DOMESTICATION AND THE EVOLUTION OF THE CHICKEN INTESTINE

by

Janet deMena

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 Animal Science

Summer 2014

DOMESTICATION AND THE EVOLUTION

OF THE CHICKEN INTESTINE

by

Janet deMena

Approved: _____

Dr. Carl Schmidt, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Dr. Limin Kung, Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____

Dr. Mark Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____

James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

It is with immense gratitude that I acknowledge the help and support of Dr. Carl Schmidt who acted as a reviewer, advisor, and guiding light throughout this entire journey. Without his encouragement and support, I would never have succeeded and grown in the ways that I have. I consider myself incredibly lucky to have had the opportunity to work alongside someone with such a brilliant mind, who also knew when to lighten up the mood when I was being too hard on myself.

In addition, I would like to thank Dr. Robert Dyer and Dr. Erin Brannick for their contributions and guidance. Working with them was always a positive experience and inspired me to constantly strive to learn more. A big thanks also goes out to all of my labmates in Dr. Carl Schmidt's lab. It was great to work as part of such an intellectually stimulating group. I would also like to acknowledge Allison Rogers and Elizabeth Pritchett for always volunteering their assistance to Schmidt lab, and for constant entertainment and laughter.

I appreciate the help from all of our necropsy teams and collaborators at the University of Illinois, Mountaire Farms in Millsboro, DE, and the Delaware Biotechnology Institute. Finally, I would like to thank NIFA for funding this project.

TABLE OF CONTENTS

LIST OF FIGURES v
ABSTRACT vi

Chapter

1 INTRODUCTION 1

2 HYPOTHESIS 4

3 MATERIALS AND METHODS 5

4 DIFFERENTIAL GENE EXPRESSION ANALYSIS 8

5 HISTOLOGICAL SECTION RESULTS 15

6 DISCUSSION 18

REFERENCES 26

Appendix

A AGRICULTURAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM 30

LIST OF FIGURES

Figure 1 Size comparison of male Illinois (left) and male Ross (right) at five weeks. 2

Figure 2 Figure 1 Hierarchical Cluster Map generated in JMP. First split separates the Illinois birds from the Ross birds. 10

Figure 3 Cyclic AMP pathway, as it relates to the Illinois line. The seven genes listed are all enriched in the Illinois line when compared to the Ross line. Five out of the seven (first box in blue- CCR7, CYSLTR2, LHCGR, PTGER2, TSHR) are known to activate cAMP, while the other two (second box in red-C3AR1, DRD2) decrease cAMP production. 13

Figure 4 Wnt/β-catenin Pathway as it relates to the Illinois line. The six Wnt genes (Wnt2, Wnt 3a, Wnt 7a, Wnt 7b, Wnt 8a, Wnt 10a) along with the Frizzled 10 gene are enriched in the Illinois line and known to be involved in this pathway. 14

Figure 5 Cross section of male Illinois duodenum at day two with PCNA staining (brown) at 10x magnification. Image demonstrates how width measurements were captured with NIS Elements D software. 16

Figure 6 Cross section of male Ross duodenum at day two with PCNA staining (brown) at 10x magnification. Image demonstrates how width measurements were captured with NIS Elements D software. 17

ABSTRACT

Since the early 20th century, the poultry industry has greatly influenced the breeding and growth of the modern broiler chicken. Birds were selected for faster growth and higher feed efficiency. In this study, the modern day broiler selected for rapid growth was compared to the meat type bird used until the 1950s, before the influence of human directed selection. Both lines were raised in identical environments including housing, feed and water, and lighting programs. Samples were taken from the duodenum at various time points over a forty-two day period. Analyses of histological sections and differential gene expression were used to explore the effects of human directed selection on the development, function, and gene expression of the duodenum in the modern day broiler to determine whether enteric differences between the two lines contribute to growth differences.

Chapter 1

INTRODUCTION

The chicken was domesticated 7000 – 10000 years ago and has become one of the most widespread, successful agricultural sectors in the world [18]. Studies have shown that the most likely ancestor of the domestic chicken is the Red Jungle Fowl (*Gallus gallus*), although there appears to have been introgression of genes from other subspecies including the Gray Jungle Fowl [38]. Domestication led to selection affecting multiple traits including docility, size, appetite, immunity, feed efficiency, and fecundity along with many other parameters [38]. The poultry industry has broadly selected for two distinct types of birds: egg layers and meat producers (broilers). Over the past 80 years, humans have increased selective pressure on the chicken by selecting for important production traits.

Both egg layers and broilers have been selected for reproductive capabilities and disease resistance. Many major organ systems including digestive, nervous, cardiovascular, integumentary, muscle, and immune have been affected by selection for the desirable traits. One way to identify how selection has modified these organ systems is to compare the growth of modern broilers with unselected heritage broilers. One such heritage line is maintained at the University of Illinois, developed by H.M. Scott. This line is the result of the cross of a New Hampshire male line and Columbian female line, both inbred since the late 1940s. This line that has not been selected for increased meat production since its initial development in the 1950s is referred to as “Illinois” and represents the heritage broilers in this study. Comparing birds from Illinois and Ross (modern) lines will provide insight into how selection has affected the modern broiler.

It is clear that human-directed evolution has led to growth differences among various chicken lines. This becomes evident when comparing the growth of three lines: Red Jungle

Fowl (RJF), Illinois and Ross708. By day 35 post-hatch RJF mass is approximately 300g [14], Illinois around 1046g, and finally Ross averages 1800g [34]. This suggests that the first several thousand years of selection from RJF to Illinois type birds led to a 3-fold increase in mass at 5 weeks post hatch. Further selection over the past 60-70 years has led to a further 1.8 fold increase when comparing between the Illinois And Ross birds. In addition, 9% of the RJF and Illinois' body mass is comprised of breast muscle while, 18% of the Ross' body mass is breast muscle. This indicates that selection through the early 20th century did not change the allometric relationship between the breast muscle and the overall body mass, while more recent selection has changed this relationship.

Figure 1 Size comparison of male Illinois (left) and male Ross (right) at five weeks.

Comparing birds of the same age from the Illinois and Ross lines (Figure 1) reveals the impact of selection for increased meat production. Most striking is the significant increase in breast muscle, along with the broadening of the rib cage, and overall increase in skeletal size. This leads to the question: how has the digestive tract been changed to provide the necessary nutrients required for the rapid growth of the modern broiler? The avian intestine is made up of a small and large intestine, with the small intestine playing the major role in nutrient absorption. The small intestine consists of a duodenum, jejunum and ileum. The pancreas supplies the duodenum with digestive enzymes and bicarbonate, while the liver sends the duodenum bile through the gall bladder. The duodenum is responsible for secreting additional digestive enzymes and the bulk of the nutrients are absorbed in the jejunum and ileum.

Morphometric studies comparing the intestine of Ross and Illinois birds uncovered that the jejunum and ileum were both approximately 20% longer in the Ross line than in the Illinois line [34]. This may allow for the modern line to have increased nutrient absorption, leading to larger mass. In contrast, the duodenal segment did not differ in length. Further investigation proved that although the lengths were similar, the cross sectional diameter of the Ross line was greater than that in the Illinois line. This increased diameter included both thickness of the wall and length of the intestinal villi. This leads to the question of what causes this increased size. Some possibilities include hypertrophy, hyperplasia, or a combination of both. In addition, what genes might be responsible for these differences?

The purpose of this study was to compare gene expression patterns of duodenal segments taken from Ross and Illinois birds. The objective was to identify differentially expressed genes, and group such genes into functional categories using pathway, ontological, and text mining tools, as well as explore histological differences in the intestine. This analysis should provide insight into how human directed evolution has affected the development of the duodenum.

Chapter 2

HYPOTHESIS

Given the more rapid growth and larger size of the birds from the Ross compared to birds from the Illinois line, we hypothesize that there will be morphometric and gene expression differences between the duodenums of the lines. Selection will have increased the overall surface area of the Ross duodenum. Comparing gene expression patterns between the two bird lines will suggest genes that may play a role in these morphometric differences.

Chapter 3

MATERIALS AND METHODS

Animal Rearing

Two strains of broiler chickens were chosen for this study. The first was Ross708, a broiler line donated by Mountaire Farm in Millsboro, DE. This breed, which represents the modern broiler line, is a popular bird currently used in the broiler production industry. The second line, referred to as the Illinois line. has been maintained since the 1950s. Eggs were purchased from Chet Utterback at the University of Illinois and used to represent the broilers utilized in the early 20th century for meat production.

Twenty-five dozen eggs from each line were set in incubators at 37°C until hatch. Females were culled in order to prevent variability due to gender and simplify the experimental design. After hatch, two hundred and forty chicks from each line were placed in the same large colony poultry houses on the University of Delaware farm. Houses were pre-warmed to 33°C and reduced 3°C each week until 21 days post hatch (dph). At 21 dph, the temperature of the houses was maintained at 24°C for the following three weeks. Chicks were raised according to commercial guidelines for lighting cycles and were provided with food and water *ad libitum.* Their diet consisted of a commercial starter feed from hatch until four weeks of age, at which point they were switched to a commercial finishing feed. Animal protocols were approved by the University of Delaware Animal Care and Use Committee and can be found in Appendix A

Tissue Collection

At days 2, 7, 14, 21, 28, 35, and 42, thirty birds were euthanized by cervical dislocation for full body necropsy. Intestinal segments were separated into the duodenum, jejunum, and ileum based on gross anatomical landmarks. The duodenum was determined to begin at the caudal end of the gizzard and included the length of the intestine comprising the duodenal

loop. The jejunum began immediately after the duodenum and continued the Merckel's diverticulum. Finally, the ileum started at the end of the jejunum and ended at the junction with the ceca and cloaca. Samples were emptied, measured for length, and weighed. Three small sections (80-100mm) were cut from the middle of each intestinal segment. Tissues were flash frozen in liquid nitrogen and then stored at -80 degrees Celsius to maintain RNA integrity until ready for RNA isolation. Samples for histologic analysis were also collected from the duodenum and preserved in 10% neutral-buffered formalin.

RNA Extraction and QC

Total RNA was isolated from an approximately 30 mg sample of intestinal tissue from each individual bird using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. RNA quality was assessed using the RNA Integrity Number (RIN) provided by the RNA Nano Lab Chip run on an Agilent 2100 Bioanalyzer(Agilent Technologies). Only RNA with a RIN above 9 was used to prepare indexed libraries for high throughput transcriptome sequencing.

Transcriptome Library Synthesis and QC

RNA seq libraries were synthesized using the Ilumina TruSeq RNA Sample Preparation Kit (Ilumina, San Diego, CA) as per the manufacturers instructions. Concentrations of each library were obtained using the Qubit broad range double stranded DNA assay. The indexed libraries were pooled sent to the DBI Core Sequencing Facility (Newark, DE) where they were sequenced on the Illumina G2 and reads per kilobase per million (RPKM) values were calculated using the ERANGE software. Results were uploaded to the Big Bird database for retrieval.

Transcriptome Library Analysis

For transcript comparisons, results were downloaded from the database and all statistical analyses were done using the JMP 10 software. Differential expression was determined using the following formula:

Log2 (Ross RPKM Average/ Illinois RPKM Average)

RPKM values for gene transcripts with a 1.5- fold difference (log base 2 above .585 and below -.585) were identified as differentially expressed between the two lines. Evaluations to determine ontological terms were performed on genes identified as differentially expressed for each line with the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool. Kyoto Encyclopedia of Genes of Genomes (KEGG) pathway mapper was used to highlight enriched genes in highly expressed pathways in each line. Text mining was performed and iTerms were generated from the differentially expressed gene lists using eGIFT. In addition to DAVID, the genes were also analyzed using the 'Core Analysis' function in IPA (Ingenuity System, Inc, USA), a tool that interprets data in the context of biological processes, pathways, and networks.

Histology Slide Preparation and Analysis

For the purpose of histological studies, duodenal samples were taken from six Ross birds and five Illinois birds at two days of age. Duodenal specimens were trimmed, processed, and sectioned by Joanne M. Kramer of the College of Agriculture and Natural Resources Comparative Pathology Lab at the University of Delaware (Newark, DE). Slides were sent out to the Biomedical Research lab at Alfred I. Dupont Hospital in Wilmington, DE to be stained with proliferating cell nuclear antigen (PCNA). Paraffin-embedded enteric sections stained with PCNA from both lines at day two were studied by the author using Olympus BX40 microscope with Nikon DS-Fi2 camera and NIS Elements D Imaging Software. Slides were analyzed for PCNA staining patterns, villus width, mucosal epithelial proliferation beyond crypts, and crypts per villus by author with subsequent review by Dr. Erin Brannick, a certified veterinary anatomic pathologist of the College of Agriculture and Natural Rescources at the University of Delaware. Images were captured with a Nikon DS-Fi2 camera and villus width was measured using NIS Elements software.

Chapter 4

DIFFERENTIAL GENE EXPRESSION ANALYSIS

For this study, transcriptome libraries from four male Illinois chicks and from six male Ross708 chicks all at two days post-hatch were analyzed. A total of 13,732 chicken genes were analyzed in duodenal samples in the two different chicken lines. Of these 13,732 genes, a total of 13,509 were expressed with RPKM values greater than 0.1. The $\log_2$ values of the ratio between the Ross RPKM and Illinois RPKM were determined of each of these genes. The list was further reduced according to the genes that were more than three standard deviations from the mean of the $\log_2$ (Ross708/Illinois) shortening the list to 806 genes. This yielded 652 genes whose expression was enriched in the Illinois line, with 154 genes enriched in the Ross line.

Inspecting the top 20 most enriched genes in the Ross lines identifies genes involved in immune response, translation, mitochondria and lipid metabolism. The top enriched gene was microRNA147, or MIR147-1. MicroRNAs are small, non coding RNAs that play crucial roles in regulation of various biological processes. MIR147, in particular, has been observed to play a role in modulating immune response [21]. Multiple ribosomal protein genes were enriched including RPS23, RPS24, RPL37A, RPL17L, RPL27, RPS3A, RPS29, RPL30, EEF1A1. Another enriched class of genes was genes related to mitochondrial proteins, ATP5G3, ATP5I, NDUFB1and SLC25A6, which are involved in oxidative phosphorylation [16]. Ferritin, heavy polypeptide 1, or FTH1, is an iron storage protein, which is important to mitochondrial function which was also enriched. The final group of Ross enriched genes in the top 20 affect lipid transport. Apolipoprotein A-1, ApoA1, is a major protein of high-density lipoproteins involved in lipid metabolism. ApoA1 promotes cholesterol efflux from tissues to the liver [30]. Fatty acid binding protein 1, FABP1, is involved in fatty acid uptake, transport, and metabolism [13]. The remaining three genes in the top twenty list were PRAP1, CCDC72,

and S100A6. Proline rich acidic protein 1, or PRAP1, may play an important role in maintaining growth homeostasis in epithelial cells. S100 Calcium binding protein A6, S100A6, is involved in regulation of a number of cell processes, including differentiation and cell cycle progression. Finally, Coiled-coil domain containing 72, CCDC72, functions in translation.

The top twenty genes for the Illinois birds contained genes with various functions including G protein coupled receptors (GPCR), WNT, and homeobox containing gene products. The gene functions were explored using NCBI's Gene Online Resource. Among the GPCRs was olfactory receptor- like protein, COR1. In addition two Wnt genes, Wnt8A and Wnt 2 appeared on the list. A large majority of the genes were homeobox genes, a class of regulatory genes that code for homeoproteins which normally act as transcription factors (HOXA11, LOC100858967, LOC100859345, EVX1, FOXN1, LOC100859078, EMX2). Two ATPases (ATP6V1G3 and ATP6V0D2) along with an NADH Dehydrogenase (NDUFB2) and a Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) are also included. The remaining six genes consist of a microRNA (Mir22), an Rh type C glycoprotein (RHCG), growth hormone releasing hormone receptor (GHRHR), calcium binding protein (CALN1), B cell translocation gene (BTG4), and finally a protein coding gene (KIAA1045).

Hierarchical clustering was used to examine further the relationship between expression patterns in the Ross708 and Illinois D2 samples. Two clusters were observed, one corresponding to the Ross samples and the other to the Illinois samples, indicating that the duodenums of the Ross birds are more like other Ross birds, while the Illinois birds are more similar to other Illinois birds.

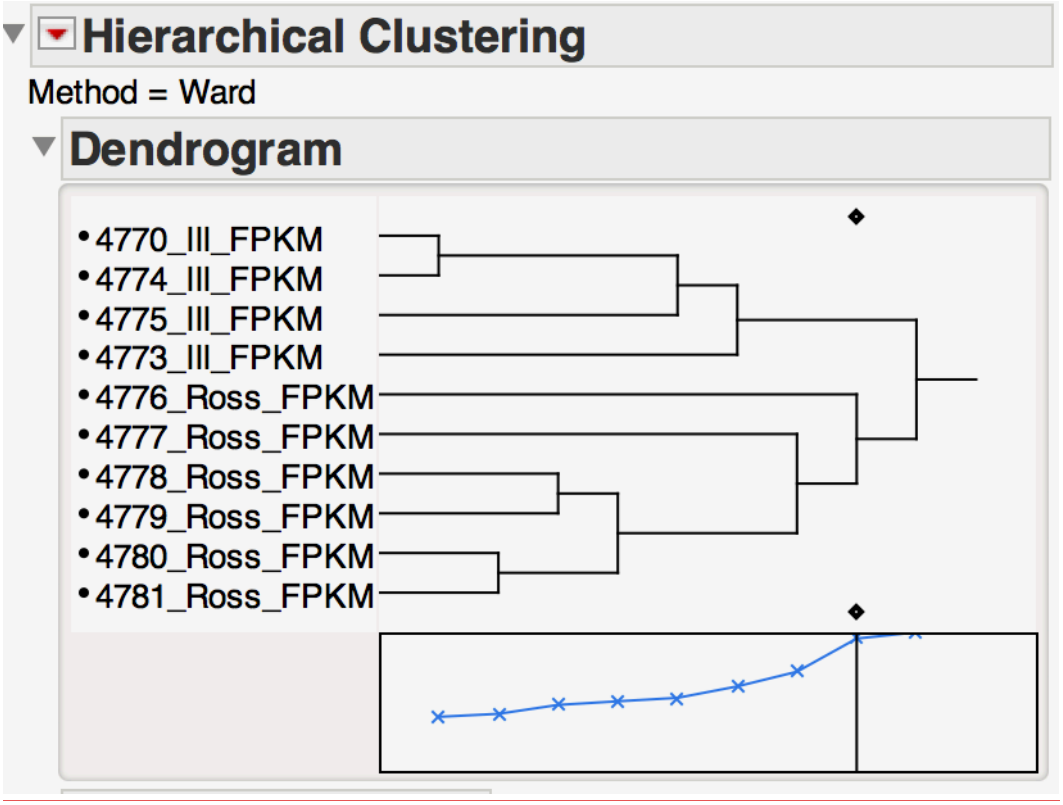


Figure 2 Hierarchical Cluster Map generated in JMP. First split separates the Illinois birds from the Ross birds.

Principle component analysis, or PCA, of the responsive genes segregated the samples based on individual differences (Illinois vs Ross). Based on the analysis, 63% of the variance was contributed to the first component, while 19% is contributed to the second component. The first component is due to individual variation between birds within lines while the second component is due to difference between the two lines.

The Functional Annotation Cluster tool in DAVID was used to highlight the most relevant gene ontology (GO) terms associated with the gene list. Those clusters with an enrichment score of 1.3 or greater are considered to be significant. In the Illinois birds, there were three main clusters generated. The first cluster, with an enrichment score of 4.16, included terms related to immunity such as cytokine activity, chemokine activity, and immune

response. With a score of 3.32, the next cluster contained terms that were related to signal transduction such as G-protein coupled receptor protein signaling pathway, cell surface receptor linked signal transduction, and transmembrane. Related to this cluster is one with a score of 1.66 that consists of cyclase activity regulation terms. Another cluster with an enrichment score of 2.24 contains terms such as visual perception, sensory perception, detection of stimulus, and opsin. The gene products included in this group were either GPCR or involved in GPCR mediating pathway events. Wnt pathway related terms made up another large cluster with a score of 1.85, while homeobox genes clustered into a group with a score of 1.65 with terms such as homeobox, developmental proteins, and transcription factor activity.

After analyzing the functional annotation clusters, DAVID was used to identify the most enriched pathways in each line by the Kegg Pathway. Further supporting data found by the clustering tool, four of the most enriched pathways in the Illinois line were all related to immune function: Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, Toll-like receptor (TLR) signaling pathway, and Cystolic DNA-sensing pathway. Jak- STAT is a pathway that helps regulate the immune system, while TLRs function in innate immunity, inflammation, and proliferation [31], [21]. In addition to immune function, pathways enriched were involved with GPCR function and Wnt.

The same Functional Annotation Clustering tool was used to generate a list of the most enriched GO terms for the Ross line. The only enriched cluster (score=1.87) contained terms relating to ribosomal activity such as ribosome and translation. This indicates that Ross duodenal tissue is likely to be more invested in protein production than the Illinois duodenal tissue. To further support these findings, the most enriched pathway in the Kegg pathway tool was Ribosome. This could support increased hyperplasia, an increase in the number of cells, and/or hypertrophy, increase in cell size, however, the absence of enrichment of pathways promoting cell cycle strongly suggests the presence hypertrophy.

In addition to JMP and DAVID, the gene lists were analyzed in Ingenuity's PathwayAnalysis (IPA). The data was input as the entire list of both enriched genes in the Ross

line as well as the Illinois line. IPA's analysis outputs lists such as top canonical pathways, top biological functions, top molecules, and top upstream regulators to explore relationships and mechanisms of relevance. From this analysis, the top canonical pathways were of interest. The three top canonical pathways were cAMP- mediated signaling with a p-value of 2.34E-06. Wnt/ β-catenin signaling with a p-value of 7.03E-06, and G-protein coupled receptor signaling with a p-value of 2.12E-05. The analysis provides a list of top molecules involved in each of these pathways as well as an indication of which line they are enriched in. From this list, it was seen that all three pathways were enriched in the Illinois birds compared to the Ross birds.

In the cAMP pathway, seven genes were enriched in Illinois when compared to Ross: C3AR1, CCR7, CYSLTR2, DRD2, LHCGR, PTGER2, and TSHR. Of these seven genes, the protein products of chemokine receptor 7 (CCR7), cysteinyl leukotriene receptor 2 (CYSLTR2), luteinizing hormone/ choriogonadotropin receptor (LHCGR), prostaglandin E receptor 2 (PTGER2), and thyroid stimulating hormone receptor (TSHR) are all known to activate cyclic AMP.

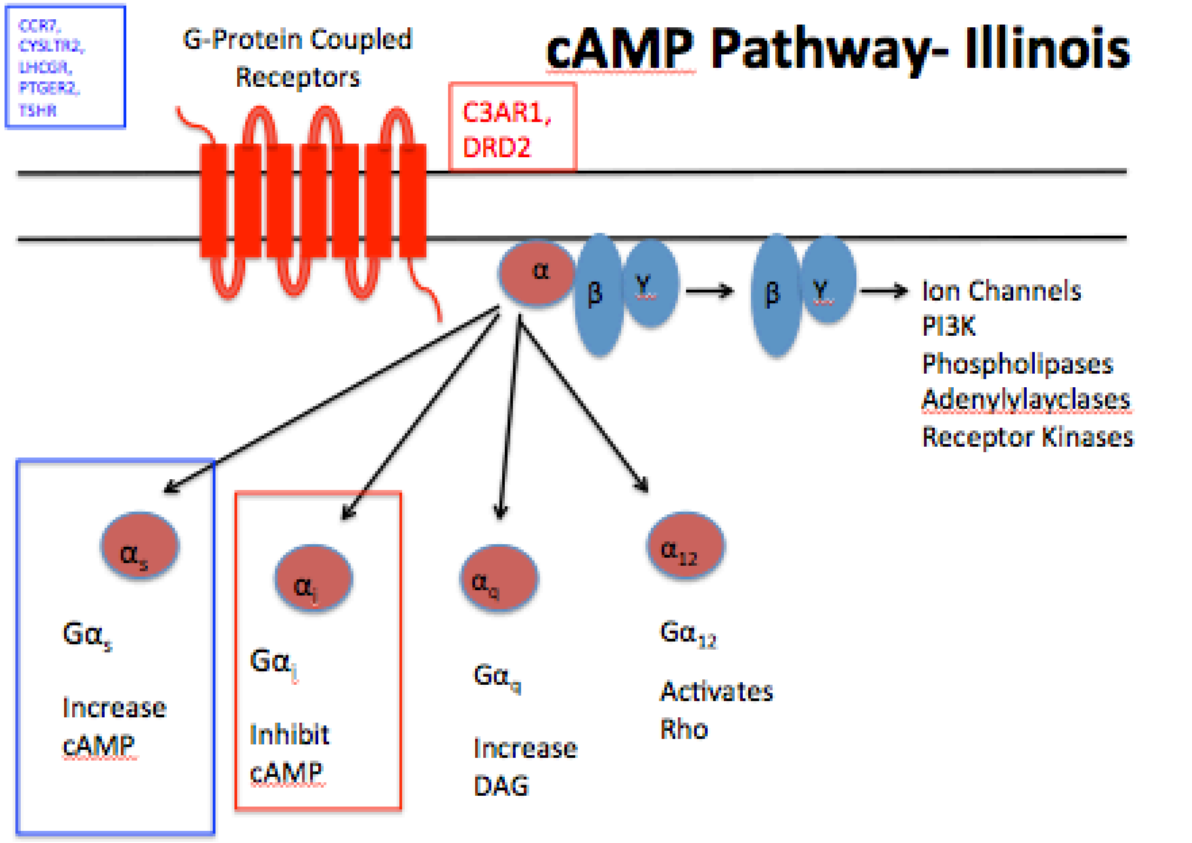


Figure 3 Cyclic AMP pathway, as it relates to the Illinois line. The seven genes listed are all enriched in the Illinois line when compared to the Ross line. Five out of the seven (first box in blue- CCR7, CYSLTR2, LHCGR, PTGER2, TSHR) are known to activate cAMP, while the other two (second box in red-C3AR1, DRD2) decrease cAMP production.

Gene products that function in the Wnt/ β-catenin signaling pathway were elevated in the Illinois lines. There are two main categories of Wnt pathways: Canonical and Non-Canonical. In this analysis, it was found that the canonical pathway, Wnt/ β-catenin signaling is present. Based on the IPA analysis, six Wnts (2, 3a, 7a, 7b, 8a, 10a) were seen to be enriched in the Illinois line along with lipoprotein receptor-related protein 1 (LRP1) and Frizzled 10 (FZD10). Frizzled 10 is a known receptor in the Wnt/ β-catenin signaling pathway

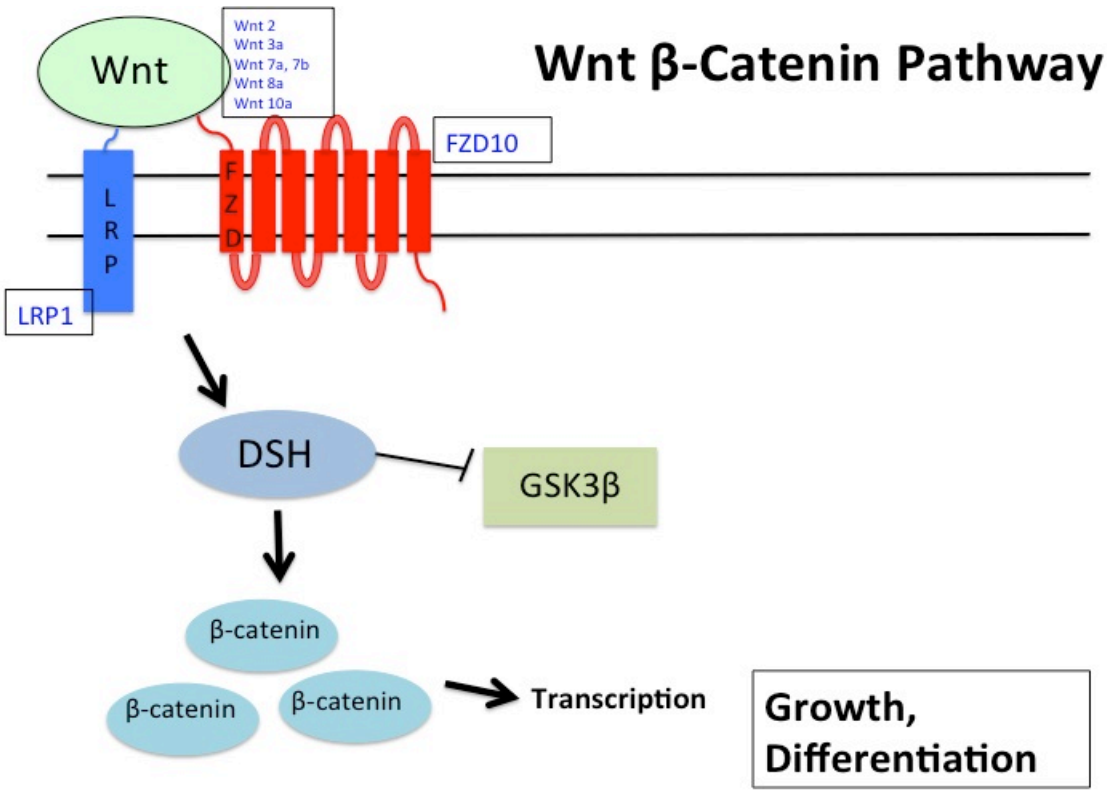


Figure 4 Wnt/β-catenin Pathway as it relates to the Illinois line. The six Wnt genes (Wnt2, Wnt 3a, Wnt 7a, Wnt 7b, Wnt 8a, Wnt 10a) along with the Frizzled 10 gene are enriched in the Illinois line and known to be involved in this pathway.

Chapter 5

HISTOLOGICAL SECTION RESULTS

PCNA stained sections from both lines at day two were studied to define the patterns of proliferating cells in the crypts and villi of the duodenum. This could indicate differences in development between the two lines. It has already been noted that unlike mammals where proliferation is contained to the crypts, proliferating cells in the chick are found in both the crypts and villi, similar to some reptiles and fish [40]. After studying the slides from six Ross birds and five Illinois birds, both lines had similar patterns of PCNA stain: the majority of the staining was in the crypts. The PCNA staining gradually faded up the length of the villus in the epithelial cells in both lines, but was seen sparsely throughout the entire lamina propria. It is possible that these cells are a mixture of both proliferating and differentiating cells [40]. Presence of PCNA staining throughout the lamina propria may also suggest proliferation of immune cells. In addition to the villus, there was scattered staining seen in both the inner circular and outer longitudinal muscle layers.

Measurements were taken of the width of each well-defined villus in each slide. These measurements were taken from right above the crypts, where the projection begins and analyzed with JMP10 by Oneway Analysis. There was a statistically significant difference in the width of the villi with the Illinois line having wider villi, with a mean of 111.1, than the Ross birds, with a mean of 85.6. It was visibly evident that based on the width differences, the Ross birds contained more villi per unit area. This discovery could positively impact the absorption capabilities of the Ross birds based on the increased surface area of absorptive cells.

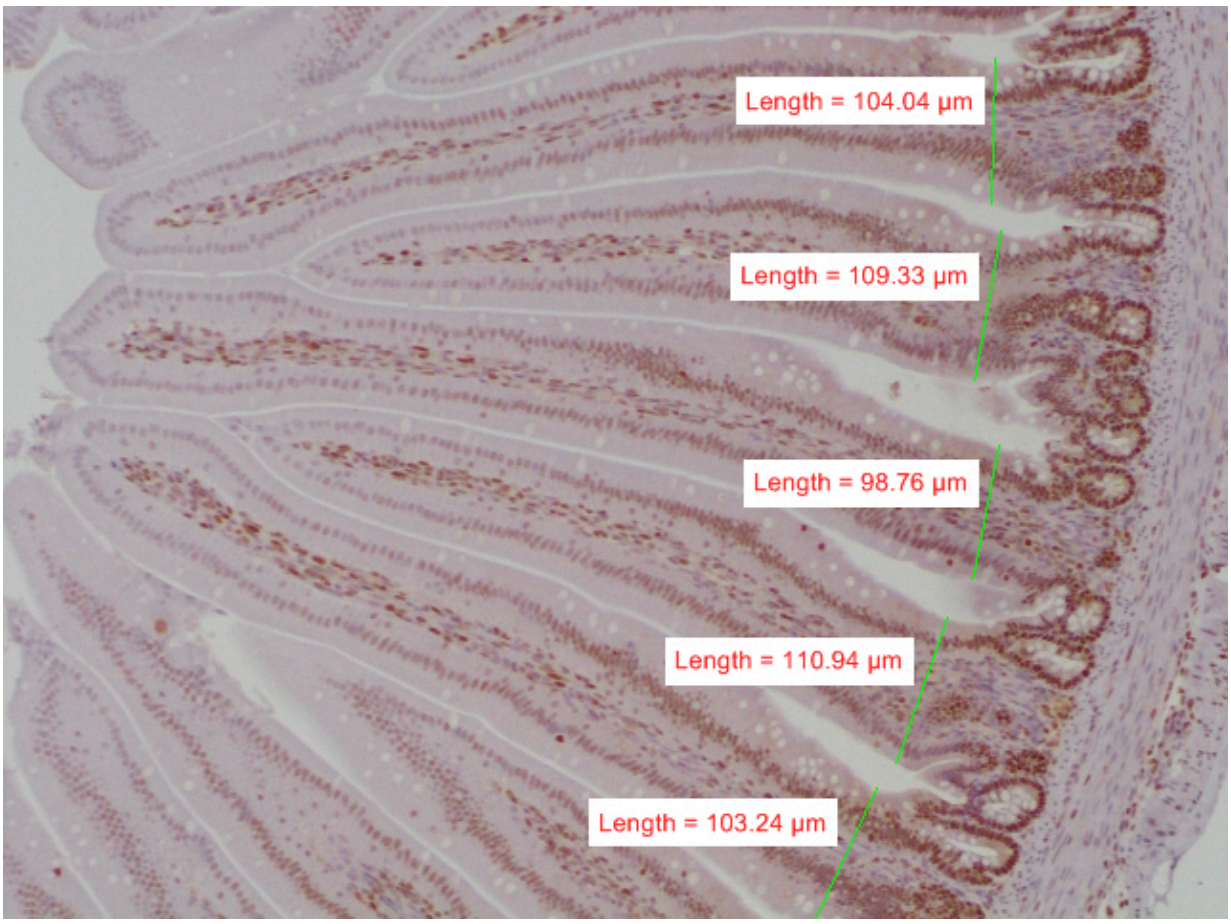


Figure 5 Cross section of male Illinois duodenum at day two with PCNA staining (brown) at 10x magnification. Image demonstrates how width measurements were captured with NIS Elements D software.

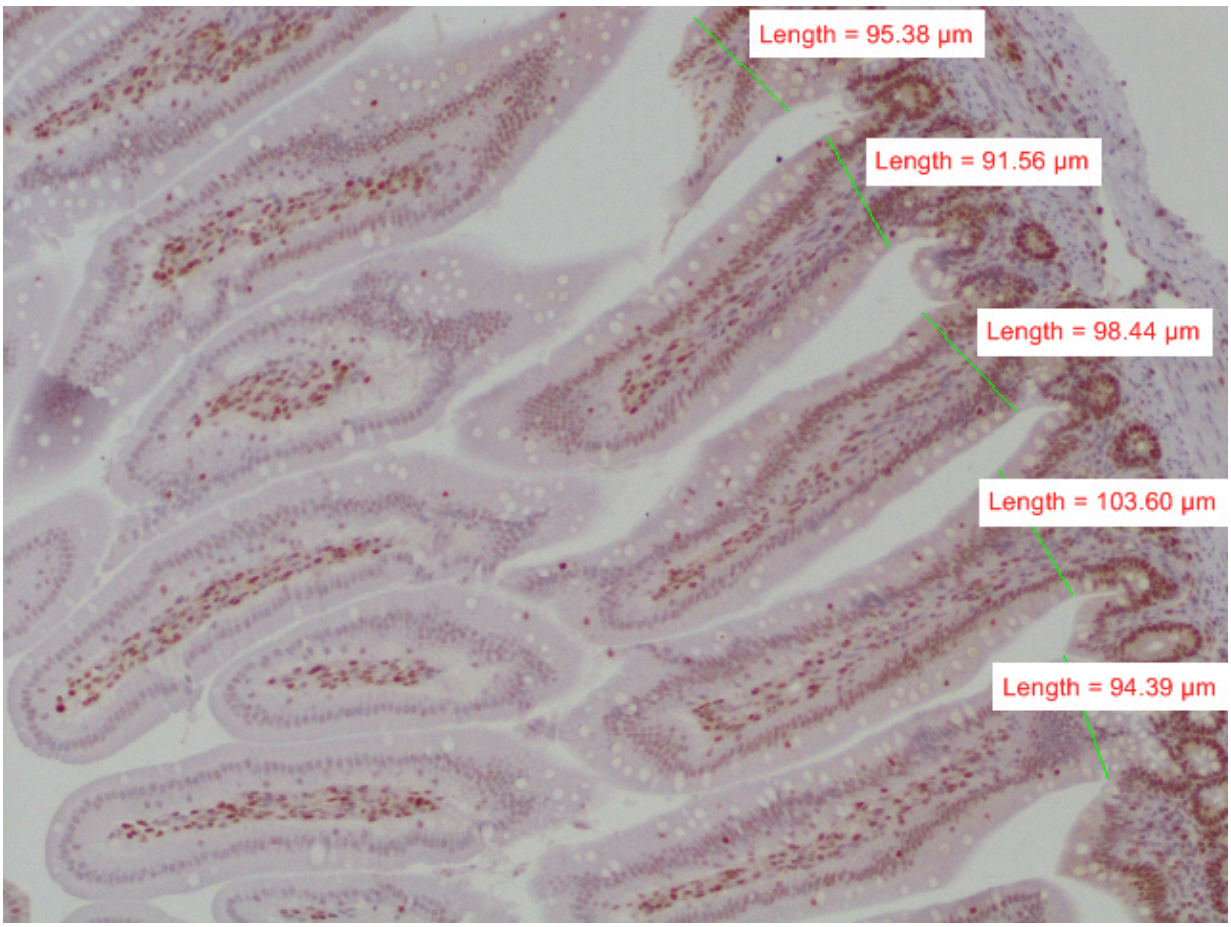


Figure 6 Cross section of male Ross duodenum at day two with PCNA staining (brown) at 10x magnification. Image demonstrates how width measurements were captured with NIS Elements D software.

Chapter 6

DISCUSSION

When comparing modern day broilers with the Illinois birds that were selected for improved growth until the 1950's, it is clear that human selection has had a significant impact on the growth of the birds. Overall growth has accelerated along with an increase in overall breast muscle mass. This discovery leads to the question of how these birds have gained the ability to convert feed more efficiently. Analyses of differential gene expression and histological sections were used to explore enteric differences between the modern day broiler and the heritage type Illinois line. One possible hypothesis would be that the Ross birds have developed in such a way that they are able to absorb and convert nutrients more efficiently, enabling them to grow more rapidly than the Illinois line. Observations made from the histological sections support this hypothesis. The Ross line's decreased villus width allows more villi to exist per unit area, meaning the overall surface area of villi epithelial cells is increased leading to increased absorptive capabilities [11]. This could improve overall feed efficiency and lead to more rapid growth.

The next question that arises after observing the histological sections and considering the correlation with absorption rate and growth is what causes this difference in villus size? It has been observed that nutrient absorptive capacity is closely correlated to morphological changes in the intestine [25]. In addition, there is evidence that an increase in villus surface area can be a result of an increase in food intake and a high fiber/ carbohydrate diet. [25], [39]. The majority of intestinal development occurs between hatch until approximately day 7 which coincides with a nutritional source shift from remaining yolk sac rich in lipids to an external diet high in carbohydrates [25]. At day two, these birds had recently been given access to pelleted food, so it is expected that food intake should be stimulating intestinal development..

This could be a contributing factor to the more rapid development of the Ross intestine when compared to that of the Illinois birds.

The villi of the duodenum consist of lamina propria containing capillaries that transport absorbed nutrients into portal circulation. A single cell epithelial layer containing absorptive enterocytes surrounds the lamina propria. When looking at the histological sections, it appears that although the villi width differs between the lines, the overall thickness of epithelial layer seems to be consistent in both. Based on this observation, it appears that the lamina propria is the portion that affects the overall width of the villus. Assuming that the Illinois line represents the ancestral state of villus structure, then one adaptation for increased feed efficiency in the Ross line is a thinner lamina propria. This structural change allows for more villi per unit area, thereby increasing overall surface area and absorptive capacity. The thinner lamina propria also means that nutrients can be absorbed through the enterocytes and travel through the capillaries into the portal circulation more rapidly in the Ross birds than in the Illinois line, enabling nutrients to be used more efficiently, although this is purely speculative at this point.

Previous studies observed that the jejunum and ileum segments in the Ross birds are longer than in the Illinois birds [34]. Based on this, it could be hypothesized that increased surface area due to villi is systematic throughout the entire small intestine. The jejunum and ileum were calculated to be 20% longer [34], therefore allowing more villi to be packed into the segments, leading to more rapid absorption of nutrients.

According to the analysis performed using JMP, DAVID, and IPA, the gene list corresponding to Ross has a prominent trend of ribosomal related terms. This observation suggests a high occurrence of translational activity and protein synthesis. Ribosomal activity has been shown to correlate to both hyperplastic and hypertrophic growth. Based on this, it would be expected that when looking at the histological sections, a difference would be seen in the cell number or size between the two lines. According to the increased number of villi seen in the Ross line, a reasonable hypothesis would be that hyperplasia of epithelial cells is occurring in order to cover the greater surface area. Elevated protein synthesis would also

contribute to supporting this hyperplastic growth, as well as possible hypertrophy occurring simultaneously. Both of these processes would be expected to require increased translational activity.

Few genes in the Ross line were enriched besides those involved in protein synthesis. Conversely, the Illinois birds were enriched in expression of genes affecting several different processes. The most informative way to interpret these genes is by studying the pathways in which they are involved. The pathways strongly implicated in development at this time point include the canonical Wnt/ β-catenin signaling pathway, the cAMP- mediated signaling pathway, and G-protein coupled receptor signaling pathway.

There are two main categories of Wnt pathways: canonical (β-catenin) and non-canonical. According to analysis by DAVID and IPA, the canonical pathway is enriched in the Illinois birds. In the absence of Wnt, β-catenin is phosphorylated within a degradation complex. This makes it a target for ubiquitation and proteolytic destruction [36]. However, when Wnt is present, it disrupts the complex so that β-catenin is not destroyed. The Wnt ligand binds to the Frizzled receptors and the LRP complex attaches to the Wnt bound Frizzled. This leads to the the activation of Disheveled. Since the destruction complex is no longer degrading β-catenin, it is able to accumulate in the cytoplasm. From the cytoplasm, β-catenin eventually moves to the nucleus where it acts as a transcriptional co-activator [36].

The canonical Wnt pathway is a part of many important biological processes, including development. Activation of the Wnt pathway is known to promote proliferation, regulate specification, control tissue patterning, regulate cell adhesion, and affect differentiation [12], [24], [37]. At hatch, the intestine in the chicken is anatomically complete but absorptive surface, rate of enterocyte proliferation, and crypt depth continues to increase post-hatch [39]. Wnt signaling is often found in the regions of proliferation during embryonic development [24]. Based on this, it is reasonable to think that it is also present in the proliferative cells on day two. It was seen that the lamina propria of the Illinois birds was thicker than that of the Ross. With the knowledge of the Wnt pathways involvement with

proliferation of cells, a rational hypothesis would be that the increase in Wnt signaling in the Illinois birds could possibly stimulate greater proliferation in the crypts as well as the non crypt areas including the lamina propria and the epithelial lining of the villi, leading to a greater villi width.

In order to test this hypothesis, staining studies could be performed to determine if members of the Wnt pathway are expressed where they are predicted to be. Use of antibodies specific to various Wnts could be used to locate where they are being expressed throughout the duodenal villi. Using in situ hybridization, probes can be utilized for Wnt specificity. According to the proposed hypothesis, it would be predicted that staining for Wnt would be greater in the Illinois when compared to the villi of the Ross line. It would also be informative to explore the proliferative expression in a three dimensional sense. Based on the structure of the villi, it could be hypothesized that gene expression could be radially gradient.

The cyclic AMP mediated signaling pathway and G-protein coupled receptor signaling pathway are the other two pathways enriched in the Illinois birds. These pathways are connected and can be considered as one in this context. The cascade is triggered by G-protein coupled receptors when a ligand is bound to the GPCR. The signal is sent through the GPCR to either activate or inactivate cellular effectors such as adenylyl cyclase. If adenylyl cyclase is activated, it uses ATP to produce cyclic AMP. This pathway is involved in cellular communication, promoting cellular proliferation, ion transport, and suppressing apoptosis [6]. Cell differentiation in the epithelium leads to attenuation of the cAMP pathway. Down-regulation of adenylyl cyclase isoforms and Gs alpha subunit may release cells from cAMP promoted anti-apoptosis in order to allow for gradual terminal differentiation [6]. As cells migrate, they lose their proliferative capabilities and begin to differentiate, gaining absorptive and digestive function [6]. Down regulation of the cAMP response may be necessary for the shift from proliferation to apoptosis to occur. Since intestinal epithelium is dynamic, the cAMP pathway's role in balancing proliferation and apoptosis is vital to epithelial development.

Intestinal epithelium is critical for uptake of nutrients and digestion, as well as existing as the single cell layer of protection that separates the body from microbes and toxins in the lumen.

Based on knowledge of the cAMP/GPCR pathways, it is hypothesized that the Ross line, with down regulation of the pathway, would have an increase in genes related to absorptive capacity, such as transporters versus digestive enzymes. In addition, it is plausible that based on its enrichment in the cAMP/GPCR pathway, the Illinois line would have upregulation of GPCR genes coupled to the stimulation of the cAMP cascade.In order to explore these hypotheses, tests must be performed to determine which receptors are expressed where. For instance, it has been seen that the least differentiated crypt epithelial cells had the highest levels of Gs alpha, and as differentiation occurs, levels drop [6]. Once these distributions are explored, more postulations can be made based on where cAMP appears in the maturing gut.

In addition to functioning as triggers for the cyclic AMP pathway, G-protein coupled receptors are known to have a role in chemosensation, the physiological response of an organ to a chemical stimulus. These GPCRs are nutrient sensors of the major macronutrients, lipids, proteins, and carbohydrates [20] The intestine has enteroendocrine cells, endocrine cells that are specialized to secrete hormones in response to nutrient stimuli, directly into the bloodstream instead of into ducts [20] It is essential to an animal's life to be capable of nutrient extraction, absorption, and digestion. Enteroendocrine cells are able to "taste" nutrients in order to regulate proper absorption and digestion [20] The Illinois line was determined to have upregulation of GPCRs in addition to annotation clusters relating to chemosensation: glucose sensing, receptor-mediated signaling, and ion channels [32].

Results from data analysis, including enriched pathways and GO terms, as well as PCNA patterns in the villi, also provided insight into the stage of development of the immune system and microbiome in the intestine at this time period. It was seen that the Illinois line had enrichment for multiple immune functions and pathways including the JAK-STAT, Toll-Like Receptor, and Tight Junction pathways. The JAK-STAT pathway is involved in the regulation

of immunity, growth, and proliferation. Interleukin-6 (IL6), secreted by T cells and macrophages to stimulate immune response, was upregulated in the pathway in the Illinois line, along with IFN, which is known to be involved in innate immunity. The Toll-Like Receptor pathway consists of Toll like receptors (TLRs) that enable inflammatory cells to recognize distinct pathogen associated molecular patterns and play a critical role in innate immune responses [21]. They participate in the first line of defense against invading pathogens and play a significant role in inflammation, immune cell regulation, survival, and proliferation.

The Tight Junction pathway is responsible for regulating cell barriers, controlling what substances are allowed in or out of the cell. A family of proteins, called Claudins, are considered the most important component of the tight junction pathway and are upregulated in the Illinois birds. This enrichment of claudins may be in response to inflammation promoted by the other pathways at this time point. Inflammation causes the cell barrier to break down, so an increase in claudins will better maintain the tight junctions needed to keep the barrier functioning properly.

Previous studies have suggested that the innate immune system is functional at hatch [3]. Immature heterophils and innate effector mechanisms are found along the gut in the hatchling to provide protection during the period needed for adaptive immune system maturity. At hatch, it is rare to find mature heterophils/granulocytes in the lamina propria, but they become evident by day two. Around this time, there is also a gradual decrease in genes coding for granule contents, which may suggest that the heterophils mature rapidly post-hatch [3].

The intestine continues to develop for three to four weeks post hatch. During the first few days post hatch, extensive enterocyte proliferation takes place. Increased production of enterocytes in the crypt and loss from villus tips is one of the most dramatic responses to inflammation. Changes in the number and cellular composition of immune cells occurs around hatch, partly as a result of the export of adaptive immune cells such as thymic T cells and bursal B cells [8]. This indicates the development of the adaptive immune system in addition to the already developing innate immune system. This development is vital as the immune system

must rapidly adapt to the shift from yolk to adult diet and colonization of bacteria in the gut. The ability to respond appropriately to food derivatives and non pathogenic microorganisms, whether through oral tolerance or oral ignorance, is paramount for immune system success [8].

There is still much speculation regarding what triggers immune system development. Immune protection at hatch may be a result of maternal antibodies passed through the yolk, or it may simply be developed enough at hatch in order to be functionally sufficient [3]. There is also the question of whether immune function is independent of exposure to feed and bacteria or if environmental stimuli is necessary in order to trigger the response.

Understanding the triggers for immune function and gut development is crucial for production application. It is important to study the effect of the separation of the hatchery and production facilities. This separation means that the chicks will have a period of time during transport without feed and water. Although the chicks are able to survive due to the remaining yolk sac, the delay in ingestion of adult diet, even for a short amount of time, could have substantial negative effects on both the gut development and immune function. It is suggested that antigens/ exposure to food may be necessary in order to stimulate full differentiation of primary immune cells. This means that until the primary cells are fully differentiated, no secondary immune structures will be able to develop, stunting certain immune functions [9].

The results from the data analysis suggest that at day two post-hatch the Illinois line is enriched in immune function when compared to the Ross line. The up-regulation of the Wnt, GPCR, and cAMP pathways support this. It appears that human directed selection for faster growing, more feed efficient birds has impacted not only the growth, but also the development of vital systems such as the immune system. It would be reasonable to speculate that at hatch, the majority of the energy in the Ross birds is being used for rapid growth in some systems (musculoskeletal, etc.) and not for development of other systems. This may mean that basic immune function has been established pre-hatch in the Ross birds, while the Illinois birds continue to use energy for further development of the immune system, both innate and adaptive, post-hatch.

This study has generated more questions than answers at this point, and there are many possible experiments to further explore the underlying genetic differences between the two lines. Performing studies on these birds from a wider range of time points, starting from the embryonic stage and continuing through the first couple weeks post hatch, may provide great insight into the wave of immune system development and intestinal growth and function. Histologically, staining for individual Wnts, cell types, and claudins will help explain mechanisms and investigate the specific functions of processes occurring in the intestine at this time. In addition, performing absorption studies may reveal differences in absorptive capabilities in the duodenum between the two lines. This could support the data found in these preliminary studies regarding a difference in absorptive capacity, and its contribution to overall growth.

The findings of this study could prove to have a great impact on research and industry related to chicken selection and poultry production. It is important to understand the positive, as well as possible negative, effects that human directed selection has made on the growth, development, and overall health of these meat type broilers. By comparing modern day broilers with a line not influenced by human directed selection, it is possible to explore changes in differential gene expression and its effect on development. In addition, it is crucial to unveil any negative impacts on growth or immune function, such as the possible delay of intestinal growth and/or immune function during the transition from hatchery to grow-out facility. This information could provide to be useful for the poultry industry to adjust the process in order to produce the most efficient bird possible.

REFERENCES

1. Balakrishnan, A., Tavakkolizadeh, A., & Rhoads, D. B. (2012). Circadian clock genes and implications for intestinal nutrient uptake. *The Journal of Nutritional Biochemistry, 23*(5), 417-422.

2. Bar-Shira, E., & Friedman, A. (2006). Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. *Developmental and Comparative Immunology, 30*(10), 930-941.

3. Bar-Shira, E., Sklan, D., & Friedman, A. (2003). Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental and Comparative Immunology, 27*(2), 147-157.

4. Berna, M. J., Tapia, J. A., Sancho, V., Thill, M., Pace, A., Hoffmann, K. M., et al. (2009). Gastrointestinal growth factors and hormones have divergent effects on akt activation. *Cellular Signalling, 21*(4), 622-638.

5. Breer, H., Eberle, J., Frick, C., Haid, D., & Widmayer, P. (2012). Gastrointestinal chemosensation: Chemosensory cells in the alimentary tract. *Histochemistry and Cell Biology, 138*(1), 13-24.

6. Choi, L. J., Jenikova, G., Hanson, E., Spehlmann, M. E., Boehling, N. S., Kirstein, S. L., et al. (2010). Coordinate down-regulation of adenylyl cyclase isoforms and the stimulatory G protein (G(s)) in intestinal epithelial cell differentiation. *The Journal of Biological Chemistry, 285*(17), 12504-12511.

7. Crosnier, C., Stamataki, D., & Lewis, J. (2006). Organizing cell renewal in the intestine: Stem cells, signals and combinatorial control. *Nature Reviews.Genetics, 7*(5), 349-359.

8. Davison, F., Kaspers, B., & Schat, K. A. (2008). The avian mucosal immune system. *Avian immunology* (First ed., pp. 241-250) Elsevier Ltd.

9. Dibner, J. J., Knight, C. D., Kitchell, M. L., Atwell, C. A., Downs, A. C., & Ivey, F. J. (1998). Early feeding and development of the immune system in neonatal poultry. *J. Appl. Poultry Res., 7*, 425-436.

10. Dyer, J., Vayro, S., & Shirazi-Beechey, S. P. (2003). Mechanism of glucose sensing in the small intestine. *Biochemical Society Transactions, 31*(Pt 6), 1140-1142.

11. Geyra, A., Uni, Z., & Sklan, D. (2001). Enterocyte dynamics and mucosal development in the posthatch chick. *Poultry Science, 80*(6), 776-782.

12. He, X. C., Zhang, J., Tong, W. G., Tawfik, O., Ross, J., Scoville, D. H., et al. (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of wnt-beta-catenin signaling. *Nature Genetics, 36*(10), 1117-1121.

13. Hughes, A. L., & Piontkivska, H. (2011). Evolutionary diversification of the avian fatty acid-binding proteins. *Gene, 490*(1-2), 1-5.

14. Jackson, S., & Diamond, J. (1995). Ontogenetic development of gut function, growth, and metabolism in a wild bird, the red jungle fowl.. *Am J Physio, 269*

15. Kaji, I., Karaki, S. I., & Kuwahara, A. (2013). Taste sensing in the colon. *Current Pharmaceutical Design,*

16. Kanamaru, Y., Sekine, S., Ichijo, H., & Takeda, K. (2012). The phosphorylation-dependent regulation of mitochondrial proteins in stress responses. *Journal of Signal Transduction, 2012*, 931215

17. Kanginakudru, S., Metta, M., Jakati, R. D., & Nagaraju, J. (2008). Genetic evidence from indian red jungle fowl corroborates multiple domestication of modern day chicken. *BMC Evolutionary Biology, 8*, 174-2148-8-174.

18. Komiyama T., Ikeo K., Tateno Y., Gojobori T.2004. Japanese domesticated chickens have been derived from Shamo traditional fighting cocks. Mol. Phylogenet. Evol. 33:16–21.

19. Kosinski, C., Li, V. S., Chan, A. S., Zhang, J., Ho, C., Tsui, W. Y., et al. (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15418-15423.

20. Liou, A. P. (2012). G protein-coupled receptors in nutrient chemosensation and gastrointestinal hormone secretion. *Journal of Animal Science,*

21. Liu, G., Friggeri, A., Yang, Y., Park, Y., Tsuruta, & Abraham, E. (2009). miR-147, a microRNA that is induced upon toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Pnas, 106*(37), 15819-15824.

22. Logan, C. Y., & Nusse, R. (2004). The wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology, 20*, 781-810.

23. Mathger, L. M., Roberts, S. B., & Hanlon, R. T. (2010). Evidence for distributed light sensing in the skin of cuttlefish, sepia officinalis. *Biology Letters, 6*(5), 600-603.

24. McBride, H. J., Fatke, B., & Fraser, S. E. (2003). Wnt signaling components in the chicken intestinal tract. *Developmental Biology, 256*(1), 18-33.

25. Mitjans, M., Barniol, G., & Ferrer, R. (1997). Mucosal surface area in chicken small intestine during development. *Cell and Tissue Research, 290*(1), 71-78.

26. Nelson, W. J., & Nusse, R. (2004). Convergence of wnt, beta-catenin, and cadherin pathways. *Science (New York, N.Y.), 303*(5663), 1483-1487.

27. Noah, T. K., Donahue, B., & Shroyer, N. F. (2011). Intestinal development and differentiation. *Experimental Cell Research, 317*(19), 2702-2710.

28. Pinto, D., & Clevers, H. (2005). Wnt, stem cells and cancer in the intestine. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 97*(3), 185-196.

29. Provencio, I., Jiang, G., De Grip, W. J., Hayes, W. P., & Rollag, M. D. (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proceedings of the National Academy of Sciences of the United States of America, 95*(1), 340-345.

30. Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackleford, J. E., Lebherz, H., & Lusis, A. J. (1987). Structure, evolution, and regulation of chicken apolipoprotein A-I. *The Journal of Biological Chemistry, 262*(15), 7058-7065.

31. Rawlings, J. S., Rosler, K. M., & Harrison, D. A. (2004). The JAK/STAT signaling pathway. *Journal of Cell Science, 117*(Pt 8), 1281-1283.

32. Reimann, F., Tolhurst, G., & Gribble, F. M. (2012). G-protein-coupled receptors in intestinal chemosensation. *Cell Metabolism, 15*(4), 421-431.

33. Saaf, A. M., Halbleib, J. M., Chen, X., Yuen, S. T., Leung, S. Y., Nelson, W. J., et al. (2007). Parallels between global transcriptional programs of polarizing caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer. *Molecular Biology of the Cell, 18*(11), 4245-4260.

34. Schmidt, C.J., Persia, M. E., Feierstein, E., Kingham, B., Saylor, W.W. (2009). Comparison of a modern broiler line and a heritage line unselected since the 1950s. *Poultry Science, 88*(12), 2610-9.

35. Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N., et al. (2007). Distinct wnt signaling pathways have opposing roles in appendage regeneration. *Development (Cambridge, England), 134*(3), 479-489.

36. Teo, J. L., & Kahn, M. (2010). The wnt signaling pathway in cellular proliferation and differentiation: A tale of two coactivators. *Advanced Drug Delivery Reviews, 62*(12), 1149-1155.

37. Theodosiou, N. A., & Tabin, C. J. (2003). Wnt signaling during development of the gastrointestinal tract. *Developmental Biology, 259*(2), 258-271.

38. Tixier-Boichard, M., Bed'hom, B., & Rognon, X. (2011). Chicken domestication: From archeology to genomics. *Comptes Rendus Biologies, 334*(3), 197-204.

39. Uni, Z. (1999). Functional development of the small intestine in domestic birds: Cellular and molecular aspects. *Poultry and Avian Biology Reviews, 10*(3), 167-179.

40. Uni, Z., Platin, R., & Sklan, D. (1998). Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. *Journal of Comparative Physiology.B, Biochemical, Systemic, and Environmental Physiology, 168*(4), 241-247.

Appendix A

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: (27) 12-22-10R

TITLE OF PROJECT: Scientific Investigation into the response of Broiler Chickens to heat stress by transcriptome analysis

INSTRUCTOR/PRINCIPAL INVESTIGATOR: Carl Schmidt

New or Three Year Review (mark one)

NEW X□ **THREE YEAR □**

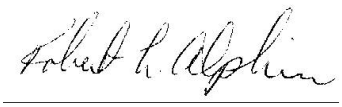
If this is a 3 year renewal, what is the assigned existing protocol number? _____

(This section for Committee use only)

Application Approved (date): 1-5-2011

Application Rejected (date): _____

Reason for Rejection: _____



Signature, Animal Care and Use Committee **1-5-2011 Date**

APPLICATION INFORMATION:

Title: Scientific Investigation into the response of Broiler Chickens to heat stress by transcriptome analysis

Principal Investigator(Research): Carl J. Schmidt

Address: 107 Allen Lab, 601 Sincock Lane, University of Delaware, Newark, Delaware 19716

Telephone: (302)-831-1334 Email: schmidtc@udel.edu

Proposed start date: February 1 2011 End date: January 31, 2014

Teaching/Outreach □ Research X

If TEACHING box was checked, select from the following:

Demonstration □ Laboratory □ Student Project □

If student project, please define project: _____

Have all participants listed above reviewed the application and is familiar with the proposed work?

YES X□ NO □

If no, identify those needing to review application.

_____ _____ _____

Are all proposed animal care management procedures 1) defined as "pre-approved" by the Animal Care and Use Committee, or 2) part of the Standard Operating Procedures developed by the Animal Care and Use Committee for that particular species?

YES X□ NO □ To be determined by AACUC □

Have all participants been trained? YES X□ NO □

Which participants have not been trained?

_____ _____ _____

Name the person responsible for conducting the training.

If after hours participation is required by students, please describe how this is being handled. (e.g. supervisors, assistants, etc.) Please include the times and days that students may be on site.

ANIMAL INFORMATION:

Common Name of the Animal Requested: Chickens

Amount Being Requested: 1600

Source of Animals: Allen Family Foods and Chet Utterback at the University of Illinois

Where are the animals being held: UD Poultry Farm

Briefly Describe the Goals or Objectives of this Application (use additional space as needed).

The goal of this study is to determine the ability of the modern broiler chicken to handle heat stress compared to the heritage variety. Following treatment, birds will be euthanized by cervical dislocation and organs harvested for transcriptome analysis.

Rationale for scale of study: This is a new area of research, using new genomic approaches to understand how birds respond to heat stress. The large numbers of birds are necessitated in order to achieve statistical significance in our gene mapping studies.

Birds: Heritage birds will be obtained from Chet Utterback at the University of Illinois and the Ross708 birds from a local supplier. Birds will be wing tagged and randomly placed into control and experimental groups as described below (Heat Shock Scheme). In each experiment 100 birds from each line will be included in each experimental group. The size of the facilities at the University of Delaware limit the number of birds per chamber, hence we anticipate multiple replicates over time to a total of 1600 birds per line. Blood will be taken from each bird for DNA extraction prior to heat stress. Also, 12 birds from each group will be removed on post hatch days 2, 7 and 21, euthanized (cervical dislocation) and tissues harvested. Blood biomarker data using the iSTAT will be collected from these birds prior to euthanasia. Chambers will be monitored on a daily basis to insure adequate feed and water and to remove any sick or dead birds.

Heat Shock Scheme: Controls are hatched from eggs incubated at 37^{o}C (99^{o}F) while thermal conditioned embryos will be incubated at 39.6^{o}C (103^{o}F) from embryonic days 10-18, then returned to 37^{o}C. Following hatch through day 21, they will be kept at ambient temperatures. At day 22, the original Control birds will be split into two populations (Control A and B) and the *In Ovo* Heat-conditioned bird also split into two groups (*In Ovo* Heat Conditioned A and B). The A populations will be kept at ambient temperatures while the B populations will be heat stressed at 35^{o}C (95^{o}F) or 7 hours per day for 21 days. There will be 20 birds per chamber. Multiple replicates (hatches) will be conducted. At the end of the trial (6 weeks from hatch), birds will be euthanized and tissues collected.

Attached below is additional protocol information.

Does this procedure involve surgery? YES NO X□

If yes, explain in detail the surgery.

Are drugs, vaccines and/or medications being used? YES □ NO X□

If yes, describe what is being used. Include dosages and routes of administration.

How often are animals monitored and how are sick or injured animals being handled?

The birds will be checked daily and given food and fresh water *ad libidum*. Sick or injured animals will be euthanized by cervical dislocation.

What is the method of euthanasia, if specified in the protocol?

Cervical dislocation as per AVMA Guidelines on Euthanasia 2007

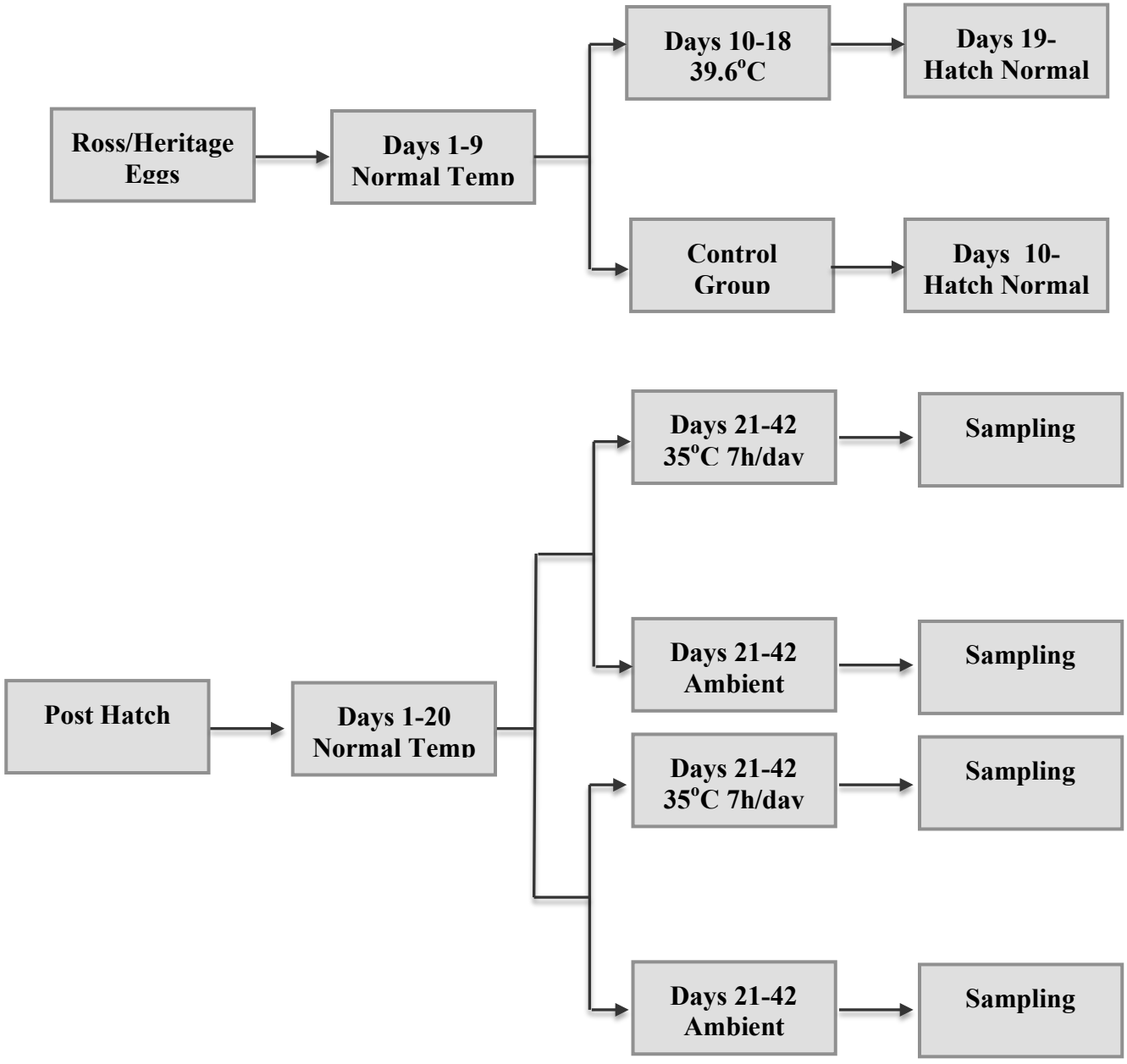
List the veterinarian who is on-call:

Name: Miguel Ruano Telephone: 302-831-1539

Does this application require approval from Occupational Health & Safety (OHS)? YES □ NO X□

If yes, what form(s) are attached?_____

NOTE: OHS approval is required for experiments involving the use of hazardous substances such as radioactive materials, highly toxic or carcinogenic materials, human reproductive hazards, or zoonotic or human pathogens.



Ross Heritage heat stress experiment: Eggs will be either heat stressed or maintained as controls from embryonic days 10-18, and then returned to normal temperatures. Subsequently, both heat stressed and control birds will be split into two groups each, with one group heat stressed from days 21-42 post-hatch, with the second group kept at ambient temperatures to function as a control. So, there will be a total of 8 groups at the end of each experiment.

Tissue Samples: Genomic DNA & RNA:

- Blood
- Brain
- Heart
- Liver
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Ceca (and contents)
- Fat pad
- Breast muscle
- Spleen

Weekly Measurements:

- iSTAT metabolic measurements
- Weight

Day 21/42

- Shank length
- Shank Width

Morphometric:

- Liver
- Spleen
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Breast muscle
- Heart

Samples are needed for:

- RNAseq

- microRNA
- Genomic DNA
    - SNP
    - CVN
    - Epigenetics