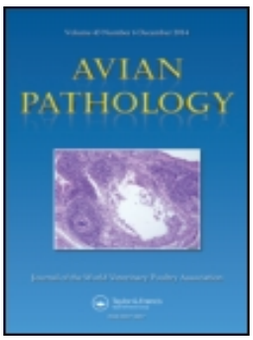

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Immune responses following experimental infection with *Ascaridia galli* and necrotic enteritis in broiler chickens

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Abstract

Broilers commonly suffer from necrotic enteritis (NE). Other gastrointestinal infectious diseases affect poultry, including nematode infections which are considered a re-emerging disease in barn and free-range systems. The aim of this study was to characterise the immune response of broilers after artificial infection with NE and contrast these with responses to the nematode *Ascaridia galli* and determine whether immune parameters measured during the course of infection can be used to distinguish infected from uninfected birds.

A total of 96 one-day-old male Ross 308 broiler chickens were used in this study. At 10 days of age, broilers were randomly assigned to one of the following treatment groups: control birds (n = 32), *A. galli* infected birds (n = 32), or necrotic enteritis infected birds (NE; n = 32) and inoculated with the appropriate infective agents. The immune response of birds was monitored through evaluation of haematology parameters, acute phase protein production, and intraepithelial intestinal lymphocyte population changes at 11, 16, 20 and 32 days of age.

T-helper cells ($CD4^+CD8^-$) increased significantly over time, and were significantly higher in *A. galli* and NE compared to day 10 controls. In conclusion, α-1 glycoprotein levels can distinguish birds with NE from other birds, inlcuding those infected with *A. galli*, also T-helper cell numbers can distinguish both NE and *A. galli* from uninfected birds and thirdly, 10 days post infection is the best time point to evaluate the bird's immune response for *A. galli* infections.

Introduction

Clinical outbreaks of disease are challenging to eradicate and are frequently associated with both reduced welfare status and significant economic losses. Of major concern to the industry is necrotic enteritis (NE), a common disorder of the gastrointestinal tract in broiler chickens with a prevalence of up to 32.8 % among the broiler industry (Hermans & Morgan, 2007). While several predisposing factors increase the likelihood of a fatal clinical outcome, the netB toxin producing *Clostridium perfringens* is the major cause for focal necrosis and, in severe cases, abrasion of the villi and mucosal layer (Keyburn et al., 2008; Shojadoost et al., 2012; To et al., 2017). Subsequently, the blood barrier can be easily crossed by pathogens. Clinical signs include depression, loss of appetite and sudden death. In many countries, NE has been controlled effectively using antimicrobial growth promoters in the feed. Concerns about increased antibiotic resistance in human pathogens have led to restrictions on antibiotic use in animal feed. Therefore, it is a common perception in many countries that NE is a re-emerging disease. Scoring of intestinal lesions is a common method to evaluate the degree of host response after infection. However, lesion scoring systems are not always consistent (Shojadoost et al, 2012). Therefore, the use of inflammatory markers such as acute phase proteins or the concentration of intraepithelial lymphocytes could be an alternative method to confirm the results from the scoring system when the levels of NE infection in the challenged birds are ambiguous. The cellular and humoral components of the immune system are a major defence mechanism against pathogens. In poultry, changes in the immune system including blood parameters, acute phase proteins and intestinal lymphocyte subpopulations can be used to detect subclinical diseases and disease infestations prior to clinical signs being observed (Chen-Io et al., 1998; Hong et al., 2006; Murata et al., 2004; Rose et al., 1979). Measurements of acute phase proteins have been used in ruminants to determine calf herd health (Ganheim et al., 2007). These early disease indicators would be very useful to monitor the health status of commercial poultry. Gastrointestinal diseases of various types occur in poultry production systems, and therefore it would be important for markers of NE to be distinguishable from other disease types. One of the other classes of gastrointestinal pathogens of increasing interest is helminth parasites. Due to an increased demand in alternative housing

systems such as floor and free-range housing, parasitic infections are considered a re-emerging problem (Höglund & Janssen, 2011; Yazwinsky et al., 2003) with a prevalence of 69.5 % reported in organic poultry production in European countries (Thapa et al., 2015). In broiler breeder farms in the USA, the prevalence of *A. galli* was 63% (Yazwinsky et al., 2013), and in single tiered floor systems in Sweden, the prevalence was 44% (Jansson et al., 2010), so access to an outdoor range is not a requisite for infection with this parasite. Infections with *A. galli* become patent (nematode eggs detectable in excreta) around 8 weeks post infection, so infections in broiler sheds are not easy to detect. Parasitic infections, including those caused by the nematode parasite *A. galli*, can have a negative impact on the health, welfare and productivity of poultry (Dahl et al., 2002; Gauly et al., 2007). A recent study conducted in Denmark revealed that hen mortality was twice as high on farms with helminth infection (> 200 eggs/g excreta) (Hinrichsen et al., 2015). The modulated immune response related to *A. galli* infections and subsequent increased severity of bacterial infections in dual infections with both helminth and bacteria is suspected to be responsible for the fatal outcome associated with *A. galli* infections (Dahl et al., 2002; Degen et al., 2005; Eigaard et al., 2006; Permin et al., 2006; Schwarz et al., 2011; Pleidrup et al., 2014).

On the other hand, intestinal nematodes are considered as potential therapeutics for gastrointestinal inflammatory diseases in humans, working by modulating the immune system (Szkudlapski et al., 2014). It has been demonstrated that the effects of intestinal helminths on the host immune system are complex and omni-directional, and involve the modulation of Toll-like receptor (TLR) expression, proliferation and activation of Th2 lymphocytes, proliferation of regulatory T-cells and production of immunomodulatory proteins (Smits et al., 2010; Zaccon et al., 2008). In poultry, the onset of immune responses to *A. galli* starts within the first two weeks post infection (Schwarz et al., 2011). In modulating the humoral and cellular immune response, *A. galli* infections are know to influence vaccine-induced immunity in laying hens (Pleidrup et al., 2014). However, limited information is available regarding the immune response directed against helminths. Detailed information about immune responses induced by nematode infection with *A. galli*, or NE could provide baseline information for further analysis of the interaction between these poultry diseases. An improved understanding of disease interactions in poultry

is expected to identify strategies aimed at improving gastrointestinal health. Therefore the aim of this study was to examine the effect of parasite infection and NE on immune cell and haematological parameters and determine the best time point to evaluate the relevant parameters.

Materials and Methods

The study was approved by the Animal Ethics Committee of the University of New England. Animals were housed and treated in accordance with the Model Code of Practice for the Welfare of Animals, Australia (CSIRO, 2002).

Birds and housing. A total of 96 male broiler chickens (Ross 308) were obtained from a commercial hatchery (Baiada Country Road Hatchery Ltd., Tamworth, NSW, Australia) at one day of age and raised indoors in a temperature controlled and ventilated room on wood shaving litter. Lighting and housing temperature were maintained according to Ross 308 standard guidelines (Aviagen, 2014).

After a period of 10 days, broilers were randomly assigned to one of the following groups: control (n = 32), *A. galli* (n = 32), or NE (NE; n = 32). The health status of all birds were assessed twice daily by visual inspection. Records of the room temperature, feed and water availability as well as any possible clinical signs of illthrift were maintained daily. Broilers in the *A. galli* group were inoculated *per os* at 10 days of age with a total of 1000 embryonated *A. galli* eggs as described by Sharma et al. (2017). Prior to inoculation, *A. galli* mature nematodes were sampled from the intestine of naturally infected laying hens. Mature nematodes were transferred into RPMI media at 37°C (with 0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/mL Amphotericin B) and cultured for three days, changing the media every 24 hours. Eggs shedded into the media were collected from the spent medium by centrifugation after each 24 hour period and concentrated eggs were diluted in 0.05 M H_2SO_4 and kept at 26°C for up to six weeks. Embryonation under these conditions was judged to have occurred after 3 weeks of culture when fully formed nematodes were visualised within the shell. For inoculation, the embryonated eggs were diluted in an equal volume of 0.1 M Na_2HCO_3 and then diluted in 0.1 M NaCl to the desired

concentration (2000 eggs/mL).

Broilers of the NE group were infected as previously described (Rodgers et al., 2014; Shojadoost et al., 2012; Wu et al., 2010). Broilers were inoculated *per os* at 10 days of age with a suspension of 5000 sporulated oocysts prepared from attenuated field strains of *Eimeria acervulina* and *E. maxima*, 2500 sporulated oocycts of *E. brunetti* and 2000 sporulated oocysts of *E. necatrix* (Eimeria Pty Ltd, Werribee, VIC, Australia) in 0.5 ml sterile phosphate-buffered saline (PBS). On day 14 and day 15, broilers were challenged with 10^8 CFU/ml *C. perfringens* type A strain EHE-NE36 (CSIRO, Geelong, VIC Australia) in 0.5 ml thioglycolate broth containing starch and pancreatic digest of casein.

Sampling

Whole blood was collected from broilers (control, *A. galli*, and NE: n = 10/group) at 10, 11, 16, 20, and 32 days of age. At each time point, the same individuals were sampled and the blood analysed using a CELL-DYN 3700 analyser (Abbott, USA, Suljevic et al., 2003). Haematology parameters measured included total leucocytes (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), total erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volums (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT). Means for each treatment group within each time point were calculated.

Production of anti- *A. galli* antibody and acute phase proteins in serum in response to infection were assessed using ELISA. Serum was prepared from blood collected from 6 broilers (n = 6) per group at 10, 11, 16, 20, and 32 days of age. At each time point, the same individuals were sampled. Concentration of the acute phase protein, α-1 glycoprotein was determined using a commercial ELISA kit: Chicken α-1-acid glycoprotein (α-1-AGP) ELISA, (Life Diagnostics, Inc., West Chester, PA, USA). An in-house ELISA was developed to assess levels of anti- *A. galli* IgY antibody in serum collected from birds. Briefly, ELISA plates were coated (100µL/well) with *A. galli* antigen extract diluted to a final concentration of 1 µg/mL in carbonate buffer (pH 9.6) and incubated at 4°C overnight. Coating solution

was flicked from plates and wells were then blocked (250µL/well) with blocking solution (PBS with 0.5% BSA, pH 7.4) and incubated at RT for 2 hours. Following washing with washing solution (PBS with 0.05% Tween 20, pH 7.4, 250µL/well), chicken serum samples and control samples (positive and negative) were serially diluted across the plate in blocking solution (final volume of 100µL/well) and plates incubated for 1 hour at room temperature. Following washing (×5 times), goat anti chicken IgY conjugated to horse radish peroxidase (HRP) (goat anti Chicken IgG(Fc):HPR, BioRad, Hercules, CA, USA) was added to wells (100µL/well) and plates incubated for 1 hour at RT. Finally, plates were washed (×5 times) and 100µl substrate solution (TMB – tetramethylbenzidine dihydrochloride, Sigma-Aldrich, St. Louis, MO, USA) added. Plates were incubated in the dark for 10-12 minutes and color development then stopped with the addition of H_2SO_4. Colour development was quantified by reading the absorbance of individual wells at 450nm with a 650nm reference wave length. A pool of positive (post-infection) and negative (pre-infection) control serum was prepared for use in ELISA procedures. Concentrations of coating antibody and conjugated antibody used in the ELISA procedure were optimised to maximise differences in observed titres for the positive and negative control samples. Titres were calculated using software developed in-house and all titre values corrected based on the value of the positive control sample run on every plate.

For measuring intraepithelial lymphocytes (cytotoxic T-cells: $CD4^-CD8^+$; T-helper cells: $CD4^+CD8^-$; double positive cells: $CD4^+CD8^+$), 4 broilers per group (n = 4) were sacrificed and sampled at days 10, 11, 16, 20, and 32 of age. Intraepithelial lymphocytes were isolated from the small intestine of chickens according to the method used by Röhe (2014). Mouse anti-Chicken CD3-AF647, mouse anti-Chicken CD4-FITC, mouse anti-chicken CD8a-PE, mouse IgG1-PE, and mouse IgG1-AF647 were purchased from Southern Biotech (Birmingham, Alabama, USA). The crude preparations of intraepithelial lymphocytes were resuspended in ice-cold Hanks balanced salt solution (HBSS) without calcium or magnesium at approximately 1×10^6 cells per mL. The cells were incubated with the primary anti-CD4, anti-CD8 cocktail or isotype control antibody preparation for 30 min on ice and in the dark. The cells

were centrifuged three times at 200g for 5 minutes at 4°C and resuspended in ice-cold PBS with 0.1% Bovine Serum Albumin (BSA). The centrifugation and wash steps were repeated twice. After the final centrifugation the cell pellets were resuspended in 200 µl of ice-cold PBS with 0.1% BSA and cell surface marker data acquired on a FlowSight flow cytometer (Amnis, Seattle, USA). The flow cytometer was compensated using the AbC anti-Mouse Bead Kit conjugated to fluorophore isotype control antibodies (Thermoscientific, Carlsbad, USA).

Statistical analyses.

Statistical method. Haematology and acute phase protein data were collated and analysed with a repeated measurements analysis using the method of residual maximum likelihood (REML), which is also sometimes called restricted maximum likelihood, in Genstat (16^{th} edition, VSNi international Ltd, Hemel Hempstead, UK). Heterogeneity over time was allowed to account for unequal variances where they occurred, and the irregular time point spacing option was also selected, the Power - city block distance (+ scalar) method was used. Each parameter, the time 0 value for the parameter, and time, were analysed as variables in conjunction with the fixed effects of treatment group and individual bird, and the interaction between time and treatment group. A separate analyses for each parameter was undertaken, and no attempts to combine parameters were made because some of these measurements are calculated from one another, therefore model optimisation was not conducted. A *post hoc* analysis where predicted means from the model were compared in a pair wise manner was performed, also within the REML module of Genstat. Means for each treatment group within each time point were calculated using the model and the differences between means were reported as significant if the values were greater than twice the standard error difference apart.

CBC. LYM, MCV and HCT data were normally distributed, RBC, WBC, EOS, MHC, MONO, BASO, PLT and NEU data were Log_{10} transformed to achieve a normal distribution, HCT data were transformed using a cube root transformation, but MCHC data could not be transformed to approach a normal distribution, so this parameter was not analysed. Day 0 values were used as a covariate in the model to

remove the influence of variation evident before onset of the experiment. Ten birds per group were sampled at 10 days of age before treatments were applied, then at days 1 (age 11), 4 (age 16), 10 (age 20) and 22 (age 32) post-treatment. Broilers of the NE group experienced 100 % mortality after day 16, hence no data for day 20 and 32 was available for this group. Serum antibodies against *A. galli* antigen were assessed by ELISA, but the number of missing data points precluded a formal statistical analysis.

FACS. Flow cytometric data were analysed using IDEAS application software version 6.0 (Amnis, Seattle USA). Populations of intact single cells were gated by analyzing forward scatter side scatter plots and confirmed by fluorescent micrograph analysis of cells from imaging flow cytometric data. Within these single cell populations, cells that were positive for CD4 or CD8 or double positive compared to isotype controls were gated and quantified. Statistical analysis of flow cytometric data were performed using Prism version 5 (GraphPad, USA). Two-way ANOVAs were performed with challenge and age as main effects. P-values < 0.05 were considered significant.

Results and Discussion

The health status of all broilers from the control group and the *A. galli* group was unremarkable. However, all broilers from the NE group became severely moribund after infection with *C. perfringens* on day 14 and 15 and most individuals died peracute at 15 and 16 days of age. This exceeded our expectations as mortalities in birds affected with NE has been reported to vary between 1 and 40 % (Shojardoost et al., 2012; McDevitt et al., 2006). Post mortem examination of the dead birds was performed and severe gastrointestinal haemorrhagia, as well as a complete loss of mucosal and submucosal structure as well fibronecrotised intestines were observed. These clinical signs are typical lesions that are observed in birds affected from severe NE (Uzal et al., 2016).

Blood analysis (CBC).

Results are displayed in Table 1. The number of erythrocytes (RBC, p = 0.004), blood haemoglobin concentration (HGB, p = 0.025) and the mean corpuscular volume (MCV, p < 0.001)) differed significantly between the groups (Table 1, Figure 1).Some of these parameters decreased in a linear or LOGlinear fashion with age, MCV (R^2 = 0.71, slope = -0.69/day), logRBC (R^2 = 0.44, slope = 0.005/day) and LYM (R^2 = 0.36, slope = -0.68/day), whilst the other parameters did not. MCV declined with time in all groups but this occurred at a slower rate in the *A. galli* infected group (from 121 fL on day 10 to 105 fL at day 32) and a faster rate in the NE group (from 117 fL to 107 fL at day 16) relative to controls (121 fL on day 10 to 106 fL at day 32). RBC increased over time in the control and *A. galli* infected birds (RBC of control birds at day 10 and day 32: $1.99x10^9$ cells/ml and $2.52x10^9$ cells/ml respectively, RBC of *A. galli* infected birds at day 10 and day 32: $1.99x10^9$ cells/ml and $2.57x10^9$ cells/ml respectively). Although there were no significant treatment group differences, birds in the NE group had substantially higher HGB and RBC levels (14.2 g/dL and $2.59x10^9$ cells/ml) than birds from the other groups at 6 days post infection (age 16 days); however, it should be noted that this effect was observed in just 2 moribund surviving birds from the necrotic enteritic group. Eosinophilia is expected in parasitic infections as larval stages of nematodes can be killed by eosinophils but the development of the response due to larvae migrating can take several days (Johnson et al., 1999). I However, in this study, both the *A. galli* and control group EOS means increased in broilers at day 32 of age but the effect of time was not significant. A reduced development of the immune response in younger birds compared to older ones can be speculated as the cause for the insignificant differences observed between the groups.

The evaluation of *A. galli* antibodies and acute phase proteins.

The level of the acute phase protein α-1-AGP was significantly increased in the NE birds immediately prior to death (324 µg/ml at day 10 to 643 µg/ml at day 16; Table 1), but did not differ significantly between control and *A. galli* infected birds at any time point. Tosi (2005) concluded that increasing levels of pro-inflammatory cytokines in humans such as IL-1 and IL-6 and TNF-α following infection stimulate liver production of acute phase proteins resulting in an increased concentration in the blood. A similar

mechanism may be at play in birds with elevation of alpha-1 glycoprotein observed in birds challenged with *C. perfringens.* The α-1-AGP concentration in broilers of the control group ranged from 247 – 290 µg/ml. Stable plasma α-1-AGP concentration were also reported by Takahashi et al (2007) when evaluating broilers older than 14 days of age. However, in these broilers the level of α-1-AGP was similar (240 ±33 µg/ml). While broilers that were injected with *Escherichia coli* lipopolisaccharides experienced a 5 – fold increase in the α-1-AGP concentration (Takahashi et al., 2007), broilers of the present study did not demonstrate any significant change in α-1-AGP levels when being exposed to *A.galli*. The peak of α-1-AGP response after lipopolysaccharide exposure could be observed 24 hours after toxin injection (Takahashi et al., 1998), which might be one of the reasons that the response in the present study was observed in the acutely diseased NE birds only, while the chronically infected birds of the *A.galli* group failed to respond. Due to the fact that the standard NE infection model always includes the concurrent use of *Eimeria* and *Clostridium*, information of the individual component on the immune parameters is limited.Our preliminary assessment is that the acute phase protein, α-1-AGP has very limited diagnostic value for the two diseases studied.

Antibodies against *A. galli* antigen were also detected by ELISA, but a high background meant that many data points were excluded from the analysis. One *A. galli* infected bird had a high and increasing titre, peaking at 20 days of age, but the sample could not be analysed at 32 days of age. Another *A. galli* group bird had an undetectable antibody level at the age of 16 and 20 days, but a high titre at day 32. Ten out of 12 birds assessed at the beginning of the experiment prior to infection had detectable antibody levels possibly due to the presence of maternal antibodies. In general, maternal antibodies in broiler chickens have a relatively short life span with a half-life of 4-6 days, depending on their specificity and can be present until 10 days of age (Gharaibeh & Mahmoud, 2013; Patterson et al., 1962).

Expression of interepithelial lymphocytes response.

Intestinal intraepithelial $CD4^+$ and $CD8^+$ lymphocyte populations were monitored during an *A. galli* or NE infection using flow cytometry (Figure 2). Significant ($P < 0.05$) time-dependent increase in $CD4^+$ (T

helper) and $CD8^+$ (cytotoxic) T-lymphocytes were observed in the intestines of broiler chickens with NE compared to uninfected broilers through days 10 to 16 post infection. This could be due to increased expression of IL-10 in NE-challenged birds (Collier et al., 2008; Park et al., 2008). IL-10, involved in innate and adaptive immune responses, hinders the IL-12 production by activated macrophages, and influences $CD4^+$ lymphocyte as well as and $CD8^+$ cytotoxic T-lymphocyte production in broilers with *Eimeria* infection. (Groux & Powrie, 1999; Rothwell et al., 2004). More precisely, increases in intestinal T cell subpopulations following an infection of birds with *E. maxima* infection resulted in a significantly increase of CD3+ , CD4+, and CD8+ cells at days 8, 6, and 7 post-primary infection, respectively, but only CD4+ cells remained elevated following secondary infection (Hong et al., 2006). Lee et al., (2010) found that the high level of IFN-γ was correlated with protective immune responses to enteric coccidial infections. Similarly results from the current study showed there was a significant ($P < 0.001$) time-dependent increase in the number of intraepithelial $CD4^+$ T helper cells in *A. galli* infected birds, peaking at day 20 post infection. This time-dependent increase accounted for approximately 42% of the observed variation. These findings are in line with findings from Schwarz et al., 2011, where T-helper cells were significantly elevated in laying hens experimentally infected with *A. galli*. There was a small but significant ($P < 0.05$) time-dependent decrease in the number of intraepithelial $CD8^+$ cytotoxic T-cells during the *A. galli* infection. However, for both NE and *A. galli* infection groups the changes in $CD4^+$ T helper and $CD8^+$ cytotoxic intraepithelial T-cells only approached significance relative to uninfected control birds. Based on the research conducted in laying hens reported by Schwarz et al. (2011), significant differences in the cytotoxic T-cells would be expected around 14 days post infection equivalent to when broilers were 32 days of age in the current study. Collectively, these data suggest that host responses to NE or *A. galli* involve an increase in intraepithelial $CD8^+$ cytotoxic or $CD4^+$ T helper populations respectively. These trends in the data can be explained by the fact that necrotic enteritis infection are cleared by a cytotoxic T-cell response (i.e. Th1 response). The parasite *A. galli* is a multicellular pathogen which is commonly controlled by Th2 T-helper cell response. In order to characterise the immune response further and investiage the regulatory pathways, the Th1 and Th2

response in infected broilers warrants further investigation. Due to the significant ($P < 0.05$) time-dependent increase in $CD4^+$ (T helper) and $CD8^+$ (cytotoxic) T-lymphocytes in the intestines of broiler chickens with NE compared to uninfected broilers through days 10 to 16 post infection, as well as based on the evaluation of the acute phase protein α-1-AGP that significantly increased in the NE birds immediately prior to death we can conclude that 10 days post infection is the best time point to evaluate for *A. galli* infections.

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Table 1: The effect of age and treatment on CBC profile and alpha glycoprotein levels for broiler chickens that were healthy (control), infected with *A.galli* (*A. galli*) or infected with necrotic enteritis (NE). Ten birds per group were sampled at 10 days of age before treatments were applied, then at days 1 (age 11), 4 (age 16), 10 (age 20) and 22 (age 32) afterwards. Broilers of the NE group experienced 100 % mortality after day 16, hence no data for day 20 and 32 is available for this group. Data are means of actual data not predictions from the statistical model.

Measurement	**Group**	**Age (days)**					**p-values**		
(units)		**10**	**11**	**16**	**20**	**32**	**Treatment**	**Time (Age)**	**Interaction**
WBC*	Control	47.43	39.60	61.72	33.52	40.88	0.764	<0.001	0.105
10^6 cells/mL	*A. galli*	43.85	44.93	66.21	30.31	37.37			
	NE	41.10	39.50	101.48					
NEU*	Control	4.62	5.43	6.51	7.47	6.52	0.509	<0.001	0.436
10^6 cells/mL	*A. galli*	3.94	4.85	6.82	7.88	7.70			
	NE	4.22	5.25	9.37					
LYM	Control	36.10	30.79	26.97	22.52	19.79	0.301	<0.001	<0.001
10^6 cells/mL	*A. galli*	35.51	36.70	22.27	18.36	13.88			
	NE	32.72	31.64	41.12					
MONO*	Control	3.26	1.96	14.82	2.31	4.74	0.601	<0.001	0.612
10^6 cells/mL	*A. galli*	2.26	1.86	14.75	2.66	4.84			
	NE	2.29	1.39	19.76					
EOS*	Control	0.004	0.004	0.011	0.003	0.038	0.400	0.059	0.627
10^6 cells/mL	*A. galli*	0.009	0.008	0.014	0.006	0.086			
	NE	0.006	0.009	0.020					
BASO*	Control	3.44	1.42	13.41	1.22	9.79	0.801	<0.001	0.217
10^6 cells/mL	*A. galli*	2.13	1.51	22.35	1.41	10.87			
	NE	1.86	1.22	31.20					
RBC*	Control	1.99	1.85	2.14	2.23	2.52	0.004	<0.001	<0.001
10^9 cells/mL	*A. galli*	1.99	1.95	2.13	2.27	2.57			

	NE	2.13	1.98	2.59					
HGB*	Control	14.3	10.4	12.5	12.4	14.0	0.025	<0.001	0.008
g/dL	*A. galli*	16.9	10.9	12.2	12.6	14.3			
	NE	13.8	11.0	14.2					
HCT§	Control	24.0	22.6	24.5	25.1	27.0	0.074	<0.001	<0.001
% (v/v)	*A. galli*	24.0	23.7	23.7	25.3	27.0			
	NE	24.8	23.7	27.7					
MCV	Control	121	123	115	113	106	<0.001	<0.001	<0.001
fL	*A. galli*	121	122	112	112	105			
	NE	117	119	107					
MCH*	Control	72.1	56.4	58.3	55.6	55.8	0.091	<0.001	0.356
pg	*A. galli*	87.2	56.2	57.6	55.8	55.7			
	NE	64.8	55.5	55.0					
PLT*	Control	0.37	0.64	2.53	0.58	1.81	0.960	<0.001	0.965
10^6 cells/mL	*A. galli*	0.59	0.23	1.17	0.74	5.32			
	NE	0.89	0.54	1.54					
α-1 GP*	Control	5/5	6/6	4/6	3/6	3/6			
Birds	*A. galli*	5/5	6/6	6/6	2/6	3/6			
evaluated/sampled	NE	5/5	5/6	4/6	0/6				
α-1 GP	Control	247	253	290	286	322	0.023	0.083	0.518
µg/ml	*A. galli*	351	305	308	265	435			
	NE	324	322	643					
	Control	9	9	10	10	10			
Number of birds evaluated	*A. galli*	10	10	10	10	10			
	NE	10	10	2					

* $\log_{10}$ transformed
§ cube root transformed
p-values are from repeated measures analysis REML via GenStat (16th edition). F-test outcomes are reported.

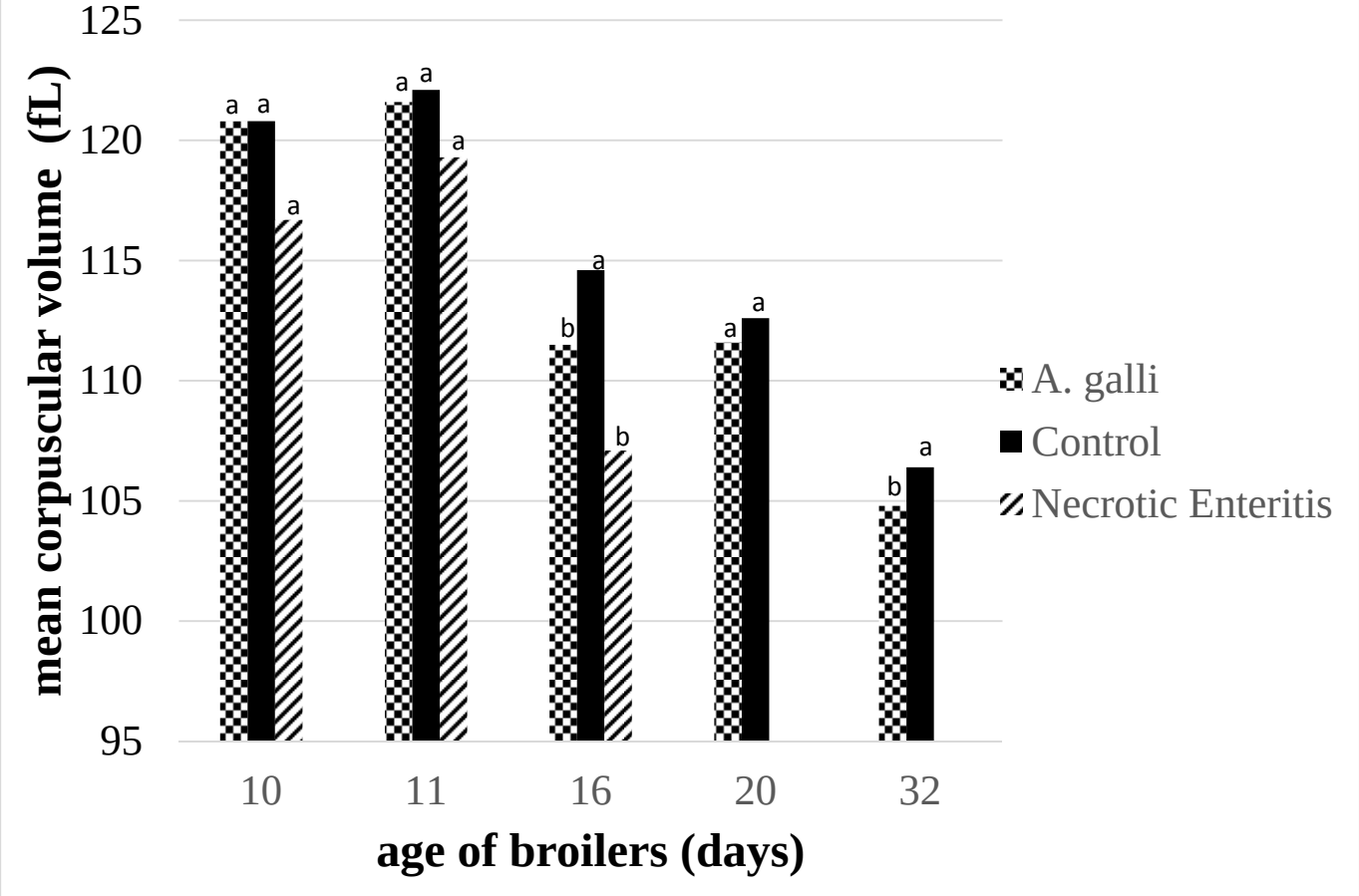

Figure 1: MCV in blood cells decrease over time.

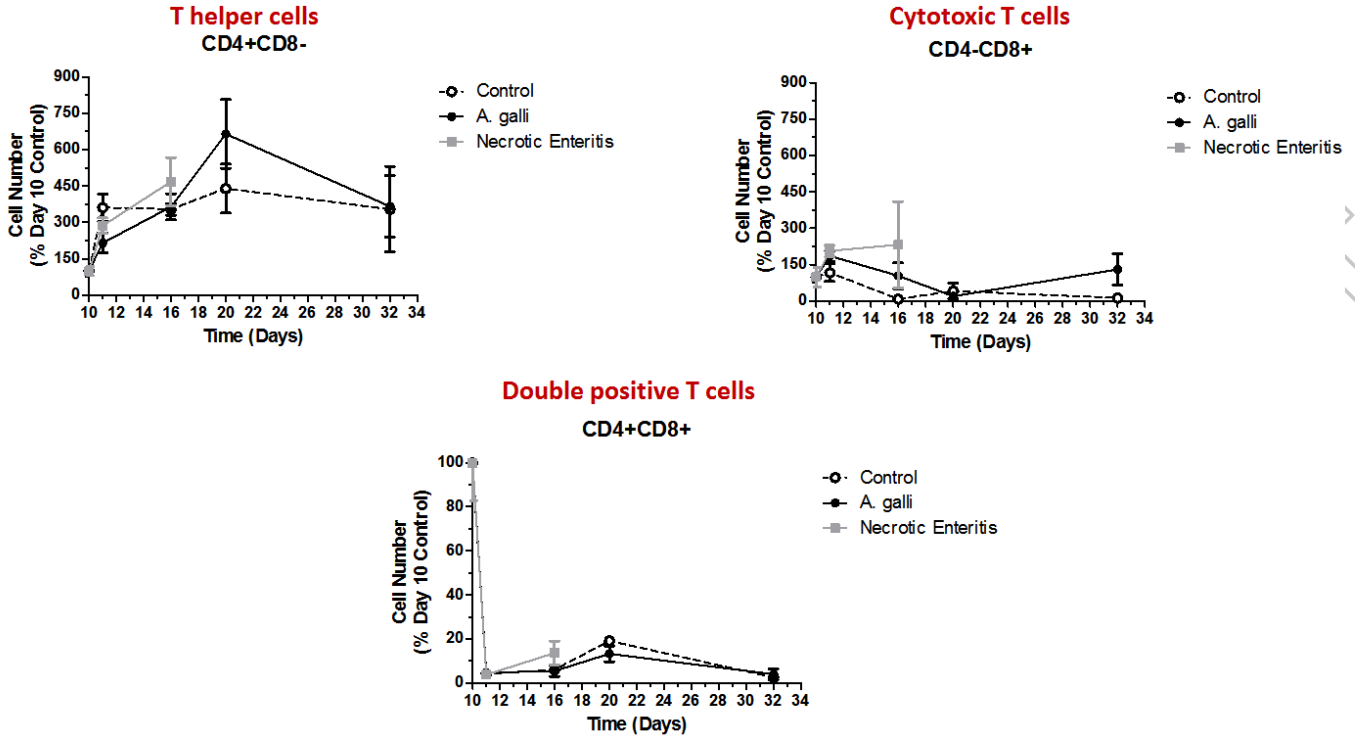

Figure 2: The cell numbers of T-helper cells, cytotoxic T-cells, and double positive T-cells in broiler chickens at various time points.