., 2005). However, Archer et al. (2017) found White Leghorn, broiler and Pekin duck hatching eggs exposed to LED light with white and red bulbs at 250 lux for 12 h d^{-1} had an improvement in hatchability. The lower percentage of early embryo mortality in White Leghorn, broiler and Pekin duck is a possible explanation for higher hatchability when illuminated with LED light. They concluded that hatchability was affected by the combination of white and red light. Light spectrum also plays an important role in affecting hatching performance. Hluchý et al. (2012) reported that a 3-4% increase of hatchability for Ross broiler eggs with white light as compared to red and blue light. But only 540 eggs were used in their study (108 eggs per treatment) and the number of eggs may not meet the requirement of comparing hatchability among treatments. The mechanism for this improvement in hatchability and embryo mortality is not clear. The hatchability of fertile eggs was not statistically improved (P-value: 0.533) by the exposure to a photoperiod during incubation in the current study. However, hatchability of fertile eggs was numerically higher for blue (89.18%), red (89.33%) and white light (89.02%) treatments compared to dark (87.71%). Differences in hatchability and embryo mortality between those studies and the current study could be influenced by several factors or their combinations, including the type of light source, the composition of light spectrum, strain of bird, breeder age, and eggshell characteristics such as thickness and pigment deposition.

In the current study, no differences in chick length, average navel scores or the percentage of navel scores of 1 (completely closed and clean) were found among treatments (Table 3.6). Some previous studies also reported that no effects on chick weight or chick quality at hatch when providing light illumination during incubation (Zhang et al., 2012; Tong et al., 2018). Chick quality, including appearance, activity, characteristics of eye, leg, navel closure condition and other parameters, was not affected by use of green light during the setter phase for Ross broilers (Tong et al., 2018). Several studies find improved navel condition at hatch when incubated under a lighting program. A photoperiod of 12L:12D decreased the percentage of chicks with unhealed navels in comparison to those incubated under dark (Huth and Archer, 2015; Archer, 2017; Archer et al., 2017). Their findings suggested that accelerated embryo development with photostimulation during incubation may result in improved maturation of the navel. However, this was not the case in the current experiment as incubating broiler hatching eggs with blue, white or red light for 12 h d^{-1} affect embryo development or newly hatched chick quality.

No differences in yolk-free body weight, relative yolk-free body weight or relative spleen were found among treatments (Table 3.7). This result was consistent with previous reports (Fairchild and Christensen, 2000; Özkan et al., 2012). Relative bursa of Fabricius weight was affected by the two-way interaction between wavelength and gender (Table 3.7). Compared to those incubated under dark, male chicks illuminated with blue light had lower relative bursa of Fabricius weight. No differences in the relative bursa of Fabricius weight in female day-old chicks were found among lighting treatments. The result indicated that the response in bursa of Fabricius weight of neonatal chicks to

photostimulation depended on gender, which has not been studied or reported before.

Bursa of Fabricius is the primary immune organ for the development and maturation of B lymphocyte in avian species. The relative bursa of Fabricius weight can be affected by diet, environmental stress or photostimulation (Xie et al., 2008; Quinteiro-Filho et al., 2010; Li et al., 2014). Li et al. (2014) reported that providing green light during the grow-out period promoted melatonin secretion and its secretion correlated with B lymphocyte proliferation in bursa of Fabricius in broilers. The different results between their study and ours could be the age of animals (2 wk broilers vs. embryo) or the light characteristics (light spectra and intensity) utilized by embryos. However, the mechanism of light spectra, in combination with photoperiods, affecting the structure and function of the primary immune organ of chicken embryos remains unclear. Immunohistochemcial staining for proliferating cell nuclear antigen can be investigated and provide a better understanding in lymphoid organ development with photostimulation.

The relative liver weight of chicks hatched under red LED light tended to be higher than those under white light (Table 3.7). Increased embryo weight at ED 15, 18 and 21, and accelerated liver development at ED 21 in Arbor Acres broiler embryos may be related to higher plasma melatonin level and expression of melatonin receptor 1c in liver when illuminated with continuous LED green light (Wang et al., 2014). However, neither liver nor heart weight differed between dark and photo-stimulation groups in turkey poults when illuminated with incandescent light for 12 h d^{-1} (Fairchild and Christensen, 2000) and Ross 308 broilers exposed to cool white fluorescent light for 16 h d^{-1} (Özkan et al., 2012). Therefore, the difference between findings on relative liver weight may relate to different strains or light regimes. Relative liver weight showed an increased trend

(P=0.054) in the current study, which could have been due to increased gluconeogenesis and increased need for mobilization of glucose during late embryogenesis until hatch. Maatjens et al. (2016) reported an increase in liver weight of broilers from internal pipping to hatch when incubated at a lower temperature than optimum during the last week of incubation. In combination with the lower air cell temperature found in embryos incubated with red light illumination, the differences in liver development may be confounded by embryo temperature. A previous study found higher gene expression of peroxisome proliferator activated receptor-γ coactivator 1 α (PGC-1α) when chicken embryos were incubated at 35 °C when compared with the 38 °C treatment group (Walter and Seebacher, 2007). Their findings suggested that lower incubation temperature induced PGC-1α gene expression and activated gluconeogenesis in chicken embryos. Wu et al. (2001) reported that illuminated chicken embryos with 12 mW cm^{-2} light had a 220% increase in embryo movement compared to 1 mW cm^{-2} light stimulation. The increase in embryo movement with light stimulation may have a higher requirement for glucose as the energy source. Our results indicated that chicks incubated under red light for 12 h d^{-1} may have increased gluconeogenesis in the liver and the effects of light spectra on embryo movement and liver gluconeogenesis need to be investigated in future research.

In conclusion, the results of this study demonstrate that illumination with different colors of LED light for 12 h d^{-1} throughout incubation did not affect hatchability, embryo mortality, spread of hatch or day-old chick quality. The higher relative liver weight found in the red light stimulation treatment suggests that photostimulation during incubation may affect embryo activity and the requirement of energy. The effects of

photostimulation on post-hatch growth and physiology need to be investigated in the future.

3.5 Acknowledgements

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Table 3.1 Effects of the application of colored photoperiodic light during incubation on egg weight loss (EWL) (%), the weights of embryo (% of egg weight) and organs (g kg^{-1}), embryo length (mm), the diameter of embryo (mm) and eyeballs (mm), and the relative embryo diameter (mm g^{-1}) and length (mm g^{-1}) at embryonic day (ED) 10, 14 and 18 of incubation in Ross 308 broiler embryos.

Item	n[1]	EWL	Relative embryo weight[2]	Yolk weight[3]	Embryo diameter	Relative embryo diameter[4]	Embryo length	Relative length[5]	Eyeball diameter	Relative heart weight[6]
Variation source (ED10)										
Dark	7	6.53	4.50	-	31.0	10.76	-	-	8.2	8.36
Blue	8	7.07	4.41	-	30.8	10.99	-	-	8.2	8.03
Red	8	6.90	4.44	-	30.7	10.95	-	-	8.1	8.35
White	8	6.54	4.39	-	30.8	10.93	-	-	8.1	8.36
SEM		0.211	0.048	-	0.31	0.149	-	-	0.05	0.157
Lighting treatment		0.237	0.443	-	0.865	0.745	-	-	0.372	0.379
Block		0.686	<0.0001	-	0.0001	<0.0001	-	-	<0.0001	0.001
Variation source (ED14)										
Dark	7	9.38	22.14	18.6	53.9	3.80	104.8	7.4	10.9	7.62
Blue	8	8.92	22.53	18.6	54.9	3.78	103.5	7.1	10.9	7.67
Red	8	9.51	22.26	18.5	53.9	3.79	103.0	7.2	10.8	7.76
White	8	9.45	22.39	18.6	54.6	3.78	103.5	7.2	11.0	7.62
SEM		0.220	0.314	0.20	0.87	0.070	0.75	0.10	0.07	0.108
Lighting treatment		0.248	0.847	0.986	0.816	0.994	0.457	0.319	0.590	0.753
Block		0.001	<0.0001	<0.0001	0.901	0.072	<0.0001	<0.0001	0.003	0.002
Variation source (ED18)										
Dark	7	11.86	46.69	13.4	56.7	1.89	159.1	5.3	11.9	7.29
Blue	8	11.81	46.57	13.6	56.8	1.90	157.4	5.2	11.9	7.49
Red	8	12.23	46.19	13.3	57.1	1.92	158.8	5.3	11.9	7.47
White	8	11.69	46.69	13.3	57.3	1.91	159.3	5.3	11.9	7.45
SEM		0.300	0.270	0.25	0.54	0.02	0.77	0.03	0.06	0.084
Lighting treatment		0.626	0.537	0.782	0.886	0.861	0.320	0.309	0.992	0.415
Block		0.012	<0.0001	<0.0001	0.037	<0.0001	<0.0001	<0.0001	0.0002	0.002

[1]Number of experimental units. Experimental unit = 8 eggs per incubator per sampling day. [2]Relative embryo weight = Embryo weight / egg weight * 100%. [3]Yolk = yolk weight / day 0 egg weight *100%. [4]Relative diameter to embryo weight = Embryo diameter / embryo weight * 100%. [5]Relative length to embryo weight = Embryo length / embryo weight * 100%. [6]Relative heart weight = Heart weight / embryo weight * 100%.

Table 3.2 Effects of the application of colored photoperiodic light during incubation on egg weight loss (%) and air cell temperature (°C) from day 7 to 18 of incubation in Ross 308 broiler embryos.

Wavelength	n[1]	Egg weight loss 0-18	Egg weight loss 7-18	Air cell temperature
Dark	5	12.34	7.38	37.8[a]
Blue	6	11.12	6.43	37.8[a]
Red	6	12.04	7.08	37.7[b]
White	6	12.23	7.20	37.8[a]
SEM		0.549	0.396	0.01
ANOVA P-value				
Wavelength (W)		0.411	0.382	<0.0001
Embryo Age (A)		N/A	N/A	<0.0001
W x A		N/A	N/A	1.000
Block		0.333	0.288	<0.0001

[1]Number of experimental units. Experimental unit = 10 eggs per incubator in trial 1, 3 and 4.

[a,b]Means within column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 3.3 Effects of the application of colored photoperiodic light during incubation on time in hours to reach a specific percentage of hatch (set time to hatch time) in total hatched Ross 308 broilers.

Wavelength	n[1]	% of chicks hatch						
		5%	10%	25%	50%	75%	90%	95%
Dark	7	483.2	486.4	491.0	495.6	500.0	504.2	506.8
Blue	8	480.8	484.3	489.6	494.8	499.8	504.4	507.0
Red	8	482.5	485.9	490.9	495.9	500.6	504.9	507.3
White	8	482.2	485.4	490.3	495.0	499.7	504.2	506.9
SEM		1.19	1.10	1.00	0.94	0.91	0.87	0.83
P-value								
Wavelength		0.562	0.629	0.744	0.844	0.900	0.938	0.976
Block		0.001	0.0004	0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]Number of experimental units. Experimental unit = 1 incubator containing 238, 208, 212 and 200 eggs in trial 1, 2, 3 and 4, respectively.

Table 3.4 Effects of the application of colored photoperiodic light during incubation on the spread of hatch (hours interval between specific percentage of hatch) in total hatched Ross 308 broilers

Wavelength	n[1]	% of chicks hatch			
		5%-95%	10%-90%	25%-75%	50%-75%
Dark	7	23.6	17.9	9.1	4.5
Blue	8	26.3	20.0	10.2	5.0
Red	8	24.8	19.0	9.7	4.7
White	8	24.8	18.7	9.5	4.7
SEM		0.94	0.76	0.41	0.19
P-value					
Wavelength		0.319	0.319	0.323	0.321
Block		0.348	0.429	0.490	0.431

[1]Number of experimental units. Experimental unit = 1 incubator containing 238, 208, 212 and 200 eggs in trial 1, 2, 3 and 4, respectively.

Table 3.5 Effects of the application of colored photoperiodic light during incubation on broiler hatching performance including set egg weight (g egg^{-1}), egg weight loss (%) during the first 18 days, fertility (%), hatchability of fertile eggs (%), chick hatch weight (g bird^{-1}), early, middle and late embryo mortality rate (%) in Ross 308 broiler hatching eggs.

Wavelength	n[1]	Egg weight	Egg weight loss[2]	Fertility[3]	Hatchability of fertile[4]	Hatch weight	Early dead[5]	Middle dead[6]	Late dead[7]
Dark	7	64.5	12.67	93.36	87.71	45.2	7.32	0.63	4.32
Blue	8	64.6	11.96	93.81	89.18	45.2	6.73	0.37	3.84
Red	8	64.2	12.40	93.93	89.33	44.6	5.62	0.62	4.43
White	8	64.5	12.05	94.02	89.02	45.3	6.61	0.94	3.43
SEM		0.18	0.320	0.560	0.801	0.26	0.668	0.247	0.736
ANOVA P-value									
Wavelength		0.457	0.428	0.8623	0.533	0.248	0.392	0.464	0.766
Block		<0.0001	0.108	<0.0001	<0.0001	<0.0001	<0.0001	0.373	0.001

[1]Number of experimental units. Experimental unit = 1 incubator containing 238, 208, 212 and 200 eggs in trial 1, 2, 3 and 4, respectively.

[2]Egg weight loss percent = (d0 egg weight – d18 egg weight)/d0 egg weight × 100.

[3]Fertility = (number of fertile eggs/number of eggs set) × 100.

[4]Hatchability of fertile = (number of eggs hatched/number of fertile eggs set) × 100.

[5]Early mortality = (number of dead embryos between 1 to 7 d of incubation/number of fertile eggs set) x 100.

[6]Middle mortality = (number of dead embryos between 8 to 14 d of incubation/number of fertile eggs set) x 100.

[7]Late mortality = (number of dead embryos between 15 d of incubation to external pipping/number of fertile eggs set) x 100.

Table 3.6 Effects of the application of colored photoperiodic light during incubation on chick length (mm), average navel score, and percent of score 1 (%) in Ross 308 broiler chicks at hatch.

Wavelength	n[1]	Chick length		Average navel score		Percentage of score 1	
		Female	Male	Female	Male	Female	Male
Dark	7	18.8	18.8	1.8	1.6	29.36	39.03
Blue	8	18.9	18.9	1.8	1.7	28.38	36.57
Red	8	18.8	18.7	1.7	1.7	30.92	36.62
White	8	18.9	18.8	1.8	1.7	27.21	36.31
SEM		0.05	0.05	0.03	0.03	2.510	2.510
P-value		Chick length		Average navel score		Percentage of score 1	
Wavelength		0.143		0.728		0.767	
Gender		0.100		<0.0001		<0.0001	
W x G		0.933		0.562		0.871	
Block		<0.0001		<0.0001		<0.0001	

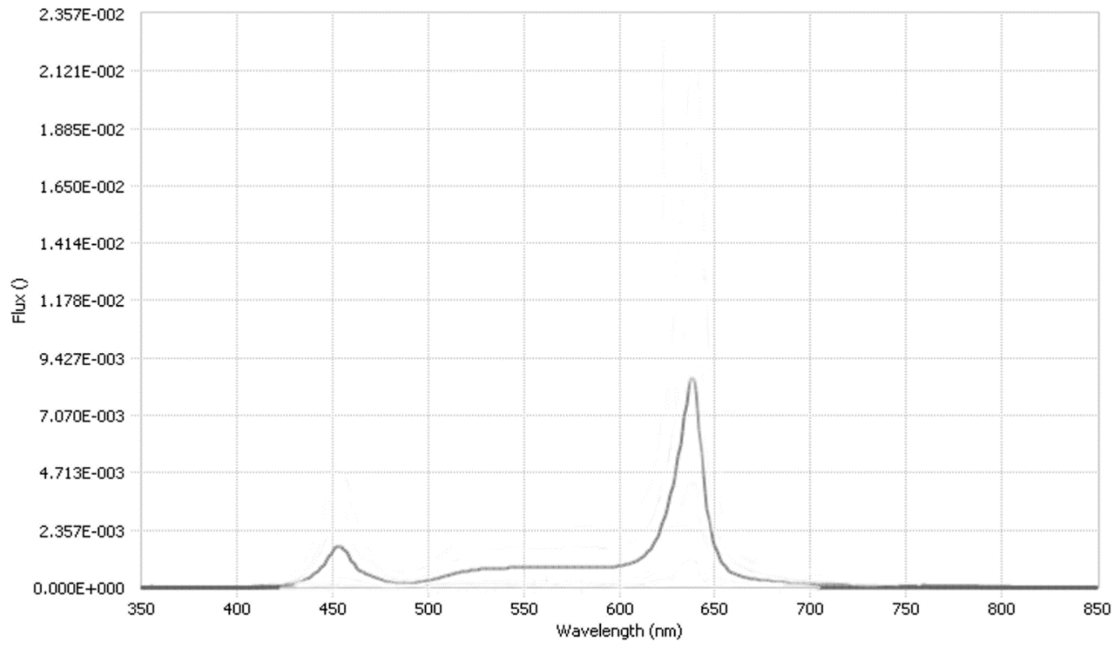
[1]Number of experimental units. Experimental unit = 40 birds per gender hatched from each incubator.

Table 3.7 Effects of the application of colored photoperiodic light during incubation on yolk-free body weight (YFBW) (g), relative YFBW (%), relative weight (g kg^{-1}) of spleen, bursa of Fabricius and liver in Ross 308 broiler chicks at hatch.

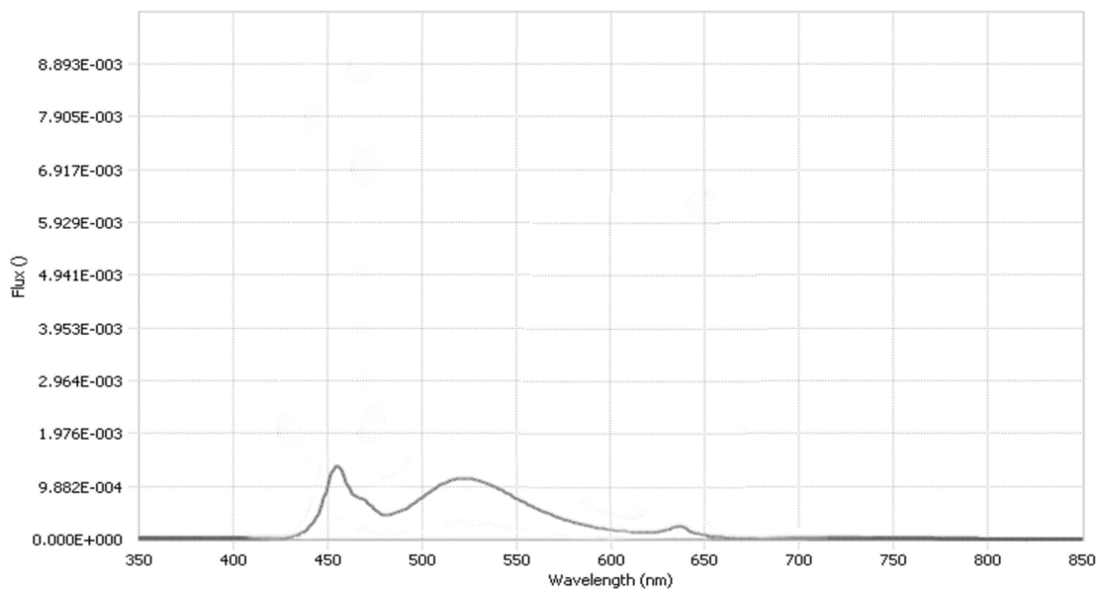
Wavelength	Gender	n[1]	Yolk-free body weight	Relative YFBW	Relative spleen weight	Relative bursa of Fabricius weight	Relative liver weight
Dark	Female	7	37.9	86.89	0.52	1.28^{c}	38.59
	Male	7	38.8	86.69	0.57	1.53^{a}	36.09
Blue	Female	8	39.6	87.69	0.60	1.44^{abc}	38.60
	Male	8	39.2	86.50	0.54	1.31^{bc}	36.07
Red	Female	8	38.6	88.53	0.57	1.39^{abc}	39.61
	Male	8	38.9	87.07	0.55	1.46^{ab}	37.44
White	Female	8	38.7	87.31	0.55	1.28^{c}	36.21
	Male	8	39.1	87.68	0.58	1.49^{ab}	35.38
SEM			0.66	0.600	0.025	0.065	0.943
ANOVA P-value							
Wavelength (W)			0.411	0.326	0.754	0.861	0.054
Gender (G)			0.470	0.134	0.918	0.029	0.006
W x G			0.821	0.335	0.212	0.014	0.786
Block			0.0002	<0.0001	0.009	0.0003	0.007

[1]Number of experimental units. Experimental unit = 40 birds per gender hatched from each incubator.

[a,b,c]Means within a column with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).



(a)



(b)

Figure 3.1 Spectrum of red (a) LED light dimmed at 60% and blue (b) LED light dimmed at 40% at egg level within incubator.

CHAPTER 4. PROVIDING COLORED PHOTOPERIODIC LIGHT STIMULATION DURING INCUBATION: 2. EFFECTS ON EARLY POST-HATCH GROWTH, IMMUNE RESPONSE, AND PRODUCTION PERFORMANCE IN BROILER CHICKENS*

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4.1 Abstract

Previous findings have reported that providing light during incubation can affect hatchability and chick quality. This study was conducted to investigate the effects of providing light during incubation on post-hatch broiler production parameters, thermoregulation and immune response. Lights with different wavelengths were used over the course of four separate hatches. Ross 308 broiler hatching eggs were randomly distributed into 4 lighting treatments for each hatch. The incubation lighting treatments included: dark as control, white, red or blue lights for 12 h d^{-1} (200 lux at egg level). Broilers hatched from each incubator with the same gender were placed into one of eight sets of pens (3 pens/set) and raised under 18 h d^{-1} photoperiod. Six birds per pen were immunized intraocularly with AviPro® ND-IB Polybanco vaccine on d 10 and 21 post-hatch. Chicks hatched under white and blue lights had heavier ($P<0.05$) body weight and higher ($P<0.05$) feed consumption than the control group during the first 6 h post placement. No differences in vent temperature were found among treatments at 24 h post-hatch ($P>0.05$). Chicks hatched with light stimulation however had more stable ($P<0.05$) cloaca temperature at 36 h post-hatch. No differences in average body weight gain, feed consumption or feed conversion ratio were found among lighting treatments between d 7 to d 35. On d 14 of age, birds hatched from red light had higher ($P<0.05$) total IgG concentration than those hatched under dark, blue or white light. These results indicated that *in ovo* light stimulation with different wavelengths did not affect growth parameters of broilers at market age. Providing photoperiodic blue and white light during incubation improved the production parameters of broilers during the first week post-hatch.

Key words: photostimulation, light color, incubation, thermoregulation, IgG

4.1 Introduction

Significant changes in genetics, nutrition and management have taken place in the broiler industry during the past decades. Broilers can achieve double their body weight in half the time when compared to broiler production in the 1950s (Havenstein et al., 2003). The National Chicken Council (2020) reported that the average slaughter age (at a weight of 2.86 kg) of a broiler is 47 days, which means the broiler spends almost one-third of its life as an embryo inside the incubator. Therefore, providing the optimum incubation environment is crucial for successful broiler production.

Light is an important exogenous environmental factor for controlling many physiological and behavioral processes in birds. It has been reported that avian species have a wider visual spectrum range than humans (Prescott and Wathes, 1999). Artificial lighting programs are a management tool for improving birds' behavior, welfare and production efficiency of commercial poultry (Rozenboim et al., 2004b; Olanrewaju et al., 2006; Schwean-Lardner et al., 2013; Sultana et al., 2013). Light intensity, the composition of the spectrum and photoperiod (daily pattern of light and dark exposure) are three parameters of light that need to be considered when used as tools to manage poultry production. Effects of providing different colors of light during rearing period on growth rates have been well documented. Broilers reared under blue or green light gained more weight than birds exposed to red or white light (Wabeck and Skoglund, 1974; Rozenboim et al., 1999, Rozenboim et al., 2004b). Soliman and Hassan (2019) reported that broilers reared under blue and white light gain significantly more weight and improve feed conversion ratio than birds reared under red light. The increased body weight might be due to increased satellite cell proliferation when exposed to light with a short wavelength

(Halevy et al., 1998). Whether providing light illumination with blue light to chicken embryos had a similar effect on growth rate of broilers post-hatch was investigated in this study.

The concept of providing illumination during incubation has been a subject for many years. As far back as the late 1960s, accelerated embryo development was found when White Rock eggs were stimulated with continuous light exposure (Siegel et al., 1969). Conventional light sources, fluorescent and incandescent bulbs, however, may produce additional heat and alter the incubation environment. Light emitting diode (LED) fixtures have become widely available as they feature durability, low heat production and energy efficiency. LED bulbs are available in monochromatic colors and capable of being dimmed. Several studies have demonstrated that providing light with LED bulbs during incubation has positive effects on embryo development (Shafey and Al-mohsen, 2002; Rozenboim et al., 2004a; Wang et al., 2014), chick quality at hatch (Huth and Archer, 2015; Archer, 2017; Archer et al., 2017) and welfare post-hatch (Huth and Archer, 2015; Archer and Mench, 2017). *In ovo* intermittent light illumination (15 min light, 15 min dark) during incubation increased the number of skeletal muscle cells and enhanced satellite cell proliferation in broilers on d 1 and 3 post-hatch (Halevy et al., 2006). Their results suggested that light stimulation during incubation could affect myogenic activity by modulating energy metabolism and interact with hormones involved in growth control. Plasma growth hormone concentration has been found to reach peak levels at late embryonic and early post-hatching development, and then gradually declined until close to sexual maturation (Scanes et al., 1992). Light spectrum has varying effects on humoral and cellular immune responses on broilers during rearing period. For example, T-

lymphocyte (Xie et al., 2008b) and splenocyte (Xie et al., 2008a) proliferation were the highest in broilers exposed to white light through the entire experimental period. However, broilers reared under green light had higher anti-Newcastle disease virus (NDV) antibody production than those under white light at d 42 of age (Xie et al., 2008b). Higher anti-NDV antibody titer was found in broilers under green and blue light than those under white and red light for the entire production period (Zhang et al., 2014). However, Firouzi et al. (2014) reported that green light only promotes anti-NDV antibody production at the early growth stage, compared to red light. No differences in humoral response were found among light color treatments on d 30 and 42. These findings suggest that light source, broiler strain, vaccination and sampling day may account for the variation in the effects of light wavelength on immune response. Those studies were focused on the effects of light illumination during rearing period. The study conducted by Archer and Mench (2013) found that broilers incubated with a light program of 12L:12D had a stronger humoral immune response to keyhole limpet hemocyanin, which is a nonpathogenic protein antigen and has often been used to evaluate humoral immune response. The information regarding the effects of providing light with different spectra during incubation on post-hatch immune response on broilers is limited and will be tested in the current study.

A bird's development during brooding stage is important for their future performance. After transferring to the grow-out facility, the changes in nutritional resources and environmental factors (temperature, humidity, light exposure and handling) may be challenging for young chicks. Provision of a photoperiod during incubation may entrain the circadian rhythms of melatonin secretion in broiler embryos and provide positive

effects on the endocrine, neuronal, and immune systems and improve behavioral processes at a young age. Optimal light regimes during incubation need to be thoroughly investigated as a potential environmental tool to improve young chicks' adaptation to the post-hatch environment.

The objective of this study was to evaluate the effects of providing a photoperiod light during incubation on early growth performance, production parameters and immune response in broilers. We hypothesized that providing blue and white LED lights for 12 h d^{-1} during incubation have positive effects on body weight gain, food intake, and thermoregulation of newly hatched chicks during an early age and humoral immune response to Newcastle-bronchitis vaccine in Ross 308 broilers.

4.2 Materials and methods

The experimental protocol was carried out in according with the Canadian Council of Animal Care Guideline (CCAC, 2009). Ross 308 hatching eggs were incubated under white (color temperature 4100K, Canarm, Brockville, ON), red (Once Innovations, Plymouth, MN) or blue (Once Innovations, Plymouth, MN) LED lights for 12 h d^{-1} at 200 lux for the entire incubation period, and the dark incubation condition served as control. The study was conducted in four repeated hatch trials (n = 2,176, 1,664, 1,696 and 1,600 eggs for four trials, respectively). In each trial, eggs were randomly assigned to four lighting treatments with two replicate single-stage incubators (ChickMaster G09, Cresskill, NJ). The details of the incubation treatments and hatching performance are given in Li et al. (2021a)

4.2.1 Animals and husbandry

After hatch, broilers were placed into 48 pens with all chicks within a pen from the same incubator. There were 25 ± 1 birds of the same lighting treatment and gender in each pen. The birds were raised under an 18 h d^{-1} photoperiod (0700 to 0100 h, which turned on at the same time as during the incubation period) with a photophase light intensity of 20 lux (Philips F17T8/TL835 17-W fluorescent tubes, color temperature at 3500K) during the first 4 d and gradually decreased to 5 lux on d 9 of age. The brooding temperature was 30-32 °C during the first 6 d. Each pen (2.19 m x 1.00 m) was prepared with new, clean, wood shavings at a depth of 4 cm. The stocking density was 0.08 m^2 $bird^{-1}$. Groups of three pens with the same treatment combination (incubation lighting x gender) from the same incubator were used as an experimental unit for the post-hatch performance portion of the research. The diets were formulated to meet or exceed National Research Council (1994) nutrient requirements. All birds were fed the same diets *ad libitum* within three growth phases. The nutritionally balanced starter diet in crumble form was supplied from d 0 to 14. The grower and finisher diets in pellet form were supplied from d 15 to 25 and d 26 to 35, respectively. The starter diet was provided in a plastic 50 cm trough feeder (Little Giant®, Miller Manufacturing, Eagan, MN) during the first 5 d after placement. After d 5 of age, feed was provided from tube feeders. Water was provided *ad libitum* from two nipple drinkers per pen.

4.2.2 Broiler growth performance

Following completion of chick processing at the hatchery (at 1 p.m.), chicks were group weighed and placed in stages with a 5 min interval between pens. Each round of placement consisted of all eight treatment combinations to minimize the effects of placement time. Chicks were placed on the litter behind the water line (Figure 4.1)

without guiding them to find feed and water. At exactly 6 h post placement, body weight and the remaining feed were weighed in the same order as placement to calculate the feed consumption and body weight gain during the first 6 h of access to feed and water. Chicks in each pen were weighed every 24 h and feed consumption was measured daily during the first 7 d. The birds were group weighed on d 14, 25 and 35. The feed remaining in the feeders was weighed on each weigh day and as mortality occurred. Mortality was recorded and sent to a veterinary pathologist for necropsy (Animal Health Laboratory, Truro, NS, Canada). Growth performance was evaluated using feed consumption, body weight, body weight gain and feed conversion ratio (FCR).

4.2.3 Body temperature

Cloaca temperature of 5 randomly selected chicks per pen was measured with a digital thermometer (Braun IRT-4020, Braun®, Kronberg, Germany) at 24 h post-hatch (8 AM). This process was repeated 12 h later (at 8 PM).

4.2.4 Vaccination, organ and blood Sampling

Six birds per pen received a AviPro® Newcastle-Bronchitis vaccine (B1 Type, B1 Strain, Mass. and Conn. Types, Live Virus, Elanco, Guelph, ON) via topical ocular at one drop/dose/chicken on d 10 and a booster vaccine was applied on d 21 in the second, third and fourth trials, according to the manufacturer's instructions.

One vaccinated chicken per pen (3 birds per experimental unit) was weighed individually at d 10, 14, 21, 25 and 35, and euthanized with an electric stunning knife. Blood samples were collected from the jugular vein immediately after euthanasia. Approximately 5 mL of blood was collected into nonheparinized tubes, incubated at room temperature for 4 h,

and centrifuged at 2,000 *x g* for 10 min. The serum was separated and stored at -80°C until further analysis. The liver, spleen and bursa of Fabricius were excised and weighed and expressed on a relative body weight basis (g kg^{-1}). A drop of blood collected from birds at d 35 of age was smeared on a glass slide. The smears were fixed and stained using Hema-3 (Fisher Science). One hundred leukocytes per slide were counted twice and the heterophil to lymphocyte (H/L) ratio was calculated.

4.2.5 Serum immunoglobulin

The total IgG concentration in serum was measured by using chicken IgG sandwich enzyme-linked immunosorbent assay (ELISA) quantitation and Starter Accessory Kits (Bethyl Laboratories, Montgomery, TX) following the manufacturer's procedure. The plates were read using a microplate reader (Bio-Tek Instrument Inc., Wonooski, VT) at 450 nm. The four-parameter logistic model was found to be the best model to describe the IgG concentration and absorbance readings. The concentration of serum IgG was calculated. The inter- and intra-assay % CV were both under 5%.

4.2.6 Statistical analysis

The experiment was a randomized complete block design with a set of 4 incubators as one block (2 blocks per trial). Three pens of birds hatched from the same incubator per trial were used as the experimental unit with 8 blocks for each treatment combination (lighting x gender). One incubator (dark-control) in trial 2 was not functioning as consistently as the other seven incubators and was removed from the analysis. Growth performance during the first 6 h, vent temperature on d 1 of age, organ development, H/L ratio, and serum IgG concentration were analyzed using the Mixed Procedure of the SAS

v. 9.4 (SAS Inc., Cary, NC, 2013). All broiler body weight, feed consumption, body weight gain and feed conversion ratio were analyzed as repeated measures by using the Mixed Procedure of SAS v. 9.4 (SAS Inc., Cary, NC, 2013). In repeated measure analysis, four covariance structures, compound symmetry, variance components, first order autoregressive and unstructured covariance, which provided the smallest corrected Akaike Information Criterion and Bayesian Information Criterion absolute values, were selected to conduct the ANOVA test. The residuals of error met the assumptions of normal distribution, independently distribution with mean zero and had constant variance. In all cases, if significant effects were found, the Tukey-Kramer test was applied to differentiate the means at 5% level of significance.

4.3 Results and discussion

4.3.1 Broiler growth performance during an early age

Chick body weight at the time of placement and FCR during the first 6 h were not affected ($P>0.05$) by the lighting treatment, gender or their combinations. Body weight ranged from 44.3 to 44.7 ± 0.21 g bird^{-1} at placement. However, the body weight following 6 h of accessing feed and water was affected ($P<0.05$) by the incubation lighting treatment (Table 4.1). Chicks hatched from white and blue light illumination during incubation had significantly higher body weight at 6 h than those hatched under dark, and red light group was intermediate. Body weight gain during the first 6 h post placement was affected ($P<0.05$) by lighting treatments and gender (Table 4.1). Chicks hatched under white light with 12 h d^{-1} had significantly higher body weight gain than those hatched under dark. Females had higher ($P<0.05$) body weight gain than males.

Furthermore, higher ($P<0.05$) feed intake was found during the first 6 h in white and blue groups than control (Table 4.1).

Providing photoperiodic white lighting during incubation positively affected the chick growth during the initial part of the growing period in the current study. The feeding and exploratory behavior might be affected by the entrained circadian rhythms during incubation and lead to higher feed intake and body weight gain. Archer et al. (2009) reported that chicks hatched under dark had lower feed intake than those hatched with light exposure. The physiology and behavior of newly hatched chicks change rapidly from hour to hour during the post-incubation period (Balážová and Baranyiová, 2010). Balážová and Baranyiová (2010) reported that male chicks had higher visual orientation behavior, while females had higher horizontal locomotor activity at d 3 of age during the first 10 min exposure to a novel environment. Our study also found that during the first 6 h in the grow-out pen, female chicks had higher body weight gain than males, which is considered increased exploratory behavior related to learning to eat. In order to test the hypothesis of behavior changes from incubation lighting treatment, an open-field behavior test would be useful to examine the response of newly hatched chicks to a novel environment in a future study. The mechanism of light illumination during incubation improving growth rate in neonatal chicks is still unclear. The higher body weight gain could be due to a higher amplitude of growth hormone secretion, which can be influenced by melatonin. There is evidence indicating melatonin influences growth by modulating growth hormone synthesis post-hatch in avian species. A positive correlation between plasma melatonin concentration and growth hormone has been reported for broilers exposed to monochromatic light (Zhang et al., 2016). Exogenous melatonin

administration increased plasma growth hormone concentration in Japanese quail (Zeman et al., 1999), mature pigeon (McKeown et al., 1975) and turkey (Fehrer et al., 1985). The development and maturation of photoreceptors starts during incubation. The enzymes involved in melatonin synthesis become functional during incubation and reach a peak before hatch (Espinar et al., 1994). Providing photoperiod for the whole incubation period resulted in a higher blood melatonin level in broiler chickens than those incubated under dark when placed in an environment with continuous light (Özkan et al., 2012). It is also possible that providing a photoperiod during incubation can lead to cerebral lateralization of visual pathways and alter the post-hatch feeding behavior (Roger, 2008). Archer and Mench (2014a) found the melatonin rhythm on d 19 of incubation had a carry-over effect on the rhythmicity of general activity over a 6-wk growth period in broilers. Furthermore, increased plasma growth hormone and prolactin levels in broiler embryos have been found with *in ovo* green light exposure (Dishon et al., 2017). But it is important to note that the light regime applied during incubation in their study was 15 min light/ 15 min dark to avoid overheating of the eggs. A short duration of light exposure (3 min) during the scotophase decreased melatonin production by inhibiting NAT activity in rats (Klein and Weller, 1972). For avian species, as little as 5 min light exposure during the dark phase decreased NAT activity in both the retina and pineal gland and the suppressive effect of light exposure was spectrum related (Zawilska et al., 1995). Zawilska et al. (1995) reported that NAT activity in the pineal gland decreased by approximately 20% when exposure to white or green light for 5 min and NAT activity started to increase after 15 min of returning to darkness and reached the control level by 1 h for red light and 2 h for blue and green light. Pineal NAT activity was still lower than

the control level after 2 h when chicks were exposed to white light for 5 min. Our study found that chicks hatched under white light had higher body weight gain at 6 h than those hatched under dark, which supports the hypothesis that providing photoperiodic blue light during incubation would positively affect body weight gain and feed consumption for young chicks.

Body weight was measured every 24 h during the first week to investigate the effects of photoperiodic light during incubation on growth performance during the brooding stage. Average body weight during the first week was significantly affected ($P<0.05$) by the incubation lighting treatments (Table 4.2). No differences ($P>0.05$) in body weight gain, feed intake or FCR were found among lighting treatments or their combination with other factors (gender or day). The white and blue groups had higher ($P<0.05$) body weight than those incubated under dark (Table 4.3). Chicks in red group had an intermediate average body weight during the first week as we found the same trend at 6 h post placement. Continuous green light stimulation during incubation has been shown to accelerate broiler weight gain and pectoral muscle weight by d 6 of age without improving feed intake (p-value: 0.08) and FCR (p-value: 0.93) (Zhang et al., 2016). It is possible that increased weight gain was associated with enhancing proliferation of skeletal muscle satellite cells by both green and blue light stimulation (Halevy et al., 1998). In our case, providing an *in ovo* photoperiod with shorter wavelength (blue light) may provide stronger stimulation to the photoreceptor cells than red light and dark, and entrained the rhythmic expression of core clock genes. Di Rosa et al. (2015) found that zebrafish larvae illuminated with blue light had higher *clock1* and *per1b* expression during the scotophase than those under white, red and dark on d 7 post-fertilization. The circadian rhythmicity

is the output of rhythmic expression of core clock genes by positive and negative transcriptional feedback (Reppert and Weaver, 2002). The circadian rhythm of pineal melatonin synthesis can provide the internal temporal cue to the target tissue and regulate the production of somatotropic axis hormones (growth hormone and insulin-like growth factor-I) and the expression of growth hormone receptors (Dishon et al., 2017).

The source, spectrum, intensity and photoperiod are artificial light characteristics influencing modern poultry management. The effects of light spectra on chicken production, health and welfare have been well documented (Rozenboim et al., 2004b; Xie et al., 2008a; Xie et al., 2008b; Sultana et al., 2013; Li et al., 2015, Dishon et al., 2017). Broilers reared under blue light, green light or their combination had higher body weight than those under white or red light (Rozenboim et al., 1999; Rozenboim et al., 2004b). Similar effects were reported for turkeys (Rozenboim et al., 2003). In broiler production, constant or near-constant light was commonly applied during an early age to maximize the visual access to feed and water. Early studies showed a reduction in feed consumption and body weight gain immediately after introduction of a dark period (Classen and Riddell, 1989). However, the impact of introducing darkness on growth rate decreased with a longer introduction time. Riddell and Classen (1992) found that male broiler chickens raised under continuous light had higher body weight gain than those with photoperiods before d 42, especially during the first 21 d. However, at d 63 of age, the birds reared under increasing lighting treatment, which gradually increases the light period from d 7 to d 42, were heavier than those reared under continuous light. Rozenboim et al. (1999) also found a higher body weight gain after d 49 of age when broilers were reared under an increasing light schedule (23L:1D from d 1 to d 4, 8L:16D

from d 5 to 14, then gradually increased to 16L: 8D by d 48). These results suggested that an increased duration of darkness resulted in lower initial body weight gain, followed by a faster compensatory growth later in the grow-out period. In addition, embryonic light stimulation has been shown to result in decreased stress level with lower corticosterone response to crating stressor (Archer and Mench, 2014b). Our study showed that providing white and blue photoperiodic light during incubation can effectively introduce the day-night cycle even before hatch and minimize the effect of introducing darkness post-hatch, as improved chick growth was found during the first week.

4.3.2 Overall growth performance

Providing white, blue or red light for 12 h per day during incubation did not affect (P>0.05) body weight, body weight gain, feed consumption or FCR after one week of age (Table 4.4). In the current study, the chicks hatched under lighting treatments lost their weight advantage after d 7 of age (Table 4.5), which may be due to the presence of grow-out photoperiod modulating rhythms of somatotropic axis hormones. Many studies have investigated the effects of providing light during incubation on post-hatch growth performance, but the effects remain inconclusive. Some studies reported that light stimulation during incubation did not affect post-hatch production parameters in broilers (Archer et al., 2009; Archer and Mench, 2014a; Huth and Archer, 2015; Archer, 2017) and Japanese quail (Sabuncuoglu et al., 2018; Hanafy and Hegab, 2019). In contrast to our results, improved body weight at market age by incubation with light was found in broilers (Zhang et al., 2016; Van der Pol et al., 2017) and turkey hens (Rozenboim et al., 2003). Interesting results were found by Zhang et al. (2012), who reported that continuous green light stimulation during incubation increased the body weight and feed

intake post-hatch, as compared to dark and blue light. The enhanced production parameters may be associated with increased number of satellite cells and proliferation and differentiation of myoblasts. The stimulatory effect on post-hatch muscle growth depends on light spectrum, but not depend on photoperiod (Halevy et al., 2006). In addition, the transmittance of light through the shell may be influenced by light characteristics, spectrum and intensity, as well as characteristics of the eggshell, thickness and pigmentation. Further studies are needed to investigate the light characteristics of different wavelengths penetrating through the eggshell and received by chicken embryo and whether blue light and green light are involved differently in regulation of growth hormone secretion in broilers. The combination of embryonic and post-hatch lighting regimen would be a valuable topic to investigate the long term effects of light components on bird health, behavior and growth performance.

Total mortality from d 1 until d 35 was 2.55%, and it did not differ (P>0.05) among incubation lighting treatments and did not interact with gender.

4.3.3 Body temperature

An important finding from our study was the cloaca temperature at 36 h post-hatch was affected by provision of photoperiodic lighting during incubation. No differences in cloaca temperature were found among treatments at 24 h post-hatch (Table 4.6). However, the cloaca temperature at 36 h post-hatch was lower (P<0.05) in chicks hatched under dark than those hatched under white, red or blue light (Table 4.7). Birds maintained a relatively stable core body temperature via regulating a variety of thermoregulatory responses to support the balance between heat production and heat loss (Yahav, 2015). Newly hatched birds may require more than 3 d for endothermic thermoregulation to be

fully functional (Wiebe and Evans, 1994). The ability to maintain a stable body temperature at young age may be due to an advanced embryo development and had a better ability to balance heat production and loss. Increased feed intake has been found to be associated with higher heat production (Zhou and Yamamoto, 1997). Heat conserving behavior may also account for the lower feed intake in chicks hatched under dark as being clustered to avoid heat loss may reduce the frequency of feeding behavior. The feeding behavior of neonatal chicks can be regulated by the daily rhythmic expression of orexigenic and anorexigenic neuropeptides in the hypothalamus (Furuse et al., 2001; Kaiya et al., 2009; Mishra et al., 2016). In addition, melatonin synthesis and secretion can regulate thermoregulatory mechanisms and energy metabolism in birds (Saarela and Heldmaier, 1987; Zhou and Yamamoto, 1997; Murakami et al., 2000; Isobe et al., 2002). Underwood and Edmonds (1995) reported that body temperature and activity were synchronized by cyclic administration of melatonin via drinking water in Japanese quail. The entrained circadian rhythm in body temperature was maintained up to 12 d. *In ovo* light stimulation changes the rhythmic secretion of melatonin and it may have an effect on thermoregulation in the bird after hatch. Our findings relate to the results obtained by Sinkalu et al. (2015) that broilers treated with exogenous melatonin via drinking water had lower and less fluctuating cloaca temperatures than those reared under continuous light from d 14 to 42 of age. The smaller differences in cloaca temperature between 24 and 36 h post placement in birds hatched under photoperiodic light illumination indicated that lighting treatments during incubation may have positive effects on thermoregulation of neonatal chicks. Maintenance of a relatively stable core temperature provides a protective internal environment for efficient functioning of the cells, tissue and organs

(Nakamura, 2011). Although we did not measure the body temperature continuously during the brooding stage, considering the higher feed intake after placement and improved growth performance, we speculated that providing photoperiodic light stimulation during incubation increased the metabolic rate and heat production in broiler chicks during the photophase at a young age.

To the best of our knowledge, this is the first report on evaluating the effects of photoperiodic light stimulation during incubation on growth performance at placement, body temperature and daily chick growth in broilers during the first week of production. The positive effects on chick growth demonstrated that providing photoperiodic blue and white light illumination during incubation can improve the adaptation of newly hatched chicks to grow-out environment. The effects were not found after d 7 of age, which may be a result of establishement of new circadian rhythm, which is entrained by the longer photoperiod during grow-out period.

4.3.4 Organ weight, H/L ratio and total IgG

Relative spleen weight was only affected ($P<0.05$) by incubation lighting treatments on d 10 of age (Table 4.8). No differences ($P>0.05$) in relative spleen weight were found among dark and light color treatments. We only found that birds hatched under red light had heavier ($P<0.05$) relative spleen weight than those hatched under blue light (Table 4.9). Most photostimulation studies on immune function have focused on photoperiod or day length. Generally, a longer dark period increased the immune response and it was associated with increased melatonin secretion. Kliger et al. (2000) found increased splenic lymphocyte proliferation when broilers were given an intermittent lighting schedule compared to constant light exposure post hatch. We did not find an accelerated

spleen development as measured by weight between dark and incubation lighting treatments. The relative weight of spleen and bursa of Fabricius can be affected by environmental factors, such as light (Li et al., 2015) and temperatures (Quineteiro-Filho et al., 2010). Lighter relative spleen and bursa of Fabricius were found in broilers exposed to a heat stress environment (Pardue et al., 1985). We hypothesized that birds hatched under red light may have higher cell proliferation in spleen than stimulated with short wavelength such as blue light according to the relative spleen weight on d 10. Heterophil to lymphocyte ratio (H/L) is used as an index to evaluate levels of stress in poultry. This ratio can increase during stressful situations, including environmental changes such as social stress (Gross and Siegel, 1983) or even lighting programs (Huth and Archer, 2015). A higher H/L ratio in blood was found when birds were raised under constant light compared to 12 h d^{-1} light treatment (Zulkifli et al., 1998). No differences in H/L ratio ($P>0.05$) among incubation lighting treatments in the current study suggest that providing light illumination up to 12 h d^{-1} did not induce stress level during rearing period.

Male birds hatched under red light had heavier ($P<0.05$) relative bursa of Fabricius weight than male birds hatched under blue light on d 35 of age (Table 4.10). No differences ($P>0.05$) in relative bursa of Fabricius weight were found among light color treatments in female broilers. Interestingly, we observed the lower relative bursa of Fabricius weight in day-old male chicks when illuminated with blue light, but no differences in relative bursa of Fabricius weight were found in female birds (Li et al., 2021). Thus far, the effects of incubation lighting on bursa of Fabricius development are still unclear, especially for the difference between males and females. Birds hatched

under red light had higher total IgG concentration than blue, white and dark groups on d 14 of age (Table 4.11). Having green or blue lights in broiler houses having positive effects on both humoral and cellular immune response have been reported by many studies (Xie et al., 2008a; Xie et al., 2008b; Firouzi et al., 2014; Zhang et al., 2014). However, providing light exposure with short wavelength (in this case, blue light) during incubation did not increase total chicken IgG level post vaccination in our study. Differences in animal age, time of light exposure and immunoglobulin specificity may explain differences observed.

In conclusion, our study shows that providing photoperiodic LED light illumination with different colors did not affect production or health parameters of broilers at market age, but we did find that *in ovo* photostimulation with blue or white light resulted in an increased feed consumption and body weight, and smaller difference in cloaca temperatures between morning and evening for chicks at a young age, which gives birds a better start for early post-hatch development. Furthermore, red light may alter humoral immune response to Newcastle-bronchitis vaccine on d 14 of age. The effects of illumination with different colors during incubation on bird performance can provide useful information for hatchery managers when they practice post-hatch feeding programs within the incubator.

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Table 4.1 Effects of providing different colored LED lights during incubation on body weight (g bird^{-1}) at 6 h post placement, body weight gain (g bird^{-1}) (% of placement body weight) and feed consumption (g bird^{-1}) during the first 6 h post placement in broilers.

Light	n[1]	Body weight at 6 h post placement			Body weight gain during the first 6 h post placement			Feed consumption during the first 6 h post placement		
		Male	Female	Average	Male	Female	Average	Male	Female	Average
Dark	7	49.0±0.30	49.0±0.30	49.0±0.22[b]	4.0±0.36 (8.88%)	4.6±0.36 (10.41%)	4.3±0.26 (9.64%)[b]	3.2±0.16	3.4±0.15	3.3±0.11[b]
White	8	50.0±0.28	50.0±0.28	50.0±0.20[a]	5.1±0.34 (11.38%)	5.4±0.34 (12.18%)	5.2±0.24 (11.78%)[a]	3.8±0.14	3.8±0.15	3.8±0.10[a]
Red	8	49.4±0.28	49.5±0.28	49.4±0.20[ab]	4.9±0.34 (11.12%)	5.4±0.34 (12.31%)	5.1±0.24 (11.71%)[ab]	3.5±0.15	3.7±0.14	3.6±0.10[ab]
Blue	8	49.4±0.28	50.3±0.28	49.9±0.20[a]	4.7±0.34 (10.56%)	5.6±0.34 (12.73%)	5.2±0.24 (11.65%)[ab]	3.7±0.15	3.9±0.14	3.8±0.10[a]
Average		49.4±0.14	49.7±0.14		4.6±0.17 (10.48%)	5.2±0.17 (11.91%)		3.6±0.07	3.7±0.07	
Effect		ANOVA P-value								
Lighting (L)		0.006			0.039			0.007		
Gender (G)		0.164			0.017			0.257		
L x G		0.313			0.768			0.883		
Block		<0.0001			<0.0001			<0.0001		

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).
[x,y]Means within a row with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).

Table 4.2 ANOVA table of the effects of providing colored photoperiodic lighting treatments during incubation on first week growth performance in broiler chickens.

Effect	Body weight	Body weight gain	Feed consumption	FCR
Light (L)	0.001	0.7691	0.1496	0.7946
Gender (G)	0.487	0.9139	0.0069	0.0522
L x G	0.164	0.9340	0.5440	0.9402
Day (D)	<.0001	<.0001	<.0001	<.0001
L x D	1.000	0.9999	0.7404	0.9462
G x D	0.430	0.4481	0.3494	0.2466
L x G x D	1.000	1.0000	0.9998	0.9994
Block	<.0001	<.0001	<.0001	<.0001
Covariance structure	Variance components with square root transformation	First order autoregressive	First order autoregressive with log 10 transformation	First order autoregressive with log 10 transformation

Table 4.3 Effects of providing different colored LED lights during incubation on average body weight (g $bird^{-1}$) during the first week in broiler chicks.

Light	n[1]	Male	Female	Average
Dark	7	101.8±0.47	101.2±0.47	101.5±0.34[c]
White	8	103.1±0.44	102.9±0.44	103.0±0.31[a]
Red	8	101.7±0.44	101.9±0.44	101.8±0.31[bc]
Blue	8	101.9±0.44	103.0±0.44	102.5±0.31[ab]
Average		102.1±0.22	102.2±0.22	

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator.

[a,b,c]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 4.4 ANOVA table of the effects of providing colored photoperiodic lighting treatments during incubation on growth performance of broiler chickens during a 35-d period in the 2nd, 3rd and 4th trials.

Effect	Body weight	Body weight gain	Feed consumption	FCR
Lighting (L)	0.3082	0.4891	0.4676	0.7518
Gender (G)	<.0001	<.0001	<.0001	<.0001
L x G	0.9878	0.9579	0.9972	0.9658
Day (D)	<.0001	<.0001	<.0001	<.0001
L x D	0.9983	0.9951	0.9619	0.9700
G x D	<.0001	<.0001	<.0001	0.2820
L x G x D	0.9995	0.7645	0.7267	0.8160
Block	<.0001	<.0001	0.0001	<.0001
Covariance structure	Variance components with log 10 transformation	First order autoregressive with square root transformation	First order autoregressive with square root transformation	Variance components

Table 4.5 Effect of providing different colored LED lights during incubation on body weight (g bird^{-1}) of broiler chickens.

		Day									
Light	n[1]	1	2	3	4	5	6	7	14	25	35
Dark	7	52.8	65.9	83.7	104.2	126.0	153.0	181.3	474.4	1390.4	2523.9
White	8	54.0	67.3	85.4	105.9	127.7	155.0	183.6	484.0	1394.5	2537.2
Red	8	53.4	66.5	84.3	104.8	126.5	153.5	181.0	481.1	1395.3	2539.2
Blue	8	54.0	67.2	85.1	105.3	126.8	154.1	182.5	480.6	1395.3	2535.7
SEM		2.3	2.6	3.6	4.2	3.8	6.0	9.5	14.3	72.1	176.2

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator.

Table 4.6 Effects of providing different colored LED lights during incubation on cloaca temperature (°C) at 24 h post hatch in broiler chickens.

Light	n[1]	Male	Female	Average
Dark	7	39.9±0.03	40.0±0.03	39.9±0.02
White	8	39.9±0.03	40.1±0.03	40.0±0.02
Red	8	39.9±0.03	40.0±0.03	39.9±0.02
Blue	8	39.9±0.03	40.1±0.03	40.0±0.02
Average		39.9±0.02[b]	40.1±0.02[a]	
Effect		ANOVA P-value		
Lighting (L)		0.180		
Gender (G)		<0.0001		
L x G		0.963		
Block		<0.0001		

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator.
[a,b]Means within a row with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).

Table 4.7 Effects of providing different colored LED lights during incubation on cloaca temperature (°C) at 36 h post hatch in broiler chickens

Light	n[1]	Male	Female	Average
Dark	7	39.7±0.04	39.8±0.04	39.7±0.03[b]
White	8	39.8±0.04	40.0±0.04	39.9±0.03[a]
Red	8	39.8±0.04	40.0±0.04	39.9±0.03[a]
Blue	8	39.9±0.04	39.9±0.04	39.9±0.03[a]
Average		39.8±0.02[y]	39.9±0.02[x]	
Effect		ANOVA P-value		
Lighting (L)		0.001		
Gender (G)		<0.0001		
L x G		0.171		
Block		<0.0001		

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).
[x,y]Means within a row with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 4.8 ANOVA table of the effects of providing colored photoperiodic lighting treatments during incubation on relative organ weight of broiler chickens during a 35-d period in the 2nd, 3rd and 4th trials.

Effect	Day 10			Day 14			Day 21			Day 25			Day 35		
	Spleen	Bursa	Liver	Spleen	Bursa	Liver	Spleen	Bursa	Liver	Spleen	Bursa	Liver	Spleen	Bursa	Liver
Lighting (L)	0.038	0.388	0.566	0.901	0.817	0.281	0.662	0.304	0.700	0.305	0.498	0.101	0.358	0.160	0.827
Gender (G)	0.457	<.0001	0.009	0.393	0.006	0.264	0.354	0.052	0.556	0.629	0.011	0.294	0.123	0.019	0.002
L x G	0.485	0.810	0.254	0.539	0.518	0.924	0.543	0.744	0.815	0.053	0.157	0.275	0.939	0.006	0.179
Block	0.013	0.002	0.238	0.644	0.514	0.111	<.0001	0.0009	0.002	0.084	0.004	0.001	0.073	0.014	0.010

Table 4.9 Effects of providing different colored LED lights during incubation on relative spleen weight (g kg^{-1}) of broiler chickens on day 10 of age in the 2nd, 3rd and 4th trials.

Light	n[1]	Male	Female	Average
Dark	5	0.70±0.033	0.73±0.033	0.72 ± 0.024^{ab}
White	6	0.68±0.033	0.66±0.033	0.67 ± 0.024^{ab}
Red	6	0.78±0.033	0.70±0.033	0.74 ± 0.024^{a}
Blue	6	0.65±0.033	0.64±0.033	0.65 ± 0.024^{b}
Average		0.70±0.017	0.68±0.017	

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator in trial 2, 3 and 4.

[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).

Table 4.10 Effects of providing different colored LED lights during incubation on relative bursa of Fabricius weight (g kg^{-1}) of broiler chickens on day 35 of age in the 2nd, 3rd and 4th trials.

Light	n[1]	Male	Female
Dark	5	1.69±0.093^{ab}	1.32±0.093^{b}
White	6	1.66±0.084^{ab}	1.51±0.084^{ab}
Red	6	1.74±0.084^{a}	1.44±0.084^{ab}
Blue	6	1.30±0.084^{b}	1.53±0.084^{ab}
Average		1.60±0.043^{x}	1.45±0.043^{y}

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator in trial 2, 3 and 4.
^{a,b}Means within a column with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).
^{x,y}Means within a row with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).

Table 4.11 Effects of providing different colored LED lights during incubation on serum IgG concentration (ng mL^{-1}) of broiler chickens on day 14 of age in the 2nd, 3rd and 4th trials.

Light	n[1]	Serum IgG concentration
Dark	5	547898±33381[b]
White	6	601279±29660[b]
Red	6	721158±29660[a]
Blue	6	570200±29660[b]
Effect	**ANOVA P-value**	
Lighting (L)	0.0015	
Gender (G)	0.2871	
L x G	0.0887	
Block	0.0115	

[1]Number of experimental units. Experimental unit = 3 pens of birds hatched from the same incubator in trial 2, 3 and 4.
[a,b,c]Means within a column with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$)

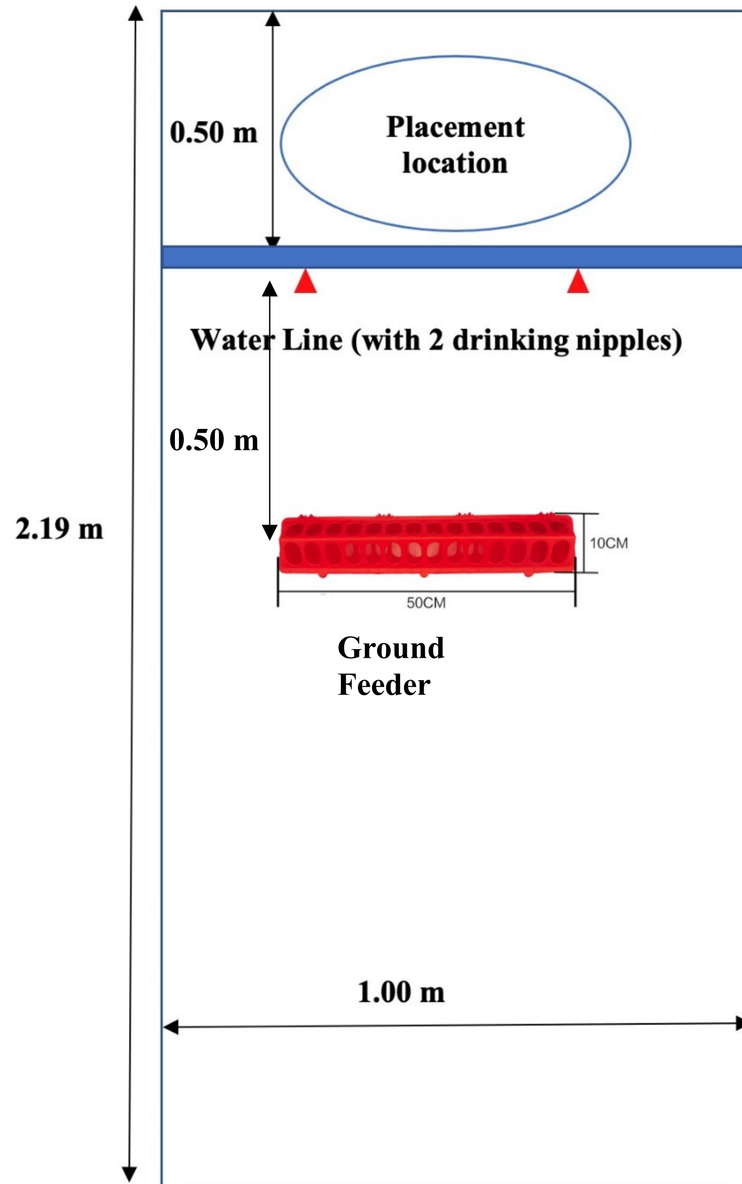

Figure 4.1 Floor pen dimension and chick placement location

CHAPTER 5. EFFECT OF PHOTOPERIOD DURING INCUBATION ON EMBRYONIC TEMPERATURE, HATCH TRAITS AND PERFORMANCE OF TWO COMMERCIAL BROILER STRAINS*

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5.1 Abstract

Provision of light during incubation has shown the potential to enhance hatching traits and affect post-hatch productivity, physiology and behavior. In this study, two repeated trials were conducted to investigate the effect of photoperiod and strain on the embryo temperature, hatching traits and post-hatch growth performance of two commercial strains of broilers (Ross 308 and Cobb 500). In each trial, hatching eggs were randomly distributed into six incubators with 3 photoperiod treatments: blue LED light for 12 h d-1 (12L:12D) or 18 h d-1 (18L:6D) during entire incubation were compared with no illumination condition (DARK). Data were analyzed as a 3 x 2 factorial arrangement with the trial as the blocking factor. Embryos incubated under 12L:12D and 18L:6D had lower air cell temperature ($P<0.05$) than the DARK embryos from d 13 of incubation onwards except on the day of candling. The response of air cell temperature to periodic illumination differed between two strains. Cobb embryos had lower air cell temperature in 12L:12D than those incubated with 18L:6D from d 16 of incubation onwards, whereas lower air cell temperature was found in Ross embryos when illuminated with 18L:6D photoperiod compared to those under 12L:12D. The 12L:12D treatment was associated with improved ($P<0.05$) navel closure condition of hatchlings. There were no differences in hatchability, embryo mortality, body weight or length at hatch among photoperiod groups or its combination with strain. No differences in production parameters were found between DARK and illuminated groups. However, 12L:12D had heavier ($P<0.05$) body weight on d 14 of age and higher ($P<0.05$) body weight gain than 18L:6D from d 7 to 14 of age. The results of this study indicate that providing blue LED light up to 18 h d-1 has no detrimental effect on production of broilers, however, 12L:12D light regime

improved chick quality at hatch compared to DARK and resulted in heavier birds by d 14 compared to 18L:6D.

Key words: incubation lighting, photoperiod, embryo temperature, chick quality, productivity

5.2 Introduction

The main goals of a hatchery are to obtain optimal hatchability, better uniformity and more chicks with good quality for the poultry farm. A good quality hatchling is critically important for successful poultry production. The effects of several environmental variables including temperature, relative humidity, egg turning and air composition on embryo development, hatching traits, bird health and productivity have been examined in-depth (Decuypere et al., 1979; Yahav et al., 2004; Bergoug et al., 2013). Several studies have reported that *in ovo* illumination could play an important role in circadian rhythm entrainment and accelerating the embryonic development (Zeman et al., 2004; Yu et al., 2018; Drozdova et al., 2019). However, the effect of *in ovo* illumination with graded photoperiods on chick quality and post-hatch performance is still underexplored. In natural incubation, chicken embryos can receive a certain degree of light exposure when the hen leaves to feed/drink or turn the eggs (Archer and Mench, 2014a). However, most hatcheries incubate eggs and hatch chickens under dark condition, which leads to limited or no access to light for chicken embryos. Chickens are equipped with the most complex circadian time measurement system among all animals with the presence of photoreceptors and oscillators in multiple organs and tissues (Gwinner et al., 1997). Light can be transformed into neuroendocrine signals through phototransduction pathways to the suprachiasmatic nucleus of the hypothalamus (SCN) or perceived by the deep brain photoreceptors located in the hypothalamus directly (Kumar, 2015). Therefore, periodic illumination during incubation has received increasing attention as an important environmental stimulus for embryo development. Early research reported that providing light illumination for the entire incubation period accelerates embryo development and

shortens incubation time without affecting hatchability or body weight at hatch (Siegel et al., 1969; Walter and Voitle, 1972; Lauber, 1975). It has been speculated that the response might be a confounding effect of light and heat production from the conventional light source. With the advancement of lighting technology, the heat effect could be excluded by using light emitting diodes (LED). LED is a great light source of illumination with low energy consumption, extended lifespan and can provide both full spectrum and monochromatic colors with minimum heat production.

The intensity, wavelength and duration of photoperiod are three main adjustable components when providing artificial lighting in animal production. Evidence suggests that embryo development and post-hatch growth can be affected by the light spectrum. Photoperiodic illumination during incubation can stimulate melatonin synthesis and improve hatchability and chick quality (Huth and Archer, 2015; Archer, 2017; Archer et al., 2017; Drozdova et al., 2019; Yameen et al., 2020). However, there are contradictory results regarding the effects of *in ovo* photostimulation on embryo development and hatching traits. It was reported that providing illumination for either the first 18 days of incubation or the entire incubation period did not influence or have negative effects on hatchability and chick quality at hatch (Walter and Voitle, 1973; Rozenboim et al., 2003; Shafey et al., 2005; Sabuncuoğlu et al., 2018; Tong et al., 2018; Li et al., 2021a). Regarding spectrum, previous studies found that green light during rearing period stimulated muscle growth during the early stage and blue light accelerated the growth in the later stage (Rozenboim et al., 1999; Cao et al., 2008). Similarly, broiler embryos incubated under continuous or intermittent green light illumination showed accelerated growth during both incubation and posthatch period (Zhang et al., 2012). It was

concluded that shorter wavelength could improve muscle cell proliferation by altering the production of somatotropic axis hormones (growth hormone and insulin-like growth factor-I) and the expression of growth hormone receptors in liver (Rozenboim et al., 2004; Halevy et al., 2006; Dishon et al., 2017). A recent study demonstrated that continuous blue light illumination during incubation had a similar growth-promoting effect as green light (Bai et al., 2016). A higher body weight and pectoral muscle weight were found in the later stage of development when incubated under green and blue light. However, limited information is available regarding the effects of periodic blue light on chick quality and production parameters. Our research group demonstrated that illumination with white and blue light under a 12L:12D photoperiod during incubation improved growth performance and reduced body temperature fluctuation during the brooding period (Li et al., 2021b). The provision of periodic illumination during incubation may stimulate the development of the thermoregulatory system of chicken embryos and potentially make newly hatched chicks more adaptable to rearing environmental changes. Hill et al. (2004) first showed that *in ovo* light-dark cycle stimulation can be served as the zeitgeber to entrain the circadian rhythm in body temperature of hatched chicks. However, it is not known whether periodic illumination with blue light during incubation would entrain the circadian rhythm of embryo temperature before hatch.

Despite the available evidence, it appears to be no research on determining the optimal length of photoperiod with blue LED light for embryo development, hatching traits and growth parameters. Therefore, the present study aimed to investigate the effects of blue light (peak wavelength at 455 nm) with different photoperiods mimicking the temperate

(18L:6D) and tropical (12L:12D) latitudes during incubation on the entrainment of circadian rhythm in body temperature, hatching traits and productivity of two commercial broiler strains. We hypothesized that 1) periodic illumination with blue light could effectively entrain the circadian pacemaker system; 2) air cell temperature could be used as a non-invasive biomarker to monitor the entrainment of circadian rhythm toward the final stage of embryogenesis and would change in anticipation of the photoperiods; 3) periodic photostimulation during incubation would improve chick quality and early productivity with a matching photoperiod during rearing period; 4) the effects of light stimulation on embryo temperature, hatching traits and productivity might be influenced by broiler strains.

5.3 Materials and methods

The experimental design and protocol were carried out in accordance with the Canadian Council of Animal Care Guideline (CCAC, 2009).

Animals husbandry

The experiment consisted of two repeated trials with a total of 2,028 Cobb 500 and 2,124 Ross 308 hatching eggs (Trial 1: 1,086 Cobb 500 eggs from 49-wk breeders and 1,086 Ross 308 eggs from 60-wk breeders; Trial 2: 942 Cobb 500 eggs from 58-wk breeders and 1038 Ross 308 eggs from 35-wk breeders) obtained from a commercial hatchery. Six identical ChickMaster single-stage incubators (ChickMaster G09, Cresskill, NJ) with a maximum capacity of 1,188 eggs were used and operated with standard environmental condition (37.5°C and 55% RH). Eggs were placed on the setter trays and turned to an angle of 90 degrees every 90 minutes from embryonic day (ED) 0 to 18. On ED 18.5, all eggs were candled and eggs with viable embryos were transferred to the hatch basket

without turning and returned to the original incubator until the end of incubation period (512 h of incubation) under the recommended temperature and relative humidity. Following completion of the hatchery processing, chicks were transported approximately 4 km to the Atlantic Poultry Research Center and reared in two identical production room with 48-floor pens. The lighting program during the rearing period was 18 h of light and 6 h of dark through a 34-d period. All chickens were fed the same starter diet (0-14 d) containing 3,000 kcal/kg ME and 23% CP in crumble form, grower diet (15-25 d) containing 3,100 kcal/kg ME and 21.5% CP in pellet form and finisher diet (26-34 d) 3,200 kcal/kg ME and 19.5% CP in pellet form. Water was provided *ad libitum* from two drinking nipples per pen.

Light management

Hatching eggs from each of the strains were randomly distributed into three treatment groups with 2 replicate incubators per trial. The first group was incubated without light illumination as control (**DARK**), and the remaining two groups were illuminated with blue LED light (Once Innovation, Plymoputh, MN) with a photoperiod of 12 h (**12L:12D**) or 18 h (**18L:6D**) per 24 h for the entire incubation period. Four LED strips were mounted on the left side of the incubators and light intensity was adjusted to 200 lux at egg level as measured light intensity over the whole egg setter tray using a digital luxmeter (61-686 Digital Light Meter, Ideal, Ajax, Canada). Lights were turned on at 07:00 am for all 12L:12D and 18L:6D incubators. No other light source was present in the incubation room except for two short periods each day to check eggs using a small head torch with low light intensity (5 lux) red light. The time of checking incubation conditions was on a random schedule from day to day to eliminate potential temporal

cues. In each trial, eggs from each of the strains (Trial 1: 176 Cobb 500 and Ross 308 hatching eggs; Trial 2: 152 Cobb 500 and 168 Ross 308 hatching eggs) were equally placed in four setter trays located in the center of each incubator to provide uniform light distribution.

Air cell temperature

In each trial, an additional 30 eggs within a narrow weight range (±2.50 g) from each of the Cobb and Ross strains were selected for measuring air cell temperature. The selected eggs were randomly placed on the top tray in each of the six incubators. All eggs were spaced within the tray to provide uniform illumination and airflow. On ED 10, all eggs were candled and 4 eggs per strain per incubator with viable embryos were implanted with thermistors (NTC thermistors, Product #: B57551G0103F000, EPCOS, Munich, Germany) in the air cell by drilling an opening with a diameter approximately 2 mm on the blunt end. The opening was then sealed with paraffin wax. Thermistors were connected to a multichannel interface (USB-6001, National Instruments Inc., Austin, TX). Air cell temperature recordings were monitored by using LabVIEW software (LabVIEW 2018, National Instruments Inc., Austin, TX) and recorded in Microsoft Excel at 10 min intervals until external pipping occurred (Figure 5.1) with an air cell temperature drop of more than 2 °C (Romanini et al., 2013). The average of air cell temperature recordings every 24 h from ED 11 to 20 was calculated. Thermistors were disconnected during egg candling for 2 h on ED 18.5.

Embryonic mortality and hatchability

At d 18 and the end of incubation, non-viable eggs were broken open to evaluate fertility status visually and categorized as early (ED 0-7), middle (ED 8-14) and late (after ED 15)

death. The fertility rate was calculated as the percentage of fertile eggs out of total set eggs. The embryonic mortality percentage was calculated out of total fertile eggs. The number of hatched chicks per incubator was counted and weighed when the incubation process terminated. The quality of the hatched chicks was assessed by scoring navel closure condition (Tona et al., 2003) for all hatched chicks and chick length (from the beak tip to the left middle toe including the nail) was measured for 40 chicks per strain per incubator.

Growth performance

Upon arrival at the growing facility, mixed gender chicks per strain hatched from the same incubator were group weighed and placed in stages with 5 min intervals between pens (4 pens/lighting treatment/strain). Each round of placement consisted of all six treatment combinations to minimize the effects of placement time. At exactly 6 h post placement, body weight and remaining feed were weighed in the same order as placement to calculate the body weight gain and feed consumption during the first 6 h of access to feed and water. On d 2 of age, 12 chicks (one chick/lighting treatment/strain/replicate) were euthanized every 3 h over a 24-h period by cervical dislocation (16 chicks per treatment combination in total) and chick weight, yolk sac weight, yolk-free body weight, and liver weight were measured. Birds in each pen were weighed on d 7, 14, 24 and 34. The feed remaining in the feeders was weighed on each weigh day and as mortality occurred. Mortality was recorded and deceased birds were sent to a veterinary pathologist for necropsy (Animal Health Laboratory, Truro, NS, Canada). Growth performance was evaluated using feed consumption, body weight, body weight gain and feed conversion ratio (FCR).

Statistical analysis

The experiment was a randomized complete block design with the trial as a blocking factor, which resulted in a total of 4 replicate incubators per lighting treatment. Incubator was used as the experimental unit for air cell temperature and hatching traits data analysis. Four pens of birds per strain hatched from the same incubator were used as the experimental unit for post-hatch data analysis. No rhythmic changes in air cell temperature were detected between the subjective day and night. Therefore, the average of air cell temperature every 24 h was calculated and analyzed by day. The embryo mortality, air cell temperature at different days of incubation, hatchability, chick quality, organ development and growth performance at different ages were analyzed using ANOVA in a generalized linear mixed model procedure (Proc GLIMMIX) of SAS v. 9.4 (SAS Inc., Cary, NC, 2013) with a statistical model including lighting, strain, 2-way interaction between lighting and strain, and trial. The residuals of error met the assumptions of a normal distribution, independent distribution with mean zero and constant variance. In all cases, if significant effects were found, the Tukey-Kramer test was applied to differentiate the means at 5% level of significance.

5.4 Results and discussion

5.4.1 Air cell temperature

The air cell temperature recordings obtained from 82 out of 96 hatching eggs, which were implanted with thermistors and hatched successfully, were used for embryo temperature analysis. No differences ($P>0.05$) in incubation temperature were observed among photoperiod treatments (Figure 5.2). The effects of photoperiod and strain on air cell temperature were summarized in the actograms (Figure 5.3). Overall, no circadian

rhythm in embryo temperature was detected when illuminated with photoperiodic blue light treatments by 492 h of incubation.

The average air cell temperature from ED 11 to 20 was affected by lighting treatment ($P<0.05$). The DARK group had a higher ($P<0.05$) average air cell temperature than both illuminated groups over the testing period (Table 5.1). Illumination up to 18 h d^{-1} did not cause an over-heating effect on both strain embryos.

Air cell temperature was affected by a two-way interaction between photoperiod and strain on ED 11. Both strains had lower ($P<0.05$) air cell temperature when illuminated with 18L:6D than those under DARK and 12L:12D but no difference ($P>0.05$) in air cell temperature within each of the strains was found between DARK and 12L:12D. The difference in strains showed up in their response to photoperiod. Cobb embryos had a higher ($P<0.05$) air cell temperature than Ross embryos when exposed to 12L:12D. In contrast, the air cell temperature in Cobb embryos was lower ($P<0.05$) than Ross embryos when illuminated with 18L:6D (Table 5.1). On ED 12 and 13, air cell temperature was only affected ($P<0.05$) by the photoperiod. On ED 12, embryos incubated with 18L:6D had a lower air cell temperature than those in the DARK and 12L:12D, but no difference ($P>0.05$) in air cell temperature was found between the DARK and 12L:12D. On ED 13, the DARK had a higher ($P<0.05$) air cell temperature than the 18L:6D, and the 12L:12D group was intermediate (Table 5.1). A significant interaction effect ($P<0.01$) of photoperiod and strain on air cell temperature was found from ED 14 onwards (Table 5.1). Embryos in both illuminated groups had a lower ($P<0.05$) air cell temperatures than the DARK. No difference ($P<0.05$) in air cell temperature of Cobb embryos was found between 12L:12D and 18L:6D from ED 14 to

16. From ED 17 to 20, Cobb embryos illuminated with a longer scotophase (12L:12D) had a lower ($P<0.05$) air cell temperature than those under 18L:6D. Different from the Cobb embryos, Ross embryos had a lower ($P<0.05$) air cell temperature when exposed to a longer duration of photophase from ED 14 to 20 (Table 5.1).

The lower air cell temperature in the 18L:6D at the early embryonic development stage in the current study was unexpected as the efficiency of endothermic reactions is very low (Nichelmann and Tzschentke, 2002). Embryo temperature is the result of a balance between heat production and heat loss. There is limited information regarding the effects of *in ovo* photostimulation on the embryo temperature or the ontogeny of thermoregulation during embryogenesis. Recently a lower rectal temperature was reported in newly hatched chicks when incubated under continuous illumination with blue light as compared to white light when embryos were exposed to 40 °C for 4 h a day from ED 14 to 18 (Zeng et al., 2022). The authors speculated that blue light could reduce heat production by reducing metabolic rate with less yolk material being utilized as an indicator. Another possible mechanism that resulted in a lower air cell temperature is incubation light improving the heat transfer between the egg and the environment by changing eggshell conductance. van der Pol et al. (2017, 2019) reported that *in ovo* photostimulation improved bone growth and leg health. The periodic illumination may increase the carbon dioxide concentration and eggshell conductance via calcium bicarbonate mobilizing from the eggshell. Increased vascularization of chorioallantoic membrane (CAM) during the second week of incubation was reported when chicken embryos were exposed to hypoxia (Strick et al., 1991). Increased CO_2 concentration tends to achieve similar effects as hypoxia and may enhance the growth of blood vessels

in CAM to improve heat transfer between the egg and incubation environment. The slight difference in incubation air temperature and ventilation can also affect evaporative heat loss. Unfortunately, the role of photostimulation on heat production and heat loss during embryogenesis remains poorly understood. Monitoring temperature distribution between the incubation chamber and the air cell temperature under photoperiodic condition could shed more light on this phenomenon.

The lower air cell temperature found in both illuminated groups after ED 14 could be attributed to three mechanisms: 1) Photostimulation during embryogenesis may increase the hypothalamus-pituitary-thyroid (HPT) and hypothalamus-pituitary-adrenal (HPA) axes activity. The HPT and HPA axes of chicken embryos become functional at around ED 14 and play major roles in the thermoregulation of chickens via mobilizing internal energy reserves (Thommes and Hylka, 1977; Jenkins and Porter, 2004). An increased plasma T4 was observed when chicken embryos exposed to a cold temperature (25°C) after ED 12 (Thommes et al.,1988). The anaerobic glucose catabolism increased during the last phase of incubation due to the limitation of available oxygen. Periodic illumination may reduce metabolic rate of chicken embryos by altering the hypothalamus-pituitary pathways; 2) Periodic illumination during incubation may stimulate the development of the thermoregulatory system by increasing the number of thermal sensing neurons. Providing photostimulation during incubation increased brain weight and neuron numbers in the hypothalamus of chicken embryos (Abdulateef et al., 2021); 3) Melatonin synthesis may provide a confounding effect on regulating embryo temperature. A light-dark illumination during incubation stimulated melatonin synthesis during the scotophase and reduce heat production by depressing the metabolism of lipid

and protein (Zeman et al., 1993). Hill et al. (2004) found that providing only one 24-h light-dark cycle as early as ED 13 was sufficient to entrain the circadian rhythm of body temperature during the first 5 d post-hatch under continuous light illumination. Study has shown that day-night differences in melatonin synthesis can be detected in isolated pineal cells from 13-d-old embryos under light-dark illumination (Akasaka et al., 1995). This finding concurs with the result of our study with no differences in air cell temperature between DARK and 12L:12D before ED 13. The observation that embryos in 12L:12D had a lower air cell temperature than 18L:6D after ED 17 suggests that longer duration of darkness can result in higher melatonin production and reduce metabolic heat production. No differences ($P>0.05$) in air cell temperature were detected between the two strains when incubated under DARK for the majority of the incubation period. When illuminated with 12L:12D, Ross embryos had higher ($P<0.05$) air cell temperature than Cobb embryos from ED 16 onwards. However, illumination with blue light for 18L:6D resulted in the opposite response for two broiler strains. A higher ($P<0.05$) air cell temperature in the Cobb embryo was found from ED 15 to ED 20 (Table 5.1). The differences in air cell temperature response to photostimulation between the two strains might be related to the shell characteristics, nutrient deposition in the eggs or the sensitivity to light. Abdo et al. (2017) reported that Cobb chickens kept under blue light showed a better ability to regulate body temperature than those illuminated with white light when under heat stress, whereas, no difference in rectal temperature were observed in Ross chickens when illuminated with white and blue lights. Compared to white light, blue light lowered the liver MDA content and the responses to heat stress differed between two commercial broiler strains. Ross chickens had a lower antioxidant enzyme activity and MDA content

than Cobb chickens when under heat stress condition. Blue light also induced upregulation of heat shock protein (HSP) 70 in both strains, while increased HSP 90 expression was only detected in Cobb chickens under heat stress (Abdo et al., 2017). Those findings demonstrated that there is a variation in the response to blue light stimulation between the two broiler strains. The data from the current study provide the first evidence that the interaction effect on air cell temperature is independent of egg weight in these two strains as all eggs used for air cell temperature measurement were within a narrow weight range and incubated in the same incubators. The variation in air cell temperature responses to photoperiod treatments suggests that perhaps an optimal lighting program during incubation needs to be established for different strains in order to maintain a more consistent embryo temperature and obtain chicks with better quality. The changes in air cell temperature on ED 18, when eggs were exposed to room temperature for a 30-min candling process, are interesting. On ED 17, embryos of both strains incubated under DARK had a higher ($p<0.05$) air cell temperature compared to the illuminated groups. However, Cobb embryos illuminated with the 18L:6D had a higher ($P<0.05$) air cell temperature than 12L:12D group, and the air cell temperature of both illuminated groups was higher than those incubated under DARK on ED 18. Ross embryos incubated with 12L:12D had a higher air cell temperature than the DARK on ED 18. The air cell temperature of Ross embryos in 18L:6D group remained the lowest among all treatments (Table 5.1). The results suggest that *in ovo* photostimulation may improve the thermoregulation of Cobb embryos with a better ability to maintain embryo temperature when exposed to a suboptimal temperature. The current study provided novel information of how embryo temperature of broiler strains responds to periodic blue

lighting during incubation. Additional studies that involved monitoring oxygen consumption and carbon dioxide production can provide direct evidence of metabolic rate changes during embryogenesis and evaluate the effect of *in ovo* photostimulation on heat production.

5.4.2 Hatchability and chick quality

No differences (P>0.05) in egg weight at set, egg weight loss % from ED 0-18, fertility rate, hatchability of fertile, or embryonic mortality were found among incubation lighting treatments or its interaction with strain. These findings are in agreement with other researchers (Özkan et al. 2012a; Archer and Mench, 2014b; van der Pol et al., 2019; Geng et al., 2021) who found that light stimulation with different photoperiods during incubation had no impacts on embryonic growth, hatchability or body weight at hatch. In contrast, some studies reported that provision of light during incubation had a positive effect on hatchability of fertile eggs due to lower embryonic death (Shafey and Al-mohsen, 2002; Shafey, 2004; Archer, 2018). Lower embryonic death might be associated with accelerated growth with increasing cell population when illuminated with light at high light intensity during the first 40 h of incubation (Ghatpande et al., 1995). However, the total embryonic mortality (11.90%, 18.55% and 20.07% in Shafey and Al-mohsen, 2002, Shafey, 2004 and Archer, 2018,, respectively) in those studies was higher than the current study (8.82%). Light stimulation during incubation may provide a benefit in reducing early embryonic mortality of eggs with poor quality.

Body weight or chick length at hatch did not differ (P>0.05) among lighting treatments or its interaction with strain (Table 5.2). Similar findings were reported in broilers (Özkan et al. 2012a; Zhang et al., 2012), layers (Archer et al., 2017) and Japanese quails

(Sabuncuoğlu et al., 2018). Body weight, chick length and navel condition at hatch were affected ($P<0.05$) by strain (Table 5.2), which was expected since there is a difference in initial egg weight between strains. Egg weight is commonly correlated with chick weight. In the current study, Ross chicks had a lower weight and a shorter chick length but a better navel closure score than chicks in the Cobb strain. The heavier egg with a larger yolk may contribute to the poorer navel condition found in Cobb chicks at hatch as a larger yolk is more difficult to fully withdraw into the body cavity than a smaller yolk. Navel closure condition was affected ($P<0.05$) by the photoperiods. Chicks in the 12L:12D group exhibited a superior navel condition with a higher percentage ($P<0.05$) of chicks that scored 1 (closed and clean navel area) and fewer chicks scored 2 (black button up to 2 mm or black string) compared to the DARK group. The navel closure condition in the 18L:6D group was intermediate. No difference ($P>0.05$) in the percentage of chicks with the poorest navel condition was found among lighting treatments (Table 5.2). Overall, providing a 12L:12D lighting schedule during incubation resulted in an improved navel closure condition compared to the conventional dark condition. Improved navel quality of newly hatched chicks has been previously observed when hatching eggs were exposed to a periodic illumination (Huth and Archer, 2015). Poor navel quality is an indicator of inefficient nutrient utilization from the yolk sac caused by suboptimal incubation temperature or poor ventilation (Molenaar et al., 2010). An unhealed navel increases the risk of omphalitis, reduction of intestinal villi growth and subsequent performance (Fasenko and O'Dea, 2008; Kawalilak et al., 2010). The improved navel health could be related to the positive effect of periodic illumination on embryo development. Considering the lower air cell temperature observed in the photostimulation

groups, we speculate that providing periodic blue light stimulation during incubation can regulate embryo temperature via altering HPT axis activity and improve navel maturation. In the current study, blue LED illumination for 12 h d^{-1} demonstrated a potential benefit to chick quality, while longer illumination up to 18 h d^{-1} did not result in detrimental effects on embryo mortality and hatchability compared to the DARK.

5.4.3 Growth performance

Cobb chicks had heavier ($P<0.01$) body weight at placement and 6 h post-placement than Ross chicks. Cobb chicks also had higher ($P<0.01$) feed consumption and percentage of body weight gain after 6 h of accessing feed and water than Ross chicks (Table 5.3). Chicks' body weight at placement and weight gain during the first 6 h of access to feed and water did not differ ($P>0.05$) among lighting treatments. However, chicks hatched under 18L:6D had a lower ($P<0.01$) feed consumption than those in the DARK and 12L:12D groups. No interaction effect ($P>0.05$) between photoperiod and strain on placement performance were found in the current study (Table 5.3). The depressed feed consumption observed in the 18L:6D group suggests that the duration of the scotophase during incubation should be more than 6 h d^{-1}. Continuous illumination during incubation may have caused some degree of stress and negatively impacted chicks' quality and early growth performance. Lower body weight at hatch in both Ross and Cobb broilers was found following incubation with constant illumination compared to the intermittent lighting schedule or constant dark (Yameen et al., 2020). Lower feed intake found in the 18L:6D group at the early stage might be associated with the disruption of eye development. Prolonged photoperiod induced ocular abnormalities in broiler (Lewis and Gous, 2009) and turkey (Leis et al., 2017) during the rearing period. Archer et al. (2009)

also found heavier eye weight in constant lighting conditions (0L:24D & 24L:0D) than those incubated under 12L:12D. However, no lesions in the epithelial cells of the retina were observed in any lighting treatment. Heavier eye weight associated with the characteristic lesion of light-induced avian glaucoma was found in chicks exposed to duration of photophase longer than 16 h d^{-1} (Oishi and Murakami, 1985). These observations suggest that more research should be conducted to evaluate the effects of light illumination during incubation on eye health and behavior at an early age. The result of no differences in body weight gain percent during the first 6 h access to feed and water was unexpected, especially for those chicks hatched from the 12L:12D group. Even though the 12L:12D showed a higher numerical body weight gain percent (10.91%) than the DARK (9.11%) and 18L:6D (8.51%) in the current study. Previous studies from our research group observed consistent results of higher body weight gain and feed consumption when chicken embryos were provided with blue LED light for 12 h day^{-1} (Li et al., 2021b; Henry, 2020). The enhanced production traits in our previous studies might be associated with changes in lateralized brain function by light stimulation during embryogenesis and the improvement of epigenetic adaption to the rearing environment. The variation in parent flock age and genotype can affect eggshell characteristics (thickness and eggshell pigmentation), which may also influence light transmission through the eggshell and the quality of light perceived by embryos (Shafey et al., 2004; Maurer et al., 2015). Rozenboim et al. (2003) showed that female turkeys incubated with green light had a higher body weight after d 28 post-hatch than those incubated under dark, whereas no difference in body weight was observed in male turkeys. It follows that

the effect of gender on the adaptive response to the rearing environment should be considered when introducing a lighting program during incubation.

There were no differences (P>0.05) in body weight and relative liver weight among lighting treatments on d 2 of age (Table 5.4). However, the yolk-free body weight of chicks hatched under light illumination (12L:12D and 18L:6D) approached significantly heavier (P=0.06) than those hatched under DARK. Light illumination during incubation may prime the light-dark cycles to the embryos and enhance the sensitivity of young chicks to the diurnal changes. Cobb chicks had a heavier (P<0.05) body weight and yolk-free body weight than Ross chicks, while the relative liver weight showed no difference (P>0.05) between strains.

For the 34-d grow-out trial, photoperiod only affected the body weight on d 14 of age (Figure 5.4). Birds hatched under 12L:12D had a higher (P<0.05) body weight than those hatched from 18L:6D. No differences (P>0.05) in body weight were found between DARK and blue light illuminated groups after d 14 of age. Inconsistent results regarding the effects of *in ovo* photostimulation on post-hatch growth have been reported. In our current study, neither 12L:12D nor 18L:6D stimulated the post-hatch growth at market age. A similar finding was reported in broilers (Archer et al., 2009; Archer and Mench, 2014b; Dishon et al., 2018, 2021), layers (Sindhurakar and Bradley, 2010) and turkeys (Rozenboim et al., 2003). Similarly, Sabuncuoğlu et al. (2018) found that no difference in the body weight of Japanese quail when incubated under dark compared to being exposed to continuous blue light. Our finding contradicted several studies that showed *in ovo* photostimulaiton accelerated the muscle growth of broilers (Rozenboim et al., 2004; Özkan et al., 2012b; Zhange et al., 2012; Bai et al., 2016). The growth-promoting effect

of green light might be achieved by regulating the activity of the somatotropic axis to enhance the proliferation of muscle satellite cells (Halevy et al., 2006`). The contradictory results among studies can be attributed to the differences in species and illumination conditions. However, it is not clear how light illumination affects the somatotropic axis activity. Dishon et al. (2021) found that green light upregulated the expression of hypothalamus growth hormone releasing hormone, growth hormone, insulin-like growth factor1 (IGF-1), and their receptors in the target tissues. The effect of photostimulation on somatotropic axis activity can be achieved by green light stimulation without the presence of a photoperiod (Zhang et al., 2012; Bai et al., 2016). Unfortunately, information on the optimal photoperiod of blue light illumination during incubation on post-hatch performance is limited. It is not known if a periodic blue light illumination would regulate the activity of somatotropic, HPA and HPT axes in broilers. The only information is from our previous study (Li et al., 2021b) with no growth-promoting effect when illuminated with 12L:12D blue light, and Drozdova et al. (2021) who reported that photoperiodic (12L:12D) red light illumination during incubation resulted in a higher body weight as compared to blue light. Drozdova et al. (2021) speculated that blue light during incubation had a long-term effect on the asymmetrical development of visual pathways and promoted the active behavior with higher energy expenditure from d 18 onwards.

Neither feed consumption nor feed conversion ratio were different ($P>0.05$) among lighting treatments (Table 5.5). Body weight gain from d 8 to 14 of age was significantly higher ($P<0.05$) for birds hatched under 12L:12D than those in 18L:6D, and the control group was intermediate (Table 5.5). The role of light stimulation during incubation on

post-hatch growth might be related to IGF-1, which is mainly secreted by the liver in association with melatonin (Wang et al., 2014). Broilers exposed to constant or near-constant (23L:1D) light illumination had lower melatonin production than those reared with 12 or 14 h of darkness (Schwean-Lardner et al., 2014). The duration of scotophase is critical when introducing a periodic lighting program. Unfortunately, no generally accepted standards or recommendations related to the duration of photostimulation during incubation have been made. To our knowledge, no study has investigated the duration of photoperiod during incubation, which matches the recommended duration of darkness (4-6 h darkness after d 7 of age) of Ross 308 specification (Avigen, 2018). The current study provided the first evidence of comparing the long-day lighting schedule (18L:6D) and 12L:12D of blue LED light illumination and found no marked effects on the growth performance of birds reared with a matched light schedule between incubation and rearing periods. The duration of scotophase less than 6 h d^{-1} resulted in a lower body weight on d 14 of age and less body weight gain during the second week of rearing period than those hatched under a 12L:12D photoperiod. It is unclear whether the difference in body weight gain between 12L:12D and 18L:6D is associated with impairments of vision, negative impacts on brain lateralization, insufficient to entrain melatonin synthesis or a combination of these due to prolonged illumination.

In conclusion, periodic blue light during incubation had no effects on hatchability or embryo mortality, but improved chick quality in terms of better navel closure condition was observed in the 12L:12D group compared to the control group. Lower embryo temperature in photostimulated embryos suggested that the application of periodic illumination during incubation may provide a novel approach to improve the resilience of

broilers to heat stress. Furthermore, the 18L:6D group had lower feed consumption during the brooding period and lower body weight on d 14 of age than those incubated under a 12L:12D photoperiod, which indicate more than 6 h d^{-1} of scotophase should be provided during *in ovo* photostimulation of broiler hatching eggs. Our results suggest that periodic blue light with an appropriate length of scotophase during incubation can improve chick quality and early growth, but this effect was not apparent during later development. The impact of photostimulation during incubation still needs more science-based research before making recommendations to commercial hatcheries.

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Table 5.1 Least squares means[1] for average air cell temperature (°C) every 24 h in two strains of eggs (Cobb and Ross) between ED 11 through ED 20 when incubated with blue LED light illuminated for 0 (Dark), 12 (12L:12D) or 18 h day^{-1} (18L:6D).

Light	Strain	Embryonic day										
		11	12	13	14	15	16	17	18	19	20	Average[2]
Dark	Cobb	37.58^{ab}	37.61^{a}	37.65^{a}	37.71^{ab}	37.77^{a}	37.81^{a}	37.84^{a}	37.79^{c}	38.14^{a}	38.38^{a}	37.83^{a}
	Ross	37.58^{ab}			37.72^{a}	37.76^{a}	37.81^{a}	37.84^{a}	37.80^{b}	38.08^{b}	38.38^{a}	
12L:12D	Cobb	37.59^{a}	37.60^{a}	37.64^{b}	37.70^{c}	37.73^{b}	37.77^{c}	37.79^{c}	37.80^{b}	38.04^{d}	38.24^{d}	37.80^{b}
	Ross	37.58^{b}			37.70^{bc}	37.74^{b}	37.78^{b}	37.81^{b}	37.83^{a}	38.04^{d}	38.27^{c}	
18L:6D	Cobb	37.55^{d}	37.58^{b}	37.62^{c}	37.69^{cd}	37.74^{b}	37.78^{bc}	37.80^{b}	37.84^{a}	38.06^{c}	38.30^{b}	37.78^{b}
	Ross	37.57^{c}			37.68^{d}	37.72^{c}	37.75^{d}	37.78^{c}	37.77^{d}	37.98^{e}	38.19^{e}	
SEM		0.003	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.005	0.006	0.008
ANOVA *P*-value												
Light (L)		*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.002*
Strain (S)		*0.730*	*0.552*	*0.640*	*0.895*	*0.203*	*0.220*	*0.873*	*<0.001*	*<0.001*	*<0.001*	*0.528*
L x S		*<0.001*	*0.286*	*0.123*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.251*

[1]n = 4. Experimental unit = 1 incubator containing 4 eggs of each of the two strains.
[2]Average: The mean of air cell temperature from ED 11 - 20.
[a-e]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 5.2 Least squares means[1] for hatch weight (g bird^{-1}), chick length (cm bird^{-1}), percentage of navel score 1 (%), 2 (%) and 3 (%) and average navel score varied by lights and strains.

Variation source	Chick weight	Chick length	Navel closure condition			
			1	2	3	Average[2]
Light (L)						
Dark	48.0	18.7	56.57^{b}	40.37^{a}	3.06	1.47^{a}
12L:12D	47.4	18.7	67.81^{a}	30.43^{b}	1.76	1.34^{b}
18L:6D	47.6	18.8	61.66^{ab}	36.72^{ab}	1.62	1.40^{ab}
SEM	0.97	0.07	2.74	2.32	0.95	0.03
P-value	*0.887*	*0.636*	*0.033*	*0.024*	*0.516*	*0.050*
Strain (S)						
Cobb	49.7^{a}	19.0^{a}	58.41^{b}	38.88^{a}	2.71	1.44^{a}
Ross	45.6^{b}	18.4^{b}	65.62^{a}	32.79^{b}	1.59	1.36^{b}
SEM	0.79	0.05	2.24	1.89	0.78	0.03
P-value	*0.002*	*<0.001*	*0.036*	*0.036*	*0.324*	*0.044*
L x S *P*-value	*0.899*	*0.920*	*0.975*	*0.979*	*0.991*	*0.972*

[1]n = 4. Experimental unit = 1 incubator containing 176 eggs of each of the two strains in trial 1, and 152 Cobb and 168 Ross eggs in trial 2.
[2]Average navel score= (number of chicks scored 1 x 1 + number of chicks scored 2 x 2 + number of chicks scored 3 x 3)/total number of chicks.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 5.3 Least squares means[1] for placement performance including placement body weight (g $bird^{-1}$), 6 h post-placement body weight (g $bird^{-1}$), body weight gain percent (%) and feed intake (g $bird^{-1}$) for the 6 h access to feed and water varied by lights and strains.

Variation source	Placement body weight	Body weight at 6 h	Body weight gain percent	Feed intake
Light (L)				
Dark	47.7	52.0	9.11	3.1[a]
12L:12D	47.3	52.5	10.91	3.4[a]
18L:6D	47.5	51.5	8.51	2.7[b]
SEM	0.96	0.64	1.16	0.09
P-value	*0.969*	*0.542*	*0.334*	*<0.001*
Strain (S)				
Cobb	49.4[a]	55.1[a]	11.54[a]	3.3[a]
Ross	45.7[b]	48.9[b]	7.48[b]	2.8[b]
SEM	0.79	0.53	0.94	0.08
P-value	*0.004*	*<0.001*	*0.007*	*<0.001*
L x S *P*-value	*0.978*	*0.864*	*0.980*	*0.385*

[1]n = 4. Experimental unit = 4 pens of birds per strain hatched from the same incubator.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 5.4 Least squares means[1] for body weight (g bird^{-1}), yolk-free body weight (g bird^{-1}), relative yolk sac weight (g/100g yolk-free body weight) and relative liver weight (g/100g yolk-free body weight) on d 2 of age varied by lights and strains.

Variation source	Body weight	Yolk-free body weight	Relative yolk sac weight	Relative liver weight
Light (L)				
Dark	63.2	60.9	3.84	4.41
12L:12D	65.2	63.0	3.53	4.42
18L:6D	65.4	63.4	3.52	4.58
SEM	0.78	0.71	0.265	0.077
P-value	*0.120*	*0.061*	*0.629*	*0.266*
Strain (S)				
Cobb	68.5[a]	66.6[a]	3.07[b]	4.49
Ross	60.7[b]	58.3[b]	4.19[a]	4.46
SEM	0.63	0.58	0.216	0.063
P-value	*<0.001*	*<0.001*	*<0.001*	*0.740*
L x S *P*-value	*0.632*	*0.565*	*0.389*	*0.388*

[1]n = 4. Experimental unit = 4 pens of birds per strain hatched from the same incubator.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

Table 5.5 Least squares means[1] for body weight gain (g bird^{-1} day^{-1}), feed consumption (g bird^{-1} day^{-1}) and feed conversion ratio over a 34-day period varied by lights and strains.

	Body weight gain				Feed intake				Feed conversion ratio			
Period (d)	0-7	8-14	15-24	25-34	0-7	8-14	15-24	25-34	0-7	8-14	15-24	25-34
Variation source												
Light (L)												
Dark	18.9	38.9[ab]	78.1	111.7	20.3	50.9	106.6	174.5	1.07	1.31	1.37	1.56
12L:12D	19.2	40.3[a]	79.4	113.2	20.6	52.4	109.1	177.7	1.07	1.30	1.38	1.57
18L:6D	18.7	37.9[b]	78.0	112.2	19.9	50.3	106.6	175.8	1.06	1.33	1.37	1.57
SEM	0.20	0.50	0.59	0.77	0.53	0.67	0.89	1.59	0.021	0.008	0.005	0.009
P-value	*0.281*	*0.013*	*0.189*	*0.393*	*0.648*	*0.091*	*0.067*	*0.397*	*0.926*	*0.056*	*0.269*	*0.829*
Strain (S)												
Cobb	19.7[a]	39.9[a]	81.7[a]	115.2[a]	20.9[a]	52.1[a]	110.9[a]	181.2[a]	1.06	1.31	1.36[b]	1.57
Ross	18.2[b]	38.2[b]	75.3[b]	109.5[b]	19.6[b]	50.2[b]	103.7[b]	170.8[b]	1.08	1.32	1.38[a]	1.56
SEM	0.16	0.41	0.48	0.63	0.43	0.54	0.72	1.30	0.017	0.006	0.004	0.007
P-value	<0.001	0.008	<0.001	<0.001	*0.042*	*0.023*	*<0.001*	*<0.001*	*0.474*	*0.307*	*0.007*	*0.300*
L x S *P*-value	*0.407*	*0.198*	*0.137*	*0.441*	*0.662*	*0.378*	*0.417*	*0.379*	*0.504*	*0.499*	*0.070*	*0.709*

[1]n = 4. Experimental unit = 4 pens of birds per strain hatched from the same incubator.
[a,b]Means within a column with different letters differ significantly according to Tukey-Kramer test (α = 0.05).

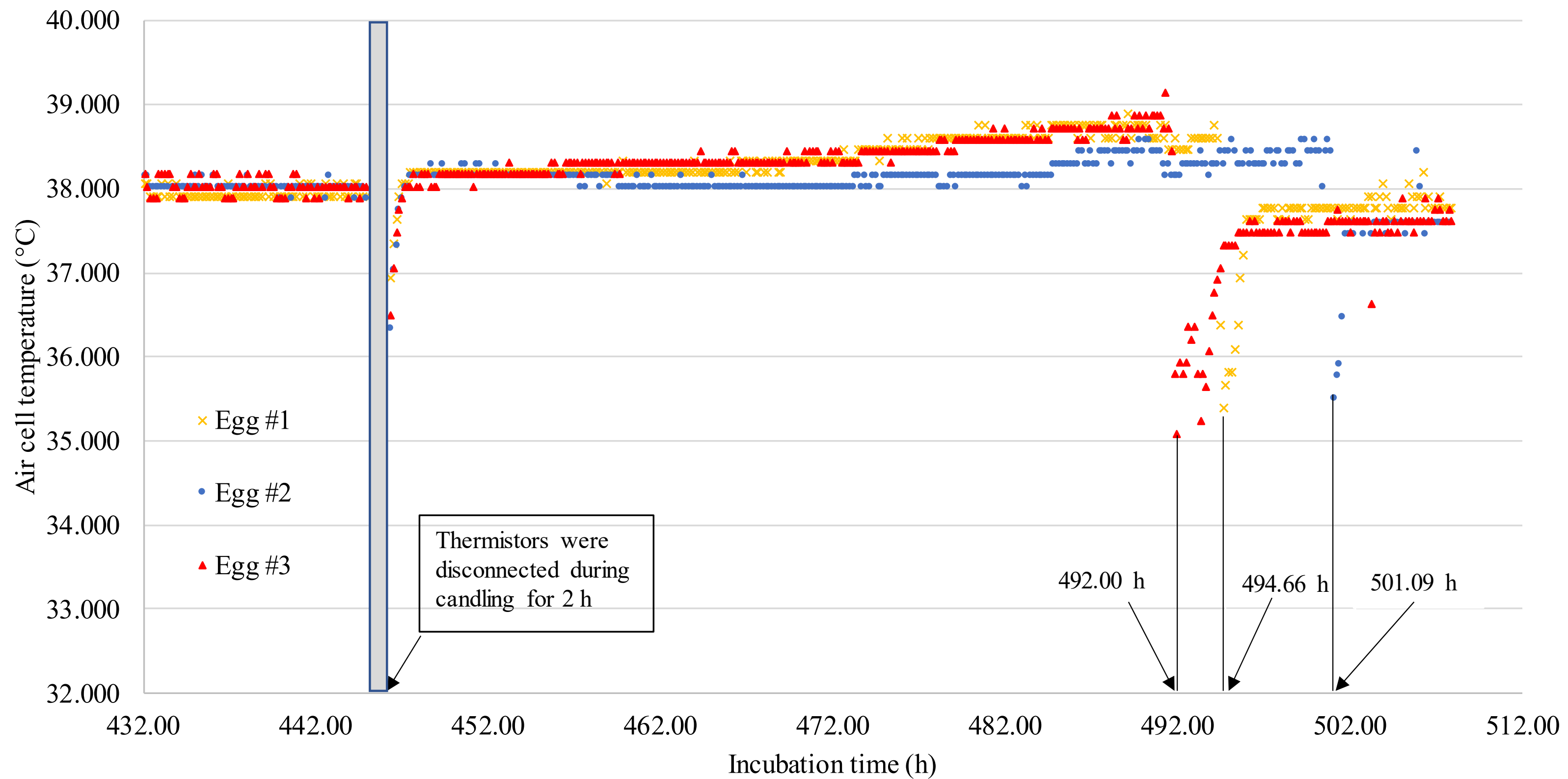

Figure 5.1 Example of air cell temperature (°C) recordings of 3 hatching eggs during incubation. The gray shadow area represents the duration when thermistors were disconnected during egg candling.

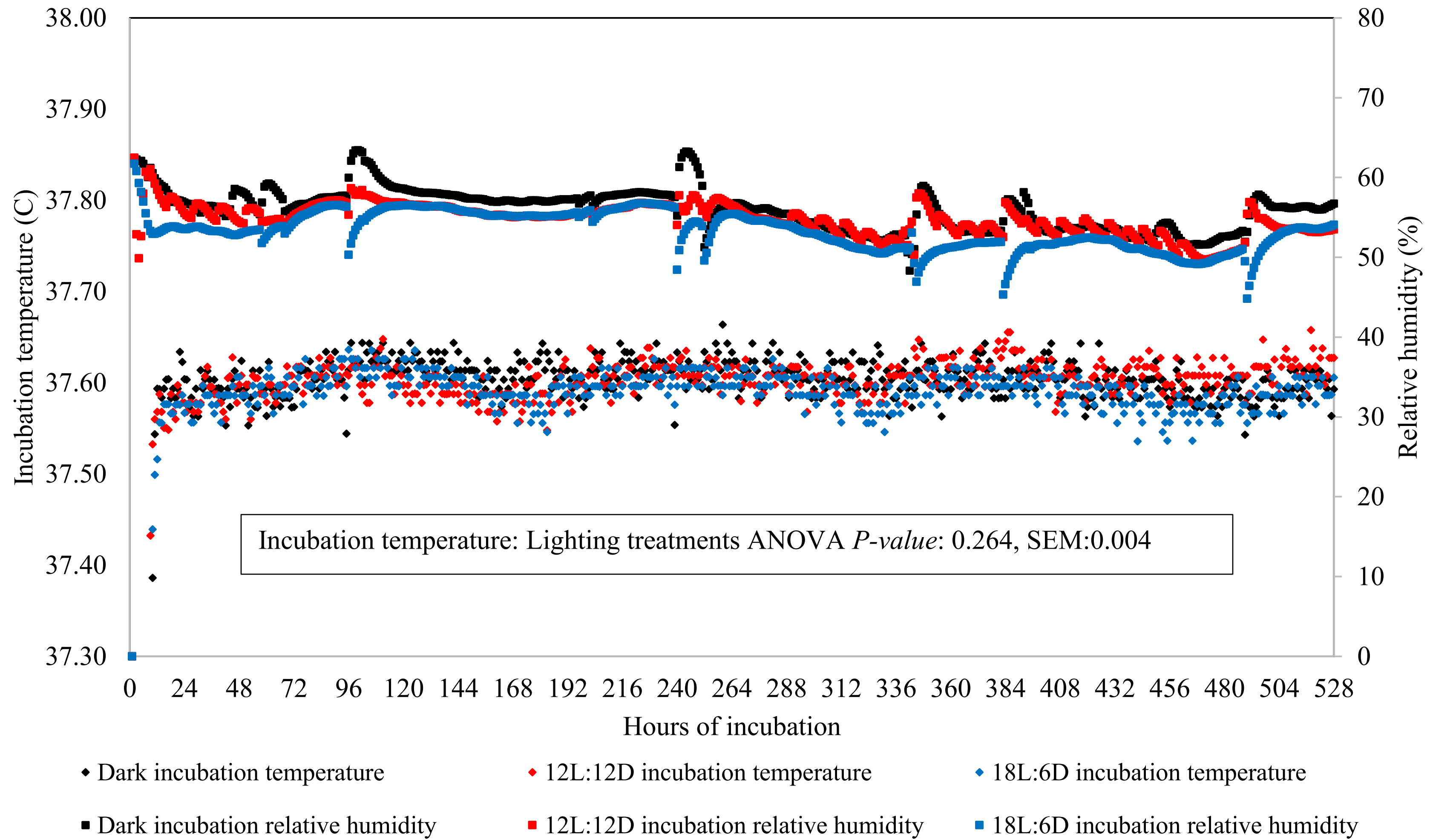

Figure 5.2 Incubation temperature (°C) and relative humidity (%) of incubators during a 512-h incubation period.

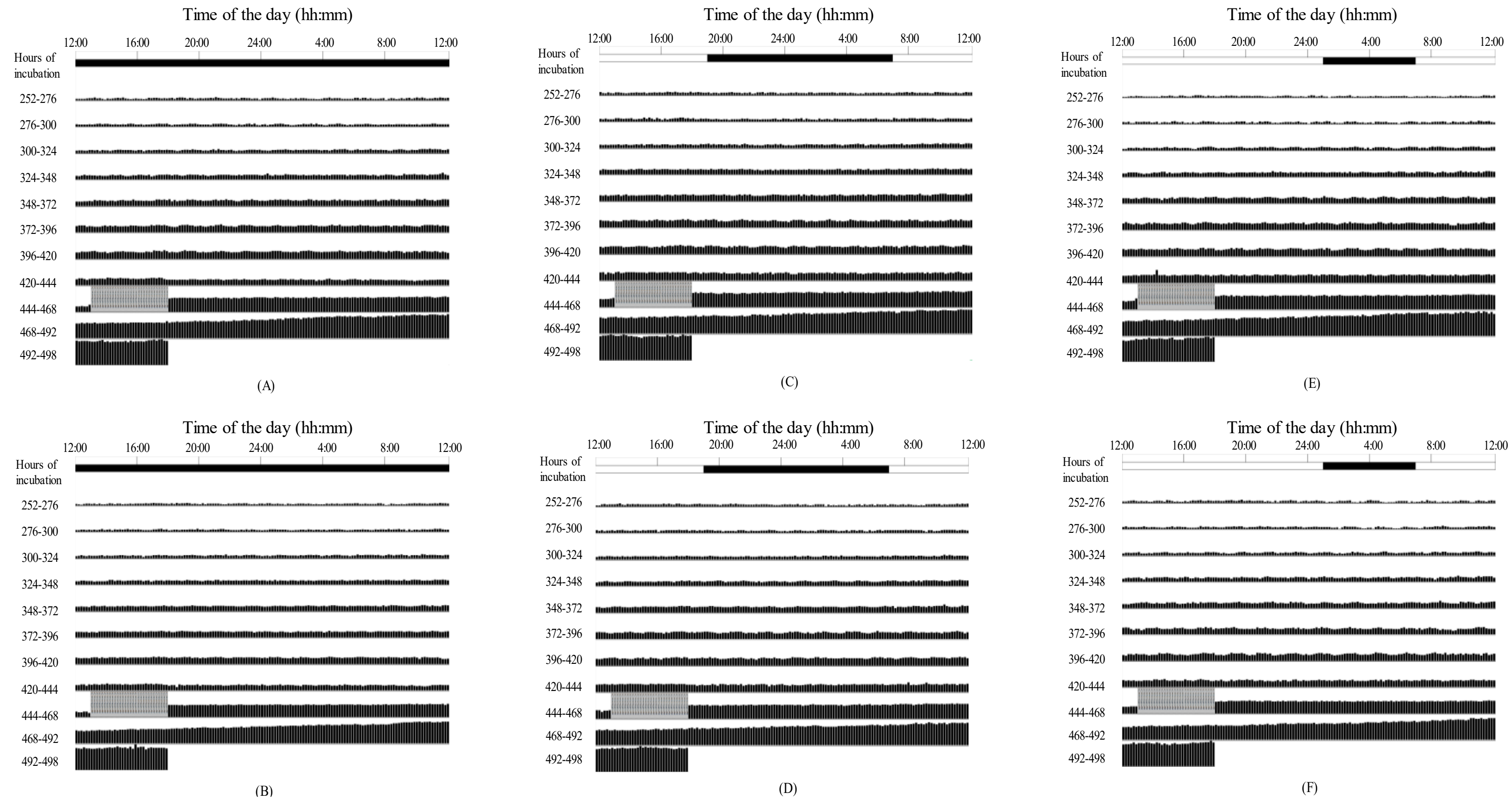

Figure 5.3 Actograms of air cell temperature (°C) of 96 broiler embryos (pooled per treatment combination) that were incubated in dark (panel A: Cobb and panel B: Ross), illuminated with blue LED light for 12 h d^{-1} (panel C: Cobb and panel D: Ross) or 18 h d^{-1} (panel E: Cobb and panel F: Ross). In each actogram, time of day is indicated on the horizontal axis and hours of incubation on the vertical axis. The horizontal white and black bars under the time of the day indicate the duration of the photophase and scotophase of the light-dark cycle. The amplitude of each bar represents the recordings of air cell temperature per 10-min from 252 to 498 h of incubation. The gray shadow area represents the duration when thermistors were disconnected during egg candling.

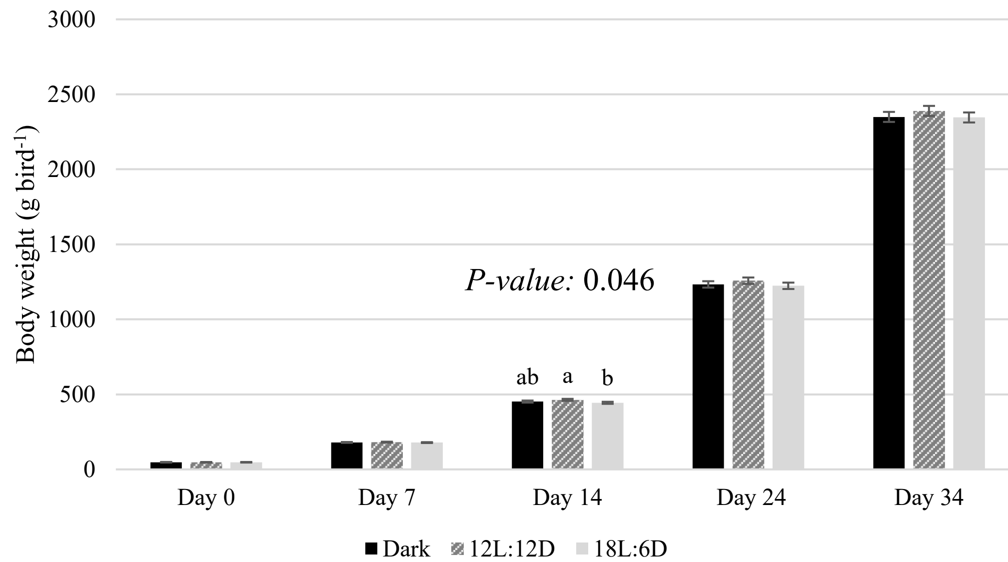

Figure 5.4 Effects of incubation lighting treatments and age on body weight (g bird^{-1}) of broiler during a 34-d rearing period.

$^{a-b}$Means with different letters differ significantly according to Tukey-Kramer test ($\alpha = 0.05$).

CHAPTER 6. GENERAL DISCUSSION

Naturally brooded eggs received varying degrees of illumination when the mother hen turns the eggs and leaves the nest for feeding and drinking (Archer and Mench, 2014). Providing illumination during incubation has caught attention from science and industry to optimize the incubation environment in attempt to fully express the genetic potential of commercial broilers. As the illumination is completely provided by artificial light source in commercial poultry production, the light components (intensity, wavelength and photoperiod) must be carefully considered to achieve the specific production objectives. Several studies have reported that *in ovo* photostimulation could play an important role in circadian rhythm entrainment and accelerating the growth of broilers (Drozdova et al., 2019; Yu et al., 2018; Zeman et al., 2004). However, the majority of the past research on providing illumination during incubation has compared one specific lighting program to another, with little attempt to develop a comprehensive approach to understand the impacts of periodic illumination on embryonic development, hatching traits and productivity in the same study. Those studies provided a general insight of lighting effects, but they failed to demonstrate the impacts of light illumination with different wavelengths, photoperiods and their combination on a wide range of outcome variables. To report the impacts of *in ovo* photostimulation on broilers, data obtained from the current study were divided into two sections: hatching traits and productivity.

6.1 Hatching traits

In previous research on the effects of *in ovo* photostimulation, the common objectives were to evaluate its impacts on embryonic development and hatchability. Illumination with various light colors did not affect embryo physical parameters during embryogenesis

or hatch traits in the current study. However, the effect of light stimulation on embryo development was not consistent in past studies. Some studies found advanced embryo development with higher embryo/chick weight when illuminated with green or blue lights (Shafey and Al-mohsen, 2002; Tong et al., 2018; Wang et al., 2014). The accelerated embryo development was considered to be related to the production of growth hormone stimulated by periodic illumination. Rhythmic light stimulation stimulates pineal melatonin secretion, then melatonin is transferred to the endocrine system and serves as the internal temporal input to the peripheral tissues and regulates the expression of hypothalamic growth hormone releasing hormone (GHRH). An increase in GHRH can further modulate growth hormone synthesis in the pituitary gland and growth hormone receptor expression and IGF-1 production in liver to regulate the muscle growth. No differences in embryo mortality or hatchability were found in this project. Even though, the beneficial impact of longer wavelength on hatchability was reported in previous studies (Archer et al., 2017; 2018), the mechanism for the improvement in hatchability is still unknown and requires future research.

There is limited information on the effect of illumination on embryo temperature and spread of hatch in previous studies. In general, most previous studies count the number of chicks at different time point during the last three days of incubation and calculate the average incubation time. In the current study, a novel statistical approach using an non-linear regression model was proposed to analyze the hatch time data and provide more detailed information on the spread of hatch. This method is beneficial for further research focusing on evaluating the effects of incubation environment on the hatching process. To our knowledge, this study was the first to investigate the impact of lighting programs on

embryo temperature by monitoring air cell temperature during incubation period. Overall, LED light illumination did not cause an over-heating effect on developing embryos. In the light color study, lower air cell temperature was found when illuminated with red LED light for 12 h d^{-1} compared to white, blue or darkness. The light with longer wavelength is better at penetrating the eggshell and activating the deep brain photoreceptors to stimulate the synthesis of melatonin and results in reduced embryonic heat production. In the photoperiod experiment, the air cell temperature was monitored continuously and demonstrated that periodic blue LED light reduced the heat production of developing embryos. The differences in air cell temperature found between two experiments might be due to the eggshell characteristics affected by breeder age, the frequency of measurement and the sampling method. In the light color experiment, eggs were taken out from the incubator every 12 h, while in the photoperiod experiment, the air cell temperature was measured automatically using the NTC-thermistors, which minimize the changes in temperature and eliminated the potential temporal cue from the cyclic exposure to room temperature. It was noted that providing blue LED illumination with a 12L:12D photoperiod resulted in a higher percentage (67.81%) of chicks with superior navel condition than those under dark in the photoperiod experiment. The response was not found in the light color experiment with lower percentage chicks with perfect navel under the same lighting treatment (32.48%). Taking hatcher buckets out of incubator to count the number of hatched chicks may affect the temperature, relative humidity and slow down the hatching process. Using a camera to record the hatching process would be useful to examine the response of the spread of hatch to *in ovo* photostimulation in future studies.

6.2 Productivity

An enhanced early growth performance was observed when embryos were exposed to periodic illumination with different colors in Experiment 1. A similar result was reported by Özkan et al. (2012b) using white light during incubation. They reported that *in ovo* photostimulation increased body weight gain of broilers during the first week post-hatch. Improved epigenetic adaption to the rearing environment might be attributed to the establishment of circadian rhythm from periodic illumination. This may also improve the sensitivity of chicks to a lighted environment. Positive impacts of light stimulation during incubation on brain lateralization and exploratory behavior have been reported (Chiandetti et al., 2005). However, a growth-promoting response was not observed in our photoperiod experiment. A difference between our two studies, which may introduce the environmental stress to newly hatched chicks was the process of counting hatched chicks during the last days of incubation for the wavelength experiment and not the photoperiod experiment.

There is evidence that periodic light stimulation can have positive effects that allow chicks to better adapt to post-hatch stressors. Differences in body weight gain found between two commercial broiler strains when illuminated with graded photoperiod of blue light suggests that the response of productivity is influenced by genotypes. It is important to take into consideration the importance of the strain variation before implementation of lighting program during incubation.

6.3 Practical application

In recent years, early feeding programs have been developed to provide feed and water with continuous illumination in the hatcher for newly hatched chicks. But prolonged

illumination during incubation may cause some degree of stress and negatively impact chicks regarding overall quality and early growth performance. Lower body weight at hatch in both Ross and Cobb broilers was found when eggs were incubated with constant illumination compared to those under intermittent lighting or constant dark (Yameen et al., 2020). Few conclusions can be drawn from recent studies regarding the color and duration of light illumination during incubation as a potential environmental tool to improve newly hatched chick quality and adaptation to the post-hatch environment. This project provides novel information from *in ovo* blue and white illumination with a 12L:12D photoperiod. Results indicate that an improvement in early growth and reduced fluctuation in cloaca temperatures between morning and evening for chicks at a young age. This improvement can result in a better start for early post-hatch development of broiler chicks.

The capacity of thermogenesis is important for the survival of newly hatched chicks. Recent studies have suggested that thermal conditioning during the final stage of incubation induces an improvement in the acquisition of thermotolerance and growth of neonatal chicks (Collin et al., 2005; Piestun et al., 2008). Provision of photostimulation during incubation may also improve chick's thermotolerance acquisition by modulating the activity of HPT axis, which becomes functional from around d 14 of incubation, coinciding with the rhythmic secretion of melatonin (Jenkins and Porter, 2004). Thyroid hormones play important roles in metabolism and thermogenesis. Melatonin inhibits the production of thyroid hormones and corticosterone, which can lower the threshold point of the HPT axis when exposure to suboptimal temperatures. The results from this study demonstrated that white and blue light illumination under a 12L:12D photoperiod

improves the adaptability of newly hatched chicks to rearing environment changes with improved growth performance and reduced body temperature fluctuations during the brooding period. Both Ross and Cobb broilers incubated with periodic blue light illumination had lower air cell temperatures than those under constant darkness from d 14 of incubation onwards, indicating that *in ovo* photostimulation may accelerate the development of endothermy of chicken embryos and may have beneficial impacts on expanding the thermoneutral zone of broilers at an early age. The advanced development of thermoregulatory system by light stimulation could reduce thermal challenge associated damage to health and welfare and improve bird health and productivity in subsequent life stages.

6.4 Conclusion

In conclusion, illumination with various colors of LED light for 12 h d^{-1} did not seem to impact embryonic development or hatching parameters of broilers. But light with full-spectrum and short wavelength demonstrated positive impacts on post-hatch adaptive response with increasing feed intake, body weight gain and reduced fluctuation in body temperature during the brooding stage. Blue light illumination under a 12L:12D photoperiod has potential benefits in terms of better chick quality with a superior navel condition and improved productivity. The depressed production parameters found in the 18L:6D group suggest that more than 6 h d^{-1} of darkness are required when providing a periodic illumination program during incubation. This study has practical applications in the industry, especially for improving chick quality and early performance of broilers.

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