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Effect of Lighting Schedule during Incubation on Embryonic Development and Bone Gene (*Col10a1*, *Spp1*, *Bglap*, and *Alp*) Expression in Broiler Hatchlings

Research Article

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ABSTRACT

We studied the effect of lighting during 19 days of incubation on embryonic bone characteristics, gene expression, thyroid hormones, and glucose in Cobb broiler hatchlings. Eggs (2160) were incubated under darkness (control) and green and white lighting (16D:8L and 12D:12L). On 19th day, tibiotarsal length increased under eight hours of white light, and femoral ossified length was longer under eight hours of white and green light. In femur, expression of *Alp*, *Col10a1*, and *Spp1* increased by 12 h white and 8 h green lighting, and *Bglap* expression decreased under white. In tibiotarsus, expression of *Alp* decreased by 8 h lighting (green or white) while *Bglap* increased by 12 h lighting (green or white). Tibiotarsal expression of *Col10a1* increased by 12 h lighting (white and green), and in 8 h green lighting, and *Spp1* expression was higher under light illumination. The hatchling blood triiodothyronine concentration in 8 h green lighting was higher than that in control, whereas, thyroxine concentration was lower under 12 h white lighting compared with other treatments. Femoral organic matter to mineral (OM:M) ratio and calcium concentration were higher in the eight-hour white light group than in the control, but tibiotarsal OM:M ratio and calcium and phosphorus concentrations were higher under 12 h white light. The findings indicated that lighting during incubation may impact, both positively and negatively, bone morphology and gene expression. More studies are needed to find the most beneficial lighting system during incubation in chickens.

KEY WORDS chondrocyte, collagen, crystallization, endocrine, light color, osteocalcin.

INTRODUCTION

Genetic selection for increasing body weight and a short rearing period have resulted in disproportionate growth in bone mass and painful leg abnormalities in poultry (Williams *et al*. 2000; Yair *et al*. 2012; Fornari *et al*. 2014; Nalon and Stevenson, 2019). The incubation period covers almost one-third of the broiler's production life, *i.e.*, from incubation to slaughter (Groves and Muir, 2017), and chicken embryos respond to light from the early stages of life (Cooper *et al*. 2011); therefore, lighting the hatcheries might affect embryonic development. In this regard, there has been an increased interest in research concerning the effects of light intensity, duration, and wavelength on embryo development in birds (Sindhurakar and Bradley, 2012; Archer, 2017; Dishon *et al*. 2017).

Organ development in chicken embryos depends on hormones and the availability of metabolites and energy substrates (Varga *et al*. 2004; Geng *et al*. 2021). Assuming a circadian rhythm in bone growth, as occurs in many meta-

bolic phenomena, the effect of a dark/light schedule was investigated during the incubation of fertilized eggs on the embryonic and postnatal development of chicken legs (Van der Pol *et al.* 2019b). Thyroid hormones (TH) are essential for ossification (Varga *et al.* 2004; Geng *et al.* 2021) but their effects on bone development during incubation lighting on chicken embryos are not clear. However, an increase in blood triiodothyronine (T3) concentration and triiodothyronine to tetraiodothyronine ratio (T3/T4) in quail chicks was observed by lighting the eggs during incubation (Khalil, 2009). Thyroid hormones also play a role in the expression of *Col10* and *Spp1* genes. Collagen-10 is the main index of chondrocyte differentiation into a hypertrophied state, while osteopontin, a glycophosphoprotein, indicates cartilage-to-bone differentiation (Varga *et al.* 2004). *Spp1* gene expression and phenotypic differentiation of chondrocytes are related to alkaline phosphatase (ALP) activity (Farquharson and Jefferies, 2000). The alp gene is necessary for bone mineralization (Anderson *et al.* 2004) and its activity is positively correlated with the levels of bone ash and phosphorus (Shao *et al.* 2019). The circadian rhythm of bone synthesis may be related to the circadian rhythm of osteocalcin concentration (Shao *et al.* 2019). Thyroid hormones (Power and Fottrell, 1991; Duncan and Williams, 2003) and estrogens (Nys and Le, 2018) are involved in bone mineralization via osteocalcin biosynthesis by osteoblasts. Hence, proper functioning of the thyroid gland is necessary to maintain optimal bone mineralization and strength (Gogakos *et al.* 2010), while the amount of bone ash is positively correlated with the bone fracture force (Yildiz *et al.* 2009). In addition, calcium and phosphorus, as the main mineral elements in the bone, are important for bone strength (Onyango *et al.* 2003).

The most beneficial lighting program for embryonic development has not been determined, and it is unclear which light spectrum might be suitable for each stage of embryonic life. To the best of our knowledge, the effect of changes in the secretion of TH during incubation illumination on embryonic bone development has not been investigated. Also, little is known about the changes in the expression of genes that impact bone formation due to programmed lighting during incubation. In this regard, this research aimed to study the influence of several lighting schedules (green and white light for 8 and 12 h *vs.* darkness) on the bone development of embryonic chicks, expression of some related genes (*Spp1, Col10a1*, and *Alp*) in the femur and tibiotarsus, and blood concentration of thyroid hormones (T3, T4) and glucose in broiler chicks at hatching. We also investigated the effects of lighting regimens on the body weight as well as the length and weight, and calcium and phosphorus concentrations in the femur and tibiotarsus at birth.

MATERIALS AND METHODS

The Animal Care and Welfare Committee of the Department of Animal Science, School of Agriculture, Shiraz University (Shiraz, Iran) approved all protocols and the experimental design.

Experimental setup

This study was carried out using 2160 fertilized eggs (62-64 g; mean eggshell thickness=0.40 mm) from a young commercial flock of Cobb 500 broiler breeder hens (39 wk.). Five incubators from a commercial hatchery (Shiraz, Fars, Iran), equipped with Jamesway (multi-stage setter and hatcher) machines, were illuminated using LED lamps for 19 days in the setter. Strip lamps were installed in rows on the roof of the first-row trays of the setter. The setters were calibrated for temperature, humidity, and other conditions. The temperature and humidity were set at 37.05 ˚C and 88%, respectively. The window in front of the machine was occluded, and the setter lights were turned off when the intended lights illuminated the setter. A completely random design was used consisting of five treatments, each with three replicates, and 144 eggs per replicate. The treatments were 24-hour darkness (24D) as the control group and green and white LED lights at two durations (16D:8L and 12D:12L). Light intensity was measured using the photoreceptor sensor of an auto-digital lux meter (Victor 1010A). The intensities of the white and green lights were 70–350 lux and 20–150 lux, respectively.

Embryonic data

Little research has been conducted on embryonic ossification during illumination, but it has been shown that the difference in the ossification of the tibiotarsus is evident after the 13th embryonic day (Van der Pol *et al.* 2019b); therefore, sampling started from the 12th day of hatching. On days 12, 15, and 19 of incubation, five eggs were randomly selected from each treatment group for evaluation of femur and tibiotarsus ossification to differentiate bone from cartilaginous tissue using Alcian blue and Alizarin staining (Van der Pol *et al.* 2019b), with some modifications (Figure 1). First, the desired bones were separated from the skin and muscle tissue by placing them in warm water for a short time and then slowly rubbing them. The bones were fixed in formalin (for 24 hours) and stored in 70% alcohol. At the time of measurement, the samples were immersed in distilled water for 24 h and then in an Alcian dye solution (80 ml ultrapure anhydrous ethanol, 20 ml acetic acid, 10 mg Alcian blue 8GS) for 48 h. The bones were then placed in 70% ethanol for 6-8 hours, in 1% potassium solution for 24 h, in Alizarin solution (100 mL 05% hydroxide potassium and 10 mg alizarin red S) for two weeks, and finally in

glycerin and ethanol solution (Figure 1). The samples were photographed using a Zeiss stereomicroscope with the Dino-Lite digital and graticule lens and Dino Capture 2 software. The femur and tibiotarsus were used to measure the total length, length, and width of the ossified region (mid-diaphysis). The percentage of ossification was calculated by dividing the ossification length by the length of the entire bone.

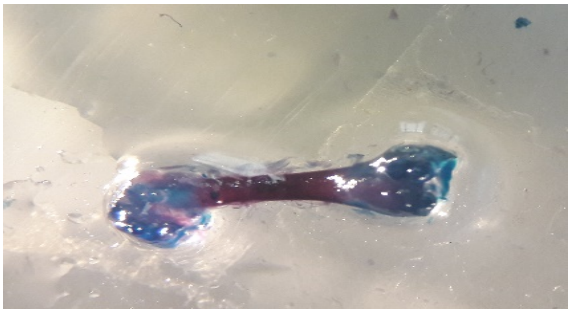

Figure 1 Femur stained with Alizarin red and Alcian blue (day 12 embryo incubated under illumination)

Hatching characteristics

On the day of hatching, five chicks were randomly selected from each replicate for each treatment (n=75). The chicks were weighed using a digital balance (A±0.01 g). The crown-rump length was measured as an index of embryonic development using a flexible tape measure, and the beak and right digit length were measured as indicators of skeletal growth using a digital calliper (Tolsen, 0.01mm).

Bone anatomy

After decapitation, the soft tissues covering the femur and tibiotarsus of the right leg were dissected using a fine pair of surgical scissors. The width (at the middle part of the bone shaft) and length of the femur and tibiotarsus of the right leg were measured with a digital calliper and weighed (referred to as the fresh bone weight). The Seedor index was calculated as a bone density index by dividing bone weight by bone length, and bone robusticity was measured by dividing the bone length by the third root of bone weight. The bones were placed in plastic zip-top bags and stored at -20 ˚C for later determination of calcium and phosphorus in ash.

Hatching characteristics

On hatching day, five chicks were selected randomly from each replicate in each treatment (n=75). The chicks were weighed on a digital balance (±0.01 g). The crown-rump length was measured as an index of embryonic development by using a flexible tape measure. Also, the beak and the right digit leg length were measured as indicators of skeletal growth using a digital calliper (Tolsen, 0.01mm).

The ratio of mineral to organic tissues in the femur and tibiotarsus

The bones were placed in acetone for 12 hours to remove the lipids, oven-dried at 105 ˚C for six hours, and the bone-dry weight was measured on a digital balance. Then, the dried bone was ashed at 600 ˚C for ten hours and weighed (Yair *et al*. 2012).

Measurement of calcium and phosphorus in bone characteristics

Calcium and phosphorus were measured by flame atomic absorption and the vanadate-molybdate colorimetric procedure, respectively (AOAC, 1990).

Gene expression in bone

On the day of hatching, the femur and tibiotarsus of the left leg from five chicks in each replicate per treatment (n=75) were used for gene expression analysis. After dissecting the muscle tissue and skin, the bone was instantly placed in a cryotube, frozen in liquid nitrogen, and then stored at -80 ˚C.

Quantitative real-time PCR (QRT-PCR)

The SINACLON kits (Sinaclon, Inc., Iran) were used to assess the genes. Frozen bones were crushed at -80 ˚C and homogenized for RNA extraction. The RNA quality was evaluated on 1% agarose gel (Ye *et al.* 2012), and appropriate kits (Yekta Tajhiz, Iran) were used to make cDNA. Initially, the set-up of genes was performed using real-time PCR reactions.

Dedicated SYBR Green PCR primers (made by Yekta Tajhiz Company) were used. The PCR protocol included: denaturation at 95 ˚C for 10 min, followed by 35 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s (Ye *et al*. 2012). The Livak method ($2^{-\Delta\Delta Ct}$method) was used for analysing the mRNA level of each gene (*Col10a1*, *Spp1*, *Bglap*, and *Alp*), and the gene expression levels were compared to *GAPDH* gene as the internal control. The primer characteristics are shown in Table 1.

Measurement of thyroid hormones (T3, T4) and glucose in blood serum

On the hatch day, before the decapitation of the randomly-selected chicks (five chicks from each replicate in each treatment, n=75), blood was drawn from the jugular vein for glucose and thyroid hormones analysis. After blood centrifugation for 10 min at 3600 × g, the serum was stored at -20 ˚C. Commercial kits (Padtan Gostar Isar, Iran) were used to measure the concentration of thyroid hormones. The sensitivity of the kit was 10 and 0.2 ng/mL, for T4 and T3, respectively. Serum glucose was measured by Pars Azmoun commercial kits, Iran (sensitivity, 5 mg/dL).

Table 1 The primers used for gene expression analysis (Sinagen Company, Iran)

Gene (gene symbol)	Primers sequences (5'→3')	Orientation
Collagen type X alpha1 chain (*Col10α1*)	CCACAACATTTGAGGACGGA	Forward
	CCCCTTGATGCTGGACTGTT	Reverse
Osteopontin (*Spp1*)	GAAAAATACGACCCCAGGAGC	Forward
	TGCTGAAGTGAAGCCAGGTC	Reverse
Osteocalcin (*Bglap*)	TAAAGCCTTCATCTCCCACCG	Forward
	TCAGCTCACACACCTCTCGT	Reverse
Alkaline phosphatase (*Alp*)	GTCAAAGCCAACGAGGGGAC	Forward
	TTCATCCTTAGCCCAGCGGA	Reverse
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	TGAAAGTCGGAGTCAACGGAT	Forward
	ACGCTCCTGGAAGATAGTGAT	Reverse

Statistical analysis

The GLM procedure (SAS, 2002) was used for data analysis. The Shapiro-Wilk test confirmed the normality of the data. Mean comparisons were performed (P≤0.05) by the Tukey-Kramer test. Data are reported as means ± SD.

RESULTS AND DISCUSSION

On the 12th day of incubation (E12), the mean comparison test showed that the length of femur and tibial ossification percentage were not significantly different between treatment groups, although analysis of variance had indicated a significant effect of treatments on these characteristics (Table 2). The difference between light regimens could have been significant should one have increased the number of eggs used for these measurements.

On the 15th day of incubation (E15), the length, width, femoral ossified length, femoral ossification percentage, tibial width, and tibial ossification percentage were not affected by the lighting system (Table 3). However, Van der Pol *et al*. (2019b) reported that 24 h lighting caused a decrease in the tibiotarsal length compared with the control (24 h darkness) and 12 h white light provision during the whole incubation period, while the data of the current experiment showing that the length of tibiotarsus in15-day-old embryos of the lighting group was greater than in the in the control in darkness (Table 3). The trend in increasing the length of the tibiotarsus continued to day 19 (E19) in the eight-hour white light group (Table 4) which is in line with the higher percentage of tibiotarsal ossification. The femoral ossified length in 19-day-old embryos was longer in eight-hour light groups than in the control group. It was found that eight hours of lighting, specifically white lighting, had a greater effect on the femoral and tibial length compared with the control (Table 4); this is conceivable considering that hens do not leave the nest for feeding or excretion while incubating their eggs. Thus, the eggs are not exposed to light for several hours during natural incubation. Other characteristics of embryonic bones (E19) were not affected by the lighting program.

Despite our extensive search for comparable research, we could not locate any that would enable us to compare our findings during the embryonic stage. This study provides unparalleled insights into the research area. The body weight of hatched chicks at 24D was higher than that in the 12L:12D green lighting group; however, indices of skeletal growth (the beak and the digit) and embryonic development (crown-rump length) did not change significantly (P>0.05, Table 5). On the other hand, Tong *et al*. (2015) reported that the use of light treatment resulted in longer crown-rump, beak, and digit lengths in hatchlings. This discrepancy may be attributed to the intensity and duration of the light in this and the current studies. The body weight of one-day-old chicks has a positive correlation with femur length and body weight at four days old (Van der Pol *et al*. 2015), and it is a predictor of body weight at slaughter age (Willemsen *et al*. 2008). In the current experiment, the body weight of chicks in the 12-hour green light group decreased compared to the dark group (Table 5). This result contradicts the results of some previous studies (Willemsen *et al*. 2008; Zhang *et al*. 2016) that a light program did not affect the body weight of chicks. Green lighting has been proven to enhance the proliferation and differentiation of myoblasts during incubation, increasing the weight of pectoral muscles (Halevy *et al*. 2006). In this regard, Yu *et al*. (2018) reported increased body weight of hatchlings of a Chinese commercial broiler line (Lingnan Huang) that were incubated under low-intensity green light; however, our results are inconsistent with these reports, which might be attributed to the strains used in these studies. To address the cause of this discrepancy, muscle histology should be integrated into the studies. This issue should be considered in future research. The analysis of variance showed a significant effect of lighting treatments on fresh, dry, and ash weight of the femur, as well as the organic matter to mineral (OM:M) ratio, Seedor index, and femoral ash calcium; however, there was no significant difference between lighting and darkness groups in terms of mean fresh, dry femoral weight, Seedor index, and femur phosphorus (P<0.05, Table 6).

Table 2 The effect of light color and duration on bone characteristics of 12-day embryos in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Femoral length (mm)	6.87±0.33[a]	7.31±0.33[a]	6.81±0.08[a]	6.72 ±0.15[a]	7.19 ±0.31[a]	0.046[†]
Femoral ossified length (mm)	3.30±0.14	3.68±0.34	3.23±0.04	3.77±1.06	3.47±0.25	0.66
Femoral ossification (%)	48.05±1.10	50.43±5.21	47.48±0.09	55.87±14.54	48.24±1.46	0.584
Femoral width	0.58±0.08	0.68±0.09	0.63±0.15	0.63±0.05	0.63±0.00	0.716
Tibial length (mm)	8.83±0.54	9.073±0.32	8.08±0.26	8.54±0.35	8.68±1.02	0.348
Tibial ossified length (mm)	4.99±0.31	5.13±0.185	4.57±0.15	3.77±1.06	4.91±0.58	0.087
Tibial ossification (%)	56.56±0.04[a]	56.57±0.01[a]	56.56±0.03[a]	43.50±11.43[a]	56.56±0.03[a]	0.044[†]
Tibial width (mm)	0.67±0.10	0.68±0.09	0.63±0.00	0.58±0.08	0.58±0.08	0.419

Despite significant F values, Tukey's test did not show any significant differences amongst the means (P>0.05).

Table 3 The effect of light color and duration on bone characteristics of 15- day- old embryos in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Femoral length (mm)	12.87±0.62	12.39±0.17	12.87±0.31	12.41±0.14	12.81±0.14	0.167
Femoral ossified length (mm)	6.94±0.31	7.99±2.27	3.23±0.04	7.47±0.84	6.92±0.17	0.577
Femoral ossification (%)	53.94±0.20	64.39±17.81	54.07±0.07	60.14±6.18	54.07±0.04	0.472
Femoral width	0.62±0.00	0.72±0.17	0.62±0.00	0.62±0.00	0.72±0.17	0.580
Tibiorasal length (mm)	14.84±0.47[b]	16.56 ±0.94[a]	16.62±0.25[a]	15.15±0.15[a]	17.68±0.18[a]	0.0002
Tibial ossified length (mm)	11.12±0.47[ab]	11.38±1.50[ab]	11.88±0.60[ab]	10.61±0.72[b]	12.90±0.09[a]	0.041
Tibial ossification (%)	74.96±0.91	68.78±5.91	71.48±3.49	70.06±4.72	72.96±4.41	0.462
Tibial width (mm)	0.72±1.79	0.72±1.17	0.62±0.00	0.62±0.00	0.62±0.00	0.580

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Table 4 The effect of light color and duration on bone characteristics of 19- day- old embryos in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Femoral length (mm)	17.94±0.79	20.46±1.09	19.10±0.93	18.43±0.31	19.28±1.47	0.087
Femoral ossified length (mm)	12.73±0.44[b]	15.88±1.40[a]	14.00±0.01[a]	12.60±1.18[b]	13.49±1.19[ab]	0.015
Femoral ossification (%)	70.96±0.82	77.60±3.76	73.45±3.76	68.47±7.44	69.93±1.51	0.186
Femoral width	1.40±0.15	1.30±0.08	1.35±0.17	1.19±0.09	1.25±0.00	0.301
Tibial length (mm)	22.48±0.61[b]	25.93±0.31[a]	24.91±0.60[ab]	24.06±0. 94[ab]	25.42±2.32[ab]	0.03
Tibial ossified length (mm)	18.63±0.51	20.97±0.25	20.64±0.50	20.08±0.79	21.06±1.93	0.071
Tibial ossification (%)	82.86±0.01[a]	80..86±0.15[b]	82.84±0.01[a]	83.45±0.54[a]	82.83±0.01[a]	< 0.0001
Tibial width (mm)	1.25±0.00	1.25±0.00	1.50±0.09	1.19±0.09	1.45±0.35	0.167

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Table 5 The effect of light color and duration on body weight and skeletal characteristics of the chicks on the hatch day in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Body weight (g)	47.3±2.3[a]	45.4±2.1[ab]	45.0±2.7[ab]	48.2±3.7[a]	40.2±1.9[b]	0.006
Crown-rump length (cm)	18.77±1.22	19.87±1.71	20.72±10.05	19.15±0.37	18.45±0.41	0.112
Beak length (mm)	9.92±0.28	10.09±1.04	10.21±0.58	9.81±0.62	9.62±1.13	0.851
Digit length (mm)	17.80±0.74	18.89±0.99	18.05±0.49	18.64±1.84	18.97±2.26	0.716

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Table 6 The effect of light color and duration on femoral characteristics on the hatch day in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Length (mm)	22.71±0.33	22.87±0.26	22.87±0.32	22.45±0.40	22.55±0.33	0.227
Width (mm)	1.92±0.03	1.88±0.10	1.79±0.12	1.97±0.08	1.92±0.12	0.169
Fresh weight (g)	0.23±0.02[ab]	0.25±0.02[a]	0.22±0.01[b]	0.21±0.00[b]	0.21±0.00[b]	0.011
Dry weight (g)	0.065±0.005[a]	0.067±0.005[a]	0.060±0.000[a]	0.067±0.005[a]	0.057±0.005[a]	0.025
Ash weight (g)	0.020±0.000[a]	0.012±0.005[b]	0.020±0.000[a]	0.017±0.005[ab]	0.020±0.000[a]	0.017
Organic matter to mineral ratio	0.914±0.007[b]	0.949±0.024[a]	0.908±0.004[b]	0.918±0.024[ab]	0.908±0.002[b]	0.012
Robusticity	36.8±1.3	36.2±0.8	37.9±1.0	37.4±0.8	37.5±0.8	0.177
Seedor index	0.010±0.0008[a]	0.011±0.0008[a]	0.010±0.0006[a]	0.009±0.0004[a]	0.009±0.0003[a]	0.027
Ash phosphorus (mg/kg)	389.84±207.71	605.46±206.12	431.25±99.44	419.53±221.00	341.40±62.41	0.296
Ash calcium (mg/kg)	165.52±34.55[b]	340.88±107.34[a]	157.52±33.51[b]	213.44±93.61[ab]	145.23±34.33[b]	0.010

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

The femoral ash weight in the 8-hour white light chicks was less than in the darkness and green lighting groups (Table 6); nevertheless, calcium concentration and OM:M in femoral ash were higher in the former group compared to the 24D and green light groups (Table 6). In this study, the effect of lighting on the length of femur was not significant (Table 6); this is inconsistent with a previous report indicating an increase in the weight and length of the femur and tibiotarsus when eggs were incubated under the light (Van der Pol *et al*. 2019a; Van der Pol *et al*. 2019b). This may be explained by the fact that most (99.8%) of the light incident on the egg is absorbed by the eggshell (Shafey *et al*. 2002), and the shell thickness affects the amount of light that passes through it (Shafey *et al*. 2004). The femoral ash weight of the eight-hour white lighting group was less than that of the dark group, although the OM:M ratio and the concentration of calcium in the ash were higher (Table 6).

The effect of treatments on the length, width, dry weight, ash weight, OM:M ratio, and phosphorus and calcium ash concentration in the tibiotarsus was significant (P<0.05, Table 7), but; there was no difference between the length and width of the tibiotarsus between the lighting and darkness groups (P>0.05, Table 7). In all lighting groups, the dry weight of tibiotarsal decreased compared to the control group. The weight of tibial ash decreased in the 12-hour white light group compared to the control group. However, the OM:M ratio of the 12-hour white light group increased, which is in agreement with the increase in tibial ash calcium and phosphorus (Table 7). It was reported that the 24-hour lighting during incubation caused an increase in femoral minerals compared to the 24-hour dark (24D:0L) and eight-hour dark (8D:16L) lighting programs; nevertheless, as in the current study, the tibial seed or index was not affected by the lighting program (Van der Pol *et al.* 2017). To the best of our knowledge, there are few studies on the changes in bone mineral content due to illumination during chicken embryonic life. According to Van der Pol *et al.* (2017), compared to 24-hour dark (24D:0L) and eight-hour dark (8D:16L) lighting programs, 24-hour lighting during incubation increased the femoral minerals; however, as in the current study, the density and tibial mineral content were unaffected by the lighting program. We also examined the robusticity of bones, which largely depends on the mineral density and cortical bone size (Cui *et al.* 2019). The densitometric and dimensional properties of bones are crucial for the movement and protection of chickens (Choruta, 2013). The calculation formula of robusticity takes into account the weight and size of the bones, which can affect the value of this parameter. According to the findings of the current research, the effect of lighting on bone robusticity was not significant (Tables 6 and 7).

We measured serum levels of thyroid hormones which are important in bone development (Varga *et al.* 2004; Lu *et al.* 2007), and glucose which is the primary energy source for development (Moran, 2007). In the current study serum T3 content was higher in the 8-hour green light chicks compared with incubation of eggs in the control group, but there was no difference between other lighting groups and control (P>0.05, Table 8), which is in line with some reported research findings. It was reported that quail chicks incubated in white light had higher serum T3 level, T3/T4 ratio, and glucose than those that were set in darkness (Khalil, 2009); however, green lighting did not have a significant impact on the blood levels of T3, T4, and testosterone in Arbor Acres broilers (Zhang *et al.* 2014). Incubated chicks exposed to low green lighting (50 lux) showed increased blood thyroxine (T4) concentration, growth performance, and hatchability (Yu *et al.* 2018). In the current investigation, hatchlings exposed to 12-hour white lighting had lower blood serum thyroxine concentrations than the other groups (Table 8); moreover, there was no significant effect of light on T3/T4 ratio and blood glucose concentration (P>0.05, Table 8). Given that fertilized eggs are rarely exposed to prolonged periods of light during natural incubation, it would seem natural that exposure to long periods of light may have decreased the T4 content of the 12-hour white light experimental groups. The equatorial region and the small end of the egg, respectively, based on the pigments of the shell, are more suitable for irradiating light than the larger end of the egg (Shafey *et al*. 2004; Yu *et al.* 2018). The eggs were arranged in the setter trays in the usual way at hatcheries in the current study and the lighting direction was the wide end of the eggs. In addition, the number of lamps may not have been sufficient to illuminate the eggs.

Some hormones (melatonin, GH, and IGF-I) were investigated in chicks incubated with a lighting program (Zeman *et al.* 1999; Van der Pol *et al.* 2019a; Van der Pol *et al.* 2019b). It was shown that melatonin secretion followed a circadian rhythm, at least during the last days of incubation in chicks incubated with a rhythmic lighting program (Zeman *et al.* 1999). Therefore, light may be more effective in bone growth during the final days in the hatchery because GH secretion increases and somatotropes respond to growth hormone-releasing hormone (GHRH) from embryonic day 16 (Porter *et al.* 1995). It should be noted that in our experiment, the eggs were illuminated only in the setter machines, and due to limitations, only the changes in plasma thyroid hormones were investigated. Other researchers have investigated melatonin, GH, and IGF-I in chicks incubated with a lighting program (Zeman *et al.* 1999; Van der Pol *et al.* 2019a; Van der Pol *et al.* 2019b).

Table 7 The effect of light color and duration on tibiotarsus characteristics on the hatch day in Cobb broiler chickens (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Length (mm)	31.94±0.18^{ab}	32.31±0.69^{a}	32.22±0.69^{ab}	30.52±1.52^{b}	30.79±0.58^{ab}	0.017
Width (mm)	1.91±0.13^{ab}	1.77±0.09^{b}	1.81±0.10^{ab}	1.99±0.07^{ab}	1.80±0.07^{a}	0.030
Fresh weight (g)	0.40±0.03	0.37±0.04	0.37±0.02	0.36±0.01	0.36±0.01	0.283
Dry weight (g)	0.15±0.02^{a}	0.10±0.05^{bc}	0.12±0.010^{b}	0.09±0.00^{c}	0.09±0.01^{c}	< 0.0001
Ash weight (g)	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.01±0.00^{b}	0.02±0.00^{a}	0.001
Organic matter to mineral ratio	0.914±0.007^{b}	0.920±0.006^{ab}	0.908±0.004^{b}	0.942±0.022^{a}	0.907±0.002^{b}	0.006
Robusticity	43.1±0.8	44.7±0.7	44.7±1.5	42.8±2.1	43.3±2.3	0.338
Seedor index	0.0120±0.0008	0.0110±0.0009	0.0110±0.0007	0.0110±0.0007	0.0110±0.0007	0.529
Ash phosphorus (mg/kg)	386.7±189.3^{b}	578.9±66.6^{ab}	535.9 ±89.5^{ab}	957.0±398.0^{a}	395.8±178.1^{b}	0.018
Ash calcium (mg/kg)	165.52±34.55^{ab}	193.34±12.86^{ab}	132.52±81.61^{b}	296.28±96.83^{a}	145.23±34.33^{b}	0.018

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Table 8 The effect of light color and duration on blood serum concentrations of thyroid hormones and glucose on the hatch day in Cobb broilers (mean±SD)

Characteristic	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Glucose (mg/dL)	197.25±13.961	208.50±14.977	204.75±14.430	187.50±15.864	196.67±21.548	0.425
Triiodothyronine (T3) (ng/mL)	0.350±0.057^{b}	0.550±0.173^{ab}	1.05±0.264^{a}	0.350±0.129^{b}	0.667±0.416^{ab}	0.003
Thyroxin (µg/dL)	2.25±0.63^{a}	2.10±0.60^{a}	2.60±0.310^{a}	0.87±0.27^{b}	3.10±0.10^{a}	0.0002
T3/T4 ratio	0.020±0.008	0.033±0.000	0.040±0.008	0.028±0.006	0.020±0.013	0.085

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Melatonin secretion follows a circadian rhythm in chicks incubated with a rhythmic lighting program, especially during the last days of incubation (Zeman *et al.* 1999). This suggests that light may be more effective in promoting bone growth during the final days in the hatchery, as GH secretion increases and somatotropes respond to growth hormone-releasing hormone (GHRH) from embryonic day 16 (Porter *et al.* 1995).

By exposing the eggs to light during the final days of incubation, it may be possible to enhance bone growth in chicks. However, further research is necessary to fully understand the effects of lighting on hormone secretion and bone growth in chickens. Simultaneous measurement of thyroid hormones, melatonin, growth hormone, and IGF-I may help clarify the lighting mechanism of the embryonic development during incubation.

Gene expression can have long-term effects on embryonic development and life after birth. Success in bone formation depends on changing the number and conditions of the cells, expression of specific genes, and mediators of their effects such as transforming growth factor- β (Stevens *et al*. 2000; Barnard *et al*. 2005; Mello and Tuan 2006), and endocrine factors such as thyroid hormones and vitamin D (Varga *et al.* 2004). The effect of lighting was significant on the relative expression of the selected genes (alkaline phosphatase, osteocalcin, collagen 10a1, and osteopontin) in the femur (Table 9) and tibiotarsus (Table 10, P≤0.01).

The femoral *Alp* gene expression level in 8-hour green lighting and 12-hour white lighting hatchlings was higher than in the control group. The white LED light decreased the relative expression of the *Bglap* gene in the femur compared to the control group.

Expression levels of *Col10a1* and *Spp1* genes in the femur were lower in 24D and 8-hour white lighting treatments than in other treatments (Table 9). Illumination (8-hour white lighting and 8-hour green lighting) resulted in lower expression of *Alp* gene in tibiotarsus compared with other groups (Table 10). However, the relative expression levels of tibial *Bglap* in 12-hour white lighting and 12-hour green lighting were higher than in other groups. Tibial *Col10a1* gene expression was higher in 12-hour lighting (both green and white) and 8-hour green lighting than in control and 8-hour white lighting. The tibial relative expression of *Spp1* gene was higher in white groups (both 8- and 12-hours lighting) and 8-hour green lighting than in the control group (Table 10). Tibial ALP is positively correlated with tibial ash content (Shao *et al.* 2019); therefore, the elevated *Alp* gene expression may indicate an increase in osteogenesis. This might align with the relatively lower *Alp* gene expression in the eight-hour white light group, which had low ash weight and dry tibiotarsus. To be more precise, it is crucial to assess the levels of alkaline phosphatase in the blood and the state of its transporters. The expression levels of *Alp* and *Col10α1* genes in the femur increased significantly in the group exposed to green light for eight hours. On the other hand, the groups exposed to white light and the control group showed a decrease in the relative expression of the femoral *Col10α1* and *Spp1* genes. There is a possibility that the transformation of hypertrophic chondrocytes into osteoblasts and a decrease in their number led to these changes. These changes are accompanied by the expression of *Col10α1* gene and the subsequent apoptosis of the cells before they become bone-forming cells.

Table 9 The effect of light color and duration on the expression of alkaline phosphatase, osteocalcin, collagen 10a1, and osteopontin genes in the femur on the hatch day in Cobb broiler chickens (mean±SD)

Gene	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Alkaline phosphatase (*Alp*)	1.00±0.04^{c}	1.01±0.11^{c}	3.12±0.12^{a}	2.77±0.19^{b}	0.83±0.06^{c}	< 0.0001
Osteocalcin (*Bglap*)	1.00±0.03^{a}	0.62±0.05^{b}	0.95±0.14^{a}	0.59±0.07^{b}	0.98±0.06^{a}	< 0.0001
Collagen 10a1 (*Col10a1*)	1.00±0.06^{c}	0.97±0.2^{c}	2.92±0.22^{a}	2.52±0.39^{ab}	1.86±0.29^{b}	< 0.0001
Osteopontin (*Spp1*)	1.00±0.01^{b}	0.74±0.13^{b}	2.28±0.29^{a}	2.34±0.38^{a}	1.70±0.22^{a}	< 0.0001

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

Table 10 The effect of light color and duration on the expression of alkaline phosphatase, osteocalcin, collagen 10a1, and osteopontin genes in the tibiotarsus on the hatch day in Cobb broiler chickens (mean±SD)

Gene	24D	8WL:16D	8GL:16D	12WL:12D	12GL:12D	P-value
Alkaline phosphatase (*alp*)	1.00±0.04^{a}	0.32±0.06^{c}	0.74±0.08^{b}	0.94±0.06^{a}	1.10±0.06^{a}	< 0.0001
Osteocalcin (*Bglap*)	1.00±0.15^{b}	1.11±0.13^{b}	1.22±0.12^{b}	2.54±0.36^{a}	2.40±0.12^{a}	< 0.0001
Collagen 10a1 (*Col10a1*)	1.00±0.09^{c}	1.29±0.11^{c}	1.93±0.19^{b}	2.45±0.16^{a}	2.22±0.11^{ab}	< 0.0001
Osteopontin (*Spp1*)	1.00±0.07^{c}	2.48±0.2^{a}	1.69±0.15^{b}	1.90±0.26^{b}	1.39±0.22^{bc}	< 0.0001

The means within the same row with at least one common letter, do not differ significantly (P>0.05).

The reduction in the number of hypertrophic chondrocytes, which specifically express collagen (Shen, 2005), may be associated with osteoblast leakage and osteogenesis. To better understand and interpret these changes, one should consider bone histology in future research.

Movement effectively improves and maintains the health of muscles and bones (Roddy *et al.* 2011); hence, outdoor systems of raising broiler chickens might be a solution for developing their bone quality (Kolakshyapati *et al.* 2019). However, it may not apply to countries with a shortage of pasture; thus, lighting may help grow chickens in a closed-house system. Higher wavelengths of light, such as red, penetrate the egg more (Shafey *et al.* 2002); therefore, higher wavelengths and intensities may have more profound effects on chick embryos. Research in this field is scarce, and numerous physiological factors affect chicken bone development, which make it difficult to interpret the effects of illumination.

There are many uncertainties regarding the effects of lighting, especially green light. To better understand the effects of light during incubation, it would be better to increase the light intensity throughout the incubation period and nurture chicks at least until the age of seven days. Examining the gene expression of some proteins in the bone tissue of chickens incubated under lighting may help to address the mechanism of the effect of light on embryonic bone growth.

CONCLUSION

The eight-hour white light schedule increased the size of the femur and tibiotarsus in chicken embryos, although the effect of the increase in bone size on bone-breaking strength is unclear. Considering the changes in the ash weight, organic matter to mineral ratio, and calcium and T3 concentration in the blood of chickens in the eight-hour white light group, white light may beneficial to bone development. In the femur, expression levels of the *Alp*, *Col10a1*, and *Spp1* increased by 12 h white and 8 h green lighting, and osteocalcin gene (*Bglap*) expression decreased under white. In tibiotarsus, the expression level of *Alp* decreased by 8 h lighting (green or white) while *Bglap* expression increased by 12 h lighting (green or white). Tibiotarsal expression of *Col10a1*, and *Spp1* increased by 12 h lighting (white and green) and in 8 h green illumination during incubation. The findings indicated that lighting during incubation could impact, both positively and negatively, on bone morphology and gene expression; therefore, more studies are needed to find the most beneficial lighting system during incubation for broiler chickens.

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