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Research Article
Effect of Light Spectrum on Stress Susceptibility and *Salmonella* Status of Laying Hens

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Abstract

Background and Objective: Light-emitting diode (LED) light bulbs are becoming more prevalent in layer production and there is discussion on whether the spectrum of light that is produced by the bulb can affect stress and health of laying hens. To investigate if differences between how different wavelengths of light affect these factors we raised hens under either bulbs that produced mainly blue light (BLUE), or mainly red light (RED) or mainly a white light (WHITE). **Methodology:** Each treatment consisted of 30 white leghorn hens and the experiment was replicated twice. Stress susceptibility was measured using bilateral asymmetry (ASYM), plasma corticosterone concentrations (CORT) and secondary antibody production to I.M. KLH injection (KLH). The birds were also subjected to a *Salmonella* challenged. Hens were broken into groups that were Unvaccinated/unchallenged (UVUC), *Salmonella enteritidis* (SE) vaccinated/unchallenged (VUC), Unvaccinated/ SE challenged (UVC) and SE vaccinated/SE challenged (VC). The ceca were enumerated. **Results:** RED birds had lower ASYM (1.43±0.12 mm) than both BLUE (1.85±0.14 mm, $p = 0.03$) and YELLOW (1.86±0.13 mm, $p = 0.03$). RED (13.8±1.7 ng mL^{-1}, $p = 0.03$) and YELLOW (12.7±1.7 ng mL^{-1}, $p = 0.01$) birds had lower CORT than BLUE (21.1±1.8 ng mL^{-1}). RED birds (401562±22013 U mL^{-1}) had higher KLH titers both BLUE (338312±18272 U mL^{-1}, $p = 0.03$) and YELLOW (333814±18790 U mL^{-1}, $p = 0.03$). Lighting did not affect ceca counts or serum titers in either trial ($p>0.05$). Differences were observed in the Salmonella titers between the vaccinated versus unvaccinated groups ($p<0.05$). **Conclusion:** The results indicate that spectrum of LED light can affect the stress susceptibility and but not *Salmonella* status.

Key words: Layers, lighting, *Salmonella*, stress, light stimulation, LED light

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Poultry are highly susceptible to light stimulation as they process light stimuli via not only their retina but the pineal gland other extra-retinal photoreceptors[1,2]. Poultry have evolved highly specialized visual systems to aid in their survival and much of poultry behavior is mediated by their vision[3]. Poultry have a wide range of vision as a result of the 4 types of single-cone photoreceptors in their eyes[4]. These provide the birds with the ability to see light in the human visual spectrum as well as the ultraviolet range, meaning they can see light of wavelengths between approximately 350-700 nm with maximum visual sensitivity at 415, 455, 508 and 571 nm[5]. As a result poultry perceive light differently than humans with some light sources appearing brighter to them than humans[6]. Poultry and birds in general also have extra-retinal photoreceptors which are located in the pineal gland and the hypothalamus[1,7]. The pineal gland and the retina are responsible for circadian rhythm control in poultry via the hormone melatonin[8-11]. The pineal gland is directly sensitive to light and is capable of synchronizing its melatonin output to cyclic light input, as well as rapidly inhibiting melatonin release during the entrained normal dark periods if exposed to light[12]. Additionally, the photoreceptors on the hypothalamus, which is located deep within the brain tissue, directly control or is involved in the control of most homeostatic and physiological processes, including reproduction.

It has been well documented that light has effective impact on immune response of poultry[13-15] but the effect of light color on the immune response is poorly understood. Xie *et al*.[16] found that broilers reared under white light had the highest peripheral blood lymphocyte proliferation response compared to blue, green, or red lights. However, Xie *et al*.[16] also found that blue and green light helped promote greater antibody production and immune function, compared to red light. Zhang *et al*.[17] also found an increase in antibody titers in broilers reared under blue/green light. However, Firouzi *et al*.[18] found no effect of light color (red, green, blue, or yellow) on New Castle's Disease Virus titers. But in yet another study Hassan *et al*.[19] found that broilers had higher levels of IgG and IgA when they were exposed to monochromatic yellow, green or blue light.

Alterations in the immune function of poultry may result in diminished ability to fight off infections by pathogens such as *Salmonella*. Furthermore, it is known that stress can increase salmonella shedding in poultry[20,21] and also that differing lighting spectrums can affect stress susceptibility differentially in poultry[22].These two factors combined with altered immune responses could result in the inability of poultry to effectively fight off infection as well as affect the robustness of any vaccination program depending on the spectrum of light the birds are reared under. To determine this we conducted an experiment to determine how three different lighting spectrums affected stress, antibody titers and *Salmonella* resistance in laying hens. It was hypothesized that spectrums would affect these parameters differentially and one would likely be optimal for lowered stress and increased health.

MATERIALS AND METHODS

Animals and husbandry: This experiment involved 3 treatments: Overdrive (WHITE, LsA19DIM 3000K, Overdrive, Clifton, NJ) LEDs and two Once, Inc. LEDS (RED, MLL and BLUE, MLBG; Once, Inc., Plymouth, MN). Light intensity level was 20 lux at feeder level as measured with a light meter (MK350, UPRTek, Jhunan Taiwan). A comparison of spectra between these bulbs can be seen in Fig. 1. Each treatment consisted of 25 cages each containing a W-36 Single Comb White Leghorn. Each treatment was housed within a light tight room outfitted with one of the light sources. Ventilation was provided by a single pass air system and heating and cooling was controlled via a central forced air system. Each of the cages measured 304.8 mm wide×520.7 mm deep×381 mm high. The experiment was replicated (Trial 1 and 2) and the treatments were randomly rotated to a new room. The birds in Trial 1 had not been vaccinated for *Salmonella*; however, the birds in Trial 2 were vaccinated prior to the study for *Salmonella*. They were vaccinated with AviPro® Megan® Egg (Elanco, Greenfield, IN) on day 23 and 42 of life and Avipro® 329 ND-IB_2-SE_4 (Elanco, Greenfield, IN) at 8 weeks of age. The birds were managed according to the guidelines set forth in the Guide for the Care and Use of Agricultural Animals in Research and Teaching[23] and methods were approved by the USDA institutional animal care and use committee (AUP #2014005). Birds were reared in from 18-42 weeks of age in Trial 1 and from 18-33 weeks of age in Trial 2.

Stress measures

Composite asymmetry score: Physical asymmetry of each bird was measured at 30 weeks of age, following the protocol outlined in Archer *et al*.[24]. Using a calibrated Craftsman IP54 Digital Caliper (Sears Holdings, Hoffman Estates, IL), the middle toe length, metatarsal length and metatarsal width were measured for both the right and left legs. The composite asymmetry score was calculated by taking the sum of the absolute value of left minus right of each trait, then dividing by the total number of traits. Thus, the formula for this trial would be:

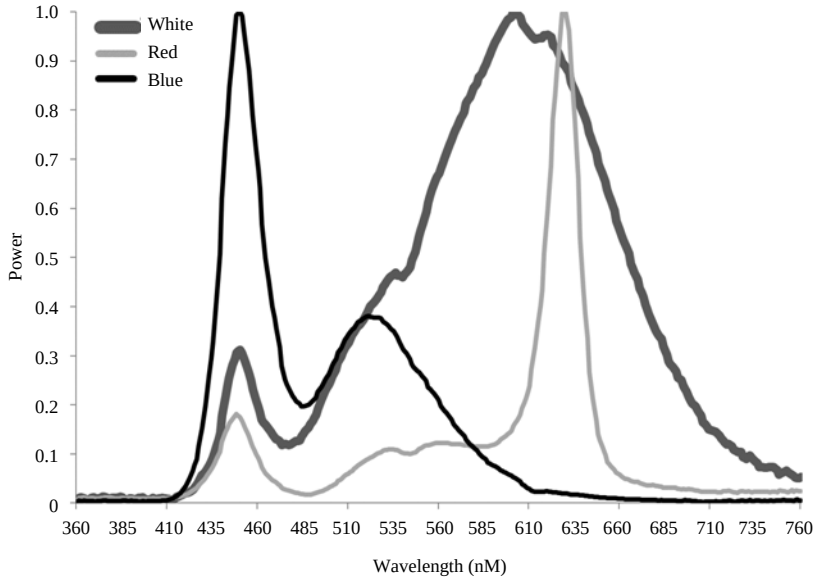

Fig. 1: Spectrum of the lighting treatments used

$$(|L\text{-}R|_{MTL}+|L\text{-}R|_{ML}+|L\text{-}R|_{MW})/3 = \text{Composite asymmetry score}$$

Plasma corticosterone: At 30 weeks of age blood samples were collected from 10 random birds per treatment via the wing vein. Between 1-2 mL of blood were collected from each bird. The blood was injected into a plasma separation gel and lithium heparin vacutainer (BD 368056, BD, Franklin Lakes, NJ), which was temporarily stored in an ice bath. Once all samples had been taken, the vacutainers were spun down in an Eppendorf AG Centrifuge 5804 (Eppendorf, Hauppauge, NY) for 15 min at 4000 RPM to separate the cells from the plasma. The plasma was poured off into 2 mL microcentrifuge tubes and stored at -19°C until further analysis. Plasma corticosterone concentrations were measured using a commercially available ELISA kit (Enzo Life Sciences, ADI-901-097, Farmingdale, NY). The inter and intra-assay CV% were both under 5%.

Antibody production: At 28 weeks of age and then again 1 week later eleven birds per treatment were injected I.M. in the leg with 0.1 mL of 1 ng KLH/0.1 mL of saline. Two weeks after the initial injection each bird had 1 mL of blood collected from the wing vein. The blood samples were centrifuged and plasma was removed and stored at 4°C until analysis. The plasma was analyzed for anti-KLH IgG titer using a commercially available ELISA (Alpha Diagnostic Intl. Inc., San Antonio, Texas, USA). The inter and intra-assay CV% were both under 5%.

Salmonella resistance

Salmonella preparation: Pure culture of Novobiocin (NO) and Nalidixic Acid (NA) resistant *Salmonella enteritidis* (SE) was retrieved from -80°C freezer, thawed and transferred using loopful into a test tube which contained 10 mL of Tryptic Soy Broth (TSB)+25 µg of NO+20 µg of NA. The TSB culture was incubated at 35°C for 8 h, then 10 µL was transferred to another test tube that contained 10 mL of TSB and was incubated at 37°C for 8 h and finally 10 µL of the second culture of TBS containing *Salmonella enteritidis* was transferred to a third test tube containing 10 mL of TBS, was incubated at 37°C for 8 h. The culture was washed with Phosphate Buffer Saline (PBS) and was centrifuged at 5000 rpm for 10 min twice. Once the washing of NO and NA resistant *Salmonella enteritidis* was completed, a tube containing 5 mL of PBS was spiked with the suspension of NO and NA resistant *Salmonella enteritidis* and the absorbance of the suspension of the NO and NA resistant *Salmonella enteritidis* was measured with the aid of spectrophotometer (Spectronic 2OD by Milton Roy Company, Ivyland, PA) at wavelength of 625 nm. The absorbance level of the pathogenic suspension was adjusted by adding more PBS until the absorbance level is equal to 1.58 (10^8 CFU mL^{-1} of NO and NA resistant *Salmonella enteritidis*).

Salmonella challenge (SE): In Trial 1, half of the birds in each lighting treatment were vaccinated subcutaneously in the neck with 0.5 mL of Layermune SE (CEVA, Lenexa KS) at

35 weeks of age. Then at 39 weeks of age, half of the vaccinated and half the unvaccinated birds were challenged with 0.5 mL of *Salmonella enteritidis* (1×10^8 CFU mL^{-1}) and returned to their cage. In Trial 2, all birds had been previously vaccinated for *Salmonella* so no further vaccination was performed; however, half of the birds were challenged with *Salmonella* as per Trial 1.

In Trial 1, on day 2, 4, 6, 8 and 12 days post SE challenge one bird per treatment (Table 2) was euthanized via cervical dislocation and had its ceca removed. The population of the *Salmonella* in the ceca was determined by transferring 0.25 g of a cecum content into 2.25 mL of PBS and 1 mL of the diluted cecum content was used to prepare a serial dilution. And 0.1 mL of the serially diluted cecum content was plated on Xylose-Lysine-Tergitol 4 (XLT4) agar (containing 25 µL and 20 µL of NO, NA respectively) and was incubated at 37°C for 24 h and the typical colony of *Salmonella* was counted and observed respectively. In Trial 2, two birds per treatment (Table 3) were euthanized so organs could be collected so that *Salmonella* shedding and enumeration could be calculated following the same procedures as in Trial 1. However, in Trial 2 this occurred on days 2, 5, 7, 9 and 12 post SE challenge.

Salmonella titers: In trial one, between 1-2 mL of blood was collected via the jugular vein on day 2, 4, 6, 8 and 12 days post SE challenge from one bird per treatment (Table 2). Blood was also collected from two birds per treatment (Table 3) in Trial 2 on days 2, 5, 7, 9, 12 post SE challenge. The blood was collected into a plasma separation gel and lithium heparin vacutainer (BD 368056, BD, Franklin Lakes, NJ), which was temporarily stored in an ice bath. Once all samples had been taken, the vacutainers were spun down in an Eppendorf AG Centrifuge 5804 (Eppendorf, Hauppauge, NY) for 15 min at 4000 RPM to separate the cells from the plasma. The plasma was poured off into 2 mL microcentrifuge tubes and stored at -19°C until further analysis. The SE antibody titers were measured using a commercially available ELISA kit (BioChek, CK117 Salmonella Group D, Scarborough, ME). The inter- and intra-assay CV% were both under 5%.

Statistical analysis: Stress measures, *Salmonella* enumeration and *SE* antibody titers were all analyzed using the GLM procedure in Minitab 18.1.0 (State College, PA). The model for the stress data and Trial 1 *Salmonella* data tested for the effects of lighting treatment, vaccine status and challenge status and the interactions between the three. The Trial 1 *Salmonella* data used the model lighting treatments, challenge status and their interaction. Significant differences were considered at $p<0.05$. Mean separation was performed using the LSD post hoc procedure.

RESULTS

Stress measures: Lighting treatments differed in composite asymmetry scores, plasma corticosterone concentrations and secondary antibody titers. Data is presented in Table 1. The RED treatment had lower composite asymmetry scores and higher antibody titers that both the other treatments ($p<0.05$). The RED and WHITE treatments had lower plasma corticosterone concentrations than the BLUE treatment ($p<0.05$). Trials only differed in antibody titers with the first trial (412511 ± 9946 U mL^{-1}) had higher titers ($p<0.001$) than the second trial (315031 ± 16868 U mL^{-1}). There were no interaction effects observed in any of the measures collected ($p>0.05$).

Salmonella challenge: The data for Trial 1 are presented in Table 2. There was no effect of lighting treatment, lighting×vaccination status, lighting×challenge status or lighting×vaccination status×challenge status ($p>0.05$) on either *Salmonella* counts in the ceca or SE antibody titers. There was an effect of vaccination status, challenge status and their interaction ($p<0.05$) on both *Salmonella* counts in the ceca and SE antibody titers. Unvaccinated birds had higher (2.57 ± 0.58 Log_{10} CFU g mL^{-1} cecal contents, $p<0.001$) *Salmonella* counts than vaccinated birds (0.66 ± 0.29 Log_{10} CFU g mL^{-1} cecal contents). Challenged birds had higher (3.12 ± 0.54 Log_{10} CFU g mL^{-1} cecal contents, $p<0.001$) *Salmonella* counts than unchallenged birds (0.09 ± 0.09 Log_{10} CFU g mL^{-1} cecal contents). The RED UVC had higher ($p = 0.04$) *Salmonella* counts (6.15 ± 1.20 Log_{10} CFU g mL^{-1}

Table 1: Stress response (composite asymmetry score (mm), plasma corticosterone concentrations (pg mL^{-1}) and secondary antibody response titers (U mL^{-1})) of layers reared under different spectrums of LED lights

Light treatments	Composite asymmetry score	Plasma corticosterone	Antibody titer
WHITE	1.86^{A}	12646^{A}	338147^{A}
RED	1.43^{B}	13793^{A}	401562^{B}
BLUE	1.85^{A}	21134^{B}	338312^{A}
SEM	0.08	1103	11827
Treatment	$F_{2,139}=3.37$, $p = 0.0.04$	$F_{2,54} = 6.78$, $p = 0.002$	$F_{2,60}=4.40$, $p = 0.02$
Trial	$F_{1,139} = 3.21$, $p = 0.08$	$F_{1,54} = 0.84$, $p= 0.36$	$F_{1,60} = 24.35$, $p<0.001$
Interaction	$F_{2,139} = 1.14$, $p = 0.32$	$F_{2,54} = 0.21$, $p = 0.81$	$F_{2,60} = 0.21$, $p = 0.81$

Different superscripts within column indicate significant differences ($p<0.05$)

Table 2: Response of *Salmonella* vaccinated and unvaccinated laying hens reared under differing spectrums of LED lighting to salmonella challenge (Ceca *Salmonella enteritidis* Enumeration (Log_{10} CFU g mL^{-1} cecal contents) and *Salmonella enteritidis* serum titers): Trial 1

Light treatments	Challenge treatment	Treatment code	CECA	SE TITER
WHITE	Unvaccinated/unchallenged	WUVUC	0.50^{c}	0.305^{d}
	SE Vaccinated/unchallenged	WVUC	0.00^{c}	0.560^{abc}
	Unvaccinated/SE challenged	WUVC	4.74^{ab}	0.443^{c}
	SE Vaccinated/SE challenged	WVC	1.00^{c}	0.562^{ab}
RED	Unvaccinated/unchallenged	RUVUC	0.00^{c}	0.272^{d}
	SE Vaccinated/unchallenged	RVUC	0.00^{c}	0.569^{a}
	Unvaccinated/SE challenged	RUVC	6.15^{a}	0.488^{abc}
	SE Vaccinated/SE challenged	RVC	1.55^{c}	0.571^{a}
BLUE	Unvaccinated/unchallenged	BUVUC	0.00^{c}	0.222^{d}
	SE Vaccinated/unchallenged	BVUC	0.00^{c}	0.5559^{ab}
	Unvaccinated/SE challenged	BUVC	4.03^{b}	0.460^{bc}
	SE Vaccinated/SE challenged	BVC	1.27^{c}	0.558^{ab}
SEM			0.354	0.021
Lighting treatment × challenge status			$F_{2,46} = 0.98$ p = 0.39	$F_{2,46} = 0.43$ p =0.65
Vaccination status × challenge status			$F_{1,46} = 18.29$ p<0.001	$F_{1,46} = 19.79$ p<0.001
Lighting treatment × vaccination status × challenge status			$F_{2,46} = 0.46$ p =0.64	$F_{2,46} = 0.49$ p =0.62

Different superscripts within column indicate significant differences (p<0.05)

Table 3: Response of *Salmonella* vaccinated laying hens reared under differing spectrums of LED lighting to salmonella challenge (Ceca *Salmonella enteritidis* Enumeration (Log_{10} CFU g mL^{-1} cecal contents) and Salmonella Enteritidis serum titers): Trial 2

Light treatments	Treatment code	CECA	SE TITER
WHITE	WUC	0.00^{b}	0.487^{abc}
	WC	2.25^{a}	0.540^{a}
RED	RUC	0.00^{b}	0.477^{bc}
	RC	1.80^{a}	0.535^{ab}
BLUE	BUC	0.00^{b}	0.460^{c}
	BC	2.44^{a}	0.492^{abc}
SEM		0.26	0.009
Lighting treatment		$F_{2,59} = 0.18$ p = 0.84	$F_{2,59} = 1.83$ p = 0.17
Challenge status		$F_{1,59} = 23.40$ p<0.001	$F_{1,59} = 7.84$ p = 0.007
Lighting treatment × challenge status		$F_{2,59} = 0.18$ p = 0.86	$F_{2,59} = 0.22$ p = 0.80

Different superscripts within column indicate significant differences (p<0.05)

cecal contents) than the BLUE UVC (4.03±0.21 Log_{10} CFU g mL^{-1} cecal contents) with the WHITE UVC (4.74±1.13 Log_{10} CFU g mL^{-1} cecal contents) being intermediate. Unvaccinated birds had higher (0.365±0.029, p<0.001) SE titers than vaccinated birds (0.563±0.002). Challenged birds had higher (0.513±0.020, p<0.001) SE titers than unchallenged birds (0.408±0.033).

The data for Trial 2 are presented in Table 3. There was no effect of lighting treatment or lighting×challenge status (p>0.05) on either *Salmonella* counts in the ceca or SE antibody titers. There was an effect of challenge status (p<0.05) on both *Salmonella* counts in the ceca or SE antibody titers. Challenged birds had higher *Salmonella* counts (2.162±0.43 Log_{10} CFU g mL^{-1} cecal contents, p<0.001) and higher SE Titers (0.523±0.010, p<0.001) than unchallenged birds (0.000±0.000 Log_{10} CFU g mL^{-1} cecal contents and 0.475±0.014, respectively).

DISCUSSION

The laying hens reared under light with a high level of red included in the spectrum exhibited less stress susceptibility when compared to the other two light spectrums used. This was consistent over two separate trials. This is contrary to what has previously been observed in monochromatic lighting. Monochromatic red LED light has been observed in laying hens to increase heterophil/lymphocyte ratios and tonic immobility duration when compared to blue LED light[25]. While in broilers Olanrewaju *et al.*[26,27] concluded that LED lighting of different color temperatures did not affect stress. However, Xie *et al.*[16] observed that that blue light may play a role in alleviating stress response in broilers due to reduction in the level of serum IL-1β. Furthermore, Abdo *et al.*[28] also observed that monochromatic blue light reduced the stress susceptibility of broilers during heat stress. Blue light has also been shown to improve immune response and alleviate the stress response in broilers[17]. Fear and stress have also been shown to be affected by different spectra of light and given that spectral output can vary drastically from light source to light source[22,29].

It is possible that the reason this current study found that red light reduced stress susceptibility compared to blue light is because these were laying hens and not broilers. Genetic background plays important roles in stress resistance in

poultry[30] and lighting can affect different strains of birds differently[31]. Furthermore, broilers are sexually immature while laying hens like those used in this current study are sexually mature. Red light plays an important role in the reproduction of poultry[32-37] and green/blue light can actually delay in rate of sexual maturity, lower egg production and lower levels of steroids[1,37-40]. So, it is possible that at differing ages and levels of sexual maturity the wavelength of light affect stress susceptibility, though this requires additional investigation.

Lighting treatments had no effect on cecal *Salmonella* counts or anti-*Salmonella* antibody titers in either trial. While anti-KLH titers were higher in the RED treatment compared to the other two treatments the anti-*Salmonella* titers were not different between treatments. This could be due to the anti-KLH titers being a secondary antibody response while the anti-*Salmonella* titers in trial one were a primary response. However, in trial 2 the anti-*Salmonella* titers were from a third exposure to a *Salmonella* vaccine so it is unclear as to why differences in humoral immunity were found in one method but not the other. The KLH titers are also inconsistent with Xie *et al.*[16] which observed that blue and green light helped promote greater antibody production and immune function, compared to red light. However, the anti-*Salmonella* titers are consistent with Firouzi *et al.*[18] who found no effect of light color (red, green, blue, or yellow) on New Castle's Disease Virus titers. Though other factors of lighting have been observed to improve immunity[13-15] light spectrum did not affect the birds in this current study ability to reduce cecal *Salmonella* populations.

While this current study hypothesized that light spectrum would affect *Salmonella* status that was not observed. However, a reduction in stress susceptibility was observed in the birds reared under light containing a large amount of red light. While this improved stress resistance did not relate to improved *Salmonella* resistance it is still an important discovery. Poultry are faced with many types of stressors during production and this study showed that lighting can reduce the physiological indicators of stress. Furthermore, previous research in broilers had concluded blue light was needed for this but in this study red light was the best indicating that age and strain of bird may be a factor as well. While these results open new questions, it is clear that lighting spectrum is important to the proper and optimum management of poultry.

SIGNIFICANCE STATEMENT

This study discovered that Color of LED lighting affected stress susceptibility of laying hens. Color of lighting did not affect *Salmonella* status. Red light can be used to improve the welfare of laying hens by reducing stress susceptibility in the early lay period.

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