**Summary:**

The NE-1834 group members work on a wide variety of projects involving the identification of pathogen specific immune responses and their genetic basis, genetic basis of disease resistance, and physiological and nutritional components affecting disease resistance with the goal to improve genetic selection of poultry. The use of advanced molecular biology techniques such as Next generation sequencing, functional annotation and others is contributing to significant amount of data being produced. Several members of the group are active collaborators and the publication and presentation list demonstrates the productivity of the group. Ongoing concerns are the conservation of genetic stocks, particularly many valuable chicken lines that are not being maintained due to the lack of funding and lack of cryopreservation techniques that are workable solutions. The members of the group will try to communicate with each other to prevent lines from being destroyed without trying to relocate them to another facility.

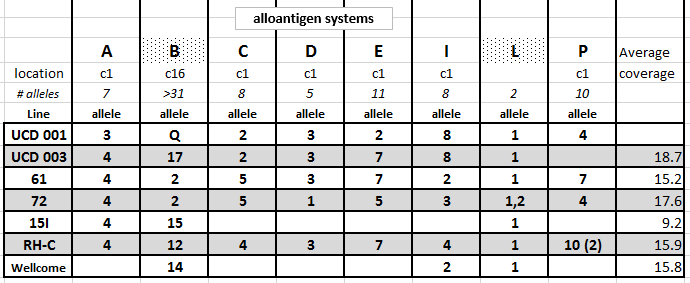
**Accomplishments:**

*Objective 1. Identify and characterize genes and their relationships to disease resistance in poultry with an emphasis on the major histocompatibility complex as well as other genes encoding alloantigens, communication molecules and their receptors and other candidate systems.*

**Robert Taylor Jr:**

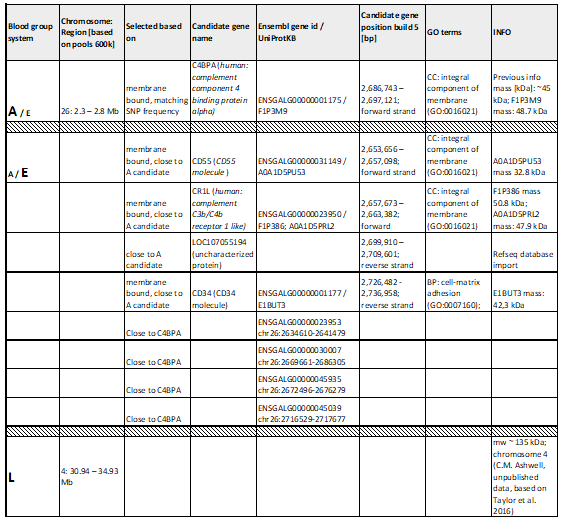
**a. Identifying chromosomal locations and products of alloantigen genes** (Taylor, Fulton, Ashwell, Kopulos, Drobik-Czwarno, McCarthy). Thirteen alloantigen systems have been identified on chicken nucleated red blood cells (*A, B, C, D, E, H, I, J, K, L, N, P, R*) [Taylor et al., 2016]. Recipients produce specific alloantisera following immunization with donor RBC mismatched for a single allele of one alloantigen system but matched for multiple systems. The antisera are tested via agglutination. Despite many studies demonstrating that alloantigens influence economic traits (reviewed by Taylor et al., 2016), alloantigen *B,* the chicken MHC, is the sole system whose precise chromosomal location and gene product have been identified. These studies seek to find the chromosomal locations and to identify the products of alloantigen genes.

Existing genome sequence and historic alloantigen data from inbred lines were used. **Table 1** lists these lines including those from Roslin (Kranis et al., 2013), UCD (provided by Mary Delany) and ADOL (provided by Hans Cheng). Commercial lines from Hy-Line which had sequence information and alloantigen type were also utilized. The chicken reference genome, Line UCD 001, version 5 (galGal5.0) was used to align sequence reads for SNP detection. Sequence for lines having known alloantigen types were compared to search for a SNP pattern consistent with the segregation of alloantigen differences among lines.



**Table 1**. Alloantigen types found in seven inbred lines (Briles et al., 1979).

Specific genotypes were used to create DNA pools for genotypes within each alloantigen system. Most systems had three genotypes: opposite homozygotes and the component heterozygote (i. e. *SS, ST, TT*) but some instances had one homozygote and one heterozygote (*FF, FG*). The same genotype was represented by multiple pools containing unique individuals. Samples were typed using a 600 K SNP panel (**funded by Genome Coordinator Funds**). Using the SNP identified via sequencing or the SNP chip, bioinformatic analyses for alloantigens *A, C, D, E, I, L* were conducted. Criteria set for this analysis included that SNP: 1) were in exons; 2) were in genes that are expressed on the cell surface; 3) could change the amino acid or truncate the protein; or 4) could change the 3-D structure of the protein. **Table 1** shows candidates identified for the *A*, *E* and *L* alloantigens.

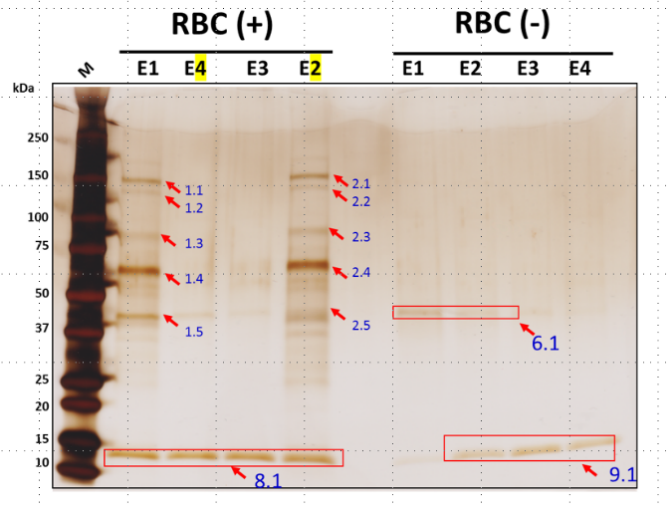


**Table 1**. Candidate genes for alloantigen systems *A*, *E*, and *L*

**BP** = Biological Process **CC** = Cellular Component (Source: Quick GO / Unipro)

Another method relied upon isolation of alloantigen proteins through a monoclonal or polyclonal antibody-coated column (**Figure 1**). Samples that are positive or negative for antibody reaction will be compared via proteomic analyses to determine molecular weight. The monoclonal antibody against alloantigen *A* and polyclonal antibodies against alloantigen *E* and *L* were used.

The results from these methods will be combined to identify specific genes identified with the different alloantigens. Subsequent work will examine the candidate SNP alleles within samples of known alloantigen alleles to determine if there is correlation between the SNP alleles and the phenotype.

**Figure 1.** Red blood cell (RBC) proteins eluted through a column coated with a monoclonal antibody against alloantigen A. Silver-stained eluants are from RBC positive (+) or negative (-) for reaction with the antibody

**b. Selection for high or low pro-inflammatory mediators IL-6, CXCL8 and CCL4** (Swaggerty, Kogut, Ashwell, Taylor). Cooperative work reported by Dr. Christi Swaggerty, USDA-ARS, College Station, TX.

**Mark S. Parcells (Delaware)**

1. Role of Exosomes in MDV-mediated Immune Suppression and Vaccine Responses. In the study of serum exosomes from vaccinated and tumor-bearing chickens:

A.In vaccine-associated serum exosomes (VEX) a.We found miRNAspredicted totarget key proliferation pathways (MAPK, growth factor signaling) b.We found mRNAs mapping to the entire MDV genome (including many structural genes). c.We identified proteins common to VEX that may represent biomarkers. B. In tumor-bearing bird-associated exosomes (TEX)a.We found miRNAs predicted to target phosphatidyl inositol signaling, suggestive of a role in blocking T-cell activation b.We found that mRNAs mapped primarily to the latency/transformation-associated regions of the genome (Meq, RLORF4, etc.) c.We identified proteins common to TEX that may represent immunosuppressive biomarkers

2. Role of Itaconate in IRG-1 (ACAD1)-associated Immune Functions. As part of a Summer student project, we have been following-up on genome-wide association studies performed by the late Pete Kaiser on the role of IRG-1 in MDV susceptibility and resistance. In 2011, their group reported that alleles of IRG-1 correlated with MD resistance and susceptibility. No mechanism was proposed at this time. As it turns out, IRG-1 (immunoresponsive gene 1, aka aconitate decarboxylase 1) is an enzyme that generates itaconate (itaconic acid) from citrate and this metabolite has multiple functions.

To examine the role of IRG-1 and itaconate on MDV infection, we have performed dose-responses of the effect of itaconate (ITA) and a cell-permeable derivative (4-octyl itaconate, 4IO) on MDV replication in CEF and in spleen cells.

**Huajiun Zhou:**

Improving food security in Africa by enhancing resistance to Newcastle disease virus and heat stress in chickens. This USAID funded Feed the Future Innovation Lab for Genomics to Improve Poultry project (H. Zhou, PI) through a partnership of the University of California at Davis (H. Zhou, R. Gallardo, T. Kelly), Iowa State University (S.J. Lamont, J. Dekkers), Sokoine University of Agriculture(SUA)-Tanzania, the University of Ghana(UOG), and International Livestock Research Institute (I. Baltenweck, E. Ouma)was renewed for the second phase (2018-2023).The five-year research program applied advanced genetics and genomics approaches to sustainably enhance innate resistance to Newcastle disease virus (NDV) and heat stress in chickens to improve poultry production in Africa. There are five specific objectives on the second phase: Assess correlations of crucial production traits with disease resistance traits: egg production, growth rate; Select and breed genetically enhanced local ecotypes; Characterize circulating strains of NDV; Determine and monitor the effectiveness of genomic selection; Conduct value chain assessment and business plan development; Develop a training toolkit on application of genetic selection platform.

Development of low density SNP Panel: To develop a 5K low-density SNP panel, SNPs were acquired from various sources, including 4500 SNPs across the chicken genome, significant/suggestive SNPs from GWAS analyses, MHC SNPs, SNPs from important genomic regions from other collaborative studies on GWAS on Hy-Line Brown and RNA-seq on inbred lines at Iowa State University and UC Davis, and SNPs that directly affect a gene product.

Interferon regulatory factor 7 (IRF7) is known as the master transcription factor of type I interferon response in mammalian species along with IRF3. Birds yet only have IRF7 while missing IRF3 with a smaller repertoire of immune-related genes which leads to a distinctive immune response of chickens compared to mammals. In order to understand the functional role of IRF7 in the regulation of antiviral response against avian influenza virus in chickens, we generated IRF7-/-chicken embryonic fibroblast (DF-1) cell lines and respective control (IRF7wt) by utilizing the CRISPR/Cas9 system. IRF7 knockout resulted in increased viral titers of low pathogenic avian influenza viruses.

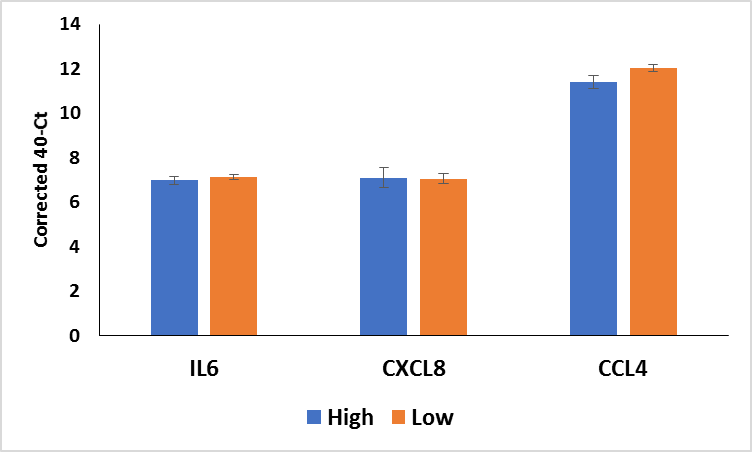
**Christina Swaggerty (USDA ARS FFSRU):**

**A. Selection for high or low pro-inflammatory mediators based on the sire** (Swaggerty, Pevzner, Kogut). Commercial broiler line sires were selected for high or low pro-inflammatory mediators: IL6, CXCLi2 (now CXCL8), and CCLi2 (now CCL4) (*Poult Sci* 93:535, 2014). Peripheral blood leukocytes were screened for these mediators using qRT-PCR of total RNA. Sires identified as high or low were mated to unselected broiler dams. The high line was more resistant to *Salmonella enteriditis* (*Poult Sci* 93:535, 2014)*, Eimeria tenella*, (*Poult Sci* 94:37, 2015), necrotic enteritis (*Poult Sci* 95:370, 2016) and *Campylobacter jejuni* (*Poult Sci* 96:16, 2017).

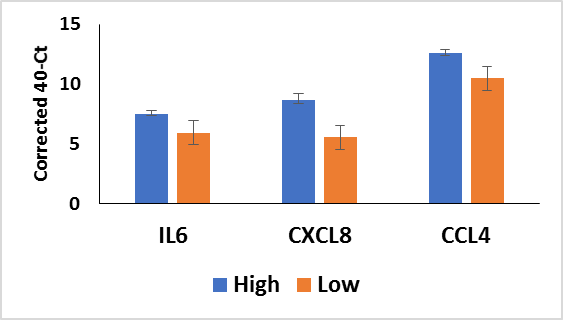
**B. Selection for high or low pro-inflammatory mediators based on the sires and dams** (Swaggerty, Ashwell, Taylor). We are using a genetics approach to reduce *Salmonella* colonization in broilers by selecting for birds with an enhanced innate immune response. The goal of this project is to develop two immunologically divergent lines of broilers based on selecting for a high and low phenotype of key innate immune markers in both sires and dams. Additionally, the role of the gut microbiome will be examined in the founder and the selected High and Low lines enabling us to begin to address the complex interplay between host genetics, the gut microbiome, selection pressures, and *S.* Enteritidis colonization.

A new selection was initiated with founder Athens Canadian randombred (ACR) sires, a 1957 broiler population and founder commercial broiler line for the dams. The initial selection format is shown in **Figure 1**. A screenshot of a cell phone

Description automatically generated **Figure 1**. Initial selection format for pro-inflammatory mediators. Founder Athens Canadian randombred sires and founder commercial broiler line dams will be identified for high or low pro-inflammatory mediators in peripheral blood leukocytes (see below).

****After four generations of selection based on high/low pro-inflammatory mediators in both the sires and dams, performance declined markedly (poor fertility, hatchability, etc.). After much discussion, the decision was made to proceed with selection based solely on the sire. The existing high and low dams were combined and randomized moving forward with no additional selection pressures. The fifth generation of progeny were to be tested in January 2019, but due to a partial government shutdown, Dr. Swaggerty was unable to receive eggs and blood samples for challenge trials and screening, respectively. Generation 6 high (n=22) and low (n=57) sire blood samples were collected and IL6, CXCL8, and CCL4 mRNA expression levels analyzed in the summer of 2019 and the data shown in Figure 2.

**Figure 2. IL6, CXCL8, and CCL4 in total RNA purified from the peripheral blood leukocytes collected from High and Low sires (Summer 2019).**



The high and low sires were ranked and the 10 highest and 10 lowest (based on composition rank) were identified and will be used to produce the next generation of progeny. The average of the high and low ranked sires is shown in Figure 3.

**Figure 3. IL6, CXCLi2 and CCLi2 values of sires that will produce the next generation.**

**Henk Parmentier (Wageningen):**

In summary, the ‘selection on NAb’ project revealed the following results:

1. It is possible to select and breed for high or low levels of NAb and natural auto-antibodies (NAAb.
2. Heritability was approximately 0.12 for IgTotal, 0.25 for IgM and 0.11 for IgG NAbs
3. About 3% of the difference in NAb can be attributed to differences between mother hens (maternal environmental effects), resulting potentially in 0.7 titer point differences between extreme mothers.
4. The response to selection was cumulative.
5. There were high genetic correlations between the NAb antibody isotypes.
6. There were low and non-significant genetic correlations between NAb and production traits.
7. The NAb titers (IgM) and total IgM levels were strongly affected by TLR1A variants on chromosome 4 explaining 60% of the genetic variation. This suggests a general effect of TLR1A on B cell development in the studied elite layer line.
8. TLR1A CC variants and CG variants were related with high NAb levels whereas the GG variant was associated with low NAb titers – indicating full dominance.
9. Other suggestive regions were GGA9 and GGA18 for IgA and GGA4 for IgTotal, for IgG no region was found.
10. The CC variant transfected in HeLA57A and HEK293 reporter cells was as functional as wild type chTLR1A variants when stimulated by TLR1A ligands, whereas this was not true for the GG variant, suggesting that the GG variant is non-functional.
11. IgM antibodies binding self-antigens are affected by the same TLR1A region and ‘follow the same principles as NAb’.
12. Selective breeding for NAb revealed increased resistance and reduced morbidity to avian pathogenic *E. coli* (APEC) in the high NAb line. Interestingly, broilers with high levels of NAb binding KLH were more resistant then broilers with low levels of NAb binding KLH to APEC.
13. It was suggested in the PhD thesis from Tom Berghof that the full dominance of the TLR1A C variants maintained the CG variant and thus GG variants in the elite base population kept under very hygienic conditions. It is hypothesized that when the elite line is crossed as maternal line with the cock line that has no GG variants, therefore no negative effect on NAb levels in the commercial cross breeds are observed.

**Sue Lamont (Iowa):**

Genomics and immunology of host response to avian pathogenic *E. coli* (APEC) Our research on infection with avian pathogenic *E. coli* (APEC) has an overall objective to identify genes, signaling pathways and biological networks associated with infection and resistance to APEC in chickens. Our current research was supported by a now-ended US-UK Collaborative grant (funded by USDA and BBSRC). At the University of Edinburgh, Roslin Institute, collaborators are L. Vervelde, M. Stevens and P. Kaiser (deceased, original coPI).

We used RNA-sequencing (RNA-seq) to characterize transcriptome responses to APEC infection in the spleens from F1 progeny of reciprocal crosses between broiler (disease- susceptible) and Fayoumi (disease-resistant) chicken lines. Chicks were inoculated with APEC O1:K1:H7 or sterile PBS at 14 days of age and spleens were collected 1 or 2 days post infection (DPI) generating four treatment groups that differed by inoculation type and DPI of tissue harvest. Serial dilutions of individual spleen homogenates were plated to determine each bird’s bacterial load.

RNA-seq reads were generated on the HiSeq 3000 (n = 12 libraries/group) and mapped onto the chicken genome. The splenic transcriptome revealed innate immune pathways that could be potential targets to modulate resistance to APEC. Detection of allele- specific expression (ASE) in these RNA-seq datasets is ongoing and aims to identify *cis*- regulation of the responses to APEC.

Genomics of host response to Newcastle Disease virus (NDV) Our research on infection with Newcastle Disease Virus has an overall objective to determine the genetics and genomics of host response to NDV. This research is part of a USAID-funded “Feed the Future Innovation Lab for Genomics to Improve Poultry” (H. Zhou, PI) with the overall goal to improve disease resistance of local ecotypes of chickens in Africa. Studies conducted in Africa are reported in the UC-Davis reports.

To conduct studies in the US in well-controlled conditions and with genetic lines of relevance for the mid-size producer in Africa, we studied commercial layer chicks (Hy- Line Brown) produced from NDV-vaccinated dams. We investigated gene expression changes by RNA-seq at day 2 (dpi2) and (dpi6) 6 after infection with Newcastle disease virus LaSota strain. Comparing NDV challenged and nonchallenged groups, 313 differentially expressed genes (DEGs) were identified at 2dpi, but only 38 by 6dpi. IPA analysis of 2dpi DEGs predicted inhibition of multiple signaling pathways involved in cell proliferation and migration in NDV challenged chickens. When combined with previous genome-wide association study (GWAS) results on the same population, several significant GWAS SNPs were found within 500kb of seven DEGs, increasing confidence in the detected effects of these genes/regions. The identified pathways and DEGs provide potential targets for breeding for NDV resistant chickens.

**Yvonne Drechsler & Ellen Collisson (WesternU):**

Impact of MHC on innate immune responses to viral infection

## Drechsler: Resistance to respiratory pathogens, including coronavirus-induced infection and clinical illness in chickens has been correlated with the B (MHC) complex and differential *ex vivo* macrophage responses. The longterm goal is to evaluate the differences of B2 and B19 haplotype chickens in response to coronavirus infection on the epigenetic level in peripheral immune cells and target tissues. RNA seq and ATAC seq have been analyzed in SPF chickens. Tissue and viral strain specific epigenetic changes have been observed. B2 and B19 are now being provided by Dr. Robert Taylor from West Virginia University. Macrophages will be differentiated as previously done for RNA seq and WGBS, ATAC seq and ChiP seq will be performed to correlate with RNA seq data.

Collisson: A new type of vaccine is being tested for IBV using a peptide (S1) to the S1 region that is highly conserved and induces neutralizing antibody to IBV. The peptide has been incorporated into liposomes that also carry the adjuvant MPL (monophosphoryl lipid A) a Toll- like receptor agonist. Two doses of the liposomes with MPL and the S1 peptide were given to chicks. Sera collected after the second dose induced neutralization of IBV propagated in vitro. Birds challenged with the vaccine were also protected. However, birds given only the liposomes with the MPL were also protected when challenged within two weeks after vaccination. The potential for longer-term protection from the liposome-MPL-SE1 peptide are now being examined. In addition to these studies, tobacco mosaic virus (TMV) has been engineered to express the SE1 peptide of the S1 gene. Expression has been shown using dot blot assays. The TMV-S1 will also be used in in vivo studies. The TMV constructs were generated through a collaboration with Drs. Larry Grilll and Kelvin Phiri at the KECK institute in Claremont, CA. Vaccine will be eventually compared in birds with different haplotypes.

**Marcia Miller (City of Hope):**

Completion of the MHC-*Y* region genomic sequence study (Goto, Warden, Zhang, Wu, Kang, McPherson, Delany, Shiina, Hosomichi, Inoko, & Miller). This portion of our studies - to provide a detailed analysis of the sequence within the MHC-*Y* genomic region - continues to move forward. We are annotating and analyzing 649 kbp of the MHC-*Y* region. The sequence has many repetitive sequences of several types. CR1 (LINE-type retroelements) and LTR retroviral elements fill 43% of the sequence space within the determined sequence. Amid these repetitive elements are over 100 genes. With continued analysis in the past year, additional pseudogenes have been identified. The genes now include 49 class I-like genes (8 are pseudogenes) and 38 C-type lectin-like genes (17 are pseudogenes), 8 class IIβ (5 are pseudogenes , 9 LENG9 (5 are pseudogenes), and 5 zinc finger protein genes (1 pseudogene). We are working to gain insights into the relationship between how the genes and repetitive elements evolve within the region.

Evaluating the relationship between MHC-*Y* haplotypes and immune responses in experimental inbred and highly selected lines (Zhang, Goto, Taylor, Honaker, Siegel, and Miller). We have investigated the relationship between MHC-Y polymorphism and immune responses in two experimentally selected chicken populations. There is clear evidence that MHC-*Y* haplotypes have become fixed in the Virginia Tech pedigreed HAS and LAS lines during the selection for high and low antibody responses to immunization with an experimental antigen, sheep red blood cells. To evaluate whether this is likely to have occurred by chance, we typed birds in a second, similar experiment conducted at Wageningen University. In this population too, we found that MHC-*Y* haplotypes differentially distributed in the lines selected for high and low antibody responses. Finding similar results in these two independent experiments adds significantly to the likelihood that MHC-*Y* polymorphism influences immune responses.

Evaluating the relationship between MHC-*Y* haplotypes and colonization of chickens by *Campylobacter jejuni* (Zhang, Goto, Psifidi, Stevens, and Miller). We now have results from a small sample set indicating that MHC-*Y* haplotype likely contributes to the strong genetic influence governing colonization of chickens by *Campylobacter jejuni*. We are looking at this further in an advanced intercross population containing the same MHC-*Y* haplotypes.

**Rodrigo Gallardo (UC Davis):**

Cytokine responses in tracheas from MHC congenic chicken lines with distinct susceptibilities to infectious bronchitis virus. The chicken MHC B locus has been linked to disease resistance to infectious diseases. We have provided evidence that the congenic chicken lines 331/B2 and 335/B19 differ in susceptibility to infectious bronchitis virus (IBV) strains M41 and ArkDPI in *in vivo* challenge experiments. Innate immune responses can be difficult to measure *in vivo* since they are nonspecific and can be triggered by environmental factors. In an attempt to address this issue, we used tracheal organ cultures derived from 331/B2 and 335/B19 birds to study local cytokine production after *in vitro* challenge with IBV M41. IFN-β, IL-1β, IL-6 and IL-10 gene expression and production were assessed. Tracheal organ cultures derived from 335/B19 birds presented an increased inflammatory response compared to 331/B2. However, it was not possible to discriminate between cytokine responses in IBV-infected and PBS-treated tracheal organ cultures. Since tracheal processing entails physical damage to the trachea, it is possible that the tracheal organ cultures presented high levels of inflammation regardless of the IBV challenge. To demonstrate the effects of IBV in the MHC congenic chicken lines, we performed an additional *in vivo* experiment that focused on cytokine gene expression and production in tracheas up to 60 hours after a challenge with IBV M41. Our results corroborate previous *in vivo* observations that suggest that detrimental local inflammatory responses in 335/B19 birds might be associated with their susceptibility to IBV, and that inflammation does not necessarily lead to the assembly of an appropriate adaptive immune response.

Effects of Chicken MHC Haplotype on Resistance to Distantly-Related Infectious Bronchitis Viruses. The major histocompatibility complex (MHC) B locus of chickens has been associated with resistance to different viral diseases. We previously provided evidence that chicken lines expressing MHC haplotypes B2 and B19 exhibit different resistance to a challenge with infectious bronchitis virus (IBV) Massachusetts 41 (M41). In the current study, we attempted to determine if those differences were true for genetically diverse IB viruses, i.e., IBV M41 and Arkansas-Delmarva poultry industry (ArkDPI). Clinical, pathologic, molecular, and immunologic outcomes were compared. Our results showed subtle clinical and pathologic differences between the two MHC chicken lines tested. Clinical differences were observed in respiratory signs at 2 days postinfection (dpi) in M41-infected birds. Pathologic differences were detected in viral load at 2 dpi in M41-infected birds and in tracheal epithelial thickness at 6 dpi in ArkDPI- infected birds. Substantial differences were observed in antibody responses at 14 dpi. The transcriptome analysis showed that B19 chickens highly expressed genes related to inflammatory and innate immune responses. This increased immune gene expression detected in B19 birds at 6 dpi did not lead to enhanced antibody production at 14 dpi. On the other hand, B2-haplotype chickens highly expressed genes related to cell responses, suggesting that B2 is able to diligently control the infection. Although not identical, genes triggered by M41 and ArkDPI are part of communal pathways and suggest similar immune and cell responses to both IBV genotypes.

Intestinal tropism of an IBV isolate not explained by Spike protein binding specificity. An infectious bronchitis virus (CalEnt) with unusual enteric tropism was isolated from a California broiler flock exhibiting runting-stunting syndrome. IBV was detected in the small intestine, but not in respiratory tract or kidney. During virus isolation in embryos, it did not replicate in chorioallantoic membrane (CAM), but could be recovered from intestines. Its S1 protein showed 93% amino acid sequence identity to a California variant isolated in 1999 (Cal99). Intestinal lesions were reproduced following ocular/nasal inoculation of specific pathogen free (SPF) chickens, but respiratory signs and lesions were also present. The virus was detected in both respiratory and intestinal tissues. To determine whether the novel tropism of IBV CalEnt was due to an increased ability of its S1 protein to bind to the intestinal epithelium, we compared the binding of soluble trimeric recombinant S1 proteins derived from CalEnt and Cal99 to chicken tissues. Contrary to expectations, CalEnt S1 protein did not bind to small intestine and Cal99 S1, did not bind to the respiratory epithelium or CAM. Using only the CalEnt S1-N-terminal domain or including the S2-ectodomain (lacking membrane and cytoplasmic domains), which have shown improved ArkDPI S1 binding, did not lead to detectable binding at the standard protein concentration to any tissue tested. Our results indicate no/poor binding of the CalEnt spike protein to both respiratory and intestinal tissues and thus do not support better attachment to intestinal epithelial cells as a reason for CalEnt’s extended tropism. These results might reflect shortcomings of the assay, including that it does not detect potential contributions of the S1-C- terminal domain to attachment. We used bioinformatic approaches to explore the possibility that the unique tropism of CalEnt might be a result of functions of the S protein in cell entry steps subsequent to attachment. These analyses suggest that CalEnt’s S2-coding region was acquired through a recombination event and encodes a unique amino acid sequence at the putative recognition site for the protease that activates the S protein for fusion. Thus S2 activation by tissue-specific proteases might facilitate CalEnt entry into intestinal epithelial cells and compensate for poor binding by its S1 protein.

Partial Molecular Characterization and Pathogenicity Study of an Avian Reovirus Causing Tenosynovitis in Commercial Broilers This study focuses on the molecular characterization of avian reoviruses (ARVs) isolated during an outbreak in commercial chickens between 2015 and 2016. In addition, a pathogenicity study of a selected ARV strain isolated from a field case of viral tenosynovitis in commercial broiler chickens was performed. The investigated sequences were distributed in 5 distinct genotypic clusters (GCs), namely GC1, GC2, GC3, GC4 and GC6. SPF and commercial broiler chickens were challenged with the GC1 genetic variant MK247011, at 14 days of age via the interdigital toe web. No significant effects in body weight gain and feed conversion were detected in both chicken types. The Δ interdigital web thickness peak in severity was noticed 4 days post challenge (DPC) in both chicken types. The inflammation in SPF birds, was slightly higher compared with broilers. Neither mortality nor clinical signs occurred in the infected groups during the length of the experiment, despite the presence of significant microscopic lesions in challenged birds. Microscopic changes of tenosynovitis became evident at 3 DPC,with the highest incidence and severity detected at 14 and 21 DPC, respectively. Seroconversion against ARV occurred 3 weeks post challenge and the microscopic lesions detected in tendon and heart sections were highly compatible with those described in the field. Differences suggestive of significance were detected between tenosynovitis and epicarditis lesions in infected and control groups. While SPF and broiler chickens showed comparable responses to the challenge with an ARV genetic variant, detected lesions were subclinical denoting the limitations of our challenge approach. The age selected in this experiment, possibly influenced the course of the infection.

Genotypic Characterization of Emerging Avian Reovirus Genetic Variants in California This study focuses on virus isolation of avian reoviruses from a tenosynovitis outbreak between September 2015 and June 2018, the molecular characterization of selected isolates based on partial S1 gene sequences, and the full genome characterization of seven isolates. A total of 265 reoviruses were detected and isolated, 83.3% from tendons and joints, 12.3% from the heart and 3.7% from intestines. Eighty five out of the 150 (56.6%) selected viruses for sequencing and characterization were successfully detected, amplified and sequenced. The characterized reoviruses grouped in six distinct genotypic clusters (GC1 to GC6). The most represented clusters were GC1 (51.8%) and GC6 (24.7%), followed by GC2 (12.9%) and GC4 (7.2%), and less frequent GC5 (2.4%) and GC3 (1.2%). A shift on cluster representation throughout time occurred. A reduction of GC1 and an increase of GC6 classified strains was noticed. The highest homologies to S1133 reovirus strain were detected in GC1 (~77%) while GC2 to GC6 homologies ranged between 58.5 and 54.1 %. Over time these homologies have been maintained. Seven selected isolates were full genome sequenced. Results indicated that the L3, S1 and M2 genes, coding for proteins located in the virus capsid accounted for most of the variability of these viruses.

*Objective 2. Identify and characterize environmental, dietary and physiological factors that modulate immune system development, optimal immune function and immune system related disease resistance and welfare in poultry genetic stocks.*

**Ryan Arsenault (Delaware):**

CpG Administration and the development of the broiler immune response Modern broiler breeds selected for rapid growth are more susceptible to pathogen challenge than older genetic lines, such as heritage chickens, that have not been selectively bred for a specific trait since 1957. We hypothesize this is due to an inappropriate immune response in modern strains when stimulated. To test this, we injected modern broiler and heritage chickens, Athens-Canadian Random Breds (ACRB), intra-abdominally with a known immunostimulatory agent to observe immune response differences between the two bird breeds. Birds were injected at multiple time points throughout the grow-out period with 25 µg of either CpG or a GpC control, and tissue samples were collected from each group prior to and 24 hours post-injections. Kinase activity in the tissues was assessed using a species-specific kinome peptide array to measure degree of phosphorylation of target peptides in key immunometabolic signaling pathways, which was then visualized by staining the array with phosphoprotein-specific fluorescent dye so that fluorescence could be quantified. The raw fluorescence data were collected and normalized using variance stabilizing normalization in the vsn package in R, and normalized fluorescence in the treatment birds’ tissues was compared to control birds’ tissues using a paired t-test to generate a fold-change value and p-value. Normalized fluorescence fold changes with p-values <0.05 are considered either significantly more or significantly less phosphorylated in the treatment birds compared to the control birds’ tissues. Five birds in each treatment group were weighed, sacrificed and tissue samples were snap frozen or stored in RNAlater. We analyzed immunometabolic signaling changes in cecal tonsil and jejunal tissue samples on days 3, 15, and 34 post hatch. We performed qRT-PCR with RNA extracted from the same cecal tonsil and jejunum samples and primers for key cytokines: IL-1beta, IFN-gamma, IL-6, and IL-18. The modern birds treated with CpG had significantly lower body weights than control birds on days 18, 21, and 29 post-hatch (p values = 0.02, 0.02, and 0.02 respectively), whereas this difference was not seen in ACRB birds. In the CpG treated modern birds on Day 15 there is a decline in activity in the cecal tonsil in an important immunometabolic pathway, PI3K-Akt, when compared to control birds, and this is not seen in the ACRB birds. In the ACRB birds’ cecal tonsils on day 15 there is NF-kB activation and insulin signaling activation. In the modern birds, insulin signaling is deactivated and there are indicators of oxidative stress leading to apoptosis. The qRT-PCR results showed an initial elevation of expression of all four cytokines in the cecal tonsils and jejunums of both modern and ACRB birds at day 3 in response to the CpG treatment. These results show a decrease in expression of IL-6 and IFN-gamma in the treated modern birds’ cecal tonsils on day 15. Targeting intermediates of the insulin sensitivity and mitigating oxidative stress pathways could be potential targets for therapeutic intervention in the modern birds to bolster their immune responses as they age. Ideally, this would maintain the performance observed in modern broiler chickens without sacrificing immune robustness.

Next we wanted to observe how this pattern may change when birds are treated multiple times with CpG. In the heritage birds, the significantly differentially phosphorylated peptides unique to post-injection (day 16) compared to pre-injection (day 15) indicate increased T cell receptor signaling and activation. Specifically, key proteins such as ZAP70, NFATC1, PAK2 were unique to this time point and indicated T cell activation. In the modern birds, there was inhibition of fatty acid synthesis as well as destabilization of PGC-1α indicating a lack of fatty oxidation unique to the modern birds’ cecal tonsils post-injection. 4E-BP1 was uninhibited in the modern birds post- injection, which leads to suppression of protein synthesis; this suppression was also seen in inhibited PI3KCD signaling. Due to CpG treatment the heritage birds exhibited adaptive immune activation, whereas we observed more metabolic changes in the modern bird.

Postbiotic Immunomodulation and *Clostridium perfringens* Challenge With the reemergence of poultry diseases such as necrotic enteritis following the restriction of in- feed antibiotics, the search for antibiotic alternatives has become critically important. Postbiotics are non-viable bacterial products or metabolic byproducts from probiotic microorganisms that have positive effects on the host or microbiota. These are a promising alternative to antibiotics. We endeavored to describe the mechanism of action of a postbiotic in the context of a *Clostridium perfringens* (*C. perfringens*) challenge model. By using performance measurements and a peptide array kinome analysis, we describe the kinotypes and signal transduction changes elicited by the postbiotic with and without *C. perfringens* challenge. The postbiotic improves lesion scores, *C. perfringens* counts and mortality compared to challenge groups without the postbiotic, and it improves weight gain in the most severely challenged birds. The postbiotic predominantly affects the innate immune response and appears immunomodulatory. In the context of infection, it reduces the proinflammatory responses and generates a homeostatic-like response. We determined this postbiotic is a viable alternative to antibiotics to improve poultry health in the context of *C. perfringens* pathogen challenge.

Calmodulin signaling and *Salmonella* Calcium (Ca2+) is a pivotal intracellular second messenger and calmodulin (CaM) acts as a multifunctional Ca2+-binding protein that regulates downstream Ca2+ dependent signaling. Together they play an important role in regulating various cellular functions, including gene expression, maturation of phagolysosome, apoptosis, and immune response. Intracellular Ca2+ has been shown to play a critical role in Toll-like receptor-mediated immune response to microbial agonists in the HD11 chicken macrophage cell line. The role of that the Ca2+/CaM pathway plays in the intracellular survival of Salmonella in chicken macrophages has not been reported. In our study, kinome peptide array analysis indicated that the Ca2+/CaM pathway was significantly activated when chicken macrophage HD11 cells were infected with *S.* Enteritidis or *S.* Heidelberg. Further study demonstrated that treating cells with a pharmaceutical CaM inhibitor W-7, which disrupts the formation of Ca2+/CaM, significantly inhibited macrophages to produce nitric oxide and weaken the control of intracellular Salmonella replication. These results strongly indicate that CaM plays an important role in the innate immune response of chicken macrophages and that the Ca2+/CaM mediated signaling pathway is critically involved in the host cell response to *Salmonella* infection.

Development of a kinome tool to study waterfowl Our lab designed and developed an *Anas*‐specific kinome peptide array that can be used to study the immunometabolic responses of mallard and American black duck to pathogens, contaminants, and environmental stress. The peptide arrays contain 2,642 unique phosphorylate‐ able peptide sequences representing 1,900 proteins. These proteins cover a wide array of metabolic and immunological processes, and 758 Gene Ontology Biological processes are statistically significantly represented on the duck peptide array of those 164 contain the term “metabolic” and 25 “immune.” In addition, we conducted a comparison of mallard to American black duck at a genetic and proteomic level. Our results show a significant genomic and proteomic overlap between these two duck species, so that we have designed a cross‐reactive peptide array capable of studying both species. This is the first reported development of a wildlife species‐specific kinome peptide array.

**Lisa Bielke (Ohio State):**

Impact of *in ovo* administered pioneer colonizers on intestinal proteome in day

of hatch chicksPioneer colonization of the gastrointestinal tract (GIT) by bacteria is thought to have major influence on neonatal tissue development. Previous studies have shown *in ovo* inoculation of embryos with saline (S), species of *Citrobacter* (C, C2), or lactic-acid bacteria (LAB) resulted in an altered microbiome on day of hatch (DOH). The current study investigated GIT proteomic changes at DOH in relation to different inoculations. Embryos were *in ovo* inoculated with S, or ~102 CFU of C, C2 or LAB at 18 embryonic days. On DOH, GIT was collected, and tissue protein extracted for analysis via tandem mass spectrometry. A total of 493 proteins were identified for differential comparison to S at p ≤ 0.10. Different levels were noted in 107, 39, and 78 proteins in C, C2, and LAB groups, respectively, which were uploaded to Ingenuity Pathway Analyses to determine canonical pathways and biological functions related to these changes. Three members of the cytokine family (IL1β, IL6, and OSM) were predicted to be activated in C2, indicated with Z-score ≥ 1.500, suggested an overall pro-inflammatory GIT condition. This was consistent with the activation of the acute phase response signaling pathway exclusively in C2 (Z-score = 2.000, p < 0.001). However, activation (Z-score = 2.000) of IL-13, up-regulation of PRDX1, SOD1 in addition to the activation of nitric oxide signaling in the cardiovascular system in LAB treated groups may predict a state of increased antioxidant capacity and decreased inflammatory status. The NRF2-mediated oxidative stress response (Z-score = 2.000, p < 0.001) was predicted to be up-regulated in C which suggested that chicks had a dys-regulated inflammatory state and associated oxidative stress, but the impact of these pathways differed from C2. These changes in the proteome suggest that pioneer colonizing microbiota may have a strong impact on pathways associated with GIT immune and cellular development.

A proteomic view of the cross-talk between early intestinal microbiota and poultry immune systemProteomics has been used for investigating cross-talk between the intestinal microbiome and host biological processes. In this study, an *in ovo* technique and proteomics approach was used to address how early bacterial colonization in the gastrointestinal tract (GIT) could modulate inflammatory and immune responses in young broilers. Embryos at 18 embryogenic days were inoculated with saline (S), 102 CFU of *Citrobacter freundii* (CF), *Citrobacter* species (C2), or lactic acid bacteria (L) into the amnion. At 10 d post-hatch, ileum samples from 12 birds per treatment were selected for tandem mass spectrometry analysis. Our further findings indicated that treatment-specific influences on early GIT microbiota resulted in different immune responses in mature broilers. Predicted functional analyses revealed activation of inflammation pathways in broilers treated *in ovo* with L and CF. Exposure to L enhanced functional annotation related to activation, trafficking of immune cells, and skeletal growth based-network, while CF inhibited biological functions associated with immune cell migration and inflammatory response. These results highlighted that proper immune function was dependent on specific GIT microbiota profiles, in which early-life exposure to L-based probiotic may have modulated the immune functions, whereas neonatal colonization of *Enterobacteriaceae* strains may have led to immune dysregulation associated with chronic inflammation.

Intestinal pioneer colonizers as drivers of ileal microbial composition and diversity of broiler chickensGiven that recent advances in metagenomics have highlighted the importance of intestinal microbes for poultry health, there has been a corresponding search for early manipulation strategies of intestinal microbiota in order to advance immune system development and optimize functional properties of growth. In this study, we used the *in ovo* technique as an experimental model to address how early bacterial intestinal colonization could affect the development and establishment of the mature ileal microbiota. Inoculations containing one of the following: 0.2 ml of 0.9% sterile saline (S), approximately 102 cells of *Citrobacter freundii* (CF), *Citrobacter* species (C2) or lactic acid bacteria (L) were administered via *in ovo* into the amnion. Results showed that *Enterobacteriaceae* abundance was negatively correlated with aging, although its high population at day of hatch affected the microbiota composition, delaying mature microbiota establishment. L treatment increased colonization of butyrate-producing bacteria by three and 10 days, and segmented filamentous bacteria in the lower ileum by 10 days. On the other hand, L probiotic decreased the population of *Enterococcaceae.* In addition, L and C2 microbial communities were less diverse at 10 days than at three days in the upper ileum. Importantly, these findings provide a valuable resource for a potential study model for interactions between microbial colonization and associated immune responses. In conclusion, our analysis demonstrates that intestinal pioneer colonizers play a critical role in driving the course of microbial community composition and diversity over time, in which early-life exposure to L based probiotic supported selection alongside greater colonization of symbiotic populations in the ileum of young broilers.

**Gisela Erf (Arkansas):**

AR Research on autoimmune vitiligo in the Smyth chicken model focused on treatment application to the target tissue, the growing feather (GF) pulp and studies on mechanism of breaking/reestablishing immunological tolerance.

Efforts are also underway at AR to characterize cellular and molecular mechanisms in the autoimmune pathology of autoimmune scleroderma/systemic sclerosis and Hashimoto’s thyroiditis in UCD200/206 and OS chickens, respectively. Using the novel GF cutaneous test in conjunction with blood sampling,

AR examined the effects of dietary trace mineral supplementation on the acute phase of the LPS-induced inflammatory response in broilers simultaneously at the local injection site (GF pulp) and systemic level (blood) in the same individuals. This study established this dual approach as an important tool to investigate effect of dietary treatments on both humoral and cellular immune system activities in vivo.

**Robert Beckstead (NC State):**

Effect of *Histomonas* *meleagridis* on broiler breeder egg production and quality. The objective of this study was to determine if inoculation with *Histomonas meleagridis* would alter broiler breeder hens’ egg production and egg quality. Previously, we reported that inoculating broiler breeder hens with *H. meleagridis* caused limited morbidity and no mortality. The onset of lay was also not altered due to infection when compared to the control flock. To test whether infection with *H. meleagridis* would alter broiler breeder egg production or quality, 520 breeder hens were transferred from a rearing to a production house at 21 weeks of age. Control and *H. meleagridis* infected hens were split into four pens with 56 hens per pen. Based on sampling the control birds were negative for the parasite and the infected hens were still positive for the parasite. Both flocks started laying at 24 weeks where egg collection occurred twice daily. Feed intake and egg production were analyzed per period (28 days) with 10 periods in this trial. Six eggs per pen were measured for: shell strength, shell thickness, egg weight, Haugh Units and vitelline membrane on weeks 30, 34, 39, 42 and 56. One hundred and eighty eggs per pen were analyzed for hatchability on weeks 27, 37 and 60 while fertility was analyzed on weeks 27 and 37. At 64 weeks, individual bird weights were recorded and cecal samples collected to determine presence of *H. meleagridis*. Differences between treatments were analyzed in JMP Pro 14 using a T-test (P≤ .05). The number of eggs produced per hen differed significantly during one period (p=0.021), where the infected birds produced more eggs per hen (17 vs 13 eggs) and had a lower FCR (3.12 vs 3.47) (p=0.0347). On week 30, the infected birds produced heavier eggs (59 vs 57 g) (p=0.04) and on week 42 they produced eggs with a thicker shell (0.35 mm vs 0.34 mm) (p=0.0305). Hatchability and fertility did not differ between treatments. At 64 weeks, the inoculated birds weighed significantly less than the control (4.16 vs 4.29 kg) (p=0.0016) and H*. meleagridis* was observed in the cecal samples analyzed via light microscopy. This study indicates that *H. meleagridis* has limited effects on broiler breeder egg production and quality. Recovery of the parasite a year after infection infers the broiler breeder as an ideal carrier of *H. meleagridis*.

Characterization of growth performance changes and lesion development in commercial toms infected with intestinal parasite *Tetratrichomonas gallinarum. Tetratrichomonas gallinarum* is an intestinal parasite commonly found in commercial poultry. While prevalent in the turkey industry, its virulence has yet to be determine. Clinical signs attributed to *T. gallinarum*, which include lesion formation in the ceca and liver of birds. However, the validity of these reports has been disputed due to diseased birds also being positive for *Histomonas meleagridis*. The purpose of this study was to determine if *T. gallinarum* field isolates caused lesions in the birds and as a result affected the growth performance of turkeys. Two separate trials were conducted to test the effects of different field isolates of *T. gallinarum* on growth performance in commercial toms. Weekly BW, BWG, and FCR data were collected for both trials and lesion scoring was conducted. The first trial demonstrated no statistical differences (p<0.05) in growth performance parameters or lesion formation between infected and uninfected groups. We did observe that infected turkeys had lower BW and BWG. To determine if this was relevant, a second trial was conducted with an increase in sample size to improve statistical power and two different *T. gallinarum* isolates were used. In addition, a decreased threonine diet was used to add nutritional stress on the birds. Birds fed the decreased threonine diet consistently had lower BW and BWG, but there was not an interaction effect of decreased threonine and infection, regardless of field isolate type used. Differences in BWG, feed intake, and FCR due to infection were observed during weeks 3-5, but there were significant changes in cumulative data. Similar to the first experiment, there was not a consistent effect of isolate type on production parameters or lesion development. The results from these bird trials suggest that *T. gallinarum* has limited pathology. It is possible that under alternative conditions, such as a co-infection with a secondary microorganism, *T. gallinarum* may exacerbate enteritis in turkeys.

Genetic screen for blackhead (histomoniasis) resistant turkeys. Turkeys are more susceptible than chickens to a blackhead outbreak, with mortality reaching up to 100%. High mortality in turkeys is thought to be the result of the turkey’s failure to mount an effective immune response. Some outbreaks in turkeys result in low level of mortality, suggesting that the field isolate of *H. meleagridis* may be less virulent or the strain of turkeys may be able to mount an immune response. We hypothesize that some turkeys may possess the genetic makeup to mount an effective immune response to *H. meleagridis* and thus be resistant to the disease. Last year we carried out a genetics screen with 4,000 parental line turkeys provided by a breeding company. That screen identified 5.1% of the birds had no lesions on day 80 after being infected with *H. meleagridis* on days 17, 38 and 59. A similar genetic screen was carried with a second company looking at 2,000 parental line turkeys. In this screen birds were inoculated 4 times and the trial was terminated on day 86. Similar to the first experiment, 3.75 of the turkeys had no signs of blackhead disease. Ceca from surviving birds with no signs of disease were sampled for the presence of H. meleagridis. Based on culture analysis, 80% of the turkeys with no signs of disease had live histomonads in their ceca, suggesting that like chickens, they had mounted an immune response to the parasite. ELISA assays will be run on the blood collected from these birds and will be compared to turkeys not infected with this parasite to confirm the hypothesis. SNP analysis and GWAS studies are ongoing for both genetic screens.

**Ramesh Selvaraj (Georgia):**

Experiments were conducted to study Regulatory T cell (Treg; CD4+CD25+) properties during *Salmonella* *enterica* serovar Heidelberg infection in broiler chickens. Day-old broiler chicks were orally gavaged with 5x106 CFU/mL *S.* *enterica* serovar Heidelberg or sterile PBS (control). Samples were collected at 4, 7, 10, and 14 d post infection. There was a significant (P < 0.05) increase in the number of CD4+CD25+ cells by day 7 post infection that increased steadily throughout the course of the 14 days of infection, whereas the number of CD4+CD25+ cells in the non-infected controls remained steady throughout the study. CD4+CD25+ cells from cecal tonsils of *S.* Heidelberg-infected birds had a higher (P < 0.05) IL-10 mRNA content than CD4+CD25+cells from the non-infected controls at all-time points studied. At a lower effector/responder cell ratio of 0.25:1, CD4+CD25+cells from cecal tonsils of *Salmonella*-infected birds suppressed T cell proliferation at days 14 post *S.* Heidelberg infection, while CD4+CD25+ cells from non-infected control groups did not suppress T cell proliferation. In conclusion, a persistent intestinal *S.* Heidelberg infection increased the Treg percentage, suppressive properties, and IL-10 mRNA amounts in the cecal tonsils of broiler birds.

**Henk Parmentier (Wageningen):**

Our studies focus on the following topics:

1. Effects of husbandry (hygienic conditions, organic feed, housing, e.g. battery cage versus free range- like) on immune responsiveness of chickens and chicken breeds.
2. Immuno-modulation of the immune response, especially via the innate immune system, with special emphasis on *Natural* antibodies, probiotics and *PAMP’s*.
3. *Natural* antibodies and *natural auto*-antibodies in chickens (and other species: bovine and pig).
4. Divergent selection of layers to KLH, see above

# Immunity and behavior

1. Immuno-development
2. Transgenerational priming of innate immunity
   * 1. Immunity and natural (auto-) antibodies (H.K. Parmentier, M. Bao, H. Bovenhuis, J. vander Poel) Chickens, like mammals have ‘natural auto-antibodies’ which may be directed to neo-epitopes. These NAAb at 16 wks of age show heritabilities alike the heritability of natural antibodies binding KLH. IgG and IgM auto-antibody profiles were also studied in plasma from adult hens, their eggs and their hatchlings from the KLH-NAb selection lines using ELISA and Western blotting. IgG NAAb were found in hens, and yolk, and hatchings, with High line birds have higher levels as compared to the other compartments. No transfer of IgM NAAb was observed, but IgG NAAb appeared to be transferred from mother to hatchling via the yolk. (NAb and NAAb profiles were also studied for other species: bovine and pig (Parmentier)).

An important target for NAAb in many mammals are phosphoryl (PC-)-protein conjugates originating from membranes from dying or apoptotic cells. Epidemiological studies in bovines revealed that levels of anti-PC antibodies predicted subsequent (metabolic and inflammatory) disorders. The presence of such antibodies in chickens and their involvement in chicken diseases is topic of future studies in cooperation with Mieke Matthijs who studies resistance to infections in broiler breeds.

Divergent selection of layers to KLH Nabs: effect on disease resistance (T. Berghof, M. Matthijs, M. Visker, H.K. Parmentier, H. Bovenhuis, and J. vander Poel) Collaboration with prof. Jos van Putten and Carlos Voogdt (Veterinary Medicine, Utrecht University) was initiated to further investigate the genetic variant in *TLR1A* that showed highly significant association in the GWAS. The aim was to express ‘our’ *TLR1A* variants in their *in vitro* system based on human cell lines and test dose-responses of different ligands, in order to unravel the mode of action of ‘our’ SNP. The sequence of the *TLR1A* gene and relevant haplotypes in the High and Low NAb line hens were established. DNA of the most frequent haplotype comprising the *TLR1A* C-allele and of the most frequent haplotype comprising the *TLR1A* G-allele is currently being used in Utrecht to build constructs for testing in the *in vitro* system. A new project on the resistance of elite layer bird lines to *E. coli* infections was rejected.

Immunity and behavior (Aart Lammers and Jerine van Eijck). Selected layer lines with higher levels of aggressive behavior showed higher levels of anti-KLH IgG NAb, whereas higher levels of IgM NAb were found in birds with less aggressive behavior.

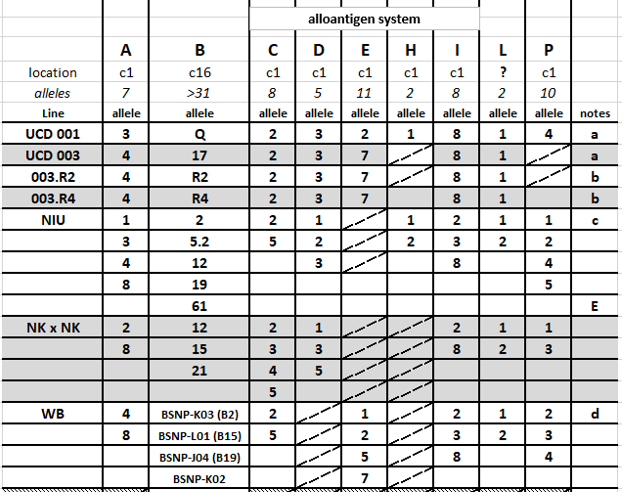
Transgenerational epigenesis (M. Verwoolde, A. Lammers and H.K. Parmentier) *In vitro* stimulation assays with PAMP’s such as β-glucans and LPS using HD11 cells and primary phagocytes from various tissues including bone marrow revealed ‘innate training’ as indicated by NO production and cytokine PCR after second challenges with PAMP’s *in vitro*. This will be studied also *in vivo* using dietary treatments and finally over generations. Lines selected for behavior not only differed in NAb levels, but innate training of their macrophages was also different.

*Objective 3. Develop, evaluate and characterize methodologies, reagents and genotypes to assess immune function and disease resistance to enhance production efficiency through genetic selection in poultry.*

**Robert Taylor Jr (West Virginia):**

1. **WVU research antisera (**Taylor, Kopulos, Yates). Northern Illinois University (NIU) transferred selected alloantisera produced by Dr. W. E. Briles West Virginia University (WVU). These antisera are available for collaborative studies.
2. **WVU genetic stocks (**Taylor, Kopulos, Delany, Ashwell). WVU research stocks are listed in **Table 2.**  University of California-Davis inbred lines UCD 001 (Jungle fowl, reference sequence) and UCD 003 (white Leghorn) were sent by Mary Delany. MHC recombinants in congenic lines developed at the University of New Hampshire were provided by Chris Ashwell. A 225 bp insert in the 3’ UTR of *BG1* present in Line 003.R4 but not in Line 003.R2 associates with lower responses against Rous sarcoma virus (RSV) and Marek’s disease (MD). Line crosses are shown in **Table 3**.

A cross of the synthetic Whiting Blue line [blue egg gene (*O*-)] and NIU stock segregated for the following MHC haplotypes: *BSNP-K03 (B2),* *B5.2,* and *BSNP-J04 (B19).* This stock, now designated WVU 1952, has other alloantigen systems segregating. Two rare breeds, Golden Sebrights and Lakenvelders are no longer held at WVU. Despite at least one unique MHC haplotype in each breed, the challenge of reproducing the lines was too great.

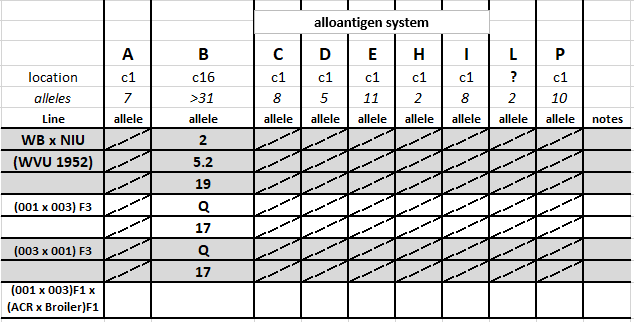
**Table 2**. Alloantigen alleles found in West Virginia University (WVU) genetic stocks.

**NIU** = Northern Illinois University **b** = non-B types inferred from 10 BC to UCD 003

**WB** = Whiting blue **c** = alloantisera typing

**NK x NK** = Hy-Line International **d** = MHC SNP type (Fulton *et al*., 2016)

**a** = antisera typing (Briles *et al*., 1979) **E** = lost haplotype or line

**Table 3**. Line crosses held at West Virginia University.

Northern Illinois University (NIU) transferred selected alloantisera produced by Dr. W. E. Briles. West Virginia University(WVU). These antisera are available for collaborative studies.

Northern Illinois University (NIU) and UC Davis lines have been transferred to West Virginia University.

**Mark Parcells:**

1. Cloning and Expression of Chicken Innate Sensors, Signaling Molecules and Meq-interacting Proteins. These are available to NE-1334 members, upon request. These are useful for protein-protein interaction, co-localization and mobility studies. Additions this year include the cytokines IL-4 and GM-CSF.

2. Modeling of MDV Evolution of Virulence. Develop models for MDV lytic infection, latency establishment and reactivation, and transformation.

**Matt Koci (NC State):**

In collaboration with: H. M. Hassan, J. N. Petitte, R. Barrangou

Understanding the molecular mechanisms by which the avian immune system recognizes, and controls different bacteria is critical to our ability to eliminate bacteria that pose a risk to poultry health and/or cause foodborne disease. Two of the most important ways the innate immune controls bacteria is through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS is primarily made by macrophages and heterophils by the NADPH oxidase enzyme NOX2, while the majority of RNS is produced by enzyme inducible nitric oxide synthase (iNOS). Studies using iNOS and/or phox null mice have demonstrated NO and ROS play major roles in limiting Salmonella’s ability to replicate inside macrophages in vitro, and the absence of these genes significantly increases susceptibility to Salmonella-induced death as compared to wild type mice.

Given the overall importance of these enzymes in the innate immune response in general, and their known association controlling Salmonella infections in mice, it is important to characterize the role these enzymes play in the regulation of Salmonella colonization in poultry. To do this we have begun to develop and characterize a panel of chicken macrophage cell lines that have been CRISPR genome edited to be functionally deficient for one or both of these genes. To this point we have developed 4 novel cell lines (XHD4, XHD7, XHD9, and XHD22) that have been sequence confirmed as having homozygous deletions in the NOX2 gene, and to be functionally incapable of producing ROS through this pathway. These cell lines were also screened for potential off-target genome mutations at 15 loci, however, no mutations were identified beyond those in NOX2 gene.

**Gisela Erf (Arkansas):**

Rescue, establishment and characterization of UCD200/206 and Obese-strain chicken populations at the University of Arkansas, Division of Agriculture, Fayetteville, AR. Avian autoimmune disease models such as the Smyth-, University of Davis (UCD) 200/206-, and the Obese Strain (OS)-lines of chicken have contributed much insight into the multifactorial non-communicable diseases in poultry as well as provided new insights into aberrant and normal functions of the avian immune system. The systemic sclerosis/scleroderma-like disease in UCD200/206 chickens exhibits all the hallmarks of the human disorder, including vascular pathology which is first evident at 1-2 weeks of age in the form of erythema, edema, necrosis and loss of the comb (self-dubbing) similar to Raynaud’s syndrome in humans. Skin lesions on the neck, legs and feet, as well as swollen joints and joint stiffness are also observed. Antinuclear antibodies, endothelial cell apoptosis, and mononuclear cell infiltration have been shown to precede the fibrosis of skin and internal organs in these animals. The spontaneously developing Hashimoto’s thyroiditis-like hypothyroidism in OS chickens is due to autoimmune destruction of thyroid tissue. Visual characteristics of disease expression include reduced body size, short shanks, small combs, hair-like feathers, and plump body shape due to excessive fat deposition. Development of the disorder is associated with mononuclear cell infiltration in thyroids, autoantibodies in the blood, thyroid hormone deficiency, and lipidemia.

Our efforts in establishing breeding populations of both UCD and OS chickens over the past 3 years have been very successful. The AR-UCD and OS lines exhibit the phenotypic and immunopathologic traits of their respective autoimmune diseases. Dr. Anthony’s graduate student Joseph Hiltz has established a pedigree breeding and selection program, MHC-typed all birds with the help of Dr. Janet Fulton and has made non-invasive measurements and visual assessment regarding the characteristics of both the OS and UCD lines. Husbandry protocols also have been optimized, which was particularly critical for the OS line. To the best of our knowledge, the AR lines are the only live populations of the autoimmune disease prone UCD and OS (and SL) chickens in the World. Collectively these lines provide excellent opportunity to study spontaneously occurring multifactorial, non-communicable disease, abnormal immune system development and function, immunopathology, loss or re-establishment of tolerance, mechanisms underlying fibrosis, etc., as well as epigenetic regulation and the role of the microbiome in health and disease. These lines will be maintained and available for collaborative studies for the foreseeable future.

Tools to monitor and assess in vivo cellular/tissue responses: the growing feather as an “in vivo test-tube”. We continue our research on developing the growing feather as an “in-vivo test-tube” and window into cutaneous in vivo immune activities to intradermally injected test-materials (Erf and Ramachandran, 2016). Special focus is on refining the method and demonstrating its uses. Included are tissue/cellular innate immune response activities, demonstration of primary and secondary effector and recall responses, simultaneous assessment of tissue/cellular responses to several test-materials in the same individual, within bird variation of responses induced in individual GF, as well as examination of effects of dietary or systemically applied treatments on immune system activities initiated in the growing feather pulp.

**Ramesh Selvaraj (Georgia):**

a. *In* *vitro* and *in* *vivo* experiments were conducted to study the effects of synbiotic supplementation on *Salmonella* *enterica* ser. Enteritidis (SE) proliferation, cecal content load, and broiler carcass contamination. *Lactobacillus* *reuteri*, *Enterococcus* *faecium*, *Bifidobacterium* *animalis*, and *Pediococcus* *acidilactici* culture supernatants decreased (P < 0.05) the *in* *vitro* proliferation of SE at 1:1 supernatant: pathogen dilution. A total of 240 Cobb-500 broiler chicks were randomly allotted to three treatment groups (8 replicates/group with 10 birds/replicate): control (basal diet), antibiotic (Virginiamycin at 20 mg/kg feed), synbiotic (PoultryStar® ME at 0.5 g/kg feed containing *L. reuteri, E. faecium, B. animalis, P. acidilactici* and a Fructooligosaccharide) from day of hatch. At 21 d of age, all birds in experimental groups were orally inoculated with 250 µl of 1 X 109 CFU SE. Antibiotic supplementation increased (P < 0.05) body weight and feed consumption, compared to the control group. Birds in the synbiotic supplementation had intermediate body weight and feed consumption that were not significantly different from both the control and antibiotic group at 42 d of age in SE infected birds. No significant effects were observed in feed efficiency at 42 d of age among the groups. Antibiotic and synbiotic supplementation decreased (P < 0.05) SE load in cecal contents by 0.90 and 0.85 log units/ g and carcass SE load by 1.4 and 1.5 log units/mL of rinsate compared to the control group at 42 d of age (21 dpi). The relative abundance of IL-10, IL-1, TLR-4, and IFNγ mRNA was decreased (P < 0.05) in the antibiotic and synbiotic supplementation groups compared to the control birds at 42 d of age (21 dpi). It can be concluded that synbiotic supplementation decreased SE proliferation in vitro and decreased SE load in the cecal contents and broiler carcass.

b. The continuing circulation of multiple serovars of *Salmonella* in poultry flocks, along with increasing reports of human Salmonellosis, warrants the necessity of developing control methods to decrease *Salmonella* load in poultry production. *Bacillus Subtilis* and *Bacillus licheniformis* are potential probiotics and are currently used as a probiotic in poultry production. The overall objective in this proposal is to determine the effects of *B. subtilis* and *B. licheniformis* probiotic supplementation on performance, cecal *Salmonella* load, and *Salmonella* contamination in meat of broilers challenged with *Salmonella enterica serovar Enteritidis*. A total of 360 one-day-old broiler chicks were randomly distributed to four experimental groups a 2 X 2 factorial set up of treatments; Control, Control + Challenge, Probiotics (10 mg/Kg feed of *B. subtilis* strain HU58; HU58TM plus 100 mg/kg feed of *B. licheniformis* SC307*;* PreproTM; Microbiome LABS, Saint Augustine, FL), and Probiotics + Challenge. Each treatment was replicated in 6 pens (n=6) with 15 chicks per pen. At 21 d of age, all birds in Challenge groups were inoculated orally with 250 µl of 1 X 109 CFU *S. enteritidis*. At 28 d of age, birds challenged with *Salmonella* had the 11% decreaed (P < 0.05) body weight gain compared to the control groups. Birds that were supplemented with probiotics in the salmonella challenged groups had only 5.1% decrease in body weight compared to the control group. Birds challenged with *Salmonella* had 1.99, 1.93 and 1.71 log *Salmonella* CFU/g of cecal contents. Birds supplemented with probiotics and challenged with salmonella had 0.73, 1.59, and 1.32 log decreased *Salmonella* CFU/g of cecal contents at 5, 12, and 21 d post-*Salmonella* infection. Birds challenged with *Salmonella* had higher (P < 0.05) titers of anti-*Salmonella* IgA in the bile. Birds supplemented with probiotics and challenged with *Salmonella* had further increased (P < 0.05) anti-*Salmonella* titers compared to the birds with *Salmonella* infection. At 21d post-salmonella infection, birds challenged with *Salmonella* had decreased (P < 0.05) villi height compared to the control groups. Birds supplemented with probiotics had the longest villi height. Birds supplemented with probiotics and challenged with *Salmonella* had comparable villi height compared to the control group. Increased villi height and crypt depth can improve nutrient digestibility and absorption and explain the improved production performances in probiotic supplemented birds. It can be concluded that *B. subtilis* probiotic can be a tool in decreasing *Salmonella* loads in broiler intestine and *B. subtilis* supplementation can be expected to decrease broiler carcass contamination with *Salmonella*.

**Sue Lamont (Iowa):**

Genetic lines and transcriptome response to inflammatory stimulus and heat stress In a now-ended USDA-AFRI Climate Change project (with C. Schmidt, UDEL), we investigated the interaction of two stressors: heat stress and exposure to an inflammation- inducing PAMP (bacterial lipopolysaccharide, LPS). Birds of two distinct and highly inbred lines (broiler, Fayoumi) that were either exposed to daily cyclic heat episodes or kept at control temperatures were injected with either LPS or saline. Bioinformatic analyses of RNA-seq generated from tissues from birds of each of the four treatment groups was conducted to identify genes and pathways associated with response to the stressors. In the past year, a manuscript on the thymus transcriptome response was drafted, submitted and accepted for publication.

Genetics and function of host defense peptides in chickens To characterize the impact of host genetics on the expression and function of host- defense peptide (HDP) genes in the chicken, we studied the expression levels over time of HDP genes in fibroblasts and in bone-marrow derived cells from Fayoumi and Leghorn lines, after exposure to LPS or to poly I:C. Analysis of data is on-going.

Genetic population development, maintenance, and characterization Iowa State University maintains eight unique chicken genetic lines, which are of three basic genetic structures: (a) highly inbred lines, (b) advanced intercross line (AIL), or (c) outbred, closed population. Highly inbred lines (70-100 generations of sib or first-cousin- mating) of defined MHC type are maintained, with the inbreeding of the earliest line (Line 8) starting in 1925. Lines are primarily of egg-type origin at the time the lines were sourced, but also include the non-commercial Fayoumi and Spanish lines, and one closed line of broiler genetics from about 25 years ago. The advanced intercross line was initiated by crossing a single outbred broiler male with highly inbred Fayoumi females. Birds of the MHC-defined inbred lines are serologically typed each generation with line- specific anti-erythrocyte antisera to verify line purity; all birds are typed as chicks and then potential breeders are typed a second time before mating takes place. These lines are used in research at Iowa State, and shared as chicks, fertile eggs, tissues or DNA with collaborating researchers, including researchers within NE-1334. Generation interval is 9 to 10 months. Move-in date for the new chicken house is expected around March, 2020. This will cause disruption over the next months, but the long-term outcome will be housing for the ISU chicken genetics lines with higher biosecurity, although less space.

**Yvonne Drechsler & Ellen Collisson (WesternU):**

Yvonne Drechsler in collaboration with Dr. Hawkins at the University of Washington is funded to functionally annotate the chicken genome in several immune cells and tissues. A total of 20 cells/tissues will be profiled until 2023 and will be combined with research looking at epigenetic regulation of differential immune responses in chickens of different genetic backgrounds. The investigators are currently mapping the cis-regulatory elements in macrophages, T-cells, B-cells, reproductive tissues and lung macrophages in the Michigan 6x7 F1 line and are working with the FAANG group to coordinate efforts across species and assays, as well as data collection. Additional tissues have been collected and are in early processing stages such as bursa, thymus, intestinal sections, spleen macrophages/T cells and kidney macrophages.

In another project we have identified differential peaks in chromatin accessibility in response to IBV in chickens, which is tissue and viral strain specific. RNA seq is currently in progress to complement this data.

We are also starting a collaboration with Dr. Erf to analyze epigenetic changes associated with chicken autoimmune disease.

**Marcia Miller (Hope):**

PCR-based MHC-*Y* genotyping (Miller, Goto, Zhang).We have moved our method of PCR- based typing for MHC-*Y* from gels to ABI sequencer. Typing is now much more reliable, routine and more reasonable for others to adopt.

**Impact:**

**Objective 1.**

1. Chicken immune responses are affected by alloantigens.
2. Identifying both the chromosomal location and the product of alloantigen genes will contribute to effective immunity as well as genetic improvement.
3. The finding of interaction of Meq splice variant-derived proteins, proteins that accumulate as MDV establishes latency, with the Polycomb Repressive complex, ties MDV latency directly to a pathway associated with cellular transformation and tumor progression. This work also connects MDV-induced lymphomagenesis to EBV-associated Hodgkin’s lymphoma, as well as other human leukemias and lymphomas.
4. Identification of genes that are associated with resistance to heat stress and Newcastle disease virus and can be used to genetic enhancement of disease resistance of chicken in adaption to hot climate.
5. Understanding function of transcription factor on the role of regulation of host response to avian influenza virus.
6. A breeding strategy that produces birds that are naturally more resistant to *Salmonella* would be of interest to poultry breeders, growers, and consumers. We propose to develop a selection strategy based on a phenotype characterized by enhanced innate immune markers that could provide the poultry industry with a viable option as they pursue selection for improved robustness, livability, and resistance against foodborne and poultry pathogens. Selection of high or low constitutive pro-inflammatory gene expression will reveal the role in critical immune responses.
7. Detailed knowledge of immune gene structure and functional genomics, and associations of SNPs and biomarkers with specific immune traits, will allow genetic selection to enhance innate disease resistance in poultry stocks, thereby improving bird health and production.
8. Identifying crucial genes in biological response pathways will aid in the rational design of vaccines, and in determining which genes or pathways are expected to have broad versus narrow protective effects.
9. An ideal mechanism for controlling disease in poultry is to breed birds with natural resistance. We are identifying mechanisms for this resistance. Innate immune functions, particularly activation of macrophages, has consistently shown to be different in disease resistant versus susceptible birds. We are investigating the role of the host epigenome in immune evasion of viruses and disease resistance and susceptibility to develop a deeper understanding of the genetic processes involved. Characterization of gene regulatory elements in the chicken genome will aid in the selection of markers for disease resistance in breeding.
10. The genomic sequence determination for MHC-*Y* in the RJF reference genome will provide a base for further studies devoted to MHC-*Y*.
11. Data are emerging supporting the hypothesis that genes within the highly polymorphic MHC-*Y* region are involve in guiding immune responses in chickens with apparently different haplotypes associated with different phenotypes.
12. This work provides further insight into the increased susceptibility of 335/B19 birds to infectious bronchitis.
13. This work provides modest evidence for differential resistance to IBV by chickens displaying different MHC haplotypes as well as insights into the expression of a variety of genes after IBV replication in the host
14. These results suggest that the basis of tissue tropism might be related by proteins encoded by genes other than S1 in IBV
15. Data from this study is important to understand the genotypic diversity of isolates in California and better strategize pathogenicity studies to characterize ARV variants causing clinical disease in the field.
16. The information generated in the present study helps the understanding of the epidemiology of reoviruses in California. In addition, provides insights on how other genes that are not commonly studied add variability to the reovirus genome.

**Objective 2.**

1. Pioneer colonizing bacteria impact innate immune response of chickens for a prolonged period of time.
2. Influencing microbiota at hatch may affect ability to resist disease, especially opportunistic pathogens.
3. Our finding that mutations in the C-terminus of the Meq oncoprotein, which have been associated with the MDV virulence level, affect the innate sensing and signaling in infected cells. This is the first direct, causal association of meq mutation and a mechanism affecting one aspect of MDV virulence; namely, the evasion of the innate immune system. In follow up to this, we have submitted Meq immunoprecipitations from MDV-infected spleen cell lysates from an in vivo study, as well as tumor cell lines from JM10, RB-1B, RB-1B-based recombinants, and MK and TK strain-transformed T-cell lines to further characterize Meq-binding proteins during lytic and latent infections.
4. The determination that Day 15 post-hatch represents an immunological low point during the grow out of the modern broiler, and this is not observed in the ACRB line, points to a time of therapeutic intervention in a production setting. In addition, the mechanism being a decline in PI3K-Akt and NFkB responses allows a tailored approach to jump starting the broiler immune response during the potentially immunocompromised time.
5. From the multi-CpG dose trial, we observed that the ACRB line generates an adaptive immune response to repeated TLR ligand exposure while the modern line continues to produce an innate, proinflammatory response. This points to a lack of adaptation to repeated challenge that may need to be addressed either through in-feed treatment or a breeding program to reestablish an adaptive response that is less energetically costly and likely more effective over growout.
6. Postbiotics have been used for years in animal agriculture but the mechanism of action at the gut level was unclear. Our work shows a mechanism of benefit for fermentates and shows a benefit during aggressive disease challenge using *C. perfringens*.
7. We have developed a novel tool for the study of waterfowl with our species-specific peptide array for the *Anas* genus. This provides a kinomic and proteomic tool for avian researchers that has to date been exclusive to humans and certain agricultural species.
8. The autoimmune disease-prone Smyth, UCD-200/206, and Obese strains of chickens are important genetic models to study the cause-effect relationship between a genetically controlled disease (complex, non-communicable disease), immune function, and environmental factors.
9. The autoimmune disease-susceptible chicken lines provide unique opportunity to study immunopathological mechanisms in poultry and examine the interrelationship between genetic susceptibility, immune system components and environmental triggers in the etiology and progression of multifactorial, non-communicable diseases.
10. Identified that a persistent intestinal *S*. Heidelberg infection increased the Treg percentage, suppressive properties, and IL-10 mRNA amounts in the cecal tonsils of broiler birds.

**Objective 3:**

1. Our finding that viral mRNAs, but not virus DNA, are present in serum exosomes in vaccinated and protected chickens suggests that these exosomes are conferring systemic immunity through CTL-priming by macrophages and dendritic cells that have taken up these exosomes and expressed these proteins. This observation, coupled with our small RNA transcriptomic analysis, may provide the very basis of systemic immune protection elicited by MD vaccines. The finding that we can, in fact, generate mature dendritic cells with IL4, GM-CSF, LPS and IFNpermits a careful and methodical analysis of the role exosomes play in mediating long-term, systemic CTL responses.
2. The cell lines represent novel resources which will be made available to the community for use in studies to assess the role the NOX pathway plays in macrophage function in vitro.
3. The development of the “*in* *vivo* test-tube system” using the growing feathers as a dermal test-site provides an important tool to monitor and evaluate local cellular immune system activities in a complex tissues and explore the immunological mechanisms underlying disease susceptibility and resistance in poultry.
4. The GF in vivo test-system together with sampling the peripheral blood or other body fluids provides a minimally invasive, two-window approach for comprehensive monitoring and assessment of local and systemic immune system activities.
5. Identified that *B. subtilis* probiotic can be a tool in decreasing *Salmonella* loads in broiler intestine and *B. subtilis* supplementation can be expected to decrease broiler carcass contamination with *Salmonella*
6. Identified that synbiotic supplementation decreased SE proliferation in vitro and decreased SE load in the cecal contents and broiler carcass.
7. Studies on the host response to food-safety bacteria may decrease the potential for microbiological contamination of poultry products.
8. Knowledge on the interactions of heat stress and inflammatory response may inform methods for better management of poultry health and production in hot climates, and identify *in vitro* tests to substitute for live-bird research trials.
9. Moving PCR-based typing to the ABI sequencer has greatly improved MHC-*Y* genotyping. Findings are can now far more easily be compared across experiments and populations.