EFFECTS OF POST HATCH DELAYED-FEEDING STRESS ON ENTERIC NERVOUS ACTIVITY AND INTESTINAL NUTRIENT TRANSPORT IN BROILER CHICKENS

by

Lydia C. Schlitzkus

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Summer 2022

EFFECTS OF POST HATCH DELAYED-FEEDING STRESS ON ENTERIC NERVOUS ACTIVITY AND INTESTINAL NUTRIENT TRANSPORT IN BROILER CHICKENS

by

Lydia C. Schlitzkus

Approved: _____
Yihang Li, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Velia M. Fowler, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____
John A. Pelesko, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

ACKNOWLEDGMENTS

Completing a master's degree has been an eye-opening experience and completing it during a pandemic has had its challenges, but it wouldn't have been possible without everyone in my life that supported me through this experience.

First, thank you to my advisor, Dr. Li, who helped coordinate my trials and helped me to learn the lab skills needed to complete the project. Thank you to my lab members, Liang-en Yu and Sofia Bialkowski for your training and help throughout my time in the lab. In addition, Lorik Tamazian and Famatta Perry have helped to make a great support network both inside and outside of lab. I would not have been able to collect all my data and learn so many skills without everyone's guidance and help. Thank you to all the undergraduate students who have come through the lab, who not only helped me with my project, but also helped me to learn how to teach and train others.

Thank you to Betty Cowgill, the Biology Department Mom, who was always available to help solve problems and guide us in the right direction. Without you I wouldn't have made it here. Thank you to my committee members, Dr. Selva, Dr. Duncan, and Dr. Arsenault for your time and guidance as I learned what it meant to be a researcher.

Academia brings so much knowledge, but I will also be forever grateful for the relationships it has allowed me to form. To all my friends that I met in graduate school, thank you! You were all there when I needed advice or help with everything. We laughed, cried, and complained together and most importantly, we grew together. To my friends from undergrad, you might have been farther away, but your support did not go unnoticed. Thank you for our endless FaceTime calls and for your support through everything.

I would be remised if I didn't thank my family. Throughout my life they have supported me, even when I didn't believe in myself. To my parents, your unwavering support is more than I could ever ask for. Without it, I would not be able to have completed this chapter, or any other chapters of my journey.

To the rest of my family, thank you for always being the foundation I can come back to when I need it. Without your love and support over the years, I wouldn't have been able to come this far. To my Grammy and Pappy, thank you from the bottom of my heart. From countless hours spent traveling to pig shows, to coming to school events, your support did not go unnoticed. You always encouraged me to push myself forward, and for that I am forever grateful. To my Omi, Opa, and Grandmom, thank you for supporting me and showing me that hard work, persistence, and struggles really do pay off.

To my pets, Walter, Bayley, Lucy and especially Pippa, thank you for showing me unconditional love anytime that I needed it.

I think Mark Twain said it best when he said “Twenty years from now you will be more disappointed by the things you didn’t do than by the ones you did do. So, throw off the bow lines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.”

Thank you to the University of Delaware for this chapter. I have learned so much about myself, others, and the world. So, as I prepare to set sail into the next chapter of my life, I know that my experiences at UD have given me the tools I need to continue to grow and become a better citizen of the world.

Thank you to everyone who has helped me in every chapter of my life. It is because of all of you that this was even possible!

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Early life stress alters gastrointestinal (GI) development leading to functional disorders (such as leaky gut, malnutrition, etc.). This leads to negative consequences later in life. The enteric nervous system (ENS) that coordinates many functions of the intestine is sensitive to stress and has been overlooked in agricultural animal's health. Post-hatch delayed feeding in the broiler industry is a common stressor which impairs the intestinal development and health of birds. This project serves to evaluate the effects of post-hatch delayed feeding stress on enteric nervous activity, and intestinal nutrient transport in broiler chickens. Ross 308 birds were incubated, hatched, and immediately allotted into either a normal feeding (NF) or delayed feeding (DF) group. DF birds did not receive feed for 72 hours post-hatch, while NF birds had immediate access to feed. Both groups had ad libitum access to water throughout the entire trial and following the 72 hours, DF birds and NF birds had ad libitum access to the same commercial diet until day 42 of age. At day 42 of age, a subset of each group experienced a 24-hour period of feed restriction to serve as a secondary stress event. Growth performance parameters were collected weekly throughout the 6 week grow out period. At day 3, 14, and 42 birds were euthanized, and intestinal samples were taken. Intestinal function was evaluated using the Ussing Chambers.

It was found that experiencing early life stress triggered immediate and long lasting changes in the intestinal functions and ENS activities of broiler chickens. Growth performance was reduced after the initial 72 hour feed delay, but by day 42 the DF birds are able to recover. The DF group had a lower FCR which suggests that there may be a mechanism such as increased nutrient absorption to allow for increased feed efficiency and potential compensatory growth.

These changes can predispose the birds to a higher risk of later life GI infectious diseases, especially under suboptimal growth conditions with secondary stress. The stressed birds were more adaptive to a secondary stress in terms of less ENS neural mediated responses and enhanced nutrient transport functions. An enhanced electrogenic nutrient transport function in the stressed birds could be the mechanism for compensatory growth seen in this group. Understanding the link between ENS activities and intestinal functions in stressed animals could give insight into developing novel targets that lead to the improvement of GI health and animal production.

Chapter 1

INTRODUCTION

The gastrointestinal tract plays vital roles in digestion including absorption of nutrients and communication with the body to ensure proper homeostasis for the animal. The gastrointestinal tract is also one of the largest mucosal barriers serving to separate the internal host environment from external components (Peterson and Artis 2014). This mucosal barrier is selective, meaning that it allows for the passage of nutrients and limits the movement of molecules that may be harmful to the body like microbes and antigens that are present in the lumen (Cavin et al. 2020). The selection of movement is particularly helpful when considering the many environmental factors that can affect the function of the intestine in addition to the impact on the intestinal epithelial layer. These environmental factors can range from early life stress to feed restriction, to disease pathogens that are present in the grow out houses (Careghi et al. 2005, H. Willemsen et al. 2010).

In general, the intestine is comprised of crypt-villus structures composed of a single layer of epithelial cells. These villi structures are crucial for increasing the surface area of the gut. They are finger-like projections that face the lumen of the intestine. This increase in surface area allows for maximal nutrient digestion and absorption into the bloodstream (Suzuki 2020).

These villus-crypt structures give rise to the epithelial cell layer which contains several cell types that allow for many of the functions that occur in the intestine. There are four main types of cells present here and they include the enterocytes, goblet

cells, Paneth cells, and enteroendocrine cells. The most abundant cell type is the enterocyte responsible for about 80% of the epithelial layer. This is followed by goblet cells that secrete mucus making up 5-10%, antimicrobial secreting Paneth cells comprise of less than 5% and finally enteroendocrine or hormone secreting cells make up 1% (Cheng and Leblond 1974). Due to the proximity to the lumen, all these cells come into close contact with digesta, and the first line of defense against any damage or potential disease pathogens is the mucus layer which is secreted by the goblet cells (Peterson and Artis 2014). The enterocyte cell population is the primary point of nutrient transport. These cells contain the machinery and transporters that allow for absorption of amino acids, glucose and other essential nutrients (Camilleri et al. 2012).

Within this one component of the intestine, many elements interact with each other including the protective mucus layer, absorptive enterocyte cells, the enteric nervous system, and the microbiome and immune system (Camilleri et al. 2012). These interactions are dynamic, and this semipermeable barrier allows for the movement of nutrients along with water and electrolytes while still protecting the host from any possible pathogens found in the luminal contents or produced from gut microbes. Various factors can affect this dynamic function of the gut, and this may result in the movement of toxic materials across the gut barrier and into the bloodstream. At this point, the entire body can become impacted, and complications can occur. Consequently, intestinal health and integrity are crucial for overall homeostatic animal health (Camilleri et al. 2012, Albert-Bayo et al. 2019).

Early Life Stress Model: Cause of Disease, Health Concerns, Acute Starvation Stress on Changing Gut Barrier Function, Other Animal Examples

It has been widely studied that early life stress is a major contributor not only to gut health concerns but also to overall health concerns for the body (Awad, Hess and Hess 2017, Camilleri et al. 2012). Within the poultry industry, there are a variety of major stressors that play a role in contributing to early life stress. From industry practices like vaccination or sex sorting to social stressors like transportation in groups, these factors have been widely associated with developmental and functional disorders (Careghi et al. 2005). In addition, there has been concern for not only short-term changes to gut health, but also long-term health concerns. These changes and their consequences for the host organism have been widely studied and characterized as the Developmental Origins of Health and Disease.

This concept was first described by David Barker in his role in understanding human development. He observed in newborns exposed to adverse environments at critical time points, like infancy, developed immediate and long-term changes that impact their survival. Barker and his colleagues noted that the first one thousand days of life have a prolific impact on a variety of body systems (Barker 2007). This includes the intestine, largely due to the interactions that it has with other body systems. For example, the group noted that early life stress causes differences in bacterial localization which leads to changes within the immune system both in the gut and throughout the body. In addition, there is a higher incidence of overall gut dysbiosis along with conditions like obesity and hypertension when stress occurs in these critical periods (Butel, Waligora-Dupriet and Wydau-Dematteis 2018). While

this theory was first discussed using humans, there has since been studies to show the same patterns in model organisms like mice, pigs, and chickens (Carpinello, DeCherney and Hill 2018). These patterns demonstrate that early life stress during critical points of development creates a variety of serious concerns for an organism and the intestine is no exception to this.

The poultry industry faces challenges that lead to early life stress for chicks. Forms of this stress can be physiological, psychological, and social. Due to recent increased demand for poultry products within the US and other countries, hatcheries are producing larger numbers of chicks resulting in delays in processing for these animals (Wang et al. 2014). These practices most commonly include sexing, sorting of birds, vaccination, and transport to grow-out houses (Nelson et al. 2018). It is all of these factors that can result in up to 72 hours without adequate access to food for these animals.

These challenges present some components to consider when evaluating feed delay in broiler chickens. The length of feed delay, age of bird, and specific breed of broiler chicken all impact the effect of a feed delay. In birds measured up to seven days of age, it has been reported that birds subjected to this delay in feed (DF) compared to the normally fed (NF) birds given immediate access to feed, have diminished growth performance and a higher mortality in their post hatch period (Careghi et al. 2005, Geyra, Uni and Sklan 2001). DF birds also have been shown to have delayed gut development following this period.

When considering the effects of feed delay for longer periods of time, the body weights of birds at day 21 and 42 were correlated to the body weight at day 7 where the delayed fed birds had a lower body weight. In addition, birds subjected to 36

hours of feed restriction post hatch had overall lower measurements for small intestine length and weight compared to birds given immediate feed access (Gonzales et al. 2003).

Despite these adverse effects seen in birds, some studies show that birds exposed to feed restriction post hatch exhibit compensatory growth by the end of the grow out period and they are able to reach similar body weights as compared to normally fed birds (Zhan et al. 2007). In some cases, feed restriction has been found to result in a better feed conversion ratio, higher efficiency, and body weight. In these studies, the birds were restricted from ad libitum feed access for various time points up to 48 or 72 hours. Up to 24 hours, there was an overall increase in broiler performance seen, however at later time points of restriction resulted in decreased performance (Santoso 2002, Zubair and Leeson 1996).

However, long term effects especially surrounding secondary stress exposure or potential disease exposure are much less understood in broiler chickens (Gonzales et al. 2003, Careghi et al. 2005). It is important to consider the intestinal changes both short and long term that these birds experience to understand the potential mechanisms that this early life stress imposes.

In addition, many studies used various types of diets as well as different chicken breeds, and they found that there are stark differences between male and female birds (Gonzales et al. 2003, van der Klein et al. 2017). Male birds grow faster initially, making their measurements initially higher, but female birds, which typically grow more slowly, exhibit compensatory growth at later time points in the grow out period resulting in differences in feed efficiency and overall performance (Shinde Tamboli et al. 2017, Gonzales et al. 2003).

Currently within the commercial broiler chicken industry in the United States, broiler birds are often fed a multistage diet that is tailored to the precise nutrient requirement for each stage of growth. This typically is a 3-5 stage diet that is presented to the birds in phases as they mature (Warren and Emmert 2000). The protein content and amino acid profile along with other crucial nutrients will vary in these diets (Moss et al. 2021). However, with rapid growth occurring over just 42 days, there is often concern that nutrients may be over or under supplied throughout the growing period. This then causes challenges for the birds regarding deficiencies in certain crucial nutrients like lysine and other essential amino acids (Fisher 1998). In addition, over supply particularly can lead to concern for environmental pollution. This leads to sustainability challenges for poultry producers (Cambra-López et al. 2022). With more research becoming available, there has been a dramatic increase in technology that allows for precise nutrition to be given in commercial broiler bird production settings where these staged diets are typically used (Méda et al. 2021). This along with more recent research on feed additives makes the nutritional status of broiler birds ever changing. This makes studying feed restriction even more important especially when considering the potential for benefit or harm on intestinal function which has a major impact on overall animal health.

When considering feed restriction, the type of feed restriction must first be defined. There are two types of feed restriction: quantitative and qualitative. Quantitative restriction is the process of reducing the feed intake compared to the ad libitum amount of feed that is consumed by the animal (Khetani et al. 2009). Qualitative restriction is the process of reducing the energy content or focusing on a specific nutrient composition in the diet (Urdaneta-Rincon and Leeson 2002). The

most common type of feed restriction that is investigated in the broiler chicken industry is quantitative restriction based on its ability to give an understanding of overall growth performance, an important measurement for poultry producers (Lunedo et al. 2019, Zhan et al. 2007). Additionally, due to the current practices used within the poultry industry, quantitative restriction aids in helping to understand the full impact on intestinal health and function along with overall broiler bird health (van der Klein et al. 2017).

These practices within the industry result in delayed feeding. This is a result of the practices like vaccination, sex sorting, and transportation which are all required to get the broiler birds to grow out houses (Careghi et al. 2005). It is during this time that the birds can have upwards of 72-hours without access to feed. It has been found that this feed-delay can lead to reduced growth performance in early life and altered intestinal development and function (Geyra et al. 2001).

Other Animal Examples: Rodents and Pigs

Rodents:

When early life stress occurs, it is known to impact a critical period for development in many animals including rodents like mice (DeMeo et al. 2002). This stress leads to increased risk of intestinal dysfunction and an increase in deficiency and disease in addition to affecting overall behavior of the intestinal microbiota (O'Mahony et al. 2009). Since rodents are mammals and give birth to live young, they are a helpful model to understand neonatal maternal separation (NMS) (Gareau, Jury and Perdue 2007). NMS has been associated with a variety of changes to rodents normal physiological and psychological patterns (O'Mahony et al. 2009). Studies

have shown changes to the cell populations that reside in the intestinal mucosa layer after NMS (Santos et al. 2001, Wong et al. 2019). This includes an enhanced population of cells like enteroendocrine and Paneth cells that contribute to the secretory function within the intestine. This increase in cell populations leads to an increased risk of enteroendocrine cell hyperplasia and increased production of serotonin. This leads to visceral hyperplasia which can impact the function of the gut. Serotonin released within the intestine activates many neural reflexes which help to regulate key functions of the GI tract like motility and secretion. In human cases of IBD, an increase in serotonin has been found especially in patients with increased pain and diarrhea symptoms (Kidd et al. 2008). The full mechanism for the increase in these cell populations and the role of serotonin is not fully known(Wong et al. 2019). However, the rodent model proves to be helpful in understanding these relationships and potential mechanisms and therapeutic treatments in intestinal dysfunction disorders.

Pigs:

Piglets are used to study early life stress for a multitude of reasons. They have many similarities to human organ systems. In addition, the pork production industry within the US exposes piglets to physiological, environmental and social stress upon weaning from the mother which has an impact on the overall growth and health of the animals (Campbell, Crenshaw and Polo 2013). One of the biggest challenges is that within the industry there are a variety of different practices used for weaning time points, diets may vary, and transportation and housing can vary as well (Boyd et al. 2019). When studying early life stress in piglets, two of the most common stress types are early and late weaned stressed piglets. This can result from industry practices that

are aimed at increasing animal productivity, which causes piglets to be weaned at an earlier age (Campbell et al. 2013). Early weaning stress, resulting from weaning between 15 to 21days of age, has been found to result in changes to the GI tract that create not only short-term changes, but also long-term changes that result in overall disruptions to gut function. Early weaning resulted in a reduction of transepithelial resistance, and a leakier gut shown by a higher flux rate from the mucosal to serosal side of the gut from ages five to nine weeks (Smith et al. 2010). These changes are thought to be a result of the quick development that is occurring during and immediately following the stress period. Namely changes to signaling pathways that can change the cell populations and neuronal input like the corticotropin releasing factor (CRF) pathway which is impacted when stress events occur. (Moeser et al. 2007). In comparison, late weaned groups of piglets, weaned at days 23 to 28 of age, appear to show less severe changes to the GI tract. In fact, some recent studies suggest that late weaning can help to give components of the gut time to develop especially the microbiota which can be helpful when considering future challenges the animal might face like the presence of disease (Pohl et al. 2017).

Secondary Stress: Exacerbating gut dysfunction

The effect of secondary stress and its relation to gut dysfunction has been widely described in certain cases, especially human conditions like Irritable Bowel Disease (IBD) and Crohn's Disease (Santos et al. 2001, Bennett et al. 1998). Particularly, the study of recurring or chronic stress has been thoroughly described in human and rat models. When looking at clinical studies following human patients with a higher level of chronic life stress there was a higher incidence of IBS cases. These patients were also more prone to flares in the conditions leading to a leakier gut

and more inflammation (Bennett et al. 1998, Sun et al. 2019). This results in health concerns for people. While this research has been completed in humans, the effect of secondary stress on broiler chickens has been much less widely studied. Much of the work in this area explores stress factors like heat and oxidative stress (Wu et al. 2018).

It is important to note that these types of stress vary in their characterization. When considering stress and the changes that it makes on the intestine and body it is important to be sure to consider the type of stress. While the effects of stress have been studied and reported, the specific underlying mechanism remains unclear (Santos et al. 2001). This is especially important when it comes to the possible mechanisms that could be contributing to things like leaky gut or epithelial barrier changes that lead to overall gut dysfunction.

Intestinal Function: Nutrient Absorption

The intestinal mucosa serves to absorb virtually all nutrients from the diet. Nutrients make their way from the lumen across the highly polarized intestinal epithelial mucosa layer and into the bloodstream via various nutrient transporters (Kiela and Ghishan 2016). The GI tract relies on its anatomical, functional, and histological specializations to properly regulate nutrient transport. The villus-crypt structures on the mucosa of the intestine are particularly important specializations of the GI tract (Camilleri et al. 2012). Villi contain epithelial cells that house absorptive cell types and allow for an increase in surface area to achieve proper nutrient transport. The GI tract participates in both passive and active nutrient transport (Kiela and Ghishan 2016). Together, these mechanisms allow for the nutrient requirement to be met and any changes to the gut function may impact this causing deficiency for the animal (Ferraris and Carey 2000).

One of the most impactful nutrients that is transported across the intestinal barrier is sodium. Sodium is particularly important when also considered the movement of other nutrients as well as the movement of water which is driven when the sodium gradient changes (Frizzell, Field and Schultz 1979). There are three main ways that sodium is moved throughout the small intestine. Nutrient coupled sodium absorption occurs through a variety of sodium-dependent transporters. This moves either amino acids or sugars in addition to sodium utilizing the gradient of sodium and its tendency to move from areas of high to low concentration. Additionally, sodium moves throughout the GI tract through electrochemical movement. This process is electroneutral since the movement is primarily mediated through the sodium/hydrogen exchange pump (Kato and Romero 2011). Lastly, the distal small intestine contains sodium channels within epithelial cells that also allow for electrogenic movement of sodium (Powell et al. 1985). Chloride often moves with sodium through the electrogenic pathway. The movement of sodium and chloride across the epithelial barrier has been shown to change when inflammation in the gut occurs. There is increased incidence of gut dysfunction through epithelial barrier dysfunction, increased levels of inflammation, and changes to gut microbiota (Kiela and Ghishan 2016). One of the biggest concerns with changes in sodium and chloride absorption is the increased incidence of diarrhea that comes with a higher rates of sodium absorption. Sodium and chloride serve to work to ensure that normal homeostatic fluid levels are maintained within the body. Changes in levels of absorption result in increased diarrhea and therefore lead to fluid loss causing dehydration (Powell et al. 1985). This is a major concern when an animal is experiencing stress and can be much more vulnerable to other diseases. These symptoms are often seen in cases of

IBD, and it is thought that there are several factors like neural stimulation and other nutrient absorption, that result in full gut dysfunction (Kiela and Ghishan 2016).

Within the diet, carbohydrates are often a major source of energy consumed by the animal (Moss et al. 2021). Sugars and starches are the primary basis of these carbohydrate sources. The most commonly fed sugars are monosaccharides like glucose and fructose. Commonly used disaccharides include maltose, sucrose, and lactose which are further digested into monosaccharides (Navarro, Abelilla and Stein 2019). These sugar types are broken down by various digestive enzymes that disrupt the linkages making them into a simpler glucose structure. This glucose structure is then able to be transported across the apical side of the intestine where it crosses the basolateral membrane and can be utilized by the body in various pathways like glycolysis which produces ATP energy for cellular processes (Kiela and Ghishan 2016, Chen, Tuo and Dong 2016).

The Sodium Glucose Linked Transporter or SGLT1 is a primary driver of the movement of glucose and galactose across the intestinal membrane. It is found on the brush border membrane (BBM) of enterocyte cells located in the small intestinal mucosa (Koepsell 2020). This transporter utilizes the movement of sodium to also move sugars, due to the concentration gradient pushing sodium. This gradient of sodium is maintained by the basolateral sodium-potassium ATPase pump (Lehmann and Hornby 2016). During this process, 2 sodium ions move along with one glucose molecule moving the glucose against its concentration gradient. In addition to the SGLT transport of glucose, there is also a family of GLUT transporters which are responsible for moving glucose by facilitated diffusion. This transport occurs across the basolateral membrane of the enterocytes (Chen et al. 2016). The amount of

glucose absorbed within the intestine can be adjusted using these two transport systems. However, the mechanism of this change remains to be fully elucidated in addition to working to understand the role that this absorption process plays in conditions like obesity and diabetes (Chen et al. 2016, Pfannkuche and Gäbel 2009).

Intestinal Function: Amino Acid Absorption

Proteins are comprised of amino acids which are the basic building blocks for many essential processes in the body, making amino acid absorption a key process in the body. For broiler chickens there is a crude dietary protein requirement and if there is no limitation on energy density, the quality and quantity of the protein source can impact overall broiler bird growth performance (Toghyani et al. 2020). Therefore, understanding amino acid absorption is key to understanding the overall impact of diet in broiler bird growth.

Amino acids are classified into essential and non-essential amino acids. Essential amino acids must be supplied in the diet as the body cannot process them otherwise. Essential amino acids are also considered to be limiting in the diet, since they must be supplemented for biological processes to occur at the proper rate. For broiler chickens, the most notable essential amino acids are methionine and lysine (Bröer and Fairweather 2018).

For nutrient transport to take place in the intestine, absorptive cells, located in the intestinal epithelium, are used. Absorptive cells in the intestine are enterocyte cells that line the epithelium of the lumen of the small intestine. These cells have been shown to have a clear role in digestion by facilitating the movement of water, amino acids, ions, vitamins, and carbohydrates along with playing a role in the immunological function of the gut (Mourad et al. 2009).

One major class of nutrient transporters in the intestine are neutral amino acid transporters. These amnio acids rely on the presence of sodium ions to move across the apical membrane of the intestinal epithelium. This transport is generally referred to as co-transport as it moves sodium and an amino acid. One of the most prominent transport systems that complete this movement is the B0AT transport system (Bröer and Fairweather 2018).

B0AT1 is a major focus of the B0AT transporter family due to the number of essential amino acids that it transports. These include leucine, isoleucine, valine, methionine, phenylalanine, tryptophan, threonine, and histidine. This transporter relies on a sodium gradient that is produced by the sodium-potassium ATPase pump present in cells within the intestine. For each sodium ion that is transported one amino acid is also transported (Bröer and Fairweather 2018). Once the amino acids are co-transported across the apical side of the epithelial cells, they then move into the bloodstream via the hepatic portal vein. It is here that the nutrient components can be utilized fully by the body.

Another key system involved in the movement of nutrients is the peptide transporter system, PepT1. This system is primarily expressed in the Brush Border Membrane (BBM) of enterocyte cells in the intestine. Its presence is most common in the jejunal and ileal segments of the small intestine. Under normal physiological conditions, PepT1 is found on the apical side of intestinal epithelial cells, moving di- and tri-peptides present in luminal dietary contents (Ingersoll et al. 2012). This system is key to moving larger peptide components that other systems cannot move. These larger peptides are still crucial for use in the body. However, when stress and inflammation occur to an animal and the intestinal tract is impacted there has been

noted changes to this transport system (Ihara et al. 2000). This includes a change in the expression profile of PepT1. In studies that included patients with chronic inflammation like IBD, there is a higher incidence of PepT1 present in the colon (Ingersoll et al. 2012). In addition, PepT1 changes proinflammatory pathways and hormone levels related to stress are also upregulated which may be correlated to the increase in PepT1 that is seen (Ingersoll et al. 2012, Cavin et al. 2020). This suggests that the ability to absorb key components of the diet is changed when an animal experiences stress or has changes to their nutritional status (Hayden and Carey 2000).

The intestine also plays a key role in absorbing water- and fat-soluble vitamins, minerals, and fats, all of which are critical to maintaining homeostasis for the organism. By working with other systems like the ENS, it responds to certain conditions that an animal may be experiencing, namely stress. It has been found that one of the biggest changes to the intestinal epithelium is the change in permeability (Cavin et al. 2020, DeMeo et al. 2002). With increased inflammation and disruption of the epithelium during stress periods comes greater permeability. While this may be beneficial for the animal and increase nutrient absorption, but often this is at a cost of other functions for the animal. This includes a greater chance of risk of diseases and infection from pathogens that make their way into the bloodstream and therefore negatively impact the entire body (Camilleri et al. 2012, DeMeo et al. 2002).

Enteric Nervous System (ENS) and its Role in Nutrient Absorption and Gut Function

The ENS is a part of the autonomic nervous system and includes a number of neural circuits that control motor functions, local blood flow, mucosal transport, and secretions, and modulates immune and endocrine functions (Goyal and Hirano 1996).

It is a center for the bidirectional flow of information from the body and sends signals from the intestine. This is important so that the intestine can communicate with the rest of the body. This becomes very important when the animal experiences things like stress that result in the release of certain neurotransmitters like serotonin and acetylcholine. These neurotransmitters can modulate gut activities like increasing or decreasing blood flow, changing motility rates, or impacting muscle activities (Cavin et al. 2020). The ENS functions like many other neural networks, where a nerve must reach its full action potential before being fired. This firing then results in the activation of a receptor in the gut which elicits a response to effect change in the intestine (Heanue, Shepherd and Burns 2016).

It has been found that while luminal nutrients and ENS activity both affect ion flux in the intestine, they generally work independently of each other. However, there is a need for ENS activity to regulate permeability in response to luminal nutrients (Cavin et al. 2020). In addition, previous work has shown that fasting events enhance neural control of basal ion transport and increase paracellular permeability in porcine intestinal segments. Cholinergic nerves help to restore the fasting-induced increase in the paracellular permeability (Hayden and Carey 2000).

While the role of the ENS in intestinal function has been evaluated, there is little information on the ENS and its role in nutrient absorption and the role that it plays when animals experience stress in the form of feed delays (Rao and Gershon 2018).

One area that has been studied is the application of Glucagon-Like Peptide-2 (GLP-2). It is known that GLP-2 is expressed in enteric neurons. It functions to help regulate gastric motility, intestinal secretions, and sugar transport (Drucker 2001).

GLP-2 is secreted from enteroendocrine cells in response luminal sugars. This then in turn helps to regulate the number of receptors like Sodium Glucose Linked Transporter 1 (SGLT1) which is a key receptor to moving glucose into the body from the luminal dietary components (Lehmann and Hornby 2016). In a study using mice intestinal tissue segments, it was found that GLP-2 cannot work in a paracrine manner because it is expressed in enteric neurons. When using an electric field stimulation technique, there was an increase in SGLT1 expression. This increase was eliminated when Tetrodotoxin was treated to the tissue. Therefore, there must be a connection between components of the ENS and the absorptive cell line and receptors in the small intestine (Moran et al. 2018). This study helped to show the connection of the ENS to nutrient absorption within the intestine. Future work in this area and in other species, like chickens, will allow for a better holistic understanding of the connections that the ENS has to nutrient absorption and intestinal functioning as a whole.

The ENS mediated secretomotor response in the intestine is key to maintaining proper intestinal function (Fuentes and Christianson 2016). It is key to ensuring the continuous mixing and hydration of luminal feed contents. The ENS uses its connections by neural reflex pathways in addition to the cell types that reside in the intestine (Cooke 2000). Within the intestinal epithelium, there are many ion channels that are expressed, and they regulate almost every function of the digestive process. This includes motility, fluid secretion, absorption and even the perception of pain within the intestine. When secretomotor neurons are fired acetylcholine or vasoactive intestinal peptide (VIP) is released(Nezami and Srinivasan 2010). This results in the secretion of chloride and bicarbonate ions which stimulates the secretion of fluid from the epithelial layer. Changes in these signaling processes can lead to motility

disorders and symptoms like diarrhea which can impact nutrient absorption ability within the intestine(Nezami and Srinivasan 2010, Fuentes and Christianson 2016).

Certain drug treatments can be used to target the ENS to help understand the effect of early life adversity on intestinal function. Veratridine is a drug of interest due to its mechanism of action and the resulting understanding of intestinal function. Veratridine treatment in the intestine results in voltage gated sodium ions channels to stay opened during sustained membrane depolarization (Ulbricht 1998). This activation allows for the measurement of veratridine-evoked short circuit current responses. These responses can be measured as an index of electrogenic ion transport that is induced via secretomotor neurons (Medland et al. 2016).

Veratridine can be paired with other nerve acting drugs to understand the role of the ENS further. Tetrodotoxin (TTX) is a sodium channel blocker (Cavin et al. 2020). This combined with veratridine during experiments, gives insight into the full effect of the ENS on secretomotor function in the small intestine.

Cholinergic signaling in the intestine also plays a large role in integrating the ENS and functions in the intestine (Hirota and McKay 2006). Similar to previously mentioned systems, the cholinergic division of the ENS relies on neurotransmitters to allow for the resulting changes in intestinal function. Acetylcholine is critical in controlling epithelial ion transport and water movement throughout the GI tract (Cooke 2000). The cholinergic nervous system affects basal ion movement and can also be measured through short circuit current. Within the cholinergic nervous system there are receptors that allow for the function of the ENS (Cheadle et al. 2014). Acetylcholine works on muscarinic receptors along with choline acetyltransferase (ChAT). Muscarinic receptor 3 is thought to aid in increases in chloride secretion

(Delvalle et al. 2018). Homeostatic levels of ChAT are critical to maintaining gut motility and ensuring the proper secretomotor response. It has been shown that without ChAT, there is intestinal dysmotility and dysbiosis (Johnson et al. 2018, Chen et al. 2018).

One of the most widely studied models for enteric nervous activity in relation to stress is the early life stress (ELS) model in pigs and rodents due to their similarity to humans (Gresse et al. 2017, O'Mahony et al. 2009). In a porcine study comparing early and late weaned pigs at both half and full maturity, it was found that there were markers of increased cholinergic signaling and a downregulation of muscarinic receptor 3 in the ELS group. This study also used ileal tissue with treatment of veratridine and saw that ELS animals had a heightened secretomotor response. In addition, this response was only seen in female, not male, pigs. This suggests that ELS in pigs induces long term, sex specific hypersensitivity of secretomotor neuron function. This may be due to the upregulation of the ENS, which suggests that there may be a mechanistic link between ELS and lifelong susceptibility to intestinal dysfunction (Medland et al. 2016).

This same pattern is also seen in rodents exposed to maternal separation stress. Western blotting and immunohistochemistry showed that there was an increased expression of ChAT. This indicated higher synthesis of acetylcholine. Together this suggests that ELS may predispose these animals to develop mucosal barrier dysfunction and intestinal hypersensitivity through the ENS. This may lead to conditions like IBD where the intestine does not function as it should leading to concerns like leaky gut and an overall decreased nutrient absorption (Gareau et al. 2007, Nakamori et al. 2021). While the impact of the ENS and ELS have been studied

in other animal models, the chicken model is much less widely studied. This lead to the hypothesis that a similar pattern could be seen in broiler chickens that experience ELS.

Electrophysiology: The Ussing Chamber and its Functional Measurements

The Ussing Chamber originates from the Danish zoologist, Hans Ussing. Ussing invented the technique and device in the early 1950s to measure short circuit current which can be interpreted as the net ion movement across a mucosal surface like the intestine (Ussing and Zerahn 1951). One important aspect of the Ussing Chamber system is that it allows for *ex vivo* measurement of permeability using fluorescent probes and electrophysiological measurements of epithelial tissue or cellular monolayers. The set-up of the chamber system allows for two chambers that isolate each side of the material that is being investigated. In the case of the intestine, the tissue is mounted on a slider that fits between the two sides of the chamber. This exposes both the mucosal and serosal sides while allowing for separate physiologic environments. Each chamber half contains a physiological buffer and is supplied with gas, typically a 95% O2/5% CO2 mixture, at a low pressure to represent physiologic conditions. The chambers are also maintained at physiological temperature via a water jacket (Clarke 2009). By maintaining these conditions, various changes can be made that allow for investigation into different areas. For example, changing the temperature of the system gives an idea of heat stress and its effect on the intestine. Another example is the use of the addition of nutrients or drugs to see their effect on the intestine or other mucosal barriers (Thomson et al. 2019).

One of these drugs is veratridine, a drug used to activate sodium gated ion channels. This leads to neuron depolarization. This change in the neuronal state allows for measurement of electrogenic ion transport that is induced by secretomotor neuron activity (Cavin et al. 2020). This paired with another drug, Tetrodotoxin can inhibit this effect due to the blocking of these channels. In studies completed using pigs as models for intestinal disease and damage, veratridine showed that experiencing ELS caused a heightened secretomotor response. In addition, this response was only seen in female, not male, pigs. This suggests that ELS in pigs induces long term, sex specific hypersensitivity of secretomotor neuron function. This may be due to the upregulation of the ENS, which suggests that there may be a mechanistic link between ELS and lifelong susceptibility to intestinal dysfunction (Medland et al. 2016).

In addition to the neuronal input on secretomotor function, studies have also shown a neuronal impact on nutrient absorption which can be measured on the Ussing chamber. In a study completed using mice small intestine samples, it was found that activation or inhibition through drug treatment resulted in changes to intestinal permeability to certain nutrients. There were acute changes in response to glucose and glutamine and significant changes when lipids were given. Samples were exposed to lipopolysaccharide (LPS). The resulting permeability was measured and it was found that there was a direct relationship between permeability and ENS activity (Cavin et al. 2020). This suggests that the ENS helps to regulate intestinal epithelial homeostasis, however there are many factors that contribute to this relationship like region of intestine, condition of the tissue, and environmental factors (Hayden and Carey 2000).

The Ussing Chamber system has several benefits. One of the main advantages of the system other than the *ex vivo* measurement ability is the precise measurement that it allows for. The system measures the physiology of live, polarized, and intact intestinal epithelial tissue (Clarke 2009). Since the tissue structure is kept intact and full tissue segments are utilized, there is an ability to understand how complex interactions between cell types occur. The system gives a chance to see how all cell types and structures interact to work within an animal system like the intestine(Arnold et al. 2019). In addition to this, the chamber system allows for the use of specific regions of the intestine to be investigated across different specimens. This precise control over the location of segments is helpful when comparing the different regions of the intestine and their specific functions (Thomson et al. 2019).

With advantages come disadvantages. The biggest disadvantage for the Ussing Chamber system is the limitation of replicates that exist in most systems. This means that there cannot be an analysis of a large set of intestinal segments either from one animal or comparing multiple animals in one experiment (Westerhout et al. 2014). This is due to the size of the current systems being offered which are typically 8 chamber systems. In addition, it can be difficult to mount live tissue onto the chamber in a quick enough manner to create consistent conditions for all tissue on the chamber.

However, there have been advancements made in this area allowing for up to twenty-four chamber systems. In addition to this, another disadvantage is the viability of tissue samples outside of the body. There is a limited time that the tissue will remain viable while on the chamber system even with the precise monitoring of physiological conditions (Sjöberg et al. 2013, Rozehnal et al. 2012).

Hypothesis and Project Aims

Hypothesis:

Early life delayed-feeding stress in broiler chickens alters the development of enteric nervous activities and intestinal functions, as well as GI adaptive responses to a secondary feed restriction stress in later life.

Aims:

1. Determine the influence of early life delayed-feeding stress on the growth performance, intestinal barrier function and nutrient transport function of chickens.
2. Determine the effects of delayed-feeding stress on GI mucosal neural receptors and neurotransmitters expression, and ENS mediated intestinal secretory function.
3. Evaluate how the altered intestine, by early post hatch delayed feeding stress, respond to a 24h-feed restriction stress in later life, in terms of intestinal function and ENS activities.

Chapter 2

MATERIALS AND METHODS

Animals, Housing, and Experimental Design

A total of 150 eggs were obtained from a local commercial hatchery (Moyer's Chicks, Inc., Quakertown, PA). They were incubated at 99.5 °F and 60% humidity for 21 days. Beginning on day 20, the incubator was checked every 4 hours and cleared of hatched chicks to prevent prolonged delays in access to feed. Over the next 24/48 hours, 2 batches of chicks were removed from the incubator, weighed, and assigned to one of ten isolators. In total there were 6 normal feed (NF) groups and 4 delayed feed (DF) groups. The chicks were assigned in a way that ensured that the starting average of the isolators was equal. The NF group had immediate access to feed and the DF group had delayed access for 72h post-hatch. All groups had immediate ad libitum access to water. No in-ovo or in-hatchery vaccinations were administered. Each isolator was maintained around 90°F for the first week and then decreased by 5°F weekly until a final temperature of 70°F was reached (Shinde et al. 2015). The birds were provided with 24 hours of light for the entire length of the grow out (Payne, Proszkowiec-Weglarz and Ellestad 2019). At day 42 of age, a subset of both groups, NF and DF, experienced a 24-hour feed restriction period. Feed was removed from each isolator, and birds continued to have ad libitum access to water. All procedures and protocols were approved by Institutional Animal Care and Use Committee of University of Delaware.

Animal Growth Performance Data

Bird body weight and feed weights were taken weekly on days 0, 3, 7, 14, 21, 28, 35, and 42. This was used to determine the body weight (BW), average daily gain

(ADG), average daily feed intake (ADFI) along with feed conversion ratio (FCR) over the entire grow out period for all birds.

Sample Collection

Tissue and blood samples were collected at d3, 14, 15, 21, 28, 35, 41, 42, and 43 after birds were euthanized by cervical dislocation and exsanguination. The entire GI tract was removed and the small intestine was located. The jejunum and ileum were separated using the yolk sac and Meckel's diverticulum as landmarks between the 2 segments. Middle jejunal and ileal segments were flushed using PBS and then mucosal and muscle tissue were separated before being immediately frozen in liquid nitrogen for further use. Distal ileal segments were put in ice-cold Ringer's Solution which was then mounted to the Ussing Chamber.

Ussing Chamber Data Collection

The Ussing chamber was used to measure differences in intestinal electrophysiology along with treating tissues with various drug treatments. There was a total of 4 replicates completed. As previously mentioned, the distal ileum was used, and it was identified using Meckel's diverticulum. After dissection, the tissue was immediately put into a tray with ice-cold Ringer's solution. This solution was pre-incubated with carbogen-gas (95% O_2-5% CO_2). For each ileal sample, the tissue was dissected further using a dissecting microscope. The tissue segments were opened at the mesentery and rinsed to remove digesta particles. The open tube was pinned down and the muscle layer was stripped. The total area of each tissue sample mounted to the chamber was 0.3cm^2 or 0.5cm^2. The apical and basolateral sides of the tissue were each incubated in 5 mL of 40°C Ringer's solution. Both the mucosal and serosal sides were continuously gassed with carbogen to allow for oxygenation and circulation of

the buffer by gas lift. The temperature was maintained at 40°C by a circulating thermostatic water jacket.

Every Ussing chamber apparatus was connected to 2 pairs of dual-channel current and voltage Ag-AgCl electrodes. These were connected via 3.5% agar bridges filled with broiler Ringer's solution. The tissue was alternatively hit with a positive or negative pulse of 20 μA and 100 ms duration. After 15-20 minutes of equilibration under open-circuit conditions, the tissue was short-circuited by clamping the voltage to zero. The potential difference (mV), short-circuit current (I_{SC}, μA/cm^2), and transepithelial resistance (R_T, Ω x cm^2) were continuously measured by the chamber software.

In addition, 10 mM glucose was added to the serosal side and 10 mM mannitol was added to the mucosal side of the tissue samples on the chamber. After allowing for stabilization, each chamber was treated with one of the following nutrients: di-peptide (Gly-Sar), alanine, lysine, or glucose. In addition, various inhibiting or activating drugs were used to investigate the neural relationships in the intestine. Transepithelial resistance (TER) is an overall measurement of gut integrity. The resulting difference in this voltage measurement is recorded as the TER, and this can be interpreted as the tightness of the epithelium. Barrier integrity was measured using the Ussing chamber and adding certain treatments that give an understanding of the movement occurring across the intestine. Fluorescein isothiocyanate dextran (FITC-d) was added and the flux rate was measured by taking samples from the chamber. NBD-labeled glucose was added in the same manner to the chamber. Potential different and short circuit current were measured across the intestinal epithelium.

RNA Isolation and cDNA

A commercially available kit (RNeasy Mini kit, Qiagen) was used for RNA isolation. All purification steps were performed according to the manufacturer's instructions with the total RNA eluted in 40µL RNase-free water. Then a Nanodrop was used to determine RNA quantity Additionally, a 3% agarose gel denatured with formaldehyde was used to check RNA quality. Synthesis of single-stranded complementary DNA (cDNA) from total RNA was performed using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Cat. No. K1672). In each DNase reaction there was a total of 4000ng RNA, 1µL dsDNase buffer, 1µL dsDNase, and up to 10µL of nuclease-free water. Each cDNA reaction contained 4µL of 5x reaction mix, 2µL maxima enzyme mix, and 4µL nuclease-free water for a total combined volume of 20µL.

The reactions were placed in PCR tubes and then a Veriti 96-well thermocycler (Applied Biosystems) was used to run the reaction. The DNase reaction was incubated at 37°C for 3 minutes. The cDNA reaction was incubated at 25°C for 10 minutes followed by 65°C for 30 minutes. The reaction was terminated by heating the samples to 85°C for 5 minutes. The resulting cDNA products were stored at -20°C until further analysis.

qRT-PCR

Real-time quantitative PCR of cDNA was performed using a QuantStudio™ 3 Real-Time PCR system (Thermo Fisher Scientific) 96-well PCR plates were used. All reactions were made at a 20uL final volume and consisted of 10uL PowerUp™ SYBR™ Green Master mix (Applied Biosystems, Cat. No. A25742), forward and reverse primers each at a final concentration of 300nM or 600nM (0.3µL or 0.6µL of a

10µM stock), 5µL of cDNA (15ng/µL per reaction), and nuclease-free water (Invitrogen, ThermoFisher Scientific, Cat. No. 10977-015) for a final reaction volume up to 20µL. Each reaction was performed in duplicate. Primers are found in Appendix A. The amplification program used was a hold stage for one cycle of 50°C for 2 min and 95°C for 2 min, a PCR stage for 40 cycles of 95°C for 1s and 60°C for 30 seconds, and a melt curve stage with one cycle of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. The melt curve was analyzed at the end of the run to determine single product amplification. The comparative Ct method (ΔΔCt method) was used to quantify PCR results. The Ct values of each gene were compared to the geometric mean of two housekeeping genes (HMBS and TBP) (Schmittgen and Livak 2008). Gene expression data were calculated as fold-change of target genes expressed in each sample relative to the mean of the control group (NF birds).

$\Delta C_T = C_{T\ target} - C_{T\ reference}$

$\Delta\Delta C_T = \Delta C_T$ sample – ΔC_T control (NF bird average)

Fold change= $2^{-\Delta\Delta C_T}$

Statistics

Statistical analysis was completed using Microsoft Excel 365 (Redmond, WA, USA) and GraphPad Prism 9 (San Diego, CA, USA). All data from birds at d3 to d35 of age were analyzed by Student's t-test between DF and NF groups. Two-way ANOVA and post hoc multiple comparison were used to analyze data from secondary feed restriction stress experiments, between NF and DF groups, and between starved and non-starved groups. Data are presented as means ± SEM. A *P*-value less than or equal to 0.05 (*, $P \leq 0.05$) is considered significant. A *P*-value less than or equal to 0.10 (#, $P \leq 0.10$) represents a trend in the data.

Chapter 3

RESULTS

3.1 Delayed Feeding Stimulates Compensatory Growth in Later Life for Broiler Birds

Body weight was determined by weighing the animals at various points throughout the 42 day grow out period. The data showed that delayed feeding significantly reduced the body weight ($P \leq 0.05$) until day 28. By day 42 of age, no significant differences were observed. The delayed fed (DF) birds were numerically heavier than the normal fed (NF) birds **(Fig. 1)**.

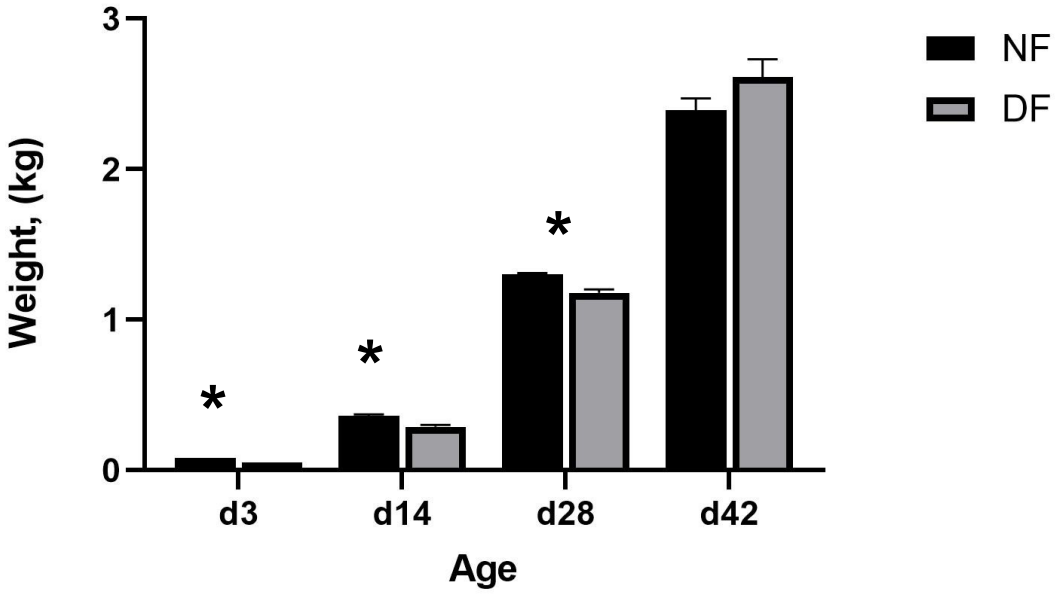


Figure 1. **Body weight analysis over a 42 day grow out period.** Effects of delayed feeding on body weight (d3~d42). Measurements of (10~12) birds were averaged in each pen, n = 4~6. Data analyzed using Student's t-test. Data are presented as mean ± SEM. * $P \leq 0.05$

Average daily gain was analyzed over the 42 day grow out period to determine the body weight gained in both the NF and DF groups **(Fig. 2)**. The DF birds had a significantly lower ADG from day 3 until day 28-35 whereas the DF birds had a significantly higher ADG ($P \leq 0.05$). From day 28, the DF birds continued to surpass the NF birds which was reflected by the higher ADG ($P \leq 0.10$). Comparing NF and DF groups throughout the entire grow out, there is no significant difference **(Fig. 2)**.

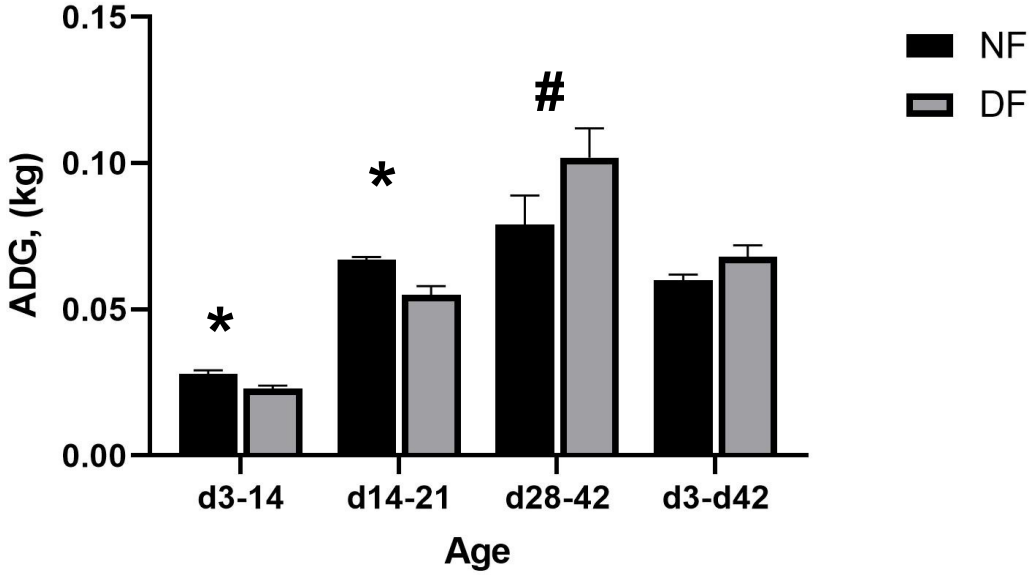


Figure 2. **Average daily gain (ADG) analysis over a 42 day grow out period.** Effects of delayed feeding on average daily weight gained (d3~d42). Measurements of (10~12) birds were averaged in each pen, n = 4~6. Data analyzed using Student's t-test. Data are presented as mean ± SEM. * $P \leq 0.05$, # $P \leq 0.10$.

Average daily feed intake was analyzed over the 42 day grow out period to determine the daily feed intake in both the NF and DF groups **(Fig. 3)**. The DF birds had a significantly lower ($P \leq 0.05$) ADFI from day 3 to day 14. By day 17 to 21 the DF birds consumed more feed than the NF birds until day 35 when the NF birds had a higher ADFI. ADFI overall the entire grow out period while not significantly different, was numerically lower in the DF birds. This indicates that while they ate less feed, there may be a mechanism for increased body weight seen in Figure 1.

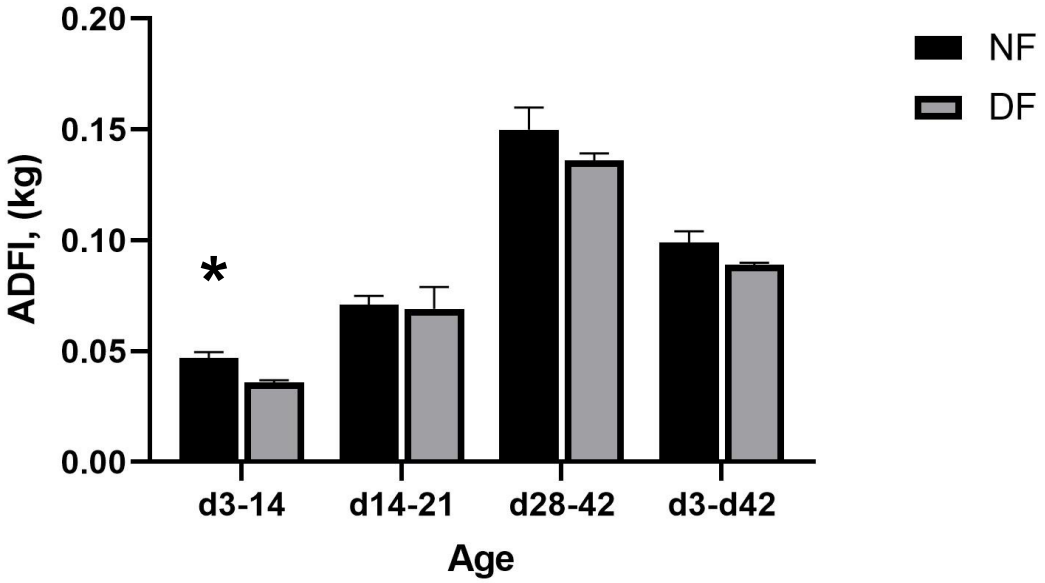


Figure 3. **Average daily feed intake (ADFI) analysis over a 42 day grow out period.** Effects of delayed feeding on average daily feed intake (d3~d42). Measurements of (10~12) birds were averaged in each pen, n = 4~6. Data analyzed using Student's t-test. Data are presented as mean ± SEM. * $P \leq 0.05$.

The FCR was significantly ($P \leq 0.05$) reduced from d3-14. While there was not a significant difference in FCR overall during the entire grow out, the DF birds had a numerically overall lower FCR **(Fig. 4)**. This data suggests that an early life stress event in the form of a feed delay does impact the birds, and the DF birds may have an increased feed efficiency.

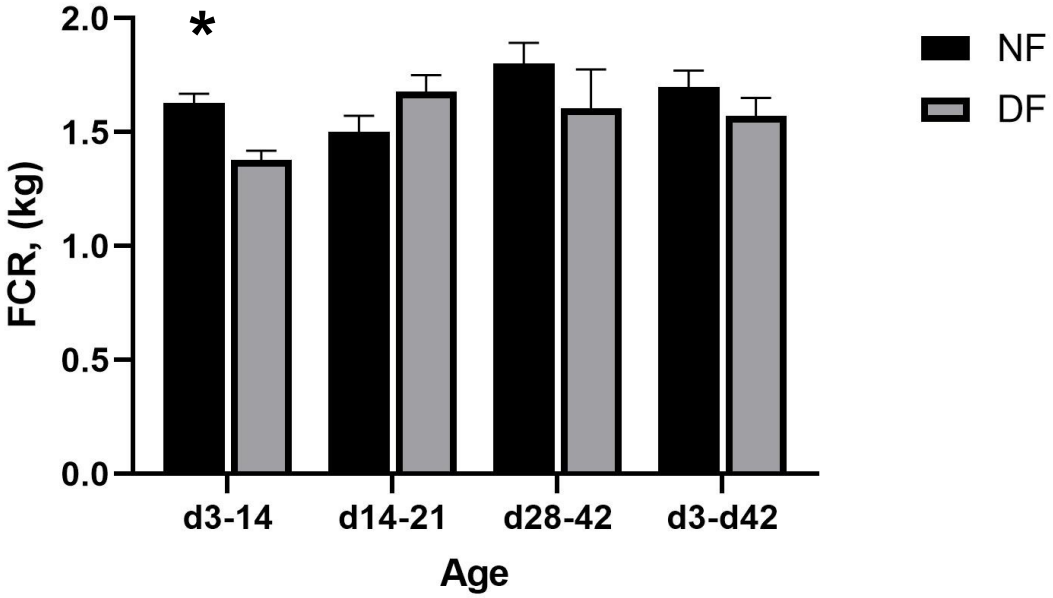


Figure 4. **Feed conversion ratio (FCR) analysis over a 42 day grow out period.** Effects of delayed feeding on feed conversion (d3~d42). Measurements of (10~12) birds were averaged in each pen, n = 4~6. Data analyzed using Student's t-test . Data are presented as mean ± SEM. * $P \leq 0.05$

3.2 Delayed Feeding Acutely Activates Enteric Nervous Activity and Alters Intestinal Functions Early in Life (d3 and d14)

To measure intestinal function, the Ussing chamber, an *ex vivo* based electrophysiological analysis was used. This technique allows for the evaluation of live intestinal barrier integrity in the ileal tissue. The addition of FITC-Dextran (FD4) can be used as a marker of flux from the mucosal to the serosal side of the tissue **(Fig. 5 A)**. Short circuit current was measured in response to the addition of a nutrient dose to the luminal side of the tissue **(Fig. 5 B**). This gives an understanding of the movement of ions throughout the mucosal tissue. In addition, the short circuit current was also measured when drug treatments were added to the Ussing chamber system. Veratridine was used to activate the voltage-gated sodium ion channels. This activation leads to neuron depolarization and subsequent changes in short circuit current which can be measured as an index of electrogenic ion transport from secretomotor neurons (Medland et al. 2016). The changes in current that resulted from Veratridine treatment give insight into the potential for nerve stimulated ion movement **(Fig. 5 C)**. As a result, there is an understanding of the role that enteric nerves play on ion movement in addition to the movement of other nutrients like amino acids

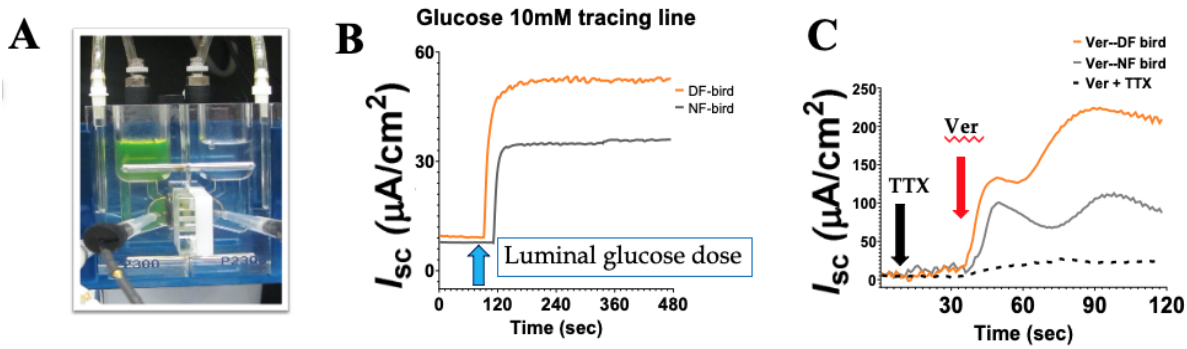


Figure 5. **Ussing chamber technique for intestinal function measurements. A**: Addition of FITC-Dextran 4k (FD4) in a Ussing chamber mucosal side used to detect FD4 Flux. **B**: short circuit current change in response to luminal glucose dose in both NF and DF fed birds. **C**: short circuit current change in response to nerve activating drug, Veratridine, and nerve inhibiting drug Tetrodotoxin in both NF and DF birds.

Effects of delayed feeding on intestinal functions after the 72-hour feed delay at 3 days old, modeling acute stress were also measured. Basal short circuit current was measured representing the chloride secretion or sodium absorption across intestinal tissue. In other words, this represents the basal ion movement. This was found to be significantly lower in the DF birds **(Fig. 6 A)**. DF birds had a significantly lower ($P \leq 0.05$) TER value, which indicated a leakier gut in the DF group **(Fig. 6 B)**. Additionally, the flux rate of FD4 was not significantly different between the two groups **(Fig. 6 C)**.

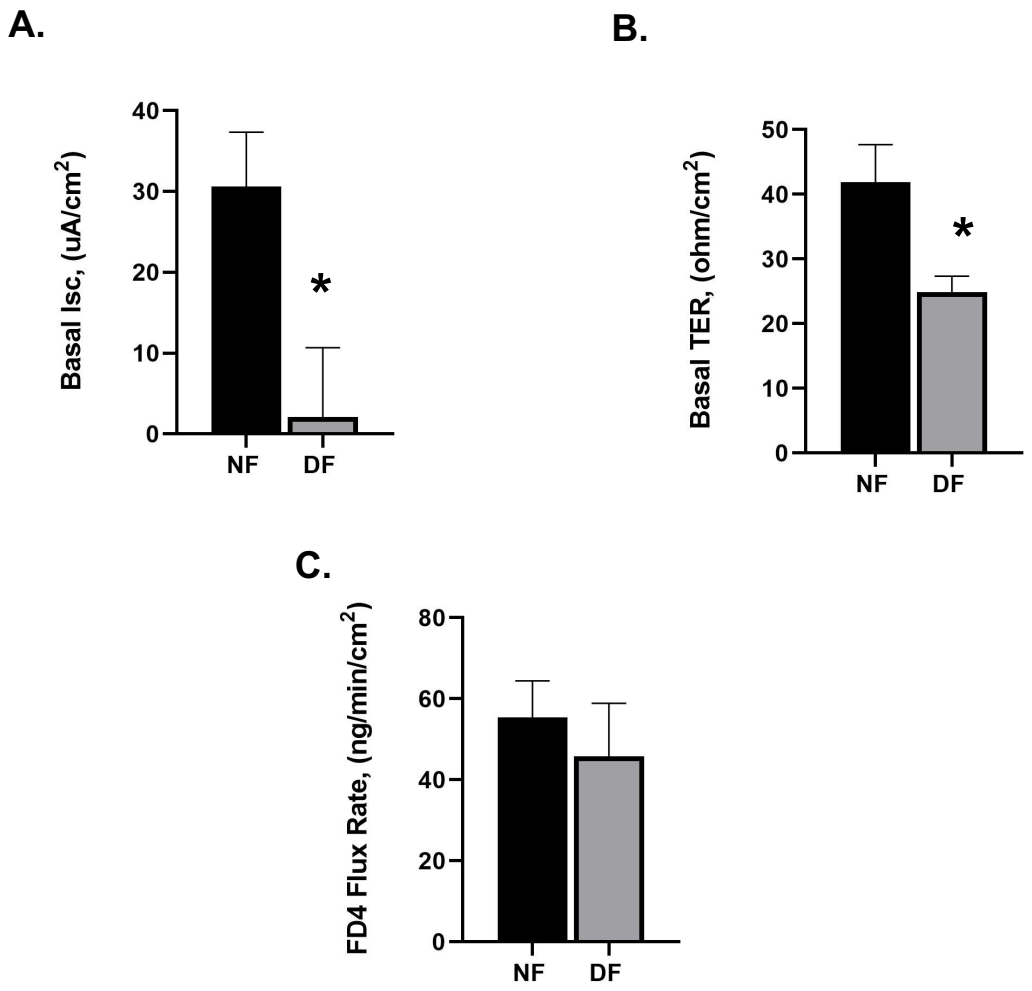


Figure 6. **Effects of delayed feeding on intestinal barrier functions at day 3 of age.** Initial barrier function was indicated by A: basal Isc (uA/cm^2), B: TER (ohm/cm^2), and C: FD4 flux rate (ng/min/cm^2). Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$

The Ussing chamber was used to measure the effects of delayed feeding on intestinal electrogenic nutrient transport at day 3 of age. Nutrient transport of glucose, di-peptide, alanine, and lysine were all significantly higher in the DF birds ($P \leq 0.05$)

as measured by the change in short circuit current on the chamber **(Fig. 7)**. This measurement represents Na-dependent electrogenic transport of nutrients.

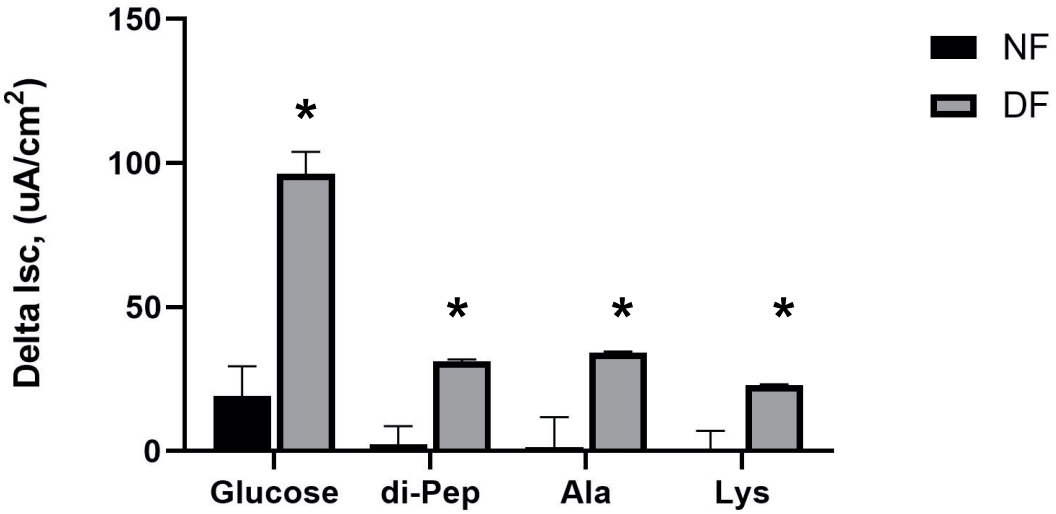


Figure 7. **Effects of delayed feeding on intestinal nutrient transport at day 3 of age.** Isc changes in response to electrogenic nutrients (glucose, di-peptide, alanine, lysine) transport (uA/cm^2).). Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$

To investigate the ENS 72-hour feed delay at 3 days old, modeling acute stress, changes were found in intestinal nervous tone and epithelial barrier function, and nutrient absorptive function. Ileal tissue from NF and DF birds was mounted to the Ussing chamber and treated with Veratridine, a neural activator of sodium gated ion channels. The resulting short circuit current was recorded. It was found that the DF

birds were more significantly more ($P \leq 0.01$) sensitive to this activation by Veratridine treatment. When the same tissue was treated with a neural inhibitor, Tetrodotoxin, the effect was neutralized **(Fig. 8)**. This shows that acute stress experienced by the DF birds makes the ENS more sensitive, especially when it comes to the electrogenic secretion.

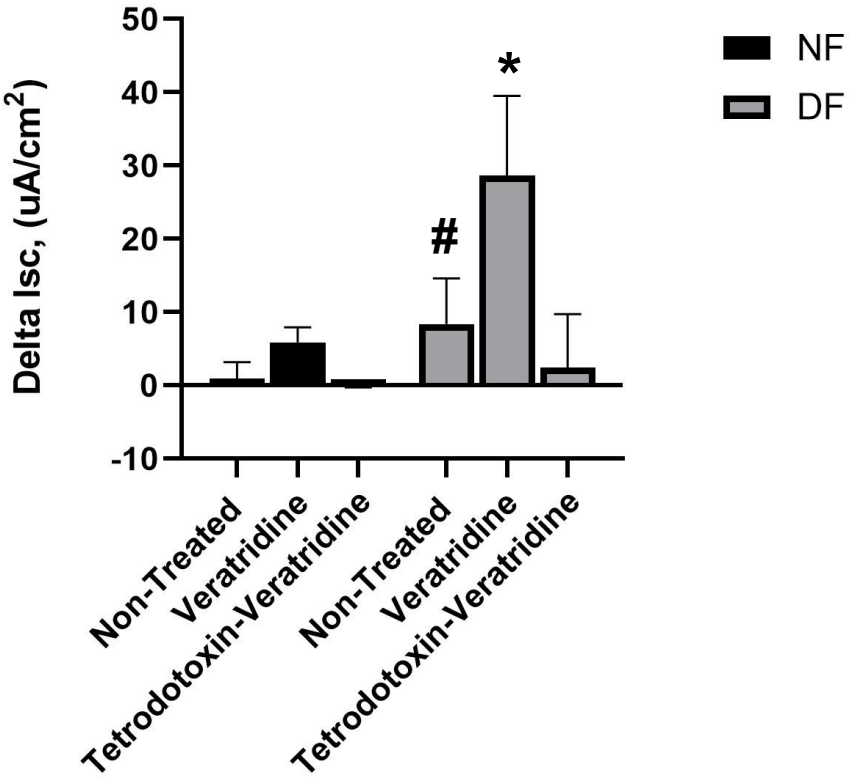


Figure 8. **Enteric nervous activity at day 3 of age.** ENS activity was measured by Veratridine stimulated short circuit current change (Isc, uA/cm^2) with or without sodium-channel inhibitor Tetrodotoxin. Drugs were given at the following doses: Tetrodotoxin 10μM, Veratridine 50μM. Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$, # $P \leq 0.01$

In the same manner as day 3, the Ussing chamber was used to measure the effects of delayed feeding on intestinal function at day 14 of age. The basal short circuit current, representing the basal ion movement across the intestinal barrier was to not significantly different between treatment groups **(Fig. 9A)**. The basal TER was also not significantly different, and the groups have numerically similar TER by day 14 **(Fig. 9B)**. The flux rate of FD4 was numerically higher ($P \leq 0.10$) in the DF birds as compared to the NF birds. This indicates the movement of molecules from the mucosal to serosal side of the intestine **(Fig. 9C)**.

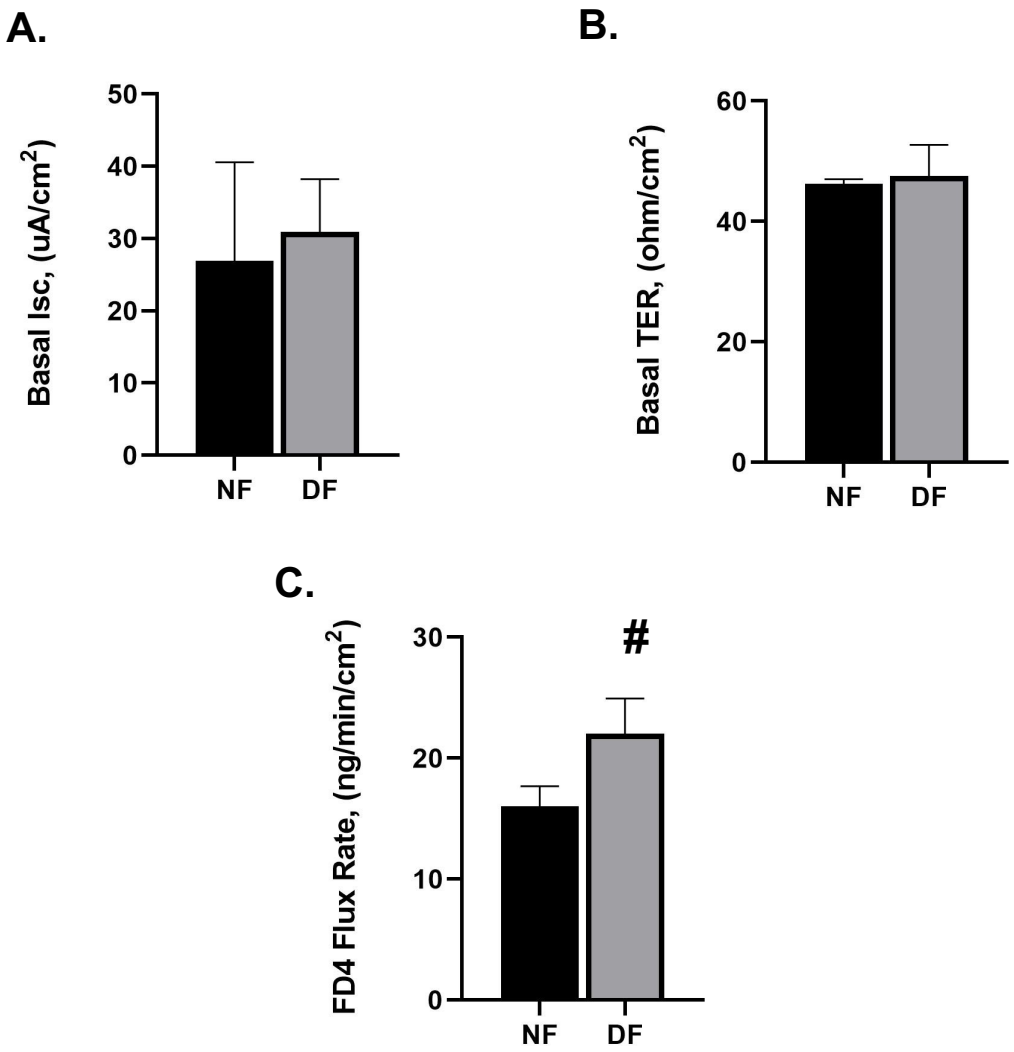


Figure 9. **Effects of delayed feeding on intestinal barrier functions at day 14 of age.** Initial barrier function was indicated by A: basal Isc (uA/cm^2), B: TER (ohm/cm^2), and C: FD4 flux rate (ng/min/cm^2).). Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. # $P \leq 0.010$

The Ussing chamber was used to determine electrogenic movement of glucose at day 14 of age in the same manner as day 3. By day 14 there is a

significant ($P \leq 0.05$) increase in glucose transport in the NF birds. There is no significant difference in the glucose transport when the tissue was treated with Veratridine although the NF glucose absorption is numerically higher **(Fig. 10)**.

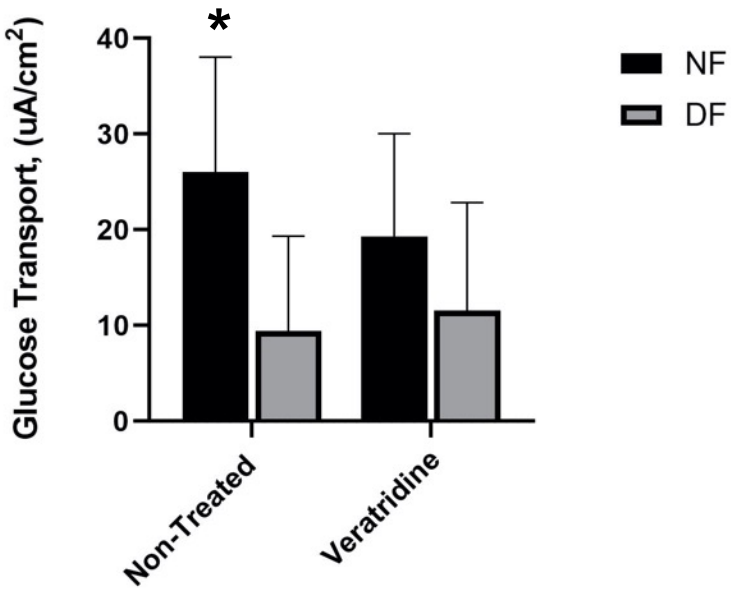


Figure 10. **Effect of delayed-feeding on nutrient transport at day 14 of age: Glucose**. Nutrient stimulated Isc changes, in response to electrogenic nutrient transport of glucose (uA/cm^2) were recorded. Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$

When looking at both treatment groups at 14 days of age, the Ussing chamber was once again implemented. The same drugs, Veratridine and Tetrodotoxin were

used to treat the tissue and either activate or neutralized the ENS respectively. Both of the groups appear to have lower delta short current values as they did at 3 days of age and there is no significant difference between NF and DF tissue. By day 14, the NF birds are more sensitive to neural activation compared to the DF birds **(Fig. 11)**. When looking at the delta short circuit current, or the change in short circuit over the course of the experiment, there is a significantly lower ($P \leq 0.05$) delta short current when the tissue is treated with Veratridine **(Fig. 11)**. This suggests that the birds may be able to overcome the effects of the stress experienced in early life regarding enteric neural activation as delta short circuit current is an indication of ENS mediated secretory function.

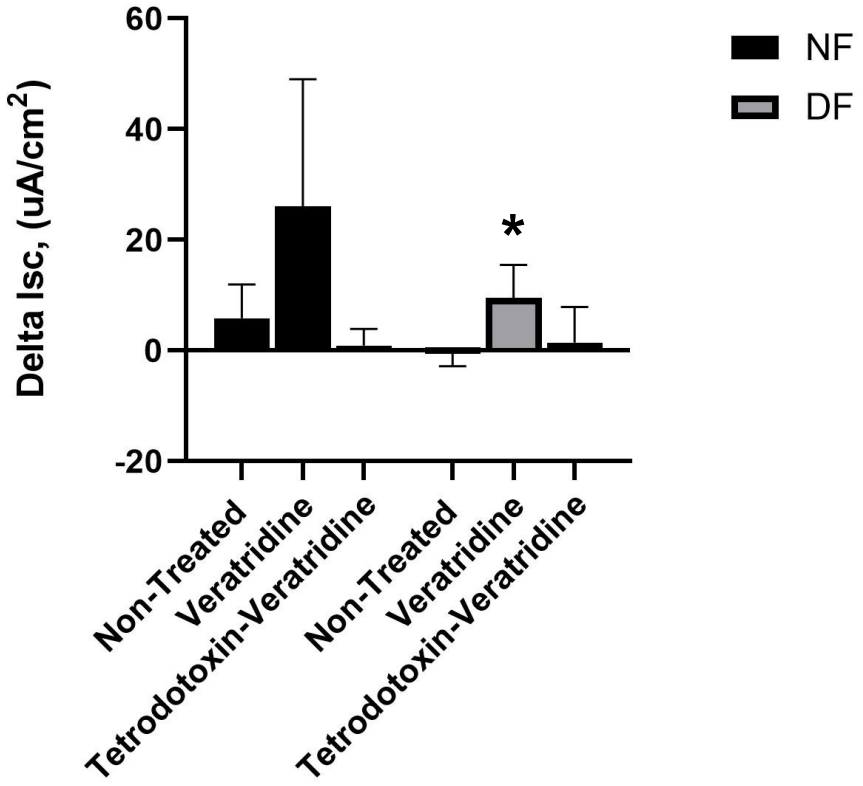


Figure 11. **Enteric nervous activity at day 14 of age.** ENS activity was measured by Veratridine stimulated short circuit current change (Isc, uA/cm^2) with or without Sodium-channel inhibitor Tetrodotoxin. Drugs were given at the following doses: Tetrodotoxin 10μM, Veratridine 50μM. Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$

3.3 Early Life Stress Damages Intestinal Barrier Functions and Adaptations to a Secondary Stress in Later Life (d42)

The effect of delayed feeding on intestinal barrier function later in life at day 42 of age with and without secondary feed stress was measured using the Ussing chamber. When examining the relative basal short circuit current, there was a significant decrease ($P \leq 0.01$) in basal short circuit current in the NF birds after the secondary stress event. There was also a significant decrease ($P \leq 0.05$) in relative basal short circuit current in the DF birds when they experienced the secondary stress event **(Fig. 12)**. This indicates that acute feed restriction/starvation decreased basal ion movement across the intestinal barrier.

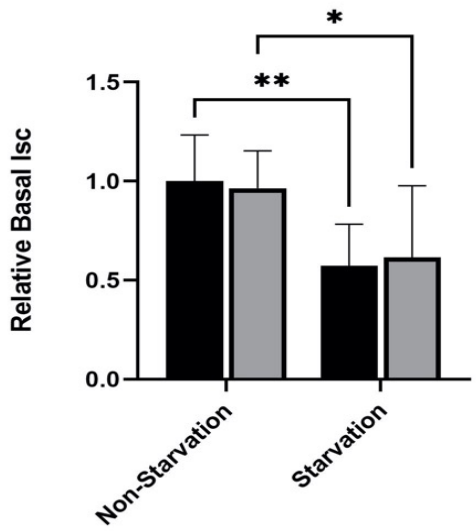


Figure 12. **Effects of delayed feeding on intestinal barrier function later in life at day 42 of age with or without secondary stress.** Initial barrier functions were indicated by Relative Basal Isc. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks * $P \leq 0.05$, ** $P \leq 0.01$

Relative potential difference (PD) across the intestinal epithelium was measured using the Ussing chamber. The NF birds that experienced starvation had a significantly lower ($P \leq 0.001$) relative PD. When comparing the DF birds, there was also a significant decrease ($p \leq 0.05$) in the relative PD **(Fig. 13)**.

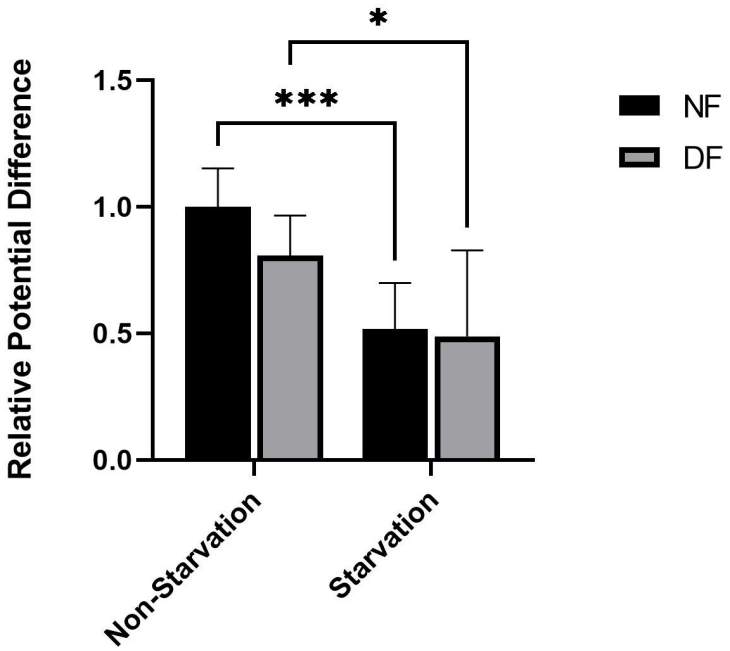


Figure 13. **Effects of delayed-feeding on intestinal functions with or without a secondary starvation stress at day 42 of age.** Relative Potential difference (PD), was measured on the Ussing chamber. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks *p≤ 0.05, *** $P \leq 0.001$

Relative transepithelial resistance (TER) was measured at day 42 and after the 24-hour feed-restriction stress period. By day 42 of age there was no significant difference in relative TER, however, experiencing starvation resulted in a significant reduction ($P \leq 0.05$) in relative TER in the feed restricted birds **(Fig. 14)**. The reduction in TER seen in the feed restricted DF birds indicates that the gut may be leakier because of the disruption of tight junctions within the intestinal epithelium.

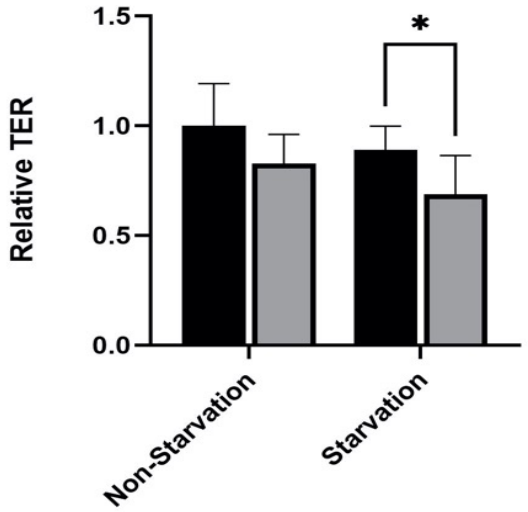


Figure 14. **Effects of delayed-feeding on intestinal barrier integrity with or without a secondary starvation stress at day 42 of age**. Relative Transepithelial resistance (TER), was measured as an indicator of gut barrier integrity. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks. * $P \leq 0.05$

In addition to the results from Figure 14, the results after the tissue was treated with FITC Dextran showed a similar pattern. While there were no significant differences, the DF birds had a numerically higher relative FD4 flux rate initially, and after feed restriction compared to the NF birds **(Fig. 15)**. This indicates that acute early life stress impacts gut barrier function initially and in the long term for broiler birds.

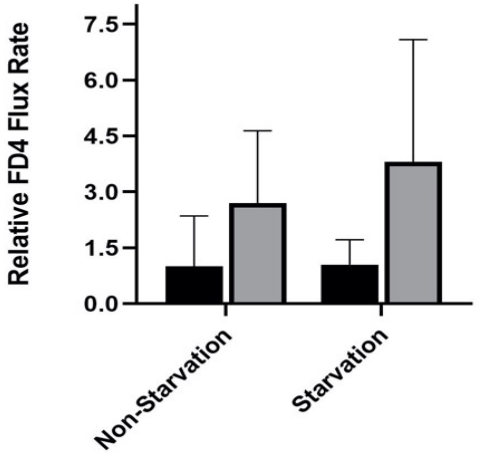


Figure 15. **Effects of delayed-feeding on intestinal barrier integrity at day 42 of age.** FIT C Dextran (FD4) was added to the Ussing chamber and the resulting fluctuation from mucosal to the serosal side was measured. This resulted in the Relative FD4 flux rate. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks.

In order to investigate the effect of early life stress on enteric neural function later in life, the Ussing chamber was used to record changes in short circuit current reported as (delta Isc) values with veratridine treatment. The tissue was treated with Veratridine as a neural activator in the same manner as previously mentioned. At day 42 of age there was a significant increase ($p \leq 0.01$) in the delta Isc of DF non-starved birds. Indicating that there is enhanced neural activity at later life stages. There is a significant increase ($p \leq 0.05$) in the delta Isc of Non-starved and starved NF birds. **(Fig. 16)**. This indicates a hyperactive ENS after acute starvation stress in the control animals.

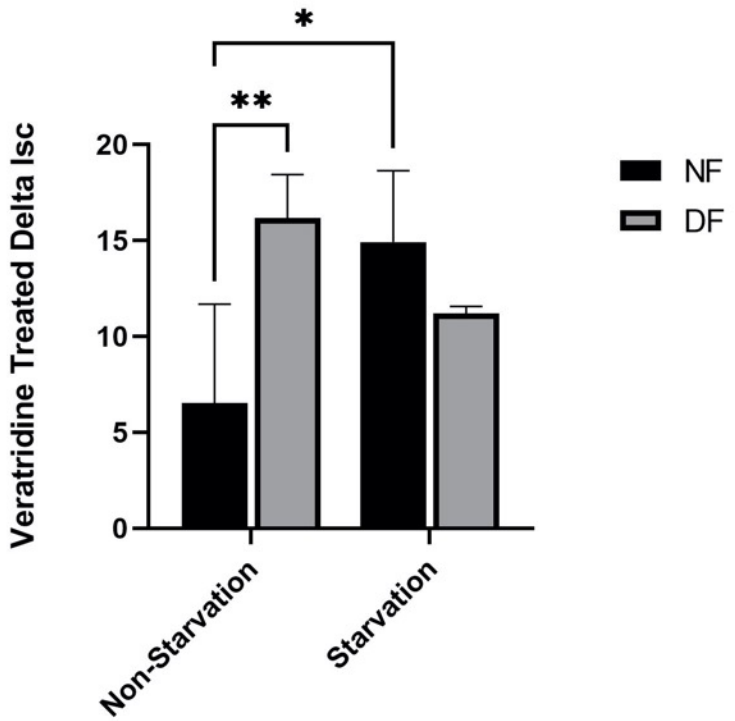


Figure 16. **Effects of delayed-feeding on enteric neural function at day 42 of age with and without starvation.** ENS activity was measured by Veratridine stimulated short circuit current change(Delta Isc). Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks. * $P \leq 0.05$, ** $P \leq 0.01$.

After treatment with Veratridine, glucose transport was measured **(Fig. 17)**. DF birds have a significantly lower ($P \leq 0.01$) transport of glucose after the secondary starvation event. In addition, the DF birds that experienced starvation had a significantly lower ($P \leq 0.001$) lower transport of glucose compared to the NF birds that experienced the stress **(Fig.17).**

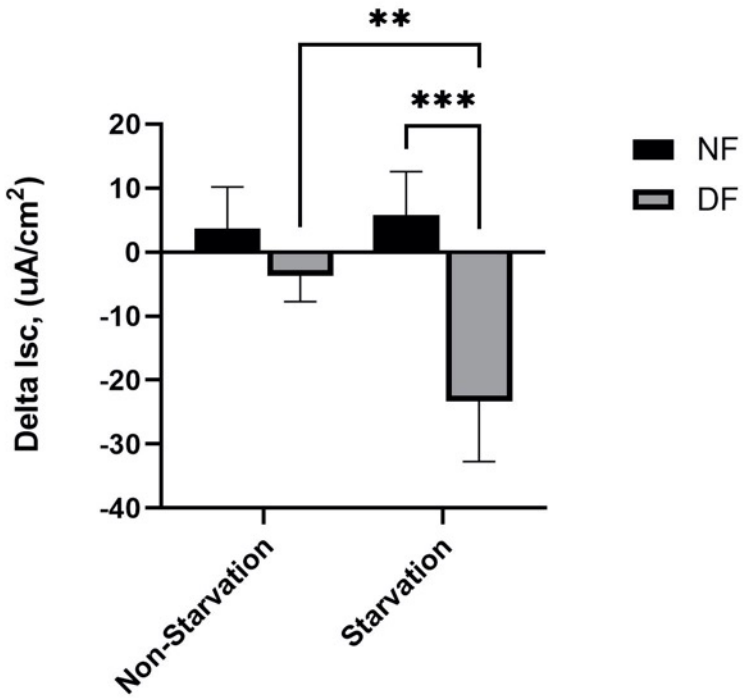


Figure 17. **Electrogenic Glucose Transport in Veratridine Activated Tissue at day 42 of age**. Isc change in response to glucose transport from Veratridine activated tissues minus non-treated tissue Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks, ** $P \leq 0.01$, *** $P \leq 0.001$

3.4 Early Life Stress Enhances Intestinal Nutrient Transport and Adaptations to a Secondary Stress in Later Life (d42)

The effect of a feed delay on nutrient absorption was measured using the Ussing chamber by adding various nutrients to each chamber and recording the resulting changes. Relative NBD-labeled glucose flux rate was measured as an indicator of total glucose absorption occurring in the intestinal tissue samples. While there was no

significant difference between the non-starved NF and DF birds, there was a numerical difference, and the DF birds had a higher flux rate of the labeled glucose molecule **(Fig. 18)**. Together this shows that electrogenic glucose movement was lower in both groups after starvation.

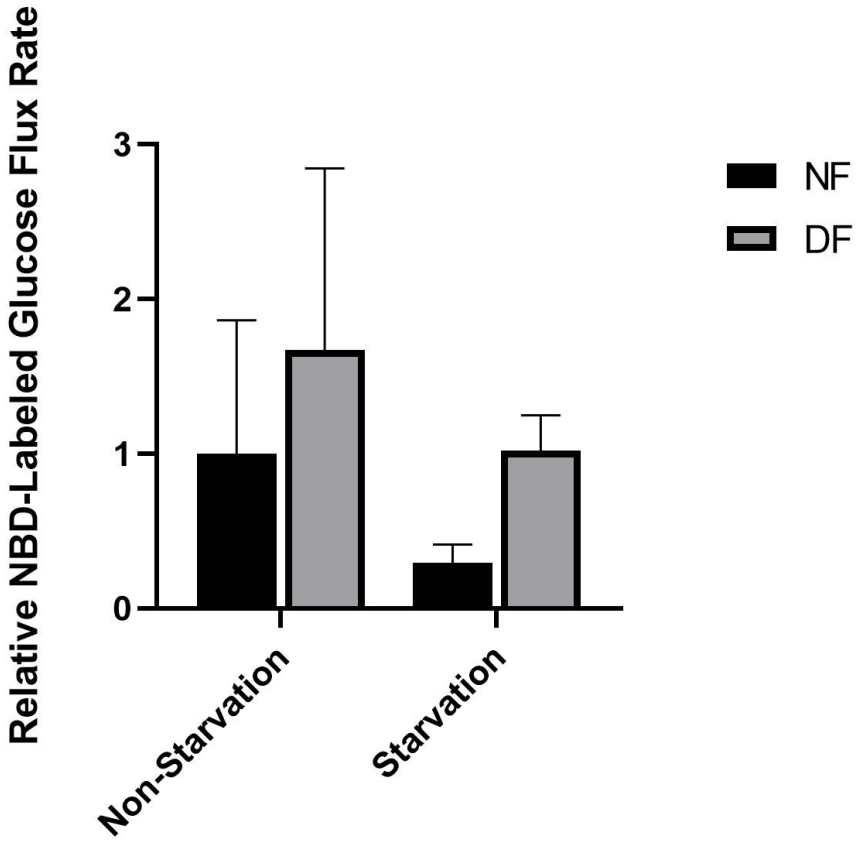


Figure 18. **Effect of delayed-feeding on total absorption of NBD-labeled glucose at day 42 of age.** Tissue on the chamber was treated with NBD-labeled glucose and the relative corresponding flux rate was measured. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks.

In addition, to the NBD-labeled glucose molecule, nutrient transport of specific nutrients, including glucose, was measured on the Ussing chamber. At day 42 of age. There was a no significant difference in glucose transport in the non-starved NF and DF groups. The feed restricted groups also did not have a significant difference, although there was a small numerical increase in the starved DF birds. The NF birds had a significantly higher ($P \leq 0.05$) glucose transport after the feed-restriction period (**Fig. 19)**. While this difference is not statistically significant, it does indicate that starvation may contribute to increased glucose transport in the intestine.

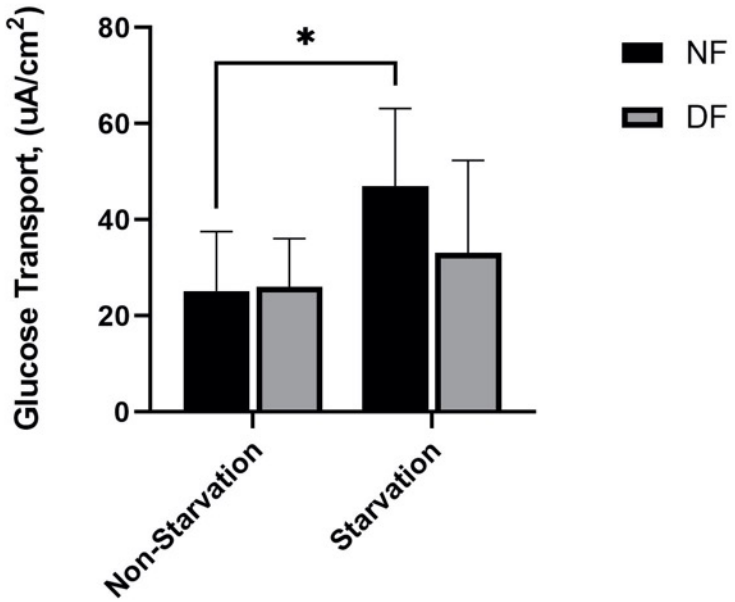


Figure 19. **Effect of delayed-feeding on nutrient transport at day 42 of age: Glucose**. Nutrient stimulated Isc changes, in response to electrogenic nutrient transport of glucose (uA/cm^2) were recorded. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks, * $P \leq 0.05$.

A di-peptide was added to investigate the effect of delayed feeding on nutrient transport in the intestine of DF and NF birds as well as a subset that also underwent a secondary feed restriction event. The resulting relative short circuit current changes were recorded. There was no significant difference between the NF non-starved and starved birds. The DF groups showed a similar pattern **(Fig. 20)**.

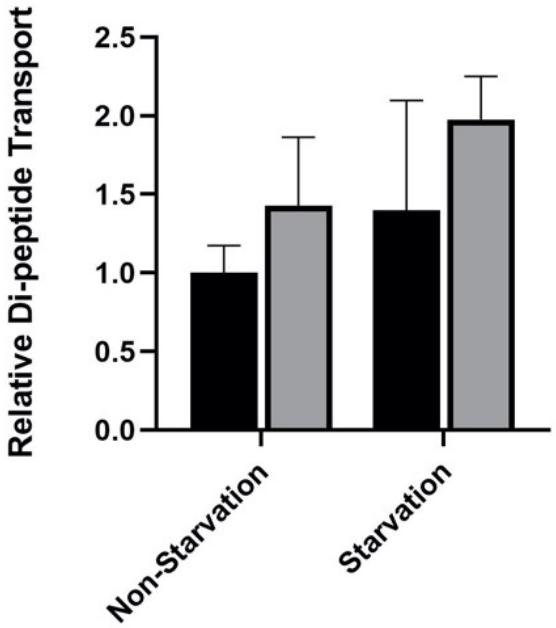


Figure 20. **Effect of delayed-feeding on nutrient transport at day 42 of age: Di-peptide**. Nutrient stimulated Isc changes, in response to relative electrogenic nutrient transport of a di-peptide were recorded. Data are presented as mean ± SEM. n = 4~6. 2-way ANOVA analysis completed, and significance denoted with asterisks.

Lysine, an essential amino acid, was added to the Ussing chamber and changes in short circuit current were measured reflecting relative transport rates. There was no significant difference among any of the groups although there was a trend ($P \leq 0.10$) towards higher relative lysine transport in DF birds. There was a numerical increase the non-starved NF birds and the starved NF birds. This same pattern was seen in both DF groups **(Fig. 21)**. Comparing the DF groups to the NF groups there was a

numerical increase in lysine transport. This indicates that stress may increase lysine transport within the intestinal mucosa.

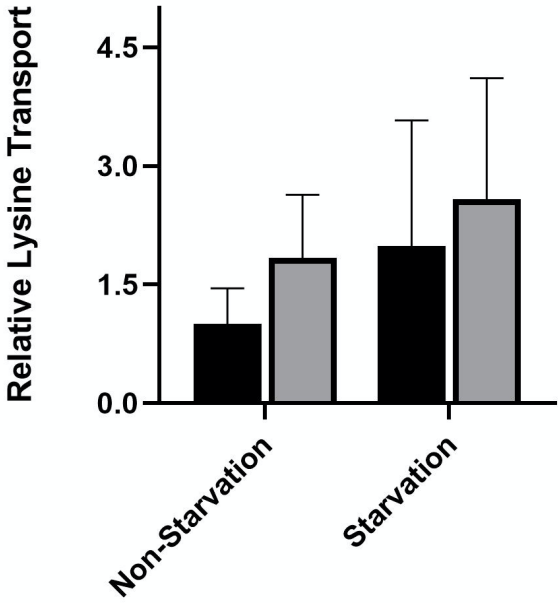


Figure 21. **Effect of delayed-feeding on nutrient transport at day 42 of age: Lysine**. Nutrient stimulated Isc changes, in response to relative electrogenic nutrient transport of lysine were recorded. Data are presented as mean ± SEM. 2-way ANOVA analysis completed, and significance denoted with asterisks.

Alanine is a non-essential amino acid for broiler chickens. It was added in the same manner that lysine was, and the resulting response was measured to determine relative amino acid transport. While there was no significant difference between the

non-starved NF and DF birds, there was a numerical increase in relative alanine transport in DF birds. There was a significant ($p \leq 0.05$) increase in the relative alanine transport in DF feed restricted birds **(Fig. 22)**. This suggests that electrogenic transport of the amino acid alanine is higher in the DF birds and may be enhanced by secondary stress events.

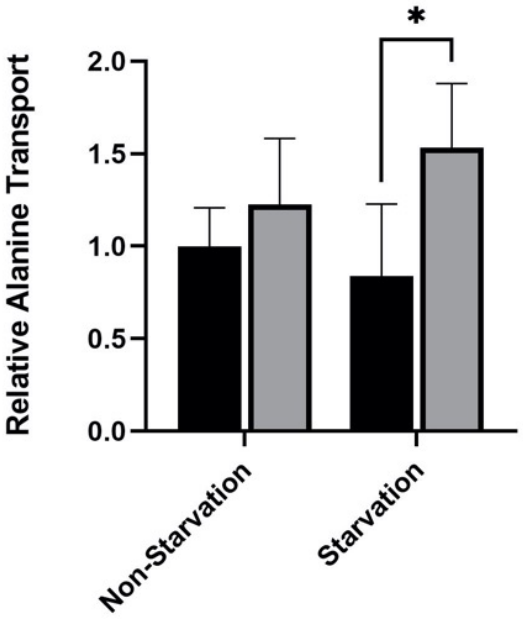
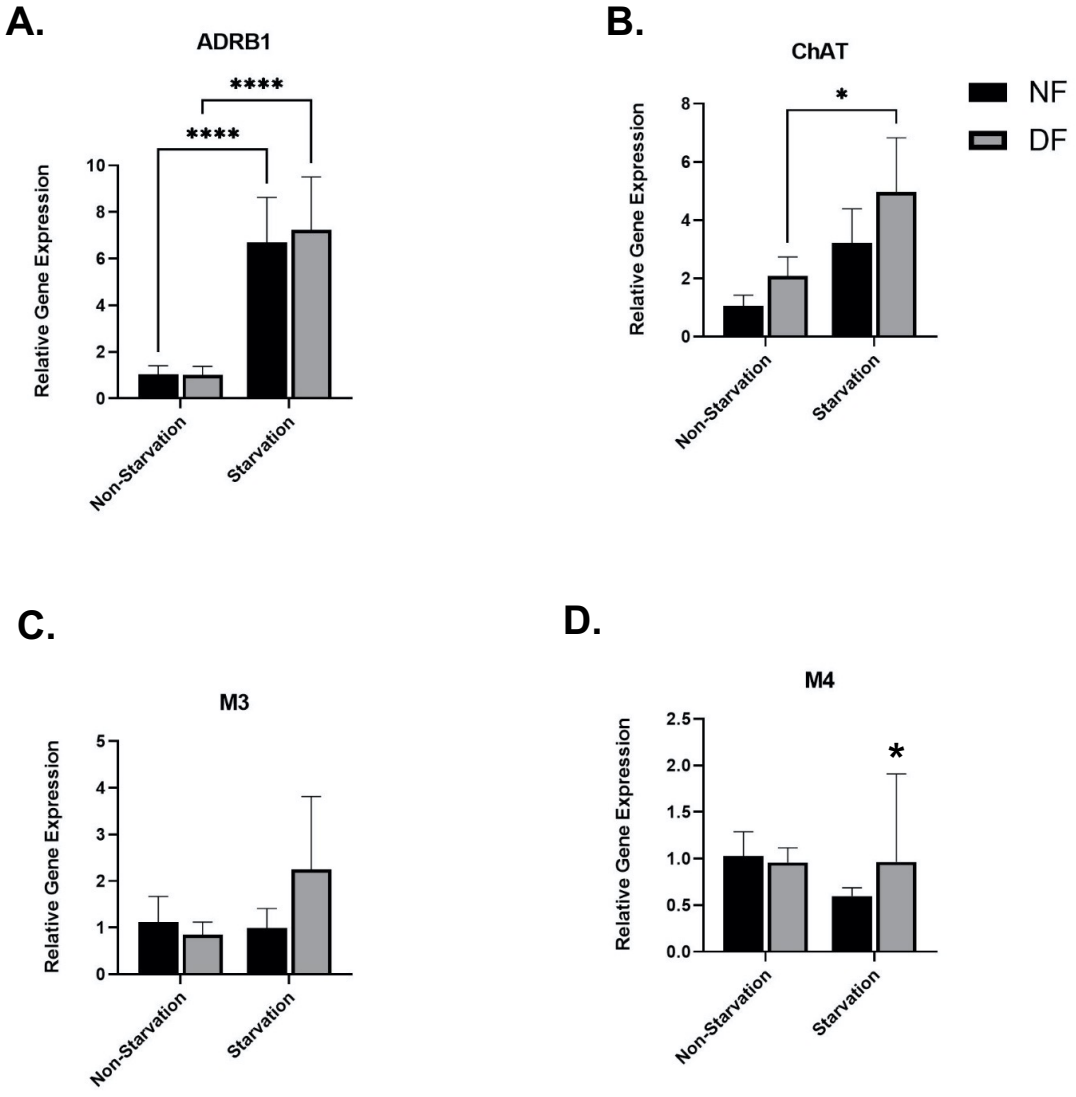


Figure 22. **Effect of delayed-feeding on nutrient transport at day 42 of age: Alanine**. Nutrient stimulated Isc changes, in response to relative electrogenic nutrient transport of alanine were recorded. Data are presented as mean ± SEM. 2-way ANOVA analysis completed, and significance denoted with asterisks. * $P \leq 0.05$.

3.5 Delayed Feeding and Later Life Stress Alters Gene Expression of Enteric Nervous System (d42)

Relative mRNA expression was evaluated for several enteric neural receptors. Adrenoreceptor beta 1, choline acetyltransferase (ChAT), cholinergic muscarinic receptors 3, 4, and 5 (M3,4,5), Nicotinic a-7 receptor (Na7), and acetylcholinesterase (ACHE) were evaluated in both NF and DF groups as well as in the secondary starved conditions. The DF birds and the DF starved birds had an upregulation of genes associated with the cholinergic nervous system. ADRB1 expression levels were significantly ($p \leq 0.0001$) increased in both starved NF and DF groups compared to the non-starved groups **(Fig 23 A)**. There was a significant ($p \leq 0.01$) increase in the expression of ChAT between the non-starved NF birds and the DF starved birds **(Fig. 23 B)**. There was also a significant increase in ChAT expression in the non-starved and starved DF birds **(Fig. 23 B)**. There was no significant changes in gene expression levels of muscarinic receptors that were investigated in this experiment **(Fig. 23 C,D,E)**. There were no significant changes in ACHE expression in all four treatment groups **(Fig. 23 F)**. Na7 gene expression was numerically increased after starvation in both the DF and NG groups. There was a significant increase in expression in the DF starved group as compared to the NF non-starved group **(Fig. 23 G)**. Together this suggests that the 24-hour starvation period acutely influences the expression of enteric nervous receptors. Therefore, this may affect GI function via neural activities through these enteric neurotransmitters and neural receptors.


A.
ADRB1
****
****
Relative Gene Expression
10
8
6
4
2
0
Non-Starvation
Starvation
B.
ChAT
*
NF
DF
Relative Gene Expression
8
6
4
2
0
Non-Starvation
Starvation
C.
M3
Relative Gene Expression
5
4
3
2
1
0
Non-Starvation
Starvation
D.
M4
*
Relative Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
Non-Starvation
Starvation

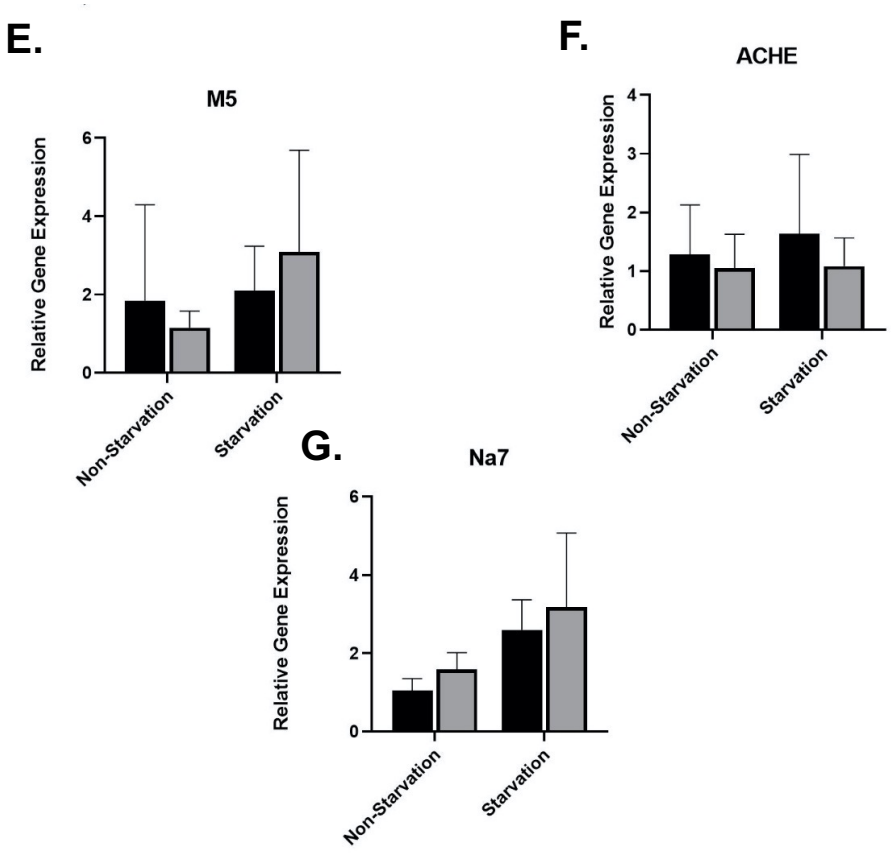


Figure 23. **Effects of delayed-feeding on ENS gene expression with or without secondary starvation stress at day 42 of age.** Adrenoreceptor beta 1 (ADRB1), choline acetyltransferase (ChAT), cholinergic muscarinic receptors 3, 4, and 5 (M3,4,5), Nicotinic a-7 receptor (Na7), and acetylcholinesterase (ACHE) were calculated relative to the non-starve NF group. Data are presented as mean ± SEM. n = 4~6. Data analyzed using Student's t-test. * $P \leq 0.05$. 2-way ANOVA analysis completed, and significance denoted with asterisks. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Chapter 4

DISCUSSION

4.1 General

The GI tract is a crucial body system that allows for appropriate nutrient absorption and protection from potential pathogens and disease agents. This multi-functioning system can be changed by a variety of factors including stress and dietary access to feed along with other body system interactions like input from the ENS and immune systems (Careghi et al. 2005, Hayden and Carey 2000). Therefore, the importance of this system has led to many ways of investigation to help understand how these factors change and ultimately influence the growth and health of animals, especially broiler chickens. The overall integrity of the intestinal barrier can be investigated using the Ussing chamber and its measurements of transepithelial resistance and permeability (Aguanno et al. 2021, Clarke 2009). Generally, increased permeability is associated with benefits like increased nutrient absorption, but it comes at a cost to the animal causing challenges for overall animal health (Awad et al. 2017). Due to the dynamic nature of the intestine and its interactions with various other body systems, the role of this study was to examine early life stress and how this alters the development of enteric nervous activities and nutrient transport functions. This was investigated in both early life and secondary stress conditions.

4.2 Delayed Feeding Stimulates Compensatory Growth in Later Life for Broiler Birds

Following an early life stress event, stressed birds have impacts to their growth performance compared to non-stressed birds. This impact may reflect compensatory growth (Figures 1-4). This change in DF birds suggests that there may be a mechanism that allows for increased efficiency, and this may be resulting from ENS input. This pattern has been seen in other studies with broiler chickens. After a seven-day feed restriction period, birds had a lower body weight, and after the remainder of the grow out period, the birds had a numerically similar body weight. These birds also were found to be more efficient compared to the control birds (Palo et al. 1995). It was also found that these birds, while they did not fully outgrow the control birds were numerically similar by the end of the grow out period. In addition, the restricted birds had a better overall feed efficiency. This suggests that while these birds were not larger, they still were more efficient and if given a few more days they may have been able to fully compensate for the restricted period (Palo et al. 1995). Another study with similar conditions showed the same pattern where full compensatory growth was achieved (Zhan et al. 2007). Taken together this suggests that the intestine can respond more quickly to restricted feeding and therefore is able to develop compensatory mechanisms that allow for recovery from restricted feeding periods (Palo et al. 1995, Zhan et al. 2007, Wang et al. 2014). It appears that this pattern suggests that feed restriction may be beneficial for broiler birds. However, there is potential for these benefits to be counteracted. This includes the potential damage that occurs in the gut when the feed restriction occurs. This may allow for increased movement in the gut, making nutrient absorption occur at quicker rates, but

the gut may also be leakier to pathogens that can cause systemic concern for the animal (Awad et al. 2017).

In addition, in the presented study, the DF group had a lower FCR which suggests that there may be a mechanism such as increased nutrient absorption to allow for increased feed efficiency and potential compensatory growth. One proposed mechanism for this are changes to the ENS which may change intestinal function. Previous work using a porcine model suggests that early life adversity induced long-term changes and upregulation in the cholinergic nervous system and overall increased the secretomotor neuron responses seen in both long- and short-term cases (Medland et al. 2016). These increased secretomotor neuron responses could contribute to compensatory growth via increased nutrient absorption. However, this study looked at sex-specific responses and found that female pigs exhibited heightened secretomotor neuron responses (Medland et al. 2016). Other broiler chicken studies have found that there are differences in male and female bird growth performance parameters. Male birds take a longer time to mature compared to female birds and therefore respond at different rates to early life adversity (Fontana et al. 1992, Cavin et al. 2020). These sex-specific differences could have impacted the timing at which the compensatory growth is seen in the present study since mixed sex broiler birds were used.

4.3 Delayed Feeding Acutely Activates Enteric Nervous Activity and Alters Intestinal Functions Early in Life (d3 and d14)

Experiencing early life stress causes immediate and long-term changes in intestinal ENS activities of broiler chickens. The increase in permeability of the intestine suggests that this may be a benefit of early life stress in the form of a feed

delay. Nutrient absorption was higher in all nutrients given on the Ussing Chamber at day 3 of age in the DF birds **(Fig. 7)**. In addition, acute stress resulted in increased ENS sensitivity, especially regarding electrogenic secretion **(Fig. 6)**. This combined with signs of a leakier gut **(Fig. 8)** indicates that there is less movement in the DF birds. When the tissue was mounted in the Ussing Chamber, there was increased nutrient absorption seen. This suggests that there are changes occurring in the intestinal development during the acute 72-hour stress period that increase nutrient absorption capability. This has been seen in other studies where GLP-2 and the ENS have been shown to be components of the enteroendocrine-absorptive pathway. This plays a role in intestinal glucose transport (Moran et al. 2018). There are often other consequences for these animals. For example, early life stress can induce chronic diarrhea (Pohl et al. 2017). These types of stress events can also lead to chronic inflammation and changes to many factors of intestinal homeostasis (Wong et al. 2019). In addition, these changes may impact birds by predisposing them to a higher risk of later life GI infectious disease especially when there are suboptimal growth conditions or a secondary stress event life like an additional feed delay. Together this suggests that while early life stress may be beneficial for these birds in terms of electrogenic nutrient transport, there may be aspects of this that outweigh the benefits in many scenarios.

4.4 Early Life Stress Enhances Intestinal Nutrient Transport and Adaptations to a Secondary Stress in Later Life While Damaging Intestinal Barrier Function (d42)

Early life stress and adversity changes intestinal development and cause long-term effects on intestinal functions. The present work used a 72-hour feed delay upon

hatching and an additional 24-hour feed restriction later in life to represent the stress events. There was no significant difference by day 42 of age in the NF and DF birds short circuit current **(Fig. 12**). However, there was a reduction in the short circuit current. This is the same pattern seen at day 3 of age **(Fig. 6A)**. This suggests that with less food present there is less secretion and absorption occurring which is not beneficial for the birds. The reduction is less severe in the DF feed restricted birds, suggesting that there is increased capability for adaptation after ELS events like post hatch feed delays. Other works has suggested that there is a benefit to ELS, resulting in the ability for increased movement or absorption of nutrients across the epithelial barrier (Fontana et al. 1992, Hayden and Carey 2000). Delayed feeding and secondary stress appear to allow the birds to adapt their ENS-mediated response, leading to enhanced nutrient transport functions. This combined with growth performance data shows that there are mechanisms that must lead to compensatory growth. One way this may be happening based on current results is through an enhanced electrogenic nutrient transport function in the delayed fed birds. Reducing the ability of tight junctions to hold adjacent enterocytes together within the intestinal epithelium allows for increased movement of materials across the intestinal barrier including nutrients (Suzuki 2020). In addition, there is a significant reduction of potential difference in both NF and DF groups after feed restriction. The decreased PD indicated a reduced electrochemical gradient across the epithelium which may came from reduced Na/KATPase activity or from the leaky tight junction function. Such adaptations may contribute to energy preservation and paracellular nutrient transport under starvation.

Overall, this reduction in tight junctions and potential difference does damage the intestinal structure and function, especially when experienced for prolonged

amounts of time (Awad et al. 2017). Therefore, this increase in intestinal permeability may be beneficial for the birds up to a point, when it then may become damaging and even cause an increase in mortality especially in on-farm settings (Fontana et al. 1992, Méda et al. 2021).

4.5 Delayed Feeding and Later Life Stress Alters Gene Expression of Enteric Nervous System (d42)

Delayed feeding and secondary stress change gene expression which may indicate altered nerve activity. The stressed birds were more adaptive to secondary stress in terms of fewer ENS mediated responses and enhanced nutrient transport functions. One potential mechanism for this compensatory growth is enhanced electrogenic nutrient transport in the delayed fed birds which is supported by increased gene expression in nicotinic receptors, choline acetyltransferase, and adreno beta receptors **(Fig. 23)**. The receptors associated with the cholinergic nervous system like Na7, ChAT, and ACHE, all play a role in restoring the fasting-induced increase in paracellular permeability (Hayden and Carey 2000). Therefore, an increase in gene expression of these receptors may be indicative of increased gut motility and electrogenic nutrient absorption following a stress event (Delvalle et al. 2018). Interestingly there is a similar pattern between levels of ChAT and ACHE both before and after feed restriction **(Fig, 23)**. This indicates that while there may be an increase in acetylcholine activity due to increased secretomotor neuron activity, there is also increased enzymes present like ACHE to break down acetylcholine (Cooke 2000). This shows that ELS and short-term feed restriction impact ENS activity which has an impact on nutrient absorption.

4.6 Conclusions and Future Directions

While the adaptations seen in this study were beneficial as measured by compensatory growth for these birds, some concerns come along with the increased permeability and stress that is experienced. Although our trial showed numerically higher body weight in the DF birds at the end of the 42-day grow out, there is some concern that these benefits may not be achieved in on-farm settings. In our controlled research environment, the birds likely were not exposed to severe pathogens or adverse growing conditions. However, there are incidences of on-farm infectious diseases that even mild, could more greatly impact stressed DF birds.

By investigating this connection between the ENS and intestinal function, we can gain insight into developing novel targets that lead to the improvement of GI health and animal production. Future work includes the investigation of protein expression of nutrient transporters through methods like western blotting (Ihara et al. 2000, Jando et al. 2017). This would give insight into the potential differences that stressed birds have in their ability for nutrient absorption outside of paracellular nutrient movement. In addition, future studies will help to evaluate the specific mechanisms that the ENS uses to regulate nutrient transport. This will help to further the understanding of the ENS's role in overall animal health and productivity.

REFERENCES

Aguanno, D., B. G. Postal, V. Carrière & S. Thenet (2021) Use of Ussing Chambers to Measure Paracellular Permeability to Macromolecules in Mouse Intestine. *Methods Mol Biol,* 2367**,** 1-11.

Albert-Bayo, M., I. Paracuellos, A. M. González-Castro, A. Rodríguez-Urrutia, M. J. Rodríguez-Lagunas, C. Alonso-Cotoner, J. Santos & M. Vicario (2019) Intestinal Mucosal Mast Cells: Key Modulators of Barrier Function and Homeostasis. *Cells,* 8.

Arnold, Y. E., J. Thorens, S. Bernard & Y. N. Kalia (2019) Drug Transport across Porcine Intestine Using an Ussing Chamber System: Regional Differences and the Effect of P-Glycoprotein and CYP3A4 Activity on Drug Absorption. *Pharmaceutics,* 11.

Awad, W. A., C. Hess & M. Hess (2017) Enteric Pathogens and Their Toxin-Induced Disruption of the Intestinal Barrier through Alteration of Tight Junctions in Chickens. *Toxins (Basel),* 9.

Barker, D. J. (2007) The origins of the developmental origins theory. *J Intern Med,* 261**,** 412-7.

Bennett, E. J., C. C. Tennant, C. Piesse, C. A. Badcock & J. E. Kellow (1998) Level of chronic life stress predicts clinical outcome in irritable bowel syndrome. *Gut,* 43**,** 256-61.

Boyd, R. D., C. E. Zier-Rush, A. J. Moeser, M. Culbertson, K. R. Stewart, D. S. Rosero & J. F. Patience (2019) Review: innovation through research in the North American pork industry. *Animal,* 13**,** 2951-2966.

Bröer, S. & S. J. Fairweather (2018) Amino Acid Transport Across the Mammalian Intestine. *Compr Physiol,* 9**,** 343-373.

Butel, M. J., A. J. Waligora-Dupriet & S. Wydau-Dematteis (2018) The developing gut microbiota and its consequences for health. *J Dev Orig Health Dis,* 9**,** 590-597.

Cambra-López, M., P. J. Marín-García, C. Lledó, A. Cerisuelo & J. J. Pascual (2022) Biomarkers and De Novo Protein Design Can Improve Precise Amino Acid Nutrition in Broilers. *Animals (Basel),* 12.

Camilleri, M., K. Madsen, R. Spiller, B. Greenwood-Van Meerveld & G. N. Verne (2012) Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterol Motil,* 24**,** 503-12.

Campbell, J. M., J. D. Crenshaw & J. Polo (2013) The biological stress of early weaned piglets. *J Anim Sci Biotechnol,* 4**,** 19.

Careghi, C., K. Tona, O. Onagbesan, J. Buyse, E. Decuypere & V. Bruggeman (2005) The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven days of age. *Poult Sci,* 84**,** 1314-20.

Carpinello, O. J., A. H. DeCherney & M. J. Hill (2018) Developmental Origins of Health and Disease: The History of the Barker Hypothesis and Assisted Reproductive Technology. *Semin Reprod Med,* 36**,** 177-182.

Cavin, J. B., H. Cuddihey, W. K. MacNaughton & K. A. Sharkey (2020) Acute regulation of intestinal ion transport and permeability in response to luminal nutrients: the role of the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol,* 318**,** G254-g264.

Cheadle, G. A., T. W. Costantini, V. Bansal, B. P. Eliceiri & R. Coimbra (2014) Cholinergic signaling in the gut: a novel mechanism of barrier protection through activation of enteric glia cells. *Surg Infect (Larchmt),* 15**,** 387-93.

Chen, L., B. Tuo & H. Dong (2016) Regulation of Intestinal Glucose Absorption by Ion Channels and Transporters. *Nutrients,* 8.

Chen, L., B. Yu, D. Luo & M. Lin (2018) Enteric motor dysfunctions in experimental chronic pancreatitis: Alterations of myenteric neurons regulating colonic motility in rats. *Neurogastroenterol Motil,* 30**,** e13301.

Cheng, H. & C. P. Leblond (1974) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat,* 141**,** 537-61.

Clarke, L. L. (2009) A guide to Ussing chamber studies of mouse intestine. *Am J Physiol Gastrointest Liver Physiol,* 296**,** G1151-66.

Cooke, H. J. (2000) Neurotransmitters in neuronal reflexes regulating intestinal secretion. *Ann N Y Acad Sci,* 915**,** 77-80.

Delvalle, N. M., D. E. Fried, G. Rivera-Lopez, L. Gaudette & B. D. Gulbransen (2018) Cholinergic activation of enteric glia is a physiological mechanism that contributes to the regulation of gastrointestinal motility. *Am J Physiol Gastrointest Liver Physiol,* 315**,** G473-g483.

DeMeo, M. T., E. A. Mutlu, A. Keshavarzian & M. C. Tobin (2002) Intestinal permeation and gastrointestinal disease. *J Clin Gastroenterol,* 34**,** 385-96.

Drucker, D. J. (2001) Glucagon-like peptide 2. *J Clin Endocrinol Metab,* 86**,** 1759-64.

Ferraris, R. P. & H. V. Carey (2000) Intestinal transport during fasting and malnutrition. *Annu Rev Nutr,* 20**,** 195-219.

Fisher, C. (1998) Lysine: Amino acid requirements of broiler breeders. *Poult Sci,* 77**,** 124-33.

Fontana, E. A., W. D. Weaver, Jr., B. A. Watkins & D. M. Denbow (1992) Effect of early feed restriction on growth, feed conversion, and mortality in broiler chickens. *Poult Sci,* 71**,** 1296-305.

Frizzell, R. A., M. Field & S. G. Schultz (1979) Sodium-coupled chloride transport by epithelial tissues. *Am J Physiol,* 236**,** F1-8.

Fuentes, I. M. & J. A. Christianson (2016) Ion channels, ion channel receptors, and visceral hypersensitivity in irritable bowel syndrome. *Neurogastroenterol Motil,* 28**,** 1613-1618.

Gareau, M. G., J. Jury & M. H. Perdue (2007) Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. *Am J Physiol Gastrointest Liver Physiol,* 293**,** G198-203.

Geyra, A., Z. Uni & D. Sklan (2001) The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. *Br J Nutr,* 86**,** 53-61.

Gonzales, E., N. Kondo, E. S. Saldanha, M. M. Loddy, C. Careghi & E. Decuypere (2003) Performance and physiological parameters of broiler chickens subjected to fasting on the neonatal period. *Poult Sci,* 82**,** 1250-6.

Goyal, R. K. & I. Hirano (1996) The enteric nervous system. *N Engl J Med,* 334**,** 1106-15.

Gresse, R., F. Chaucheyras-Durand, M. A. Fleury, T. Van de Wiele, E. Forano & S. Blanquet-Diot (2017) Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health. *Trends Microbiol,* 25**,** 851-873.

H. WILLEMSEN , M. DEBONNE , Q. SWENNEN , N. EVERAERT , C. CAREGHI , H. HAN , V. BRUGGEMAN , K. TONA & E. DECUYPERE (2010) <h1 data-v-4c963415="" style="box-sizing: border-box; margin: 0.2rem 0px 12px; padding: 0px; border: 0px; font-variant-numeric: inherit; font-variant-east-asian: inherit; font-stretch: inherit; line-height: 1.29em; font-family: "noto sans", Helvetica, Roboto, Arial, sans-serif; font-size: 28px; vertical-align: baseline; color: rgb(51, 51, 51); text-rendering: optimizelegibility; background-color: rgb(255, 255, 255);">Delay in feed access and spread of hatch: importance of early nutrition. *World's Poultry Science Journal* 66**,** 177-188.

H. Willemsen, M. Debonne, Q. Swennen, N. Everaert, C. Careghi, H. Han, V. Bruggeman, K. Tona & E. Decuypere (2010) Delay in feed access and spread of hatch: importance of early nutrition. *World's Poultry Science Journal* 66**,** 177-188.

Hayden, U. L. & H. V. Carey (2000) Neural control of intestinal ion transport and paracellular permeability is altered by nutritional status. *Am J Physiol Regul Integr Comp Physiol,* 278**,** R1589-94.

Heanue, T. A., I. T. Shepherd & A. J. Burns (2016) Enteric nervous system development in avian and zebrafish models. *Dev Biol,* 417**,** 129-38.

Hirota, C. L. & D. M. McKay (2006) Cholinergic regulation of epithelial ion transport in the mammalian intestine. *Br J Pharmacol,* 149**,** 463-79.

Ihara, T., T. Tsujikawa, Y. Fujiyama & T. Bamba (2000) Regulation of PepT1 peptide transporter expression in the rat small intestine under malnourished conditions. *Digestion,* 61**,** 59-67.

Ingersoll, S. A., S. Ayyadurai, M. A. Charania, H. Laroui, Y. Yan & D. Merlin (2012) The role and pathophysiological relevance of membrane transporter PepT1 in intestinal inflammation and inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol,* 302**,** G484-92.

Jando, J., S. M. R. Camargo, B. Herzog & F. Verrey (2017) Expression and regulation of the neutral amino acid transporter B0AT1 in rat small intestine. *PLoS One,* 12**,** e0184845.

Johnson, C. D., A. J. Barlow-Anacker, J. F. Pierre, K. Touw, C. S. Erickson, J. B. Furness, M. L. Epstein & A. Gosain (2018) Deletion of choline acetyltransferase in enteric neurons results in postnatal intestinal dysmotility and dysbiosis. *Faseb j,* 32**,** 4744-4752.

Kato, A. & M. F. Romero (2011) Regulation of electroneutral NaCl absorption by the small intestine. *Annu Rev Physiol,* 73**,** 261-81.

Khetani, T. L., T. T. Nkukwana, M. Chimonyo & V. Muchenje (2009) Effect of quantitative feed restriction on broiler performance. *Trop Anim Health Prod,* 41**,** 379-84.

Kidd, M., I. M. Modlin, B. I. Gustafsson, I. Drozdov, O. Hauso & R. Pfragner (2008) Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile salts, amines, tastants, and olfactants. *Am J Physiol Gastrointest Liver Physiol,* 295**,** G260-72.

Kiela, P. R. & F. K. Ghishan (2016) Physiology of Intestinal Absorption and Secretion. *Best Pract Res Clin Gastroenterol,* 30**,** 145-59.

Koepsell, H. (2020) Glucose transporters in the small intestine in health and disease. *Pflugers Arch,* 472**,** 1207-1248.

Lehmann, A. & P. J. Hornby (2016) Intestinal SGLT1 in metabolic health and disease. *Am J Physiol Gastrointest Liver Physiol,* 310**,** G887-98.

Lunedo, R., L. R. Furlan, M. F. Fernandez-Alarcon, G. H. Squassoni, D. M. B. Campos, D. Perondi & M. Macari (2019) Intestinal microbiota of broilers submitted to feeding restriction and its relationship to hepatic metabolism and fat mass: Fast-growing strain. *J Anim Physiol Anim Nutr (Berl),* 103**,** 1070-1080.

Medland, J. E., C. S. Pohl, L. L. Edwards, S. Frandsen, K. Bagley, Y. Li & A. J. Moeser (2016) Early life adversity in piglets induces long-term upregulation of the enteric cholinergic nervous system and heightened, sex-specific secretomotor neuron responses. *Neurogastroenterol Motil,* 28**,** 1317-29.

Moeser, A. J., C. V. Klok, K. A. Ryan, J. G. Wooten, D. Little, V. L. Cook & A. T. Blikslager (2007) Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *Am J Physiol Gastrointest Liver Physiol,* 292**,** G173-81.

Moran, A. W., M. A. Al-Rammahi, D. J. Batchelor, D. M. Bravo & S. P. Shirazi-Beechey (2018) Glucagon-Like Peptide-2 and the Enteric Nervous System Are Components of Cell-Cell Communication Pathway Regulating Intestinal Na(+)/Glucose Co-transport. *Front Nutr,* 5**,** 101.

Moss, A. F., P. V. Chrystal, D. J. Cadogan, S. J. Wilkinson, T. M. Crowley & M. Choct (2021) Precision feeding and precision nutrition: a paradigm shift in broiler feed formulation? *Anim Biosci,* 34**,** 354-362.

Mourad, F. H., K. A. Barada, C. Khoury, T. Hamdi, N. E. Saadé & C. F. Nassar (2009) Amino acids in the rat intestinal lumen regulate their own absorption from a distant intestinal site. *Am J Physiol Gastrointest Liver Physiol,* 297**,** G292-8.

Méda, B., F. Garcia-Launay, L. Dusart, P. Ponchant, S. Espagnol & A. Wilfart (2021) Reducing environmental impacts of feed using multiobjective formulation: What benefits at the farm gate for pig and broiler production? *Animal,* 15**,** 100024.

Nakamori, H., K. Noda, R. Mitsui & H. Hashitani (2021) Role of enteric dopaminergic neurons in regulating peristalsis of rat proximal colon. *Neurogastroenterol Motil,* 33**,** e14127.

Navarro, D., J. J. Abelilla & H. H. Stein (2019) Structures and characteristics of carbohydrates in diets fed to pigs: a review. *J Anim Sci Biotechnol,* 10**,** 39.

Nelson, J. R., D. R. McIntyre, H. O. Pavlidis & G. S. Archer (2018) Reducing Stress Susceptibility of Broiler Chickens by Supplementing a Yeast Fermentation Product in the Feed or Drinking Water. *Animals (Basel),* 8.

Nezami, B. G. & S. Srinivasan (2010) Enteric nervous system in the small intestine: pathophysiology and clinical implications. *Curr Gastroenterol Rep,* 12**,** 358-65.

O'Mahony, S. M., J. R. Marchesi, P. Scully, C. Codling, A. M. Ceolho, E. M. Quigley, J. F. Cryan & T. G. Dinan (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry,* 65**,** 263-7.

Palo, P. E., J. L. Sell, F. J. Piquer, M. F. Soto-Salanova & L. Vilaseca (1995) Effect of early nutrient restriction on broiler chickens. 1. Performance and development of the gastrointestinal tract. *Poult Sci,* 74**,** 88-101.

Payne, J. A., M. Proszkowiec-Weglarz & L. E. Ellestad (2019) Delayed access to feed alters expression of genes associated with carbohydrate and amino acid utilization in newly hatched broiler chicks. *Am J Physiol Regul Integr Comp Physiol,* 317**,** R864-r878.

Peterson, L. W. & D. Artis (2014) Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol,* 14**,** 141-53.

Pfannkuche, H. & G. Gäbel (2009) Glucose, epithelium, and enteric nervous system: dialogue in the dark. *J Anim Physiol Anim Nutr (Berl),* 93**,** 277-86.

Pohl, C. S., J. E. Medland, E. Mackey, L. L. Edwards, K. D. Bagley, M. P. DeWilde, K. J. Williams & A. J. Moeser (2017) Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. *Neurogastroenterol Motil,* 29.

Powell, D. W., H. M. Berschneider, L. D. Lawson & H. Martens (1985) Regulation of water and ion movement in intestine. *Ciba Found Symp,* 112**,** 14-33.

Rao, M. & M. D. Gershon (2018) Enteric nervous system development: what could possibly go wrong? *Nat Rev Neurosci,* 19**,** 552-565.

Rozehnal, V., D. Nakai, U. Hoepner, T. Fischer, E. Kamiyama, M. Takahashi, S. Yasuda & J. Mueller (2012) Human small intestinal and colonic tissue mounted in the Ussing chamber as a tool for characterizing the intestinal absorption of drugs. *Eur J Pharm Sci,* 46**,** 367-73.

Santos, J., P. C. Yang, J. D. Söderholm, M. Benjamin & M. H. Perdue (2001) Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut,* 48**,** 630-6.

Santoso, U. 2002. Effects of Early Feed Restriction on the Occurrence of Compensatory Growth, Feed Conversion Efficiency, Leg Abnormality and Mortality in Unsexed Broiler Chickens Reared in Cages. 1319-1325 Asian Australasian Association of Animal Production Societies.

Schmittgen, T. D. & K. J. Livak (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3**,** 1101-8.

Shinde, A. S., A. Goel, M. Mehra, J. Rokade, P. Bhadauria, A. B. Mandal & S. K. Bhanja. 2015. Delayed Post Hatch Feeding Affects Performance, Intestinal Morphology
and Expression Pattern of Nutrient Transporter Genes in Egg Type
Chickens. Journal of Nutrition and Food Sciences.

Shinde Tamboli, A. S., A. Goel, M. Mehra, J. J. Rokade, P. Bhadauria, A. S. Yadav, S. Majumdar & S. K. Bhanja (2017) Delayed post-hatch feeding affects the performance and immunocompetence differently in male and female broiler chickens. *Journal of Applied Animal Research,* 46**,** 306-313.

Sjöberg, Å., M. Lutz, C. Tannergren, C. Wingolf, A. Borde & A. L. Ungell (2013) Comprehensive study on regional human intestinal permeability and prediction of fraction absorbed of drugs using the Ussing chamber technique. *Eur J Pharm Sci,* 48**,** 166-80.

Smith, F., J. E. Clark, B. L. Overman, C. C. Tozel, J. H. Huang, J. E. Rivier, A. T. Blikslager & A. J. Moeser (2010) Early weaning stress impairs development of mucosal barrier function in the porcine intestine. *Am J Physiol Gastrointest Liver Physiol,* 298**,** G352-63.

Sun, Y., L. Li, R. Xie, B. Wang, K. Jiang & H. Cao (2019) Stress Triggers Flare of Inflammatory Bowel Disease in Children and Adults. *Front Pediatr,* 7**,** 432.

Suzuki, T. (2020) Regulation of the intestinal barrier by nutrients: The role of tight junctions. *Anim Sci J,* 91**,** e13357.

Thomson, A., K. Smart, M. S. Somerville, S. N. Lauder, G. Appanna, J. Horwood, L. Sunder Raj, B. Srivastava, D. Durai, M. J. Scurr, V. Keita Å, A. M. Gallimore & A. Godkin (2019) The Ussing chamber system for measuring intestinal permeability in health and disease. *BMC Gastroenterol,* 19**,** 98.

Toghyani, M., L. R. McQuade, B. V. McLnerney, A. F. Moss, P. H. Selle & S. Y. Liu (2020) Initial assessment of protein and amino acid digestive dynamics in protein-rich feedstuffs for broiler chickens. *PLoS One,* 15**,** e0239156.

Ulbricht, W. (1998) Effects of veratridine on sodium currents and fluxes. *Rev Physiol Biochem Pharmacol,* 133**,** 1-54.

Urdaneta-Rincon, M. & S. Leeson (2002) Quantitative and qualitative feed restriction on growth characteristics of male broiler chickens. *Poult Sci,* 81**,** 679-88.

Ussing, H. H. & K. Zerahn (1951) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol Scand,* 23**,** 110-27.

van der Klein, S. A., F. A. Silva, R. P. Kwakkel & M. J. Zuidhof (2017) The effect of quantitative feed restriction on allometric growth in broilers. *Poult Sci,* 96**,** 118-126.

Wang, Y., Y. Li, E. Willems, H. Willemsen, L. Franssens, A. Koppenol, X. Guo, K. Tona, E. Decuypere, J. Buyse & N. Everaert (2014) Spread of hatch and delayed feed access affect post hatch performance of female broiler chicks up to day 5. *Animal,* 8**,** 610-7.

Warren, W. A. & J. L. Emmert (2000) Efficacy of phase-feeding in supporting growth performance of broiler chicks during the starter and finisher phases. *Poult Sci,* 79**,** 764-70.

Westerhout, J., E. van de Steeg, D. Grossouw, E. E. Zeijdner, C. A. Krul, M. Verwei & H. M. Wortelboer (2014) A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. *Eur J Pharm Sci,* 63**,** 167-77.

Wong, H. L. X., H. Y. Qin, S. W. Tsang, X. Zuo, S. Che, C. F. W. Chow, X. Li, H. T. Xiao, L. Zhao, T. Huang, C. Y. Lin, H. Y. Kwan, T. Yang, F. M. Longo, A. Lyu & Z. X. Bian (2019) Early life stress disrupts intestinal homeostasis via NGF-TrkA signaling. *Nat Commun,* 10**,** 1745.

Wu, Q. J., N. Liu, X. H. Wu, G. Y. Wang & L. Lin (2018) Glutamine alleviates heat stress-induced impairment of intestinal morphology, intestinal inflammatory response, and barrier integrity in broilers. *Poult Sci,* 97**,** 2675-2683.

Zhan, X. A., M. Wang, H. Ren, R. Q. Zhao, J. X. Li & Z. L. Tan (2007) Effect of early feed restriction on metabolic programming and compensatory growth in broiler chickens. *Poult Sci,* 86**,** 654-60.

Zubair , A. K. & S. Leeson 1996. Compensatory growth in the broiler chicken: a review. 189 - 201. World's Poultry Science Journal

Appendix A

PCR PRIMERS

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
HMBS	GGTTGAGATGCTCCGTGAGTTT	GGCTCTTCTCCCCAATCTTAGAA
RPL1	TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG
TBP	CTTCGTGCCCGAAATGCT	GCGCAGTAGTACGTGGTTCTCTT
ADRB1	TGAGGACGCTCGCTTTCTGT	CCTTCTGCACCAAGGACCCA
ChAT	AAGCCAGCTCTGTCGGATTCT	AGTGTTCTCGTGCCACCTCTC
M3	TCTCCTCTTCCTTGTTCATGCC	CAGGGGCAAGGCTGAACTGT
M4	TCACCAAGCAGACTGGGACC	CTGGCAAACTTCCTGGCGAC
M5	TGGCTTGTGACCTGTGGCTA	GATGCCAGCTCTTTTGGGCG
Na7	CGTCCGTTTTCCTGATGGACT	TGGCAGTGTCCCGAAGAATTGA
ACHE	ACCTTCCCGGGTTTCCAAGG	TCCAAAGACACCGAACCGCT

Appendix B

IACUC PROTOCOL

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

Title of Protocol: Evaluation of early post-hatching stress and dietary supplementation of organic acids and botanicals on growth performance, intestinal microbiome, intestinal function and development of gut-health biomarker in broilers	
AUP Number: 101R-2019-A	← **(4 digits only)**
Principal Investigator: Dr. Yihang Li	

Requested Changes

I am requesting a change to: ***(Check all that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☒ Animal Numbers *(Complete Section 2)*
- ☒ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

(Complete Section 7)

Changes MUST NOT be initiated until IACUC approval is granted

Official Use Only

IACUC Approval Signature: Steven Tallman, DVM

Date of Approval: 9/16/2020