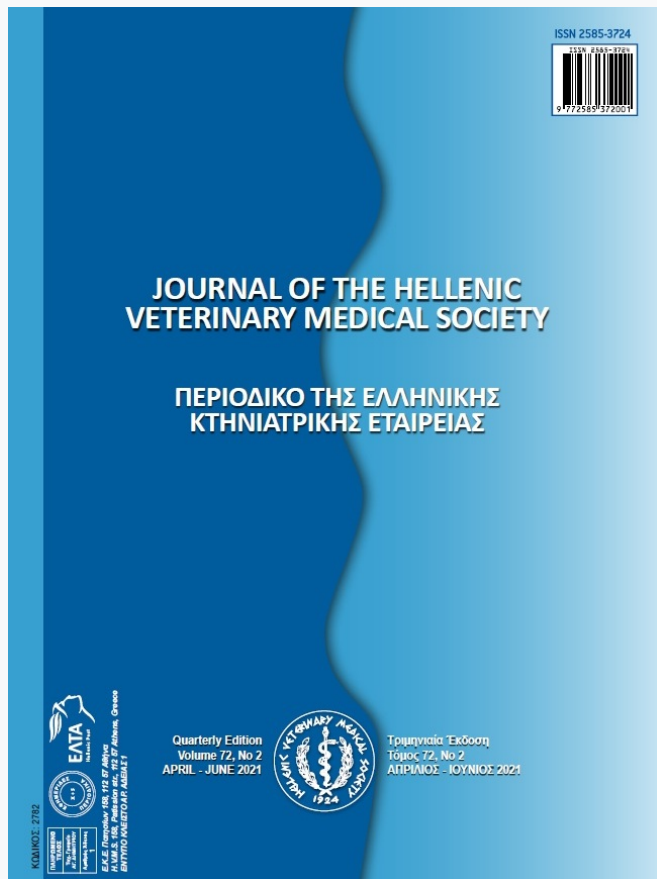


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Effect of light colour temperature on expression and serum profile of selected immune markers in layers

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ABSTRACT: This study was carried out to quantify the effects of different temperatures of light colour on expression and serum profile of selected immune markers in Fayoumi layers. A total of 165 Fayoumi healthy pullets, 17 weeks of age were used. At laying, birds were separated in well ventilated environmentally-controlled rooms and allocated into three groups of 55 birds each (5 males and 50 females) for 3 months and these birds represented the base generation (F_0). Fertile eggs were collected and the newly hatched chicks were also divided into three groups from first day of life till 3 months after laying and these represented the first generation (F_1). In the two generations, the first group (control) was exposed to cool white LED light (day light) (6500 kelvin), the second group was exposed to very cool white LED light (sky blue light) (10000 kelvin) and the third group was exposed to warm white LED light (yellow light) (2700 kelvin). Birds of each group of the two generations were evaluated for expression profile of *TLR4* and *IL10* genes and serum level of IL10. Results showed that blue light-exposed groups, in the two generations, exhibited a higher up-regulation of *TLR4* and *IL10* genes and increased serum level of IL10 compared to groups experienced either white or yellow light colour. Comparison between F_0 and F_1 individuals revealed improved genetic profiles for F_1 birds. The results therefore elucidate the benefits of using blue light in improving the immune status of layers in order to predict the most susceptible risk time for disease incidence and to build up an effective management regimen.

Keywords: Layers; gene expression; immune markers; light; Fayoumi chickens

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INTRODUCTION

Poultry production is an important component of agriculture all over the world. Chickens are considered one of the most popular types of poultry all over the world regardless culture and religion. The reason for popularity could be the high nutritive values poultry products have (Bell et al., 2002; da Silva et al., 2017; Kralik et al., 2018). Fayoumi is one of the native old breeds of chicken in Egypt. It is named for the Faiyum Governorate, southwest of Cairo and west of the Nile (Meyer, 1997; Zhou and Lamont, 2003). They are a lightweight fowl, with roosters weighing in around 2 kilograms (4.4 lb) and hens 1.6 kg (3.5 lb). In roosters, the plumage is silver-white on the head, neck, back and saddle, with the rest in a black and white barring. Hens have heads and necks in the silver-white hue, with the rest barred. Fayoumi has a single comb, red moderately large earlobes and wattles, with a white spot in the earlobes. Fayoumi also has dark horn coloured beaks, and slate blue skin (Zhou and Lamont, 2003).

Artificial light source as an external environment factor is an important aspect affecting growth and immunity in layers. It is well known that lighting factors, such as intensity, exposure time and colour affect physiology and immune competence of chickens (Foss et al., 1972; Rozenboim et al., 1999; Olanrewaju et al., 2006; Xie et al., 2008; Blatchford et al., 2009; James et al., 2018). Light colour is described by chromaticity. Chromaticity is the measure of warmth of the light source (warm light) or coolness (cool light) expressed in degrees Kelvin. The scale ranges from 2000 to 7000K. Chromaticity values of 4000 K and above are considered cool (mostly blue light), while those around 3500 K or 3600 K are called neutral, and those of about 3000 K or below are considered warm (more red light) (Knisley, 1990). Light-emitting diodes (LED) saves energy efficiently and provide sufficient brightness (Hassan et al., 2014). Natural daylight can be also effectively simulated by the application light-emitting diode (LED) than the spectral gaps of other lighting sources (El-Sabrouh and Khalil, 2017). Additionally, LEDs are potentially beneficial to the poultry industry due to long life span, moisture resistance, and narrow spectrum (Olanrewaju et al., 2015; Sharideh and Zaghari, 2017). Thus, most of the poultry producers have replaced ICD (inductively coupled discharges) lamps with LEDs. It has been established that the colour of light is a remarkable physical component of light that has a great impact on different productive, reproductive and immune parameters of

chickens (Olanrewaju et al., 2015). Moreover, as long as the longer wave lengths are possessed, the higher penetration power of light is attained (Yang et al., 2016).

Light is also a key microclimatic factor that hits chicken skull at the retinal receptors and travels through neurons to the pineal gland and hypothalamus regulating centers (Egbuniwe and Ayo, 2016). Poultry detect light through the photoreceptors of retina and the extra-retinal photoreceptors in the brain. The brains of birds are equipped with active extra-retinal photoreceptors that receive light energy and transmit it through the skull and tissues. The chicken eye is capable of discriminating light colour due to 7 photoreceptors (1 rod and 6 cones) in the eye (Hartl and Hayer-Hartl, 2002). The chicken retina consists of four types of single cones and a double cone, which are highly responsive to violet, blue, green, and red light (Bowmaker and Knowles, 1977). Photoreceptive pigments located at cones are characterized by a high sensitivity to violet (415 nm), blue (455 nm), green (508 nm), and red (571nm) (Parry et al., 2004). Therefore, light colour has been studied in poultry over the last three decades and its use has increased recently. The use of coloured lighting systems is an option to enhance production of layers in the modern layer industry. Many kinds of lights have been introduced commercially however, light emitting diode (LED) can dramatically save energy and provide adequate illumination (Rozenboim et al., 1998).

Chicken Toll-like receptor (TLR) repertoire consists of ten genes similar to that found in human and is two fewer than mouse (Higgs et al., 2006). The identified TLRs include TLR1 type 1, 2, TLR2 type 1, 2, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21. Chicken TLRs are present in different organs as thymus, liver, kidney, brain, muscle, spleen, bursa, and testis (Bekeredjian-Ding and Jegou, 2009). Toll-like receptors (TLRs) are a family of transmembrane-spanning proteins, which recognize molecules unique to microbes, discriminate self from non-self-antigens, trigger appropriate immune responses, act as sentinels of tissue damage, and mediate inflammatory responses to aseptic tissue injury (Marsh et al., 2009).

Interleukin-10 encodes a 178-aa polypeptide, with a predicted 162-aa mature peptide. It has 45 and 42% aa identity with human and murine IL-10, respectively. The chIL-10 gene structure is similar to (five exons, four introns), but more compact than, that of its mammalian. Chicken IL-10 mRNA expression

was identified mainly in the bursa of Fabricius and cecal tonsils, with low levels of expression also seen in thymus, liver, and lung (Rothwell et al., 2004). IL-10 is one type of the anti-inflammatory cytokines that could control the nature and degree of inflammation responses during infection, and also share an important role in immunity of intestine, and hemostasis (Manzanillo et al., 2015). IL-10 expression is extensively regulated at the post-transcriptional level, which may involve control of mRNA stability via AU-rich elements and by microRNAs such as let-7 or miR-106 (Sharma et al., 2009).

Research has elaborated the effects of light colour temperatures on different parameters of broiler including performance (Hassan et al., 2014; Sultana et al., 2014; Archer, 2016; Shariadeh and Zaghari, 2017; Abdel-Azeem and Borham, 2018), behaviour (Prayitno et al., 1997; Blatchford et al., 2009), welfare (Mohamed et al., 2014), health and productivity (Blatchford et al., 2009; Deep et al., 2010), heat stress (Abdo et al., 2017; Mousa-Balabel et al., 2017), carcass characteristics (Onbaşlılar et al., 2007; Olanrewaju et al., 2015), immune parameters (Hassan et al., 2014; Firouzi et al., 2014), blood properties (Firouzi et al., 2014; Seo et al., 2016), and physical traits (Alattar et al., 2019). In layers, research has focused also on many aspects including performance (Kamanli et al., 2015), egg production (Han et al., 2017; EL-Emam et al., 2019), egg quality (Er et al., 2007; Borille et al., 2013; Kamanli et al., 2015; El-Sabrouh and Khalil, 2017), behaviour (Mohamed et al., 2010; Sultana et al., 2013; Shi et al., 2019), hatching performance (Yu et al., 2018) and stress response (Liu et al., 2018; Archer, 2019).

Research carried out on the effect of light colour in broiler showed conflicting findings. Blue light colour has been suggested to improve the immune status of birds (Hassan et al., 2014; Mohamed et al., 2014; Seo et al., 2016; Guo et al., 2018; Soliman and Hassan 2019). However, warm light colour has been reported to enhance immunity (Sharideh and Zaghari, 2017). There is a little information regarding the effect of light colour temperature on immune status of layers. Additionally, no previous studies have considered how environmental factors such as the temperature of light colour may affect immunity of layers through successive generations, as well as how these effects can be explored through the approach of gene expression profile of immune markers.

Therefore, the objectives of this study were to

evaluate the effect of different light colour temperatures on the immune status of Fayoumi layers by investigating the expression pattern of *TLR4* and *IL10* genes and the serum profile of IL10.

MATERIALS AND METHODS

Experimental birds and design

Base generation (F₀)

A total of 165 Fayoumi healthy pullets (17 weeks) with a similar body weight (900 ± 30 gram) were used in this experiment. Pullets were purchased from a governmental farm for poultry breeding in Fayoum Governorate, Egypt. All birds were housed in the same room till the time of laying at a density of 8 birds/m². The photoperiod was 12L: 12D, the relative humidity ranged from 67 to 77 % (Cao et al., 2008), and the house average temperature was 28 °C. Ventilation and temperature were checked daily and kept adjusted throughout the experiment (Rosa et al., 2019). From the 19th week the lighting schedule was gradually increased half an hour every week till it reached 16L: 8D lighting schedule at laying time (Han et al., 2017). As soon as laying started, at 24 weeks, the birds were allocated into three groups in three separate, well-ventilated, environmentally-controlled rooms according to the light colour temperature. Each room had a floor area of 9 m² (3m width x 3m depth) and was used for housing of 55 birds (5 males and 50 females). The first group (control) was exposed to cool LED white light (day light) (6500 kelvin). The second group (sky blue light) was exposed to very cool LED white light (10000 kelvin) and the third group (yellow light) was exposed to warm LED white light (2700 kelvin) till the end of the experiment. Light intensity was 25 lx (1.4-ft candle) during the light phase and 0 lx during the dark phase of the photoperiod (Mohammed et al., 2010). The intensity of light was recorded near the floor, nearly at the level of bird height. Artificial light systems were placed 10 cm above the birds using plastic crosses attached to the ceilings of the rooms. Feed intake was calculated daily according to standard farm husbandry practices to meet the nutrient recommendations for poultry of National Research Council (NRC, 1994) and drinking water were allowed ad-libitum throughout the experimental period.

First generation (F₁)

Eggs were collected daily, and egg number and egg weight were recorded daily for each group. All eggs

for incubation were sorted in order to remove cracks, morphological deformities and dirt. At 28 weeks of age, fertile eggs were collected for 5 days from each group. They were incubated in a humidified egg incubator at 37 °C and 70% RH. The newly hatched chicks (F_1) were wing banded, weighed at hatch and then every two weeks, and were inoculated based on the program of vaccination of the Local Veterinary Organization. Chicks were divided into three groups from first day of life as the base generation control cool white, sky blue light and yellow light but were subjected to a continuous artificial lighting during the first 8 weeks of age. This artificial light was decreased to 12 hours light and 12 dark at 17th week of age, then was gradually increased by one hour/month till reached 16 hours light at the 21st week of age (Han et al., 2017). Chicks were offered a ration for starters (19 % CP and 2800 Kcal/Kg) from the time of hatch to the age of 8 weeks, a ration for growers (15 % CP and 2700 Kcal/kg) from the age of 9 to 20 weeks, and then

were fed a balanced ration for layers covering their nutritional requirements (16 % CP and 2700 Kcal/kg) till the end of the experiment (Baghban-Kanani et al., 2020). Table 1 shows the ingredients and chemical composition of the diet.

Sample collection

In both base and first generations, tissue and blood samples were taken in each group from 50 females and 5 males. Tissue samples were taken from spleen for RNA extraction. The samples were put in Eppendorf containing RNA later (Qiagen, Germany), to minimize the action of endogenous RNase. The blood samples were collected without anticoagulant from wing veins into clean and dry centrifuge tubes, were allowed to clot at room temperature, and were then centrifuged at 3000 rpm for 5 min. Serum was stored at -20 °C until biochemical analysis. Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University approved the protocol of the study.

Table 1. Ingredients and chemical composition of the diet used in the experiment.

Ingredient	Starter (0-8 wk.)	Growing (9-20 wk.)	Laying (21wk.-till end of experiment)
Yellow corn (kg)	63	63	65
Soybean meal (44% cp) (kg)	30	16.50	23.3
Wheat bran (kg)	3	16.70	1.90
Di- calcium phosphate (kg)	1.80	1.30	1.50
Limestone (kg)	1.50	1.80	7.6
Nacl (kg)	0.30	0.30	0.30
Premix (vitamins minerals mixture) (kg)	0.30	0.30	0.30
Methionine (kg)	0.10	0.10	0.10
Total (kg)	100	100	100
Calculated analysis:			
Metabolizable energy k Cal /kg	2800	2700	2700
Crude protein %	19	15	17
C/P ratio	147	193	168
Calcium %	1	.90	3.30
Available phosphate %	0.45	0.40	0.40
Lysine %	0.95	0.70	0.73
Methionine %	0.38	0.30	0.32
Methionine and cystine %	0.70	0.54	0.62

Table 2. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of *TLR4*, *IL10* and β -*Actin* genes.

Gene	Primer (forward)	Product length (bp)	Accession number	Reference
<i>TLR4</i>	F:5-GAGAACCTCAATGCGATGC-3 R:5-ATAGGAACCTCTGACAACG-3	272	NM_001030693	(Lu et al., 2013)
<i>IL10</i>	GGAGCTGAGGGTGAAGTTTG-3 -5: F TAGAAGCGCAGCATCTCTGA-3 -5: R	416	AJ621254	(Lu et al., 2013)
β - <i>Actin</i>	F:5-GAGAAATTGTGCGTGACATCA-3 R:5-CCTGAACCTCTCATTGCCA-3	152	NM_205518.1	(Yuan et al., 2007)

Table 3. Reverse transcription and real time PCR program for *TLR4*, *IL10* and β -*Actin* genes.

Gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
			Secondary denaturation	Annealing	Extension	Secondary denaturation	Annealing	Final extension
<i>TLR4</i>	50°C 30 min	94°C 15 min.	94°C 15 sec	56°C 30 sec.	72°C 30 sec	94°C 1 min.	56°C 1 min.	72°C 1 min.
<i>IL10</i>	50°C 30 min.	94°C 15 min.	94°C 15 secs	59°C 30 sec.	72°C 30 sec.	94°C 1 min.	59°C 1 min.	72°C 1 min.
β . <i>actin</i>	50°C 30 min.	94°C 15 min.	94°C 15 sec.	51°C 30 sec.	72°C 30 sec.	94°C 1 min.	51°C 1 min.	72°C 1 min.

RNA extraction and real time PCR

The RNA extraction was done using RNeasy Mini Kit (Qiagen, Germany), according to the protocol of the manufacturer. To remove any contaminating genomic DNA, RNA was treated with RNase free-DNase I (Qiagen, Germany). The expression profile of *TLR4* and *IL10* genes was carried out in spleen. The relative expression was quantified using SYBR Green PCR Master Mix (2x SensiFast™ SYBR, Biorad). Primer sequences and annealing temperatures are shown in Table 2. The housekeeping β -actin gene was used as an internal control. The reverse transcription of the extracted mRNA and the real time PCR program schedule for each gene is illustrated in Table 3. The real time PCR procedures for selected immune genes were carried out according to procedures described by Ateya et al., (2019). Stratagene MX3005P software was used to determine CT values. In order to detect variation of gene expression on the RNA of different samples, CT of each sample was compared with that of the control group according to “ $\Delta\Delta C_t$ ” method stated by Yuan et al., (2006).

Biochemical analysis

IL-10 was determined using ready-made interleukin-10 (IL-10) ELISA Kits provided by Quantikine

Company according to the method described by Zdanov et al. (1996).

Data analysis

Results were expressed as means \pm standard error of the mean. Analysis was done using one-way analysis of variance (ANOVA) to test all groups' unpaired values. Duncan Multiple Range Test was used to separate the means among the treatment groups. Differences were considered to be significant at the level of ($P \leq 0.05$).

RESULTS

The impact of light colour temperature on the pattern of expression of immunity genes (*TLR4* and *IL10*) was explored in males and females of F_0 and F_1 generations (Figure 1). Blue colour light-exposed groups exhibited a significant up-regulation of the *TLR4* and *IL10* in both males and females compared to both white (control) and yellow colour light-exposed groups. Comparison of F_0 and F_1 generations revealed that F_1 generation had a higher up-regulation of *TLR4* and *IL10* genes than F_0 in both males and females.

The impact of light colour temperature on the serum profile of IL10 was explored in both males and

females of F_0 and F_1 generations (Figure 2). There was a significant effect to the light colour temperature on serum levels of IL10. Blue colour light-exposed groups exhibited a significant increase in serum IL10 values in both males and females compared to

both white (control) and yellow colour light-exposed groups. Comparison of F_0 and F_1 generations elucidated that F_1 generation had higher values of IL10 than F_0 in both males and females.

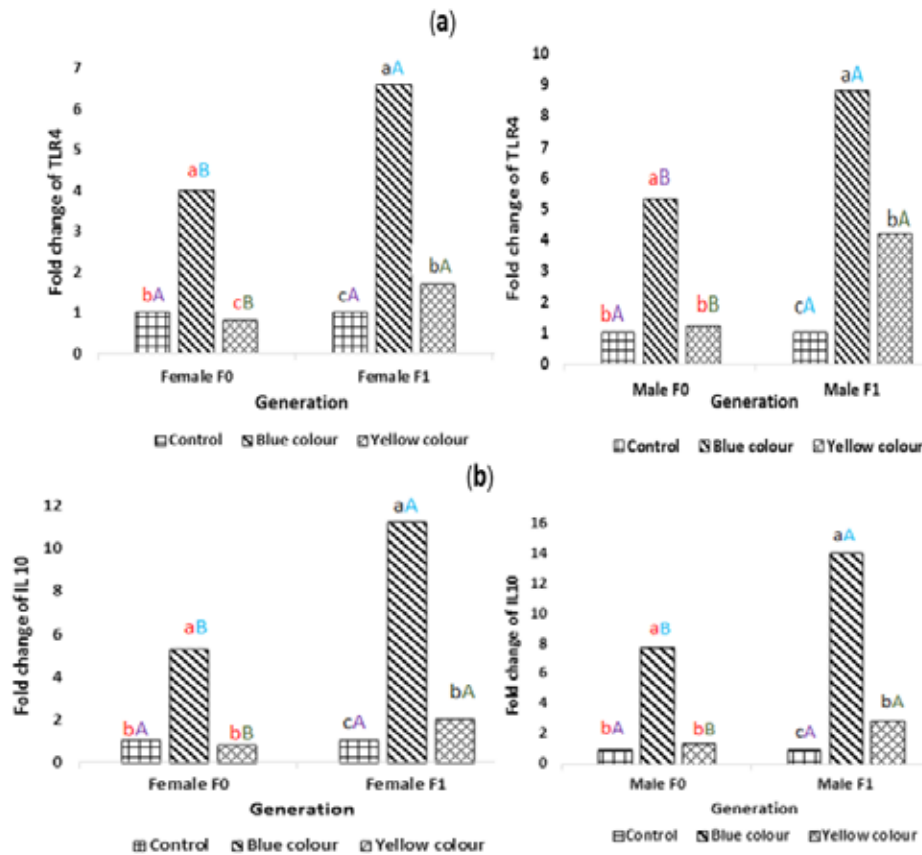


Figure 1. Relative expression of *TLR4* (a) and *IL10* (b) in males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference between groups at the same generation. Capital letter indicates a significant difference between the two generations.

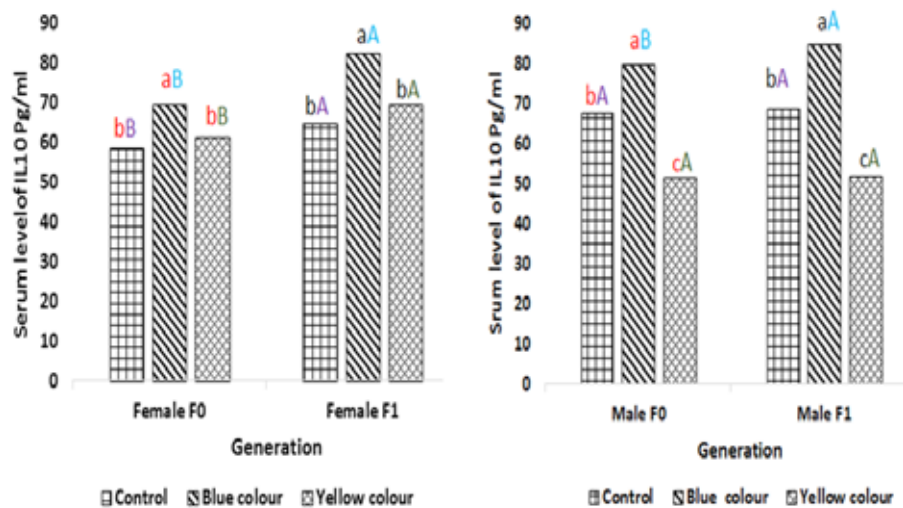


Figure 2. Serum level of IL10 in males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference between groups at the same generation. Capital letter indicates a significant difference between the two generations.

DISCUSSION

Light is as an important management tool to manipulate layer immunity. Light colour in particular is considered an important aspect of light that has been considered at one time as a management tool in poultry production (Prayitno et al., 1997). Photoperiod, wavelength, light intensity and more importantly light colour are characteristics that have to be taken into consideration in the selection of artificial light sources and the design of lighting programs for chickens (Thiele, 2010).

Maintenance of layers immune function has therefore become a necessity to avoid reduction in disease resistance and productivity. It has been shown that chicken serum composition could be modulated by lighting program (Onbaşilar et al., 2007). Toll-like receptors (TLRs) are highly conserved proteins secreted from macrophage and dendritic cells, participate in pathogen detection, enhance the production of inflammatory cytokines and up-regulate co-stimulatory molecules (Krishnan et al., 2007; Underhill and Ozinsky, 2002). In avian species, ten TLRs have been identified. Chicken *TLR4* is expressed in different locations including blood and spleen (Kogut et al., 2005). IL-10 is known as the anti-inflammatory cytokine secreted by macrophages, monocytes, and B cells (Eskdale et al., 1997). It also possesses pleiotropic effects in inflammation and immunoregulation (Pestka et al., 2004; Saraiva and O'Garra, 2010). Moreover, gene expression regulation can be exerted at a posttranscriptional level (Said et al., 2010; Haritova and Stanilova, 2012).

Findings of the current study revealed that blue colour light-exposed group had the highest up-regulation of *IL-10* and *TLR4* gene expression in both males and females of F_0 and F_1 generations. As far as we are concerned there is a lack of studies exploring the effect of light colour temperature on immune status in layers particularly those considered gene expression of immune markers. Light has been shown to have a remarkable effect on immune response (Moore and Siopes, 2000; Onbaşilar et al., 2007; Blatchford et al., 2009) however, this effect may be poorly understood (Xie et al., 2008). The improved immune profile of the birds experienced blue colour light in the current study could be attributed to specific action of the colour blue of light on immune system of birds. Blue light colour has been shown to have a remarkable positive effect on splenocyte and mononuclear cells proliferations and to increase levels of nitric oxide that activates macrophage for phagocytosis and produc-

tion of antimicrobial compounds (Seo et al., 2016). The improved immune profile emerged by blue light colour could also be due to a higher peripheral blood T-lymphocyte proliferation (Xie et al., 2008; Zhang et al., 2014; Chen et al., 2016; Guo et al., 2018), a higher H/L ratio (Mohamed et al., 2014; Mousa-Balabel et al., 2017) and its role in modifying heat shock biomarker activities toward enhancing immunity levels and reducing negative impacts of heat stress (Abdo et al., 2017). Other causes for the beneficial effect of blue light colour could be the ability of blue light to improve blood antioxidant (total antioxidant capacity, superoxide dismutase, and glutathione peroxidase), and increase B-lymphocyte proliferation in broilers (Li et al., 2015). Nevertheless, improved immune profile in Japanese quail exposed to warm white colour has been also reported (Moore and Siopes, 2003), and was referred to the release of melatonin that stimulates cellular and humoral immune response. Warm light colour was also reported to increase number of WBC (Abu Tabeekh, 2016).

In the current study we found that blue colour light-exposed group showed the highest level of IL-10 in both males and females of F_0 and F_1 generations. The results of serum profile also coincided with those of the gene expression pattern. There is also little information on serum profile of immune markers in layers exposed to different light colour temperatures. There is also controversy between the results reported in the current experiment and those of previous work. The reason for such controversy could be differences in the light source, light colour temperatures, light intensity and species/strain of the bird. It could also be that previous experiments were conducted on only one generation of birds. For instance, Abu Tabeekh, (2016) investigated how light colour affected some blood parameters of layers and reported that birds experienced warm light colour exhibited higher white blood cell counts than those received red light (RL), blue light (BL), green light (GL), and blue-green mix light (BGL).

The effect of light colour temperature was previously investigated in broiler chickens, and there were also controversies between the results. An enhanced IgG and IgA was reported in broiler receiving mixed green-blue light compared to those receiving either monochromatic green or blue light (Hassan et al., 2014). A significant enhanced proliferation of splenocyte and blood mononuclear cells was observed in chickens reared in blue compared to those reared in

green light-emitting diode (LED) (Seo et al., 2016). Similarly, blue light-exposed Cobb broiler chicks showed a significant increase in interleukin-1 β (IL-1 β) compared to those exposed to warm light (Mohamed et al., 2014). In the same respect, Guo et al., (2018) found an improved *a*-Naphthyl-acetate esterase and increased antibody production in broilers exposed to intermediate or low-intensity blue lights. Soliman and Hassan, (2019) reported also that blue light colour-exposed broiler chickens showed a significant increase in anti-Newcastle antibody titer as well as a highly remarkable decline in total bacterial count (TBC), and total Enterobacteriaceae count compared to red and white-exposed groups. On contrary, Sharideh and Zaghari, (2017) traced the effect of light emitting diodes with different colour temperatures on immune responses of male broiler and reported that warm-white light was the most suitable to provide the optimum level of immunity.

In conclusion light colour temperature has a pronounced effect on immune status of layers particularly Egyptian Fayoumi chickens. These findings recommend using blue light for better immune status in poultry farms to predict the most susceptible risk time

for disease incidence and to build up an efficient management protocol. More studies are needed to investigate the effect of light colour temperature on other chicken breeds. Expression profile of other immune genes is also needed to understand their regulation mechanisms.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

A. Ateya conceived, designed the experiment, performed the gene expression and wrote the manuscript; H. EL-Emam collected samples, contributed to doing the gene expression and writing of the manuscript. U. Abou-Ismael, I. El-Araby, and M. Fouda analyzed data and contributed to writing of the manuscript.

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