RESPONSE OF INNATE IMMUNE GENES TO NEWCASTLE DISEASE VIRUS (NDV) CHALLENGE IN CHICKEN EMBRYOS INCUBATED UNDER DIFFERENT LIGHTING REGIMENS

A Dissertation

by

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ABSTRACT

Newcastle disease (ND) is a viral disease causing severe economic losses. The extensive use of currently available ND vaccines and biosecurity appears to keep NDV under control in developed countries. However, current vaccination strategies are not fully effective under different environmental conditions. The development of new concepts for generating new ND vaccines is needed to effectively control NDV infection. The present study aimed to identify genes and mechanisms contributing to different light regimes by studying the innate immune response in chick embryos after ND virus challenge. The innate immune system of chick embryos becomes immunocompetent pre-hatching. By using chick embryos, experimental costs will be significantly reduced with larger sample sizes, minimal biosecurity is required, and the effects of confounding variables will be minimized. This study first validated the optimum pH to deliver the NDV vaccine. The results showed that the minimum reduction in NDV infectivity titer was recorded in pH 7.00 diluent held on ice. Second, by using chick embryos, we were able to uncover the immune response to *in ovo* ND virus challenge in three different lighting regimes (dark 0L:24D, blue 12L:12D, and white 12L:12D). This determined the highest virus Fold change (FC) occurs 36-h post-ND virus challenge, the dark treatment (2.84 FC) had a significantly higher virus FC than blue and white light treatments (1.03 and 1.73 FC, respectively) ($P < 0.01$). At 96-h post-challenge, the blue treatment had the lowest viral FC (0.11 FC), and the dark treatment had the highest viral FC (0.73 FC) ($P < 0.05$). The transcriptional response of innate immune genes is differentially expressed in blue light

treatment *versus* dark and white light treatments. The comparison between the dark and blue light treatments validated that they have similar differences in some immune responses by expressing similar genes in both treatments; however, both lighting treatments respond differently in viral particle enumerations. Through these studies, we were able to propose a panel of genes that are associated with the innate immune response under different light regimes, which constitutes the first line of viral defense against NDV and relies on a large family of pattern recognition receptors (PRRs), including TLR 7, which detect the viral single-stranded RNA, termed pathogen-associated molecular patterns (PAMPs). Overall, this study has provided a new tool to examine the innate immune response to NDV. Future studies would benefit from including chick embryos from different breeds and lines as well as examining other poultry pathogens using the chick embryos.

DEDICATION

This dissertation is dedicated to my family, who supported me during this long journey to achieve my degree. My father is the person who taught me the value of hard work and self-control. My mother, through her love and patience, gave me the self-confidence to achieve my dreams. To my two brothers, Marwan and Nizar, thank you for your support and your love. My charming wife and my four little kids, Leen, Tala, Lana, and Ahmed, thank you all from the depth of my heart for your tolerance, love, encouragement, and unconditional support throughout the good and bad times of my Ph.D. journey. Without you, I would not have been able to achieve this degree. You are my inspiration and motivation. God bless you all.

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NOMENCLATURE

APCs	Antigen Presenting Cells
CCL4	C-C Motif Chemokine Ligand 4
CCL5	C-C motif chemokine ligand 5
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCs	Dendritic Cells
DEGs	Differentially Expressed Genes
EID_{50}	50% Embryo Infectious Dose
ELISA	Enzyme-linked immunosorbent assay
ES	Enrichment score
F	Fusion gene
FC	Fold change
GO	Gene ontology
HN	Hemagglutinin-Neuraminidase
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL6	Interleukin-6
IPA	Ingenuity Pathway Analysis
IRF1	Interferon Regulator Factor 1
JAK	Janus Kinase

MHC	Major histocompatibility complex
mL	Milliliter
Mx1	MX Dynamin like GTPase 1
MYD88	Myeloid differentiation primary response
ND	Newcastle disease
NDV	Newcastle disease virus
NK	Natural Killer
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pattern Recognition Receptors
RBC	Red Blood Cells
RT-PCR	Real-time reverse transcription-polymerase chain reaction
SPF	Specific pathogen-free
STAT1	Signal transducer and activator of transcription 1
$TCID_{50}$	50% tissue culture infective dose
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
μL	Microliter

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Newcastle Disease Virus (NDV)

Newcastle disease (ND), the paramyxovirus prototype, is a vital viral disease of poultry, causing severe economic losses worldwide. ND virus (NDV) is an enveloped virus containing a single-stranded RNA genome of negative-sense polarity (Lamb & Parks, 2007).

History of NDV

In 1926, ND was first known in Java, Indonesia. Around the same time, the same disease symptoms were observed in England, and it was recognized in Newcastle by Doyle. Within the next ten years of the disease being discovered, the disease had spread to Australia, East Africa, Japan, India, middle Korea, and the Philippines. In 1930, a relatively mild-respiratory disease was observed in chickens in California, USA. At first, this disease was called pneumoencephalitis, but later it was found to be caused by Newcastle Disease Virus (NDV). Then, within a few years, many of the NDV that produced either mild or no disease were isolated worldwide. Such isolates were later used as live vaccines (Alexander, 1988, 1991).

There have been at least four defined panzootics recognized (Miller & Koch, 2013); the first outbreak began in 1926 and spread to most countries and continued until the late 1950s due to widespread vaccination. In the 1960s, as part of the second and third

panzootics, NDV was recognized in Central and South America, Hawaii, Canada, Mexico, Europe, China, and Middle East (Alexander, 1988).

Now, ND has been recognized in all countries of the world. It causes substantial economic losses to smallholder farmers with high morbidity and mortality rates in poultry flocks (Schilling et al., 2019). The virulent form of ND is one of the most harmful diseases of poultry. It causes 100% mortality in unvaccinated flocks, negatively impacting economic livelihoods and human welfare by decreasing food supplies (Alders, 2014; Ashraf & Shah, 2014).

NDV is responsible for significant economic losses to poultry production in developed and developing countries. For example, the 2002 NDV outbreak in California destroyed 3 million birds and financial losses of over $ 160 million (Diel et al., 2012).

NDV Pathotyping and Classification

NDV strains have been broadly classified into three major pathotypes, based on their pathogenicity to chickens, as avirulent (lentogenic), intermediate (mesogenic), and virulent (velogenic). Lentogenic NDV strains usually cause subclinical infections with low mortalities and are considered low-virulence. Mesogenic NDV strains are moderate-virulence, causing respiratory infection with mortalities up to 50 percent, while velogenic NDV strains are high-virulence, causing severe disease with high mortality rates up to 100 percent (Sharp et al., 1952).

NDV Transmission and Replication

The natural infection route of NDV includes the oral, nasal, and ocular routes. Also, the virus has been found to infect the host when vaccinated by intravenous (IV), intramuscular (IM), and intracerebral (IC) injection (Suarez et al., 2020). After infection, virus replication starts with hemagglutinin-neuraminidase (HN) binding receptors on host cell membranes. The fusion (F) protein helps the merger of the virus to the host cell membrane. Then, the nucleocapsid complex penetrates the host cell's cytoplasm, where it begins the replication process for the virus. The HN receptors for NDV bind to the receptors that contain sialic acid on the surface of red blood cells (RBCs). This binding allows RBCs agglutination and can be used as a diagnostic tool in haemagglutination assays. The HN protein can also degrade sialic acid receptors, preventing viral particles from self-binding and clumping. This is called neuraminidase activity, and it can also be used for diagnosis (Bousse et al., 2004).

NDV Biological Activity

NDV consists of two types of transmembrane glycoproteins; hemagglutination-neuraminidase activities (HN) and a fusion protein (F). During the infection process, the HN protein is responsible for binding the virus to the host cell, and the F protein causes the fusion between the host cell membranes and the virus to allow the genetic material to enter the cell (Alexander, 1991). To confirm NDV replication, the OIE Standards Committee prescribes the detection of NDV in embryonated chicken eggs using the haemagglutination assay (HA) and haemagglutination inhibition (HI) test (OIE, 2000).

Haemagglutination assay (HA) is a method that can be used to measure the presence of some enveloped viruses such as Newcastle disease virus. This assay relies on the specific feature of many viral surface glycoproteins to adsorb to red blood cells (erythrocytes) resulting in agglutination. In the absence of virus, red blood cells will settle down by gravity, forming a sharp dot in a V-bottom clear 96 well microplate. However, if viruses are present, the red blood cells become bound to the virus particles in a lattice or network (this happens because a single virus can bind multiple red blood cells) at the bottom of the well (Senne, 1998).

Avian Immune System

The immune system is present in all animal kingdom species; it acts to defend and protect the host organisms against foreign substances by recognizing and responding to antigens. Generally, the avian immune system does not differ from the mammalian immune system. The immune system is divided into the innate (also called natural or native immunity) and the adaptive (also called specific or acquired immunity) immune system. The innate immunity is initiated within hours to respond to possibly harmful substances; however, it retains no immunological memory of previous antigens. The activation of the innate immune system triggers the stimulation and release of immune molecules, ultimately leading to the activation of the adaptive immune system. Adaptive immunity takes days or even weeks to become established; however, it retains immunological memory of previous antigens. Unlike innate immunity, the adaptive immune responses are more specific to pathogens. adaptive immunity can also provide

long-lasting protection, which occurs after exposure to an antigen either from a pathogen or a vaccination. This part of the immune system is triggered when the innate immunity is insufficient to control infection. Indeed, the adaptive immune response could not be triggered without participation from the innate immune system.

Chicken Innate Immunity

The skin, mucous surfaces, ciliated, and non-ciliated epithelium act as the first line of defense against pathogens to prevent the invasion of microorganisms. In the gastrointestinal tract, low stomach pH and normal bacterial flora in the gut also provide an extra protection layer. The normal bacterial flora acts by producing inhibitory substances against the invading organisms. When abnormal conditions such as diarrhea disturb the normal bacterial flora balance, the susceptibility to an invasion of possibly harmful substances increases.

Cells that are considered major components of the innate immune system, include natural killer (NK) cells, γδ T cells, and phagocytic cells (Delves & Roitt, 2000). γδ T cells and NK cells have a lymphoid lineage origin, while phagocytic cells such as granulocytes and mononuclear phagocytes are derived from myeloid lineages (Melchers, 2010). These cells selectively recognize and kill virus-infected and tumor target cells and do not need prior antigenic exposure for target recognition. The NK cells are not MHC-restricted (Göbel et al., 1996; Telfer & Rothenberg, 2001).

Another crucial component of avian innate immunity is the monocytes-macrophage system. Macrophages are considered an essential part of the innate immune

defense, working immediately when a pathogen enters the body, limiting its growth (Qureshi et al., 2000). Furthermore, macrophages are effector cells in the late phase of the adaptive immune response.

Chicken Adaptive Immunity

The adaptive immunity is relatively naïve to foreign antigens. Defense against pathogens is mediated by the early trigger of innate immunity and the later responses of adaptive immunity. Antigen-presenting cells (APCs), including dendritic cells (DCs), B cells, and macrophages, are the bridge between innate and adaptive immunity.

Adaptive immunity is divided into humoral and cell-mediated immunity. Humoral immunity involves B cells that ultimately produce antibodies. Cell-mediated immunity involves T cell-dependent and the production of cytotoxic T-lymphocytes, activated NK cells, activated macrophages, and cytokines (Melchers, 2010).

In avian species, B cell development occurs in the bursa of Fabricius, while in mammals, B cells develop in the bone marrow. In both chickens and mammals, T cells develop in the thymus.

Newcastle Disease Vaccines

Live and inactivated vaccines have been used since the mid-twentieth century, whereas vectored vaccines became commercially available at the end of the twentieth century.

Vaccination is used to help control ND. The main goals of ND vaccination are to eliminate or decrease clinical disease, eliminate or decrease the amount of virulent virus shed, and produce protective immunity (Kapczynski et al., 2013). These three goals are essential and must be taken into consideration for manufacturing a successful and effective vaccine. Unfortunately, in current control strategies, only the first goal is considered, as field veterinarians do not have the tools to test and evaluate vaccination effectiveness and improve it to achieve the second and third goals.

Biosecurity is a critical component in preventing infection of a flock by keeping the challenge virus away. For the ND vaccination program to be successful and to achieve herd immunity against ND, at least 85% of the flock must receive an adequate dose and response to vaccination (van Boven et al., 2008).

Table 1-3 summarizes the main characteristics of live, inactivated, and vectored vaccines, which are the most commercially used ND vaccines.

Live Newcastle disease vaccines

ND Live vaccines have been broadly used since the 1950s. In 1948 the first live vaccines were licensed. These vaccines were formulated with strains, classified now as virulent, causing disease in younger chickens and were only used for chickens that are at least four weeks old and needed to be administrated with the wing-web application. In the early 1950s, two low virulence of NDV strains (B1 and LaSota) isolated from chickens from the USA were licensed for use (Goldhaft, 1980). The most common ND vaccine worldwide is the live virus vaccine from strains that were isolated in the 1940s and 1960s.

All these ND viruses belong to genotype II; the main differences between these vaccine virus strains are the tropism and replication capacity in naïve chickens, which are higher in the LaSota strain and lead to higher levels of neutralizing antibodies compared to other vaccine virus strains (Kaleta & Baldauf, 1988).

Live vaccines provide both humoral and mucosal immunity and can be administered using most routes (e.g., spray, aerosol, drinking water, eye drop, and injection). They may cause clinical respiratory disease and a decrease in egg production. They can be easily inactivated if not kept at the desired temperature (Winterfield & Dhillon, 1981).

The efficacy of live ND vaccines is associated with the vaccine dose. Under standard conditions, the 50% embryo infectious dose (EID_{50}) of 10^4-10^5 could achieve 100% protection in adult SPF chickens (Cornax et al., 2012; Miller & Koch, 2013).

LaSota vaccine doses of 10^6 EID_{50} or higher produced robust humoral immune responses, and no increase in titers was observed after the challenge, indicating little to no replication of the challenge virus (Cornax et al., 2012). Table 1 summarizes the main characteristics of live ND vaccines.

Table 1: Properties of live Newcastle disease vaccines*

Storage	Frozen, freeze-dried, chilled, and liquid
Adjuvants	No
Administration route	Spray, aerosol, drinking water, eye drop, and injection
Duration of immunity	Shorter than inactivated and vectored vaccines
Response to the vaccine	Systemic and local
Antibody immune response	IgY, IgM, IgA
Cell-mediated immune response	Stronger than inactivated vaccines
Affected by maternal antibodies	Yes, depending on the level of antibodies
Affected by pre-existing antibodies from previous vaccinations	Yes, if induced by live vaccines
Protection onset	2–3 weeks
Symptoms after vaccinations	Mild respiratory signs, depending on many factors (age, immunity, etc.)
Thermostability	No, (some strains show some thermotolerance)
Cost	Less expensive than inactivated and vectored vaccines
Genotype	I, II
Vaccine strain	I-2, V4, PHY-LMV42, Ulster, LaSota, B1, VG/GA, Clone 30

* More information on ND vaccines produced worldwide could be found at //www.poultrymed.com/Vaccines. Table modified from Dimitrov et al. (2017).

Inactivated Newcastle disease vaccines

From the middle to the end of the 20th century, ND live and inactive vaccines were the only vaccine platforms available and were used to reduce poultry economic losses (Gallili & Ben-Nathan, 1998). In 1945, inactivated vaccines became commercially available in the USA; at that time, they were not adopted by the poultry industry because the cost was expensive, and the vaccine could not prevent clinical disease to a sufficient level worthy of widespread use (Goldhaft, 1980).

One of the downsides to inactivated ND vaccines is that each vaccine requires individual administration by intramuscular or subcutaneous injection. Although inactivated vaccines give higher levels of humoral antibodies, they do not develop a robust cell-mediated response compared to birds vaccinated with live ND vaccines (Schijns et

al., 2014). Even though live and inactivated vaccines protect against clinical symptoms in SPF birds, there are persistent reports of vaccine failure under field conditions (Perozo et al., 2012; Rehmani et al., 2015). Poor vaccination response may be one of the possible causes of these failures (Kaleta & Baldauf, 1988). Inactivated vaccines require the use of adjuvants. Use of R-848 as an adjuvant for inactivated ND vaccine administered intramuscularly in chickens showed significantly upregulated expression of IL-1β, IL-4, IFN-α, IFN-β, and IFN-γ compared to two ND inactivated vaccines used alone (Sachan et al., 2015). Table 2 summarizes the main characteristics of inactivated vaccines.

Table 2: Properties of inactivated Newcastle disease vaccines*

Storage	Chilled, suspension, emulsion
Adjuvants	Yes
Administration route	Injection
Duration of immunity	Longer than live vaccines
Response to the vaccine	Systemic
Antibody immune response	IgY, IgM
Cell-mediated immune response	Weaker than live vaccines
Affected by maternal antibodies	Yes, depending on the level of antibodies
Affected by pre-existing antibodies from previous vaccinations	Depending on the level of antibodies
Protection onset	3–4 weeks
Symptoms after vaccinations	No
Thermostability	No
Cost	More expensive than live vaccines
Genotype	Any
Vaccine strain	Any

* More information on ND vaccines produced worldwide could be found at //www.poultrymed.com/Vaccines. Table modified from Dimitrov et al. (2017).

Vectored Newcastle disease vaccines

NDV is an attractive vector vaccine candidate for animal use. It is a promising candidate for rational design of live attenuated vaccines and vaccine vectors due to its modular nature of transcription, minimum recombination frequency and lack of DNA phase during replication. The genome of NDV is simple to modify using the reverse genetics system (Bukreyev & Collins, 2008; Ganar et al., 2014; Samal, 2011). The small size of the NDV genome, combined with the low probability of genetic recombination, facilitates the use of NDV as a vaccine vector. The virus can also be replicated in several mammalian species, facilitating NDV to be used as a vector vaccine against other diseases such as infectious bursal disease, influenza, West Nile disease, rabies, canine distemper, Ebola, severe acute respiratory syndrome, respiratory syncytial virus syndrome and human immunodeficiency syndrome (Kim & Samal, 2016). There is no evidence, however, of mammals acting as a biological vector for the transmission of ND to poultry, while mammals, including humans, sometimes act as mechanical vectors for the virus, including humans (Dimitrov et al., 2017). Table 3 summarizes the main characteristics of vectored vaccines.

Table 3: Properties of vectored Newcastle disease vaccines*

Storage	**Frozen, cryo-frozen (liquid nitrogen)**
Adjuvants	No
Administration route	depending on the vector *(in ovo*, eye drop, injection, subcutaneous or wing-web, spray, aerosol)
Duration of immunity	Longer than live vaccines
Response to the vaccine	Systemic and local
Antibody immune response	depend on the vector and the administration route (IgY, IgM, IgA)
Cell-mediated immune response	Strong, for Newcastle disease virus (NDV)-vectored
Affected by maternal antibodies	Yes, depending on the vector
Affected by pre-existing antibodies from previous vaccinations	Yes, if induced by live vaccines
Protection onset	4–5 weeks
Symptoms after vaccinations	NDV-inserted – no
Thermostability	No
Cost	Variable
Genotype	Any
Vaccine strain	Any

* More information on ND vaccines produced worldwide could be found at //www.poultrymed.com/Vaccines. Table modified from Dimitrov et al. (2017).

In ovo ND vaccination

In ovo vaccination, or inoculation of the vaccine to the egg before hatching, is an early and safe option for delivering the vaccine to the embryo. It was first used for vaccination against Marek's disease in 1982 (Sharma & Burmester, 1982). Due to its significant benefits over the traditional vaccine, *in ovo* vaccination has been used commercially for over 20 years to monitor avian diseases. The advantages of *in ovo* vaccination include reducing costs, stimulating earlier immunity, reducing stress, and delivering the vaccine uniformly. *In ovo* vaccinations have proven effectiveness for diseases including Marek's disease and infectious bursal disease (Sharma, 1985; Sharma & Burmester, 1982). However, current vaccine options for NDV have some limitations, such as the presence of anti-NDV maternal antibodies. Maternal antibodies can interfere

with early immunity development after a single vaccination (Czifra et al., 1998). Maternal antibodies can be detected for up to 4 weeks after hatching, although protection is only provided during the first two weeks after hatching (Partadiredja et al., 1979). Maternal antibodies can interfere with early immunity development through epitope masking and inhibition of B cell response (Siegrist, 2003). In neonates vaccinated with paramyxoviruses, T helper cell immunity is not suppressed by maternal antibodies; maternal antibodies interfere with the humoral response development but not T cell proliferation (Gans et al., 2003). Another limitation of *in ovo* vaccination is the inefficiency of existing vaccine strains to produce high levels of protective antibodies within a short period of time.

Factors Affecting the Stability of NDV Live Vaccine

The stability of the viral antigen determines the effectiveness of viral vaccines during storage and reconstitution of the lyophilized vaccine. This stability is necessary in order to provide the final vaccine for administration. For NDV vaccines, stability means preserving the infectious titers. For inactivated, recombinant, and subunit vaccines, the stability means preserving the antigenic structure and the ability of the relevant epitopes to trigger an immune response. Generally, stability of the viral structure, abnormal pH, temperature, organic solvents, suspension medium, antiseptics agents, freeze-thaw cycles, and light are factors that may cause a negative effect on the stability of viral vaccines (Peetermans, 1996).

NDV is highly sensitive to physical (temperature, extreme pH) and chemical (disinfectants, detergents) insults (Gentry & Braune, 1972). Water properties such as pH, temperature, and salinity may have a significant impact on virus infectivity (Nazir et al., 2010; Peetermans, 1996). Nazir et al. (2010) have demonstrated that extreme pH, elevated temperature, and high salinity of the water negatively affect virus infectivity. Likewise, the case for a single freeze-thaw cycle will lead to a rapid decline in the virus titer. Tariq et al. (2017) reported that the optimum water pH for effective delivery of the NDV live vaccines for administration is 7.00 to 8.00, and the virus can be effectively preserved for 3 hours after reconstitution in diluent with pH 7.00, even without the addition of any stabilizer.

In poultry farms, drinking water is usually improved by using water sanitizers such as chlorine, detergents, and organic acids. These water sanitizers may adversely affect, if not properly treated, the infectivity of the virus and lead to vaccine failure. However, the addition of chlorine neutralization or skimmed milk to the ND virus water mixture may preserve the virus infectivity to a maximum level and protect the virus from the damaging effects, even at extreme acidic and alkaline pH conditions (Tariq et al., 2017).

NDV live vaccines need chain cold transport; the purpose of the cold chain is to preserve the quality of vaccine from the time of manufacture until the administration point by ensuring that vaccines are transported and stored within recommended temperature ranges. Repeated freezing and thawing of NDV vaccines may influence the stability of the virus. Previous work by Trybała (1987) on the effect of freezing and thawing on the

biological properties of NDV has shown that repeated freezing and thawing cycles might cause a marked reduction in virus infectivity.

These characteristics of the live vaccine affect controlling the NDV, especially in developing countries.

NDV Immunity

The innate and adaptive immunity play important roles in NDV infection defense. Innate immune defense to NDV is made up of chemical and physical barriers, inflammation-related serum proteins (e.g., complement, C-reactive protein, and lectins such as mannose-binding lectin), and phagocytic cells (e.g., monocytes, macrophages) (Susta et al., 2013). The stimulation of phagocytosis initiates the release of cytokines and inflammatory mediators. Cytokines are proteins that play a significant role in both the innate and adaptive immune response. The splenic cells of NDV-infected chicken begin producing IFN-α, IFN-β, IFN-γ, and IL-6 within a few hours, NK cells begin to produce IFN-γ, which activates macrophages and stimulates cell-mediated immunity (Suarez et al., 2020). Cell-mediated immunity is detectable as early as 48-72 hours post-infection, although it may or may not protect against NDV challenge by itself (Reynolds & Maraqa, 2000). Protection with cell-mediated immunity alone has been demonstrated with other viruses, such as IBV (Seifi et al., 2014), indicating the need for better studies on the role of cell-mediated immunity during NDV infection.

The humoral immunity can protect for a long period of time against NDV. Activated B cells secrete protective antibodies that target either HN or F

glycopolypeptides. Plasma cells begin secreting these antibodies within 6-10 days post-infection (Al-Garib et al., 2003; Miller & Koch, 2013). Three types of antibodies are produced in avian species: IgA, IgY, and IgM (Miller & Koch, 2013). IgY is the major antibody in chickens. IgA induces local protection in the respiratory and intestinal tracts. Only IgG and IgM are detected when vaccinated with inactivated vaccines, which are usually given intramuscularly or subcutaneously, while live vaccination stimulates the production of IgA, IgM, and IgG. However, both vaccine types induce full protection against NDV (Al-Garib et al., 2003; Miller et al., 2009).

Cytokines Response Induced by NDV

Cytokines can be classified into categories such as chemokines, interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), transforming growth factors (TGF), etc. (Kaspers & Schat, 2012). Cytokines of innate immunity include type I interferons, which have antimicrobial activity, particularly viral pathogens, and IL-10, which has anti-inflammatory activity (Ivashkiv & Donlin, 2014; Rothwell et al., 2004).

Cytokines such as interferon-gamma (INFγ) can be a key moderator of cell-mediated immunity, killing a variety of intracellular pathogens, while others such as IL-2, IL-7, and IL-15 can be a key host immune responses against intracellular pathogens by enhancing the effector and memory T-lymphocyte responses (Barouch et al., 2004; Thompson & Staats, 2011).

Schilling et al. (2018) showed that the expression of TLR3, Mx1, and CCL5 in lung tissues of NDV challenged chick embryos was higher in the Kuroiler breed than in

the Tanzanian ecotypes lines. Also, the expression patterns of three genes that are associated with innate immune response (IL8, STAT1, and IRF-1) were examined in the Fayoumi and Leghorn sublines; the results showed that the Leghorn subline had a higher expression of all genes except IL-8 (Schilling et al., 2018).

Schilling et al. (2019) studied innate immunity genes associated with NDV load in chick embryos from inbred and outbred lines. The results showed increased expression levels of some selected innate immune genes associated with NDV, such as SOCS1, NOS2, and CCL4. These results suggested that specific conserved and differentially expressed innate immunity genes are involved in the response of highly outbred chicken lines to NDV (Schilling et al., 2019).

Lighting Regimen and NDV Infection

A circadian rhythm is a self-sustained ("built-in") biological process, and it is naturally produced. It displays an endogenous, entrainable oscillation in the behavior, physiology, and metabolism of organisms of about 24 hours. Circadian rhythm has been widely observed in animals, fungi, and plants; an endogenous timekeeper drives these rhythms called the circadian clock; this term comes from the Latin ("circa" meaning around or approximately, and "diēm' meaning day) (Vitaterna et al., 2001). Although circadian rhythms are endogenous, they are regulated by external cues (including light, temperature, and redox cycles) called zeitgebers. The term zeitgebers come from German, and the meaning is "time giver" (Peek et al., 2015).

The development of circadian rhythm during incubation has been reported to be correlated to growth enhancement, health, and welfare for the hatched chicks (Blatchford et al., 2009; Markowska et al., 2017). Current industry practice is to incubate eggs in complete darkness resulting in poor development of circadian rhythm during incubation due to lack of environmental cues (Tong et al., 2018). Providing light to embryonated eggs during incubation affects the quality of the chicks' growth development post-hatch, which leads to improved growth and hatchability and reduces defects such as an unhealed navel, leg abnormalities, weakness, or any other type of abnormality (Archer, 2017).

There are a few reports of the effect of light on the immune response. In mammals, the immune system can be affected directly by light spectra. As proposed earlier by Ferguson et al. (1992), visible spectra affect directly on intraocular T-cell interactions. In addition, rats raised in constant darkness gained more thymus weight, possibly due to an increase in the number of lymphocytes and enlarged cortical epithelial cells (Mahmoud et al., 1994). In birds, an intermittent photoperiod regimen can enhance splenocyte proliferation in broiler chickens when compared to continuous lighting (Kliger et al., 2000). These results indicate the critical role that photostimulation plays in influencing the immune response. However, fewer studies have been reported to date, and the mechanism of the effect of monochromatic light on immune responses is not fully understood in avian species.

Objectives

I. pH levels of the vaccine diluent at the time of administration

   i. Evaluate the virus infectivity titer of live attenuated NDV vaccine (B1 Type, LaSota Strain, Live Virus) reconstituted in diluent at different pH levels and holding temperatures.

   ii. Study the duration of live attenuated NDV vaccine stability and effectiveness following reconstitution in diluent at different pH levels.

Hypothesis

The hypothesis for the first project is that different pH levels of the vaccine diluent at the time of administration will affect the virus infectivity titer and duration of live attenuated NDV vaccine in chicken.

II. *In ovo* challenge with live attenuated NDV vaccine

   i. Study the progression of the virus particle enumerations after *in ovo* challenge of live attenuated NDV vaccine (B1 Type, LaSota Strain, Live Virus) under different lighting regimens.

   ii. Study innate immune response at 96 hours post-challenging with a live attenuated NDV vaccine (B1 Type, LaSota Strain, Live Virus) in lung tissue.

   iii. Determine the activities of different lighting regimens during chick embryos incubation on the innate immune response to ND virus challenge.

Hypothesis

The central hypothesis is that different monochromatic light wavelengths will affect the innate immune response in chick embryos based on circadian development. Based on the literature, the expected results are that embryos with a well-developed circadian rhythm represented by the oscillation of clock genes expression will pace the physiological processes in the chicks' body resulting in faster development of the innate immune response.

CHAPTER II

IMPACT OF DILUENT pH AND HOLDING CONDITIONS ON NEWCASTLE DISEASE VACCINE VIRUS INFECTIVITY TITER*

Introduction

ND is a viral disease causing severe economic losses worldwide. Despite routine vaccination, severe outbreaks continue to occur. Vaccine failures can result from mutations in the virus; however, virus inactivation may also occur in lyophilized Newcastle disease virus (NDV) live vaccines due to diluent properties at the time of administration (Munir et al., 2012; Tariq et al., 2017).

The present study (1) evaluated the virus infectivity titer of the NDV live vaccine reconstituted in diluent at different pH levels and holding temperatures. (2) Determined the duration of the NDV live vaccine stability and effectiveness following reconstitution in diluent at different pH levels.

Material and Methods

Chicken Embryonated Eggs

This study had used two types of eggs, (1) fertilized SPF premium eggs (Charles River Laboratories Avian Vaccine Services, Norwich, CT, USA) (*n* =124). (2) Lohmann LSL fertile eggs were obtained from a commercial laying hen flock with known low titers of anti-NDV antibodies at the Texas A&M Poultry Science Center. All eggs were incubated at the Texas A&M Poultry Science Center.

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Incubators

GQF 1502 combo incubators (GQF Manufacturing, Savannah, GA, USA) were used. All eggs were incubated under standard temperature and humidity levels of 37.5°C (99.5°F) and 58% relative humidity with tray tilting each 2 hours. Chicken embryonated eggs were inoculated at day 9 with NDV vaccine. Egg candling was performed on day 8 before virus inoculation, and on days 10, 11, 12, and 13. Mortality was regarded as non-specific within 24 hours after inoculation and was not included in the calculation for titer infectivity. Dead embryos after 24 hours were moved to 4°C until harvesting the allantoic fluid for infectivity titer calculations.

Inoculation of Embryonated Eggs

9-day-old chicken embryonated eggs were inoculated with the diluted NDV vaccine. Fertile eggs were candled before inoculation in a darkroom to verify embryo viability and to mark the air cell on the shell. The position of the embryo was noted by its spontaneous movements; a point marked 4-5 mm above the air cell on the shell with a pencil. Embryonated eggs were then sprayed with 70% ethanol; a pore was made on the marked point by using stoppers with an 18 gauge needle. Eggs were then inoculated with the diluted NDV vaccine using 1 mL disposable sterile syringes with 25 gauge, 5/8 inch needles. NDV vaccine dilutions from 10^{-3}x to 10^{-7}x were used to measure the virus infectivity titer. Eggs were inoculated with 100 µL of the inocula on day 9 of incubation (Figure 1), and the pores were sealed with the GE Silicone II sealant after inoculation to avoid evaporation. Eggs were then incubated under standard conditions and candled daily

to check for embryo death for four days. Embryo death within 24 hours post-inoculation was discarded and considered as non-specific. On day four post-inoculation, all eggs were chilled at 4 °C for at least two hours.

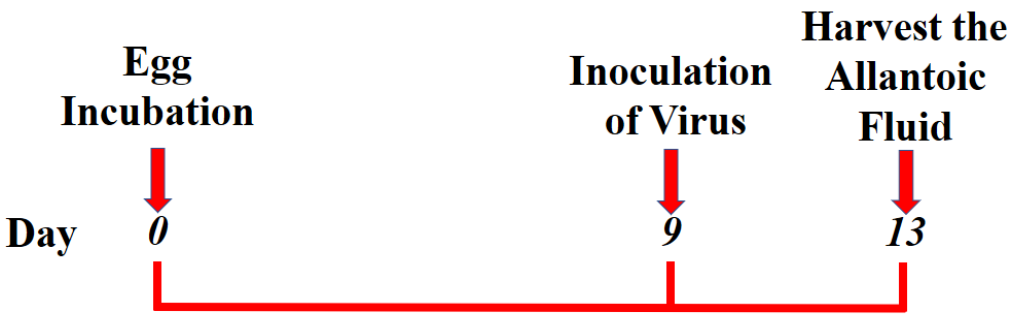


Figure 1: Experimental Timeline. Schematic illustration of the experimental timelines of experiment 1. Lohmann LSL or SPF premium fertile eggs were incubated in GQF 1502 combo incubators/hatcher under standard temperature and humidity. Dilutions from 10-3X to 10-7X of live NDV, LaSota Strain, were inoculated on day 9 of embryonic development. Allantoic fluids were harvested on day 13 of embryonic development to measure the virus infectivity titer.

pH

Phosphate buffered saline (PBS) was prepared; pH was calibrated at 5, 6, 7, 8, and 9 by using electrometric Accumet AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA). Titration was done using HCl and NaOH.

NDV Live Vaccine Strain

Newcastle Disease Vaccine, B1 Type, LaSota Strain, Live Virus, freeze-dried ampoules of 5000 dose (Product Code: ND1820, Merial) was used for the viral inoculation for the study. Dilutions from 10^{-3}X to 10^{-7}X of the recommended dose were used to measure the virus infectivity titer, 0X for the negative control group.

Dilution of the Vaccine

The freeze-dried ampoules of Newcastle Disease Vaccine, B1 Type, LaSota Strain, Live Virus was used for the viral inoculation. The vial containing 5000 doses was dissolved in 5 mL of nanopure water for inoculation, then the 5 mL was divided into 5 groups, each group was diluted in PBS at different pH levels (5, 6, 7, 8, and 9), dilutions from 10^{-3}x to 10^{-7}x of the recommended dose were used to measure the virus infectivity titer, 0X for the negative control group. Reed and Muench mathematical technique was used to calculate EID_{50}/mL of virus suspension (Reed & Muench, 1938). EID_{50} (50% Embryo Infectious Dose) is the amount of virus infecting 50% of inoculated eggs. The Reed-Muench method technique is used to calculate the endpoint for each of the inoculated eggs depending on the outcome results of the HA tests. The infectivity titre is calculated by using the below formula to calculate an index (proportionate distance) then applied to the appropriate dilution. The infectivity titre is expressed as EID_{50} per mL.

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above } 50\%) - 50\%}{(\%\text{ infected at dilution immediately above } 50\%) - (\%\text{ infected at dilution immediately below } 50\%)}$$

Harvest of Allantoic Fluid

Eggs were moved to room temperature and sprayed with 70% ethanol. Then the shell over the air cell was carefully removed using sterile forceps, and the chorioallantoic membranes ruptured. Allantoic fluid was then harvested from the 13-day old embryonated eggs with a sterile syringe into sterile tubes. Virus infectivity titer was measured by hemagglutination test.

Experimental Design

First trial: a 3x2 factorial trial was conducted, using 3 diluent pH levels (pH 5, 7, and 9). At each pH level, the reconstituted virus was held one hour at room temperature or on ice. One hundred twenty-four fertilized SPF eggs were utilized (20 eggs per group, 4 eggs for the negative control).

Second trial: a 3x2 factorial trial was conducted and repeated three times, using 3 diluent pH levels (pH 5, 7, and 9). At each pH level, the reconstituted virus was held one hour at room temperature or on ice. Two hundred seventeen fertilized eggs were utilized (35 eggs per group, 7 eggs for the negative control).

Third trial: the third trial was repeated three times, using 5 diluent pH levels (pH 5, 6, 7, 8, and 9). At each pH level, the reconstituted virus was held one hour at room temperature. One hundred eighty-two fertilized eggs were utilized (35 eggs per group, 7 eggs for the negative control).

The second and third trials' eggs came from a commercial laying hen flock with known low titers of anti-NDV antibodies. Comparison of the first trial and the other two trials verified that the commercial laying hen flock low level of anti-NDV antibodies did not affect the virus infectivity titer measurement.

Collection and preparation of RBCs

Blood was collected from the wing veins of healthy Lohmann LSL hens from a flock at the Texas A&M Poultry Science Center. Blood was collected into a 3-mL sterile syringe with Alsever's solution (Alfa Aesar, Haverhill, MA, USA) added to the blood (1

part Alsever's solution to 1 part blood). Then the RBCs were washed by centrifugation at 1000 g for 10 minutes. The supernatant was removed, and an equal volume of sterile phosphate buffer saline (PBS) was added to pack erythrocytes. This procedure was repeated at least three times, and packed erythrocytes were then diluted to prepare a 1% solution of erythrocytes for use in HA.

HA test procedure

A volume of 50 µL of allantoic fluid from each of embryonated eggs was dispensed into each well of V-bottom microwell plate. Fifty µL of PBS was used for RBCs control's auto-agglutination. Fifty µL of 1% (v/v) chickens RBCs was dispensed to each well. Then the solution was mixed by gently tapping the sides of the plate. The RBCs were then allowed to settle for 45 minutes at room temperature.

Statistical Analysis

Data were analyzed by using the generalized linear model (GLM) – analysis of variance (ANOVA) by JMP pro 14 software (SAS, Institute Inc., Cary NC). Means were compared and significance differences were identified by using all pairs Tukey-Kramer HSD at $P< 0.01$.

Results

Mean infectivity titers of NDV vaccine that was reconstituted in diluent at different pH conditions (5, 6, 7, 8, and 9) and holding temperatures (room temperature or on ice) without adding any stabilizer, are presented in Table 4. NDV vaccine diluted in pH 7 and held on ice had the minimum reduction in virus infectivity titer.

	1st trial		2nd trial		3rd trial
	Vaccine held at room temperature	Vaccine held on ice	Vaccine held at room temperature	Vaccine held on ice	Vaccine held at room temperature
pH 5	$10^{5.40}$	$10^{5.67}$	$10^{5.73}$	$10^{5.90}$	$10^{5.46}$
pH 6	-	-	-	-	$10^{5.80}$
pH 7	$10^{6.50}$	$10^{7.00}$	$10^{6.21}$	$10^{6.57}$	$10^{6.59}$
pH 8	-	-	-	-	$10^{6.16}$
pH 9	$10^{5.25}$	$10^{5.17}$	$10^{5.37}$	$10^{5.69}$	$10^{5.74}$

Table 4: Infectivity titer of NDV for all trials using 5 diluent pH levels (pH 5, 6, 7, 8, and 9) held at room temperature or on ice.
Note: All values are virus infectivity titer EID_{50}/dose, GC

Infectivity titer of NDV Vaccine in pH 5, 7, and 9 diluent held at room temperature

In samples that were diluted in pH 5, 7, and 9 and held at room temperature without adding any stabilizers, minimum reduction in virus infectivity titer was recorded in pH 7 diluent held at room temperature (1st trial: $10^{6.50}EID_{50}$/dose, 2nd trial: $10^{6.21}EID_{50}$/dose, and 3rd trial: $10^{6.59}EID_{50}$/dose). The virus was less stable at pH 5 diluent held at room

temperature (1st trial: $10^{5.40}EID_{50}$/dose, 2nd trial: $10^{5.73}EID_{50}$/dose, and 3rd trial: $10^{5.46}EID_{50}$/dose). While maximum reduction of infectivity titer was observed in pH 9 diluent held at room temperature (1st trial: $10^{5.25}EID_{50}$/dose, 2nd trial: $10^{5.37}EID_{50}$/dose, and 3rd trial: $10^{5.74}EID_{50}$/dose) (Figure 2).

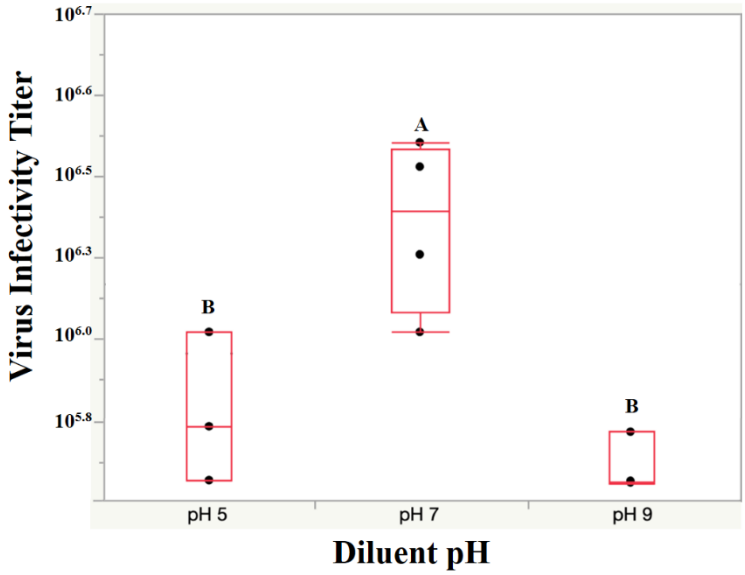


Figure 2: Infectivity titer of NDV using 3 diluent pH levels (pH 5, 7, and 9) held at room temperature for one hour. Minimum reduction in virus infectivity titer was recorded in pH 7 diluent $10^{6.21}$ EID_{50}/dose. The virus was less stable at pH 5 diluent. Maximum reduction of infectivity titer was observed in pH 9 diluent. Different letters indicate the significant differences between the treatments ($P<0.01$).

Infectivity titer of NDV Vaccine in pH 5, 7, and 9 diluent held on ice

In samples that were diluted in pH 5, 7, and 9 and held on ice without adding any stabilizers, minimum reduction in virus infectivity titer was recorded in pH 7 diluent held on ice (1st trial: $10^{7.00}EID_{50}$/dose, 2nd trial: $10^{6.57}EID_{50}$/dose). The virus was less stable at pH 5 diluent held on ice (1st trial: $10^{5.67}EID_{50}$/dose, 2nd trial: $10^{5.90}EID_{50}$/dose). While

maximum reduction of infectivity titer was observed in pH 9 diluent held on ice (1st trial: $10^{5.17}EID_{50}$/dose, 2nd trial: $10^{5.69}EID_{50}$/dose) (Figure 3).

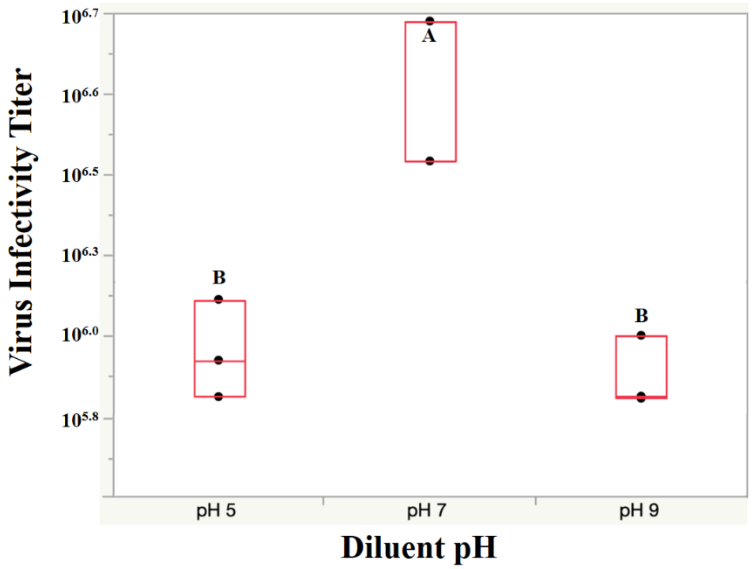


Figure 3: Infectivity titer of NDV using 3 diluent pH levels (pH 5, 7, and 9) held on ice for one hour. Minimum reduction in virus infectivity titer was recorded in pH 7 diluent $10^{6.58}$ EID_{50}/dose. The virus was less stable at pH 5 diluent. Maximum reduction of infectivity titer was observed in pH 9 diluent. Different letters indicate the significant differences between the treatments ($P<0.01$).

Infectivity titer of NDV Vaccine in pH 7 diluent held at room temperature

The linear regression model showing a decline in the infectivity titer of NDV vaccine diluted in the pH 7 condition and held at room temperature without adding any stabilizers is presented in Figure 4. Minimum reduction in virus infectivity titer was recorded in pH 7 diluent used immediately after reconstitution. While maximum reduction of infectivity titer was observed in pH 7 diluent held 4 hours at room temperature. The virus was less stable over time by holding it at room temperature at pH 7 diluent (0 hour:

$10^{6.38}EID_{50}$/dose, 1 hour: $10^{6.16}EID_{50}$/dose, 2 hours: $10^{6.00}EID_{50}$/dose, 3 hours: $10^{5.75}EID_{50}$/dose, and 4 hours: $10^{5.65}EID_{50}$/dose).

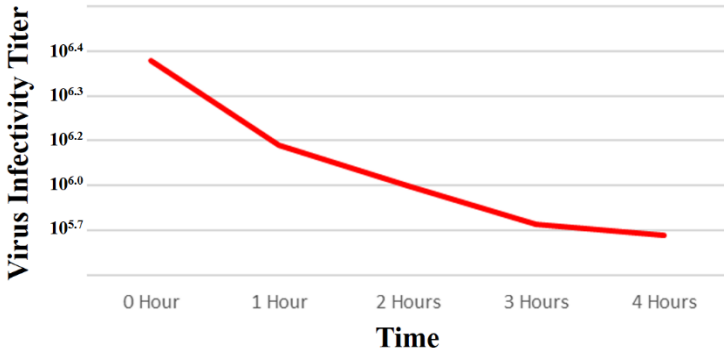


Figure 4: Linear regression model showing reduction in the infectivity titer of NDV vaccine suspended in pH 7 diluent held at room temperature for 0-4 hours. Minimum reduction in virus infectivity titer was recorded without incubate the virus. The virus losses its stability over time.

Infectivity titer of NDV Vaccine in pH 5, 6, 7, 8, and 9 diluent held at room temperature

In samples that were diluted in pH 5, 6, 7, 8, and 9 and held at room temperature without adding any stabilizers, minimum reduction in virus infectivity titer was recorded in pH 7 diluent held at room temperature ($10^{6.59}EID_{50}$/dose). The virus was less stable at pH 6, 8, and 9 diluent held at room temperature ($10^{5.80}EID_{50}$/dose, $10^{6.16}EID_{50}$/dose, $10^{5.74}EID_{50}$/dose, respectively). While maximum reduction of infectivity titer was observed in pH 5 diluent held at room temperature ($10^{5.46}EID_{50}$/dose) (Figure 5).

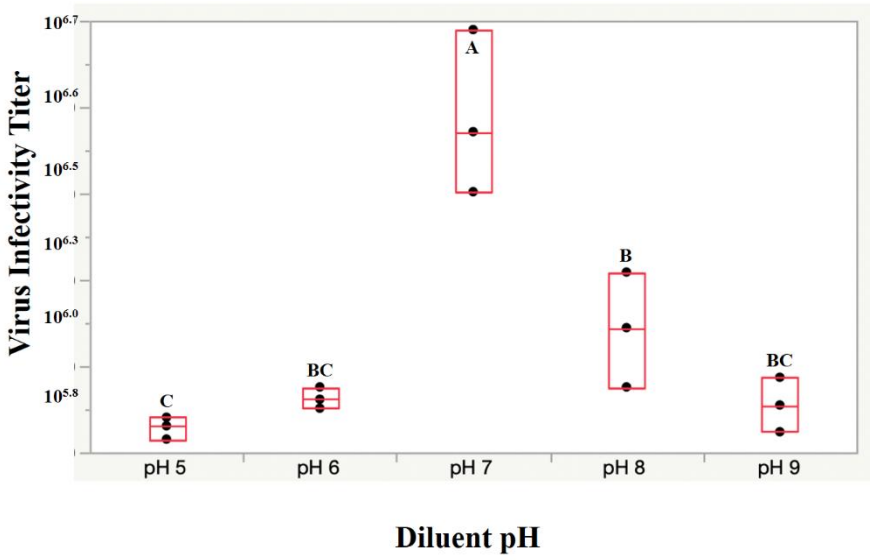


Figure 5: Infectivity titer of NDV using 5 diluent pH levels (pH 5, 6, 7, 8, and 9) held at room temperature for one hour. Minimum reduction in virus infectivity titer was recorded in pH 7 diluent $10^{6.59}$ EID_{50}/dose. The virus was less stable at pH 6, 8, and 9 diluent. Maximum reduction of infectivity titer was observed in pH 5 diluent. Different letters indicate the significant differences between the treatments ($P<0.01$).

Discussion

Lentogenic NDV such as LaSota strain is commonly used as live attenuated viral vaccine and supplied in lyophilized form in commercial poultry. Usually, these vaccines are reconstituted in a diluent and then administered through conventional routes that include: drinking water, spraying, and eye drops. However, due to the administration's cost, NDV vaccine administration is most commonly given through the drinking water route.

NDV live attenuated vaccines' efficacy depends on the infectivity of the vaccine virus, which can be lost by unsuitable cold chain management and poor storage conditions

(Rani et al., 2014). The presence of a virus inhibitor substance in the diluent also may lead to vaccine failure due to virus inactivation at the time of administration (Tariq et al., 2017). pH is one of the factors that affect stability of the NDV live vaccines during administration. Virus inactivation due to properties of the diluent at the time of administration may lead to vaccine failure.

A virus infectivity titer of live attenuated NDV vaccine was used in the present study to evaluate the effect of diluent pH on the survival of the LaSota strain of NDV. However, due to the homology of the virus genome of LaSota strain with other NDV strains virus genomes, the experimental findings might be applicable to other virus strains. It was observed that extreme pH conditions (pH 5 and pH 9) negatively affect virus infectivity titer. Infectivity titer at pH 7 was significantly higher than all of the other pH conditions. Also, this effect was apparent at pH 6 and 8 (Table 4, Figure 5).

A previous study on virus inactivation shows that the medium's pH is reflected in the loss of virus infectivity. At low pH, enveloped viruses display conformational changes in their spike glycoproteins, forming clumps and large aggregates, which distort the virus's ability to bind to the cell surface receptors (Sturman et al., 1990). An *Orthomyxovirus* was completely inactivated by exposure to pH 4 for 30 min, while infectivity was reduced by more than 90% at pH 11 immediately after exposure (Falk et al., 1997). Tariq et al. (2017) evaluated the survival of NDV live vaccine strain by determination of virus infectivity on Vero cells; they reported that extreme acidic pH conditions (pH 5) or alkaline pH conditions (pH 9) were harmful to the infectivity of NDV live vaccine.

The present study also demonstrates that extreme acidic pH conditions or alkaline pH conditions were detrimental to the infectivity of the virus (Table 4), similarly, by comparing natural water sources used as diluents on live NDV vaccine in chickens. The minimum antibody titers against NDV in the vaccinated birds were reported in the ones that the vaccine was diluted in acidic pH diluents, indicating that the vaccine's efficacy was reduced by diluting the vaccine in acidic pH diluents (Khalil & Khalafalla, 2011).

In all diluent conditions, infectivity titer at pH 7 was significantly higher than all of the other pH conditions. The results of these studies indicate that optimum diluent pH for effective delivery of the NDV live vaccines for administration is 7. However, holding virus on ice may have beneficial effects on preserving the infectivity titer of the virus, even at pH 5 or 9 conditions.

CHAPTER III

RESPONSE OF INNATE IMMUNE GENES OF THE CHICKEN EMBRYO'S LUNG TO ND VIRUS CHALLENGE UNDER DIFFERENT LIGHTING REGIMENS

Introduction

The poultry industry has been optimized over recent decades for the highly productive and economical processing of eggs and meat, with significant improvements in management practices and specialized development processes. Nevertheless, public awareness about the negative effects of intensive production on food safety and animal welfare has also increased significantly. Animal health is an essential aspect of welfare, and it is also necessary to increase productivity and food safety for human consumption (Proudfoot & Habing, 2015).

A fully functioning immune system is necessary for good animal health. Adverse environmental stimuli and other stressors could suppress the immune system and reduce its ability to prevent infections. Many environmental factors act as stressors like temperature, air quality, and the light regime, which may negatively impact the immune systems (Morgan & Tromborg, 2007).

Visible light is one of the most critical exogenous factors entraining the circadian rhythms of immune system development in birds and mammals (Engert et al., 2019; Makeri et al., 2017). Poultry light management focuses on three different light properties: light intensity, wavelength, and photoperiod (Nazar & Marin, 2011). Light regimes

stimulate immune system development in chickens and may be used as a tool to strengthen the immune system (Hofmann et al., 2020).

Several publications have studied how the post-hatching light regimes can alter immune function in poultry (Blatchford et al., 2009; Kirby & Froman, 1991; Moore & Siopes, 2000). However, few publications have evaluated the effects of pre-hatching light exposure. This study is aimed to evaluate the innate immune response in lung tissues after *in ovo* challenge of live attenuated NDV vaccine and determine the effects of different lighting wavelengths (blue, white, and conventional dark) during egg incubation. Lung tissue was chosen because NDV is an upper respiratory tract disease, and the virus particles will be higher in the lung tissue.

Materials and Methods

The Eggs

Lohmann LSL fertile eggs (n =300) were obtained from a commercial laying hen flock at the Texas A&M Poultry Science Center. The fertile eggs were incubated in Dr. Athrey's lab KLCT 419 - Texas A&M University.

The Incubators

Three GQF 1502 combo incubators/hatcher (GQF Manufacturing, Savannah, GA, USA) were used in the trial. All incubators were operating at the same time. The front windows of the incubators were obscured with opaque tape to prevent light intrusion. The control group was in an incubator with the conventional dark method of incubation

(0L:24D, zero hours of light; 24 hours of darkness). The other 2 incubators were fitted with white daylight (≥6500K) and blue (450nm) LED light sources. A light–dark cycle (12L: 12D) was provided for the whole period of incubation. Framed LED lighting panels were mounted on each of the three levels of the incubator, installed along the racks to produce an even spread of illumination on the surface of each egg and without additional heat. Blue and white photoperiods were provided from the first day of incubation in lighting treatments. All eggs were incubated under standard temperature and humidity levels of 37.5°C (99.5°F) and 58% relative humidity with tray tilting each 2h. Egg candling was performed at day 18 of incubation, and fertile eggs were moved to hatch tray in the same incubator, where eggs were incubated under standard temperature and humidity levels of 36.9°C (98.5°F) and minimum 66-75% relative humidity until hatching.

NDV Live Vaccine Strain

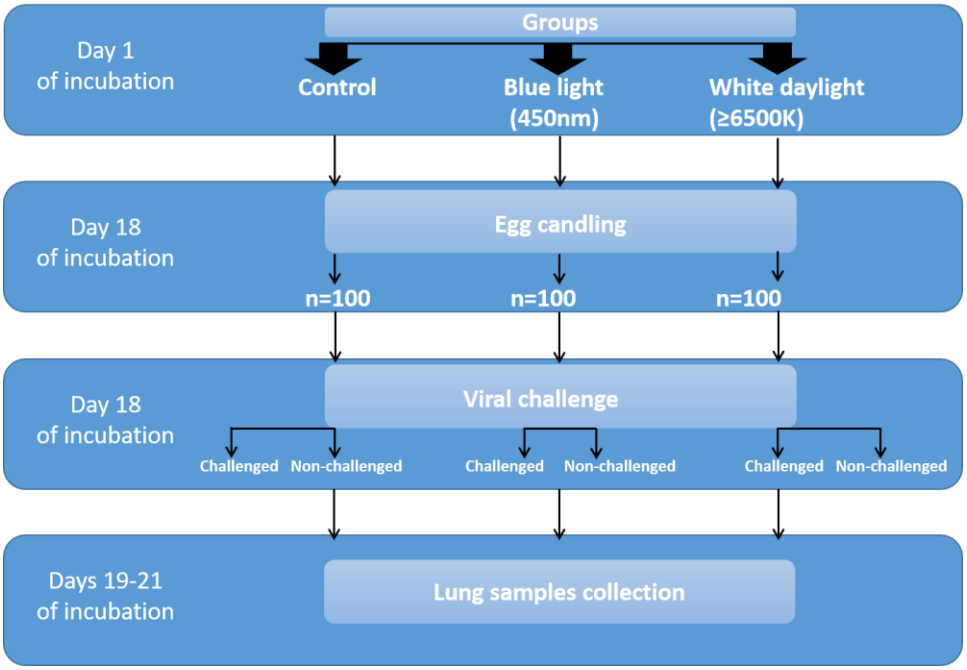
Newcastle Disease Vaccine, B1 Type, LaSota Strain, Live Virus (Product Code: ND1820, Merial) was used as the viral challenge for the study. After vaccine resuspension, the suspended virus vaccine was stored at -80°c until used. The virus vaccine was administered *in ovo* at 1X of the recommended dose ($1x10^{6.6}$ EID_{50}/dose), 0X for the negative control group. The challenge doses were administrated to birds with a needle (100 µl/egg). Marcano (2017) assessed the LaSota live vaccine strain overdose challenge, which was $10^{3.5}$ 50% embryo infectious dose (EID_{50}/egg) at 18 days of embryonation. We applied 1X of the recommended dose ($10^{6.6}$ EID_{50}/dose) because we wanted to induce marked immune response changes.

Experimental Design

Embryonated eggs were randomly divided into three main groups (n=100/ group) according to the light wavelengths (blue, white, and control). Each main group was divided into two sub-groups, either challenged or non-challenged (Figure 6). On day 18 of age, one subgroup of each main group was challenged with Newcastle Disease Vaccine, B1 Type, LaSota Strain, Live Virus (Product Code: ND1820, Merial). Lung samples were collected 24, 36, 48, 60, 72, 84, and 96 hours post-challenge (Table 5).

Challenged was done by *in ovo* inoculation with 100 µl of NDV viral suspension ($10^{6.6}$/dose EID_{50}) injected into the amniotic fluid with 21 gauge, 1 inch needle, preceded by puncturing the eggshell with an 18 gauge needle. The injection holes were sealed with food-safe grade clear silicone to prevent infection and dehydration. Challenged and non-challenged embryos were then placed back on the hatching tray in separate compartments in the same incubator.

Figure 6: Experimental design. First day of incubation all eggs were randomly divided into three main groups according to the light source. Blue and white lights were provided from the first day of incubation until the end of the study in a form of photoperiods of 12h intervals (light: dark) except for the control (24h dark) group. On embryonic day 18, challenged groups were challenged with NDV virus. Lung samples collection were performed on embryonic day 19-21.



Sample Collection

At day 19 of incubation (24 hours post challenge) lung tissue samples were collected for expression analysis from four embryos at each of seven time points over an 84-h period (12-h intervals) (Table 5). Four embryos were randomly selected from each group, checked for viability and then the eggshell was broken open and the embryo was decapitated for lung tissue harvesting. The hatched chicks were euthanized humanely using exposure to CO2, followed by cervical dislocation prior to lung tissue harvesting.

All tissues were dissected within 20 minutes post mortem and preserved directly in RNALater solution in a ratio of 1 gram tissue: 5mL RNALater solution (Ambion Inc, ThermoFisher Scientific). Tissue samples were stored at 4°C for a minimum of 24h 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). A total of 168 samples were collected during the study with four replicates at each time point from all treatments.

Table 5: Tissue sample collection schedule during incubation.

Embryonic day (ED)	Time after challenge	Tissue harvested
ED19	24 hours	Lung
ED19	36 hours	Lung
ED20	48 hours	Lung
ED20	60 hours	Lung
ED21	72 hours	Lung
ED21	84 hours	Lung
ED 22	96 hours	Lung

RNA Isolation and Quantification

RNA was extracted from the lung samples using MagMAX™ mirVana™ Total RNA Isolation Kit and a magnetic bead-based automated system using 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, 100 µl of lysate was added to 100 µl isopropanol and

20 µl of binding beads. The lysate mixture then shook for 5 minutes at 950 rpm, then 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). An initial RNA concentration measurement was done using NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific) to determine the range of RNA concentration, 260/280 ratio for protein contamination and 230/260 ratio for extraction reagents contamination. Following, the total RNA isolates was subjected to quality control step using Bioanalyzer 2100 (Agilent Technologies, USA) using Agilent RNA 6000 Nano kit (No: 5067-1511) to specify the whole sample RNA integrity number (RIN). All RNA samples that have RIN 7.0 or higher its concentration were measured using 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 passed these quality control checks were normalized by dilution 100 ng/µL using nuclease-free water (NF water) and used for library preparation.

Virus Particle Enumerations

All lung tissue samples from the experimental challenge groups were examined for the quantity of NDV through qPCR test. The qPCR-based Taqman methods was prepared using Techne qPCR kit (Techne Catalog number: EW-93970-45). A master mix

was prepared using the oasig™ lyophilized OneStep 2X RT-qPCR according to manufacturer protocols and added to each well of the 384 well plate.

RNA Library Preparation and Transcriptome Profile Generation

Library preparation for RNA sequencing (RNAseq) with Illumina was performed in Dr. Athrey's lab with the Lexogen QuantSeq 3'mRNA Library Prep Kit (Lexogen, Vienna, Austria). A total of 168 single-indexed libraries were prepared, 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™ dsDNA HS Kit (Catalog number: Q33231). Two batches of 96 and 72 libraries 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

All bioinformatics analysis was performed with open-source tools and using well established RNAseq analysis pipelines. 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). Followed by

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, Jan 2020) the short-read de-novo splice mapper STAR program, where reads mapped to exons were counted using STAR "--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 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 at any given gene less than one count per million (CPM) at least in two columns were filtered out from the generated object of the "DGEList" function in the further analysis. The estimated common dispersion was calculated 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. Tagwise dispersion was calculated for replicates pairs to assess the consistency between biological replicates in the same treatment. The quasi-likelihood F-test (QLF) likelihood ratio test 'glmLRT' function was used in full factorial design specified by

"my.contrasts" function to test for significant differential expression between 168 groups at an FDR < 0.05. A power analysis based on actual dispersion (common dispersion of 0.063) was performed in the RNAseq data using ssizeRNA 1.3.2 (Bi & Liu, 2016), which showed that our design had 98.9% power to detect Log2-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 aim of the study by revealing the impact of the light source on innate immune development.

The canonical pathways mean which signaling pathways are constitutively active (naturally occurring inside the body, tissues, or cells). While the non-canonical pathways mean inducible signaling pathways (might be from drugs or chemicals from outside of the body).

Results

RNA Sequencing Results

This study was performed to identify the effect of wavelength and photoperiods on the immune response of chick embryos with the greatest differential gene expression patterns post-challenge to ND virus. Eighteen-day old chick embryos were infected with

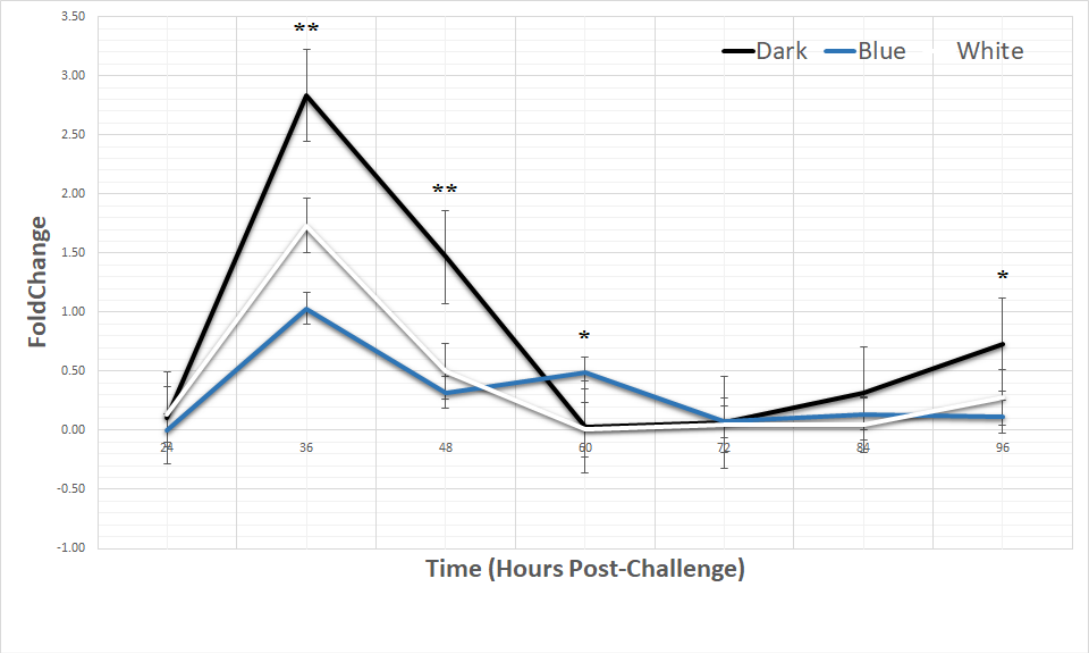
the LaSota strain of NDV via the amniotic fluid. Lung tissues were harvested at various times post-challenge; the results shown here are for two time points, 24 and 96 hours post-challenge.

NDV Particle Enumerations

ND virus particle propagation study was conducted in the lung tissue of chick embryos over seven time point's post-NDV *in ovo* challenge (24, 36, 48, 60, 72, 84, and 96 hours) under three different incubations conditions, dark (0L:24D), blue (12L:12D), and white (12L:12D). The ND virus enumeration was performed through qPCR-based Taqman methods for high specificity. The changes of ND virus propagation under three different incubations conditions are presented in Figure 7. At 24 hours post-NDV challenged, all treatments (dark, blue, and white) had the same non-significant fold change (FC) difference between each other (0.11, 0.00, and 0.13 FC, respectively), whereas the highest fold change detected at 36-h post-NDV challenge, where the dark treatment (2.84 FC) had a significantly higher fold change virus propagation than blue and white light treatments (1.03 and 1.73 FC, respectively) ($P < 0.01$). Another spike of virus propagation in blue light treatment (0.49 FC) at 60 hours post-challenge was observed. When comparing the viral proliferation at 96-h post-challenging between the light treatments, the blue treatment had the lowest viral propagation (0.11 FC), and the dark treatment had the highest viral propagation (0.73 FC) ($P < 0.05$).

Figure 7: Average virus fold change (±SE) between 24-h to 96-h post-challenge for eggs incubated under 3 different lighting conditions. The black line indicates that the 0 h of light and 24 h of darkness (0L:24D) virus fold change differed from the 12 h of light (blue or white) and 12 h of darkness (12L:12D). (* = $P < 0.05$, ** $P < 0.01$).

A.



Innate immune response in chick embryos under different lighting photoperiods and wavelengths

The RNA-seq from the challenged and non-challenged results were analyzed as described in Materials and methods to identify transcriptional changes occurring in innate immunity in response to ND virus challenge under different lighting treatments by comparing conventional dark, blue light, and white light. The transcriptome sequence data from each group were analyzed using the EdgeR. Filtering the EdgeR results for significance (FDR < 0.05) produced a final dataset including 3705 genes that were differentially expressed between challenged and non-challenged dark photoperiods groups, contains 2069 genes that are upregulated and 1636 genes that are downregulated in response to ND virus challenge (Figure 8 A). Whereas the challenged and non-challenged blue and white photoperiods groups showed 3840 and 1642 genes differentially expressed, respectively. Of these, 2118 and 905 genes that are upregulated, and 1722 and 737 genes that are downregulated, respectively (Figures 8 B and C, respectively).

Genes identified as significantly differentially expressed were further investigated through gene ontology (GO) enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.8 (Huang, Sherman, & Lempicki, 2009; Huang, Sherman, Zheng, et al., 2009); analysis was done by using available Entrez gene IDs against *Gallus gallus* references. The results of the analysis with top enriched term are presented in Table 6; these records contain GO terms indexed under different category terms for direct involvement in biological process (BP),

direct localization to cellular compartment (CC), and direct involvement in molecular function (MF), Kyoto Encyclopedia of Genes and Genomes pathways (KEGG-pathway), Uniprot Keywords (Up_Keywords), and type of the active tissue (Up_Tissue).

The clustering analysis of the conventional dark treatment genes included enriched clusters for innate immune response (19 genes), immunity (13 genes), inflammatory response (9 genes), and antigen processing and presentation (8 genes). The clustering analysis of the blue light genes included enriched clusters for innate immune response (26 genes), Immunity (15 genes), Inflammatory response (10 genes), and toll-like receptor signaling pathway (10 genes). The clustering analysis of the white light genes included enriched clusters for MHC class I-like antigen recognition (5 genes), MHC classes I/II-like antigen recognition protein (5 genes), Immunoglobulin/major histocompatibility complex, conserved site (4 genes), and Immunoglobulin C1-set (4 genes). The clustering analysis with the highest enrichment scores for dark, blue, and white lighting groups are presented in Tables 7-9.

In agreement with previous light stimulation during incubation studies (Archer & Mench, 2013), a higher immune response in light incubated embryos than dark-incubated embryos, also observed an upregulation in genes involved in innate immune response, and inflammatory response.

Figure 8: Mean abundance plots (logFC by logCPM) of differential gene expression by light treatments for both challenged and non-challenged groups between 24 and 96 hours post-challenge. Red stars indicate upregulated genes (FDR < 0.05) while dashed gray lines indicate logFC 2 and -2.

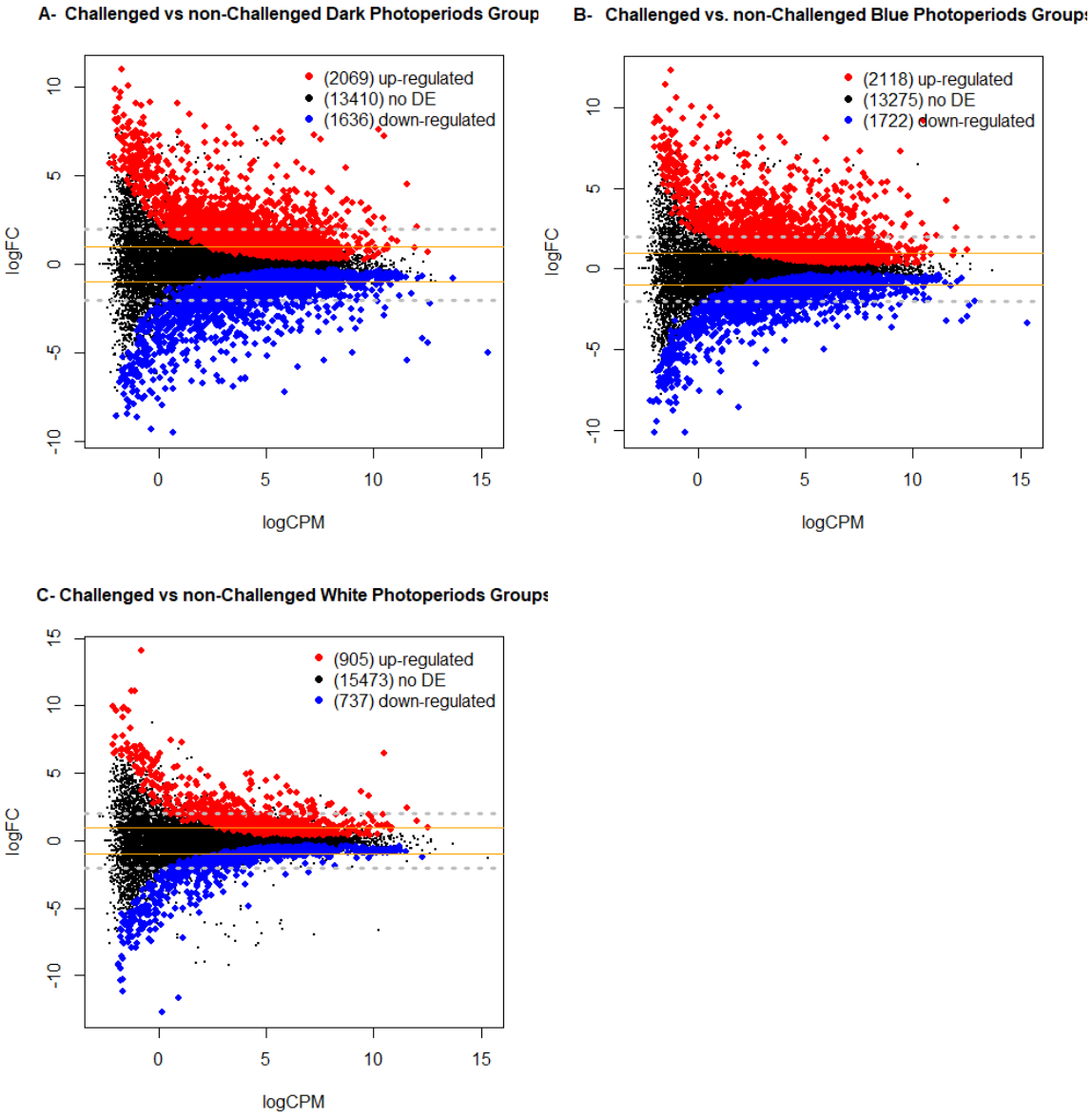


Table 6: GO terms indexed under different categories for Dark, Blue, and White treatments genes. The following categories presenting the analysis with the top enriched term resulting from DAVID-GO Functional Annotation Chart, represent the overlapping genes in both challenged and non-challenged groups

Dark Treatment	Blue Light Treatment	White Light Treatment
Biological process		
inflammatory response	inflammatory response	translation
immune response	toll-like receptor signaling pathway	glycolytic process
angiogenesis	immune response	programmed cell death
peptide cross-linking	innate immune response	ribosomal large subunit assembly
endodermal cell differentiation	proteolysis involved in cellular protein catabolic process	cytoplasmic translation
Cellular Component		
extracellular exosome	extracellular exosome	extracellular exosome
focal adhesion	focal adhesion	cytosolic large ribosomal subunit
cytosol	cytoplasm	blood microparticle
plasma membrane	membrane raft	membrane
extracellular space	extracellular space	myelin sheath
Molecular Function		
GTPase activator activity	GTPase activator activity	structural constituent of ribosome
signal transducer activity	GTP binding	poly(A) RNA binding
GTP binding	protein tyrosine kinase activity	threonine-type endopeptidase activity
tumor necrosis factor-activated receptor activity	identical protein binding	actin filament binding
structural constituent of ribosome	motor activity	identical protein binding
KEGG Pathways		
Cytokine-cytokine receptor interaction	Phagosome	Ribosome
Phagosome	Lysosome	Proteasome
Jak-STAT signaling pathway	Biosynthesis of amino acids	Glycolysis / Gluconeogenesis
Ribosome	Biosynthesis of antibiotics	Biosynthesis of amino acids
Biosynthesis of amino acids	Cytokine-cytokine receptor interaction	DNA replication
Up Keywords		
Signal	Signal	Proteasome
Ribosomal protein	Disulfide bond	Ribosomal protein
Glycoprotein	Immunity	Ribonucleoprotein
SH3 domain	Innate immunity	Cytoplasm
Phosphoprotein	Inflammatory response	Glycolysis

Table 7: Dark Treatment Genes. GO-Functional Annotation Clusters for dark treatment genes. The following clusters (1-5) resulting from DAVID-GO Functional Annotation Clustering, represent the overlapping genes in both challenged and non-challenged dark treatment groups

Cluster	ES	Category	Associated Terms	PValue	Fold Enrichment
1	8.68	UP_KEYWORDS	Transmembrane helix	3.17E-10	1.41
		UP_KEYWORDS	Transmembrane	4.27E-10	1.40
		UP_KEYWORDS	Membrane	1.99E-09	1.36
		GOTERM_CC_DIRECT	integral component of membrane	7.37E-08	1.39
2	5.11	UP_SEQ_FEATURE	signal peptide	3.86E-12	2.05
		UP_SEQ_FEATURE	disulfide bond	1.64E-04	1.70
		UP_KEYWORDS	Glycoprotein	4.41E-04	1.66
		UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	1.25E-02	1.39
3	4.15	INTERPRO	Immunoglobulin-like fold	2.24E-11	2.50
		INTERPRO	Immunoglobulin-like domain	1.00E-09	2.69
		INTERPRO	Immunoglobulin subtype	3.14E-04	2.08
		INTERPRO	Immunoglobulin V-set	3.79E-04	2.87
		SMART	IG	4.42E-04	2.02
		UP_KEYWORDS	Immunoglobulin domain	1.67E-03	2.50
		INTERPRO	Immunoglobulin subtype 2	1.87E-02	1.87
		SMART	IGc2	2.32E-02	1.82
		INTERPRO	Immunoglobulin I-set	4.79E-02	1.85
4	3.37	INTERPRO	MHC classes I/II-like antigen recognition protein	1.48E-04	6.31
		INTERPRO	Immunoglobulin/major histocompatibility complex	1.48E-04	6.31
		GOTERM_BP_DIRECT	antigen processing and presentation	5.36E-04	5.24
		INTERPRO	Immunoglobulin C1-set	6.97E-04	5.05
		SMART	IGc1	8.16E-04	4.90
		INTERPRO	MHC class I-like antigen recognition	8.43E-04	7.28
5	3.33	UP_KEYWORDS	Immunity	3.34E-06	5.23
		UP_KEYWORDS	Inflammatory response	2.10E-05	6.89
		GOTERM_BP_DIRECT	innate immune response	6.45E-05	2.93
		SMART	TIR	1.20E-04	6.44
		UP_KEYWORDS	Innate immunity	2.17E-04	5.17
		INTERPRO	Toll/interleukin-1 receptor homology (TIR) domain	2.92E-04	5.73
		GOTERM_BP_DIRECT	MyD88-dependent toll-like receptor signaling pathway	8.50E-04	7.26
		GOTERM_BP_DIRECT	toll-like receptor signaling pathway	1.52E-03	5.24
		GOTERM_MF_DIRECT	transmembrane signaling receptor activity	2.95E-03	4.01
		INTERPRO	Cysteine-rich flanking region, C-terminal	4.08E-02	2.29
		SMART	LRRCT	4.67E-02	2.22

ES = Enrichment score produced by Functional Annotation Clustering in DAVID.
Category Terms Defined: UP Keywords = Uniprot Keywords; 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; UP SEQ DIRECT = Uniprot Sequence Feature; INTERPRO = database of protein families.

Table 8: Blue Treatment Genes. GO-Functional Annotation Clusters for blue treatment genes. The following clusters (1-5) resulting from DAVID-GO Functional Annotation Clustering, represent the overlapping genes in both challenged and non-challenged blue treatment groups

Cluster	ES	Category	Associated Terms	PValue	Fold Enrichment
1	6.82	UP_SEQ_FEATURE	signal peptide	4.05E-13	2.04
		UP_SEQ_FEATURE	disulfide bond	1.12E-08	2.04
		UP_KEYWORDS	Glycoprotein	3.38E-05	1.80
		UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	3.59E-03	1.44
2	5.93	UP_KEYWORDS	Membrane	2.04E-07	1.31
		UP_KEYWORDS	Transmembrane	1.88E-06	1.30
		GOTERM_CC_DIRECT	integral component of membrane	2.16E-06	1.34
		UP_KEYWORDS	Transmembrane helix	2.26E-06	1.30
3	4.56	GOTERM_BP_DIRECT	innate immune response	2.02E-09	4.08
		UP_KEYWORDS	Immunity	5.72E-08	6.03
		INTERPRO	Toll/interleukin-1 receptor homology (TIR) domain	2.86E-07	7.96
		SMART	TIR	1.04E-06	8.01
		UP_KEYWORDS	Inflammatory response	1.95E-06	7.66
		GOTERM_BP_DIRECT	toll-like receptor signaling pathway	2.00E-06	7.62
		UP_KEYWORDS	Innate immunity	3.78E-06	6.32
		KEGG_PATHWAY	Toll-like receptor signaling pathway	7.63E-05	3.10
		GOTERM_BP_DIRECT	MyD88-dependent toll-like receptor signaling pathway	7.84E-04	7.39
		GOTERM_MF_DIRECT	transmembrane signaling receptor activity	2.80E-03	4.05
		GOTERM_MF_DIRECT	receptor activity	3.22E-02	2.39
		INTERPRO	Cysteine-rich flanking region, C-terminal	3.89E-02	2.31
		SMART	LRRCT	4.79E-02	2.21
4	4.40	INTERPRO	Immunoglobulin C1-set	1.22E-06	7.01
		INTERPRO	Immunoglobulin/major histocompatibility complex	1.28E-06	7.96
		SMART	IGc1	1.75E-06	6.70
		INTERPRO	MHC class I-like antigen recognition	4.55E-06	9.80
		INTERPRO	MHC classes I/II-like antigen recognition protein	1.47E-05	7.17
		GOTERM_BP_DIRECT	antigen processing and presentation	4.81E-04	5.33
		INTERPRO	MHC class I, alpha chain, alpha1/alpha2	1.50E-03	8.85
		GOTERM_MF_DIRECT	antigen binding	4.54E-02	4.83
5	4.06	INTERPRO	Immunoglobulin-like fold	2.88E-07	2.13
		INTERPRO	Immunoglobulin-like domain	4.83E-07	2.37
		INTERPRO	Immunoglobulin V-set	9.96E-05	3.08
		INTERPRO	Immunoglobulin subtype	1.33E-03	1.95
		SMART	IG	2.26E-03	1.87
		UP_KEYWORDS	Immunoglobulin domain	1.11E-02	2.19

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; UP SEQ DIRECT = Uniprot Sequence Feature; KEGG PATHWAY = KEGG Pathway; INTERPRO = database of protein families.

Table 9: White Treatments Genes. GO-Functional Annotation Clusters for white treatments genes. The following clusters (1-5) resulting from DAVID-GO Functional Annotation Clustering, represent the overlapping genes in both challenged and non-challenged white treatments groups

Cluster	ES	Category	Associated Terms	PValue	Fold Enrichment
1	5.60	UP_KEYWORDS	Disulfide bond	2.62E-08	2.57
		UP_KEYWORDS	Secreted	3.09E-07	3.39
		UP_SEQ_FEATURE	signal peptide	2.15E-05	2.16
		UP_SEQ_FEATURE	disulfide bond	2.19E-04	2.30
2	2.73	INTERPRO	MHC class I-like antigen recognition	6.80E-05	20.95
		INTERPRO	MHC classes I/II-like antigen recognition protein	4.17E-04	13.61
		INTERPRO	Immunoglobulin/major histocompatibility complex	5.45E-03	10.89
		INTERPRO	Immunoglobulin C1-set	1.03E-02	8.71
		SMART	IGc1	1.49E-02	7.56
3	2.41	GOTERM_MF_DIRECT	fatty acid binding	1.12E-03	18.25
		INTERPRO	Lipocalin/cytosolic fatty-acid binding protein domain	3.38E-03	12.81
		INTERPRO	Calycin	7.17E-03	9.90
		INTERPRO	Calycin-like	8.13E-03	9.47
4	2.17	INTERPRO	Ribonuclease A, active site	1.95E-03	40.84
		INTERPRO	Ribonuclease A	1.95E-03	40.84
		INTERPRO	Ribonuclease A-domain	1.95E-03	40.84
		SMART	RNAse_Pc	2.56E-03	35.42
		UP_SEQ_FEATURE	region of interest:Substrate binding	1.44E-02	7.46
		GOTERM_MF_DIRECT	ribonuclease activity	1.58E-02	15.06
		UP_SEQ_FEATURE	Proton donor	2.37E-02	6.21
		GOTERM_MF_DIRECT	endonuclease activity	4.37E-02	8.86
5	2.02	INTERPRO	Proteinase inhibitor I25, cystatin	6.57E-03	23.34
		SMART	CY	8.58E-03	20.24
		GOTERM_MF_DIRECT	cysteine-type endopeptidase inhibitor activity	1.58E-02	15.06

ES = Enrichment score produced by Functional Annotation Clustering in DAVID.
Category Terms Defined: UP Keywords = Uniprot Keywords; GOTERM MF DIRECT = GO Term for Direct Involvement in Molecular Function; UP SEQ DIRECT = Uniprot Sequence Feature; INTERPRO = database of protein families.

Pathways Activated by Differentially Expressed Genes

Ingenuity Pathway Analysis (IPA) was used to identify the most significant gene networks and to categorize differentially expressed genes (DEGs) in specific pathways and functions for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) (Table 10). The IPA results classified the specific pathways and functions into different categories; some of these categories are Top Canonical pathways, Upstream Regulators, Molecular and Cellular Functions, Physiological System Development and Function, and My Top Pathways (Table 11).

In the dark treatment, Top Canonical pathways demonstrating STAT3 pathway, EIF2 Signaling, PI3K/AKT Signaling, mTOR Signaling and Cardiac Hypertrophy Signaling. Several of the specific canonical pathways identified were repeatedly observed overall three treatments. This was also observed of the upstream regulators and Molecular and Cellular Functions by IPA (Table 11). The most observed pathways over all three treatments in the Top Pathways include NF- κB Signaling.

Table 10: Summary of the number of differentially expressed genes by IPA for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D). The table shows the up, down, and total differentially expressed genes (FDR < 0.05).

	Dark Treatment	Blue Light Treatment	White Light Treatment
DE UP	1657	1753	778
DE Down	1228	1170	504
Total DE	2885	2923	1282

Table 11: Summary of differential gene expression results from the results of the pathway analysis in IPA. The table shows the specific information for each light treatment and top pathway and function terms.

Dark Treatment	Blue Light Treatment	White Light Treatment
Top Canonical Pathway		
STAT3 Pathway	STAT3 Pathway	EIF2 Signaling
EIF2 Signaling	Iron homeostasis signaling pathway	Phagosome Maturation
PI3K/AKT Signaling	Role of Macrophages, Fibroblasts	Protein Ubiquitination Pathway
mTOR Signaling	and Endothelial Cells in Rheumatoid Arthritis	Insulin Secretion Signaling Pathway
Cardiac Hypertrophy Signaling	Axonal Guidance Signaling	Lipid Antigen Presentation by CD1
Upstream Regulators		
dexamethasone	lipopolysaccharide	MYC
TNF	dexamethasone	torin1
lipopolysaccharide	TNF	TP53
IL4	TGFB1	LARP1
beta-estradiol	beta-estradiol	HNF4A
Molecular and Cellular Functions		
Cellular Movement	Cell Death and Survival	Cell Death and Survival
Cell Death and Survival	Cellular Movement	Cellular Compromise
Cellular Function and Maintenance	Cellular Function and Maintenance	Protein Synthesis
Cellular Assembly and Organization	Cellular Development	RNA Damage and Repair
Cellular Development	Cellular Growth and Proliferation	Cellular Assembly and Organization
Physiological System Development and Function		
Hematological System Development and Function	Hematological System Development and Function	Organismal Survival
Tissue Morphology	Tissue Morphology	Hematological System Development and Function
Lymphoid Tissue Structure and Development	Organismal Survival	Hematopoiesis
Organismal Development	Immune Cell Trafficking	Tissue Development
Immune Cell Trafficking	Organismal Development	Connective Tissue Development and Function
My Top Pathways		
NF-B Signaling	NF-B Signaling	Mitochondrial Dysfunction
Rho-GTPase Signaling	MAPK	Rho-GTPase Signaling
RhoGDI Signaling	Rho-GTPase Signaling	NF-B Signaling
MAPK	RhoGDI Signaling	P53

Differentially Expressed Genes in Response to ND Virus Challenge

The results of differential gene expression analysis from EdgeR were used in the DAVID database considering only the differentially expressed genes (FDR < 0.05). The DAVID database identified several of the specific pathways that were repeatedly observed over all three different methods of incubations, dark, blue, and white. To narrow down the genes that are differentially expressed in response to ND virus challenge, a list of shared and unique genes was generated from all comparisons. First, the focus was placed on selecting out the genes that are associated with the innate immune response, immune response, inflammatory response, and defense response to virus. A comparison between the differentially expressed genes ($P < 0.05$) was used to generate unique and shared gene lists. The shared list helped identify which treatments had the better response to ND virus challenge based on the fold change for the gene expression (Figure 9).

Of interest in the shared list are multiple genes associated with innate immune response (MX1, PTX3, S100A12, CYBB, IFIH1, EXFABP, BLK, TLR2B, TLR1B, TLR4, B2M, TLR7, JCHAIN, ANXA1, EIF2AK, LYN, CYBA, TEC, JAK2) (Figure 10), immune response (CCAH221, CSF3, CCL4, CCR2, CXCL13L3, CD244, CD74, CTSS, LCP2, IL18, FYB, B2M, C7, TLR7, TNFSF15, IRF8, IL15, CXCL14, TNFRSF1A, TNFRSF11A, TNFRSF1B, ENPP2, TGFBR3, TNFSF10) (Figure 11), inflammatory response (CCL26, CCL4, S100A12, CCR2, CXCL13L3, CYBB, EXFABP, LIPA, FABP4, NOS2, TNFSF4, TLR2B, TLR1B, IL18, TLR4, PTAFR, TLR7, ANXA1, MYD88, LYN, PTGFR, TNFRSF1A, TNFRSF11A, CYBA, TNFRSF1B, HYAL2, ADAMTS12, JAK2, AHSG) (Figure 12), and defense response to virus (IFIT5, MX1,

DDX60, TLR7, CARD9, F2RL1, BNIP3, HYAL2) (Figure 13). Among the genes of the shared list, the differentially expressed genes were uniformly higher in the blue treatment than in the dark and white treatment.

Figure 9: Bar plots showing the LogFC for the shared and unique differentially expressed genes list for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).

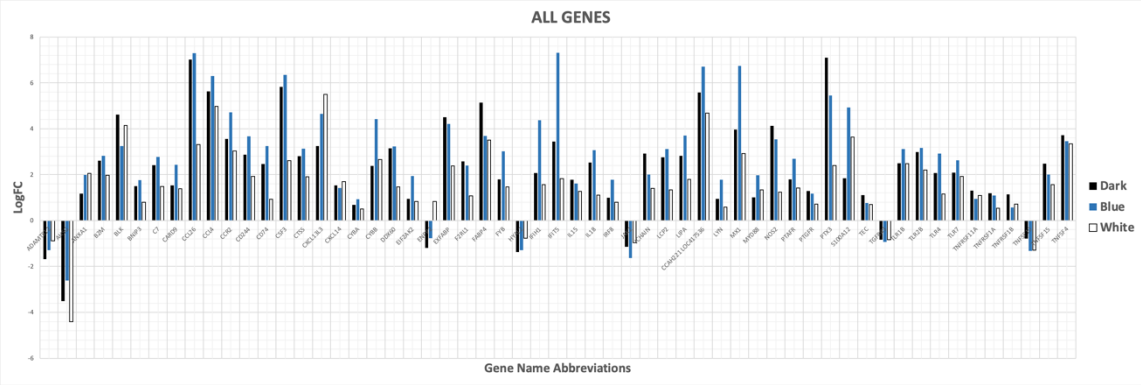


Figure 10: Bar plots showing the LogFC for the differentially expressed genes that are related to the innate immune response for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).

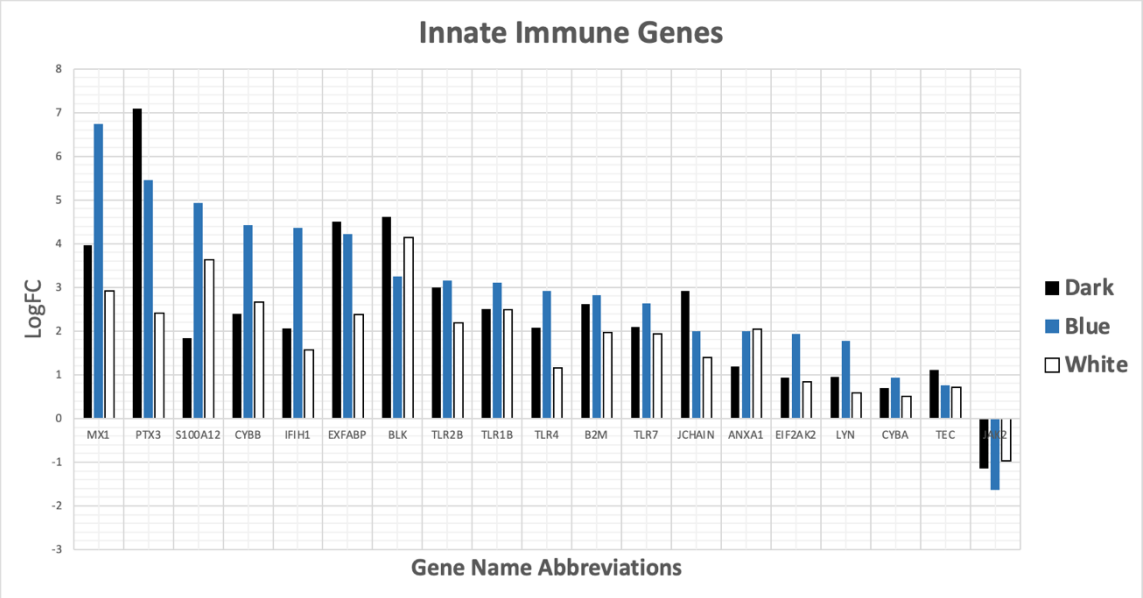


Figure 11: Bar plots showing the LogFC for the differentially expressed genes that are related to the immune response for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).

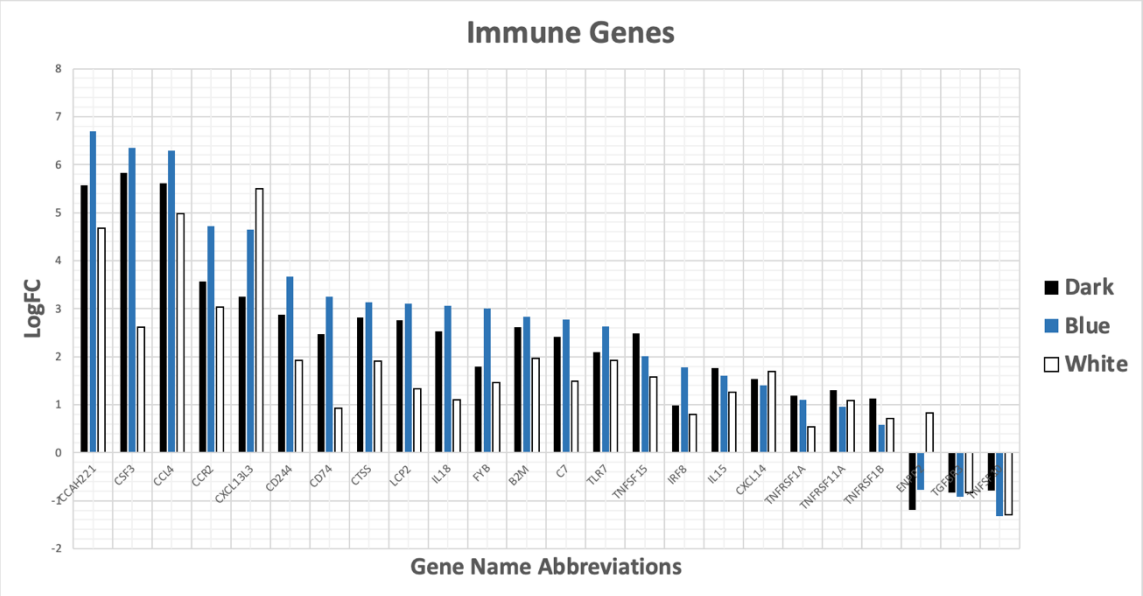


Figure 12: Bar plots showing the LogFC for the differentially expressed genes that are related to the inflammatory response for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).

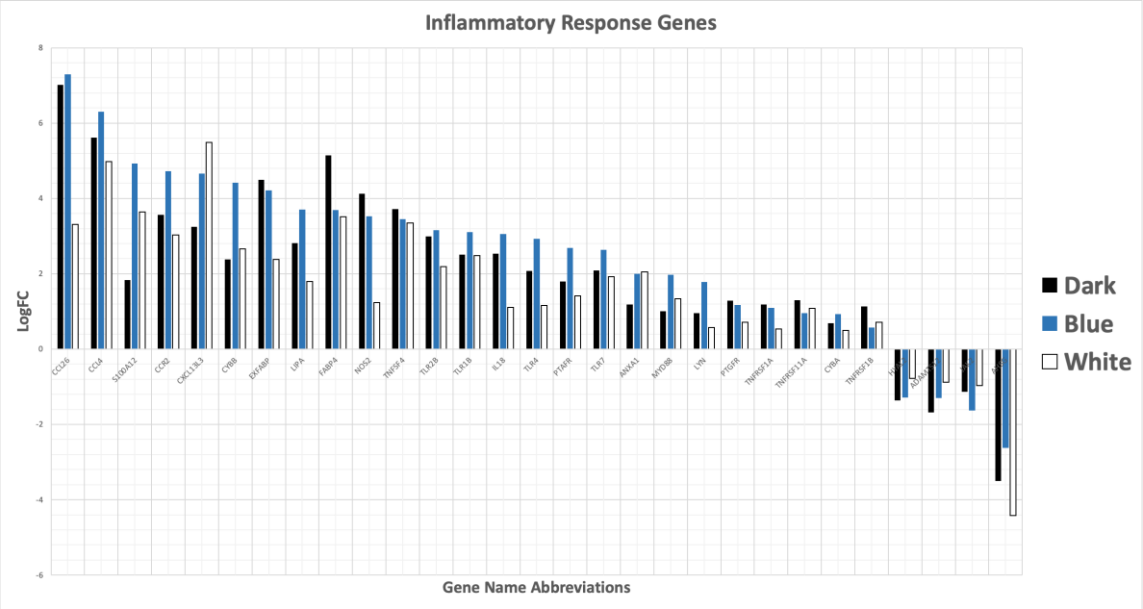
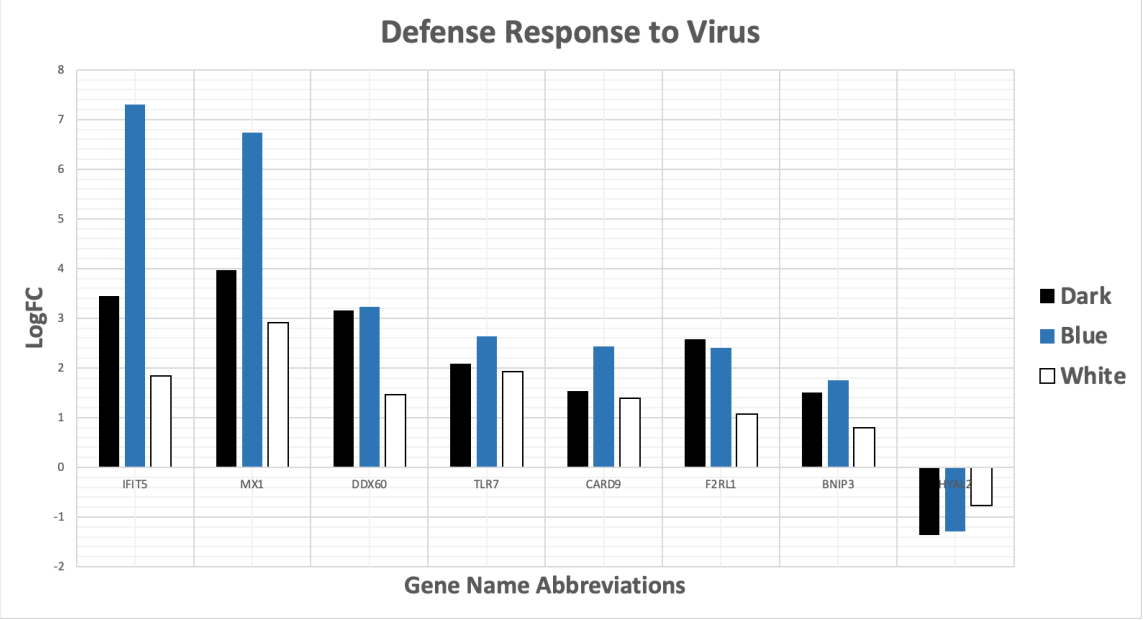


Figure 13: Bar plots showing the LogFC for the differentially expressed genes that are related with the defense response to virus for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).



Discussion

Understanding the genetics and mechanisms influencing the immune response in chick embryos to NDV infection, especially in the current rapid development of the poultry industry, will promote the genetic improvement of poultry. Thus identifying the innate immune genes that control NDV will aid to reducing susceptibility to NDV infection. Our study began to specify the innate immune genes in response to ND virus challenge under different lighting regimes in lung tissue. The results showed a variation in the level of the innate immune response for the different lighting treatments, dark (0L:24D), blue (12L:12D), and white (12L:12D). The research on ND viral response has been performed using certain phenotypic traits such as antibody response, viral load, weight, mortality, and morbidity (Deist et al., 2017). However, since the focus was on chick embryos rather than post-hatched chicks, the viral enumeration was used as our estimator to evaluate the level of susceptibility under different lighting treatments to ND virus challenge as it is a direct quantification method of the virus's ability to replicate. The viral particle enumerations were ascertained in lung tissues of chick embryos at 24, 36, 48, 60, 72, 84, and 96 hours post-challenge, when the LaSota strain of ND viral suspension was injected directly into the amniotic fluid. It was clearly significant ($P < 0.01$) that the dark treatment had the highest viral particle enumerations at 36 and 48-h post-challenge through qPCR-based Taqman methods. Also, at 96-h post-challenging, the dark treatment had the highest viral particle enumerations, whereas the blue treatment had the lowest viral particle enumerations ($P < 0.05$). Here, we showed that the blue light treatment had the lowest viral particle enumerations, followed by the white light treatment, then the dark

treatment (Figure 7). Until now, some studies have suggested that light incubated chick embryos have higher immune responses than dark incubated chick embryos (Archer & Mench, 2013; Hogshead, 2015). However, we have now provided evidence that these claims are valid, although further research is needed to support these results through studies examining the level of the immune response in post-hatching chicks to fully understand the level of NDV response.

Since the innate immunity for chick embryo becomes immunocompetent pre-hatching, we here characterized the transcriptional profiles response of innate immune genes to ND virus challenge in chick embryos incubated in different lighting treatments at 96-h post-challenge. The clustering patterns from DAVID-GO Functional Annotation Clustering showing that the dark and blue lighting treatments appear similarly related to each other than the white lighting treatment (Table 7-9). Consistent with previous studies examining the effects of different lighting treatments on immune response (Sadrzadeh et al., 2011; Xie et al., 2008).

By studying specific innate immune genes, there are differences in expression patterns between lighting treatments. Some genes stand out as being differentially expressed between dark versus blue treatment, including IFN-γ. Another set of innate immune genes, expressed differently in the dark and blue versus white, includes IL6, IL8, Interferon regulatory factor 1 (IRF1), and Nitric oxide synthase 2 (NOS2). Studies examining these genes more thoroughly may reveal important mechanisms associated with NDV response.

A remarkable gene that was differentially expressed in the blue light treatment is IFN-γ (Supplementary Table). Innate immune response to NDV initiates by expression of IFNs, which are key antiviral effector molecules and pro-inflammatory cytokines (Alkie et al., 2019; Reemers et al., 2010; Reuter et al., 2014). In this study, logFC for IFN-γ in the blue lighting treatment was significantly ($P < 0.01$) higher than the dark and white lighting treatments. This was also true of the upstream regulators identified by IPA (Figure 14). More specifically, IFN-γ is negatively correlated with the viral particle enumerations in the blue light treatment.

Another gene, IL6, is differentially expressed in both dark and blue lighting treatments rather than white lighting treatment (Supplementary Table), which may influence the innate immune response to ND virus challenge. However, the expression of IL6 is negatively associated with viral particle enumerations in the blue light treatment, but positively correlated with the white light treatment. This is interesting since high viral particle enumerations in the dark treatment are associated with higher fold changes of IL6, but on the other hand, in the blue light treatment, this is the opposite and low viral particle enumerations are associated with higher fold changes in gene expression. The upregulation of the interleukins significantly influences many pathways, including the IL6 signaling pathway, the TLR pathway, and pattern recognition receptors (PRRs) in recognition of viruses. These signaling pathways play key roles in the innate immune response to pathogens and control infectious diseases in poultry (Wigley & Kaiser, 2003). IPA identified the upstream regulators of IL6 in the dark treatment as presented in Figure 15.

Figure 14: Ingenuity Pathway Analysis (IPA) for the ND virus challenged vs. non-challenged in blue light treatment (96-h post-challenge). Pathway analyses were performed using the IPA (Qiagen Inc.). Only genes significant at FDR <0.05 were included in pathway analyses. Dashed lines indicate direct relationships, while solid lines indicate indirect relationships. The orange lines represent activation; the blue lines represent inhibition, or an unknown relationship (grey).

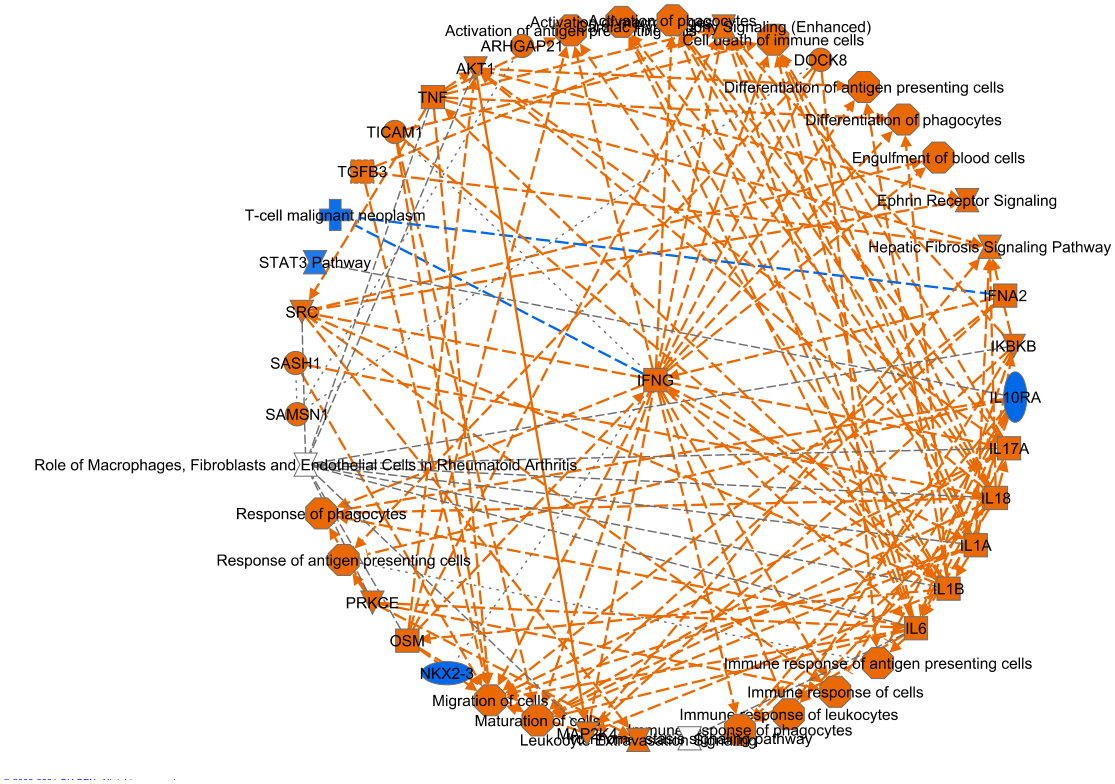
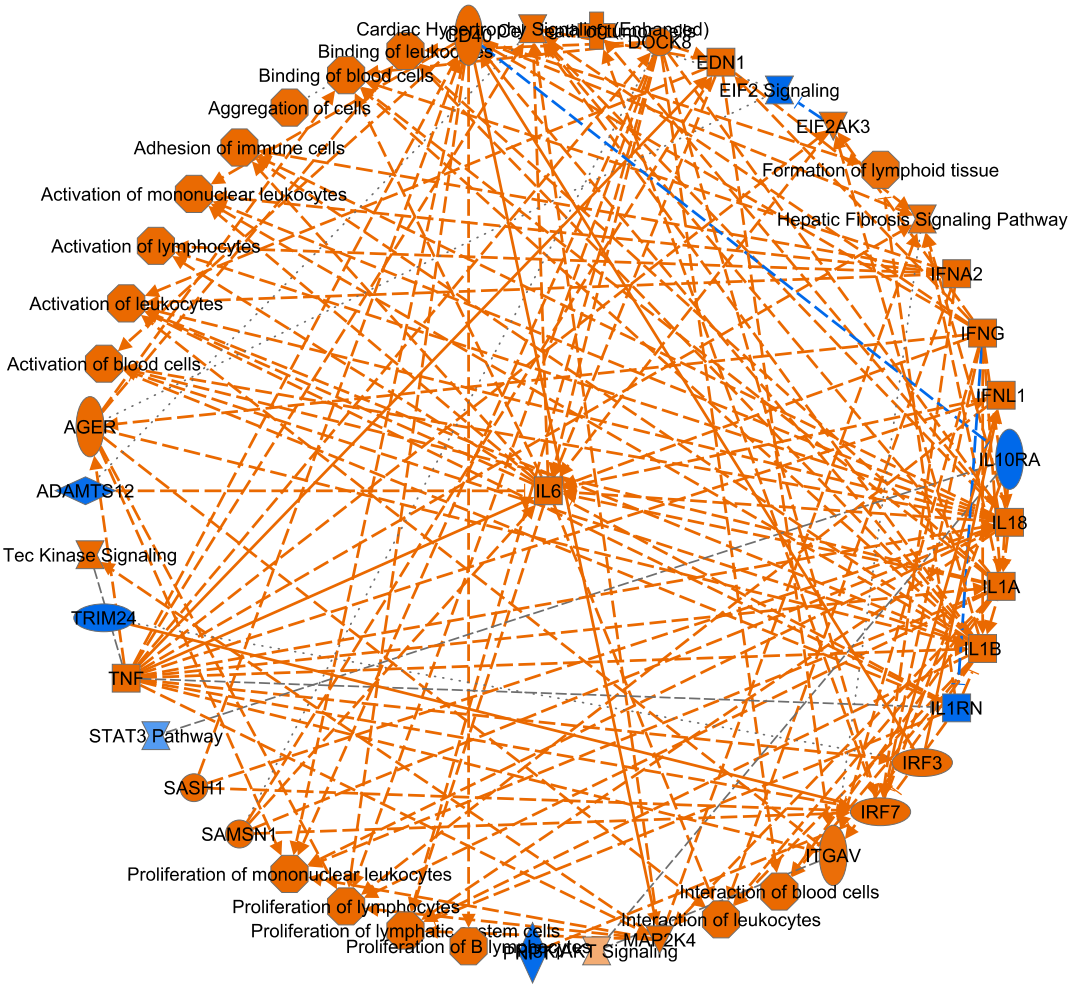


Figure 15: Ingenuity Pathway Analysis (IPA) for the ND virus challenged vs. non-challenged in dark treatment (96-h post-challenge). Pathway analyses were performed using the IPA (Qiagen Inc.). Only genes significant at FDR <0.05 were included in pathway analyses. Dashed lines indicate direct relationships, while solid lines indicate indirect relationships. The orange lines represent activation; the blue lines represent inhibition, or an unknown relationship (grey).



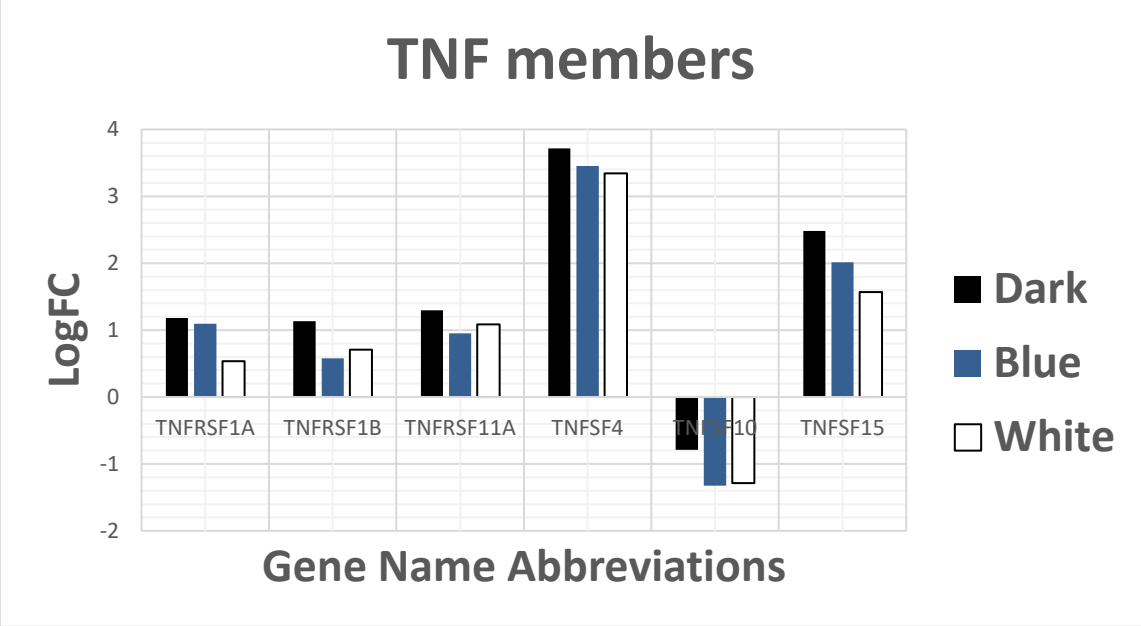
Another gene is NOS2. NOS2 is commonly upregulated in response to many virus infections. NOS2 is responsible for inducing nitric oxide (NO), a free radical linked to play multiple regulations of immune responses, particularly in the clearance of viruses

(Kapczynski et al., 2013). In this study, white light treatment demonstrated lower differential expression of NOS2 than the dark and blue light treatments. However, it is important to know that an high increase in NO production may do more harm than good (Burggraaf et al., 2011; Rue et al., 2011).

Curiously, Myxovirus (Mx1) and Interferon-induced protein with tetratricopeptide repeats 5 (IFIT5) were negatively correlated with viral particle enumerations (Figure 13). Both genes responded differently in the blue light treatment versus dark and white light treatments; they were lower differentially expressed in the dark and white light treatments. Mx1 and IFIT5 have a broad spectrum of defense responses to virus activity (Li et al., 2017; Yin et al., 2010). Those genes, mainly due to the high expression in the blue light treatment, are strong candidates for future studies to understand how the upregulated expression of Mx1 and IFIT5 plays a role in responding to ND virus challenge in the blue light incubation method.

TNFR receptor family such as TNFRSF1A, TNFRSF1B, TNFRSF11A, TNFSF4, TNFSF10, and TNFSF15. In this study, TNF members expressed differently within the same group but were a part of the conserved response between all three lighting treatments (Figure 16). TNF members were differentially upregulated in all lighting treatments except TNFSF10 which was downregulated in all lighting treatments. The expression of ND viral HN molecules leads to increased production of TNFs, which further upregulates tumoricidal activity and apoptosis (Lam et al., 2011). Demonstrating these genes may be a part of the conserved response to ND virus challenge regardless of lighting treatments.

Figure 16: Bar plots showing the LogFC for the differentially expressed TNF members for the three different methods of incubation, dark (0L:24D), blue (12L:12D), and white (12L:12D) ($P < 0.05$).



In the dark and blue light treatments, IL8 and IRF1 were part of the conserved response to the ND virus challenge (Supplementary Table). The expression of IL8 and IRF1 in the white light treatment was just below the cutoff for significance ($P < 0.05$).

Through studying these genes that are important in response to ND virus challenge in the different lighting treatments, it is interesting to note that all of these genes are crucial for the innate immune activation and defense response to the ND virus. This poses an interesting hypothesis; the basal levels of innate immune gene expression are higher in the blue light regime, contributing to higher fold changes for the innate immune genes after the ND virus challenge. Understanding the pathways and mechanisms these genes are

involved in under different lighting regimes may uncover new information that may have otherwise been overlooked when studying ND virus response.

Taking these data together, we can propose a set of genes that are differentially expressed in response to the ND virus challenge under different lighting regimes. These genes may serve as possible candidates for future studies on response to NDV to determine genes for breeding strategies to improve NDV resistance. Prospective studies could be expanded to include other tissues from the chick embryos, and possibly other time points of tissue harvest, more poultry lines (broilers, breeders, and laying hens) and study different pathogens, as well as studying the post-hatched chicks immune response are needed to fully understand the ND virus response in chickens.

CHAPTER IV

CONCLUSIONS

The extensive use of currently available ND vaccines, biosecurity, rapid diagnostics, and other containment measures appears to be keeping NDV under control in developed countries. However, as evidenced by the multiple outbreaks occurring throughout the world, current vaccination strategies are not fully effective under different environmental conditions. The development of new concepts for generating new ND vaccines is needed to effectively control NDV infection and to improve the immune response induced by the vaccines. Currently, phenotypic traits such as antibody response, viral load, weight, mortality, and morbidity are considered the standard gold method for NDV studies. However, molecular-based assays such as qPCR, RT-PCR, and RNAseq can specifically detect NDV and its response. Thus, it is crucial to investigate the chemical and physical characteristics of the ND virus as well as specifically identify the immune response.

Overall, this study has validated using the chick embryos as a model to examine the response to ND virus challenge under different lighting regimes. First, the results indicated that the optimum diluent pH for effective delivery of the NDV live vaccines for administration is 7.00. The 1X dose of the live ND vaccine ($1 \times 10^{6.6}$ EID_{50}) is considered a challenge at 18 days of embryonic development. Second, throughout this study, we were able to uncover the immune response in three different lighting regimes. We hypothesized

that the lighting regime might play a role in regulating the innate immune response to the ND virus challenge.

Among the shared list of the differentially expressed genes were uniformly higher in the blue light treatment than in the dark and white light treatments. The comparison between the dark and blue light treatments validated that they have similar differences in some immune responses by expressing similar genes in both treatments; however, both lighting treatments respond differently in viral particle enumerations. Also, this study was able to uncover specific genes that are differentially expressed between different light treatments. These genes, including (Mx1, IFIH1, IFIT5, TLR1B, TLR2B, TLR4, TLR7, JAK1, JAK2, CCL4, CXCL13L3, IL15, IL18, IRF8, NOS2, and MYD88).

This study established a framework for multiple prospective studies to examine the innate immune response of chick embryos under different lighting regimes and susceptibility to different pathogens. In order to fully understand the innate immune response described in this study, it is important to study the susceptibility of NDV on post-hatched chicks that were incubated under different lighting regimes. This will allow for further associations of the lighting regime and the innate immune genes with the level of NDV susceptibility. Overall, this study has provided a new tool to examine the innate immune response to NDV and multiple different poultry pathogens. Future studies would benefit from including chick embryos from different poultry breeds and lines as well as examining other poultry pathogens using the chick embryos. To the best of our knowledge, this is the first study on the innate immune response to an *in-ovo* ND virus challenge under different light regimes.

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APPENDIX A

SUPPLEMENTARY TABLES

Supplementary Table. logFC data for the differentially expressed genes. Only genes significant at $P < 0.05$ were included.

	Dark	Blue	White
Gene Name	logFC	logFC	logFC
IL4I1	6.77	6.02	4.45
RACK1	-0.59	-0.40	-0.72
RPL9	-0.59	-0.49	-0.47
PRNP	2.89	2.53	1.12
TMEM183A	-1.08	-0.40	-0.62
AHCYL1	-0.73	-0.70	-0.89
ACE	-1.62	-0.93	-1.46
SLC1A6	3.24	2.13	1.51
BTN1A1	3.29	3.74	3.48
ANGPTL4	2.22	1.62	2.43
PSMD7	0.41	0.50	0.62
RPL22	-0.61	-0.58	-0.94
GBP1	1.13	1.82	0.79
STX12	0.75	1.03	0.90
THEMIS2	4.13	5.59	3.26
RAB7B	2.85	3.52	2.62
CTSS	2.81	3.13	1.91
EYA3	0.51	0.73	0.35

	Dark	Blue	White
Gene Name	logFC	logFC	logFC
PSMA6	0.62	0.68	0.47
SH3D19	1.47	1.15	0.98
B4GALT2	-0.49	-0.43	-0.45
TMEM53	0.71	1.34	0.85
RPS8	-0.73	-0.50	-0.50
PLK3	1.36	1.90	1.61
TSPAN8	3.20	5.76	4.23
DOCK8	2.04	1.89	0.74
LGR5	-1.88	-1.92	-1.27
VLDLR	1.58	1.56	0.88
PNN	0.43	0.41	0.37
SLC35B2	1.21	1.79	0.75
HSP90AB1	0.58	0.54	0.55
PLK4	1.39	1.10	1.14
ENTPD5	-0.99	-0.81	-0.98
ABCD4	2.36	3.32	1.51
VRTN	3.37	4.20	4.14
PRDX1	0.91	1.46	0.85

	Dark	Blue	White
Gene Name	logFC	logFC	logFC
TGFBI	1.20	0.74	1.49
KIRREL	1.37	1.04	1.27
CXCL13L3	3.25	4.66	5.49
MMRN2	-2.07	-1.45	-0.69
SMAD7B	5.18	7.17	-6.10
SEMA3F	2.13	2.00	1.36
CHST13	2.80	4.41	2.70
C1QC	3.58	4.12	2.08
MTURN	1.68	1.25	1.36
NT5DC3	0.93	1.44	1.06
IFFO2	1.81	1.74	0.88
LMO2	-0.45	-0.87	-0.49
RPL30	-0.62	-0.47	-0.52
EPB41L4B	-1.03	-1.19	-0.95
IGSF1	2.80	3.06	1.67
HHEX	0.52	0.50	0.57
CTSB	3.16	3.32	2.12
FABP4	5.14	3.69	3.51

PTAFR	1.80	2.68	1.41	GMPS	0.43	0.56	0.54	FBXL2	-1.09	-1.04	-1.20
OAZ1	1.46	1.77	1.70	CXCL13L2	5.97	7.05	4.78	PTDSS1	1.11	0.63	0.70
DOT1L	0.74	1.02	0.63	HERC3	1.12	1.02	0.47	ADCY8	-3.83	-3.42	-1.58
GARNL3	-0.70	-0.50	-0.59	SIAH2	0.58	0.64	0.73	CD1C	4.18	5.76	3.38
SELENBP1	-1.69	-1.05	-0.61	RASGRP3	-1.30	-0.78	-0.94	PABPC1	-0.60	-0.47	-0.76
MAPKAPK2	0.72	0.61	0.99	BNIP3	1.50	1.76	0.80	IFNAR1	2.03	2.38	1.47
HBEGF	2.91	2.01	1.30	CMPK1	1.10	0.95	0.82	LOC107049174	7.70	6.82	-9.28
TPRG1L	-0.73	-0.45	-0.61	PAPSS1	0.50	0.66	0.43	TRIM27.1	-3.25	-2.77	-1.62
NEK6	1.06	1.32	0.90	EIF2AK2	0.94	1.94	0.84	TBC1D31	-0.37	-0.37	-0.37
KCNJ5	2.85	2.09	2.37	ACSL1	2.83	2.60	1.10	ADAMTS4	3.75	3.55	3.41
NCF1C	1.96	3.83	1.24	ZCCHC11	1.38	1.22	0.64	SLC19A1	0.64	0.96	0.73
SRRM1	0.57	0.73	0.38	RPA3	0.84	1.30	1.25	SEMA6D	-0.99	-1.19	-0.61
CAMK1G	2.97	2.90	2.91	ADSS	1.47	1.28	0.87	FZD6	-0.68	-0.85	-0.63
ST14	1.35	1.63	0.86	PLD5	-1.77	-0.92	-0.98	TRABD	0.95	1.09	0.81
RBL1	0.94	1.68	1.01	GALNT7	0.90	0.62	1.18	GTPBP1	-0.39	-0.58	-0.58
CSNK1A1	0.78	0.76	0.42	TMEM61	3.07	3.85	3.11	IL31RA	2.10	1.33	1.06
DAB2IP	-0.67	-0.55	-0.39	BZW2	0.34	0.63	0.53	DMTF1	-0.59	-0.62	-0.58
EFHD1	1.39	1.46	1.50	AGR2	2.26	1.91	1.79	GNAS	0.53	0.72	0.39
TTC1	0.39	0.55	0.40	G3BP2	1.14	1.07	0.55	RPLP1	-0.59	-0.58	-0.76
NTM	2.78	2.48	1.16	PAQR3	-2.46	-2.84	-2.62	PTGDS	3.50	2.51	1.21
GSN	-1.33	-1.05	-0.72	ABCB5	2.77	2.78	1.96	CSF3	5.83	6.35	2.62
ADORA3	3.37	3.34	2.15	MYF6	6.70	3.15	-6.64	CLEC3B	-0.81	-1.15	-1.18
RAP1A	-0.49	-0.58	0.50	KLHL7	-0.53	-0.64	-0.73	HMCN1	-1.49	-1.80	-1.09
FAM212B	-1.02	-1.39	-1.85	IGF2BP3	1.08	1.13	0.61	ABCB8	-1.32	-0.76	-0.95
CALCOCO2	0.70	0.99	0.82	SLC35F3	-2.47	-2.35	-2.06	PTPRM	-0.61	-1.22	-0.66
ARHGAP39	-0.48	-1.18	-0.93	PGM1	0.66	1.30	0.96	TULP1	3.05	3.77	1.53
NFS1	-0.89	-0.71	-0.64	SNX10	2.50	3.67	1.41	LOC374195	7.04	5.80	-6.55

MYO1F	2.18	3.00	1.18	PDE4B	2.15	1.68	1.13	LOC107055431	5.98	6.43	-7.97
UFD1L	-0.47	-0.60	-0.49	PSMD14	0.47	0.84	1.11	TDG	0.55	1.36	0.65
GLB1L2	-2.41	-1.89	-1.19	PLA2R1	1.85	1.59	0.67	MAFG	-1.63	-1.56	-0.73
RPL23	-0.44	-0.45	-0.56	CHN2	1.08	0.95	0.74	DDIT3	3.16	2.45	2.67
RNF122	-1.66	-1.35	-0.74	SLC25A29	1.46	1.18	0.98	RAB1B	0.48	0.94	0.56
44444	0.90	1.49	1.52	GPAT3	1.38	1.31	1.00	ICOSLG	1.24	1.91	1.40
GABRB2	-1.00	-1.75	-0.79	WARS	0.55	0.82	0.67	ARHGAP18	-1.41	-1.55	-1.03
NPTN	0.66	0.72	0.39	PLCL2	-1.50	-0.77	-0.60	RP1	-1.39	-1.82	-1.15
ACSBG2	3.72	3.03	1.53	THRB	1.13	1.09	1.00	ZP1	3.94	2.87	2.41
MFSD11	0.51	1.07	0.72	KIF25	-1.61	-3.01	-2.60	TES	0.82	1.69	0.72
BBS4	-0.59	-0.73	-0.51	DYNC1H1	0.33	0.43	0.30	LEF1	-0.90	-0.65	-0.73
SDF4	0.91	1.06	0.64	PRKAG3	-2.03	-2.65	-1.59	MYO5A	1.89	0.58	0.96
CARD9	1.53	2.43	1.39	AMN	-3.12	-2.13	-1.63	RPL34	-0.90	-0.82	-0.88
ARIH1	-1.12	-0.83	-0.70	RNASET2	2.61	2.14	0.66	MMP16	-1.55	-1.99	-0.85
TBC1D2	-0.99	-0.68	-0.45	ARPC2	0.94	1.31	1.19	MC5R	-2.57	-2.24	-1.14
PTBP1	0.35	0.58	0.33	CKB	0.55	1.28	1.34	ULK3	0.49	0.43	0.55
MED16	-1.04	-1.42	-0.49	XRCC3	0.55	0.88	0.74	NPM3	1.34	3.01	1.14
PKLR	1.47	2.73	1.29	BCL2L14	4.07	5.29	2.08	UBA5	1.02	0.78	1.82
SNCG	-2.83	-1.98	-1.66	JCHAIN	2.91	2.00	1.39	NADK	0.72	0.96	0.66
COMT	1.34	1.43	0.97	GYPC	-1.54	-0.73	-1.56	LOC100858381	3.03	3.36	2.21
DOCK2	1.80	1.07	0.60	EPB41L5	-1.96	-1.87	-1.02	GEM	3.60	2.01	1.86
CLCC1	-0.66	-0.67	-0.96	CLASP1	-0.49	-0.56	-0.69	FMNL1	2.06	2.43	0.93
EVPL	-1.10	-1.70	-0.91	ATG9A	-0.64	-0.75	-0.99	LY96	3.12	4.28	3.09
LCP2	2.76	3.11	1.33	SEMA5B	-0.75	-0.78	-0.50	CCL4	5.62	6.30	4.97
HYAL2	-1.37	-1.29	-0.77	CDCA4	0.93	0.77	0.52	ENPP2	-1.20	-0.77	0.83
RPS17	-0.52	-0.38	-0.80	HACD2	-0.92	-0.37	-0.40	MECR	2.63	3.35	1.12
B2M	2.61	2.83	1.97	MTHFD1	1.26	0.86	1.43	NCL	0.47	0.45	0.40

PLEKHB2	0.85	0.84	0.63	SYNE2	-0.62	-0.77	-0.52	ADAMTS2	0.77	1.30	0.66
NR5A2	2.72	1.87	1.23	ENGASE	-0.77	-0.60	-0.58	SLC7A3	1.06	1.12	1.03
PTPRC	2.31	3.00	1.20	FAT4	-0.88	-1.67	-1.06	ATOH8	-1.78	-1.77	-1.06
NOC2L	0.47	0.47	0.46	HIF1A	1.48	1.29	0.80	RUNX1T1	-0.53	-0.99	-0.31
ETF1	1.04	0.96	0.43	NFAM1	2.62	4.65	2.29	PSMD3	0.47	0.75	0.59
H3F3B	0.62	0.36	0.33	TOB2	-0.81	-0.98	-0.38	TGM4	6.95	5.48	3.47
AP3S1	0.45	0.64	1.05	DAAM1	-2.53	-1.57	-0.86	PQLC2	2.03	2.19	1.19
C9H3orf58	-1.19	-1.56	-0.59	ADSL	-0.71	-0.45	-0.61	FAM107B	0.71	1.19	0.72
HSPA9	0.78	0.61	0.72	DTX3L	1.56	2.36	1.15	LOC427816	-4.96	-3.39	-1.32
CA6	1.96	3.78	1.83	AP5M1	0.60	1.00	0.60	AKTIP	-0.55	-0.78	-0.44
TSPAN14	0.69	0.56	0.52	MARCO	2.81	3.50	1.75	H2AFV	-0.78	-0.87	-0.70
CFH	-3.26	-2.77	-2.51	CFI	-1.25	-1.55	-0.90	PLCG2	1.02	1.36	0.50
SLC2A5	2.95	2.93	1.51	ARL14EP	-0.52	-0.58	-0.49	ATP6V1B2	1.20	1.69	0.57
CREB3L4	1.01	1.45	0.62	ACTR3	0.64	1.02	0.73	RSL1D1	0.52	0.75	0.63
UCHL5	0.50	0.61	0.61	RPL3	-0.68	-0.70	-0.65	IFI30	3.29	3.76	1.21
MCCC2	-0.63	-0.98	-0.46	NCKAP5	-1.73	-1.80	-0.70	RALY	-1.99	-2.10	-1.10
GJA4	-1.93	-1.55	-0.63	MGAT5	0.83	1.06	0.44	CSF1	1.65	1.51	1.11
GUSB	2.28	2.11	1.35	GCH1	3.07	3.77	1.45	GOLT1B	-0.39	0.48	0.44
ARAP3	-0.76	-0.92	-0.95	RAP1GDS1	0.45	1.10	0.56	NAT8B	-0.50	-0.54	-0.60
PIK3CD	1.27	1.60	0.75	UBXN4	0.36	0.42	0.43	EEF2	-0.65	-0.56	-1.20
PPARD	1.09	0.99	0.71	MAFF	0.91	1.79	0.76	MEA1	-0.47	-0.49	-0.40
KCTD7	-0.81	-0.65	-0.58	RAB32	1.27	1.69	1.31	EIF6	0.70	0.81	0.66
SLC25A42	-0.94	-0.96	-0.54	NUP43	0.90	0.72	0.63	KCNK5	-1.33	-1.95	-0.90
SRRL	1.91	1.63	1.23	ARHGAP15	1.89	2.89	2.73	TRIM63	-1.90	1.82	-1.55
RPL10A	-0.58	-0.51	-0.53	CYTH4	1.96	4.09	2.13	TNFRSF1B	1.13	0.58	0.71
ITGAV	1.17	1.28	0.43	RAC2	1.56	2.32	0.84	VPS25	-1.17	-0.92	-1.05
NME4	-1.67	-1.70	-1.22	LRFN5	-2.23	-2.37	2.05	ZAR1L	-2.11	-2.38	-2.24

SLC16A3	2.69	2.64	1.27	EIF3D	-0.38	-0.51	-0.90	ATP6V0D2	5.32	5.13	4.44
SIRT1	-0.96	-0.49	-0.84	FOXRED2	0.96	1.09	1.14	MTHFD2	1.56	2.21	1.95
APEH	0.66	0.67	0.50	RASD2	-2.08	-1.44	-0.69	KRT10	-2.48	-1.61	1.64
PSTPIP1	3.78	4.96	1.39	HMOX1	2.33	1.71	1.08	IMPA1	0.55	0.52	0.51
PGD	0.58	1.21	0.72	CTSV	1.58	1.48	0.59	LOC427010	5.99	6.86	-7.63
RPS10	-0.65	-0.55	-0.93	PWP1	0.52	0.74	0.52	DCLK3	1.62	2.34	0.96
IFI35	1.92	2.09	1.21	CKAP4	0.58	0.58	0.52	CELF2	1.34	0.94	0.59
RPL27	-0.40	-0.37	-0.54	ALDH1L2	0.91	1.35	0.77	INHBA	2.88	3.92	2.82
RPL26L1	-0.70	-0.62	-0.67	DSP	0.83	0.69	0.70	TOX3	1.94	2.41	1.41
CASZ1	-1.29	-0.92	-0.75	TBXAS1	1.35	2.67	1.03	ALDH18A1	0.73	1.15	1.32
PRELID1	0.92	1.37	0.84	F13A1	3.92	4.43	2.65	CTGFL	-2.39	-2.42	-1.65
MASP2	-1.23	-1.31	-1.74	ECI2	0.97	1.36	1.41	USP12-like	0.66	0.37	0.45
PSMA4	0.30	0.72	0.63	TRIM24	-2.12	-1.23	-0.75	RPLP2	-0.58	-0.38	-0.42
BFAR	0.78	1.12	0.87	RIPK1	0.37	0.74	0.34	CMKLR1	1.58	1.79	0.72
RGS14	0.96	1.26	0.96	SERPINB5	3.34	4.12	2.86	ALB	-2.57	-2.98	-4.90
NCOR2	0.94	0.72	0.36	DNM1L	0.61	0.90	0.61	RPL7	-0.62	-0.63	-0.69
BRF2	-0.94	-1.67	-0.62	FAM60A	1.08	1.66	0.86	HBE	-4.72	-3.88	-3.23
CBFB	1.12	1.39	1.22	CDYL2	1.09	1.12	0.86	NCKAP1L	1.72	3.04	1.22
RNASE6	3.44	4.91	2.11	CMBL	6.86	6.31	2.83	IREB2	-0.49	-0.65	-0.54
EIF4EBP1	-0.66	-0.74	-0.54	ATP6V1E1	1.27	1.80	0.92	INPP5D	1.55	1.79	1.73
UTP6	0.58	0.71	0.60	BCL2L13	-0.78	0.61	0.53	AOAH	3.77	4.70	2.46
ADAMTS12	-1.68	-1.30	-0.87	USP41	2.99	5.36	1.43	SYNJ2BP	0.74	1.29	0.86
HDAC1	1.30	1.10	0.61	BPGM	-1.00	-0.93	-0.78	NT5DC2	-1.63	-1.51	-1.13
RILPL1	-0.96	-0.50	-0.59	UPP1	2.50	2.54	1.86	GRAMD1C	-0.55	-0.96	-0.68
NECAB2	-3.62	-1.66	-1.64	IKZF1	2.35	1.44	1.72	UBP1	0.51	0.49	0.40
PRRX1	2.36	2.38	1.63	EPS8	2.04	1.84	1.35	ACVRL1	-1.31	-1.12	-0.49
NOP16	0.73	0.90	0.94	MGST1	1.76	2.02	1.22	RPL35	-0.72	-0.72	-0.67

ACSS2	1.98	2.40	1.36	BCAT1	3.67	3.53	2.82	LOC431003	7.25	6.33	-6.36
HERC1	-0.36	-0.49	-0.42	LPCAT1	-1.13	-1.73	-0.74	SHISA9	-1.13	-1.71	-1.61
BSDC1	-0.34	-0.38	-0.46	GABBR2	-0.80	-0.92	-1.08	CD48	2.27	3.54	1.85
LOC112530206	-1.13	-0.86	-0.76	SLC39A6	0.54	0.78	0.53	ATAD3B	0.70	0.63	1.17
LOC107054267	1.51	2.32	1.68	INSRR	-2.18	-1.85	-1.10	KIAA1143	1.26	1.27	0.80
PLEKHM2	0.60	1.37	1.10	IKBKE	2.58	1.14	1.23	HINTW	7.58	6.48	-6.61
USP49	1.86	0.76	0.70	EXOC3	1.05	1.09	0.54	ALDH3B2	1.37	1.61	1.20
UCK2	2.35	1.93	0.88	PDCD6	0.68	0.81	0.69	ANGPT2	1.97	1.36	1.02
SPINK7	-4.97	-3.62	-1.93	GZMA	3.01	3.72	2.57	RAB4A	-0.40	-0.63	-0.68
SYNC	1.41	1.49	1.44	SEC61B	0.80	1.41	0.89	OAZ2	-0.95	-0.61	-0.48
DDX5	0.40	0.32	0.21	IFI6	3.14	4.47	3.31	CAP1	0.75	0.79	1.07
SLC6A2	-1.79	-1.49	-1.05	SCGN	-2.70	-3.60	-1.77	SEC61A2	1.09	0.64	0.55
FN1	1.42	0.91	0.92	ATP6V1H	1.15	1.25	0.68	AMH	4.23	3.96	3.20
ANK1	-4.44	-2.57	-3.78	IPCEF1	1.77	2.14	1.80	LOC101749223	-2.43	-1.47	-1.12
MAFB	3.63	3.16	1.39	ACOT13	0.61	1.27	1.10	FAM49B	1.00	0.88	0.63
KRT14	3.52	5.17	3.16	DLC1	-0.64	-0.81	-0.61	SYBU	-1.52	-1.25	-0.84
RNF145	-1.00	-1.58	-1.10	OPTN	0.96	1.18	0.73	EFNB1	-1.41	-1.15	-1.00
MACF1	-0.82	-0.71	-0.39	PTPN2	1.46	1.37	0.67	CXCR1	1.23	2.33	2.06
PLAT	-2.47	-1.58	-1.18	IL20RA	3.71	2.09	1.80	NDUFAF8	-0.79	-0.66	-0.86
PLCG1	-1.29	-1.10	-1.24	SEH1L	0.43	0.39	0.49	MYZAP	-0.85	-0.88	-0.73
NKD1	-1.11	-0.65	-0.53	FAM210A	0.95	0.90	0.47	UBE2L3	0.37	0.72	0.51
ANXA2P2	0.88	1.89	1.18	NAPG	-0.41	-0.61	-0.39	ADGRL2	-1.52	-1.89	-0.57
SUN1	-0.53	-0.35	-0.75	RPS12	-0.84	-0.71	-0.71	RPS27A	-0.56	-0.74	-0.69
FYB	1.79	3.01	1.46	VNN1	3.99	4.77	1.86	CUL1	0.27	0.41	0.34
ARPC3	0.52	0.88	0.56	LRMP	1.09	2.13	2.23	KCNA3	2.86	2.47	2.54
TRIT1	-0.51	-0.78	-0.65	ARNTL2	0.94	1.11	0.76	DECR1	1.06	1.24	1.08
LRRFIP1	1.94	1.56	0.79	TEC	1.11	0.76	0.71	CREBL2	-0.89	-1.14	-0.69

MFSD2A	1.12	1.38	1.17	ARFGAP3	0.75	1.12	0.88	EIF3E	-0.85	-0.87	-0.95
XPR1	0.70	0.99	0.60	KMT5B	-0.65	-0.67	-0.42	RPF2	0.39	0.77	0.68
COL6A3	1.96	1.33	1.10	LOC769000	6.77	6.46	-7.49	FAM241A	0.67	0.67	0.44
RPL23A	-0.72	-0.79	-0.93	PRR5	3.07	3.66	1.46	ZFHX4	0.87	0.67	0.54
TWF2	1.03	1.14	0.38	NUP50	0.54	0.60	0.69	LOC101752158	2.18	4.26	1.90
LYPLA2	0.44	0.84	0.55	SLC2A14	2.34	2.19	1.41	TALDO1	0.67	1.14	1.14
DHRS13	-1.04	-1.32	-1.61	ZC3HAV1L	0.88	0.59	0.62	MACROD2	-0.76	-0.74	-0.58
GPR174	2.20	1.55	1.22	RPS20	-0.92	-0.41	-0.67	LOC396477	-1.87	-1.73	-1.85
SPP2	-3.91	-2.42	-2.73	GAPDH	1.78	2.61	1.31	RPH3AL	-0.84	-1.05	-0.60
LBFABP	-9.50	-4.97	-2.95	LDB2	-1.19	-1.71	-0.66	SIPA1L2	-1.74	-1.17	-0.91
ATOX1	-1.03	-0.64	-0.64	BST1	1.91	2.25	2.90	FGFRL1	-0.75	-1.00	-0.64
IL22RA1	1.31	2.43	1.54	TPI1	1.11	1.65	0.82	PINK1	-0.50	-0.39	-0.74
IFITM5	2.38	1.51	1.47	ENO2	5.16	5.62	4.39	USF1	0.89	0.98	0.62
LOC420160	0.92	1.43	0.82	PTPN6	1.86	2.42	1.32	NECAP1	0.37	0.39	0.42
P4HA1	0.46	0.74	0.53	CCL26	7.02	7.30	3.31	COL14A1	1.92	0.85	1.17
ANXA6	-1.42	-1.43	-0.73	C1S	4.10	4.85	2.69	NBN	1.36	2.21	1.40
ABR	2.04	2.69	2.19	ERAP1	0.93	1.27	0.86	RPS19	-0.48	-0.41	-0.90
CARD11	1.80	2.51	1.00	ZYX	1.15	1.50	0.68	SLC47A1	2.43	2.79	1.43
SPECC1	1.73	1.63	1.83	KEL	-1.64	-1.84	-1.25	PRKCB	2.81	2.35	1.80
SDK1	-0.80	-0.84	-0.64	ATP6V1A	1.93	1.57	1.40	LNPK	0.78	0.67	0.86
ARSB	1.50	1.94	1.60	RPL37	-0.90	-0.71	-0.87	IL10RB	1.25	1.64	1.32
PALD1	-1.06	-0.86	-1.03	C7	2.41	2.78	1.49	GLTP	0.87	1.41	0.90
SGPL1	1.01	1.07	0.36	PLCXD3	2.49	1.72	1.77	CX3CR1	3.37	3.19	1.59
LRRC8A	0.42	0.40	-0.44	PARP8	-0.83	-0.86	0.83	NR1H3	0.49	0.89	0.45
RPS14	-0.53	-0.45	-0.73	ARL15	-0.81	-1.09	-0.55	NOS2	4.13	3.53	1.24
SULF2	-1.03	-0.81	-0.55	USP14	0.78	1.08	0.81	SMIM3	-0.55	-0.80	-1.08
CD74	2.47	3.25	0.92	ARHGEF28	1.81	1.26	0.82	NCF4	1.74	2.11	1.12

SHISA5	1.71	2.11	1.48	POLQ	-1.41	-1.14	-1.49	FNTB	0.62	0.42	0.32
SPTAN1	-1.42	-1.06	-0.52	HEXB	0.60	1.25	0.64	EFHD2	2.92	3.38	2.57
PSAP	2.19	2.85	1.61	IQGAP2	1.23	1.26	0.65	SLC20A2	0.72	0.91	0.92
C1QB	3.86	4.83	2.10	F2RL1	2.58	2.40	1.07	PLEKHA6	-1.74	-1.78	-1.69
PPIF	0.86	1.38	0.88	TRAF3IP2	1.05	1.20	0.76	MID1	-0.69	-1.03	0.73
RPS4Y1	-0.66	-0.57	-0.98	JAK2	-1.14	-1.63	-0.97	LAPTM5	2.48	3.48	2.15
RPS24	-0.60	-0.54	-0.51	SLC16A10	-1.29	-1.26	-0.94	FBXL12	0.52	-0.42	-0.47
CORO1C	0.67	1.09	0.56	TTC39C	0.54	1.14	0.61	TBX10	1.92	3.87	1.60
RPL36A	-0.38	-0.49	-0.88	B4GALT4	0.98	1.19	0.77	SYDE2	-1.45	-2.62	-0.94
GPI	1.60	1.59	0.78	KCTD1	-1.16	-0.54	-0.82	LOC100858962	3.50	1.98	1.63
AIPL1	2.75	-3.22	-3.08	ALDH1A1	-1.33	-2.24	-1.38	RASSF5	1.81	1.91	1.01
EFCC1	-1.66	-1.59	-0.59	ANXA1	1.18	1.99	2.05	FILIP1	-0.75	-1.24	-0.87
ACAD9	0.85	0.97	0.67	RAB10L	2.19	2.40	0.98	LOC427025	6.80	6.12	-5.96
MPP1	1.78	2.27	1.20	RPS15	-0.65	-0.48	-0.54	CD44	3.02	3.96	1.77
SERPINE2	2.07	1.94	1.19	CMSS1	0.52	0.72	0.51	CA8	-1.24	-1.17	-1.36
AMY1A	-1.47	-1.22	-0.75	RPL24	-0.52	-0.41	-0.64	RASAL3	2.44	2.20	1.60
PTRH2	-0.62	-0.73	-0.52	CHRNA6	1.07	1.41	1.01	AGRN	-0.86	-0.79	-0.72
VMP1	2.52	2.29	1.09	SDCBP	1.28	1.36	0.58	AXIN2	-0.82	-1.18	-0.80
SEC24C	0.43	0.33	0.31	SLC44A1	-1.18	-1.25	-0.82	NDUFA13	-0.40	0.52	0.37
CYP8B1	2.57	1.77	3.18	CD247	1.22	1.02	-1.00	COL6A2	1.07	1.30	0.87
TKTL1	1.77	1.67	0.89	ROBO2	-1.15	-1.45	-1.34	STAB2	-1.75	-1.28	-1.44
EIF3F	-0.53	-0.85	-0.77	JAKMIP1	-2.00	-2.37	-1.47	SLA	2.54	1.83	1.38
MSLN	2.29	1.60	1.43	ALDOB	-2.91	-2.72	-2.73	RNF213	2.35	2.87	1.36
CA4	1.58	1.50	1.43	GRPEL1	0.47	0.81	0.58	DDR2	0.92	1.00	0.78
HAGH	-0.60	-0.53	-0.55	VCAN	2.30	2.55	1.55	C5H15orf52	-1.89	-0.78	-1.54
AGL	-0.62	-0.95	-0.80	HAPLN1	-3.04	-3.72	-1.93	P2RY13	2.45	4.18	1.52
JMJD4	-0.56	-0.68	-0.54	FGFR3	-1.07	-0.88	-0.59	TNFRSF1A	1.18	1.09	0.54

FRRS1	-1.37	-1.48	-0.97	SLBP	1.66	3.22	1.98	LRRCC1	-0.53	-0.82	-0.47
CDH13	1.15	1.26	0.72	AKIRIN2	0.54	0.41	0.46	GSPT2	0.74	0.83	0.47
IL17RD	-1.42	-1.92	-1.03	HMGN2P46	-0.69	-0.48	-0.66	PLCE1	-0.90	-0.60	-0.61
PIK3CB	1.18	1.80	0.88	MB21D1	0.72	0.85	1.16	GFPT2	1.14	1.21	0.93
DPYD	-0.79	-1.01	-0.72	OGFRL1	0.93	1.01	0.86	PRAM1	2.10	3.07	1.49
SRSF1	0.28	0.25	0.47	RPIA	0.60	0.61	0.44	CERS5	0.34	0.40	0.34
DKK3	0.97	1.02	0.73	B3GALT5	-4.10	-2.27	-1.79	LOC112531360	-0.40	-0.38	-0.49
ADIPOQ	2.38	2.41	1.37	MX1	3.96	6.74	2.91	NKAIN2	-2.10	-2.29	-1.18
MSI2	1.77	1.69	0.89	DDX3X	-0.30	-0.52	-0.40	PLXNB2	1.05	0.60	0.46
ARF4	0.54	0.76	0.88	RPL8	-0.48	-0.44	-0.68	SLC4A1	-2.41	-2.37	-1.26
IL2RG	3.46	3.42	1.60	CYBB	2.39	4.42	2.66	CD244	2.87	3.67	1.92
USP10	0.46	0.73	0.64	RAB23	0.69	0.67	0.72	MAPK11	2.80	2.22	1.71
FLNB	1.18	1.08	1.11	DST	-1.00	-0.84	-0.65	HNRNPKL	7.29	6.43	-6.96
GARS	0.55	0.82	0.66	GSTA3	3.11	3.75	2.02	UBE2W	0.79	0.54	0.61
IRF8	0.99	1.78	0.80	AGPAT5	1.01	0.72	0.47	PSTPIP2	2.34	2.24	1.51
SEPTIN2	1.41	1.86	1.47	EIF2S3	-0.33	-0.34	-0.38	TPD52	0.92	1.30	1.69
RNF130	0.56	0.51	0.63	ITGB1BP1	-0.40	-0.74	-0.60	CEBPD	1.92	1.65	1.51
SLC7A5	4.27	3.35	2.00	NT5C1B	1.56	1.38	0.83	IVD	-1.02	-1.32	-0.58
IL11RA	-2.35	-2.40	-0.60	HS1BP3	0.49	0.70	0.85	DUSP1	-1.36	-1.82	-1.40
PGP	0.68	0.95	0.51	REPS2	-2.10	-2.42	-0.99	HERPUD2	-0.37	-0.27	-0.30
RPL5	-0.68	-0.53	-0.83	ESCO2	-1.76	-0.95	-0.91	PUDP	1.01	0.66	0.77
MYD88	1.01	1.97	1.34	TLR7	2.09	2.63	1.93	ACTBL2	-4.33	-5.29	-3.84
RPL27A	-0.67	-0.56	-0.74	ARHGAP6	1.85	1.61	0.87	SCNN1A	0.99	1.18	1.26
COL6A1	1.74	1.87	1.11	ARSH	-3.30	-1.82	-1.17	CREG1	-1.74	-1.60	-1.87
DOCK11	0.88	0.82	0.62	TDH	-4.23	-3.18	-1.50	TFEC	2.31	1.70	1.95
TGFBR3	-0.83	-0.92	-0.83	BLK	4.61	3.25	4.14	IL18R1	1.15	2.21	1.29
SOX6	-2.92	-2.21	-1.03	AvBD10	-3.36	-8.60	-5.39	METTL6	0.96	0.88	0.60

RASGEF1C	2.48	1.79	-3.99	P2RY8	1.93	2.06	0.86	SPIN1W	7.63	6.28	-7.76
PHF6	0.55	0.42	0.40	ASMTL	1.08	0.56	0.76	PRLH	6.59	7.80	6.03
NUCB2	0.73	0.78	0.55	CYP2AC1	-6.26	-5.88	-3.06	CHMP1B	0.25	0.45	0.23
SLC52A3	1.22	1.57	1.20	SLC25A6	-0.64	-0.50	-0.47	CFD	3.44	2.83	1.53
CSNK2A1	0.41	0.54	0.41	CSF2RA	2.31	4.87	2.33	WDR77	1.55	1.35	1.95
HM13	0.42	0.64	0.49	CYP39A1	-2.93	-1.92	-1.57	MRPL3	0.79	1.18	0.82
LOC100857714	1.76	2.47	0.84	TNFRSF21	1.84	1.42	1.13	SAMSN1	2.93	4.44	1.74
LDHA	4.29	4.20	2.70	PLCXD1	2.75	2.84	1.75	LOC101748577	1.83	2.60	1.26
IL21R	6.07	5.31	5.79	RP2	1.19	1.64	0.99	METTL24	-2.90	-1.82	-1.77
CHCHD4	0.47	0.48	0.60	EIF5B	0.43	0.67	0.39	HTRA1	2.15	1.89	0.73
CXCL14	1.53	1.41	1.69	AFF3	-0.98	-1.78	-1.76	NT5DC4	-2.70	-1.97	-2.24
LIPA	2.82	3.70	1.80	IL1RL1	1.02	0.96	0.86	IFIH1	2.06	4.37	1.56
ARHGEF6	0.49	0.36	-0.41	SEPTIN10	0.84	0.49	0.64	IGF2BP1	5.25	2.53	2.91
MADPRT1	5.36	5.28	2.55	RASA3	0.55	0.79	0.52	RRAGA	1.07	1.14	0.61
SKP1	-0.50	-0.52	-0.68	MYO16	-3.05	-1.43	-1.39	RRP12	0.70	0.78	0.80
BCL2A1	3.39	3.74	2.99	NAXD	0.62	0.69	0.67	ARFIP1	0.50	0.53	0.26
PROCR	-2.34	-2.39	-2.49	FARP1	-0.89	-0.68	-0.67	SDHB	0.44	0.52	0.46
VIPR2	2.70	1.45	1.22	ACOD1	3.26	3.42	3.37	DENND4A	0.85	0.57	0.97
LSP1P1	1.30	1.64	0.81	PCDH17	-2.94	-2.17	-1.22	DDX31	-0.65	-0.49	-0.51
ABHD2	2.12	1.31	1.95	WBP4	-2.04	-1.51	-1.29	ACKR4	2.67	2.42	2.40
OAF	1.07	1.07	0.87	SPERT	-2.12	-1.77	-0.82	CCDC189	-0.63	-0.63	-0.82
GPC2	-1.24	-0.82	-1.01	LCP1	2.69	3.68	1.56	IMPAD1	0.64	1.07	0.55
CDK5RAP1	3.12	3.67	1.77	ITM2B	-0.48	-0.63	-0.35	MTPN	0.66	1.06	0.62
THY1	1.58	2.58	1.14	FNDC3A	0.46	0.50	-0.48	LY6E	1.09	2.05	0.97
PLOD2	1.33	1.36	0.67	ALG5	0.98	0.55	0.49	DUSP7	0.89	1.67	1.04
PLSCR1	1.82	1.62	1.46	BRCA2	-1.82	-2.03	-0.99	GNAI3	0.57	1.16	0.39
SPATA20	-0.71	-0.82	-1.15	SLC46A3	-1.13	-1.14	-1.03	KLF3	-1.03	-0.67	-0.38

CTSA	1.78	2.21	0.63	WASF3	-0.65	-0.66	-1.13	PLA2G15	1.77	2.68	-1.85
IMPDH2	0.50	0.72	0.37	ATP8A2	-2.02	-1.71	-1.70	ANO1	2.92	2.67	1.91
ARIH2	1.13	1.09	0.83	SACS	1.04	0.58	0.71	DYNC2H1	-0.81	-0.61	-0.83
MMP9	2.51	4.90	1.96	ZDHHC20	1.52	1.55	0.96	S100A6	0.65	1.16	1.41
APOD	-1.76	-2.51	-2.12	PSPC1	0.85	0.91	0.54	MTSS1	1.04	1.08	0.56
TLR4	2.08	2.93	1.16	ELMOD1	-2.63	-2.00	-2.50	K123	4.84	5.00	4.93
IGSF6	2.82	4.24	1.88	GUCY1A2	-1.89	-1.96	-1.72	UGDH	0.55	1.22	0.88
KIF21A	1.42	0.96	0.84	MMP13	2.95	4.35	3.13	LOC422513	2.30	3.43	2.05
APOA1	3.04	2.73	1.29	TMEM123	2.99	3.12	1.78	HNRNPA1	-0.45	-0.48	-0.51
LOC101750621	4.49	4.67	2.37	CEP57	-0.63	-0.62	-0.77	LARP4	0.41	0.34	0.35
TNFSF15	2.48	2.01	1.57	CTSC	2.94	3.25	2.30	LOC107054133	-4.16	-4.65	-1.82
PTPRG	-2.43	-2.11	-0.70	VWF	1.79	0.76	0.77	AvBD5	-1.67	4.03	1.57
TMEM268	1.45	2.78	1.78	C1H12ORF4	0.80	0.83	0.77	DOCK1	-0.41	-0.70	-0.59
IL1RAP	1.93	1.71	0.92	SRSF2	0.36	0.32	0.58	CHURC1	-1.09	-0.84	-0.71
ARHGAP12	0.83	1.16	0.51	YWHAG	0.46	0.45	0.45	LYN	0.95	1.78	0.58
PYCR1	1.03	1.94	1.22	OSTF1	0.74	0.53	0.47	FAM198B	-1.05	-1.15	-0.72
PRELID3B	1.17	1.43	0.98	COTL1	1.87	2.44	0.91	BBS2	1.35	1.14	0.69
TMLHE	-1.71	-1.15	-0.90	Y_RNA	-1.44	-2.11	-2.58	ACTN1	0.57	0.85	1.10
TFRC	-1.76	-0.92	-1.59	Y_RNA	-1.61	-2.37	-1.56	LOC100857928	1.94	2.28	0.97
ITGB2	2.81	4.04	1.55	MIRLET7B	-1.80	-1.83	2.23	TMEM64	-1.66	-1.10	-0.74
ABCC3	1.26	0.68	0.57	SOD3	3.07	1.63	0.94	RSPO3	3.39	3.00	1.91
ABI1	0.92	0.71	0.96	LOC770126	-1.21	-0.75	-0.67	NBAS	-0.40	-0.47	-0.37
APBB1IP	1.38	1.81	0.55	PCGF2	-0.90	-1.84	-1.16	SMIM4	-0.86	-1.02	-0.87
PGAM1	1.40	1.77	0.91	PHF11	1.79	2.31	1.51	HEPHL1	5.23	-4.27	4.42
RPL35A	-0.51	-0.46	-0.50	LOC418667	3.19	2.53	2.00	CD1B	4.12	3.98	2.70
VEGFRKDRL	-2.44	-2.20	-0.87	LOC418170	-1.55	-0.78	-1.01	EXFABP	4.50	4.21	2.38
SLC9A3R1	1.37	1.61	0.90	CPSF6	0.51	0.67	0.46	DEDD	0.64	0.39	0.34

STAT1	2.86	3.40	1.55	CHST9	-0.72	-1.31	-0.62	SMARCD3	-1.17	-0.69	-0.65
RPS25	-0.48	-0.41	-0.69	CNDP2	1.42	1.95	0.89	ARPP21	-0.67	-1.39	-0.84
RLIM	0.92	0.86	0.75	SERPINB2	3.85	4.39	2.43	LYGL	3.45	5.32	2.10
AURKB	0.97	0.85	1.12	H3F3C	-0.74	-0.88	-0.80	EZH2	0.58	0.60	0.37
PROK2	4.46	5.58	3.13	RAPGEF2	-0.48	-0.95	-0.67	SMIM7	-0.84	-0.42	-0.42
UPRT	1.04	0.83	0.57	IL13RA2	3.86	3.24	2.20	EGFL7	-0.71	-0.51	-0.49
ATRX	-0.67	-0.60	-0.62	STK32C	2.19	1.42	1.29	SGK3	1.43	1.56	0.92
IL18	2.53	3.06	1.10	CCDC141	-3.32	-1.47	-1.42	CHMP4C	-0.81	-1.19	-0.78
STK17B	1.59	1.81	0.61	LRRC8D	1.39	1.46	1.01	PRPF18	-0.38	-0.37	-0.29
PGK2	1.88	2.53	1.36	FAM237A	2.84	4.44	3.80	BEST4	-1.49	-1.09	-0.77
ARMC7	-1.29	-0.83	-0.60	ARPC5	0.73	1.09	0.77	DAPP1	0.74	1.69	0.87
ITGA11	-0.86	-1.14	-1.08	LOC112532977	3.59	4.08	2.32	NDFIP1	0.55	0.63	0.50
CEBPB	2.31	2.52	2.75	RNF7	-0.43	-0.70	-0.54	HEXA	1.72	1.88	0.86
TAPBP	1.31	3.67	1.44	SLC7A9	-2.21	-1.79	-2.79	MSMB	6.42	3.47	4.16
LOC417013	-1.44	-2.79	-2.13	UBE2D1	0.75	0.65	0.41	LOC101749377	2.15	2.44	2.99
CORO2B	-0.89	-1.51	-0.93	CDCA9	-1.25	-1.16	-1.50	CLEC17A	1.84	2.49	1.83
HBP1	0.76	0.60	-0.70	LOC100858919	2.08	2.94	2.06	LOC426820	2.70	2.98	1.08
MTMR3	-0.45	-0.55	-0.49	ABCA9	2.56	3.57	1.89	IL13RA1	2.05	2.89	1.08
PAK3	1.31	1.97	0.79	ADAR	1.15	1.58	0.74	C18orf63	-5.13	4.07	-2.48
SPI1	3.44	4.59	2.69	C1QA	3.81	4.38	2.33	SLC51B	4.10	4.63	3.13
NT5C2	1.08	1.79	1.38	IFI27L2	2.43	3.35	1.97	PLIN3	-1.82	-1.72	-1.74
AOX1	2.33	1.18	-0.90	MIR146B	6.83	6.63	4.55	VSIG4	2.27	2.99	1.26
TPCN3	-1.53	-1.52	-0.91	LOC420107	2.40	2.58	4.13	MISP	-0.96	-1.08	-0.66
PDCD11	0.96	1.11	0.53	MOG	-3.04	-2.09	-1.26	IFIT5	3.45	7.30	1.83
IL5RA	-3.26	-1.51	-2.15	SULT1B	0.84	1.16	-1.03	FCER2	5.21	5.04	3.00
CRBN	-0.73	-0.59	-0.58	UMAD1	0.70	0.68	0.72	LOC107053353	2.26	3.42	1.76
NEURL1	1.71	2.26	0.80	RPLP0	-0.87	-0.71	-0.86	EIF4B	-0.59	-0.63	-0.89

ITPR1	1.27	0.59	0.76	TAL1	-2.26	-1.88	-1.23	PARP9	1.69	2.19	1.46
FGL2	1.60	1.64	1.07	TMEM154	1.96	3.04	1.52	PEX6	-0.80	-0.70	-0.65
BHLHE40	3.21	2.45	1.94	F2RL2	0.90	1.42	1.44	IVNS1ABP	-1.37	-1.15	-0.64
LOC772071	1.77	1.51	0.78	AGPAT2	0.80	0.87	0.73	REM1	2.84	2.18	2.29
GSTO2	1.81	1.79	1.15	ASS1	2.68	3.48	1.40	TNFRSF11A	1.30	0.95	1.09
NET1	1.15	0.83	0.58	TREM-B2	1.54	3.52	1.62	HEMGN	-5.34	-4.61	-1.85
WDR12	0.61	1.18	1.07	TREM2	2.86	4.56	2.01	SGO2	-1.02	-1.06	-0.83
MRPS25	0.56	0.61	0.87	ENTPD2L	2.57	1.68	1.73	FAM83F	5.51	4.01	4.47
GSL	2.71	1.77	0.79	G0S2	5.74	5.00	3.57	KNG1	-1.78	-1.90	-3.07
EIF4G1	0.79	0.81	0.64	LOC419851	-1.11	-1.69	-1.03	PARP12	1.09	1.85	0.75
FGD5	-1.03	-0.55	-0.55	UBAP2L	0.93	1.23	0.84	IL1R2	5.42	6.21	4.14
AHSG	-3.51	-2.62	-4.42	S100A12	1.83	4.93	3.64	LOC418355	-4.07	-2.99	-2.22
SLC39A12	-2.27	-2.65	-2.13	CCLI5	2.70	3.15	2.96	PDCD1	4.05	3.94	2.33
RPL39L	-0.64	-0.56	-0.53	NMRK2	0.65	0.57	0.51	CYP2AC2	-4.85	-4.11	-2.81
AXL	-0.95	-1.01	-0.56	SMAD6	-2.29	-2.28	-0.80	TNFSF4	3.72	3.45	3.34
ICOS	2.59	2.93	2.63	AVD	7.21	9.19	6.51	FCER1G	2.55	3.81	1.76
CST7	1.55	2.87	1.28	DIO2	4.88	3.88	1.60	DMBT1	2.69	4.60	2.69
RRBP1	0.81	0.72	0.72	LOC100857512	-0.77	-0.98	-1.03	YF5	2.72	2.62	1.93
VIM	1.06	1.16	0.54	ALDOC	2.06	2.15	1.21	GPR35L	2.56	5.53	3.89
XDH	3.12	3.65	-1.24	RBM38	-1.78	-1.79	-1.21	ASPH	0.92	0.68	0.61
ITGA8	1.26	1.23	0.74	CACNG4	-1.60	-1.82	-1.22	CCDC107	0.49	0.42	0.37
PLEK	1.60	2.06	1.08	PSMD12	0.57	0.62	0.85	LOC769729	-0.92	-0.90	-0.92
SPAM1	-4.53	-7.16	-3.63	PIGY	-1.88	-2.41	-1.60	DUS2	1.80	2.44	1.72
ACTR2	0.56	1.02	0.77	C3orf33	-1.19	-1.36	-0.68	TPRN	1.18	1.34	0.50
NBEAL1	-0.61	-0.59	-0.39	MRPS2	-0.38	-0.47	-0.95	TCN2	-1.65	-0.96	-0.68
SEC23B	0.72	0.59	0.37	TMSB4X	0.50	0.59	0.70	LOC101752211	6.94	6.99	-6.28
RPL12	-0.72	-0.86	-0.90	RPS16	-0.81	-0.64	-0.68	GBP	4.10	4.51	2.68

CROT	0.79	1.19	0.55	SNRNP25	-0.53	-0.52	-0.40	UBE2R2L	6.77	6.50	-6.97
IQUB	-1.43	-1.13	-0.93	S100A4	2.95	3.93	2.48	EIF4EBP3	0.50	0.34	-0.38
ATP6AP1	0.98	1.39	0.89	ALOX5AP	2.78	3.85	2.23	NF2L	3.18	4.67	2.94
TTLL7	0.85	0.79	0.60	SARS	0.77	1.34	0.55	PEBP4	-1.09	-0.79	-1.46
UGP2	1.50	1.24	0.68	PINLYP	1.78	3.31	1.41	TMEM170B	1.14	1.01	1.12
FXR1	-0.64	-0.81	-0.69	ARF6	0.60	0.82	0.49	WBP1	-1.17	-0.92	-1.13
PDE1A	3.51	3.36	1.84	ADD2	-3.75	-2.49	-1.65	BG8	-2.27	-2.68	-2.29
PTGFR	1.29	1.17	0.71	LOC770612	2.08	2.54	1.42	RPS28	-1.54	-0.95	-1.24
B3GNT2	1.65	1.23	1.00	RRAGD	1.30	1.80	0.88	HDGFRP3	-0.35	-0.48	-0.47
TMEM87B	0.30	0.38	0.30	RPL21	-0.75	-0.62	-0.73	LOC101751325	1.26	1.18	0.86
SESTD1	0.38	0.64	0.58	TLR1B	2.50	3.11	2.48	REEP1	-1.71	-1.60	-1.05
LBH	3.07	3.20	2.09	PTF1A	7.76	4.80	4.86	EMP2	-1.70	-1.87	-0.96
CD99L2	1.58	0.96	0.93	RSFR	2.60	5.37	2.69	CLC2DL3	-1.73	-1.36	-1.19
EHD3	1.21	1.38	0.91	LOC100859602	4.96	6.20	-6.18	LYPD1	-1.95	-1.94	-0.84
FMR1	0.93	0.94	0.65	MIR6585	-0.50	-0.48	-0.49	DUSP23	-0.35	-0.55	-0.39
TNFSF10	-0.79	-1.32	-1.29	SLC46A2	2.37	2.17	2.06	LOC112530485	5.88	6.23	-7.09
MND1	-1.11	-0.86	-0.54	LRRC71	1.40	1.71	1.78	NPM2	-3.34	-2.92	-2.44
TBC1D24	-1.01	1.84	2.73	HIVEP3	1.87	1.77	0.94	CCR2	3.56	4.72	3.03
TLR2B	2.99	3.16	2.19	P3H4	1.25	0.96	0.67	TP53TG5	5.64	8.42	4.58
PLRG1	0.48	0.65	0.54	PLEKHG5	-1.74	-1.68	-0.89	LOC107054704	2.52	2.59	1.90
PAPLN	1.75	1.44	1.68	GLRX	2.20	2.14	1.50	MRGPRH	3.02	6.04	3.22
CLDN11	-1.88	-1.25	-1.23	GM2A	1.10	1.07	0.86	LOC107050411	-1.39	-2.04	-1.55
SYNJ2BP	1.75	2.09	1.20	HPS5	5.54	4.19	2.08	LOC100859084	1.89	1.49	1.02
GRK5	-1.15	-1.20	-0.74	TROJANZ	1.96	3.60	1.64	LOC101750757	2.24	3.17	1.96
RGS10	2.52	2.42	1.16	TNFAIP8	0.75	1.03	0.68	CYP2J23	1.69	0.88	0.92
TMEM168	1.03	2.82	1.83	LOC771876	3.47	2.65	1.73	GDF15	3.29	3.63	1.90
DUSP10	1.35	1.36	0.86	CNN2	1.77	1.46	1.67	BATF3	3.11	3.87	1.86

DCAF5	-0.52	-0.58	-0.71	SOCS3	3.59	2.96	2.92	LOC427029	5.45	4.90	2.30
SAMD9L	4.92	8.11	4.04	CPE	-0.97	-1.66	-1.40	C4H4ORF50	-3.34	-3.13	-1.50
ARG2	1.40	2.47	1.39	LOC101747660	3.18	2.85	1.55	LOC107055361	4.09	5.33	2.78
44256	2.87	3.62	1.70	CISD2	0.36	0.67	0.49	ARL4C	4.14	4.31	2.04
PRICKLE1	-0.69	-0.74	-0.88	STBD1	1.62	1.37	0.54	LOC101751070	-2.86	-1.93	3.33
ATP6V1D	0.48	0.63	0.54	VHL	-1.22	0.97	-1.01	LOC417536	5.58	6.70	4.68
SRP14	0.69	0.83	0.58	CDC42SE1	0.32	0.40	0.43	LOC424199	-2.73	-2.68	-1.76
MFSD1	0.51	0.99	0.62	CTSK	1.18	1.55	1.14	ESYT1	4.53	-4.42	4.21
ACTB	0.71	1.19	0.94	WASHC4	0.37	0.53	0.58	DOK2	5.74	-4.14	6.10
DDX60	3.15	3.23	1.46	HBE1	-2.81	-2.97	-2.37	C14orf180	4.22	2.62	1.97
ARHGAP11B	1.07	1.12	1.14	PTX3	7.09	5.45	2.40	CCL28	1.80	1.56	1.54
CTBP2	-0.98	-0.82	-0.68	MMR1L4	2.40	2.56	0.81	DAZAP2	0.70	0.98	0.75
ASNS	1.78	1.63	1.57	CDKN1A	3.63	3.76	1.37	MBNL3	0.73	0.83	0.42
SLC7A11	5.56	4.44	-4.29	FAM63A	-0.59	-0.44	-0.62	LOC768350	2.36	3.41	2.58
NAA15	0.66	0.48	0.87	OC3	-1.96	-3.08	-1.46	LOC101747255	-3.69	-2.21	-4.45
RPS6KC1	0.58	0.56	-0.69	SEMA5A	-2.22	-1.64	-1.17	CD164	1.17	1.75	0.88
LPGAT1	1.11	1.21	0.56	MIR1766	-2.39	-1.81	-1.50	TRIQK	-2.34	2.11	-1.25
IL15	1.77	1.61	1.26	CHCHD10	-0.77	-1.09	-1.00	VPS37D	-3.74	-5.31	-4.29
UBR2	-0.92	-0.46	-0.45	RNF152	-1.10	-1.57	-1.08	LOC100859100	2.97	2.92	1.74
LRPPRC	0.47	0.44	0.60	TPT1	-0.81	-0.77	-0.71	RIPPLY3	3.41	1.98	-2.90
AKAP6	4.77	4.07	2.50	NUDT14	-0.64	-1.06	-0.75	LOC107052981	1.85	2.40	2.06
PRKCE	-1.05	-1.12	-1.68	CMPK2	1.87	4.62	1.26	TM4SF1	3.45	6.43	2.71
EGLN3	4.73	4.76	2.95	CLEC5A	4.92	9.97	7.45	LOC101750389	4.94	4.01	5.65
RHOQ	1.90	2.16	1.65	ISLR2	3.33	3.42	2.21	DISP1	-2.45	-2.41	-0.95
SNX32	0.55	0.60	0.66	ZDHHC3	-0.93	-0.73	-0.43	SLC13A3	-2.94	-3.31	-2.77
CNOT2	-0.42	-0.74	-0.34	GMIP	2.20	2.45	0.92				