THE FUNCTIONAL PROFILES OF CHICKEN EGGS INCUBATED UNDER MONOCHROMATIC LIGHTING

A Dissertation

by

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ABSTRACT

Poultry production remains susceptible to significant infectious disease threats such as Avian Flu, and Newcastle Disease Virus (NDV), which threaten the supply of poultry production. My dissertation research addresses this challenge by leveraging avian circadian biology to improve responses to vaccines to enhance poultry performance. The central hypothesis is that specific visible light wavelengths would enhance circadian rhythm development *in ovo*, leading to improved immune responses.

I addressed an essential question regarding the effect of providing photoperiods with different wavelengths (Blue, Green, and White) on circadian rhythm development and its interplay with the immune response following the NDV challenge in chick embryos using the RNAseq technology. Our results showed that incubating chicken embryos under blue light 450nm was most efficient in entraining the circadian rhythm in lung tissue, compared to white light or dark treatment. Blue light showed a specific impact on skeletal muscle, regulation of striated muscle contraction, Glycerolipid metabolism, and development of neurons. The white light incubation led to a photo-acceleration stimulant effect on epidermal growth factor receptor signaling pathway, ErbB signaling pathway, MAPK signaling pathway, and Insulin signaling pathway were upregulated in white light non-challenged treatment. The response to NDV challenging showed a distinct transcriptome profile between blue and white light. The blue light showed a potent innate immune response, targeting viral replication, clearly pointing to antiviral response.

The white light showed less immune response, but more pronounced cell proliferation and metabolic state, suggesting photo-acceleration as the primary process, particularly T cell development, T cell homeostasis, and T lymphocytes' quantity, suggests a rapid or ongoing transition between innate and adaptive immunity. This observation paves the way to photo-accelerated effect of providing white light during chicken egg incubation on organismal development and immune response. It is noteworthy that unvaccinated white light incubated chicks hatched 6-8 h earlier than other blue or dark incubation at the organism level.

In conclusion, this study is the first to generated high-resolution RNASeq evidence demonstrating the effect of lighting color background on the circadian rhythm development and modulation of the innate immune response of chicks’ embryos challenged with NDV.

DEDICATION

This dissertation is dedicated to my family, who have been a constant support and encouragement source during the challenges of achieving my degree and life. A special feeling of gratitude to my loving parents. My late father, Magdy Ibrahim, who gifted me the ingenuity of his hand, unfortunately, did not stay in this world long enough to see his son achieved his PhD degree. My mother, Naima El-Desoky, and Grandmother Al-Sayeda Hussain, whose words of encouragement and push for tenacity ring in my ears. My brothers, Abdelfattah, Mostafa, Mahmoud, and Ahmed Ibrahim have never left my side and are very special. My wife, Nouran Elmelighy, and my kids, Malak, Hamza, and Nour Ibrahim, who have all supported, encouraged, forbearance, and for being there for me throughout the entire doctorate program. I dedicate this dissertation to my advisor during my Master of Science degree, Professor Essam A. El-Gendy, for helping to develop my academic skills, making me able to face a lot of challenges throughout the entire doctorate program. I dedicate this dissertation to Dr. Mohammad R. Khattab, Assistant Research Scientist at Gastrointestinal Laboratory, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, for his guidance and support throughout the course of this research. I also dedicate this work to my many friends who have supported me throughout this PhD. I would not have been able to complete this degree without your help. You are my inspiration and my encouragement. Thank you all.

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Contributors

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Professor Giridhar Athrey assisted in the data analysis for "Chapter II and III." All Athrey Lab members assisted in project management and dissections.

All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

°C	Celsius
°F	Fahrenheit
AANAT	Arylalkylamine N-acetyltransferase Enzyme
APCs	Antigen Presenting Cells
APR	Acute Phase Response Signaling
AUP	Animal Use Protocol
BCV	Biological Coefficient Variation
Bmal1	Brain, Muscle Arnt-like 1
BP	Biological Process
CC	Cellular component
CD	Cluster of Differentiation
CLOCK	Circadian locomotor output cycles kaput
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DEGs	Differentially Expressed Genes
EID50	50% Embryo Infectious Dose
ES	Enrichment score
FC	Fold change
FDR	False Discovery Rate
GO	Gene ontology
IACUC	Institutional Animal Care and Use Committee

IFN-ά	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL6	Interleukin-6
IPA	Ingenuity Pathway Analysis
IRF	Interferon Regulator Factor
JAK	Janus Kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LEDs	Light Emitting Diode
LPS	lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
MF	molecular function
NDV	Newcastle disease virus
RIN	RNA Integrity Number
SCN	Suprachiasmatic Nucleus
SMART	Simple Modular Architecture Research Tool
STAT1	Signal transducer and activator of transcription 1
TIGSS	Texas A&M Institute for Genome Sciences and Society
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTFL	Transcriptional/post-translational feedback loop
WV	Wavelength

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Chickens are the most consumed source of animal protein in the world today (OECD, 2018). As a source of meat and eggs worldwide, domestic chicken is a cornerstone of animal agriculture and food sustainability. Continued improvements in poultry performance and production systems are crucial to supply the projected demands in the upcoming decades. Today, poultry production remains susceptible to significant infectious disease threats, such as Avian Flu, Newcastle Disease Virus (NDV), and Marek's disease (Hassan et al., 2016; Kuiken, 2013; Schilling et al., 2018), each of which threatens the security of this vital food source. Other issues such as skeletal health and muscle disorders remain significant concerns and add to poultry production's continuing threats. Therefore, improving poultry immune and metabolic health are essential considerations both from the point of food security and human health to control zoonotic disease outbreaks threatening public health worldwide.

Producing healthy chicks able to adapt to a farm's environmental conditions and survive potential infections is a challenging task. For a long time, the hatchery industry has focused a majority of its research on the ideal environmental conditions for incubation factors such as temperature, humidity, egg tray motion, and even carbon dioxide concentrations to produce healthy chicks with good physical characteristics, as these metrics are crucial for proper incubation procedures (G S Archer, 2017; Boleli et al., 2016; Nääs, Gigli, Baracho, Almeida Paz, & Salgado, 2008). Stimulating the development of circadian rhythm using illumination during incubation has been associated with many beneficial aspects in the hatched chicks, including growth

enhancement, improved health, adaptation, and welfare (Blatchford et al., 2009; Honda, Kondo, Hiramoto, Saneyasu, & Kamisoyama, 2017; Huth & Archer, 2015; Joy Mench et al., 2008; Markowska, Majewski, & Skwarło-Sońta, 2017). The current industry practices are to incubate eggs under complete darkness, which prevents the formation of circadian rhythms during incubation in the absence of light as a Zeitgeber (time giver) (V. J. Csernus, Nagy, & Faluhelyi, 2007; Tong et al., 2018).

Literature Review

Reported Effects of Photo-Biostimulation During Incubation

Lighting during incubation has caught the attention of biologists and its role in photostimulation during natural egg incubation. Photostimulation during incubation helps entrain the chicken embryo to the post-hatch environment (G S Archer, 2018; Gregory S. Archer, 2015a, 2015b; Ozkan, Yalçin, Babacanoglu, Kozanoglu, et al., 2012; Shafey & Al-mohsen, 2002; Zeman, Pavlik, Lamosˇová, Herichová, & Gwinner, 2004). Naturally brooded eggs receive varying durations of light based on the hen's diurnal activities and become important in the brooding period's final third (Duncan, Savory, & Wood-Gush, 1978; Rogers, 1996). Several studies have reported that providing light to chicken embryos during incubation affects the chick's development quality post-hatch. For example, providing light can improve growth and hatchability characteristics, and decrease the numbers of chicks with defects like unhealed navel, leg abnormalities, weak, dirty, or having traits a hatchery would reject or any other kind of abnormality (G S Archer, Jeffrey, & Tucker, 2017; G S Archer, 2018; Gregory S. Archer, 2015b; J. B. Cooper, 1972; Shafey, 2004). Light exposure during incubation may also modify hatch-linked hormones like thyroid T4, T3, and corticosterone which may shorten the time to hatch (Fairchild &

Christensen, 2000; Huth & Archer, 2015; Shafey & Al-mohsen, 2002; Tong et al., 2018). However, most of these studies relate the use of lights to the enhancement of the physiological, physical, and behavioral parameters of hatched chicks, or evaluated welfare parameters such as the decrease in fear responses and stress susceptibility (G S Archer, Shivaprasad, & Mench, 2009; Gregory S. Archer & Mench, 2014, 2017; Ozkan, Yalçin, Babacanoglu, Kozanoglu, et al., 2012; Ozkan, Yalçin, Babacanoglu, Uysal, et al., 2012). Only recently has the use of lighting during incubation emerged as an approach to stimulate the circadian and circadian-regulated processes.

Avian Circadian System

Circadian rhythms are the self-sustained biological process that produces endogenous and entrainable oscillations in the behavior, physiology, and metabolism of organisms in an approximately 24h cycle. Daily rhythms are synchronized due to an endogenous timekeeper called the circadian clock (from Latin ‘‘*circa*’’ = around and ‘‘*diem*’’ = day). Due to this, if the organism is isolated from external stimuli, it will still maintain the circadian rhythm that was driven by an environmental cue or ‘Zeitgeber’, for a short time (Edgar et al., 2012; Hill, Bassi, Bonaventura, & Sacus, 2004; Peek et al., 2015). Circadian rhythms are widely observed biological processes among organisms from cyanobacteria to vertebrates (Dunlap, Loros, Liu, & Crosthwaite, 1999; Edgar et al., 2012; Schultz & Kay, 2003; Tong et al., 2018; Young & Kay, 2001). The circadian pacemaker system in avian species is one of the most well-developed systems to regulate circadian rhythms among all animal species (M. J. Bailey et al., 2003; Bell-Pedersen et al., 2005; Herichová, Zeman, Macková, & Griac, 2001; Zeman, Gwinner, Herichová, Lamosová, & Kost’ál, 1999), and is more complex than in mammals. In addition to the suprachiasmatic nucleus (SCN) of the hypothalamus, the pineal gland contains self-sustained oscillators and considered as the third eye.

The pineal gland plays an integral part in the circadian organization by releasing melatonin hormone (Bernard et al., 1997; V M Cassone, 1990; Vincent M Cassone, 2014; V. J. Csernus et al., 2007; Deguchi, 1979; Ma, Wang, Cao, Dong, & Chen, 2018; Okano et al., 2001; Schomerus & Korf, 2005; Takahashi, Hamm, & Menaker, 1980; Turkowska, Majewski, Rai, & Skwarlo-Sonta, 2014). Previous studies have addressed how the lighting in the incubators can affect the chicks. The development of the rhythms in chicks was previously studied by monitoring body temperature changes, melatonin synthesis, and the rate-limiting enzyme activity, arylalkylamine N-acetyltransferase enzyme (AANAT). AANAT is a biomarker for the circadian system development and oscillations. Light served as the main Zeitgeber (time giver) cue in these studies to entrain circadian rhythms during the embryonic development of chicks (Bernard et al., 1997; Herichová et al., 2001; Hill et al., 2004; Lamosova, Zeman, Macková, & Gwinner, 1995; Zeman, Gwinner, & Somogyiová, 1992; Zeman et al., 2004).

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Clock Genes and Circadian Rhythm Regulation

Circadian studies linking the function and expression of clock genes in the avian brain have revealed the molecular mechanisms of circadian oscillations (Helfer, Fidler, Vallone, Foulkes, & Brandstaetter, 2006; Jiang, Wang, Cao, Dong, & Chen, 2017). The transcriptional feedback loop mechanism of the clock controls the circadian rhythmicity in most organisms. The expression of *Bmal1*, *Clock*, *Per2*, *Per3*, *Cry1*, *Cry2*, and *Cry4* clock genes have been used to study the development of circadian rhythm to compare the expression patterns in birds, and other species (M. J. Bailey, Chong, Xiong, & Cassone, 2002; Helfer et al., 2006; Yoshimura et al., 2000). The genes brain, muscle Arnt-like 1 (*Bmal1*), and circadian locomotor output cycles kaput (*CLOCK*) represent the positive part of the feedback loop, producing the heterodimer proteins which activate

the transcription of core clock genes. The genes period (*Per*) and cryptochrome (*Cry*) are transcription factors that regulate the clock-controlled genes in peripheral organs to produce enzymes, hormones, and induce receptor activity to comprise the negative feedback loop. The *Per* and *Cry* inhibit their own transcription by interacting with *CLOCK-Bmal1* heterodimer proteins and inhibit positive clock genes' activity (M. Bailey & Silver, 2014; Dunlap et al., 1999; Kohsaka & Bass, 2007; Young & Kay, 2001).

Ontogeny of Circadian Rhythm in Avian Species

The circadian rhythm in avian species begins independently from an early stage during embryonic development, and it can be detected clearly in the avian embryo prior to hatch (Vincent M. Cassone, 2015; Okabayashi et al., 2003; Zeman et al., 2004). Even though mammalian embryos synchronize with their mother's circadian clock via the maternal melatonin oscillation, their daily rhythm of physiology and behavior takes time to synchronize with the endogenous circadian clock later postnatally (Russel J Reiter, Tan, Korkmaz, & Rosales-Corral, 2014; Reppert & Schwartz, 1983; Zeman et al., 1999). To establish early circadian rhythms, the avian embryo needs light as an external Zeitgeber to develop circadian rhythms (Vincent M. Cassone, 2015; Vincent M Cassone, 2014; V Csernus, Faluhelyi, & Nagy, 2005; Helfer et al., 2006; Herichová et al., 2001; Paulose, Peters, Karaganis, & Cassone, 2009; Tong et al., 2018; Zeman et al., 1999).

In birds, the pinealocytes where photoreceptor activity exists, and melatonin is released, are very sensitive to light. For example, light intensities of 10 lux induce circadian rhythms in melatonin release *in vitro.* The effect of such low-intensity light suggests similar action *in vivo* as well (V Csernus et al., 2005; V. J. Csernus et al., 2007; Faluhelyi & Csernus, 2007). The high sensitivity of the avian pinealocytes may reflect the close evolutionary relationship to retinal cells

(Mano & Fukada, 2007) and explain why avian embryos develop early internal circadian rhythms without endocrine signals from the mother (Herichová et al., 2001; Okabayashi et al., 2003; Zeman et al., 1999, 2004). It has been reported that during natural incubation, the chicken embryo starts to produce melatonin from embryonic day 10 *in vitro* (Moller & Moller, 1990). Still, no routine rhythm of melatonin secretion is distinguished until the approximately embryonic day(ED) 18 (Zeman et al., 1999, 1992), or until ED 13 and 18 *in vitro* following 12h:12h light: dark cycles (Akasaka, Nasu, Katayama, & Murakami, 1995; Lamosova et al., 1995). It is clear that circadian oscillators not only regulate the synthesis and secretion of melatonin, but are synchronized to the environment in the chicken embryonic life (Akasaka et al., 1995; V. J. Csernus et al., 2007; Kommedal, Csernus, & Nagy, 2013).

Factors Affecting the Ontogeny of Circadian Rhythm in Avian Species

The development of circadian rhythm is not only photoperiod-dependent but also wavelength-dependent. The pineal photoreceptor pinopsin is more sensitive to shorter transmitted wavelengths of the visible range of the light spectrum (G S Archer, 2017; Valér Csernus, Becher, & Mess, 1999; Okano, Yoshizawa, & Fukada, 1994). Exposing an egg to green light can affect the development of photo-acceleration (Maurer, Portugal, & Cassey, 2011) and stimulates embryonic growth and development (Orna Halevy, Piestun, Rozenboim, & Yablonka-Reuveni, 2006; Rozenboim, Biran, et al., 2004; Shafey & Al-mohsen, 2002; Tong et al., 2018). In contrast, white and red light plays a role during egg hatching and post-hatch development (G S Archer et al., 2017; G S Archer, 2017; Gregory S. Archer, 2015a; Rozenboim, El Halawani, Kashash, Piestun, & Halevy, 2013; Yakimenko, Besulin, & Testik, 2002).

Circadian Rhythms and Immune System Interplay

The circadian clock is also associated with the rhythmic activity of the immune system. From an evolutionary perspective, it is thought that the circadian system evolved to anticipate diurnal changes, including times at which pathogens are encountered. The master circadian clock rhythm controls the transcriptional/post-translational feedback loop (TTFL) of immune genes (Labrecque & Cermakian, 2015; O'Neill, Maywood, & Hastings, 2013), as is illustrated in (figure1). Cytokine and chemokine secretion are synchronized with the circadian system's activity, likely enabling the host to anticipate and control microbial threats more efficiently (Hayashi, Shimba, & Tezuka, 2007; Keller et al., 2009; Scheiermann, Gibbs, Ince, & Loudon, 2018). Mice with *Bmal1* deletion in myeloid cells lacked the rhythm of cytokine response from macrophages, monocytes, and granulocytes, showing that the *Bmal1-CLOCK* TTFL feedback loops in myeloid cells are required to regulate the transcription and release of cytokines (J. E. Gibbs et al., 2012). Mice exposed to constant light or darkness showed abnormally increased peritoneal macrophages IL-6 response upon lipopolysaccharide (LPS) challenge, showing that circadian clock dysfunction alters the secretion of immune response signal mediator interleukins showing loss of the circadian gating mechanism (Castanon-Cervantes et al., 2010; Cutolo et al., 2006; Keller et al., 2009).

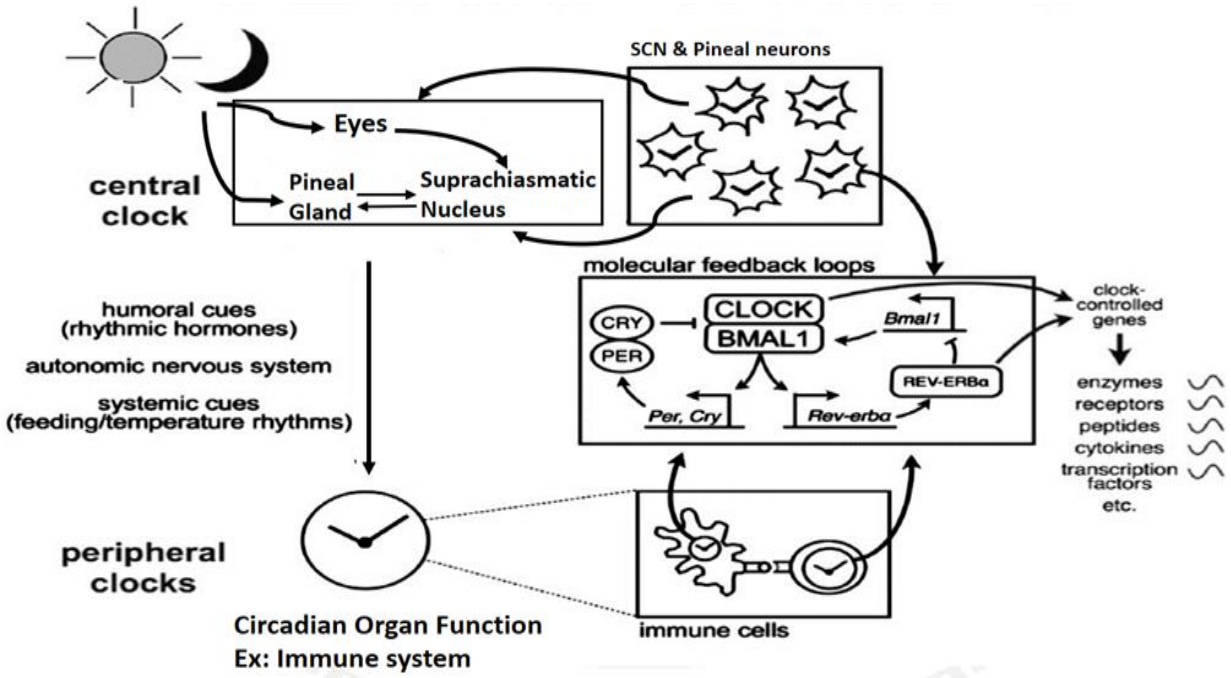

Figure 1. A modified diagram showing the interplay between the circadian system and clock molecular mechanisms based on autoregulatory of clock feedback loops that control the rhythmic expression of entrained peripheral clocks via humoral, neuronal, and systemic (Labrecque & Cermakian, 2015).

Obstructed Circadian Rhythm and Homeostasis

Circadian disturbance has been linked to the dysfunction of multiple physiological processes like energy metabolism, gut function, and immune response. These circadian interactions with metabolic, immune, and skeletal health have a high significance for livestock species (Aoyama & Shibata, 2017; Di Cara & King-Jones, 2016; Ohta, Mitchell, & McMahon, 2006; Shimizu, Yoshida, & Minamino, 2016). The gut microbiota diversity and functional activity are in synchrony with the organism circadian rhythms in healthy individuals, and circadian disruption has revealed dysbiosis in mammals (S. N. Archer et al., 2014; Maury, Hong, & Bass, 2014; Voigt et al., 2014). A recent study was done in our lab, Hieke et al. (2019) reported that

rearing chickens under extended (23/1LD) photoperiods had altered the microbiota assembly in chicks post-hatch. Regular light/dark cycles resulted in rhythmic clock gene expression and microbiota oscillation. Those findings are the first to report the potential of developing a circadian rhythm as a mechanism to engineer the colonization of beneficial microbiota in chicken. The interplay of circadian rhythm and immune response, host-microbiome, and their symbiotic resilience is receiving considerable attention in the last decade to improve poultry health and help mitigate outbreaks impacting public health worldwide (Brisbin, Gong, & Sharif, 2008; Hooper, Littman, & Macpherson, 2012; Round & Mazmanian, 2009; Scheiermann et al., 2018; Tognini, Murakami, & Sassone-Corsi, 2018). While there is a suggestion that particular wavelengths are specifically effective at stimulating circadian, little is known about which particular wavelengths, e.g., white or blue light and photoperiod combinations, will promote robust circadian entrainment *in ovo*, and in turn, kickstart immune system responses.

Objective and Central Hypothesis

My objective was to determine the effects of monochromatic green, blue, and white light during incubation on the circadian rhythm development and characterize the development of pre-hatch immune responses. My central hypothesis is that monochromatic lighting during incubation is a potent circadian stimulator, enhancing immune responses to challenge.

Study Aims

I addressed the objective in this study by pursuing two aims.

Aim 1. Determine the effects of different monochromatic light wavelengths during incubation on circadian rhythm entrainment. The working hypothesis for this aim was that the

different light wavelengths would entrain the circadian rhythm differently. The rationale was that the avian pineal gland contains pinopsin photoreceptors similar to rod retinal cells in the eye, which have similarity in the perceive and respond to light colors differently (G S Archer, 2017; V. J. Csernus, Becher, & Mess, 1999; Okano et al., 1994; Vígh, 1998) and it can result in trigger different biological developments like circadian cock (Valér Csernus et al., 1999), photobiostimulation in the embryonic growth and development and early hatching (Gregory S. Archer, 2015b; Orna Halevy et al., 2006; Rozenboim, Biran, et al., 2004; Shafey & Al-mohsen, 2002; Tong et al., 2018; Yakimenko et al., 2002).

Aim 2. Determine the role of *in ovo* circadian rhythm formation in modulating immune response following vaccination. The working hypothesis was that circadian rhythm with different entrainment backgrounds would result in a different innate and adaptive immune response 'specific antibodies production' to NDV vaccination post-hatch. The rationale for this was that the circadian pacemakers modulate the oscillations of the immune system components, acting as an essential regulator to the onset of disease impact and immune system response therapy (Arjona, Silver, Walker, & Fikrig, 2012; J. E. Gibbs et al., 2012; J. Gibbs et al., 2014; Lange, Dimitrov, & Born, 2010; Markowska et al., 2017).

Upon completing the aims, the newly generated knowledge will form the basis of identifying the most efficient light color to entrain the circadian system and result in an effective immune response among vaccinated or unvaccinated groups. Moreover, studying the canonical pathways activated based on the transcriptome profile will help clarify the cellular regulation at the gene expression level and illuminate how circadian cues affect the embryos' Biological Processes, Cellular Components, and Molecular function of the detected Gene Ontology (GO) terms analysis.

CHAPTER II

EFFECTS OF MONOCHROMATIC LIGHTING BIOSTIMULATION AND IN OVO VACCINATION ON THE SPLENIC TRANSCRIPTOME PROFILES OF CHICKEN

Introduction

The last half-century has seen more than fivefold growth in total poultry production worldwide, and this trend is expected to continue (FAO & OECD, 2016). Poultry is the world's most consumed animal protein (OECD, 2018), and the per capita consumption of poultry products is increasing worldwide. Due to this increasing demand, there is a growing emphasis on sustainable production. Morbidity and mortality rates, especially in early life, remain challenging, and approaches that improve health and performance are needed. Poultry breeders and producers value improvements in egg incubation variables to produce healthy chicks that can cope with the housing environment and overcome potential infections.

Lighting is a ubiquitous management tool that plays an essential role in poultry production, reproduction, and health. Light is a vital external abiotic cue for the chicken's physiological development, behavior, health, and welfare (Gregory S. Archer & Mench, 2017; Parvin, Mushtaq, Kim, & Choi, 2014; Raccoursier, Thaxton, Christensen, Aldridge, & Scanes, 2019). More recently, the role of lighting and photoperiods have become important tools for modulating the gut microbiota of poultry (Hieke et al., 2019; Parkar, Kalsbeek, & Cheeseman, 2019). These benefits have spurred intensive research on the use of monochromatic lighting to improve poultry health and production traits. Light-emitting diodes (LEDs), a monochromatic light source, are a promising approach to enhance avian productivity (Sultana, Hassan, Choe, & Ryu, 2013; L. Zhang et al., 2016). Like other avian species, Fowl has a unique visual system with exceptional sensitivity

to different wavelengths and a more comprehensive visible range than humans. The extra-retinal photoreceptors in the hypothalamic suprachiasmatic nucleus (SCN) and pinopsin photoreceptors in the pineal gland enable sensitivity to different light colors (Blackwell, 2002; Valér Csernus et al., 1999; Knott et al., 2010; Withgott, 2000; Yamao, Araki, Okano, Fukada, & Oishi, 1999). The transduction of light to biological signals promotes physiological and growth performance (Foster & Follett, 1985; Lewis & Morris, 2000; Mano & Fukada, 2007; Rozenboim, Robinzon, & Rosenstrauch, 1999). In poultry, the implementation of a wide variety of monochromatic light colors receives attention, as they reduce fear and stress responses and improve health and welfare (G S Archer, 2017; Sultana et al., 2013). The current industry practice is to incubate eggs under complete darkness. At the same time, lighting during incubation over recent years has been reported for its role in embryo development during incubation, hatchability, and later post-hatch chick quality.

Light exposure is beneficial, beginning at the embryonic stage. Chick embryos can sense light cues at an early stage during embryogenesis, which translates into positive effects on behavioral and physiological development (Orna Halevy et al., 2006; Rozenboim, Piestun, et al., 2004; L. Zhang et al., 2012). For example, lighting during incubation accelerates a chick's embryogenesis and shortens incubation time compared to commercial dark incubation (Adam & Dimond, 1971; Shutze, Lauber, Kato, & Wilson, 1962; Siegel, Isakson, Coleman, & Huffman, 1969; Walter & Voitle, 1973). The use of a specific range of light colors improve certain aspects of chick qualities like growth and hatchability characteristics and decrease the numbers of chicks with defects like unhealed navel, leg abnormalities, and other health defects (G S Archer et al., 2017; G S Archer, 2018; Gregory S. Archer, 2015b; J. B. Cooper, 1972; Shafey, 2004). During incubation, light exposure may also modify hatching-linked hormones like thyroid T4, T3, and

corticosterone, which may accelerate the time to hatch (Fairchild & Christensen, 2000; Huth & Archer, 2015; Shafey & Al-mohsen, 2002; Tong et al., 2018). Exposing chick embryos to warm and cool white LED lights showed a similar improvement effect on hatchability, chick quality, and decreased stress and fear responses post-hatch in broilers compared to conventional industrial dark incubation (S. Archer, 2016).

The benefits of *in ovo* lighting also translate into outcomes in the post-hatch environment. Monochromatic blue light used in poultry lighting programs significantly increases muscle growth and satellite cell proliferation after 21 days post-hatch, while the green LEDs spectrum is more effective to promote muscle growth and satellite cell proliferation during post-hatch day 1 to day 21 (J Cao et al., 2008; Liu, Wang, & Chen, 2010). On the other hand, monochromatic red light reduced the proliferation of satellite cells, changed myofiber formation and muscle growth of broilers, unlike the photostimulation by monochromatic green or blue light enhancement effect due to their role in upregulating the insulin-like growth factor 1 receptors (IGF-1R) mRNA level in skeletal muscle and plasma IGF-1 (Bai et al., 2016). Moreover, welfare and behavior are a significant perspective influenced by the type of monochromatic light used. Several studies indicated that broilers stay energetic and mobile under long wavelengths compared to short wavelengths, which might control tonic immobility in the fear response. These findings help the production and welfare parameters such as FCR, flock stress, and fear response (Gregory S. Archer & Mench, 2017; Parvin et al., 2014; Sultana et al., 2013).

Among these different monochromatic lights that had significant effects, the green spectrum showed many advantageous effects on the physiological process of chick embryogenesis development during egg incubation or either physiology or behavior post-hatch. Rozenboim et al. (2004), Zhang et al. (2012), and Zhang et al. (2014) suggested that green LED light biostimulation

during embryogenesis improves the body and breast muscle weight during incubation and post-hatch due to promoting satellite cell proliferation and differentiation in both late embryonic and newly hatched chicks. These experiments indicate that light stimulation during embryogenesis improves growth and productivity and long-term reductions in fearfulness (G S Archer, 2017; Chiandetti, Galliussi, Andrew, & Vallortigara, 2013). Greenlight promoted melatonin secretion from chicks' pineal gland during incubation (Jiang, Wang, Cao, Dong, & Chen, 2016; Jin et al., 2011; Ma et al., 2018; Ma, Wang, Cao, Dong, & Chen, 2019), and accelerates embryo development and modify hatch-related hormones, thyroid, and corticosterone resulting earlier hatching (Tong et al., 2018). Greenlight found to promote the chicks' cellular and humoral immune response, where the proliferation of peripheral blood T and B lymphocytes and the IL-2 concentration increased during the early stages post-hatch (J. Li, Wang, Cao, Dong, & Chen, 2013; Sadrzadeh et al., 2011; Xie, Wang, Cao, Dong, & Chen, 2008; Xie, Wang, Dong, et al., 2008; Z. Zhang, Cao, Wang, Dong, & Chen, 2014). In conjunction with other research, these findings show that light exposure during embryogenesis has important implications for behavioral phenotypes and chickens' welfare, immune response, and productivity. Taken together with the importance of lighting regimens for post-hatch gut health and performance, driven by circadian rhythms (Hieke et al., 2019; Wang et al., 2020), it is necessary to investigate how monochromatic lighting during incubation can improve production traits. This study investigated how green light biostimulation during incubation influences vaccination responses against Newcastle Disease Virus (NDV). Here we report our research investigating the role of lighting in influencing splenic transcriptome profiles in layer type chicken. We hypothesized that photostimulation during incubation would generate distinct responses to vaccination measured by splenic gene expression.

Methods and Materials

Animal ethics statement

We carried out all the live animal work using protocols approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP #2016-0051). Spleen samples used in this study were collected from a study investigating the role of green monochromatic light biostimulation on average plasma corticosterone in the dark vs. light exposed chicks' embryos and Newcastle disease antibody titer post-hatch with the following design and conditions.

Animals and experimental design

White layer (*Gallus gallus domesticus*) fertilized eggs (n=1728) were obtained from a commercial hatchery (Hy-Line North America, LLC) and randomly distributed among six treatments. We placed 288 eggs in each incubator (QFG 1550), with two incubators assigned for each treatment (Table 1).

Table 1. Summary of the six treatment groups in this study, showing the combination of monochromatic lights and vaccination strategies. The main groupings were based on biostimulation during incubation and the route of vaccination. The experimental birds were administered vaccines either *in ovo* on an embryonic day 18 (ED18) or day one post-hatch (Day1). Vaccinated birds were sampled on post-hatch Day 7 (PHD7).

Treatment Group	**Light Treatment**	**Vaccination Route & Age**	**Sampling Age**
Light not vaccinated (LNV)	Green Monochromatic Light	None	ED18
Dark not vaccinated (DNV)	Dark	None	ED18
Light *In ovo* vaccinated (LIV)	Green Monochromatic Light	*In ovo* (ED18)	PHD7
Dark *In ovo* vaccinated (DIV)	Dark	*In ovo* (ED18)	PHD7
Light post-hatch vaccinated (LPHV)	Green Monochromatic Light	Spray (PH1)	PHD7
Dark post-hatch vaccinated (DPHV)	Dark	Spray (PH1)	PHD7

Incubators were fitted with an LED green monochromatic light source, measured at an average of 515nm before passing the white eggshell, and an average of 517nm after passing through the eggshell (Figure 2). In effect, the shell barrier did not noticeably alter the emitted spectrum, consistent with a previous report by Archer (2017). Incubators were illuminated with two vertical LED light bars at the backside of the egg trays and two on the incubator's door (AgriShift® TLP, Junglite Green™ technology, once® Animal-Centric Lighting Systems), producing an average light intensity of 250 lux (measured at a total of 9 locations) at the egg's surface using a light meter (Extech 401 027, Extech Instruments, Nashua, NH). In contrast, the irradiance was 0.8757 W/m2 (Everfine SFIM-3000, Hangzhou, China). Glass windows on the incubators were covered with opaque sheets to prevent light intrusion from outside. In light-exposure treatments, incubators were illuminated for 24 hours a day (LD 24:0) for the entirety of incubation. For the dark treatments (LD 0:24), incubators were not illuminated and were also outfitted with opaque window covers to prevent light intrusion during incubation.

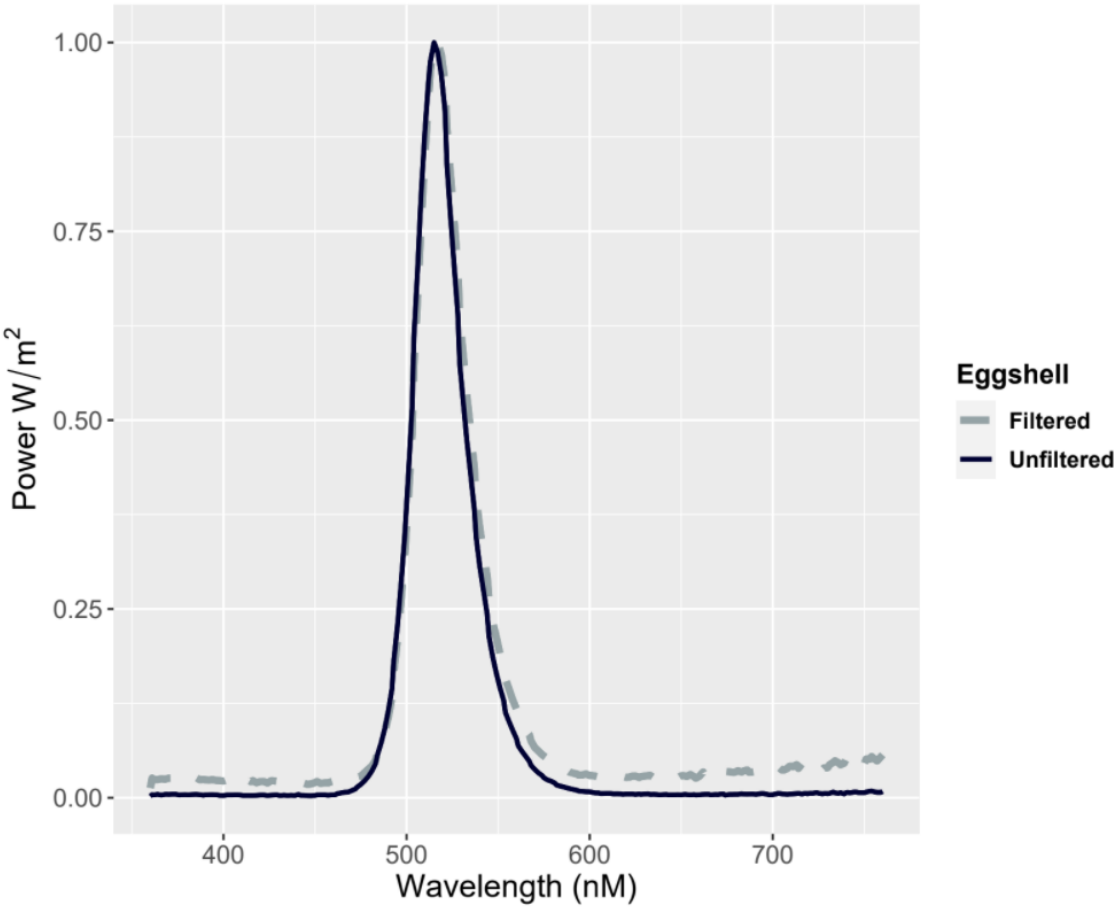

Figure 2. Light frequency spectrum for green LED light spectrum filtered by Hi-Line white eggshells. Green LED light spectrums are not changed by broiler eggshell (G S Archer, 2017).

Eggs were incubated at standard conditions of 37.5°C and 55% relative humidity for 18 days. After 18 days, eggs were transferred to the hatchers and maintained at temperature and relative humidity levels of 36.9°C and 65%, respectively. The hatchers were not fitted with illumination sources as previous research has shown that circadian rhythms are established by day 18 when incubated with illumination (Gregory S. Archer, 2015b). On an embryonic day 18 (ED18), we administered the *in ovo* vaccination with Newcastle Disease Virus (INNOVAX®-ND-SB, Intervet) to the LIV and DIV treatments. Vaccines were administered via injection of a 1x

dose in 100 µl volume of the vaccine into the amniotic fluid by one inch 21G (0.819 mm outer diameter, OD) needle, preceded by puncturing the eggshell with an 18G needle (1.270 mm OD. The injection holes were sealed with food-safe grade clear silicone to prevent infection and dehydration. For the post-hatch vaccination groups (LPHV and DPHV), NDV vaccination (NEWHATCH-C2®, B1 Type, C2 Strain, Live Virus, Intervet) was administered on Day 1 after hatch by spraying the chicks immediately before placement into rearing pens.

Regardless of the vaccination strategy, all hatched chicks were inspected, and 100 healthy and active chicks were placed in floor pens (3.34 m^2), equipped with tube feeder and nipple drinker, and raised until 14 days of age. We followed the standard recommendation for lighting given in the Hy-Line management guide, providing LD 20:4 hours of illumination in the first week (30-50 lux) followed by LD 19:5 hours of lighting a day (25 lux) during the second week. Room temperatures were maintained at 32 ± 2 °C for the first week and then decreased by 2–3 °C for the second week.

Sample collection

For the LNV and DNV treatments, we collected the embryonic spleen (ED18). Ten eggs were randomly selected from each incubator with green light and the control (dark) treatment. Embryos were dissected immediately after breaking open the eggs. Spleen samples were excised and stabilized in 1:5 volume of RNA*Later*™ (Ambion Inc, ThermoFisher Scientific) and stored at 4°C moving to long-term storage at -80°C until RNA isolation. For sampling at post-hatch day 7, ten chicks were selected randomly from LIV, LPHV, DIV, and DPHV treatments (total 40 individuals). Randomly selected chicks were euthanized humanely using exposure to CO_2, followed by cervical dislocation. We harvested tissues from euthanized chicks within 20 minutes

postmortem, and the spleen samples were stored in the same way as described above. All rearing and euthanasia procedures were performed using protocols approved by the Texas A&M University's Institutional Animal Care and Use Committee (IACUC AUP #2016-0051).

RNA isolation and quantification

From each sample, approximately 15-30 mg of spleen tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with $1cm^3$ of 1.0 mm diameter ZIRCONIA beads (cat.no. 11079124zx) using a Mini-Beadbeater-96 (BioSpec, OK, USA). We extracted total RNA, followed by an initial quantitation using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA), and estimation of protein contamination (260/280 ratio) and other organic contamination (230/260 ratio). The samples with sufficient quality and quantity were checked further with a Bioanalyzer 2100 (Agilent Technologies, DE, USA) chip reader using Agilent RNA 6000 Nano kit (No: 5067-1511) to assess the whole sample RNA integrity number (RIN) and suitability for library preparation. Total RNA samples with RIN 8.5 or higher were quantified with a Qubit™ RNA BR assay, 20–1000 ng/µL ng (Catalog number: Q10211) as well as Qubit™ dsDNA BR assay, 100 pg/µL to 1000 ng/µL to accurately determine the contamination of genomic DNA (Catalog number: Q32853). Total RNA samples passing these quality checks were normalized by dilution 400 ng/µL using nuclease-free water (NF water) and used for library preparation.

RNA library preparation and transcriptome profile generation

We used 200ng of total RNA as input for library preparation following the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina kit protocol (Lexogen, Vienna, Austria). We used

oligo (dT) primers and Illumina-specific Read 2 linker sequences to reverse transcribe mature poly-A tailed mRNA to produce a complementary first strand DNA, followed by second-strand synthesis using a random primer containing the Illumina-specific Read 1 linker sequence in the presence of DNA polymerase enzyme. We cleaned libraries using a magnetic bead-based purification step using supplied purification beads to remove impurities that interfere with library enrichment and indexing steps. Enriched single-indexed libraries were cleaned and checked using the TapeStation 2200 system and the D1000 ScreenTape assay (Agilent Technologies, Inc), and libraries were normalized to 4 nm. Twenty-four high-quality libraries (N=4/treatment) were pooled in equimolar proportions and sequenced at Texas A&M Institute for Genome Sciences and Society (TIGSS, College Station, TX) on an Illumina NextSeq (Illumina, San Diego, CA) platform. Libraries were sequenced in 75bp single-end mode, generating an average of 8.8 million reads per library.

Transcriptome data analysis

We performed all bioinformatics analysis with open-source tools and using well-established RNAseq analysis pipelines. In summary, the single-end raw reads in FASTQ format were quality checked with FastQC (Babraham Institute, Cambridge, UK) version 0.11.9 and MultiQC version 1.9 (Ewels, Magnusson, Lundin, & Käller, 2016; Martin, 2011), followed by the removal of adapter contamination and Lexogen indices. We retained only reads with a Phred quality score greater than 30 (99.9% bp signal accuracy) and over 35bp in length using Trim_Galore version 0.4.5 (Bolger, Lohse, & Usadel, 2014). Reads passing quality filters were mapped to the *Gallus gallus* genome, Galgal6 (Version 6, Ensembl Release 99 GRCg6a, Jan 2020) using the *de novo* splice mapper STAR program (version STAR_2.5.3a_modified) (Dobin et al.,

2013; Dobin & Gingeras, 2015). We counted the single-end reads mapped to exon features using HTSeq-count (version 0.9.1) (Anders, Pyl, & Huber, 2015).

We analyzed differential gene expression based on read counts using the package EdgeR (version 3.26.8) in the R statistical platform (version 3.6.2) (McCarthy, Chen, & Smyth, 2012; Robinson, McCarthy, & Smyth, 2010) using a two-factor model. Genes with uniformly low expression (<2CPM) were not included in further analysis. We applied normalization factors to correct for differences in library sizes and estimated common and tagwise dispersion (generalized linear model). We used the Exact test 'decideTestsDGE' function in the EdgeR package to test for significant differential expression between treatment groups. In addition to the global two-factor model, we ran a single-factor analysis to assess biostimulation's effects with a monochromatic green light (LNV vs. DNV, LIV vs. DIV, and LPHV vs. DPHV). The GLM approach is better at handling factorial designs with the interaction of photo-biostimulation and vaccination route, so we used the likelihood ratio test 'glmLRT' function to test for significant differential expression between groups at FDR < 0.05. We performed a power analysis based on actual dispersion (common dispersion of 0.06) in the RNAseq data using ssizeRNA 1.3.2 (Bi & Liu, 2016), which showed that our design had 97% power to detect Log_2-Fold differences at FDR≤0.05.

Pathway analyses

Differentially expressed genes were subjected to further analysis using the Ingenuity Pathway Analysis (IPA; QIAGEN Inc.) software (Krämer, Green, Pollard, & Tugendreich, 2014) to reveal canonical pathways and networks activated by these DEGs and their roles in molecular and cellular functions and physiological system development and function.

Results & Discussion

RNA sequence results and identification of differentially expressed genes.

We sequenced 24 RNAseq libraries, with four biological replicates per treatment group (six treatments), generating a total of 211.2 million reads, with an average of 8.8 million reads per library. After quality filtering and adapter trimming, we retained 96.34–97.49% of the reads per replicate (Supplementary Table S1). An average of 93.71- 95.05% mapped to the genome reference (Galgal6, ENSEMBL 99 released in January 2020)) (Supplementary Table S2), and an average of 58% of the reads mapped uniquely to exons using HTSeq-Count, (Supplementary Table S3). The common dispersion estimate for the entire dataset was low (0.069). Tagwise dispersion values in the dataset indicated that 75% of genes had a biological coefficient variation (BCV) below 0.11 (Figure S1). In contrast, the upper quartile of tagwise dispersion density estimates pointed that genes with lower expression had higher dispersion, a maximum value of 4.75. A total of 24,356 genes were detected, of which 12,769 genes were expressed at CPM>1. Of these, 11,300 genes were annotated on ENSEMBL, while the rest were novel transcripts with no annotations. Most of the expressed genes were protein-coding (91%), and the rest were assigned to long non-coding RNAs (lncRNAs, 7%), pseudogenes (0.8%), small nucleolar RNAs (snoRNAs, 0.49%), microRNA (miRNA, 0.25%), mitochondrial transfer RNA (Mt-tRNA, 0.11%), small nuclear RNA (snRNA, 0.78%).

The analysis of differential expression using EdgeR showed that the fraction of DE genes ranged from 0.5 - 55%. The highest numbers of differentially expressed genes (FDR < 0.05) were in the comparison of groups differing in the route of vaccination (*In ovo* vs. post-hatch). The effect of green light biostimulation during incubation showed fewer but revealing differences. The post-

hatch comparisons revealed the fewest differences. All comparisons made are presented in Supplementary Table S4.

Incubation with monochromatic green light stimulates gene expression important for immune response and energy metabolism in the embryonic spleen.

In the embryonic spleen (E18), we saw 217 genes differentially expressed (FDR < 0.05) between the LNV and DNV treatments. Of these, 76 were upregulated in LNV, and 141 were downregulated (Figure 2 A). Analysis of the enriched gene ontology (GO) terms with the DAVID database (using Entrez gene IDs against the chicken reference) returned 197 genes classified into three GO categories: biological process (BP), cellular component (CC), and molecular function (MF). The top enriched biological process terms were 'Plasminogen Activation', 'Positive Regulation of Protein Secretion', and 'Cell-Matrix Adhesion', for cellular components, the top enriched terms included 'Blood Microparticles', 'Fibrinogen Complex', and Extracellular Exosome', where the top enriched molecular functions were 'Metallocarboxypeptidase Activity', 'Small Molecule Binding', and 'Oxygen Binding' (Supplementary Table S4).

The Ingenuity Pathway Analysis (IPA) classified the 217 DEGs into 184 molecules annotated in its database. These molecules participate in 97 canonical pathways, of which 29 canonical pathways had a significant Z-score weighting. The top three activated pathways (based on Z-score) were 'NF-κB Signaling,' 'Sirtuin Signaling Pathway,' and 'Sumoylation Pathway.' The top three inhibited pathways included 'SPINK1 Pancreatic Cancer Pathway', 'LXR/RXR Activation,' and 'NER Pathway'. Nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway (NF-κB), a protein complex that controls transcription of DNA, cytokine production, and cell survival (Figure S2 A), was the top activated pathway with green light

incubation, involving five genes, three (*INS*, *IRAK4*, and *PIK3CG)* of which were upregulated. Two (*HDAC1* and *NFKBIA)* were downregulated.

Finding the NF-κB and the Sirtuin-signaling pathways activated in this comparison is notable. First, the circadian core oscillator gene CLOCK controls the transcription factor NF-κB (Spengler et al., 2012). This is noteworthy because the NF-κB pathway, which includes over 100 genes, is involved in regulating various biological responses, mainly related to immune responses and inflammation (Dolcet, Llobet, Pallares, & Matias-Guiu, 2005). This association between the circadian oscillator and the immune-response pathway suggests that the photostimulation during development invokes this coupled mechanism's activation. Similarly, sirtuins are known regulators of circadian transcription (Chang & Guarente, 2013; Masri, Orozco-Solis, Aguilar-Arnal, Cervantes, & Sassone-Corsi, 2015).

Green monochromatic light stimulates innate immune activity following post-hatch vaccination.

The post-hatch samples showed relatively fewer differentially expressed genes in the spleen irrespective of the vaccination method (*in ovo* or post-hatch). LPHV vs. DPHV groups revealed 116 DEGs at FDR < 0.05, where 48 were upregulated, and 68 were downregulated (Figure 2 B). The enriched GO terms were based on 107 genes annotated in DAVID. The top three enriched Biological Processes terms were 'plasminogen activation,' 'positive regulation of heterotypic cell-cell adhesion,' and 'protein polymerization'. The top three cellular components were 'blood microparticle', 'extracellular space', and 'extracellular exosome', whereas the top Molecular Function terms were 'metallocarboxypeptidase activity', 'small molecule binding', and 'hormone activity' (Supplementary Table S4).

Based on the 116 DEGs in LPHV vs. DPHV groups, IPA classified 88 to annotated molecules (39 molecules upregulated and 49 downregulated). These molecules were part of 35 canonical pathways, of which six were significant based on activation Z-score. The 'Acute Phase Response Signaling' (APR) pathway was predicted to be activated. In contrast, five pathways were predicted to be inhibited, namely 'LXR/RXR Activation', 'SPINK1, Pancreatic Cancer Pathway',' Production of Nitric Oxide and Reactive Oxygen Species in Macrophages', 'Coagulation System', and 'Intrinsic Prothrombin Activation Pathway'. The Activated APR Pathway (Figure S2 B) that have a role in a rapid inflammatory response included 12 DEGs in the LPHV and DPHV comparison group, three of which were expected to be upregulated *FGA*, *FGB*, and *FGG* and four of which were predicted to be downregulated (*ALB*, *AMBP*, *APOH*, and *TRR*).

The APR pathway is involved in various early-defense against various stressors, chief among them as an innate immune response (Cray, Zaias, & Altman, 2009). One of the modalities is invoking a local proinflammatory cytokine response, which subsequently triggers downstream processes such as protease inhibition, clotting, and opsonization (Koj, 1996; O'Brien, 2012). This innate immune activity's activation is further supported by our finding of the GO terms for 'plasminogen activation' and 'blood microparticles'. Both these suggest the associated inflammation and coagulation responses, where coagulation proteases may modulate the inflammatory response. The activated pathways observed in the LPHV versus DPHV show that biostimulation with monochromatic light during incubation promoted these enhanced immune responses to vaccination. The interleukins IL-22 and IL-6 are both regulators of the APR protein synthesis (Castell et al., 1989; Liang et al., 2010). These genes are known to have strong diurnal oscillations corresponding to circadian expression (Nilsonne, Lekander, Åkerstedt, Axelsson, & Ingre, 2016). While we did not find significant differences in CLOCK gene expression in this

comparison, the activation of the APR pathway under circadian control, suggests a role for stimulation of circadian-regulated processes. These results indicate that the embryonic circadian system was stimulated in embryos exposed to monochromatic green light, which in turn appears to activate the innate immune responses we observed here.

The *in ovo* vaccinated groups (LIV vs. DIV) showed few differences, but this is not surprising given that lighting was the only variable. We observed 62 DEGs (FDR < 0.05) at day seven post-hatch, of which 11 were upregulated in LIV, and 51 were downregulated (Figure 2 C). The top three Biological Processes were 'lipid catabolic process', 'transport', and 'positive regulation of ERK1 and ERK2 cascade; the top three Cellular Component were 'extracellular region', 'extracellular space', and 'blood microparticles'; Molecular Functions had only two enriched GO term 'metallocarboxypeptidase activity' and 'fatty acid-binding' (Supplementary Table S4).

IPA classified 46 of the DEG to annotated molecules (seven were upregulated and 39 downregulated). These molecules contributed to 19 canonical pathways, of which only one pathway was predicted to be activated based on Z-score' serine protease inhibitor Kazal-type 1' (SPINK1). SPINK1 is a protein that cleaves prematurely activated trypsin to prevent the enzyme from causing cellular damage to the organ. Nine genes were involved in this pathway (Figure S2 C), all of them were down-regulated (*CELA1*, *CLPS*, *CPA1*, *CPA2*, *CPA5*, *CPB1*, *CTRB2*, *CTRC*, and *CTRL*). The SPINK1 secretory protein is protective of pancreatic function, but can also be active in promoting tumor progression (Mehner & Radisky, 2019). In this case, as no pathologies are involved, the activation of this pathway suggests the former activity (protective). In some cancers, the SPINK1 protein modulates cancer cells' tolerance, regulates apoptosis, and maintains the body's natural immune surveillance system (Lu et al., 2011). Therefore, the involvement of

cell-mediated immunity in the context of vaccination in this study is noteworthy. The efficacy of vaccines is dependent on both a humoral and cell-mediated immune response (Amanna & Slifka, 2011). Previous studies of NDV vaccination response have noted that cell-mediated immunity contributions were crucial in decreasing disease and transmission potential (Kapczynski, Afonso, & Miller, 2013). While we would expect that both the LIV and DIV groups would elicit the same immune responses, factors that improve these responses would be highly relevant from an application standpoint. Suppose embryonic stimulation with the green light is indeed better at stimulating the cell-mediated immune component of vaccine response, as suggested by our results. In that case, this is an important outcome of our work. The observation of SPINK1 activation in the *in ovo* vaccinated, but not the post-hatch vaccinated group is another notable difference. It remains to be determined if the post-hatch environment (absence of green monochromatic lighting) contributed to this observation.

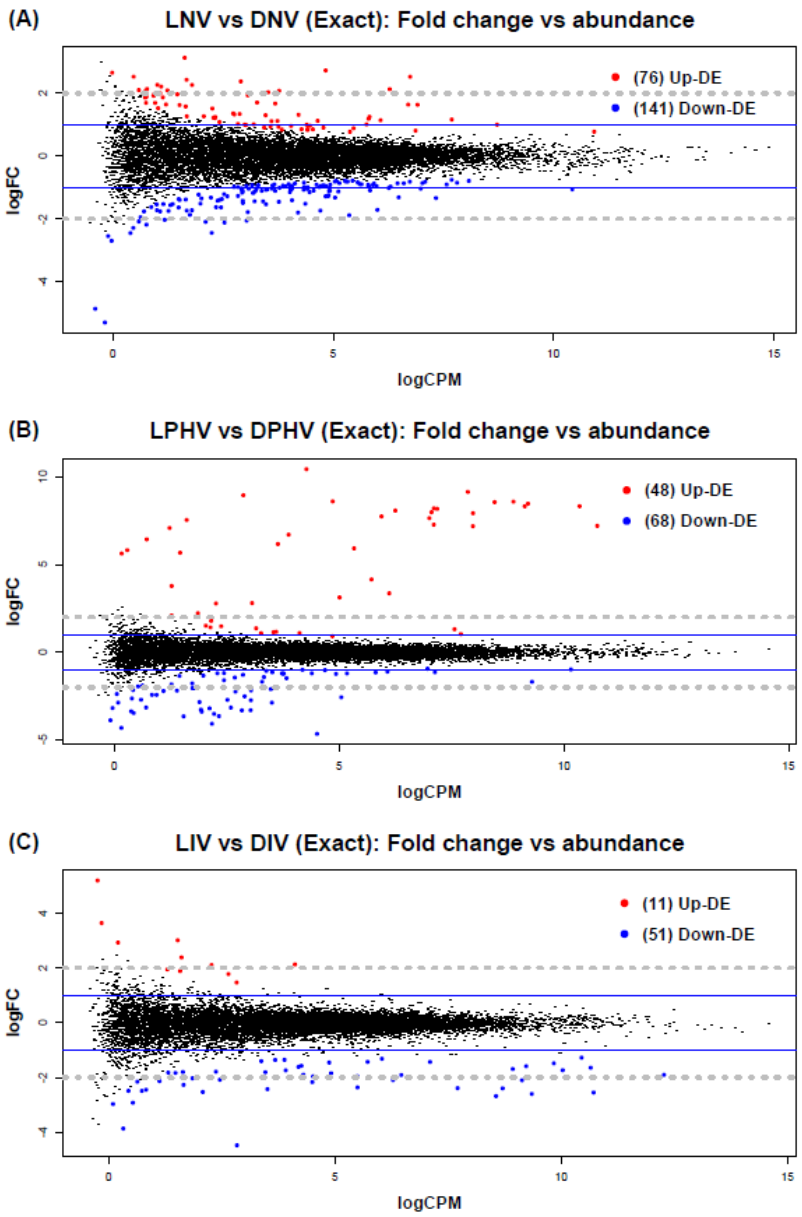

Figure 3. Mean difference (MD) plot highlighting the log fold change and average abundance of each gene in pre and post hatch spleen tissue transcriptome. Significantly up and down DE genes and their numbers are highlighted in red and blue, respectively, at FDR. Figure 3 A shows the DEGs in the embryonic spleen (E18) during green monochromatic light biostimulation pre-hatch in LNV vs DNV treatment groups. Figure 3 B shows the DEGs in spleen tissue post hatch (D7) in LPHV vs DPHV treatment groups received vaccination post hatch on day one. Figure 3 C shows the DEGs in spleen tissue post hatch (D7) in LIV vs DIV treatment group received *in ovo* vaccination E18. The Y-axis corresponds to the mean average of log10 count per million (CPM), and the X-axis displays the log2 FC at FDR < 0.05. LNV: light not vaccinated; DNV: dark not vaccinated; LPHV: light post hatch vaccinated; DPHV: dark post hatch vaccinated; LIV: light *in ovo* vaccinated; DIV: dark *in ovo* vaccinated.

Similarity of Expression networks

We assessed gene co-expression networks to characterize correlations and directionality of expression. We identified thirteen networks for the LNV vs. DNV comparison, and six networks were identified for both LPHV vs. DPHV, and the LIV vs. DIV comparisons. The networks identified from the DEGs in spleen samples pre- and post-hatch by IPA are presented in Supplementary Table S5.

The top networks (Figure 3 A) in the LNV vs. DNV comparison include 21 genes involved in cell morphology, digestive system development and function, and organ morphology. The genes that were upregulated in embryos biostimulated by green light were *DHTKD1, GOLPH3L, INS, MAGI2, mir-451, PDIA2, SEMA3D, and SYTL1*; while the downregulated genes were *APOB, APOC3, C1QTNF6, CD151, CXCL14, ENPP2, GC, HADH, INSIG1, LAPTM4B, PID1, IVA1,* and *SYTL4.* Notably, in this network, the Apolipoprotein transporters (APOB and APOC3) were downregulated while Insulin was upregulated. The reciprocity between insulin and lipoproteins is reported from various insulin resistance disorders (Åvall et al., 2015; Duivenvoorden et al., 2005; Haas, Attie, & Biddinger, 2013), but what it means for the LNV vs. DNV comparison is not clear, but perhaps suggests ongoing metabolic signaling. This interpretation is also supported by GO term and pathway analyses.

The top network in LPHV vs. DPHV comparison (Figure 3 B) contained 23 genes involved in carbohydrate metabolism, lipid metabolism, and protein Synthesis biological function. The network includes the genes *2210010C04Rik, CELA1, CELA2A, CLPS, CPA1, CPA2, CPA5, CPB1, CTRB2, CTRC, CTRL, HS6ST2,* and *RBPJL*, which were upregulated in spleen samples from chicks vaccinated post-hatch, and the genes *AHSG, AMBP, APOH, CRABP1, FGA, RBP4, SERPINC1, SFTPA1, SFTPA2,* and *TTR* were downregulated. The upregulation of several

chymotrypsin (CEL) genes indicates pancreatic activity involved in the breakdown of proteins. In this comparison, these particular genes are not readily apparent, other than suggesting digestive activity. The biostimulated chicks may have more pronounced enzymatic activity, and the finding of this activity while controlling for vaccination status supports that conclusion. As the circadian is a regulator of metabolic signaling, this network activity is not surprising, but, notably, biostimulation may produce positive benefits for metabolic performance. Further studies focusing on metabolic pathways and production traits would be necessary to confirm this hypothesis.

The top network in LIV and DIV group (Figure 3 C) showed 27 genes that imply developmental disorder, hematological disease, and hereditary disorder. Genes *EN1, FGB, and FGG,* were upregulated in spleen samples from chicks biostimulated during incubation and received *in ovo* vaccination. In contrast, *AMY2A, CAMP, CEL, CELA1, CPA1, CPA2, CPA5, CPB1, CRABP1, CTRB2, CTRC, CTRL, DMBT1, FGF13, FOXA2, Gcg, GSTM3, HS6ST2, ISL1, PDIA2, PNLIPRP1, PTPRN2, RBPJL, and SST* genes were downregulated.

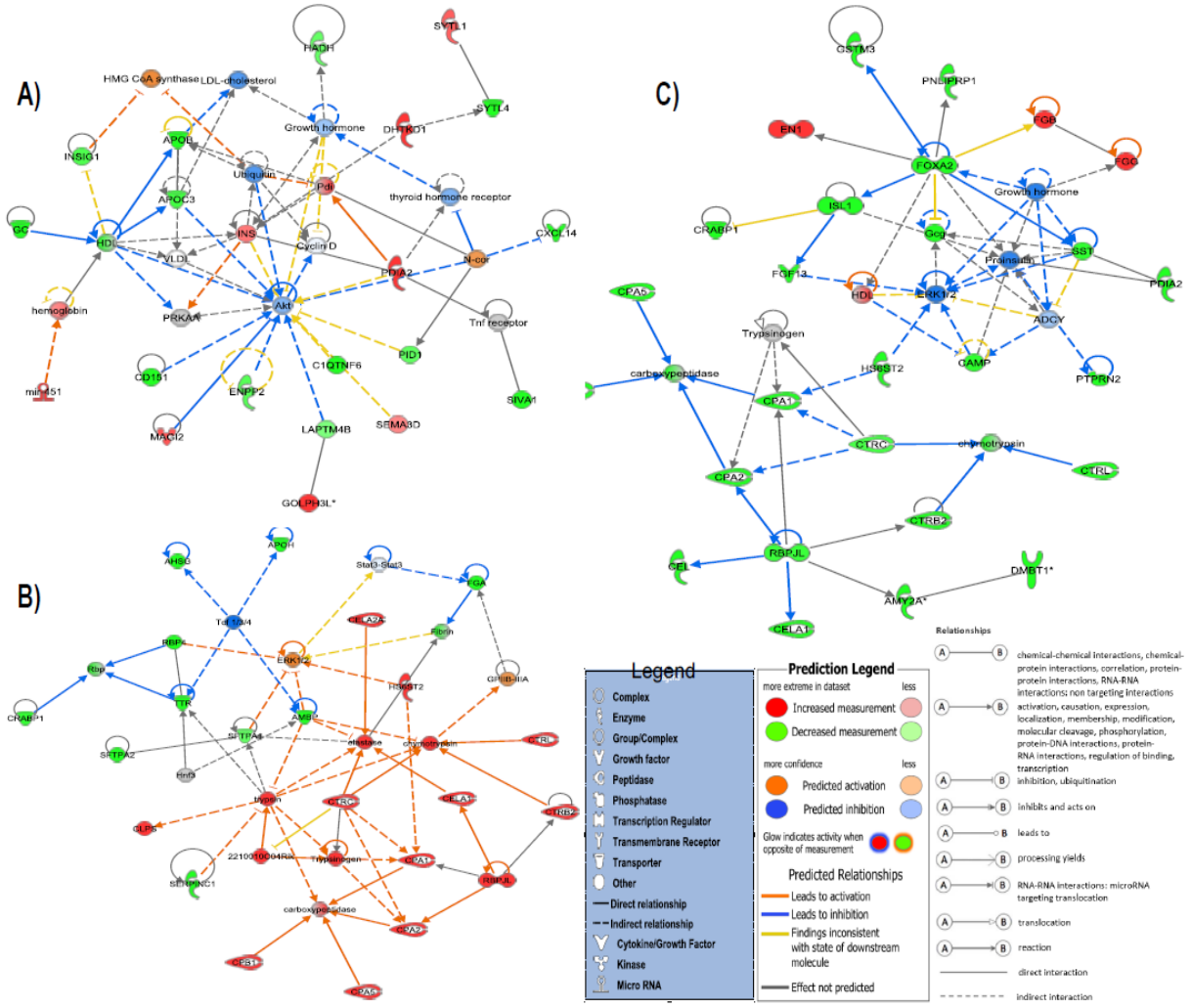

Figure 4. The top 3 detected gene networks underline the affected genes in green monochromatic light biostimulation comparisons during incubation and their interaction in potentially regulating developmental biological processes pre and post-hatch generated by QIAGEN's Ingenuity Pathway Analysis (IPA; QIAGEN Inc.) (Krämer et al., 2014). (**A**) LNV vs. DNV gene network Cell Morphology, Digestive System Development, and Function, Organ Morphology. (**B**) LPHV vs. DPHV gene network for Carbohydrate Metabolism, Lipid Metabolism, Protein Synthesis. (**C**) LIV vs. DIV gene network for Developmental Disorder, Hematological Disease, Hereditary Disorder. Differentially expressed genes in the biostimulated comparisons were used in Ingenuity Pathway Analysis, and significant gene networks based on IPA scores were identified. Genes highlighted in red were upregulated, while those highlighted in green were downregulated in all biostimulated comparisons**.**

Overrepresented GO and Pathway terms indicate lighting stimulates early life metabolic activity.

Shared pathway terms indicate metabolic and immune functions, as well as transcriptional activity controlling developmental processes. Specific canonical pathways (from IPA analysis) were observed repeatedly across the three pairwise comparisons. Similarly, there were recurring overlaps in the list of upstream regulators, molecular and cellular functions, and physiological system development and function (supplementary Table S4). These terms are summarized in Figure 4. The most observed canonical pathways are Serine protease inhibitor Kazal type 1 (SPINK1) and Acute Phase Response Signaling (Figure 4 A), while the most observed upstream regulators include HNF1A, FOXA2, and NAR5A2 (Figure 4 B). These transcription factors are expressed in several tissues and are known to be important in development, and in this case, do not provide additional context to the canonical pathways. Moreover, the most observed molecular and cellular functions include lipid metabolism, molecular transport, and small molecules biochemistry (Figure 4 C).

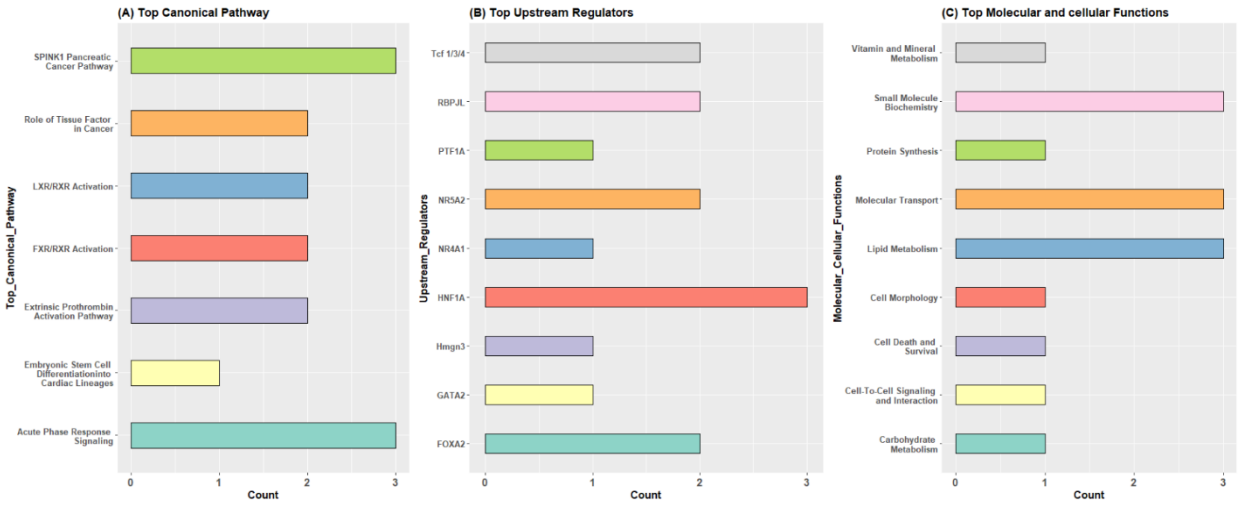

Figure 5. Most frequently observed pathway terms based on differentially expressed genes in pre- and post-hatch birds incubated in green monochromatic light versus dark. (A) Top Canonical pathway, (B) Upstream Regulator, (C) Top Molecular and Cellular Functions. All DEGs from pre (E18) and post-hatch (D7) were subjected to IPA analysis to detect ready molecules across observation, molecules with FDR < 0.05 considered significant in IPA detected Terms.

Shared GO terms all indicate ongoing developmental processes. GO analysis with DAVID also returned several overrepresented terms (shown in Figure 5). Biological process terms were repeated across the three pre- and post-hatch comparisons indicating ongoing background molecular events, including regulation of blood coagulation and vessel formation, different cell types to cell attachment, protein-polymer formation, and regulation of hormones-based protein secretion (figure 5 A, Supplemental Table S4). Blood coagulation, fibrin clot formation (GO:0072378), plasminogen activation (GO:0031639), and fibrinolysis (GO:0042730) are biological process GO terms that fall under a coagulation system, and angiogenesis is involved in wound healing. Positive regulation of exocytosis (GO:0045921), positive regulation of heterotypic cell-cell adhesion (GO:0034116), positive regulation of peptide hormone secretion (GO:0090277), and protein polymerization (GO:0051258) fall under developmental tissue state through biogenesis, regulation of hormone levels, and increase the extent of heterotypic cell-cell adhesion.

Biomolecules with specific functions in plasma membranes or adjacent areas were the most enriched and overrepresented cellular components GO terms across comparisons (figure 5 B). These GO terms include blood microparticle (GO:0072562), extracellular region (GO:0005576), extracellular space (GO:0005615), fibrinogen complex (GO:0005577), and platelet alpha granule (GO:0031091), which play a role in the organization of protein microparticles and gene products secreted from a cell but retained within the organism (i.e., released into the interstitial fluid or blood). Specific GO cellular components terms were recognized only in LIV and DIV treatment; those terms have a role in lipid metabolism include yolk (GO:0060417), chylomicron (GO:0042627), high-density lipoprotein particle (GO:0034364), and very-low-density lipoprotein particle (GO:0034361). In addition, the overlapping occurred in Up_Keywords included; 'Signal', 'Secreted', 'Disulfide bond', and 'Carboxypeptidase' terms indicated enrichment in background molecular and cellular function indicating protein synthesis and catalysis of the rearrangement of both intrachain, interchain disulfide bonds in proteins, catalysis of the hydrolysis of the terminal or penultimate peptide bond at the C-terminal end of a peptide or polypeptide, and cellular process in which a signal is conveyed to trigger a change in the activity or state of a cell (figure 5C).

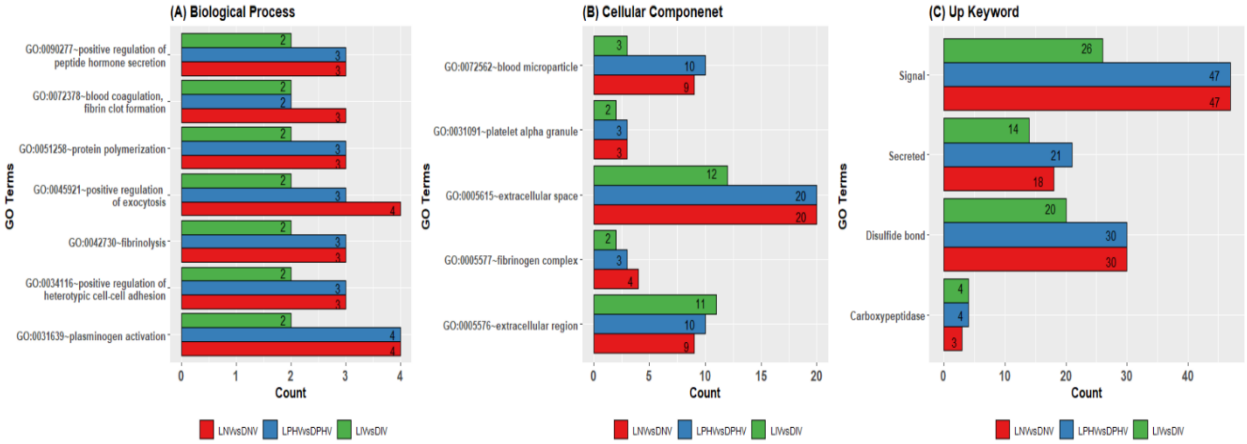

Figure 6. Overlapped Gene Ontology enrichment analysis terms in differentially expressed genes in pre and post-hatch in green monochromatic light biostimulated and dark treatments during incubation. (A) Biological process, (B) Cellular Component, (C) Up Keywords. All DEGs from pre (E18) and post-hatch (D7) were subjected to the DAVID database for Gene Ontology (GO) enrichment analysis. All the GO terms with a modified Fisher Exact P-value <0.05 and a threshold gene count of 2 were considered enriched.

We analyzed the pathways enriched for the differentially expressed genes during embryonic day 18 of incubation and post-hatch treatments on day 7 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016). Kegg Pathways were considered enriched if at least two DEGs were found in the background pathway and a modified Fisher Exact P-value < 0.05. In the embryonic spleen (LNV vs. DNV), three KEGG pathways were significantly enriched, including oxidative phosphorylation followed by Toll-like receptor signaling pathway, and RNA polymerase. In the post-hatch spleen, Metabolic pathways and PPAR signaling pathways were enriched in LPHV vs. DPHV, whereas Metabolic pathways were the only enriched pathway in LIV vs. DIV. KEGG IDs for each comparison, along with fold enrichment value and incorporated Entrez ID genes, are presented in table 2.

Table 2. KEGG pathway enrichment analysis of differentially expressed genes in spleen tissues from embryonic day 18 of incubation, LNV and DNV treatments, and day seven post-hatch, LPHV, DPHV, LIV, and DIV treatments, that exposure to monochromatic green light during incubation, were subjected to the DAVID database for pathway enrichment analysis. All the pathways with a modified Fisher Exact P-value < 0.05 and a threshold gene count of 2 were considered enriched.

Group	KEGG ID	Pathway terms	Fold Enrichment	Entrez IDs of Genes
LNV Vs DNV	gga00190	- Oxidative phosphorylation	3.05	- PPA2, NDUFA5, NDUFS6, COX7C, UQCRFS1, NDUFB5
	gga04620	- Toll-like receptor signaling pathway	2.5	- NFKBIA, PIK3CG, TOLLIP, MAPK12, IRAK4
	gga03020	- RNA polymerase	1.5	- POLR1D, POLR2F, POLR2D
LPHV vs DPHV	gga01100	- Metabolic pathways	14.3	- PNLIPRP1, PSPH, ALDOB, SIIL, PLA2G1B, ATP5A1W, CEL, FUT9, ADH1C, GATM, TCIRG1, HPD, PLA2G1BL, AMY2A, UGT1A1
	gga03320	- PPAR signaling pathway	2.9	- SCD, APOC3, FABP1
LIV vs DIV	gga01100	- Metabolic pathways	12.5	- PNLIPRP1, SIIL, PLA2G1B, CEL, PLA2G1BL, FUT9, AMY2A

The overlap between the total DEGs (217, 116, and 62) detected in LNV vs. DNV, LPHV vs. DPHV, and LIV vs. DIV comparisons are shown in Figure 6. Eighteen DEGs were overlapping in green monochromatic light biostimulation treatments compared to entire dark groups during incubation (Table 3).

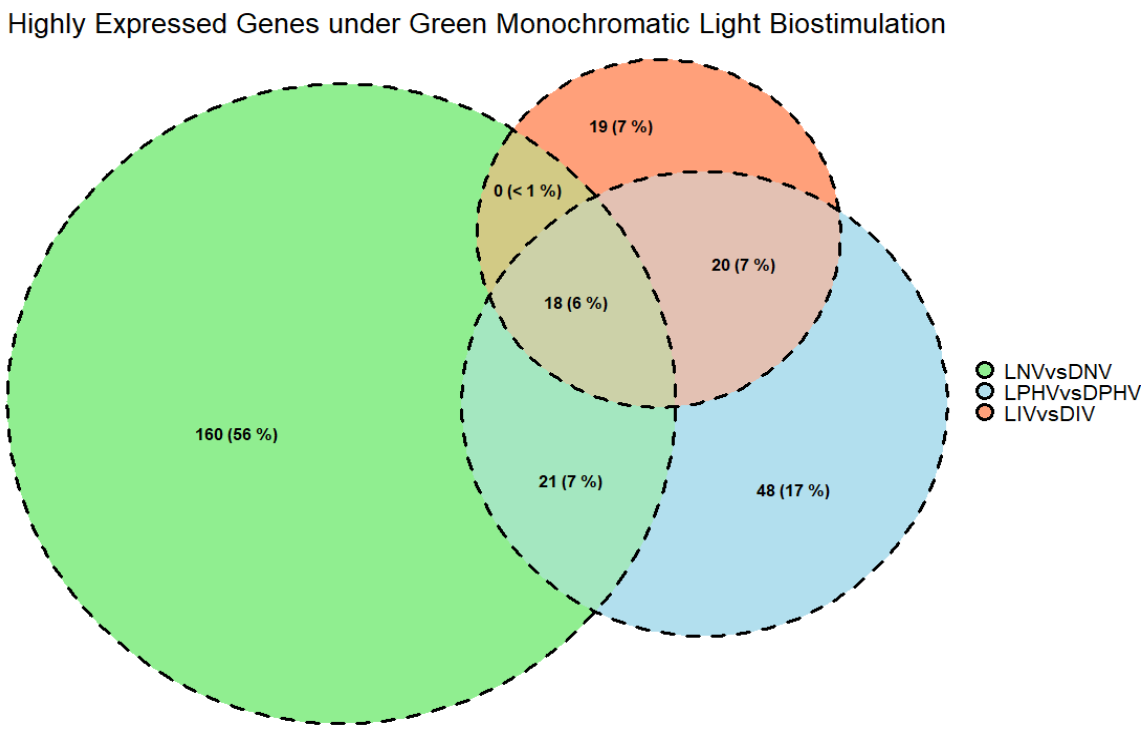

Figure 7. Number and overlapping DEGs in spleen tissues between pre-hatch (LNV vs DNV) and posthatch (LPHV vs DPHV and LIV vs DIV) treatments, stimulated by green monochromatic light during incubation. The DEGs were determined by statistical algorithms EdgeR. Notably, embryonic spleen samples had a greater number of highly expressed DEGs (FDR < 0.05) compared to post-hatch spleen samples indicating the dilution of biostimulation in the post-hatch environment (shift to standard lighting).

Table 3. Eighteen DEGs were shared in spleen tissues between pre-hatch (LNV vs. DNV) and post-hatch (LPHV vs. DPHV and LIV vs. DIV) treatments. Transcripts from spleen tissue of groups exposed to green light during incubation and groups kept in full dark were aligned to the chicken genome and mapped genes with at least fold change over one difference and FDR < 0.05 were considered differentially expressed.

Gene name	Gene description	LNV vs DNV LogFC	LPHV vs DPHV LogFC	LIV vs DIV LogFC
FGB	fibrinogen beta chain [373926]	-1.63	-3.65	1.89
HS6ST2	heparan sulfate 6-O-sulfotransferase 2 [395150]	2.08	2.23	-1.57
CPA1	carboxypeptidase A1 [395276]	2.73	8.47	-1.73
LBFABP	liver basic fatty acid binding protein [395345]	-2.45	-3.31	1.95
PIT54	PIT54 protein [395364]	-2.08	-4.07	2.11
DNASE1	deoxyribonuclease 1 [395725]	2.21	8.96	-1.90
FGG	fibrinogen gamma chain [395837]	-1.76	-3.26	2.38
ALB	albumin [396197]	-1.88	-4.65	2.13
SST	somatostatin [396279]	1.64	7.75	-1.43
CPA5	carboxypeptidase A5 [416683]	2.27	7.65	-2.40
CEL	carboxyl ester lipase [417165]	1.97	8.21	-2.10
CPB1	carboxypeptidase B1 [424888]	1.58	9.15	-1.59
CTRL	chymotrypsin like [427531]	1.87	7.27	-2.68
CTRC	chymotrypsin C [430670]	2.53	8.33	-1.27
CTRB2	chymotrypsinogen B2 [431235]	2.10	8.18	-2.39
CLPS	colipase [771102]	1.67	7.19	-1.48
PDIA2	protein disulfide isomerase family A member 2 [100857897]	2.27	5.93	-2.36
LOC101749216	uncharacterized LOC101749216 [101749216]	3.13	10.44	-1.96

We analyzed shared DEGs among photo-biostimulated groups to get a deep insight into the genes' role and the common pathways that may have participated in their enrichment using IPA. IPA mapped 15 genes out of 18 shared genes to a human orthologs genes, of which seven

genes *Colipase, Carboxypeptidase A1, Carboxypeptidase A5, Carboxypeptidase B1, Chymotrypsinogen B2, Chymotrypsin C, Chymotrypsin like* were involved in one canonical pathway 'SPINK1' that was inhibited in LNV and DNV group, and LPHV and DPHV group. At the same time, it was activated in LIV and DIV groups, where all the seven genes were downregulated or upregulated. The networks identified from the 18 shared DEGs IPA are presented in table 3 (see drive). IPA network analysis showed an overrepresented network with Lipid Metabolism, Molecular Transport, and Small Molecule Biochemistry functions in the three comparisons. Three genes *CEL, CLPS, DNASE1, and triacylglycerol lipase,* were upregulated in LNV and DNV group, and LPHV and DPHV (Figure 8 A), unlike LIV and DIV group where those genes were downregulated (Figure 8 B). The genes in this network encode for encodes for proteins that play a role in lipid metabolism, e.g., carboxyl ester lipase (CEL) secreted from the pancreas to break down cholesterol lipid-soluble vitamin ester hydrolysis and absorption, and protein metabolism. e.g., carboxypeptidase produced in the pancreas that hydrolyzes a C-terminal peptide bond in polypeptide chains, and signaling proteins that trigger an intracellular signal-transduction pathway leading to differentiation, proliferation, or photoreceptor protein that converts the light waves into signals, e.g., light-absorbing chromophores (de Freitas & Hamblin, 2016; El-Gendy, Abdelaziz, Abdelfattah, Badr, & Salama, 2015; Tiina I. Karu, 1996; Losi, Gardner, & Möglich, 2018; Shichida & Matsuyama, 2009). The activity of pancreatic tissue is predominantly upregulated, which is consistent with the embryonic developments and the need for enzymes to metabolize the fat and protein from egg yolk to support the embryogenesis process; this is consistent with the finding of Zhang et al. (2016), where he realized that the embryos in the green light group developed faster, resulting in higher nutrient consumption from the yolk, showing a lower weight percentage of yolk retention on 19 d of embryogenesis and 1 d of post-hatch.

Table 4. Gene networks from the 18 shared differentially expressed genes for Monochromatic Green Light Biostimulation groups converted to human orthologous genes**.**

ID	*contrast*	*Molecules in Network*	*Score*	*FM*	*Top Diseases and Functions*
I	LNV vs DNV	*1-O-hexadecyl-2-N-methylcarbamol-sn-glycerol-3 phosphocholine, Akt, **ALB**, AMBP, asparagine, carboxypeptidase, CEACAM8, chymotrypsin, **CPA1**, CPA2, **CPA5**, **CPB1**, CPB2, CPN1, **CTRB2**, **CTRC**, **CTRL**, ERK1/2, F13B, factor XIII, **FGB**, **FGG**, Growth hormone, homocysteine thiolactone, **HS6ST2**, Insulin, L-leucine, monooleylphosphatidic acid, Nkx2-2os, **PDIA2**, RBPJL, RXFP3, SCT, **SST**, Trypsinogen*	34	12	Developmental Disorder, Hematological Disease, Hematological System Development and Function
I	LPHV vs DPHV	*1-O-hexadecyl-2-N-methylcarbamol-sn-glycerol-3-phosphocholine, Akt, **ALB**, AMBP, asparagine, carboxypeptidase, CCKAR, CEACAM8, chymotrypsin, **CPA1**, CPA2, **CPA5**, **CPB1**, CPN1, **CTRB2**, **CTRC**, **CTRL**, ERK1/2, **FGB**, **FGG**, GHRHR, GHSR, Growth hormone, homocysteine thiolactone, **HS6ST2**, Insulin, KCNB2, Nkx2-2os, **PDIA2**, PRSS2, RBPJL, RXFP3, SCT, **SST**, Trypsinogen*	34	12	Developmental Disorder, Hematological Disease, Hereditary Disorder
I	LIV vs DIV	*1-O-hexadecyl-2-N-methylcarbamol-sn-glycerol-3-phosphocholine, Akt, **ALB**, AMBP, asparagine, carboxypeptidase, CEACAM8, chymotrypsin, **CPA1**, CPA2, **CPA5**, **CPB1**, CPB2, CPN1, **CTRB2**, **CTRC**, **CTRL**, ERK1/2, F13B, factor XIII, **FGB**, **FGG**, Growth hormone, homocysteine thiolactone, HS6ST2, Insulin, L-cysteine, L-leucine, L-phenylalanine, Mcpt1, monooleylphosphatidic acid, **PDIA2**, RBPJL, SCT, **SST***	34	12	Amino Acid Metabolism, Increased Levels of Albumin, Molecular Transport
II	LNV vs DNV	*amino acids, AMP, APCS, APOC3, bile acid, bile salt, Ca2, CCKAR, CCKBR, CD209, **CEL**, cholesterol, cholesterol ester, **CLPS**, Cr3, D-galactosyldiacylglycerol, digalactosyldiacylglycerol, **DNASE1**, EIF2AK3, GHRH, glycerol, GNRH, lipase, LPA, M6PR, NADPH oxidase, OLR1, PCSK9, PNLIP, PNLIPRP2, retinyl ester, Sod, sodium chloride, triacylglycerol lipase, VWF*	6	3	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
II	LPHV vs DPHV	*ABCB1, amino acids, AMP, APCS, APOC3, bile salt, Ca2, CCK, CCKAR, CCKBR, CD209, **CEL**, ceramide, cholesterol, cholesterol ester, **CLPS**, Cr3, D galactosyldiacylglycerol, digalactosyldiacylglycerol, **DNASE1**, EIF2AK3, GHRH, glycerol, GNRH, Ldh (complex), lipase, LPA, M6PR, Mlc, OLR1, PNLIP, PNLIPRP2, retinyl ester, sodium chloride, triacylglycerol lipase*	6	3	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
II	LIV vs DIV	*AMP, APCS, APOC3, bile acid, bile salt, Ca2, CCKAR, CCKBR, CD209, **CEL**, ceramide, cholesterol, cholesterol ester, **CLPS**, Cr3, D-galactosyldiacylglycerol, dehydroisoandrosterone, digalactosyldiacylglycerol, **DNASE1**, GHRH, glycerol, lipase, LPA, M6PR, NADPH oxidase, OLR1, PCSK9, PNLIP, PNLIPRP2, retinyl ester, Sod, sodium chloride, triacylglycerol lipase, VWF, Zn2*	6	3	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry

FM= Focus **Molecules, Red**= Upregulated; **Green**=Downregulated.

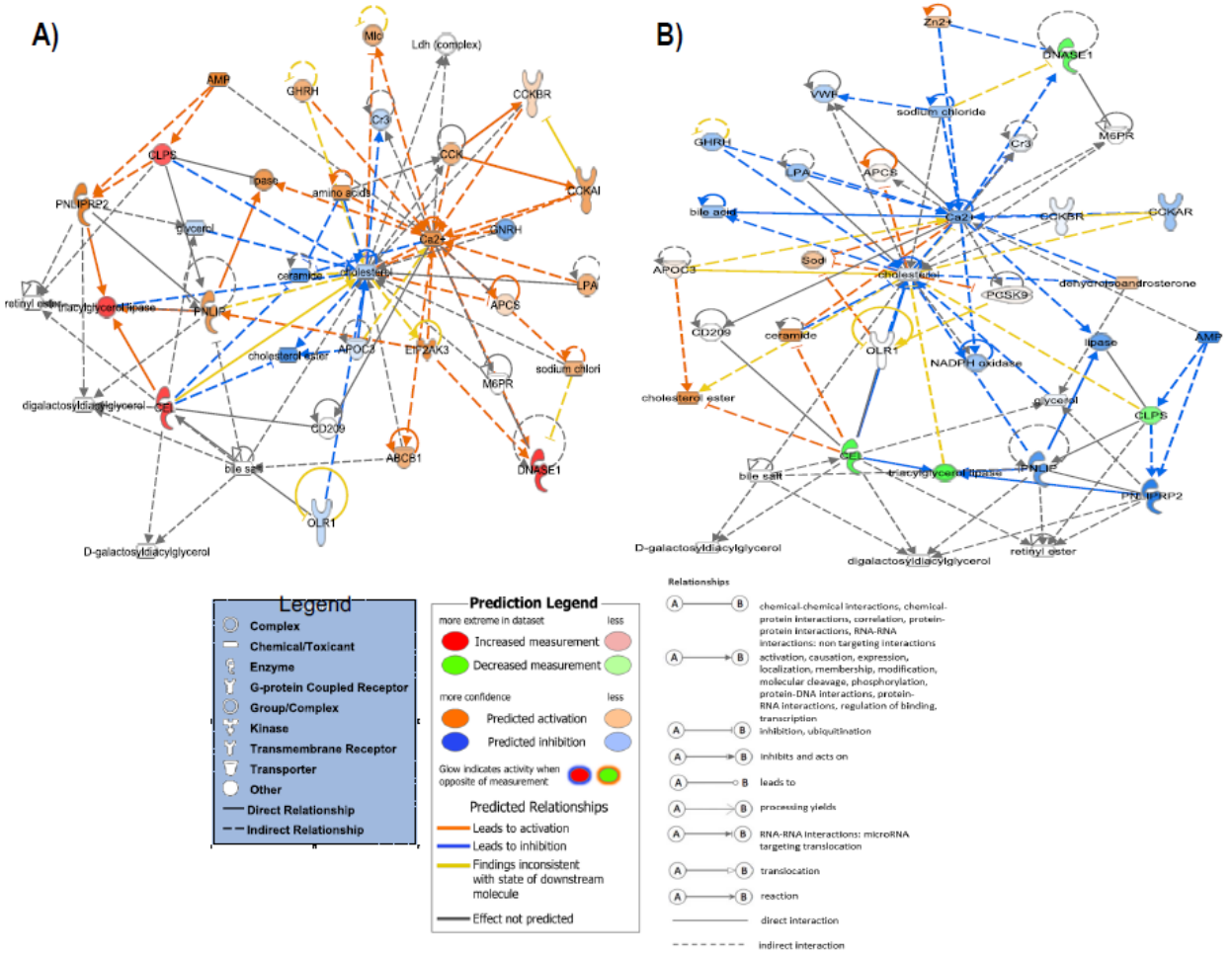

Figure 8. Activated and inhibited lipid metabolism network resulted from the shared 18 DEGs in the green light biostimulation comparisons underline the potential role of green monochromatic light biostimulation comparisons during incubation and their interaction in potentially regulating developmental biological processes pre and post hatch generated by QIAGEN's Ingenuity Pathway Analysis (IPA;QIAGEN Inc.) (Krämer et al., 2014). (**A**) LNV vs. DNV and LPHV vs. DPHV gene network activated Lipid Metabolism. (**B**) LIV vs. DIV gene network for Inhibited lipid metabolism. Differentially expressed genes in the biostimulated comparisons were used in Ingenuity Pathway Analysis and significant gene networks based on IPA scores were identified. Genes highlighted in red were upregulated, while those highlighted in green were downregulated in all biostimulated comparisons.

Limited but notable interaction of biostimulation and vaccination

The route of vaccination (*in ovo* or spraying) altered the transcriptomic profile observed in spleen tissues post-hatch regardless of lighting during incubation. In the DIV and DPHV comparison group, we saw 7076 differentially expressed genes (FDR < 0.05) between the DIV and DPHV treatments. Of these, 3845 were upregulated in DIV, and 3231 were downregulated (Figure 9 A). The enhanced DEGs were queried against the DAVID database; of the 1432 genes uploaded, 1420 genes were annotated into the GO terms biological process, cellular component, and molecular function. About 347 genes were enriched for 51 biological processes. The top enriched BP terms were 'positive Regulation of Transcription from RNA Polymerase II Promoter', 'Regulation of Rho Protein Signal Transduction', and 'Heart Development'. Nineteen cellular component terms were enriched based on 545 incorporated genes, with the top terms including 'Nucleoplasm', 'Kinesin Complex', and 'Nucleus'. Twenty-six molecular functions were enriched, among which the top MF terms were 'metal ion binding', 'zinc ion binding', and 'ATP binding'. The top GO terms categories are presented in Supplementary Table S4. KEGG pathways analysis showed 166 upregulated genes were enriched in 21 KEGG pathways, and the top three KEGG pathways included 'MAPK signaling pathway', 'Insulin resistance', and 'Regulation of actin cytoskeleton' (Supplementary Table S4).

From the DEGs between DIV vs. DPHV, 1556 genes were enhanced at LogFC greater than 2, with 197 downregulated and 1359 upregulated. In IPA, these 1556 genes were part of 263 canonical pathways, of which 235 were predicted to be activated, and 23 pathways were predicted to be inhibited based on Z-score. The topmost five significant canonical pathways include 'Protein Kinase A Signaling', 'Cardiac Hypertrophy Signaling (Enhanced)', 'Factors Promoting Cardiogenesis in Vertebrates', 'SAPK/JNK Signaling and 'Synaptogenesis Signaling Pathway'

(supplementary Table S4). The top five canonical pathways predicted to be activated include 'Cardiac Hypertrophy Signaling (Enhanced)', 'Superpathway of Inositol Phosphate Compounds', 'Cardiac Hypertrophy Signaling' '3-phosphoinositide Biosynthesis', and 'NF-κB Signaling', while the top five canonical pathways predicted to be inhibited, namely 'Pancreatic Cancer Pathway (SPINK1)', 'LXR/RXR Activation', 'PTEN Signaling', 'Endocannabinoid Cancer Inhibition Pathway', and 'RhoGDI Signaling'.

In the LIV and LPHV comparison group, we found a total of 6755 differentially expressed genes (FDR < 0.05), among which 3730 were upregulated in LIV, whereas 3025 genes were downregulated (Figure 9 B). DEGs with LogFC values greater than two were queried against the DAVID database; of the 1328 genes uploaded,1320 genes were annotated into three GO terms; biological process, cellular component, and molecular function. The top three enriched terms were 'Microtubule-Based Movement', 'Cell Migration', and 'Positive Regulation of Transcription from RNA Polymerase II Promoter'. We saw 23 cellular components GO terms enriched, and the top three terms were 'nucleoplasm', 'kinesin complex', and 'cytoplasm'. Genes (456) involved in the enrichment of molecular functions yielded the top molecular functions' zinc ion binding', ATP binding', and 'metal ion binding'. Upregulated genes (112) were enriched in 13 KEGG pathways and, with the top pathways being 'Fanconi Anemia Pathway', 'MAPK Signaling Pathway', and 'Endocytosis' (Supplementary Table S4).

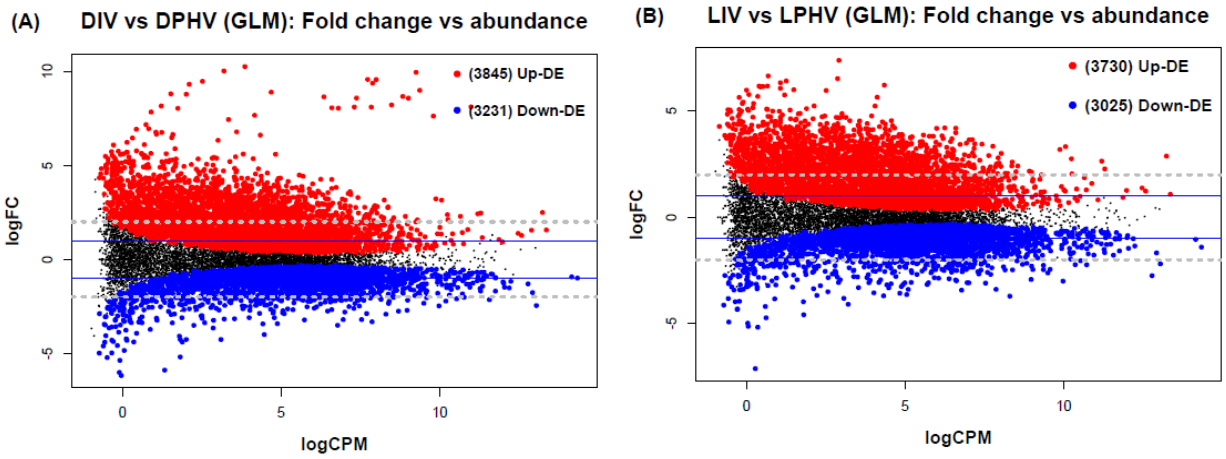

Figure 9. Mean difference (MD) plot highlighting the log fold change and average abundance of each gene, comparing the differences between the route of vaccination with or without the adding effect of green monochromatic light biostimulation in the transcriptome of spleen tissue collected post hatch at day 7. Significantly up and down DE genes and their numbers are highlighted in red and blue, respectively, at FDR < 0.05. Figure 8 A shows the DEGs in the post hatch spleen tissue (D7) received two different routes of vaccination but not biostimulated during incubation in DIV vs DPHV treatment groups. Figure 8 B shows the DEGs in the post hatch spleen tissue (D7) received two different routes of vaccination and biostimulated during incubation in LIV vs LPHV treatment groups. The Y-axis corresponds to the mean average of log10 count per million (CPM), and the X-axis displays the log2 FC at FDR < 0.05. DIV: dark *in ovo* vaccinated; LIV: light *in ovo* vaccinated; DPHV: dark post hatch vaccinated; LPHV: light post hatch vaccinated.

Using the enhanced DEGs (LogFC>2), 1476 genes were annotated in IPA, of which 1266 were upregulated. Those molecules were enriched in 252 canonical pathways, of which 227 pathways were predicted to be activated, 18 pathways were predicted to be inhibited based on the weighted Z-score, and the rest could be predicted due to zero Z-core values. The most significant five canonical pathways encompass 'Protein Kinase A Signaling',' B Cell Receptor Signaling', 'Cardiac Hypertrophy Signaling (Enhanced)', 'Superpathway of Inositol Phosphate Compounds', and 'SAPK/JNK Signaling' (Supplementary Table S4). The top five weighted with Z-score canonical pathways predicted to be activated included 'Cardiac Hypertrophy Signaling (Enhanced)', 'Super pathway of Inositol Phosphate Compounds', '3-phosphoinositide

Biosynthesis', '3-phosphoinositide Degradation', and 'D-myo-inositol-5-phosphate Metabolism'. The top five canonical pathways with negative Z-score included 'PTEN Signaling', 'Endocannabinoid Cancer Inhibition Pathway', 'PPAR Signaling', 'RhoGDI Signaling', and 'VDR/RXR Activation'.

The Cardiac Hypertrophy Signaling pathway was predicted to be activated in both comparisons presented above, with Z-scores 6.6 and 6.4 in DIV vs. DPHV and LIV vs. LPHV, respectively. Finding this as the top activated pathway in both comparisons shows that, perhaps unsurprisingly, cardiac hypertrophy was a crucial developmental process unaffected by lighting or vaccination status. However, this is valuable information from an application standpoint to know that lighting during incubation is not detrimental to normal developmental processes. Furthermore, we saw the non-canonical pathways Cancer, NF-kB Signaling, Mitochondrial Dysfunction pathway, MAPK, Rho-GTPase Signaling, P53, RhoGDI Signaling, and Circadian pathways were activated among LIV vs. LPHV and DIV vs. DPHV comparisons (Figure 10).

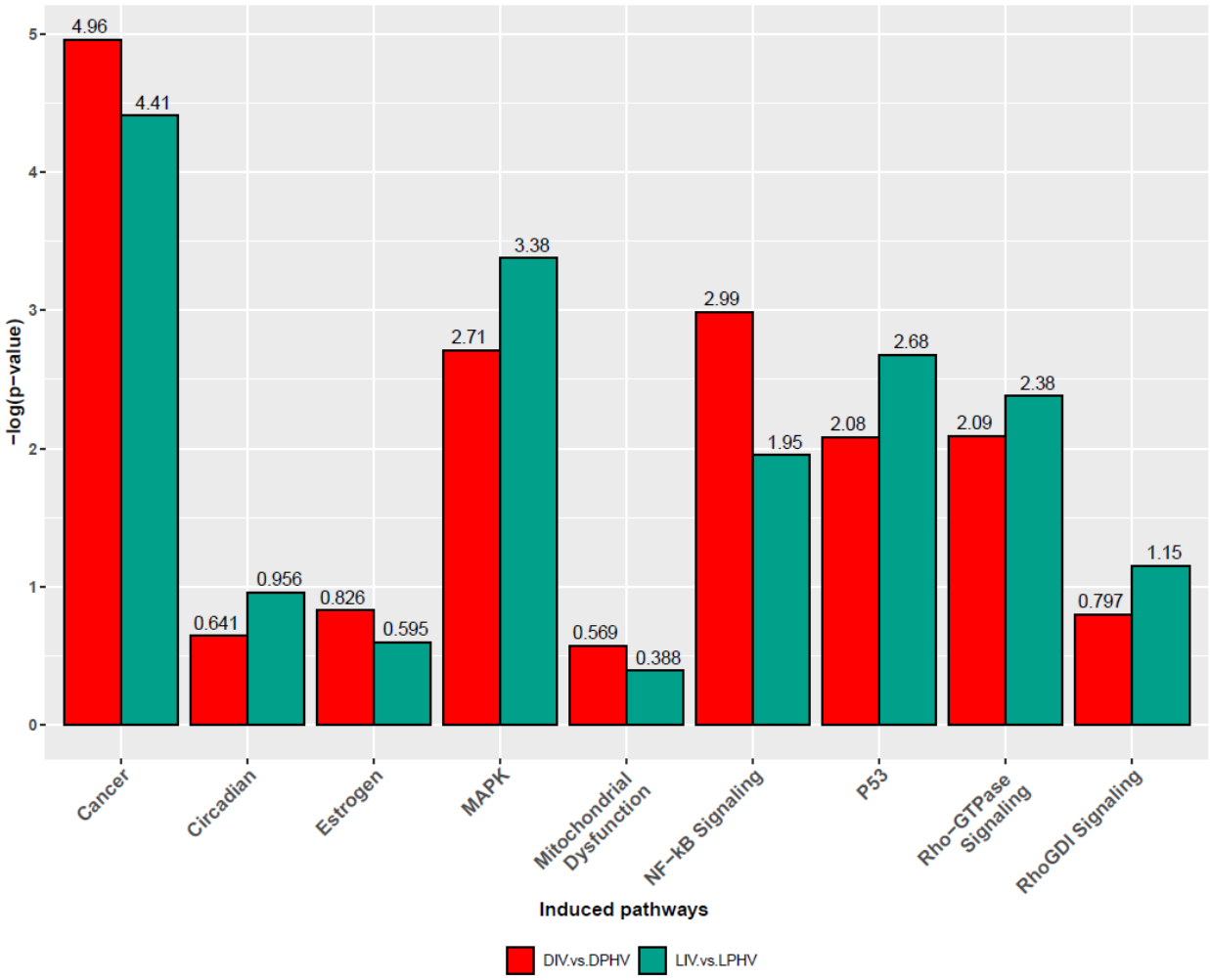

Figure 10. Similarities of non-canonical pathways induced by the interaction of route of vaccination and green monochromatic light interaction.

We analyzed the enhanced DEGs (LogFC>2) shared and unique DEGs between the DIV vs. DPHV and LIV vs. LPHV comparisons, to determine which expression differences are attributable to the light biostimulation alone. We found 381 genes unique to the LIV vs. LPHV group, after removing those shared with DIV vs. DPHV (Figure 11). Of the 381 unique DEGs, 327 genes (186 upregulated and 96 downregulated) were annotated in IPA. Thirty-six canonical pathways were predicted to be activated, whereas four were predicted to be inhibited. The top 5 most significant canonical pathways predicted to be activated include 'Integrin Signaling', 'Reelin

Signaling in Neurons', 'Amyotrophic Lateral Sclerosis Signaling', 'p53 Signaling', and 'ILK Signaling'. In contrast, the canonical pathways predicted to be inhibited are 'T Cell Exhaustion Signaling Pathway', 'Protein Kinase A Signaling', 'Phospholipase C Signaling', and 'Endocannabinoid Cancer Inhibition Pathway'. On the other hand, the non-canonical pathways or induced pathways unique to the LIV vs. LPHV group showed enrichment of 'P53', 'MAPK', 'Circadian', and 'Rho-GTPase Signaling' pathways. Two of the pathways (Integrin Signaling, and ILK signaling pathway) are central to cell adhesion, and play a role in cell proliferation and differentiation, especially during development (Harburger & Calderwood, 2009; Tarekegn et al., 2020). When considering leukocytes, integrin signaling is a mediator of leukocyte migration and activation (Abram & Lowell, 2009), and therefore this pathway may indicate a role in leukocyte signaling and function. As our data is generated with bulk RNAseq, we cannot determine if this activated pathway is informative about leukocytes specifically, or generally to the extracellular matrix of the solid tissue.

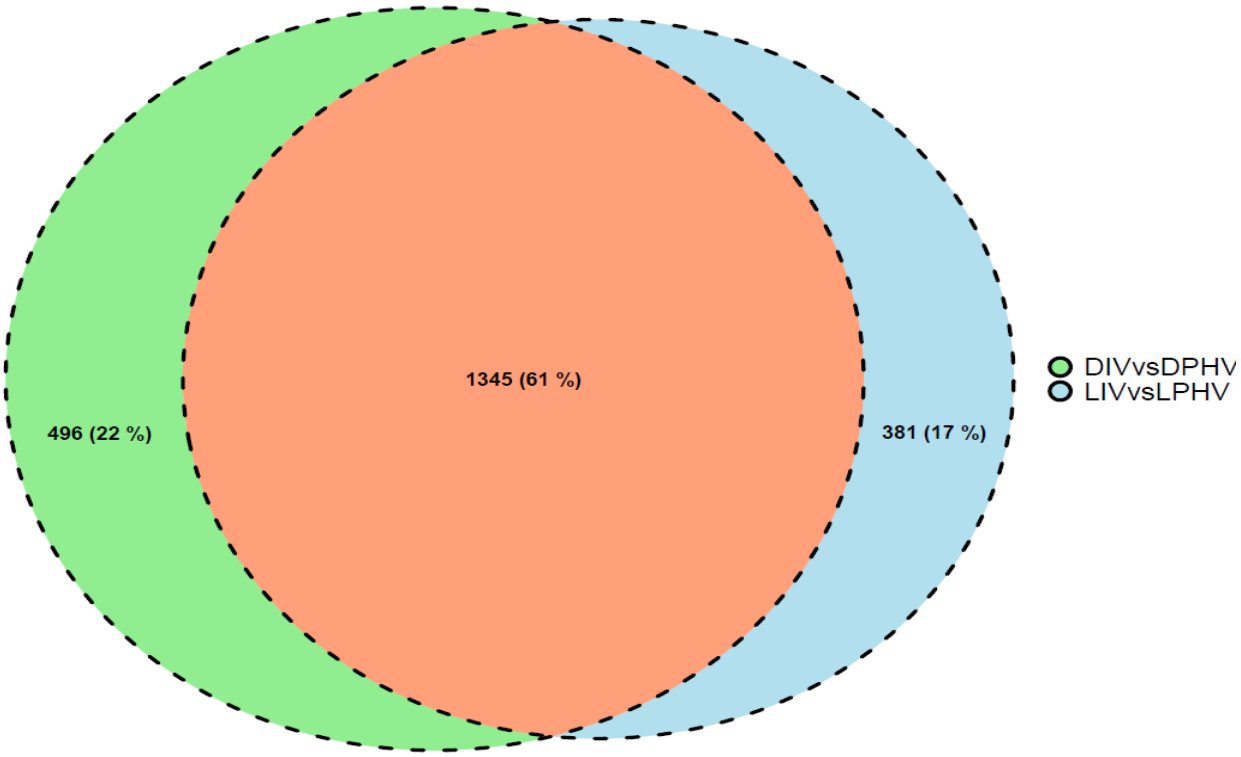

Figure 11. Distinct and overlapping DEGs between route of vaccination and the interaction of green monochromatic light photobiostimulation in spleen tissues in Posthatch Comparisons (DIV vs DPHV and LIV vs LPHV), stimulated by green monochromatic light during incubation. The DEGs were determined by statistical algorithms EdgeR (FDR < 0.05).

Ten networks emerged from the DEGs unique to the LIV vs. LPHV group that highlight the activities that are attributable to incubation with a monochromatic green light (Supplementary table S6). The networks are involved in biological functions classified into "Cell Morphology, Tissue Development, and Cell Maintenance", "Cell-To-Cell Signaling and Interaction", "Lipid Metabolism, Molecular Transport, and Small Molecule Biochemistry", and "Cell-mediated Immune Response, Lymphoid Tissue Structure, and Development". The top canonical pathway and the top induced pathway were Integrin Signaling and P53 pathways, respectively. As referenced above, Integrins play an important role in cell-to-cell and cell-extracellular matrix

(ECM) interactions, whereas p53 is a transcription factor and tumor suppressor. These classifications are supported by the network terms "Cellular Assembly and Organization", "Cellular Function and Maintenance", and "Tissue Development". Also, the role of photo-biostimulation in enhancing innate immune response continued in chicks post-hatch through the activation of the Acute Phase Response signaling pathway plays an important role in the rapid reprogramming of gene expression and metabolism in response to inflammatory cytokine signaling restoring tissue homeostasis (Venteclef, Jakobsson, Steffensen, & Treuter, 2011).

In summary, green monochromatic lighting during incubation enhanced cell signaling and cell proliferation or differentiation, following vaccination. If these enhanced activities translate to improved immune responses, it would suggest a synergistic effect of lighting on improved immune responses. This finding needs to be investigated further to identify the source of these patterns and understand their implications.

Conclusion

Our results showed that the vaccination route had a profound effect on gene expression in the post-hatch spleen, whereas the effect of green light was absent in the post-hatch comparisons. This finding is not surprising given that all birds were on the same lighting treatments after hatching. However, there was a minor but notable interaction between *in ovo* lighting and vaccination on key immune developmental processes. This suggests that continuing lighting schemes in the post-hatch environment may be necessary to reinforce the pre-hatch activity patterns. Our study shows that the effects of *in ovo* lighting dissipate if the same lighting is not maintained in the post-hatch environment. Finally, our study emphasizes the need for continued investigation of *in ovo* lighting for optimizing responses to vaccinations.

CHAPTER III

THE EFFECT OF CIRCADIAN RHYTHM DEVELOPMENT UNDER DIFFERENT MONOCHROMATIC LIGHTS ON THE TRANSCRIPTOME PROFILE OF CHICKS LUNG TISSUE CHALLENGED WITH NEWCASTLE DISEASE VIRUS DURING INCUBATION

Introduction

During the 1960s poultry industry observed that the efficacy of providing light intensities above a certain threshold in the early embryonic development led to enhanced growth rates (Isakson, Huffman, & Siegel, 1970; Shutze et al., 1962), which opened the way to other experiments to evaluate potential light-based enhancement mechanisms.

Understanding the role of specific light wavelengths in controlling circadian rhythm development and regulating the immune response are becoming highly important to maximize healthy poultry production worldwide. Circadian rhythms are the self-sustained biological processes that cycle over 24 h, synchronized by an endogenous circadian oscillator, driven by environmental cues or 'Zeitgebers', and it is a widely observed biological process among organisms from cyanobacteria to vertebrates (Edgar et al., 2012; Hill et al., 2004; Peek et al., 2015). In birds, the visual system is unique and plays a profound role in circadian rhythm development, where it can detect a broader spectrum of wavelengths than mammals (Withgott, 2000), making them highly responsive to small changes in a light color (Lind, Mitkus, Olsson, & Kelber, 2014; Withgott, 2000). This responsiveness comes from the possession of highly evolved and capable vision system among the animal kingdom; birds have six different types of photoreceptors in their retinas (Bennett & Théry, 2007; Hart & Hunt, 2007), of which are four spectral types of single rods photoreceptors responsible for generating a tetrachromatic visual perception under dim light conditions "scotopic

vision" (T. H. Goldsmith & Butler, 2005; Maier & Bowmaker, 1993; Osorio, Vorobyev, & Jones, 1999), and double cone photoreceptors associated with bright light and motion recognition "photopic vision" (v. Campenhausen & Kirschfeld, 1998; Vorobyev, Osorio, Bennett, Marshall, & Cuthill, 1998). The earliest signs of chicken embryo's ability to sense light during embryogenesis were reported at the second day of incubation, where light accelerates cell division in neural crest mesoderm, accelerating closure of the neural tube at "embryonic d1 or stage 7 of the Hamburger–Hamilton (H–H) classification of avian embryonic development" and subsequent somatic differentiation of central nervous system precursors (Isakson et al., 1970). The beneficial effect of light on embryonic development, called photo-acceleration, may begin within hours of laying an egg (C. B. Cooper, Voss, Ardia, Austin, & Robinson, 2011). This photo-accelerating effect is limited by the light ability to penetrate to the cellular level during the early stage of chick's embryogenesis day 1 to day 5 in chicken embryos (Shafey, 2004). Later, light inducing effect is mediated by photoreceptors that exist in retina, SCN, and pineal gland (C. B. Cooper et al., 2011). After pineal gland formation, a light-sensitive pineal opsin photoreceptors called "Pinopsin" form on on day three or H-H stage 17 of embryogenesis, and plays a key role in entrainment and melatonin secretion (Hill et al., 2004).

The early development of light sensitivity in a chicken's vision system during embryogenesis makes the onset of circadian rhythm a prenatal and independent event, unlike mammals (V. J. Csernus et al., 2007; Y. Li & Cassone, 2015; Okabayashi et al., 2003). While the mammals have two pacemakers in their circadian system (retina, and suprachiasmatic nuclei (SCN) of the hypothalamus), birds possessing a core circadian system consists of three independent endogenous circadian oscillators including; retina, and SCN, and pineal gland (Jing Cao, Bian, Wang, Dong, & Chen, 2017; Gwinner, Hau, & Heigl, 1997; Kumar, Singh, & Rani,

2004). This advanced and complex visual system in birds results from the cooperation of multiple photoreceptors in the circadian oscillation system by absorbing photons directly from the external environment during embryogenesis through the retina, pineal gland, and hypothalamus, unlike mammalian embryos (M. J. Bailey et al., 2002; Borges, Johnson, O'Brien, Vasconcelos, & Antunes, 2012; Ebihara, Uchiyama, & Oshima, 1984; Ma et al., 2018). Perceived light cues are transmitted through the optic nerve to the brain after converting it to neural signals which control the biological rhythm and regulate the function of central and peripheral organs. Therefore, birds are an ideal model animal to study circadian development. Faluhelyi and Csernus (2007) showed that the light-sensing system of the pinealocytes in the chicken's circadian system is already fully established by the 17th embryonic day, and a significant daily rhythm of pineal melatonin secretion in chick embryos at 18 of incubation before internal pipping (C. B. Cooper et al., 2011; Zeman et al., 1992).

Different light wavelengths stimulate the retina and pineal cells of birds in divergent ways, resulting in behavioral changes that influence growth, development, and productivity (O Halevy, Biran, & Rozenboim, 1998; Rozenboim, Biran, Uni, Robinzon, & Halevy, 1999; Tong et al., 2018; L. Zhang et al., 2016). For instance, light with high intensities was found to improve chicken embryonic cells' proliferation (Ghatpande, Ghatpande, & Khan, 1995). Exposing chicken embryos to green light (560 nm) accelerated the mesodermal differentiation early at embryonic day 5. This process is regulated by MyoD transcription factors that may have been triggered by signals of retinal or pineal photoreceptors acting on the neuroendocrine system (Orna Halevy et al., 2006). Evidence suggests that visible spectra penetrate the cellular level in the early avian embryonic stage and photobiostimulate the cytochromes in the mitochondrial transport chain and cascade the cAMP activity, subsequently initiating DNA synthesis and increasing cellular metabolism (T I

Karu, 1988). Visible light also promotes clock genes in the brain, ovary, diencephalon, liver, and skeletal muscle in chicks (Honda et al., 2017; Nakao et al., 2007), translating into behavioral changes that affect growth, development, and productivity (Nelson, Bray, Delabbio, & Archer, 2020; Z. Zhang et al., 2014). Photoperiods also play a significant role in the immune response. Photoperiods modulate the level of melatonin via reciprocal regulation of melatonin receptors in splenocytes, suggesting a mechanism for modulating avian species' immune responses (Yadav & Haldar, 2013). In contrast to a long-day photoperiod, short-day photoperiods cause an improvement in immune response (Blom, Gerber, & Nelson, 1994; Markowska et al., 2017). It is worth notable that avian photoreceptors are highly sensitive to blue light due to special photosensitive pigment in the pinealocytes (Okano et al., 1997, 1994). Moreover, Csernus et al. (1999) reported that the pineal gland has a blue light sensitivity preference resulting in a significant change in secreted melatonin in chicken *in vitro*.

It is clear that much is known about the mechanics of the avian circadian. However, little is known about whether early-life circadian stimulation has functional significance, and whether different wavelengths produce different developmental trajectories. An important unknown is whether lighting during incubation has consequences of immune responses against challenges. The objective in this study was to address this gap by comparing the differential effects of blue versus visible wavelengths on chick circadian development, and downstream consequences. I further investigated whether chicks incubated under different wavelengths showed a different innate immune response when challenged with attenuated Newcastle Disease Virus (NDV) during incubation.

Materials and Method

Animal Ethics Statement

We performed all the live animal experimental work in accordance with US guidelines for animal welfare with the ethics approval and monitoring by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University under AUP number IACUC 2020-0107.

Animals and experimental design

The goal of the experiment was to determine the effects of blue and white LED light, compared to conventional dark conditions, during incubation on circadian rhythm entrainment and its effect on modulating innate immune response following *in ovo* challenging with Newcastle Disease Virus (NDV). This circadian study consisted of six treatments, namely non-challenged blue, white, and control, and challenged blue, white, and control. We used fertilized Lohmann LSL layer eggs (n =300), provided by Texas A&M Poultry Science Research, Teaching, and Extension Center. We used the LaSota strain of NDV (Newcastle B1type/LaSota, Code: ND1820, Merial), with a mean embryo infectious vaccine dose (EID_{50}) of 10^6/ml. After virus particle resuspension, the viral suspension was kept at -80^0C for storage until administration. We randomly distributed the eggs among three incubators (n=100/incubator). For the circadian study, we modified the three GQF 1502 combo incubator/hatcher (GQF Manufacturing, Savannah, GA) by blocking the incubator's front windows with opaque tape to prevent light intrusion. During the experiment, one incubator was kept under complete dark conditions (0L:24D) and served as the control group (dark), while we fitted the two other incubators with two different color LED lights, blue (450nm), and white daylight ≥ (6500K), respectively. In light treatments, we exposed incubated eggs to a light-dark cycle of (12L: 12D) with 250 lux at egg surface for the whole period

of incubation. We used a 2-in-1 incubator/hatcher cabinet instead of moving the eggs to dark hatcher on the last 3 days as was performed in (G S Archer, 2017). We mounted the LED light panels in a frame on each of the three levels, running the length of the racks to produce an even spread of illumination on the surface of each egg and no extra heat. LED light panels were attached to metal frames, which were attached to the underside of the rack above them. The LED light panel was held up for the top rack up by a metal frame made to rest on the top rack (G S Archer, 2017; Shafey, Al-Batshan, Ghannam, & Al-Ayed, 2005; Tong et al., 2018). We provided blue and white photoperiods from the first day of incubation in lighting treatments, where the Zeitgeber Time 0 (ZT00) was set to the time of light on (Hieke et al., 2019). We maintained the incubators at standard temperature and humidity levels of 37.5°C (99.5°F) and 58% relative humidity with tray tilting every 2h. On day 18 of incubation, we candled eggs to remove unfertilized eggs and inviable embryos. Following the candling process, live embryos are divided into two halves, one half challenged by *in ovo* injection with 100 µl of NDV viral suspension (10^6/ml EID_{50}) injected into the amniotic fluid by 1-inch 21G needle, preceded by puncturing the eggshell with an 18G needle. The injection holes were sealed with food-safe grade clear silicone to prevent infection and dehydration. The remaining eggs were unchallenged and served as a control for the challenged group. Right after the candling and challenging process we placed the unchallenged and challenged eggs back on the hatching tray in separate compartments in the same incubator. We maintained the eggs at a standard temperature and humidity levels of 36.9°C (98.5°F) and a minimum of 66-75% relative humidity till the end of circadian study (G S Archer, 2017; Gregory S. Archer & Cartwright, 2012). A graphical summary is illustrating the time for ND virus *in ovo* challenging and photoperiods for different light biostimulation (figure 12).

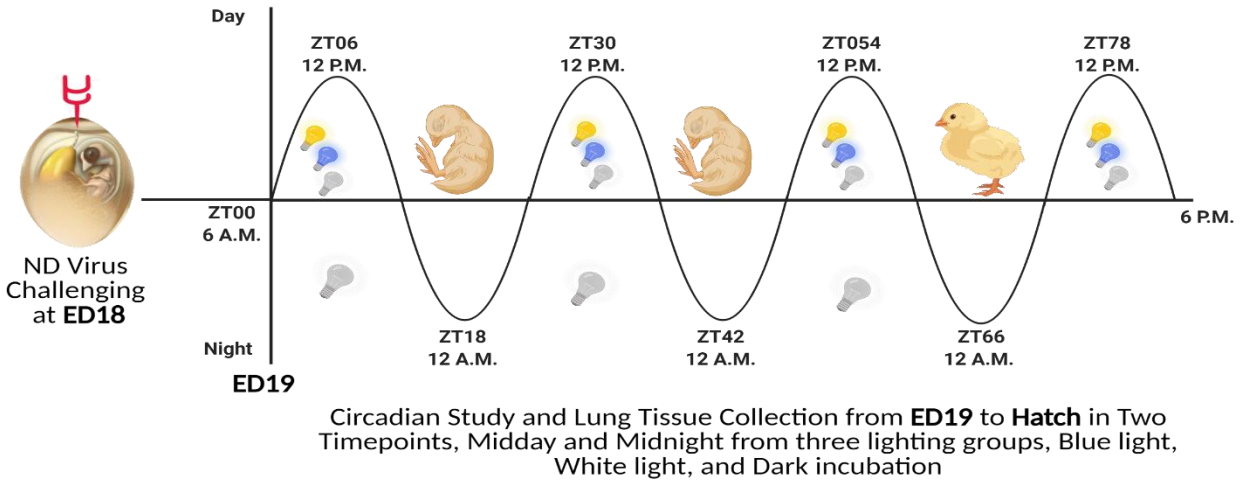

Figure 12. Graphical summary showing the circadian study timeline and the time of challenge. Blue and white lights were provided from the first day of incubation till the end of the study a photoperiod of 12h intervals (light: dark) except for the control (24h dark) group. Half of the experimental embryonated eggs were challenged with NDV virus *in ovo* on embryonic day 18 (ED18).

Sample collection for the circadian study

On day 19 of incubation and 24h post-challenge, we collected lung tissue at 12 h intervals at midday and midnight over three days with a total of seven collection time points (Table 5). At each time point, four embryos were randomly selected from each of the six treatments, checked for viability, and the eggshell was broken open, and chick embryos from each light treatment and control treatments (dark) to characterize circadian oscillations. As incubation progressed, hatched chicks were euthanized humanely using exposure to CO_2, followed by cervical dislocation prior to lung tissue harvesting. We illuminated the dissection room with dim LED red light while checking the embryos' viability to avoid disrupting the circadian rhythm. We dissected all embryos collected simultaneously within 20 minutes post-mortem for all treatments (with multiple dissectors). Harvested lung tissues were preserved in RNALater solution in a ratio of 1 gram tissue:

5mL RNALater solution (Ambion Inc, ThermoFisher Scientific). We kept samples in the RNA stabilizing solution for at least 24h at four °C and up to 1 month before discarding the RNALater and transferring the tissue for long-term storage at -80°C until RNA isolation, according to the manufacturer's guidelines (Ambion Inc, ThermoFisher Scientific).

Table 5. Treatment groups in the circadian study showing the lighting treatments along with challenged treatment groups. Blue and white lights were provided from the first day of incubation till the end of the study in the form of photoperiods of 12h intervals (light: dark) except for the control (24h dark) group. Half of the experimental embryonated eggs were challenged with NDV virus *in ovo* on embryonic day 18 (ED18). A total of 168 samples were collected during the study, with four replicates at each time point from all treatments. All euthanasia procedures were performed using protocols approved by the Texas A&M University's Institutional Animal Care and Use Committee (IACUC AUP #2020-0107).

Timepoint 12h intervals	**Light Treatment and NDV Challenging (*Rep:4*)**					
	Blue	**Blue**	**White**	**White**	**Dark**	**Dark**
ZT06 **Light on_Day**	No	Yes	No	Yes	No	Yes
ZT18 **Light of_Night**	No	Yes	No	Yes	No	Yes
ZT30 **Light on_Day**	No	Yes	No	Yes	No	Yes
ZT42 **Light off_Night**	No	Yes	No	Yes	No	Yes
ZT54 **Light on_Day**	No	Yes	No	Yes	No	Yes
ZT66 **Light off_Night**	No	Yes	No	Yes	No	Yes
ZT78 **Light on_Day**	No	Yes	No	Yes	No	Yes

RNA isolation and quantification

We extracted RNA from all samples using MagMAX™ *mir*Vana™ Total RNA Isolation Kit and a magnetic bead-based automated system, KingFisher Flex, for high purity RNA (Applied Biosystems, Carlsbad, CA, USA). Approximately 20 mg of lung tissue was homogenized in a 400 µl lysis buffer (1:20 ratio) with 0.2 cm^3 of 1.0 mm diameter ZIRCONIA beads (cat.no. 11079124zx) using a Mini-Beadbeater-96 (BioSpec, OK, USA). After homogenization, we added 100 µl of lysate to 100 µl isopropanol and 20 µl of binding beads and shook the lysate mixture for 5 minutes at 950 rpm and transferred to sterile deep well 96 plates for the automated process of wash, genomic DNA removal, rebind, and elute the RNA according to the manufacturer's instructions of KingFisher Flex (Applied Biosystems, Carlsbad, CA, USA). Following the RNA extraction, we made performed quantitation using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA), estimation of protein contamination (260/280 ratio), and other organic contamination (230/260 ratio). The samples with sufficient quality and quantity were checked further with a Bioanalyzer 2100 (Agilent Technologies, DE, USA) chip reader using Agilent RNA 6000 Nano kit (No: 5067-1511) to assess the whole sample RNA integrity number (RIN) and suitability for library preparation. Total RNA samples with RIN 7.0 or higher were quantified with a Qubit™ RNA BR assay, 20–1000 ng/µL ng (Catalog number: Q10211) as well as Qubit™ dsDNA BR assay, 100 pg/µL to 1000 ng/µL to accurately determine the contamination of genomic DNA (Catalog number: Q32853). Total RNA samples passing these quality checks were normalized by dilution 100 ng/µL using nuclease-free water (NF water) and used for library preparation.

RNA library preparation and transcriptome profile generation

We carried out the library preparation for RNA sequencing (RNAseq) on the Illumina platform in our lab using QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina kit (Lexogen, Vienna, Austria). We prepared a total of 168 (n=4) single-indexed libraries, with 500 ng of total RNA as an input for each library. The quality of enriched single-indexed libraries was checked with the Agilent TapeStation 4200 using D1000 DNA ScreenTape assay (Agilent Technologies, Inc), and concentration was determined using the Qubit^{TM} dsDNA HS Kit (Catalog number: Q33231). Two batches of 96 and 72 libraries respectively were individually barcoded. Each individually barcoded library in the two batches was normalized to 4 nM to be pooled in equimolar proportions and submitted to Texas A&M Institute for Genome Sciences and Society (TIGSS, College Station, TX), for sequencing on an Illumina NextSeq (Illumina, San Diego, CA) platform. An average of 28 million reads was generated for each library in a 100 bp single-end mode.

Transcriptome data analysis

We performed all bioinformatics analysis with open-source tools and using well-established RNAseq analysis pipelines. In summary, the quality of the single-end raw reads of the RNAseq data generated in FASTQ format was checked with FastQC (Andrews, 2010) version 0.11.9 and MultiQC version 1.9 (Ewels et al., 2016; Martin, 2011). After removing adapter contamination and Lexogen indices, only reads with a Phred quality score greater than 30 (99.9% bp signal accuracy) and over 35bp in length were retained using Trim_Galore version 0.4.5 (Bolger et al., 2014). Reads passing quality filters were mapped to the *Gallus gallus* genome, Galgal6 (Version 6, Ensembl Release 99 GRCg6a, downloaded Jan 2020) using the short-read de-novo splice mapper STAR program. We counted reads mapping to exons using the "*--quantMode*

GeneCounts" option (version STAR_2.5.3a_modified) (Dobin et al., 2013; Dobin & Gingeras, 2015).

Differential gene expression statistical analysis

The differential gene expression analysis of counts data for each treatment and across all circadian time points were analyzed in the R statistical platform (version 3.6.2) using the EdgeR package (version 3.26.8) to determine the differentially expressed genes (DEGs) (McCarthy et al., 2012; Robinson et al., 2010). In summary, differences in RNAseq libraries are corrected by calculating the normalization factors, across counts data. The sum of rows for any given gene less than one count per million (CPM) at least in two columns was excluded from further analysis. We calculated the estimated common dispersion to evaluate the overall counts' data dispersion, whereas a high value indicates a higher noise of the biological replicates and a low value indicates less noise inferring specific patterns across counts data. We calculated tagwise dispersion for replicates pairs to assess the consistency between biological replicates in the same treatment. We used the likelihood ratio test 'glmLRT' function in full factorial design specified by "my.contrasts" function to test for significant differential expression between 168 groups at an FDR < 0.05. We performed a power analysis based on actual dispersion (common dispersion of 0.086) in the RNAseq data using ssizeRNA 1.3.2 (Bi & Liu, 2016), which showed that our design had 98.9% power to detect Log_2-Fold differences at FDR≤0.05.

Gene ontology and pathway analysis

Significant differentially expressed genes underwent gene ontology and pathway analysis using the Ingenuity Pathway Analysis platform (IPA; QIAGEN Inc.) software (Krämer et al., 2014) to detect the activated canonical pathways and networks and their roles in molecular, cellular

functions, physiological system development and function between blue and white light treatments in controlling to dark treatment to address the first aim of the study by revealing the impact of the light source on circadian development across day and night time points. Hence, addressing the second aim by determining the effect of different backgrounds of circadian development on the response of the immune system in challenged chicks.

Results & Discussion

RNA sequence results and identification of differentially expressed genes.

We sequenced 168 RNAseq libraries, with seven-time points of circadian study sample collection and four biological replicates per treatment group (six treatments), generating a total of 5713.7 million reads, with an average of 34.01 million reads per library. After quality filtering and adapter trimming, we retained an average of 98.15 of the reads per replicate. An average of 91.17% mapped to the Galgal6 genome reference, and an average of 45% of the reads mapped uniquely to exons using "--quantMode Gene Counts" option in STAR program (Table 6). The common dispersion estimate for the entire dataset was low (0.086). Tagwise dispersion values in the dataset indicated that 75% of genes had a biological coefficient variation (BCV) below 0.26 (Figure S3). In contrast, the upper quartile of tagwise dispersion density estimates pointed that genes with lower expression had higher dispersion, a maximum value of 126.49. A total of 24,356 genes were detected, of which 17,115 genes were expressed at CPM>1. Of these, 11,300 genes were annotated on ENSEMBL, while the rest were novel transcripts with no annotations. Most (91%) of the expressed genes were protein-coding, and the rest were assigned to long non-coding RNAs (lncRNAs, 7%), pseudogenes (0.8%), small nucleolar RNAs (snoRNAs, 0.49%), microRNA (miRNA, 0.25%), mitochondrial transfer RNA (Mt-tRNA, 0.11%), small nuclear RNA (snRNA,

0.78%) .

Table 6. Combined quality control summary of RNASeq Reads from all treatments.

QC	Blue	Blue-Vac	White	White-Vac	Ctrl	Ctrl-Vac
M Seqs	925.4	968	959.7	923.7	958.9	978
Length	96	96	96	96	96	96
% Dups	64%	63%	66%	66%	67%	66%
% Trimmed	1.8%	1.9%	1.8%	1.8%	1.9%	1.9%
M Aligned	853.8	884	884.8	840.7	874.2	881
% Aligned	92%	91%	92%	91%	91%	90%
M Assigned	406	452	432.5	423.5	450.8	444.7
% Assigned	43%	46%	44%	45%	45%	45%

To be concise, we present the results for two successive days and nighttime to track the impact of 12h photoperiods of blue and white LEDs light, in challenged and non-challenged embryos. These two successive time points were chosen based on ND virus particles enumeration/ propagation study in lung tissue over all the seven-time points of the circadian study. We picked the interval illustrating ND virus exponential/log phase replication (Alhaj Ali et al., 2021, *in preparation*). Hence, the acquired transcriptome profiles result in all treatment groups during ZT06 (daytime) and ZT18 (nighttime) are discussed in detail in this section.

Impact of light treatments on the expression pattern in non-challenged treatments controlling for the dark group during daytime.

Blue Light Incubated vs. Dark incubated embryos at ZT06.

The transcriptome data from embryonic lung tissue in non-challenged blue light treatment showed 1037 genes differentially expressed (FDR < 0.05), when comparing against the non-challenged dark group at time point ZT06. Of these, 685 were upregulated, and 352 were downregulated (Figure 13 A). Analysis of the enriched gene ontology (GO) terms through "Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources Version v6.8" (Huang, Sherman, & Lempicki, 2009a, 2009b), using entries of 778 Entrez gene IDs against the chicken reference, returned 765 classified genes into 113 generated records, these records contain GO terms indexed under different categories, for instance, biological process (BP), cellular component (CC), and molecular function (MF), Kyoto Encyclopedia of Genes and Genomes pathways (KEGG-pathway), Annotation Summary (Up_Keywords), and Type of the active tissue (Up_Tissue). The top enriched biological process terms were 'calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules and cell Migration', 'regulation of Rho protein signal transduction', 'epidermal growth factor receptor signaling pathway', ' skeletal muscle contraction', and 'positive regulation of fat cell differentiation' for the cellular components, the top enriched terms included 'troponin complex', 'receptor complex', 'integral component of membrane', ' extracellular exosome', and 'cell surface', where the top enriched molecular functions were 'ephrin receptor binding', 'calcium ion binding', 'protein serine/threonine kinase activity' and 'transporter activity'. In addition, we analyzed the pathways enriched for the differentially expressed genes using KEGG pathway analysis (Kanehisa et al., 2016). Kegg Pathways were considered enriched if at least two DEGs were found in the

background pathway and Fisher Exact P- value < 0.05. In the embryonic lung tissue (Blue vs. Dark Non-challenged at ZT06), Two KEGG pathways were significantly enriched, including 'Glycerolipid metabolism' and 'Biosynthesis of amino acids. DAVID summary annotation for the DEGs detected in non-challenged blue light compared to dark treatment, giving the raise for some Up Keywords includes 'Muscle protein', 'Transferase activity', 'Motor Protein', and 'Disulfide bond', where the attributed tissues to the detected GO terms indicate biological activities in 'Pectoralis Muscle', 'Blood' and 'Intestine' tissues ($P < 0.05$) (Supplementary Table S7). It might be notable to detect active tissue other than lung tissue where the transcriptome data came from, but the circulation of enzymes, hormones, and synthesized proteins required for maintaining the body homeostasis and development can indicate what entities of biological processes surpassing the other body activities. Furthermore, the DEGs dataset was further clustered by GO term using the Functional Annotation Tool in DAVID, where the clustering analysis for non-challenged blue light against dark group enriched for blue copper proteins 'Cupredoxins ', 'multicopper oxidases type II&III', ' Intracellular signaling protein (Pleckstrin)', 'Muscle protein', 'Skeletal Muscle Contraction', 'Troponin Complex', 'Motor protein', 'Myosin, N-terminal, SH3-like', 'Myosin-like IQ motif-containing domain', and 'Myosin tail' protein families (Table 7).

Table 7. GO-Functional Annotation Clusters for Blue Non-challenged vs. Dark Non-challenged at ZT06 DEGs. The following clusters (1-5) resulting from DAVID-GO Functional Annotation Clustering, represent the DEGs in Blue light Non-challenged group compare to dark group (765 genes)

Blue Non-challenged vs. Dark Non-challenged at ZT06					
Cluster	ES	Category	Associated Term	p-value	No. Genes
1	1.96	INTERPRO	Cupredoxin	0.003495	5
		INTERPRO	Multicopper oxidase, type 3	0.014973	3
		INTERPRO	Multicopper oxidase, type 2	0.024104	3
2	1.92	INTERPRO	Pleckstrin homology domain	0.008276	18
		INTERPRO	Pleckstrin homology-like domain	0.013978	25
		SMART	PH	0.01427	18
3	1.61	UP_KEYWORDS	Muscle protein	0.010257	7
		GOTERM_BP_DIRECT	skeletal muscle contraction	0.033172	3
		GOTERM_CC_DIRECT	troponin complex	0.043704	3
4	1.44	UP_KEYWORDS	Motor protein	0.029468	8
		INTERPRO	Myosin, N-terminal, SH3-like	0.03278	4
		INTERPRO	Myosin-like IQ motif-containing domain	0.039439	4
		INTERPRO	Myosin tail	0.046727	4

ES = Enrichment score produced by Functional Annotation Clustering in DAVID. Category Terms Defined: UP Keywords = Uniprot Keywords; GOTERM BP DIRECT = GO Term for Direct Involvement in Biological Process; GOTERM MF DIRECT = GO Term for Direct Involvement in Molecular Function; GOTERM CC DIRECT = GO Term for Direct Localization to Cellular Compartment; KEGG PATHWAY = KEGG Pathway

The Ingenuity Pathway Analysis (IPA) classified the 1037 DEGs to 571 annotated molecules ready for analysis in its database, where across the observation, there were 346 upregulated molecules and 225 downregulated molecules. The top three activated pathways were

‘Epithelial Adherens Junction Signaling’, ‘Gap Junction Signaling’, and ‘Axonal Guidance Signaling’. Epithelial Adherens Junction Signaling “ is a pathway that form, strengthen and spread, degrade, and then re-form as their associated proteins create ephemeral connections with counterparts from adjacent cells was the top activated pathway with blue light incubation at ZT06, involving 14 genes, seven were upregulated (*MYH10, EPN2, APC, CTNND1, SNAI2, EGF, TGFBR2*) and seven genes were downregulated (*MYH2, MYL1, MYH7B, MYL3, ACTN2, CRK, TUBA1B*) these genes mainly have a role in biological processes of ‘cardiac epithelial to mesenchymal transition’, ‘positive regulation of cell migration’, and ‘muscle contraction', indicating a correlation between the blue light and development of muscle proteins of the chick’s embryos. (Supplementary Table S7).

The non-canonical pathways do not involve an intracellular accumulation of β-catenin protein and operate independently of it, but depend on other central intracellular mediator called the Wnt/Jun N-terminal kinase (*JNK*) or Wnt/calcium pathway, respectively (Rao & Kühl, 2010), showed upregulation in circadian, P53, and RhoGDI Signaling. The most upregulated pathway with blue light incubation at ZT06 was the circadian pathway consisting of 16 genes, of which 12 genes were upregulated (*MAT2A, EGR3, CSNK1E, GRIA2, ATOH7, PPARGC1A, GABRG2, PPARA, GABRB2, DPYD, KCNMA1, GSK3B*) and 4 were downregulated (*CSNK1D, PRNP, PTGDS, GABRP*). The involved genes of the circadian pathway revealed biological processes have a role in ‘regulation of circadian rhythm’, ‘circadian regulation of gene expression’, ‘positive regulation of fatty acid oxidation’, and ‘negative regulation of neuron apoptotic process’. Ion channel, Ion transport, Synapse, Cell junction, Receptor, and Transport were the Up Keywords summary functions of these genes. KEGG pathway analysis indicated enrichment of those genes into Hedgehog signaling pathway that transmits information to embryonic cells that is essential

for proper cell differentiation (Choudhry et al., 2014), and interestingly Insulin resistance KEGG pathway. Previous reports associated with blue light (~450–500 nm) short-wavelength from light-emitting diode-dependent materials, impaired glucose tolerance, and metabolism by disturbing the biological clock's natural activity, modifying sleep-wake cycles, and causing metabolic changes (Masís-Vargas, Hicks, Kalsbeek, & Mendoza, 2019). This gives rise to an important question, is the blue photoperiod provided during incubation is conflicting with the earth magnetic field (Fernie, Bird, & Petitclerc, 1999; R J Reiter, 1992, 1993), or it is only the selective preference of pinealocytes to blue light (Okano et al., 1997, 1994).

White Light Incubated vs. Dark incubated embryos at ZT06.

Eggs incubated under white light in non-challenged groups showed nearly 61% fewer DEGs than the blue light treatment at ZT06. We detected a total of 403 (FDR < 0.05) DEGs, of these, 286 genes were upregulated, and 117 were downregulated (Figure 13 B). Of the 403 enhanced DEGs queried against the DAVID database, 296 genes were annotated and enriched for 31 gene ontology terms. The top enriched biological processes were ‘microtubule cytoskeleton organization’, ‘epidermal growth factor receptor signaling pathway’, and ‘pericardium morphogenesis’. The top enriched cellular components included ‘perinuclear region of cytoplasm’, ‘neuromuscular junction’, and ‘chromatin’. Where ‘SMAD binding’, ‘nucleic acid binding’, ‘receptor signaling protein serine/threonine kinase activity’ was enriched as the top detected Molecular functions (supplementary TableS7). KEGG pathways analysis showed four enriched pathways include ‘Regulation of actin cytoskeleton’, ‘ErbB signaling pathway’, ‘Focal adhesion’, and ‘Glycerolipid metabolism’ (supplementary Table S7). The summary of GO terms detected in the white light non-challenged group when controlling to conventional dark incubation revealed

some keywords include ‘Ribosomal protein’, ‘Ribonucleoprotein’, and ‘Differentiation’ indicating an enhanced or accelerated embryogenesis process than in the dark treatment.

The Ingenuity Pathway Analysis (IPA) classified the 403 DEGs to 231 annotated molecules ready for analysis in its database, where across the observation, there were 173 upregulated molecules and 58 downregulated molecules. The most significant canonical pathways encompass ‘Glycogen Degradation II’, ‘Wnt/β-catenin Signaling’, ‘and ‘Glycogen Degradation III’ (supplementary Table S7). The top significant non-canonical pathways were ‘MAPK’, ‘Rho-GTPase Signaling’, ‘P53’, ‘NF-B Signaling’, and ‘RhoGDI Signaling’. Activation of the Mitogen-Activated Protein Kinases (MAPK) pathway indicates enhanced signals communication from the receptors on the surface of the cells to DNA in the nucleus of the cells, influencing processes like cell division. The detected MAPK pathway involved fifteen genes, ten were upregulated (*PLA2G4E, TGFBR2, DUSP4*, *SOS1, ATF1*, *PAK2, FADD, ELF2, RALB,* and *GRB2*), and five were downregulated (*H3-3A/H3-3B, RPS6KC1, MYC, TAB2, ELF5*). These genes have a role in biological processes of a developmental process, metabolic process, multicellular organismal process, response to stimulus, and signaling. Reflecting enhanced cell proliferation, and differentiation during embryonic development under white light incubation. It is notable that the MAPK pathways act as input during the entrainment of 24 hour rhythms and regulate tissue-specific expression patterns (C. S. Goldsmith & Bell-Pedersen, 2013). Therefore, this finding supports the hypothesis that lighting during incubation can stimulate circadian rhythms.

Blue Light Incubated vs. White light incubated embryos at ZT06.

When comparing the outcomes of blue versus light during incubation, we saw 193 differentially expressed genes (FDR < 0.05). Of these, 105 were upregulated in blue light, and 88 were downregulated (Figure 13 C). DAVID analysis showed biological process includes 'somitogenesis', 'regulation of axon extension involved in axon guidance', and 'exocytosis' and cellular function of 'semaphorin receptor complex', 'integral component of membrane', and 'myosin complex, whereas the molecular function encompassed 'motor activity', 'semaphorin receptor activity', and 'vasoactive intestinal polypeptide receptor activity'. KEGG pathway analysis indicated upregulation in lysosome activity. The summary of the DAVID functional annotation analysis highlighted a group of upregulated processes (Up_Keywords) included 'ANK repeat', 'Lipid transport', 'Transmembrane helix & Transmembrane', 'Membrane', and 'Myosin' as a direct effect of the blue light on the embryogenesis process comparing to providing the white light photoperiod during incubation (Supplementary Table S7). Clustering analysis for the differentially expressed genes detected in Blue vs. White treatments non-challenged groups, identified groups of enriched GO terms includes 'integral component of membrane', 'Transmembrane & Transmembrane helix', 'Ankyrin repeat-containing domain', 'ANK repeat', 'Myosin head & motor domain', 'MYSc: Myosin. Large ATPases', 'Myosin', 'Myosin complex', and 'Calmodulin-binding' (Supplementary Table S7). These results indicate that genes differentially expressed between blue and white light incubation involved in protein synthesis and conformational change, which either activates or inactivates their function through calcium-binding activity. These results also indicate muscle skeletal muscle contraction and development and are consistent with the finding (from Blue vs. Dark) that blue light during incubation stimulates muscle development.

IPA classified 89 of the DEGs in Blue vs. White, of which 55 were upregulated. The top three activated canonical pathways were ‘Glutamate Removal from Folates’, ‘Agranulocyte Adhesion and Diapedesis’, ‘Cellular Effects of Sildenafil (Viagra) (Supplementary Table S7). Glutamate Removal from the Folates pathway included the gamma-glutamic hydro lease (GGH) gene, which was upregulated 19 fold. This gene plays a vital role in the cellular homeostasis of folate and regulates intracellular folate and antifolates for optimal nucleotide biosynthesis and antifolate-induced cytotoxicity, respectively. Folates are required in various reactions (known as one-carbon metabolism) in mammalian tissues, where they act as carriers of one-carbon units in various oxidation states. These one-carbon units are utilized in the biosynthesis of various cellular components, including glycine, methionine, formylmethionine, thymidylate, pantothenate, and purine nucleotides. Another notable finding is the activation of agranulocyte adhesion and diapedesis pathway, which is essential in innate immune response, and are a primary line of protection against infection, and essential for attracting agranulocytes and granulocytes to the injury site (Xing, Cheng, Zha, & Yi, 2017). While it is not clear why injury repair mechanisms are recruited, one explanation is that we witnessed a discoloration of the downs in the blue-light incubated chicks, which can be a feature of anemia or circulatory defects and may trigger other injury signals. It was reported that melanocytes sense blue light and regulate the skin's pigmentation through non-visual opsins photoreceptors in humans (Regazzetti et al., 2018). Further research is needed to understand the blue light impact on chicks down discoloration.

Taking together, the main results from these three comparisons, the blue light photobiostimulation showed an immense effect on stimulating the circadian rhythm than white light when compared to dark incubation where the induced circadian pathway was significant in

blue light and non-significant in white light incubation, respectively *(P < 0.01)*. This supports our first hypothesis in this study, where different light wavelengths will entrain the circadian rhythm differently. These outcomes valid previous studies claimed a blue light sensitivity preference by the photoreceptors exist in pineal gland photoreceptors (Okano et al., 1997, 1994).

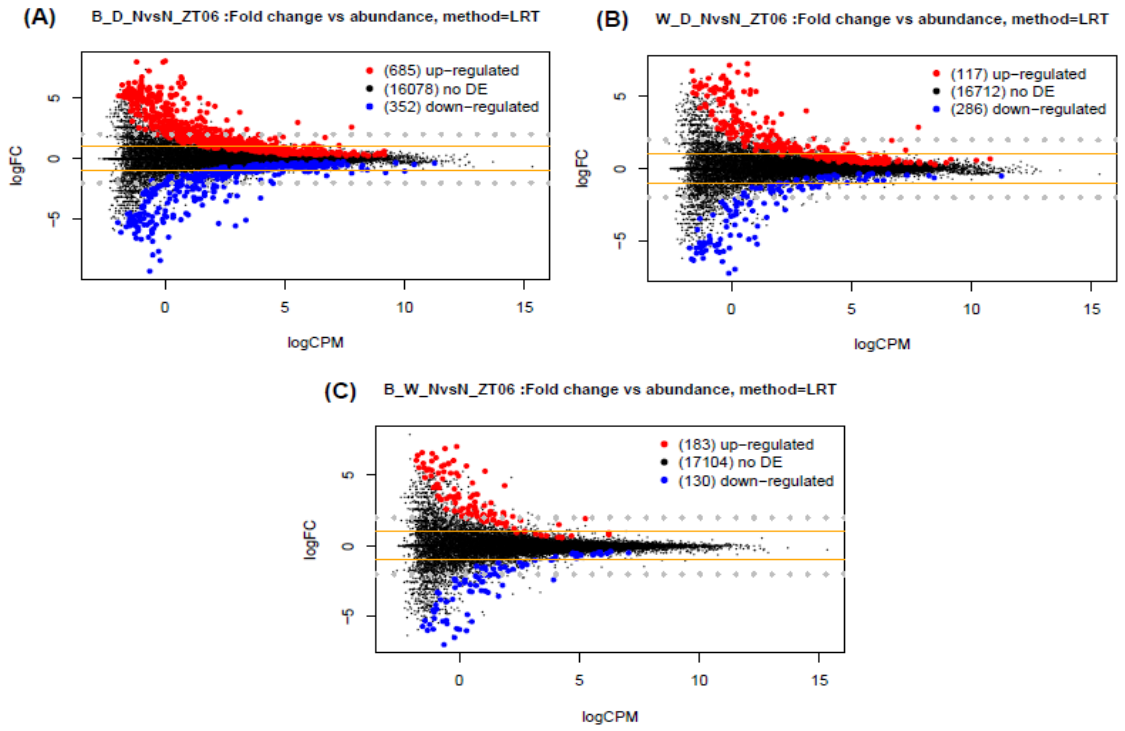

Figure 13. Mean difference (MD) plot highlighting the log fold change and average abundance of each gene, comparing the differences between blue or white light photobiostimulation to dark condition during incubation in the non-challenged ND virus group at time point ZT06 (daytime). Significantly up and down DE genes and their numbers are highlighted in red and blue, respectively, at FDR < 0.05. (A) shows the DEGs in blue light vs dark incubation during daytime. (B) shows the DEGs in white light vs dark incubation. (C) shows the DEGs in blue light vs white incubation. The Y-axis corresponds to the mean average of log10 count per million (CPM), and the X-axis displays the log2 FC at FDR < 0.05. B:Blue; D:Dark; W: White; N_N: Non-challenged in both treatments.

Impact of light treatments on the expression pattern in challenged treatments controlling to the dark group during daytime.

Dark + NDV Challenged vs. Dark + non-Challenged group at ZT06.

Embryos in the control treatment (dark) challenged *in ovo* with NDV at ED18 showed relatively few differences compared to the non-challenged embryos. Four-hundred and ninety-eight genes were differentially expressed at FDR < 0.05, with 258 up regulated and 240 down regulated genes (Figure 14 A). The enriched GO terms were based on 337 Entrez gene IDs annotated in DAVID. The top three enriched Biological Processes terms were 'muscle contraction', 'positive regulation of NF-kappaB import into nucleus', and 'defense response to virus'. The top three Cellular Component terms were 'apical plasma membrane', 'clathrin coat of coated pit', and 'clathrin coat of trans-Golgi network vesicle', whereas the top Molecular Function terms were 'peptide binding', 'phosphatidylinositol-4-phosphate binding', and 'helicase activity' (Supplementary Table S8). KEGG pathway analysis indicated upregulation in Nicotinate and Nicotinamide Metabolism, precursors of the coenzymes nicotinamide-adenine dinucleotide (NAD+), and nicotinamide-adenine dinucleotide phosphate (NADP+). These molecules are essential for redox reactions and electron transport, crucial in glycolysis, TCA cycle, pentose phosphate cycle, and fatty acid biosynthesis(Magni et al., 2004). The DAVID functional annotation analysis summary highlighted the upregulated processes (Up_Keywords) included 'Cytoplasm', 'Muscle protein', 'Hydrolase', and 'SH2 domain' (Supplementary Table S8).

We analyzed 274 classified DEGs with IPA, of which 158 were upregulated. The top three significant canonical pathways were 'Actin Cytoskeleton Signaling', 'Gα12/13 Signaling', and 'Hepatic Fibrosis / Hepatic Stellate Cell Activation'. Based on activation Z-score, the upregulated pathways were 'Role of NFAT in Regulation of the Immune Response', 'Systemic Lupus

Erythematosus In B Cell Signaling Pathway', and 'Estrogen Receptor Signaling', and significantly downregulated pathways were 'Nicotine Degradation III', 'Nicotine Degradation II', and 'Wnt/β-catenin Signaling' in the (dark) challenged group at ZT06 (Supplementary Table S8). The top significant non-canonical pathways were 'NF-κB Signaling', and 'MAPK'. Observing the transcription factor NF-κB Signaling pathway indicates the regulation of multiple aspects of innate and adaptive immune functions and serves as a crucial mediator of inflammatory responses that induce activation and differentiation of innate immune cells and inflammatory T-cells. NF-κB activation contributes to the pathogenic processes of various inflammatory diseases, in our case, the *in ovo* NDV challenge.

Blue Light + NDV Challenged vs. Dark + NDV Challenged embryos at ZT06.

Embryos incubated under blue light showed 530 DEGs (FDR < 0.05) when compared to the dark control incubation when both were challenged with NDV. Of those, 351 genes were upregulated, and 179 were downregulated (Figure 14 B). The enriched GO terms were based on 368 Entrez gene IDs annotated in DAVID. The top three enriched Biological Processes terms were 'defense response to virus', 'protein ubiquitination involved in ubiquitin-dependent protein catabolic process', and 'mitotic sister chromatid cohesion'. The detected Cellular Component terms were 'microtubule cytoskeleton', 'nucleus', 'intrinsic component of endoplasmic reticulum' and 'membrane', whereas the top molecular function 'protein serine/threonine kinase activity', 'ATP binding', and 'Wnt-protein binding' (Supplementary Table S8). KEGG pathway analysis confirmed that DEGs were remarkably enriched in pathways of 'Calcium signaling pathway', and 'Neuroactive ligand-receptor interaction'. Calcium signaling pathway upregulation indicates a surge in the use of calcium ions (Ca^{2+}) to communicate and drive intracellular processes, often as

a step in signal transduction, where the neuroactive ligand-receptor interaction pathway is a collection of receptors and ligands associated with intracellular and extracellular signaling pathways on the plasma membrane (Hou, Yang, Wang, Wang, & Zhang, 2018; Lauss, Kriegner, Vierlinger, & Noehammer, 2007). The summary of the DAVID analysis highlighted the upregulated processes 'Nucleotide-binding', 'Transferase', 'Microtubule', 'ATP-binding' and 'Cell adhesion' (Supplementary Table S8). The clustering analysis for blue challenged vs. dark challenged embryos at ZT06, did not identify specific enriched GO terms, but the most highlighted biological processes indicated immune response, negative regulation of viral genome replication, and defense response to virus, all indicating that the response to the challenge was significantly enhanced in blue-light incubated embryos.

IPA classified 250 analysis-ready molecules from this comparison, where 144 were upregulated and 106 downregulated. The top three most significantly detected canonical pathways were 'IGF-1 Signaling', 'Role of RIG1-like Receptors in Antiviral Innate Immunity', and 'Insulin Secretion Signaling Pathway'. The top upregulated pathways based on Z-score were 'Opioid Signaling Pathway', 'Synaptic Long Term Depression', 'Protein Kinase A Signaling', 'Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes', and 'Synaptic Long Term Potentiation', whereas the top down regulated canonical pathways were, 'Role of RIG1-like Receptors in Antiviral Innate Immunity', 'Activation of IRF by Cytosolic Pattern Recognition Receptors', 'JAK/Stat Signaling', 'Systemic Lupus Erythematosus In B Cell Signaling Pathway', and 'TGF-β Signaling' (Supplementary Table S8). IPA analysis revealed several highly upregulated non-canonical pathways where the top three pathways included 'RhoGDI Signaling', 'Rho-GTPase Signaling', and 'MAPK'. Notably, NF-κB Signaling, and Circadian pathways were upregulated in the blue-challenged embryos at time point ZT06. RhoGDI Signaling and Rho-

GTPase Signaling indicates cellular regulation, including morphology and migration, gene transcription, cell cycle progression and cytokinesis, phagocytosis and vesicular traffic, as well as regulation of a range of enzymatic functions, e.g., NADPH oxidase (Etienne-Manneville & Hall, 2002). The activation of the NF-κB signaling pathways in this contrast is noteworthy because NF-κB is regulated by the circadian core oscillator gene CLOCK. (Spengler et al., 2012). The NF-κB pathway, which includes over 100 genes, is involved in regulating a variety of biological responses, particularly related to immune responses and inflammation (Dolcet et al., 2005). This association between the circadian oscillator and the immune-response pathway suggests that the photostimulation during development invokes this coupled mechanism's activation.

White Light + NDV Challenged vs. Dark + NDV Challenged embryos at ZT06.

Embryos incubated under white light and challenged with NDV showed 1357 differentially expressed genes (FDR < 0.05) to the dark incubated NDV challenged embryos. Of these, 771 genes were upregulated, and 586 were downregulated (Figure 14 C). The enriched GO terms were based on 1119 Entrez gene IDs annotated in DAVID. The top three enriched Biological Processes terms were ‘enteric nervous system development’, ‘response to toxic substance’, and ‘neural crest cell migration’. In Cellular Components , the three most significant cellular components were ‘nucleoplasm’, ‘Golgi apparatus’, and ‘nucleus’. KEGG pathway analysis confirmed that DEGs were enriched in ‘Nucleotide excision repair’, ‘Cell cycle’, and ‘mTOR signaling pathway’. The top five GO terms for each category and KEGG pathways concerning white light effects on NDV challenge are displayed in (Supplementary Table S8).

IPA based on 911 annotated molecules included 439 upregulated molecules and 472 downregulated molecules. The top three most significant canonical pathways were ‘Nucleotide

Excision Repair Pathway', 'CNTF Signaling', and 'CMP-N-acetylneuraminate Biosynthesis I (Eukaryotes)'. While' UVB-Induced MAPK Signaling', 'EGF Signaling', and 'Huntington's Disease Signaling' 'PPARα/RXRα Activation' and 'Sirtuin Signaling Pathway' were the most upregulated canonical pathways based on Z-score. Contrary, 'Assembly of RNA Polymerase II Complex', 'NER Pathway', 'Cyclins and Cell Cycle Regulation', 'TGF-β Signaling', and 'Cell Cycle Control of Chromosomal Replication' were the top down regulated canonical pathway , based on Z score (Supplementary Table S8). The top three non-canonical pathways included 'Mitochondrial Dysfunction', 'Estrogen', and 'MAPK'. Finding Mitochondrial Dysfunction upregulated together with Nucleotide Excision Repair Pathway, which indicates DNA repair mechanisms associated with radiation, chemicals, and other mutagens (Griffiths, Miller, Suzuki, Lewontin, & Gelbart, 2000; Halliwell & Gutteridge, 2015). It is not clear if the enhanced DNA repair activity is in response to effects potential detrimental effects of lighting, for example, UV-B mutagenic UV-B exposure, or due to the stimulation of growth-associated pathways. Tissue development induced by the EGF signaling pathway is one of the most critical pathways in mammalian cells, which regulate proliferation, migration, differentiation, and intercellular communication during development. If light accelerates tissue development, reflecting a higher demand on mitochondrial activity, manifesting as mitochondrial dysfunction. Furthermore, the upregulation of mTOR pathways in white light incubation suggests an enhancement effect on upstream pathways, including insulin, growth factors (such as IGF-1 and IGF-2), and amino acids (Hay & Sonenberg, 2004).

Blue Light Incubation vs. White incubation in challenged group at ZT06

We observed more significant differences in this comparison than in the non-challenged comparison. We detected 584 DEGs (FDR < 0.05) between the NDV challenged embryos incubated either white or blue light. Of these, 281 genes were upregulated, and 303 were downregulated (Figure 14 D). The top enriched Biological Processes from DAVID were 'innate immune response', 'defense response to virus', 'positive regulation of epithelial to mesenchymal transition', and 'negative regulation of viral genome replication', where the top enriched cellular components are 'extrinsic component of cytoplasmic side of plasma membrane', and 'cytoplasm'. The detected molecular function showed enrichment in 'DNA helicase activity', 'ATP binding', and 'double-stranded RNA binding'. KEGG pathway analysis confirmed that DEGs were remarkably enriched in pathways of 'Herpes simplex infection', 'Purine metabolism', and 'RNA degradation'. The top five GO terms for each category and KEGG pathways are shown in (Supplementary Table S8). The most significant Up Keywords in DAVID were 'Manganese', 'Immunity', and 'Nucleotide-binding'. Based on clustering, the top enriched clusters were 'innate immune response', 'extrinsic component of cytoplasmic side of plasma membrane', ' regulation of cell proliferation', 'receptor tyrosine kinase autophosphorylation', 'ubiquitin-protein ligase activity by RING domain of Zinc finger', and 'Interferon regulatory factor'(Table 8).

Table 8. GO-Functional Annotation Clusters for Blue challenged vs. white challenged with ND virus at ZT06 DEGs. The following clusters (1-3) resulting from DAVID-GO Functional Annotation Clustering, represent the DEGs in Blue light Non-challenged group compare to dark group (569 genes).

Blue vs. White challenged with ND virus at ZT06					
Cluster	ES	Category	Associated Term	p-value	No. Genes
1	2.22	GOTERM_BP_DIRECT	innate immune response	8.46E-05	14
		GOTERM_CC_DIRECT	extrinsic component of cytoplasmic side of plasma membrane	0.005783	7
		GOTERM_BP_DIRECT	regulation of cell proliferation	0.01479	10
		GOTERM_BP_DIRECT	peptidyl-tyrosine autophosphorylation	0.02847	5
		GOTERM_MF_DIRECT	non-membrane spanning protein tyrosine kinase activity	0.03788	5
2	1.92	SMART	RING	0.006151	13
		INTERPRO	Zinc finger, RING-type, conserved site	0.0082975	10
		INTERPRO	Zinc finger, RING-type	0.0095952	14
		GOTERM_MF_DIRECT	ubiquitin protein ligase activity	0.0445339	9
3	1.49	INTERPRO	Interferon regulatory factor, conserved site	0.0262705	3
		INTERPRO	Interferon regulatory factor DNA-binding domain	0.0341647	3
		SMART	IRF	0.0383697	3

ES = Enrichment score produced by Functional Annotation Clustering in DAVID. Category Terms Defined: UP Keywords = Uniprot Keywords; GOTERM BP DIRECT = GO Term for Direct Involvement in Biological Process; GOTERM MF DIRECT = GO Term for Direct Involvement in Molecular Function; GOTERM CC DIRECT = GO Term for Direct Localization to Cellular Compartment; KEGG PATHWAY = KEGG Pathway

IPA classified the 584 DEGs to 368 annotated molecules, of which 198 were upregulated. The top three significant canonical pathways were ‘Kinetochore Metaphase Signaling Pathway’, ‘Interferon Signaling’, and ‘Coronavirus Replication Pathway’. The top upregulated canonical pathways based on Z-score were ‘Protein Kinase A Signaling’, ‘Coronavirus Replication Pathway’, and ‘Kinetochore Metaphase Signaling Pathway’. The top downregulated canonical pathways were ‘Role of PKR in Interferon Induction and Antiviral Response’, ‘Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza’ ‘Interferon Signaling’(Supplementary Table S8). The top non-canonical pathways were ‘NF-kB Signaling’, ‘Cancer’, and ‘Mitochondrial Dysfunction’. The Protein Kinase A signaling pathway has several functions in the cell, including regulation of glycogen, sugar, and lipid metabolism, in addition to the Kinetochore Metaphase Signaling Pathway, which has an important role in the cell cycle, cellular assembly and organization, DNA replication, recombination, and repair. The upregulated Coronavirus Replication Pathway in blue +NDV challenged treatment is interesting, but we perhaps observed this as both Coronavirus and NDV are single-stranded RNA. This might be a potential explanation for interferon dysregulation in blue + NDV challenged embryos, as the continued expression can lead to inflammatory or autoimmune diseases, or the more likely it is the effect of the circadian rhythm, when the inflammation is reduced during the day and activated during night time (J. E. Gibbs et al., 2012; J. E. Gibbs & Ray, 2013; Scheiermann et al., 2018; Ye & Maniatis, 2011), Our study of the virus propagation showed that the lowest fold change of ND virus propagation between ZT06 and ZT18 was detected in blue light, where it was the highest in dark treatment followed by white treatment (Figure 15) (Alhaj Ali et al., 2021, *in preparation*).

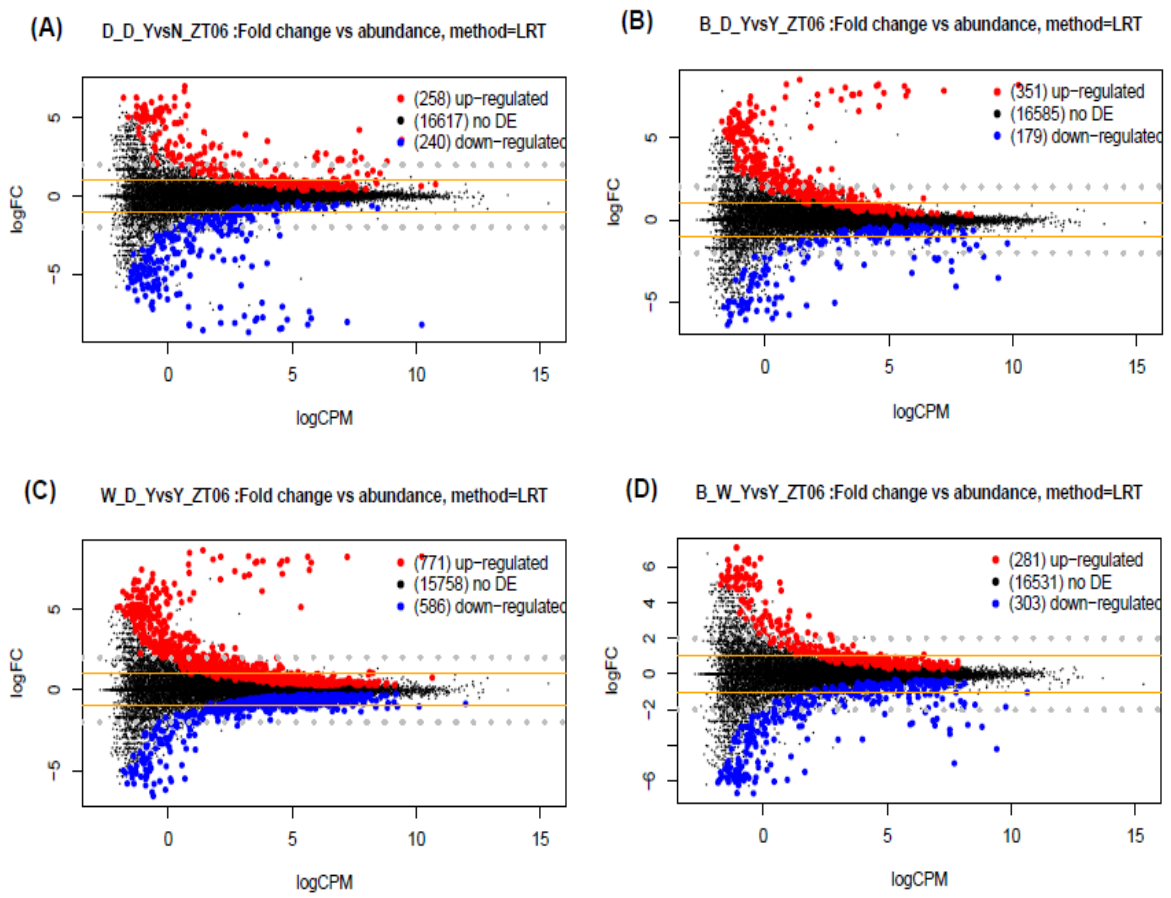

Figure 14. Mean difference (MD) plot highlighting the log fold change and average abundance of each gene, comparing the differences between blue or white light photobiostimulation to dark condition during incubation in the *in ovo* ND virus challenged group at time point ZT06 (daytime). Significantly up and down DE genes and their numbers are highlighted in red and blue, respectively, at FDR < 0.05. (A) shows the DEGs in challenged dark vs non-challenged dark incubation during daytime. (B) shows the DEGs in challenged blue light vs challenged dark incubation. (C) shows the DEGs in challenged white light vs challenged dark incubation. (D) shows the DEGs in challenged blue light vs challenged white incubation. The Y-axis corresponds to the mean average of log10 count per million (CPM), and the X-axis displays the log2 FC at FDR < 0.05. B:Blue; D:Dark; W: White; N: Non-challenged in both treatments; Y: Challenged in both treatments.

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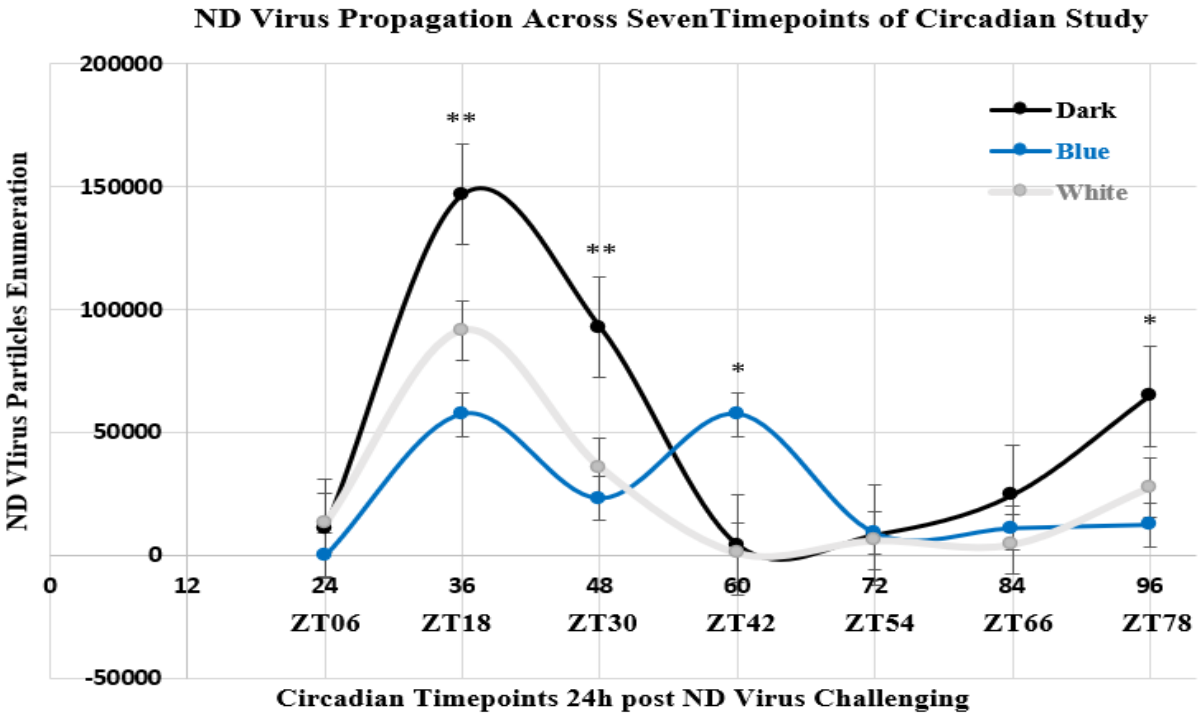

Figure 15. Multi-step propagation curves of Newcastle Disease Virus (NDV) an avian paramyxovirus 1 (APMV-1) in challenged chicken embryos exposed to three different photoperiods of photobiostimulation during the entire period of incubation; Dark no light (0L:24D), Blue photoperiod (12L:12D), and White photoperiod (12L:12D). Embryos were *in ovo* challenged with 100 µl of NDV viral suspension (10^6/ml EID_{50}) at embryonic day 18 (ED18), after 24h post challenging, propagated NDV particles were enumerated using TaqMan qPCR based method (Y axis) in circadian manner of seven timepoints, ZT06 to ZT78, with 12h intervals simulating day and night condition (X axis). Each data point represents mean +/− standard error (n = 3). * and ** denotes $p<0.05$ and $P<0.01$ respectively for the comparison between three lighting treatments at each circadian study timepoints.

Comparison of significant pathways among the three incubation strategies over a 12 hour interval.

While the main comparisons of within light and between light treatments and challenge groupings provided a detailed view of differences arising primarily due to the effect of illumination during incubation, we need to understand the continuing and downstream effects of these strategies on stimulating immune responses. As the innate immune response is the first line of defense and therefore occurs in the first few hours following challenge, we compared the pathways activated in the 12 hours following ZT06, by performing similar DEG analyses as described above, but in this instance, comparing ZT06 against ZT18 for each light and vaccination treatments. For the sake of brevity, we are only presenting the pathway analysis results from these comparisons.

We conducted an IPA comparison analysis between the detected DEGs in NDV challenged groups over the 12-hour interval between ZT06 and ZT18 timepoints within the same incubation condition dark, blue, and white light. These timepoints also reveal differences occurring between midday and midnight, potentially revealing circadian regulated processes that show diurnal oscillations (figure 16). The P53 and Rho-GTPase Signaling pathways were highly upregulated in control dark incubated group, relative to the blue and white light incubated embryos. The MAPK pathway was the most activated white light treatment, whereas the RhoGDI Signaling pathway was significantly different in the blue light incubation relative to the others. It was notable that the circadian pathway was activated in all three treatments but was significantly more so in the blue light incubated groups. Despite the control group having no illumination, we saw an activation of the circadian pathway, but this is perhaps not surprising considering that the earth's rotation and the electromagnetic field (EMFs) can also act for circadian rhythm development. It was reported that EMFs are perceived as light by birds and some mammalian species affecting circadian

production of plasma melatonin levels during the day (Fernie et al., 1999; R J Reiter, 1992, 1993). The strong response of the circadian pathway to blue light incubation supports our hypothesis different light wavelengths will entrain the circadian rhythm with different efficiencies. These outcomes validate previous studies showing blue light sensitivity preference by the photoreceptors exist in pineal gland photoreceptors (Okano et al., 1997, 1994), resulting in a significant change in secreted melatonin in chicken *in vitro (Valér Csernus et al., 1999)*. While our study confirms this, we also provide new evidence for the differential effects of wavelengths on circadian entrainment, and the consequences of this phenomenon for activation of other important pathways during development.

Blue and white light during incubation both appear to have a similar effect in upregulating the NF-kB signaling pathway, unlike the dark incubation, which indicates the regulation of multiple aspects of innate and adaptive immune functions and serves as a crucial mediator of inflammatory responses that induce activation and differentiation of innate immune cells and inflammatory T cells. The transcription factor NF-κB is regulated by the circadian core oscillator gene CLOCK. (Spengler et al., 2012), and the activation of the circadian pathways in both illuminated treatments further supports a strong correlation between these two pathways. This is noteworthy because the NF-κB pathway, which includes over 100 genes, is involved in regulating a variety of biological responses, particularly related to immune responses and inflammation (Dolcet et al., 2005).

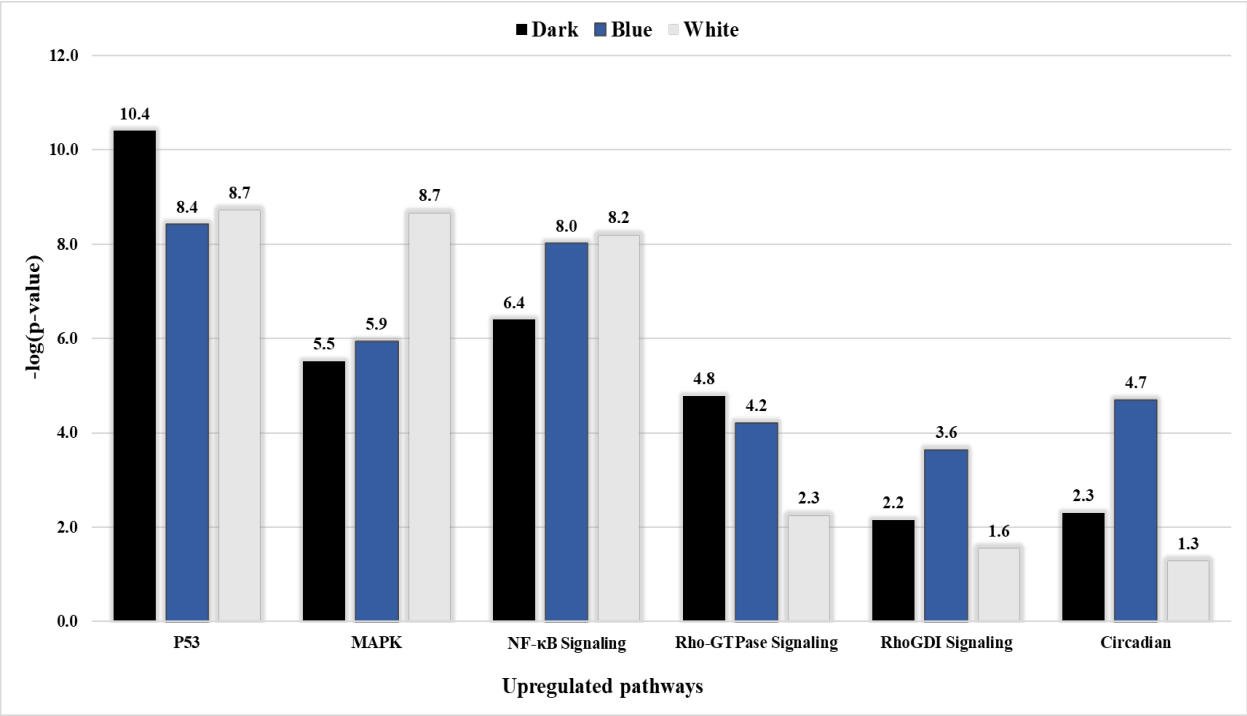

Figure 16. Induced pathways resulted from the change in DEGs over two successive timepoints (ZT06 and ZT18) timepoints within the same treatment group in challenged treatments, dark, blue ,and white light incubation. Detected pathways on the X-axis considered significant with a *-logp-value* ≥ 1.3, an equivalent of (*P <0.05*) on the Y-axis.

In conclusion, our results showed that the entrainment of circadian rhythm in chicken embryos is affected significantly by the wavelength of light used during incubation. The blue light was more effective at stimulating circadian development and circadian-regulated differential expression than white light during daytime (ZT06) in non-challenged embryos. Blue light showed a distinct impact on specific biological processes, including skeletal muscle development, satellite cell activation, stem cell development, regulation of striated muscle contraction, and Glycerolipid metabolism. The detected DEGs in blue light treatment were enriched for Up_Tissue of pectoralis muscle. Our results confirm previous studies where the blue light was found to enhance growth

and development in broilers. Additionally, our study is one of the first to provide a high-resolution understanding of pathways that are perturbed by wavelength-specific circadian activation.

Exposing chicken embryos to white light during incubation showed DEGs enriched for biological processes with a role in enhanced cell proliferation, differentiation, Translation and rRNA processing, and ATP synthesis, which give the white light more of a photo-acceleration property than its circadian stimulating effect. Epidermal growth factor receptor signaling pathway, ErbB signaling pathway, MAPK signaling pathway, and Insulin signaling pathway were upregulated in white light whether the chicken embryos regardless of challenge status.

The response to NDV challenging showed a distinct transcriptome response between blue and white light at ZT06, where the blue light treatment showed a higher expression in various molecules concerning (including interferons, cytokines, and chemokines) altogether involved in innate antiviral immunity, and DNA damage repair. The response of challenged embryos under white light showed a higher expression in molecules such as (*CAB39L, CDKN2A, MYC, RABL6, RBL1*, and *RICTOR*) indicating enrichment of metabolic and proliferative activities. Taken together, embryos under white light maintained a proliferative state even with the burden of NDV infection, whereas blue light showed a pronounced activation of innate immune responses focused toward viral nucleic acids. These divergent responses clearly show that embryos incubated under blue light developed a more specific response against NDV. Why these anti-viral responses were enhanced under blue light is not clear and requires additional investigation. One notable difference we observed between the blue and white light incubation is the efficacy of circadian entrainment and the activation of NFKB versus MAPK pathways, respectively. Both these pathways are tightly correlated with circadian rhythms, but our data suggest that these wavelength differences may perturb key molecules in these pathways that generate different functional trajectories.

In investigating the activities between the 12hr window between ZT06 and ZT18, we saw that blue light showed an upregulation in TP53, RBL1, and RBL2 molecules, and activation of the senescence pathway, arising from the activation of the circadian pathway. In contrast, white light did not induce a significant circadian rhythm but continued to show the same upregulation expression pattern of cellular proliferative between ZT06 and ZT18.

In the NDV challenged groups, we continued to observe this pattern, with blue light incubated embryos showed higher expression of innate immune genes, particularly those representing antiviral response, differentiation of antigen-presenting cells, maturation of phagocytes, *IFNG, IRF7, TNF*, autophagy of cells, immune response of antigen-presenting cells, immune response of leukocytes and phagocytes, and Interferon Signaling. These observed upstream regulators and pathways represent early stages of the innate immune response at 36h post-challenge. These also corresponded with an apparent activation of the circadian pathway genes between ZT06 and ZT18. In the white light +NDV challenged embryos, we observed upregulation of innate immune genes such as interferons, cytokines, chemokines, besides cell proliferation, differentiation, transcription regulation, and development molecules, despite weak activation of the circadian pathway. Notably, expression patterns between ZT06 vs. ZT18 in white light +NDV challenged embryos showed photo-acceleration of the innate immune response by upregulating the T cell development, T cell homeostasis, and quantity of T lymphocytes which show features that put it at the boundary of innate and adaptive immunity. The suggestion of transition between innate to adaptive immunity seen in these experiments shows promise for accelerating and optimizing immune responses during development in chicken.

This study used a combination of careful circadian experiments, and RNAseq approaches to dissect the interaction of lighting during incubation, its effects on stimulating the circadian

system, and how this benefits immune responses to challenge. We show comprehensively that blue and white light during incubation clearly stimulates circadian and circadian-associated pathways. These translated into strong signals for both developmental acceleration (MAPK) and innate immune responses (NF-κB). Furthermore, while blue light stimulated RhoGDI signaling significantly, indicating the role of this lighting source in various cell proliferation and signaling processes, suggesting accelerated growth, we also noted that in the DEG data that blue light increased DNA damage repair processes. This particular effect needs to be further investigated to understand its relevance for application in poultry. In conclusion, the ability to use lighting during incubation to stimulate innate immune responses to challenge can be a potentially valuable and economical approach to improve the immune health of poultry.

In summary, experimental work in this study reveals several key conclusions:

(i) Blue light is a more significant stimulator of the circadian rhythm entrainment process than white light and dark treatment.

(ii) White light is more significant as a photo-accelerator for cell proliferation and embryonic development.

(iii) White light advanced the innate immune response to the *in ovo* NDV challenge by upregulating the T cell development pathway.

(iv) Embryos incubated under white light treatment hatched 6-8 h early compared to blue, whereas the embryos in the control (dark) group were slowest to hatch.

CHAPTER IV

CONCLUSIONS

Poultry production remains susceptible to significant infectious disease threats such as Avian Flu, and NDV, which threaten the supply of poultry production. Therefore, improving poultry immunity is essential both from the point of food security and human nutrition. This study addresses this challenge by leveraging avian circadian biology to improve poultry responses to vaccines and infection challenges. Recent research on studying the effects of *in ovo* lighting during incubation shows a feasible and economical approach for improving chick health, growth enhancement, and improved immune responses following vaccination. However, these studies used phenotypic or physiological markers such as melatonin secretion, specific antibody titer production, or circadian clock gene expression to evaluate circadian rhythms' development photo-biostimulation during embryogenesis. Functional genomics approaches like RNASeq provide a powerful tool to develop a holistic picture of the interplay between photobiostimulation, circadian rhythm development, and its impact on improving the overall chick's immune response to challenge.

In this study, we used the RNAseq approach to investigate the effects of photo-biostimulation with different light spectrum ranges (colors) on entraining the circadian rhythm, and in turn, stimulating innate immune response against NDV challenge in chick embryos.

In the second chapter, I showed that photo-biostimulation using monochromatic green light during incubation of chicks' embryos stimulated genes related to immune response and energy metabolism in the embryonic spleen tissue and varied in relation to the route of NDV vaccination, in ovo or post-hatch vaccination. The vaccination route had a dominant and profound effect on

gene expression in the post-hatch spleen, whereas the effect of green light was absent in the post-hatch comparisons. This finding is not surprising given that all birds were on the same lighting treatments after hatching. In addition, there was a minor but notable interaction between *in ovo* lighting and vaccination on early-life cell proliferation. The green light study shows that the effects of *in ovo* lighting dissipate if the same lighting is not maintained in the post-hatch environment.

The third chapter addressed an important question regarding the effect of providing photoperiods with different wavelengths on circadian rhythm development and its interplay with immune response following NDV challenge. The hypothesis was that providing blue or white photoperiods will entrain the circadian rhythm with different efficiencies, compared to the dark incubation. I tested whether these different efficiencies translated to differential immune response following *in ovo* NDV challenge. I showed that incubating chicken embryos under blue light was most efficient in entraining the circadian rhythm, compared to both the white light or dark treatment. Also, blue light showed a specific impact on skeletal muscle, satellite cell activation, stem cell development, regulation of striated muscle contraction, Glycerolipid metabolism, and development of neurons. The white light incubation led to a photo-acceleration stimulant effect property more significant than its circadian stimulating effect, where epidermal growth factor receptor signaling pathway, ErbB signaling pathway, MAPK signaling pathway, and Insulin signaling pathway were upregulated in white light non-challenged treatment.

The response to NDV challenging showed a distinct transcriptome profile between blue and white light photobiostimulation. The blue light incubated embryos showed a potent innate immune response, specifically targeting viral replication, clearly pointing to a specialized antiviral response. In contrast, the white light incubated embryos showed a much less immune response activity, but more pronounced cell proliferation and metabolic state, suggesting photo-acceleration

as the primary process. However, I also found that the photo-accelerated affected the immune response, particularly the T cell development, T cell homeostasis, and quantity of T lymphocytes which suggest a rapid or ongoing transition between innate and adaptive immunity. This observation paves the way to the putative photo-accelerated effect of providing white light during chicken egg incubation on organismal development and immune response.

At the organism level, it is noteworthy that unvaccinated white light incubated chicks hatched 6-8 h earlier compared to blue, whereas the dark incubated eggs were last to hatch. Furthermore, the down color of chicks in the blue treatment was discolored (more bleached), suggesting that the blue light interacted with melanocytes during embryogenesis. In contrast, the down color was standard yellow in the white treatment and control treatments. In general, the vaccinated groups in all treatments had delayed hatching, but the decreased hatchability was mitigated in the white light treatment showing a promise for application. These differences in hatchability and time to hatch are perhaps related to the energetics of mounting an immune response and need to be quantified more precisely.

In conclusion, this study is the first to generated high-resolution RNASeq evidence demonstrating the effect of lighting color background on the circadian rhythm development and modulation of the innate immune response of chicks' embryos challenged with NDV. Not only does this study provide a firm scientific foundation for the potential application of circadian biology in modulating poultry immune responses, but it also establishes a platform for optimizing response to vaccination against a range of infectious agents. For future studies, post-hatch research is vital to fully understand provided light regimen and disease susceptibility and compare the effect of light wavelength with the different eggshell pigments during incubation on circadian rhythm development and immune response.

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APPENDIX A

SUPPLENTARY TABLES

Table 9. Supplementary Table S1-Combined quality control summary of RNASeq Reads from all treatments.

Sample Name	**LNV**	**DNV**	**LPHV**	**DPHV**	**LIV**	**DIV**
Raw Reads Counts	39530298	30151163	36331931	29093066	38929069	37139134
M Seqs	39.5	30.1	36.4	29.1	38.9	37.2
Length	75 bp	75 bp	75 bp	75 bp	75 bp	75 bp
% Dups	42.325	38.15	42.025	39.375	36.7	36.725
% GC	45.75	45.5	44.75	45	43.5	43
% Failed	9	9	9	9	9	9
Trimmed Reads Counts	38359095	29164359	35386720	28376252	37891576	35776767
Trimmed Reads Counts M Seqs	38.3	29.2	35.4	28.4	37.9	35.8
%Trimmed Reads Counts M Seqs	97.01	96.62	97.37	97.48	97.38	96.33
% Trimmed	4.425	4.675	4.025	3.625	4	5.2
Length	74	74	74	74	74	74
% Dups	39.075	35.15	38.875	36.075	33.4	33.475
% GC	45.75	45.25	45	45	43.25	43
% Failed	9	9	9	9	9	9

Table 10. Supplementary Table S2- Summary of total RNASeq data mapping to the chicken genome (Galgal6.0).

RNASeq libraries	Raw Reads	Trimmed Reads	No. Uniquely Mapped reads (UMR)	UMR %	% unique mapped reads to a gene feature
LNV	39530298	38359095	35944945	0.937064469	56.65
DNV	30151163	29164359	27340602	0.937466241	57
LPHV	36331931	35386720	33187981	0.93786542	60.45
DPHV	29093066	28376252	26755751	0.942892352	61.18
LIV	38929069	37891576	36014850	0.950471155	56.61
DIV	37139134	35776767	33646885	0.940467455	55.91

Table 11. Supplementary Table S3- Combined HTSeq-count output stats showing unique mapped reads to a gene feature for all treatments.

High-Throughput Sequencing Data (HTSeq-count) output stats	LNV	DNV	LPHV	DPHV	LIV	DIV
Total counted reads	37568253	28624071	34945803	27823890	37425016	35379683
Reads not assigned to a gene feature	14256117	10722846	11641820	9406439	14398969	13446389
% Reads not assigned to a gene feature	37.95	37.46	33.31	33.81	38.47	38.01
Ambiguous reads assigned to more than one feature but not counted to any	405372	302958	420167	325593	429762	419302
%Ambiguous reads assigned to more than one feature but not counted to any	1.08	1.06	1.2	1.17	1.15	1.19
% Too low quality reads	0	0	0	0	0	0
% Reads not aligned to a gene feature	0	0	0	0	0	0
Reads assigned to more than one feature not unique	1623308	1283469	1757822	1068139	1410166	1732798
%Reads aligned to more than one feature not unique	4.32	4.48	5.03	3.84	3.77	4.9
unique mapped reads to a gene feature	21283456	16314798	21125994	17023719	21186119	19781194
% unique mapped reads to a gene feature	56.65	57	60.45	61.18	56.61	55.91

Table 12. Supplementary Table S4: EdgeR results for differential gene expression analysis, Ingenuity Pathway Analysis (IPA), and DAVID annotations top results.

Treatment	**LNV**	**LPHV**	**LIV**
Control	**DNV**	**DPHV**	**DIV**
DE UP	76	48	11
DE Down	141	68	51
Total DE	217	116	62
Total genes No	11530	10998	11879
% DE	1.88	1.05	0.52
	Top Canonical Pathway		
IPA Analysis Top Results	SPINK1 Pancreatic Cancer Pathway	LXR/RXR Activation	SPINK1 Pancreatic Cancer Pathway
	FXR/RXR Activation	FXR/RXR Activation	Embryonic Stem Cell Differentiation into Cardiac Lineages
	LXR/RXR Activation	SPINK1 Pancreatic Cancer Pathway	Acute Phase Response Signaling
	Acute Phase Response Signaling	Acute Phase Response Signaling	Extrinsic Prothrombin Activation Pathway
	Role of Tissue Factor in Cancer	Extrinsic Prothrombin Activation Pathway	Role of Tissue Factor in Cancer
	Upstream Regulators		
	Tcf 1/3/4	RBPJL	RBPJL
	NR4A1	HNF1A	PTF1A
	Hmgn3	FOXA2	F0XA2
	HNF1A	Tcf 1/3/4	NR5A2
	HNF4A	NR5A2	GATA2
	Molecular and Cellular Functions		
	Carbohydrate Metabolism	Protein Synthesis	Lipid Metabolism
	Cell Morphology	Lipid Metabolism	Small Molecule Biochemistry
	Lipid Metabolism	Molecular Transport	Vitamin and Mineral Metabolism
	Molecular Transport	Small Molecule Biochemistry	Molecular Transport
	Small Molecule Biochemistry	Cell-To-Cell Signaling and Interaction	Cell Death and Survival
	Physiological System Development and Function		
	Organ Morphology	Hematological System Development and Function	Digestive System Development and Function
	Organismal Development	Digestive System Development and Function	Nervous System Development and Function

Table 12 Continued

	Renal and Urological System Development and Function	Hepatic System Development and Function	Hematological System Development and Function
	Digestive System Development and Function	Organ Development	Embryonic Development
	Cardiovascular System Development and Function	Endocrine System Development and Function	Organismal Development
	Biological process		
DAVID Gene Ontology (GO) terms	GO:0031639~plasminogen activation	GO:0031639~plasminogen activation	GO:0016042~lipid catabolic process
	GO:0050714~positive regulation of protein secretion	GO:0034116~positive regulation of heterotypic cell-cell adhesion	GO:0006810~transport
	GO:0007160~cell-matrix adhesion	GO:0051258~protein polymerization	GO:0070374~positive regulation of ERK1 and ERK2 cascade
	GO:0045921~positive regulation of exocytosis	GO:0006810~transport	GO:0050829~defense response to Gram-negative bacterium
	GO:0034116~positive regulation of heterotypic cell-cell adhesion	GO:0009615~response to virus	GO:0031640~killing of cells of other organism
	Cellular Component		
	GO:0072562~blood microparticle	GO:0072562~blood microparticle	GO:0005576~extracellular region
	GO:0005577~fibrinogen complex	GO:0005615~extracellular space	GO:0005615~extracellular space
	GO:0070062~extracellular exosome	GO:0070062~extracellular exosome	GO:0072562~blood microparticle
	GO:0005615~extracellular space	GO:0005576~extracellular region	GO:0005577~fibrinogen complex
	GO:0031091~platelet alpha granule	GO:0060417~yolk	GO:0031091~platelet alpha granule
	Molecular Function		
	GO:0004181~metallocarboxypeptidase activity	GO:0004181~metallocarboxypeptidase activity	GO:0004181~metallocarboxypeptidase activity
	GO:0036094~small molecule binding	GO:0036094~small molecule binding	GO:0005504~fatty acid binding
	GO:0019825~oxygen binding	GO:0005179~hormone activity	
		GO:0005102~receptor binding	
		GO:0004252~serine-type endopeptidase activity	
	KEGG Pathways		
	gga00190:Oxidative phosphorylation	gga01100:Metabolic pathways	gga01100:Metabolic pathways
	gga04620:Toll-like receptor signaling pathway	gga03320:PPAR signaling pathway	
	gga03020:RNA polymerase		

Table 12 Continued

Treatment	`	**LIV**
Control	**DPHV**	**LPHV**
DE UP	3845	3730
DE Down	3231	3025
Total DE	7076	6755
Total genes No	12769	12769
% DE	55.42	52.9
	Top Canonical Pathway	
IPA Analysis Top Results	Protein Kinase A Signaling	Protein Kinase A Signaling
	Cardiac Hypertrophy Signaling (Enhanced)	B Cell Receptor Signaling
	Factors Promoting Cardiogenesis in Vertebrates	Cardiac Hypertrophy Signaling (Enhanced)
	SAPK/JNK Signaling	Superpathway of Inositol Phosphate Compounds
	Synaptogenesis Signaling Pathway	SAPK/JNK Signaling
	Upstream Regulators	
	ESR1	TP53
	DAP3	HNF4A
	MIR17HG	ESR1
	ERG	camptothecin
	TCF7L2	MYC
	Molecular and Cellular Functions	
	Cellular Assembly and Organization	Cellular Assembly and Organization
	Cellular Function and Maintenance	Cellular Function and Maintenance
	Cell Morphology	Cell Death and Survival
	Cellular Development	Cell Cycle
	Cellular Growth and Proliferation	Gene Expression
	Physiological System Development and Function	
	Organismal Survival	Organismal Survival
	Nervous System Development and Function	Organismal Development
	Tissue Development	Embryonic Development
	Organismal Development	Cardiovascular System Development and Function
	Cardiovascular System Development and Function	Nervous System Development and Function

Table 12 Continued

DAVID Gene Ontology (GO) terms	**Biological process**	
	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	GO:0007018~microtubule-based movement
	GO:0035023~regulation of Rho protein signal transduction	GO:0016477~cell migration
	GO:0007507~heart development	GO:0045944~positive regulation of transcription from RNA polymerase II promoter
	GO:0019933~cAMP-mediated signaling	GO:0030705~cytoskeleton-dependent intracellular transport
	GO:0030705~cytoskeleton-dependent intracellular transport	GO:0035023~regulation of Rho protein signal transduction
	Cellular Component	
	GO:0005654~nucleoplasm	GO:0005654~nucleoplasm
	GO:0005871~kinesin complex	GO:0005871~kinesin complex
	GO:0005634~nucleus	GO:0005737~cytoplasm
	GO:0014069~postsynaptic density	GO:0015629~actin cytoskeleton
	GO:0030027~lamellipodium	GO:0005815~microtubule organizing center
	Molecular Function	
	GO:0046872~metal ion binding	GO:0008270~zinc ion binding
	GO:0008270~zinc ion binding	GO:0005524~ATP binding
	GO:0005524~ATP binding	GO:0046872~metal ion binding
	GO:0003682~chromatin binding	GO:0032454~histone demethylase activity (H3-K9 specific)
	GO:0005089~Rho guanyl-nucleotide exchange factor activity	GO:0016887~ATPase activity
	KEGG Pathways	
	gga04010:MAPK signaling pathway	gga03460:Fanconi anemia pathway
	gga04931:Insulin resistance	gga04010:MAPK signaling pathway
	gga04810:Regulation of actin cytoskeleton	gga04144:Endocytosis
	gga04520:Adherens junction	gga04070:Phosphatidylinositol signaling system
	gga04012:ErbB signaling pathway	gga04520:Adherens junction

Table 13. Supplementary Table S5- Gene networks from the 395 differentially expressed genes for Monochromatic Green Light Biostimulation groups converted to human orthologous genes.

ID	comparison	Molecules in Network	Score	FM	Top Functions
I	LNV vs DNV	*Akt, APOB, APOC3, C1QTNF6, CD151, CXCL14, Cyclin D, DHTKD1, ENPP2, GC, GOLPH3L, Growth hormone, HADH, HDL, hemoglobin, HMG CoA synthase, INS, INSIG1, LAPTM4B, LDL-cholesterol, MAGI2, mir-451, N-cor, Pdi, PDIA2, PID1, PRKAA, SEMA3D, SIVA1, SYTL1, SYTL4, thyroid hormone receptor, Tnf receptor, Ubiquitin, VLDL*	38	21	Cell Morphology, Digestive System Development and Function, Organ Morphology
I	LPHV vs DPHV	*2210010C04Rik, AHSG, AMBP, APOH, carboxypeptidase, CELA1, CELA2A, chymotrypsin, CLPS, CPA1, CPA2, CPA5, CPB1, CRABP1, CTRB2, CTRC, CTRL, elastase, ERK1/2, FGA, Fibrin, GPIIB-IIIA, Hnf3, HS6ST2, Rbp, RBP4, RBPJL, SERPINC1, SFTPA1, SFTPA2, Stat3-Stat3, Tcf 1/3/4, trypsin, Trypsinogen, TTR*	51	23	Carbohydrate Metabolism, Lipid Metabolism, Protein Synthesis
I	LIV vs DIV	*ADCY, AMY2A, CAMP, carboxypeptidase, CEL, CELA1, chymotrypsin, CPA1, CPA2, CPA5, CPB1, CRABP1, CTRB2, CTRC, CTRL, DMBT1, EN1, ERK1/2, FGB, FGF13, FGG, FOXA2, Gcg, Growth hormone, GSTM3, HDL, HS6ST2, ISL1, PDIA2, PNLIPRP1, Proinsulin, PTPRN2, RBPJL, SST, Trypsinogen*	75	27	Developmental Disorder, Hematological Disease, Hereditary Disorder
II	LNV vs DNV	*Cbp/p300, CD24, CDK8, CNTNAP1, COL9A1, CYBC1, DPY30, EEF1A1, estrogen receptor, GSE1, GTF2A2, Hdac, HDAC1, Histone h3, Histone h4, Holo RNA polymerase II, Hsp70, Hsp90, JUP, KDM2B, PCNA, Pka catalytic subunit, Pkc(s), POLR1D, POLR2D, POLR2F, RBM8A, RNA polymerase II, Rnr, RPF1, SCAMP3, Secretase gamma, SLC12A5, TOR1AIP1, Wnt*	38	21	Connective Tissue Development and Function, DNA Replication, Recombination, and Repair, Gene Expression
II	LPHV vs DPHV	*26s Proteasome, Actin, ADH1C, ALDOB, AMY2A, BAG3, C/EBP, caspase, CD34, CEL, CPNE4, DMBT1, DNASE1, F Actin, Gcg, Histone h3, HPD, Hsp70, Hsp90, Immunoglobulin, Insulin, mir-25, NFkB (complex), p70 S6k, Proinsulin, PXR ligand-PXR-Retinoic acid-RXRα, RBMX, RNA polymerase II, SPINK4, TCIRG1, Tgf beta, THRSP, TP63, Ubiquitin, UGT1A1*	37	18	Cellular Compromise, Hematological Disease, Immunological Disease

Table 13 Continued

II	LIV vs DIV	*acetic acid, Akt,* ***ALB****, asparagine, Atg5,* ***CCKAR****,* ***CCL17****,* ***CLPS****,* ***CPNE4****, Creb, ERK,* ***GATA4****, GFRA4,* ***GJA5****, Histone h3, IL12 (complex), indican, Insulin, Jnk, L-isoleucine, L-leucine, Ldh (complex), LOC100359583/Ptma, Mapk, NFkB (complex), P38 MAPK, PI3K (complex), Pka,* ***PLA2G1B****, RNA polymerase II,* ***SLC1A3****,* ***SLC9A8****,* ***SPINK4****, valine, Vegf*	16	8	Amino Acid Metabolism, Increased Levels of Albumin, Molecular Transport
III	LNV vs DNV	***AHSG****,* ***AMBP****,* ***APOH****, carboxypeptidase,* ***CELA2A****, chymotrypsin, CK1,* ***CLPS****, Collagen type IV, Collagen(s),* ***CPA1****,* ***CPA5****,* ***CPB1****,* ***CPD****,* ***CTRB2****,* ***CTRC****,* ***CTRL****, elastase, ERK1/2,* ***FBLN1****,* ***FGA****,* ***FGB****,* ***FGG****, Fibrin, Fibrinogen, GPIIB-IIIA, Hnf3,* ***HS6ST2****,* ***SFTPA1****,* ***SPINK5****, Stat3-Stat3, Tcf 1/3/4, trypsin, Trypsinogen,* ***TTR***	36	20	Developmental Disorder, Hematological Disease, Hereditary Disorder
III	LPHV vs DPHV	*Akt,* ***ALB****, AMPK, Ap2,* ***APOA4****,* ***APOB****,* ***APOC3****,* ***FABP1****, FGB, FGG, Fibrinogen, GC, Glycogen synthase, GOT, Growth hormone, HDL, HDL-cholesterol,* ***HSP90B1****, IgG, IL12 (complex),* ***INS****, Ldh (complex), LDL, LDL-cholesterol, MHC Class II (complex), ORM1, Pdi,* ***PDIA2****,* ***PLA2G1B****,* ***SCD****,* ***SLCO1A2****, STAT5a/b, VLDL, VLDL-cholesterol,* ***VTN***	32	16	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
III	LIV Vs DIV	***ADPGK****,* ***ALG9****,* ***ANKLE2****, ARHGAP1, B4GALNT1,* ***C4orf19****,* ***CANX****,* ***CCPG1****, CNTNAP3B,* ***DIPK1A****, DNASE1,* ***FKBP14****,* ***FUT10****,* ***FUT9****,* ***GAS6****,* ***GNL3****,* ***HAL****,* ***HS6ST1****,* ***ICMT****,* ***ITPRIP****,* ***LMF1****,* ***NETO2****, NPTX1, NUP155, PLXNA2, PLXNB2,* ***SEL1L****,* ***SETX****,* ***SLC12A2****,* ***STK24****,* ***TMED6****,* ***TMEM214****,* ***TRIM25****,* ***VEZT****,* ***WLS***	7	4	Cell-To-Cell Signaling and Interaction, Cellular Development, Cellular Growth and Proliferation
IV	LNV vs DNV	*20s proteasome, 26s Proteasome, calpain, caspase,* ***CD34****, Cdk,* ***CEL****,* ***CHGA****, Ctbp, Cyclin A, Cyclin E, Cytochrome bc1,* ***DZIP1****,* ***EHD4****, ERK,* ***FABP3****,* ***HBA1/HBA2****,* ***HEXA****, Hsp27,* ***LSM5****, Mitochondrial complex 1, NADH dehydrogenase,* ***NDUFA5****,* ***NDUFB5****,* ***NDUFS6****,* ***NFE2L1****,* ***PARL****,* ***POLH****, PP2A,* ***PPP1R17****, Rb,* ***RFC2****, RPA,* ***SFTPC****,* ***UQCRFS1***	33	19	Cell Signaling, Post-Translational Modification, Protein Synthesis
IV	LPHV vs DPHV	*1-O-hexadecyl-2-N-methylcarbamol-sn-glycerol-3-phosphocholine,* ***ACOX1****,* ***AKT1****,* ***AQP12A/AQP12B****,* ***ATP5F1A****, Ca2 , CCKAR, CCKBR,* ***CEL****, cholesterol,* ***CLPS****,* ***FUT9****,* ***GATM****, GHSR, GLP1R,* ***GPR157****,* ***GUCY1A2****, HNF4A,* ***HPD****, INHBE, LHPP, LHX1, mir-802, P2RY14, PNLIP,* ***PNLIPRP1****,* ***PPP1R17****,* ***PTEN****,* ***SPP2****, SSTR1, SSTR4, TACR2,* ***TM4SF4****, triacylglycerol lipase,* ***UFC1***	27	14	Lipid Metabolism, Nutritional Disease, Psychological Disorders

Table 13 Continued

IV	LIV vs DIV	***GORASP1***, ***GORASP2***, ***Tmed11***	2	1	Cellular Assembly and Organization, Cellular Compromise, Cellular Function and Maintenance
V	LNV vs DNV	***AAMDC***, ***AHSG***, ***APP***, ***C16orf70***, ***CCDC60***, *COL17A1*, ***CXCL14***, ***DIXDC1***, ***EGFR***, ***GNG5***, ***HRAS***, ***ITM2C***, ***LAD1***, *LRP10*, ***LRRIQ1***, ***LXN***, ***MGAT3***, ***MLX***, ***MPP7***, ***MPZL2***, *NDP*, *peptidase*, ***PIK3R5***, *plasminogen activator*, ***RSRP1***, ***SIVA1***, *SLC30A3*, *SYT17*, ***TMEM267***, *TMEM59*, ***TOPAZ1***, ***TRAPPC4***, ***TRPT1***, ***TSPAN12***, ***ZDHHC20***	31	18	Cancer, Neurological Disease, Organismal Injury and Abnormalities
V	LPHV vs DPHV	***ADAM10***, ***ADAMTS3***, ***AGGF1***, *ARFRP1*, ***B3GNT7***, ***C4BPA***, *CDKN2D*, ***CFD***, *CRNDE*, ***CTNNB1***, ***DIP2C***, ***EN1***, *EVX1*, ***F13A1***, ***GORASP2***, ***HHEX***, ***IL10RA***, ***MGAT3***, ***NIPBL***, ***NUP98***, *OVOL2*, ***PCSK7***, ***PDPR***, ***PRCC***, ***PSPH***, ***RAB18***, ***RNPEP***, *Saa3*, *SPON1*, ***SYCN***, ***TGFB1***, ***Tmed11***, ***TRIM25***, ***TSPAN15***, ***UBAP2***	22	12	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function
V	LIV vs DIV	*10E, 12Z-octadecadienoic acid*, ***B3GNT7***, *beta-estradiol*, ***IL10RA***, *POU5F1*, *TFCP2L1*, *TUFM*	2	1	Embryonic Development, Organ Development, Organismal Development
VI	LNV vs DNV	***ADK***, *AGR2*, ***BAG3***, ***BCDIN3D***, ***CRLS1***, ***ELAVL1***, ***FAM49B***, ***FANCD2***, ***GLIPR1***, ***GNPNAT1***, ***GPRIN3***, ***GPX8***, ***HSPD1***, ***ISOC1***, *MPEG1*, ***MRPL44***, ***MRPL46***, ***MYC***, ***MYCT1***, ***NNT***, ***NSFL1C***, ***PPA2***, *PPI*, ***PPIL3***, ***Rpl23a***, *Rpl29 (includes others)*, *Rpl32*, *Rplp1 (includes others)*, *Rps27/Rps27rt*, ***SPCS3***, ***SRD5A3***, ***TMEM123***, ***TMEM177***, ***XPNPEP3***, ***YRDC***	26	16	Cancer, Organismal Injury and Abnormalities, Respiratory Disease
VI	LPHV vs DPHV	*1-O-hexadecyl-2-N-methylcarbamol-sn-glycerol-3-phosphocholine*, *ADCY*, *Ap1*, *Ck2*, ***CNN2***, *Collagen type IV*, *Creb*, *cytokine*, *ERK*, *GHRHR*, ***GIMAP8***, *GMP*, *Ige*, *IL1*, *Integrin*, *Jnk*, *Mapk*, *N-arachidonoyl-dopamine*, *Neurotrophin*, *P38 MAPK*, *PI3K (complex)*, *Pka*, *Pkc(s)*, *PLC*, ***PSCA***, *Rac*, *Sct*, *SRC (family)*, ***SST***, ***SSTR3***, *SSTR4*, ***TAC1***, *TCR*, *Vegf*, *voltage-gated calcium channel*	9	6	Cell Signaling, Nucleic Acid Metabolism, Small Molecule Biochemistry
VI	LIV vs DIV	***CFTR***, ***DYNC1I2***, *GDI1*, *HNRNPL*, *IDI2*, ***RABL2B***, ***RASEF***	2	1	Developmental Disorder, Hereditary Disorder, Neurological Disease

Table 13 Continued

VIII	LNV vs DNV	*ABTB2, ATP5F1C, ATP5PD, ATRN, CNOT11, CRYBG3, DCP1B, DHTKD1, GDPD2, GDPD4, H2AC4, KRT8, MAP4K4, MIGA1, NANOS2, NOL9, NUDT19, PDF, PEX16, PEX3, RAI1, SNRK, SRRD, TEX2, TOMM20, TOMM22, TOMM40L, TOMM7, TRAK1, TUBGCP6, TXNL4A, VIRMA, VPS13D, XPO1, ZNF704*	24	15	Cellular Assembly and Organization, Cellular Function and Maintenance, Protein Trafficking
VII	LNV vs DNV	*ADRB, ALDOB, Calmodulin, CG, Cox7c, cytokine, FH, FSH, GNLY, GNPNAT1, Gsk3, IL1, Immunoglobulin, Insulin, Interferon alpha, KLHDC4, Lh, Mapk, MTORC1, NFKBIA, P38 MAPK, PIK3CG, Pka, PKDCC, PLC, PPP3R1, Proinsulin, RAS, Ras homolog, RIMBP2, RNF130, RPL18A, SST, TCR, Vegf*	22	14	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function
IX	LNV vs DNV	*43S Translation Preinitiation, ATOH1, BRI3, CDH24, CER1, CERKL, Ck2, COL4A6, CONNEXIN, COPS9, CTNNB1, ECRG4, EIF3I, Groucho, Hsd3b4 (includes others), LHX1, MALAT1, miR-483-5p (miRNAs w/seed AGACGGG), MYO18A, NCL, NEDD8, PDAP1, Pgk, POFUT1, PRICKLE2, PTCH2, REXO5, SP6, SPP2, TANC1, TUBB2B, UFC1, UFM1, VHL, WNT9B*	20	13	Cellular Development, Embryonic Development, Organismal Development
X	LNV vs DNV	*Ap1, BCL11B, BHLHE40, C/EBP, Calcineurin protein(s), CSF1, G0S2, GOT, GPX1, Gsta1, Ifn, IFN Beta, Iga, IgG, IgG1, Igm, IL12 (complex), IL12 (family), IRAK, IRAK4, Ldh (complex), LDL, MAP2K1/2, MAP3K13, MAPK12, MEOX1, MHC Class II (complex), MMRN1, NFkB (complex), peptidase, PLC gamma, Rxr, Tlr, Tnf (family), TOLLIP*	18	12	Cardiovascular Disease, Organismal Injury and Abnormalities, Protein Synthesis
XI	LNV vs DNV	*ABHD2, ABHD6, acylglycerol lipase, ADGRG2, ALG3, ATP2A3, CDH15, CLCA2, CLDN11, COL12A1, COL9A2, ESR1, FETUB, FSHR, GPR108, HOXB1, HOXB3, HOXB6, LMNA, MBOAT2, MMP15, NDC1, PNLIPRP2, RBMS3, S1PR4, SLC13A3, ST7L, TCTN3, TGFB1, TLCD1, TMEM203, TREML2, tretinoin, TWIST, UPK3BL1*	16	11	Cellular Growth and Proliferation, Organ Development, Skeletal and Muscular System Development and Function
XII	LNV vs DNV	*ACTG2, Actin, ALB, Alp, Alpha catenin, atypical protein kinase C, CaMKII, CD3, Collagen type I (complex), Creb, cytochrome C, DNASE1, F Actin, Fcer1, G-Actin, HBE1, HSPB7, Ige, Integrin, ITGB5, Jnk, NME3, p85 (pik3r), Pdgf (complex), PDGF BB, PFN2, PI3K (complex), PI3K p85, Pkg, Rac, Rap1, Rock, SRC (family), STAT5a/b, Tgf beta*	11	8	Cardiovascular System Development and Function, Organ Development, Organ Morphology
XIII	LNV vs DNV	*C11orf88, TLR7*	2	1	Cancer, Humoral Immune Response, Organismal Injury and Abnormalities

Red= Upregulated; **Green**=Downregulated , FM= Focus Molecules

Table 14. Supplementary table S6-Gene networks from the 327 differentially expressed genes for Unique interaction of *in ovo* Vaccination and Green Monochromatic Light Biostimulation (LIV vs. LPHV) converted to human orthologous genes.

Network No.	Molecules in Network	Score	FM	Top Functions
I	*ALDOB, Alpha tubulin, BETA TUBULIN, BICD1, C20orf27, CADPS2, CENPJ, COMMD4, DCTN1, DPP9, Dynein, E2F8, EIF4ENIF1, ENTR1, EP400, EPB41, ERK, GOLGA1, HUS1, IFFO2, MAP4, MRPS12, MYCBP2, NIN, NUP155, PLEKHO2, PRICKLE2, PSMA5, RAB2B, RAB6A, TNRC6C, TSPOAP1, TTF2, TUBB, tubulin*	57	30	Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Development
II	*26s Proteasome, Actin, Adaptor protein 2, ARHGEF16, ATG9A, BAAT, BABAM1, BRCA1, Calmodulin, CHRNB2, Clathrin, COPB1, EXO1, Hdac, Histone h3, HNRNPM, HSPA8, KIF21A, MCC, NDUFAF3, NDUFS5, POLR3E, RAS, RNF169, SCAF4, SLC38A10, SMC4, SMOC2, TBX3, TCF20, UBN1, Vegf, WBP1, WWC1, WWOX*	46	26	Cell Morphology, DNA Replication, Recombination, and Repair, Embryonic Development
III	*ADAMTS3, ANKS1B, ASAP1, ASAP3, c-Src, CFD, CHKA, collagen, Collagen type I (complex), Collagen(s), CTSH, DAB1, Eotaxin, ERK1/2, FERMT2, Integrin, Integrin alpha 3 beta 1, ITGB1BP2, ITGB3, LAMA3, LAMA4, Laminin (complex), Laminin (family), MAFA, Ncoa6, PACSIN2, PP2A, Proinsulin, RAPGEF3, SGPP1, SLC12A2, Sos, TNMD, TPP1, TRPM2*	36	22	Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Movement]
IV	*AMDHD2, ANKRD26, APBB2, ATP8A1, ATP8B1, ATP9A, CCDC142, CLCN4, FRYL, GNPTAB, HEATR5A, HEG1, ITPKB, MIGA1, MITF, NCAPD3, NCR3LG1, PDE4D, PDZD2, PLBD2, SSR3, STT3A, SVEP1, SYDE2, TEX2, TMEM30A, USP24, USP31, USP34, USP36, USP42, USP54, VIRMA, ZBED4, ZNF555*	23	16	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
V	*AAMDC, ACAP3, ANXA8/ANXA8L1, APP, ARHGAP27, ARL5A, ARL8A, ARMC9, AUP1, COL4A6, CRELD1, CYP26B1, DIP2B, EIF6, ESR2, Evi5l, FAM126B, GTPase, HIGD2A, KIF3A, ODAM, OSBPL11, Plpp1, PLPP2, PRDM15, PRUNE2, RASAL1, RBX1, SHROOM4, SLC4A1AP, TENT2, TIMM23B, TMEM126B, UHRF1BP1L, WWC3*	21	15	Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization
VI	*ACACA, ADGRL3, beta-estradiol, CNTNAP3B, DHDH, EIF2S1, FAM216A, GP2, GPRC6A, GRM2, GRM3, HARS1, HEPH, HHIPL1, IL13, KNDC1, MMP26, MON1A, PITPNM2, PTPN5, PTPRK, RAB33A, RBMS1, RTN4, SGCB, SGCZ, SHISA4, SLC26A4, Sprr2a1/Sprr2a2, TJP3, TSC22D1, UQCC3, VPS37B, WDFY3, XPO1*	20	14	Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Function and Maintenance

	Table 14 Continued			
VII	*ADRB2, BTBD10, BTBD6, BTBD7, C1QBP, CCDC158, CFAP300, CG, CLGN, CUL3, DLG5, EPAS1, ERK1/2, F2RL3, FMC1, FOS, GPR17, Gucy1b2, HCAR1, HSPD1, KLHL6, MARS2, MRPL4, MTNR1B, NUDT8, PCDH11X, PKIB, Ppp1r12a, PTGER1, RAMP3, SCUBE1, sGC, SLC16A4, SNAPC5, STARD3*	20	14	Cell Death and Survival, Inflammatory Disease, Nervous System Development and Function
VIII	*Alp, AMMECR1, ATXN7, BASP1, Cbp/p300, Cyclin E, DENND3, E2f, Focal adhesion kinase, Gstt3, H4C11, Histone h4, IFN Beta, Igm, IL12 (complex), LDL, LRP6, LTBP2, Mlc, MTORC1, NEGR1, p85 (pik3r), PDGF BB, Pdgfr, PI3K (complex), POLR3D, RAD23A, Rb, RORC, Sfk, Smad2/3, STAT5a/b, TCIRG1, Tgf beta, Ubiquitin*	18	13	Cell-mediated Immune Response, Lymphoid Tissue Structure and Development
IX	*ABCF3, BAG3, BOLA2/BOLA2B, CGNL1, COX15, CRIP2, DNAJC6, EED, EFTUD2, ERLEC1, GALK1, HSPA5, IQCC, LAMB4, LAMTOR1, MAT2B, METTL21A, MOGS, NAXE, NHP2, PIGV, PLEC, PPP2R2D, RAPSN, RNPEP, RTL9, SRPX, TCF15, TRABD2B, TTC27, TTC32, TUG1, WNT3A, WNT9B, ZNF414*	18	13	Inflammatory Disease, Inflammatory Response, Connective Tissue Disorders
X	*ABCB8, ADGRA1, ARHGEF26, CACNA1A, Ces1e, DEDD2, Dlg, DLG1, FIG4, GIPC2, GPR17, GRIN2D, GRM2, GRM3, GUCY2C, HAGHL, HNF4A, HNF4α dimer, Insulin, IPO13, MIS18BP1, NAA38, OR2L13, Pka, PLC, Ras homolog, RBFOX2, receptor protein tyrosine kinase, SLC37A4, Slco1a4, SRC (family), UNC13C, VN1R1, ZAN, ZP3*	14	11	Cell Morphology, Cell-To-Cell Signaling and Interaction, Cellular Function and Maintenance

FM= Focus molecules, Red= Upregulated; **Green**=Downregulated

Table 15. Supplementary table S7-EdgeR results for differential gene expression analysis, Ingenuity Pathway Analysis (IPA), and DAVID annotations top results for the impact of light treatments on the expression pattern in non-challenged treatments controlling to the dark group during daytime**.**

Treatment	**Blue Non-Challenged at ZT06**	**White Non-Challenged at ZT06**	**Blue Non-Challenged at ZT06**
Control	**Dark Non Challenged at ZT06**	**Dark Non Challenged at ZT06**	**White Non Challenged at ZT06**
DE UP	**685**	**286**	**105**
DE Down	**352**	**117**	**88**
DE	**1037**	**403**	**193**
Non-DE	**16078**	**16712**	**16922**
DE %	**6.06**	**2.36**	**1.13**
IPA Analysis Top Results			
Top Canonical Pathway based on -log(p-value)			
Epithelial Adherens Junction Signaling		Glycogen Degradation II	Glutamate Removal from Folates
Gap Junction Signaling		Wnt/β-catenin Signaling	p53 Signaling
Axonal Guidance Signaling		Glycogen Degradation III	Agranulocyte Adhesion and Diapedesis
Ephrin B Signaling		Glutamate Removal from Folates	Cellular Effects of Sildenafil (Viagra)
Estrogen Receptor Signaling		PTEN Signaling	Lysine Degradation II
Top Canonical Pathway based on positive z Score (Upregulated)			
CREB Signaling in Neurons		Synaptogenesis Signaling Pathway	
Synaptic Long Term Potentiation		Mouse Embryonic Stem Cell Pluripotency	
Thrombin Signaling		Protein Kinase A Signaling	
Neuropathic Pain Signaling In Dorsal Horn Neurons		Colorectal Cancer Metastasis Signaling	
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells		Natural Killer Cell Signaling	
Top Canonical Pathway based on negative z Score (Downregulated)			
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell		PTEN Signaling	
RhoGDI Signaling		B Cell Receptor Signaling	
Actin Cytoskeleton Signaling			
p53 Signaling			
LPS/IL-1 Mediated Inhibition of RXR Function			
Upstream Regulators			
ESR1		MIR17-92	LMNA
TARBP2		levodopa	IL11RA
SATB1		MMP3	TREM1

Table 15 Continued

beta-estradiol	DVL3	XRCC5
ULBP1	Sos	Klra7 (includes others)
Molecular and Cellular Functions		
Cellular Movement	Cell Cycle	Cell-To-Cell Signaling and Interaction
Cellular Function and Maintenance	Cell Morphology	Cellular Movement
Cell Morphology	Cellular Compromise	Cell Death and Survival
Cellular Assembly and Organization	Cellular Function and Maintenance	Cell Signaling
Cell Cycle	Cellular Movement	Vitamin and Mineral Metabolism
Physiological System Development and Function		
Nervous System Development and Function	Embryonic Development	Connective Tissue Development and Function
Organismal Development	Renal and Urological System Development and Function	Nervous System Development and Function
Embryonic Development	Nervous System Development and Function	Hematological System Development and Function
Organ Morphology	Organismal Development	Immune Cell Trafficking
Digestive System Development and Function	Immune Cell Trafficking	Cell-mediated Immune Response
Top Pathways		
Circadian	MAPK	P53
P53	Rho-GTPase Signaling	Cancer
RhoGDI Signaling	P53	Circadian
	NF-B Signaling	NF-kB Signaling
	RhoGDI Signaling	Mitochondrial Dysfunction
DAVID Gene Ontology (GO) terms		
Biological Process		
calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules, regulation of Rho protein signal transduction, cardiovascular system development	microtubule cytoskeleton organization	somitogenesis
skeletal muscle contraction	epidermal growth factor receptor signaling pathway	regulation of axon extension involved in axon guidance
epidermal growth factor receptor signaling pathway	pericardium morphogenesis	exocytosis
positive regulation of fat cell differentiation	integrin-mediated signaling pathway	semaphorin-plexin signaling pathway involved in axon guidance
single organismal cell-cell adhesion, endocytosis, angiogenesis	vasculogenesis	branchiomotor neuron axon guidance

Table 15 Continued

Cellular Component		
integral component of membrane	perinuclear region of cytoplasm	semaphorin receptor complex
extracellular exosome	neuromuscular junction	integral component of membrane
cell surface	chromatin	myosin complex
receptor complex	early endosome	plasma membrane
troponin complex	perinuclear region of cytoplasm	
Molecular Function		
ephrin receptor binding	SMAD binding	motor activity
calcium ion binding	nucleic acid binding	semaphorin receptor activity
protein serine/threonine kinase activity	receptor signaling protein serine/threonine kinase activity	vasoactive intestinal polypeptide receptor activity
transporter activity	potassium ion symporter activity	GO:0030246~carbohydrate binding
	potassium:chloride symporter activity	
KEGG Pathways		
Glycerolipid metabolism	Regulation of actin cytoskeleton	Lysosome
Biosynthesis of amino acids	ErbB signaling pathway	
	Glycerolipid metabolism	
	Focal adhesion	
Up Keywords		
Muscle protein	Ribosomal protein	ANK repeat
Transferase	Ribonucleoprotein	Lipid transport
Motor protein	Differentiation	Transmembrane helix, Transmembrane
Disulfide bond	Cytoskeleton	Membrane
	Coiled coil	Myosin

Table 16. Supplementary table S8 EdgeR results for differential gene expression analysis, Ingenuity Pathway Analysis (IPA), and DAVID annotations top results for the impact of light treatments on the expression pattern in challenged treatments controlling to the dark group during daytime.

Treat	**Dark Challenged at ZT06**	**Blue Challenged at ZT06**	**White Challenged at ZT06**	**Blue Challenged at ZT06**
Ctrl	**Dark Non Challenged at ZT06**	**Dark Challenged at ZT06**	**Dark Challenged at ZT06**	**White Challenged at ZT06**
DE ↑	258	351	771	281
DE ↓	240	179	586	303
DE	498	530	1357	584
Non-DE	16617	16585	15758	16531
DE %	2.91	3.1	7.93	3.41
IPA Analysis Top Results				
Top Canonical Pathway based on -log(p-value)				
Actin Cytoskeleton Signaling		IGF-1 Signaling	Nucleotide Excision Repair Pathway	Kinetochore Metaphase Signaling Pathway
Gα12/13 Signaling		Role of RIG1-like Receptors in Antiviral Innate Immunity	CNTF Signaling	Interferon Signaling
Hepatic Fibrosis / Hepatic Stellate Cell Activation		Insulin Secretion Signaling Pathway	CMP-N-acetylneuraminate Biosynthesis I (Eukaryotes)	Coronavirus Replication Pathway
Epithelial Adherens Junction Signaling		p70S6K Signaling	dTMP De Novo Biosynthesis	Activation of IRF by Cytosolic Pattern Recognition Receptors
Calcium Signaling		Systemic Lupus Erythematosus In B Cell Signaling Pathway	Assembly of RNA Polymerase II Complex	Senescence Pathway
Top Canonical Pathway based on positive z Score (Upregulated)				
Role of NFAT in Regulation of the Immune Response		Opioid Signaling Pathway	UVB-Induced MAPK Signaling	Protein Kinase A Signaling
Systemic Lupus Erythematosus In B Cell Signaling Pathway		Synaptic Long Term Depression	EGF Signaling	Coronavirus Replication Pathway
Estrogen Receptor Signaling		Protein Kinase A Signaling	Huntington's Disease Signaling	Kinetochore Metaphase Signaling Pathway
Synaptogenesis Signaling Pathway		Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	PPARα/RXRα Activation	Pyridoxal 5'-phosphate Salvage Pathway
Protein Kinase A Signaling		Synaptic Long Term Potentiation	Sirtuin Signaling Pathway	Salvage Pathways of Pyrimidine Ribonucleotides

Table 16 Continued

Top Canonical Pathway based on negative z Score (Downregulated)			
Nicotine Degradation III	Role of RIG1-like Receptors in Antiviral Innate Immunity	Assembly of RNA Polymerase II Complex	Role of PKR in Interferon Induction and Antiviral Response
Nicotine Degradation II	Activation of IRF by Cytosolic Pattern Recognition Receptors	NER Pathway	Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza
Wnt/β-catenin Signaling	JAK/Stat Signaling	Cyclins and Cell Cycle Regulation	Interferon Signaling
PI3K Signaling in B Lymphocytes	Systemic Lupus Erythematosus In B Cell Signaling Pathway	TGF-β Signaling	Activation of IRF by Cytosolic Pattern Recognition Receptors
Sirtuin Signaling Pathway	TGF-β Signaling	Cell Cycle Control of Chromosomal Replication	Spliceosomal Cycle
Upstream Regulators			
IRF7	IRF7	HNF4A	IRF7
IRF3	TRIM24	ESR1	PNPT1
PRL	PNPT1	ONECUT1	IFNL1
Interferon alpha	DUSP11	miR-1-3p (and other miRNAs w/seed GGAAUGU)	IRF3
IRGM	PRL	"2-(4-amino-1-isopropyl-1H-pyrazolo	
Molecular and Cellular Functions			
Cellular Function and Maintenance	Cell Death and Survival	Gene Expression	Gene Expression
Cellular Assembly and Organization	Cellular Assembly and Organization	Cell Death and Survival	DNA Replication, Recombination, and Repair
Cellular Movement	Cellular Function and Maintenance	Cellular Assembly and Organization	Molecular Transport
Protein Synthesis	Molecular Transport	Cellular Function and Maintenance	Protein Trafficking
Cell Signaling	Cell Signaling	DNA Replication, Recombination, and Repair	Cell Death and Survival
Physiological System Development and Function			
Skeletal and Muscular System Development and Function	Nervous System Development and Function	Organismal Survival	Tissue Morphology
Tissue Development	Organismal Development	Nervous System Development and Function	Embryonic Development
Organismal Survival	Tissue Development	Organismal Development	Organismal Development

Table 16 Continued

Organismal Development	Embryonic Development	Tissue Development	Nervous System Development and Function
Humoral Immune Response	Organ Development	Embryonic Development	Tissue Development
Top Pathways			
NF-κB Signaling	RhoGDI Signaling	Mitochondrial Dysfunction	NF-kB Signaling
MAPK	Rho-GTPase Signaling	Estrogen	Cancer
	MAPK	MAPK	Mitochondrial Dysfunction
	Estrogen, Mitochondrial Dysfunction	Rho-GTPase Signaling	P53
	P53, NF-κB Signaling, and Circadian	P53	Circadian
DAVID Gene Ontology (GO) terms			
Biological Process			
muscle contraction	defense response to virus	enteric nervous system development	innate immune response
positive regulation of NF-kappaB import into nucleus	"protein ubiquitination involved in		
ubiquitin-dependent protein catabolic process"	response to toxic substance	defense response to virus	
defense response to virus	mitotic sister chromatid cohesion	neural crest cell migration	positive regulation of epithelial to mesenchymal transition
intracellular protein transport	cellular response to insulin stimulus	cellular response to DNA damage stimulus	negative regulation of viral genome replication
Cellular Component			
apical plasma membrane	microtubule cytoskeleton	nucleoplasm	extrinsic component of cytoplasmic side of plasma membrane
clathrin coat of coated pit	nucleus	Golgi apparatus	cytoplasm
clathrin coat of trans-Golgi network vesicle	"intrinsic component of endoplasmic reticulum		
membrane"	nucleus		
centrosome		nuclear pore outer ring	
Molecular Function			
peptide binding	protein serine/threonine kinase activity	chromatin binding	DNA helicase activity
phosphatidylinositol-4-phosphate binding	ATP binding	DNA binding	ATP binding

Table 16 Continued

helicase activity	Wnt-protein binding	zinc ion binding	double-stranded RNA binding
xanthine dehydrogenase activity	DNA binding	phosphatidylinositol-4-phosphate binding	non-membrane spanning protein tyrosine kinase activity
"oxidoreductase activity, acting on the			
KEGG Pathways			
Nicotinate and nicotinamide metabolism	Calcium signaling pathway	Nucleotide excision repair	Herpes simplex infection
	Neuroactive ligand-receptor interaction	Cell cycle	Purine metabolism
		mTOR signaling pathway	RNA degradation
		Basal transcription factors	Pyrimidine metabolism
		RNA degradation	Insulin resistance
Up Keywords			
Cytoplasm	Nucleotide-binding	Coiled coil	Manganese
Muscle protein	Transferase	Nucleus	Immunity
Hydrolase	Microtubule	Ubl conjugation pathway	Nucleotide-binding
SH2 domain	ATP-binding	Zinc-finger	Coiled coil
	Cell adhesion	Lipid biosynthesis	GTP-binding

APPENDIX B

SUPPLENTARY FIGURES

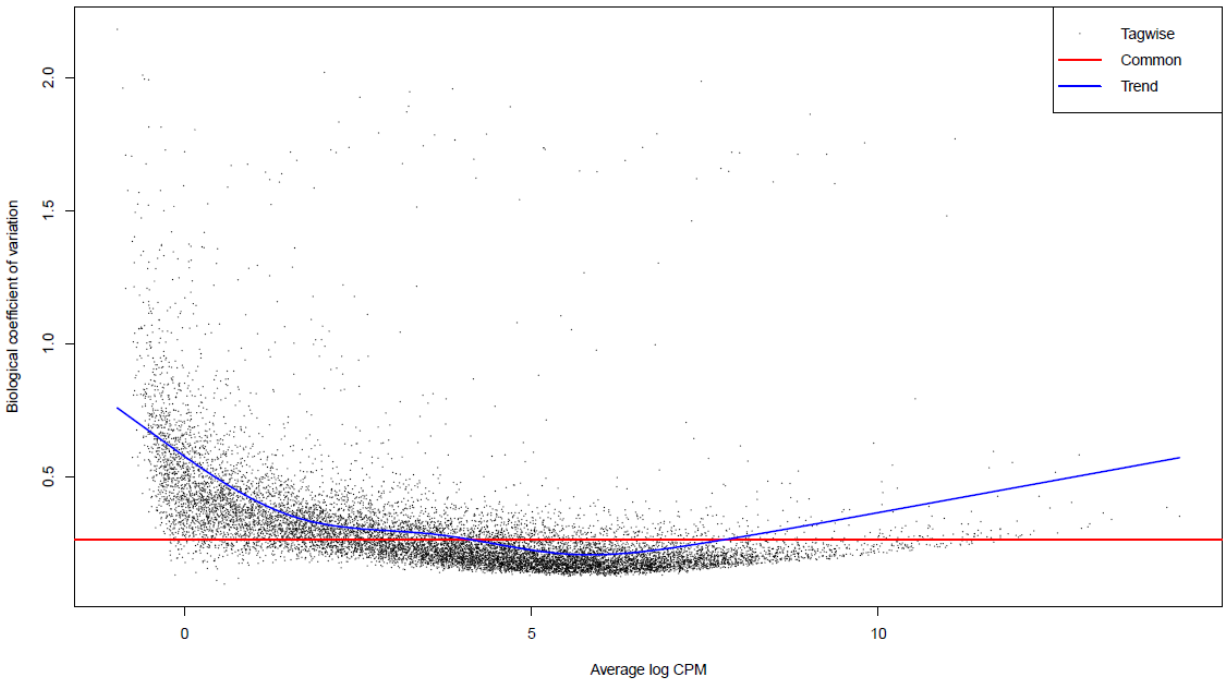

Figure 17. Supplementary Figure S1-Variance estimations for genes in the RNASeq data determined in edgeR analyses. The Red line indicates the common dispersion, black dots indicate the tagwise dispersion for each gene in the dataset, while the blue line shows the trended dispersions calculated with edgeR. BCV, biological coefficient of variance

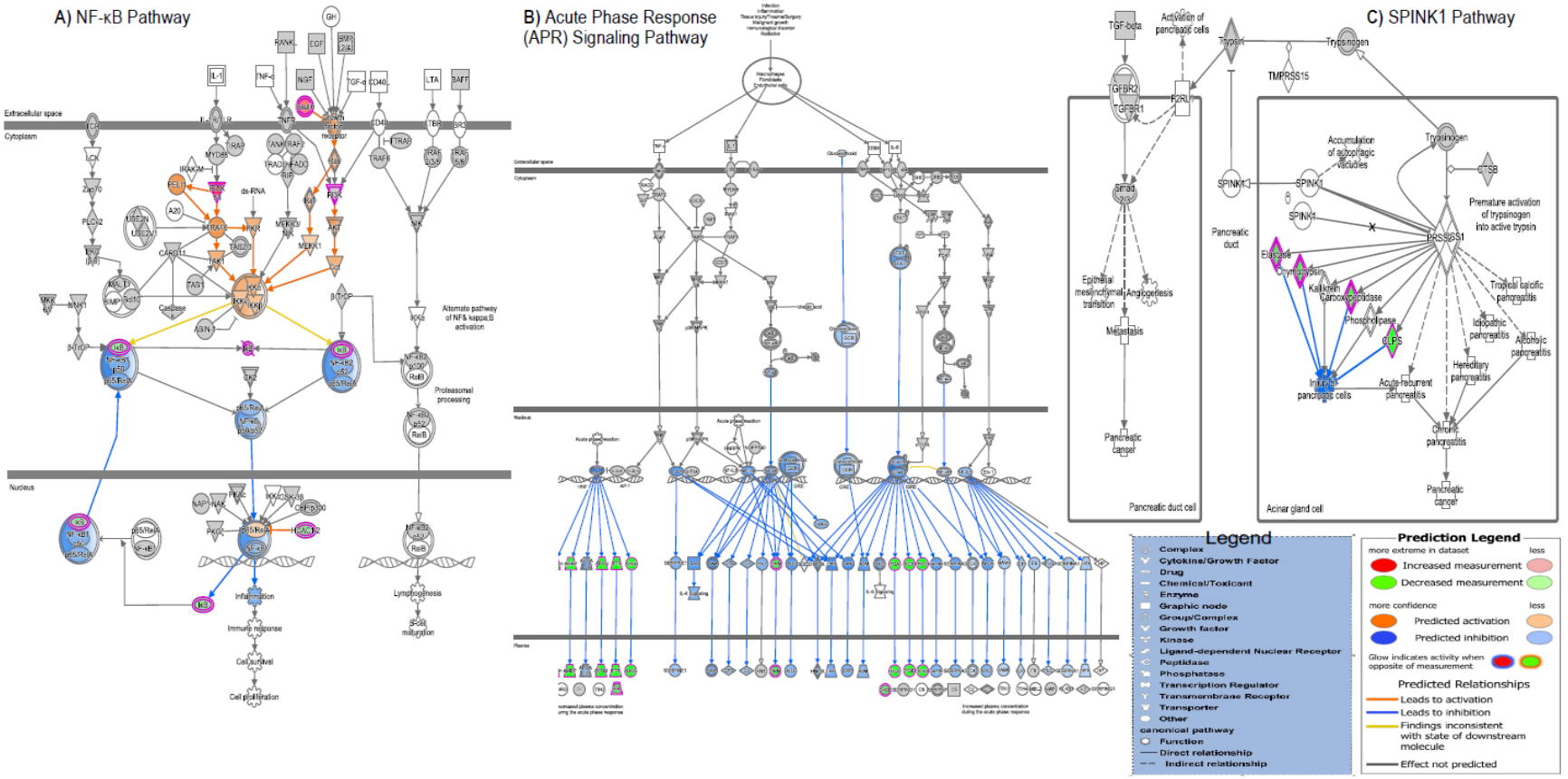

Figure 18. Supplementary Figure S2-Results of significant and activated canonical pathways associated with green monochromatic light biostimulation comparisons during incubation and their interaction in potentially regulating developmental biological processes pre and post hatch generated by QIAGEN's Ingenuity Pathway Analysis (IPA;QIAGEN Inc.) (Krämer et al., 2014). (A) LNV vs DNV, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells (NF-κB) Signaling Pathway; (B) LPHV vs DPHV, Acute Phase Response (APR) Signaling Pathway; (C) LIV vs DIV Serine Protease Inhibitor Kazal-type 1 (SPINK1) Pathway. Differentially expressed genes in the biostimulated comparisons were used in Ingenuity Pathway Analysis and significant canonical pathways based on IPA Z-scores were identified. Genes highlighted in red were upregulated, while those highlighted in green were downregulated in all biostimulated comparisons.

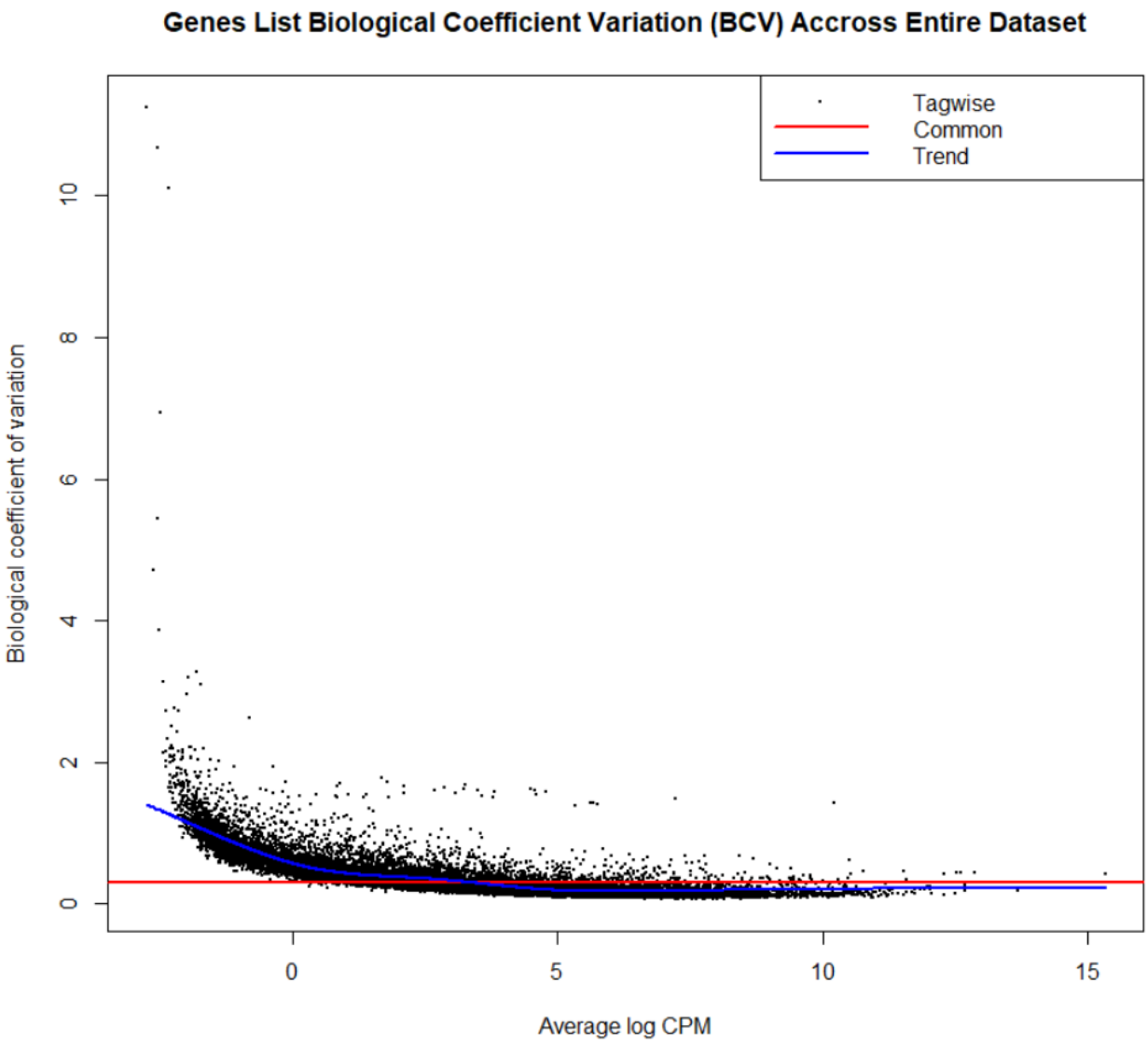

Figure 19. Supplementary Figure S3-Variance estimations for genes in the RNASeq data determined in edgeR analyses. The Red line indicates the common dispersion, black dots indicate the tagwise dispersion for each gene in the dataset, while the blue line shows the trended dispersions calculated with edgeR. BCV, biological coefficient of variance