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Executive Summary

Lameness due to “femoral head necrosis (FHN)” is reported to be a major economic and welfare problem in commercial broilers in Australia. However, the true extent of the disease in the Australian broiler industry is currently unknown, and epidemiological factors influencing the development of the condition are also poorly understood. The purpose of the current project was to ascertain the prevalence of “FHN” in Australian commercial broiler flocks, to characterise the aetiological agents involved in its development, and to investigate whether environmental, host or pathological cofactors influence the development of the disease. This study used several broiler farms in Victoria as a model to investigate the epidemiology and aetiology of “FHN” in Australia. It was found that “FHN” is a common multifactorial disease, characterized by chondronecrosis with osteomyelitis in the proximal end of the femur and tibiotarsus. Therefore, given the pathogenesis and histological features of the condition, we propose to use the term bacterial chondronecrosis and osteomyelitis (BCO) instead of FHN. Results of this study revealed that BCO occurs throughout the life of the broiler flocks at an alarmingly high rate - approximately one quarter of the mortalities and culls. The condition also frequently occurs within the tibiotarsus.

BCO is an infectious process that appears to occur most commonly as a result of bacteraemia and haematological spread of bacterial pathogens - especially *Escherichia coli* - to the bones. Results from this study also suggested that *E. coli* involved in development of BCO are primarily derived from the grower environment. We observed that almost all *E. coli* isolated from cases of BCO are avian pathogenic *E. coli* (APEC), suggesting that preventative measures should be directed at this organism.

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Introduction

Lameness is a major economic and welfare issue in commercial broiler bird production worldwide. The condition is multifactorial and influenced by infectious processes, bird genotype, and nutritional and management factors. However, it is increasingly recognised that a large proportion of lameness cases in broiler chickens are a result of the condition known as bacterial chondronecrosis and osteomyelitis (BCO). The condition is also referred to as femoral head necrosis, but this term is a misnomer, as the tibiotarsus is also commonly affected. BCO is a bacterial osteomyelitis and physitis, which often develops in conjunction with osteochondral necrosis and pathological bone fracture. The true incidence of the disease is unknown in most countries, but in Northern Ireland the mean incidence of culling due to lameness was 0.52% in male flocks and 0.38% in female flocks, with BCO the most common cause of lameness in broiler birds (McNamee & Smyth, 2000). Moreover, the researchers in this study suggested that it is likely that the true incidence of BCO is underestimated, as many lesions are not apparent on gross examination. Investigations in Bulgaria also revealed a significant problem, with lameness responsible for up to 15% of mortality in some broiler flocks, and BCO accounting for >90% of these cases (Dinev. I, 2009). For free-range production systems, data is even more limited, with no published surveys of BCO prevalence available.

Epidemiological data for Australian flocks is lacking, but lameness due to BCO is anecdotally reported to be a major welfare concern and cause of culling both in Victoria and in other states. Prevalence of this condition varies from one farm or company to another, but on severely affected farms approximately half of the culling in broiler flocks reportedly relates to lameness. In some instances, reported incidence of this condition can be as high as 3-5%. BCO occurs in all breeds and strains of commercial poultry, although some chicken breeds appear to show a higher susceptibility to the disease (Wideman *et al.*, 2013) - possibly due to increased limb stress caused by rapid growth and development of large breast musculature.

BCO-induced lameness, frequently referred to as leg weakness, is a chronic and painful condition, and affected birds are unable to easily access feed and water. Left unchecked, affected birds often die of dehydration. Culling of the affected birds is the only viable option available to growers, and review of culling records documented for RSPCA accreditation of broiler farms clearly indicates that lameness is the major cause of culling. In addition to welfare concerns, BCO has been reported predominantly in older birds, and culling of birds

close to the market age leads to significant financial loss to the farmers and processors.

The organism most commonly reported in association with BCO is *Staphylococcus aureus*, but other septicaemic pathogens have also been isolated, including *E. coli*, *Enterococcus* and coagulase-negative *Staphylococcus* (Dinev. I, 2009; McNamee & Smyth, 2000; Thorp *et al.*, 1993). In contrast, the experience of our diagnostic service is that a large proportion of lameness cases in young chicks have BCO lesions colonized by avian pathogenic *E. coli* (APEC). APEC is responsible for causing massive economic losses to the poultry industry through septicaemia (colisepticaemia) and various localised forms of infections including airsacculitis, pericarditis, perihepatitis, cellulitis, omphalitis, salpingitis, peritonitis and osteomyelitis (Barnes, 2008). APEC is a subset of extra-intestinal pathogenic *E*. *coli* (ExPEC), a pathotypic category which also includes human uropathogenic *E*. *coli* (UPEC) (Russo. T & Johnson. J. R, 2003). APEC can cause neonatal meningitis and septicaemia in humans (Johnson & Stell, 2000; Kaper *et al.*, 2004), and it is also hypothesized that APEC derived from poultry can act as food-borne cause of urinary tract infection in humans (Rodriguez-Siek *et al.*, 2005). Comparative genomic analysis of 1000 APEC and UPEC strains revealed that APEC O1 shares extensive genetic similarities with human ExPEC, supporting the notion that food-borne zoonotic transmission of APEC to humans may be possible (Timothy J. Johnson *et al.*, 2007). Thus, further clarification of the role of different pathogens in the development of BCO, and APEC in particular, could aid in prevention of poultry-related food-borne illness, in addition to reducing incidence of the disease.

Despite BCO being a major cause of culling in broilers, the pathogenesis and predisposing risk factors for this disease remain poorly understood. A range of factors have been proposed to play a role in the development of the condition, including immunosuppression and immunosuppressive disease (such as infectious bursal disease and chicken infectious anaemia virus), vascular impairment within the epiphysis, arthritis and arthritis-causing pathogens such as reovirus, trauma, nutrition and nutritional deficiencies, growth rate, bird breed and hatchery conditions (McNamee & Smyth, 2000). Based on this wide range of purported influences, a systemic examination of the epidemiology of the disease in the field is warranted. Deficits in understanding of the BCO thwart adoption of appropriate preventative strategies to minimize the development of this condition, and the major purpose of this project is characterize the epidemiology of BCO in Victorian broiler flocks, in order to determine risk factors for the development of the condition in the field.

Aims of the projects

1. Assess the influence of epidemiological factors on the incidence of the disease, including age, genotype, hatchery conditions, nutrition, grower management practices and concurrent disease processes.
2. Determine the incidence and aeiological agents in BCO amongst birds in Victorain broiler flocks.
3. Based on the findings of this work, propose procedures or strategies that mayreduce the incidence of BCO in commercial flocks.

Methodology

1 Epidemiological survey into farm and flock risk factors associated with lameness culls

1.1 Aims

To determine if any shed design, management practice or flock characteristic is associated with an increased prevalence of BCO.

1.2 Methods

A retrospective cross-sectional study was performed using RSPCA cull data collected over an 18 month period from an integrated broiler company. Data was collected from multiple flocks in 86 different sheds on 18 farms. Characteristics of each flock were recorded including the number of birds placed, the number of male and female birds, date of placement, total flock mortalities and the number of culls including the reason for culling (leg problems, runt bird, non-starter chick, disease and injury). Characteristics recorded from the flocks, sheds and farms are listed in Table 1.

Table 1. Variables measured in epidemiological survey

Variable	Character
Shed design	Floor type (earth or concrete) Shed wall construction (solid, curtain Number of feeder and drinker lines Shed length and width % litter coverage Age of shed
Flock characteristics	Reasons for culling % mortality % males Time of placement
Management Practices	Shed clean out procedure

1.3 Statistics

Farm and flock characteristics were collated in a Microsoft Access database and exported into the R statistical package (RStudio-Version 0.99.903 – © 2009-2016 RStudio, Inc) for analysis. Given that BCO is thought to be the most common cause of lameness in broiler flocks in Australia, the number of lameness culls was used as a correlate for the prevalence of BCO in each flock. The confounding influence of data from successive flocks from the same shed and farm was factored into the analysis, as flocks from the same shed were essentially repeat observations of time-invariant characteristics, and flocks from the same farm also shared common characteristics (ie. same manager and management practices). This was achieved by entering shed and flock characteristics into mixed models which controlled for both farm and shed by declaring them as random effects. One model aggregated flock data by the month of placement to look specifically at the effect of the time of placement and climatic conditions associated with the time of placement on the percentage of lameness culls. The second model investigated the effects of flock and shed characteristics on lameness culls. The significance of each characteristic was tested with the likelihood ratio test by comparing the model both with and without the characteristic as an explanatory variable.

Statistical correlation between temperature (external to shed), rainfall, male and female ratio, farm age, non-start culls and monthly lameness culls was assessed using linear regression and one-way ANOVA to visualise differences among the means of monthly lameness culls. Statistical relationship between shed design, month placed, drinker-feed line distance and lameness culls was assessed using Kruskal-Wallis test with bonferroni

adjustment, followed by Wilcoxon rank sum test if groups found to be significantly different. Statistical relationship between stocking density and lameness culls was assessed using Linear mixed model.

2 Pathological and microbiological analysis of cull birds from commercial farms

2.1 Aims

This phase of the project aimed to determine the incidence of bacterial chondronecrosis and osteomyelitis in commercial broiler mortalities and culls (including both lameness culls and other cull types) to characterize the infectious agents involved in the development of the disease, and to assess for correlation between BCO and other infectious, traumatic or developmental disease.

2.2 Methods

2.2.1 Gross examination

Culled birds and mortalities were submitted for assessment from 20 different broiler flocks at either 1 week, 4 weeks or 5 weeks of age, based on pilot information of the most common ages for outbreaks of BCO. A quarter of the flocks (n=5) were examined longitudinally at all three age points. For each submission, all culls or mortalities from a single day were examined, up to a maximum of 20 birds; a total of 325 birds were submitted for necropsy for this study. For each submitted bird, complete post-mortem examination was performed according to set criteria (Appendix 1). The birds were weighed and the skin and mucous membranes examined for any lesions, external parasitism or trauma (including foot pad injury). Limbs and joints were examined for swelling or malformation to suggest traumatic injury or arthritis. The vent was assessed for evidence of enteritis or polyuria, and the umbilicus was inspected for evidence of omphalitis. Full internal post-mortem examination was performed, with swabs collected for microbiological assessment of potential lesions (including swollen joints, swollen foot pads, and any lesions in liver, heart or air sacs). Samples of liver, duodenum, jejunum, ileocaecal junction and yolk sac remnants were collected in formalin for histopathological assessment, as well as representative tissues from other gross lesions observed. Cardiac blood was collected for serological testing. Bilaterally, the coxofemoral joints were disarticulated and the femoral heads were examined for evidence of damage, cartilage separation or abnormal synovial fluid. Femurs and tibiotarsi were cleanly bisected with a sterilized knife, and gross

evidence of osteomyelitis, dyschondroplasia or bacterial chondronecrosis was recorded. The proximal end of each femur and tibiotarsus was swabbed for microbiological culture, and the sectioned bone was fixed in formalin for histological assessment.

2.2.2 Histopathology

The bone and tissue samples were fixed in 10% neutral buffered formalin for seven days. The proximal end of each femur and tibiotarsus was decalcified and all tissues were processed and embedded in paraffin. Five micrometre sections of each tissue were prepared and stained with haemotoxylin and eosin. The histological features assessed in the bone specimens are summarized below:

- The presence of inflammation and/or necrosis, including location and character (eg. necrotizing, heterophilic, fibrinous, granulomatous).
- The presence, location and general morphology of bacteria.
- Degeneration of growth plate cartilage, indicated by the presence of non-artefactual separation or clefting of the cartilage, or regions of matrix eosinophilia (necrosis) within the cartilage.
- Presence of a retained cartilage plug within the metaphysis (chondrodystrophy).
- Degenerative changes within growth plate vascular channels.
- Cellular depletion within the bone marrow (bone marrow suppression).
- Lymphoid hyperplasia within the bone marrow (immunological stimulation).
- Evidence of synovitis within the adjacent joint (coxofemoral joint for femoral specimens, stifle for tibiotarsal specimens).

Histological sections of duodenum, ileum and colon were assessed for the presence of inflammation and coccidia. Sections of liver and yolk sac remnant/Meckel's diverticulum (where identified) were assessed for the presence of inflammation or infection.

2.2.3 Bacteriology

Swabs collected from the birds were inoculated onto sheep blood and MacConkey agars and incubated overnight. The plates were examined for the presence of bacterial colonies, colony morphology, abundance and purity. Presumptive identity of the bacteria was determined using conventional bacteriological and biochemical methods and preliminary bacteriology results were recorded (Appendix 2). Definitive identification and typing of bacteria was carried out using conventional, multiplex PCR techniques. Characterisation of

E. coli isolates was carried out based on virulence genotyping (presence of virulence genes *omT*, *hlyF*, *iss*, *iutA*, and *iroN*) using previously described methods (see section 2.2.3.1) (Mohsenifard *et al.*, 2016). Bacterial isolates were classified as avian pathogenic *E. coli* based on identification of any of these virulence genes.

2.2.3.1 Identification of avian pathogenic E. coli (APEC)

DNA was extracted from cultures grown on the sheep blood agar plates. Overnight bacterial cultures were harvested and re-suspended in 500 µl of sterile water, boiled for 10 min, pelleted at 16,200 × g for 1 min, and the supernatant (5 µl) was collected for use as template DNA for polymerase chain reaction (PCR) (Johnson & Brown, 1996). The sequence of the primers and expected size of the products used to confirm the presence of avian pathogenic *E. coli* DNA were summarised in Table 2. Each PCR was performed in a total volume of 25 µl containing 5 µl template DNA, 4 µl of 1.25 mM each dNTP mixture (Bio-39025; Bioline, Alexandria, NSW, Australia), 4 µl of 25 mM $MgCl_2$ (A351H; Promega, Auburn, VIC, Australia), 10 µl of 25 µM primer mixture, 1.5 U GoTaq polymerase (Promega), 5 µl of 5× GoTaq flexi green buffer (Promega) and 1 µl of nuclease-free water. The mixture was incubated at 94°C for 2 min, and then subjected to 24 cycles of 94°C for 20 sec, 60°C for 10 sec and 72°C for 20 sec and final extension step of 72°C for 5 min. PCR products were analysed by electrophoresis in a 3% (w/v) agarose gel.

Table 2. Oligonucleotide primer sequences used in identification of Avian pathogenic *E. coli* (APEC)

Gene	Amplicon size (bp)	Sequence	Gene Description (reference)
iroN	553	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	Salmochelin siderophore receptor gene (Johnson *et al.*, 2006)
ompT	496	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	Episomal outer membrane protease gene (Johnson *et al.*, 2006)
hlyF	450	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	Putative avian hemolysin (Morales *et al.*, 2004)
Iss	323	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	Episomal increased serum survival gene (Johnson *et al.*, 2008b)
utA	302	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	Aerobactin siderophore receptor gene

2.2.4 Serology

2.2.4.1 Collection and preparation of serum

Blood samples were collected directly from the heart of the culled birds at each sampling points (approximately weeks 1, 4 and 5). Blood samples were collected into BD Vacutainer® SST™ II advance tubes and were allowed to clot for 60 minutes at room temperature. The blood samples were centrifuged at 1200 × g for 10 minutes at room temperature. The serum samples were collected into 2 ml self-standing screw cap tubes Neptune™ and stored -20°C until use.

2.2.4.2 Reovirus and Infectious bursal disease virus indirect ELISAs

Indirect ELISAs were performed using the commercial IDEXX REO Ab Test (99-09264) and IDEXX IBD Ab Test (99-09260) (IDEXX Laboratories Pty Ltd, Australia). Briefly, for both tests, all test sera were diluted 1:500 with sample diluent and 100 µl of diluted sera were added to the individual wells of the plates and the plates were incubated at room temperature for 30 minutes. The contents of the plate were discarded then washed once with wash buffer. 100 µl of prediluted HRP anti-chicken conjugate was added and the plates were incubated at room temperature for 30 minutes. The conjugate was discarded and the plates were washed as described before. A 100 µl TMB substrate was added to each well and the plates were incubated for 15 minutes at room temperature under ambient light. The reaction was stopped by adding 100 µl of stop solution and the absorbance of each well at 650 nm was measured using a *MULISKAN FC* plate reader (Thermo scientific™). Results for individual samples were determined by calculating the ratio of sample absorbance compared to positive control. Samples with a ratio above 0.2 were considered positive.

2.2.4.3 Chicken anaemia virus blocking ELISA

A blocking ELISA was performed using IDEXX CAV Ab Test (99-08702) (IDEXX Laboratories Pty Ltd, Australia). All test sera were diluted 1 in 10 with sample diluent and 100 µl of diluted sera were added to the individual wells of the plate, incubated for 60 minutes at room temperature. The contents of the plate were discarded then washed once with wash buffer. 100 µl of prediluted HRP anti-CAV conjugate was added and the plates were incubated at room temperature for 30 minutes. The contents of the plate were discarded then washed with wash buffer. A 100 µl TMB substrate was added for to each

well and the plates were incubated for 15 minutes at room temperature under ambient light. The reaction was stopped by adding 100µl of stop solution and the absorbance of each well at 650 nm was measured using a *MULISKAN FC* plate reader (Thermo scientific^{TM}). Results for individual samples were determined by calculating the ratio of sample absorbance compared to negative ratio. Samples with a ratio below 0.6 were considered positive.

2.3 Statistical analyses

Bacterial isolates from the femur or tibiotarsus (both with and without inflammation), isolates from other sites, and numbers of bacterial isolates in different flocks and ages were compared using Fisher's exact test (IBM SPSS Statistics, version 22). Statistical association between presence of BCO and other gross, histological and bird features was assesed using Fisher's exact test (GraphPad Prism, version 6). Associations for ages and lesion locations were assessed by binary logistic regression and flock size correlations were assessed by linear regression analysis (IBM SPSS Statistics, version 22). Bodyweight data sets were analysed by Mann-Whitney u test (GraphPad Prism, version 6). Analysis of serological data was carried out using Minitab® Statistical Software version 17.1.0 (Minitab Inc). Optical densities obtained from CIAV, Reovirus and IBDV ELISAs were subjected to one-way ANOVA to visualise differences among the means of different time points for each ELISA. The paired t-confidence interval and test procedures were used to analyse the differences between means of optical densities between different groups. *p* values ≤ 0.05 were considered statistically significant

3 Results

3.1 Epidemiological survey into farm and flock risk factors associated with lameness culls

3.1.1 Temperature and rainfall and median monthly lameness culls

The frequency of percentage lameness culls by placement month is shown in Fig 1. The median percentage lameness culls from all flocks was 0.67%. The months with a statistically significant number of flocks higher than the median were January (p = 0.002), June (p = 0.004), October ($p < 0.001$) and December (p = 0.010). Other than two consecutive summer months of higher than median lameness culls, no discernible pattern could be seen with the other months of high lameness culls. There was no statistically significant relationship between external monthly average temperature or total monthly rainfall and the median percentage lameness culls for flocks placed that month (Fig 2).

3.1.2 Multivariate mixed model of flock and shed characteristics

3.1.2.1 Male to female ratio and median lameness culls

The median number of lameness culls was higher in all male flocks (1.58% compared to 0.61% and 0.67% in all female or mixed flocks respectively (Fig 3)), but the difference was not statistically significant.

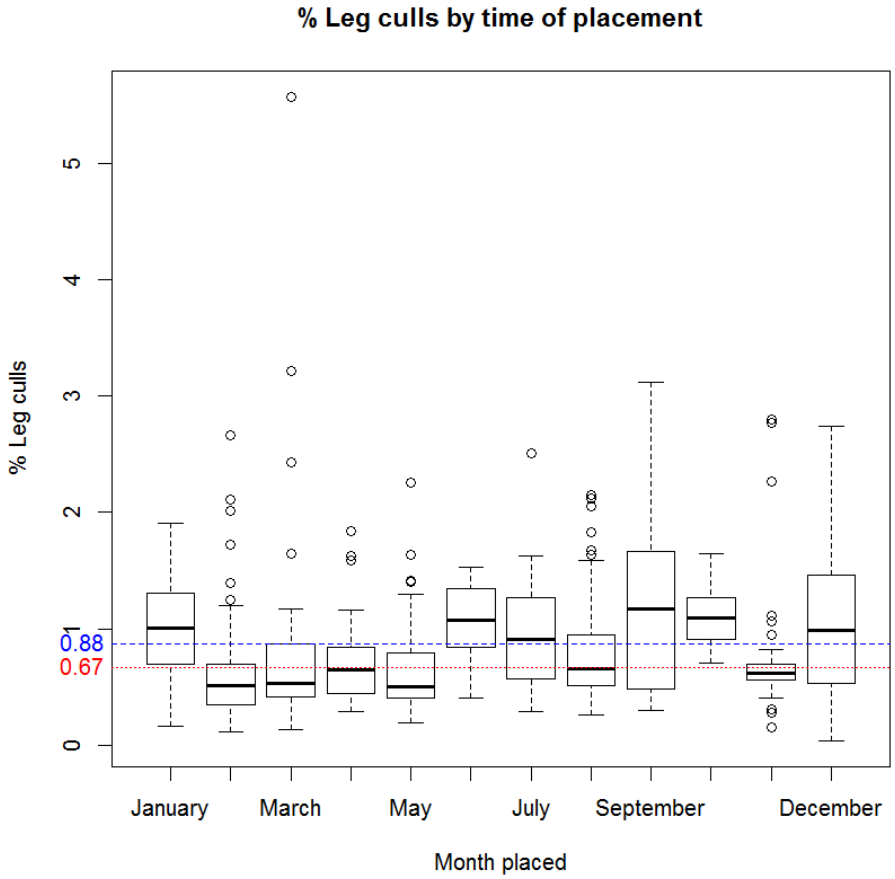

Figure 1. *Percentage lameness culls by month placed. Median percentage leg culls are shown with a red line, mean values with a blue dashed line.*

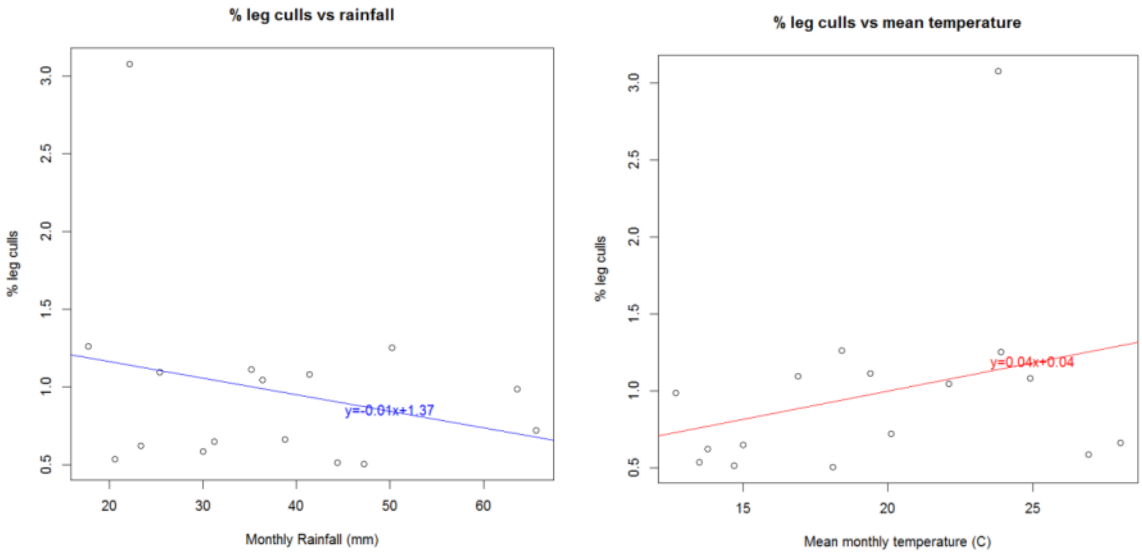

Figure 2. *Linear models for the relationship between monthly total rainfall (mm) and mean temperature and the median percentage lameness culls of flocks placed in those months. No statistically significant relationship was found.*

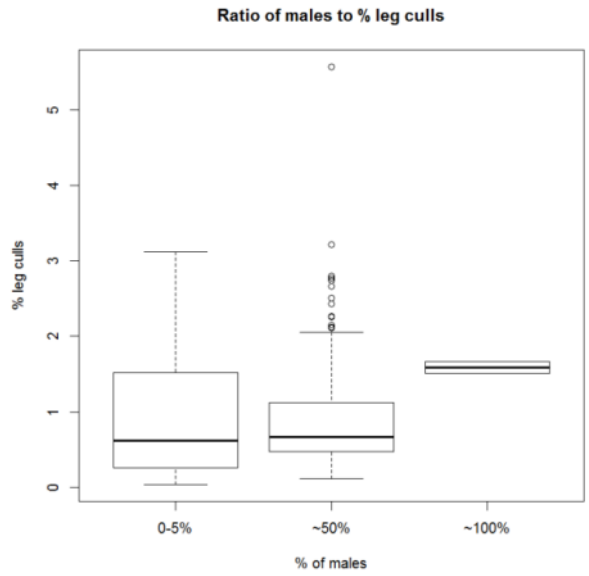

Figure 3. *Percentage lameness culls by male to female ratio. Differences between flocks were not statistically significant (p = 0.14)*

3.1.2.2 Other reasons for culling

A small positive correlation was noted between the percentage of lameness culls and the percentage of non-start culls (birds culled for non-specific poor development within first few days of production) (R^2 = 0.1, p < 0.001) within a broiler flock. No statistically significant correlation was found between the percentage of lameness culls and other reasons for culling or the percentage mortality (deaths not due to culling).

3.1.2.3 The relationship between stocking density and lameness culls

The total number of birds placed divided by the area of the shed was used to calculate the birds/m^2 housed for each flock. Sheds which were divided to run two flocks separately (e.g. males and female birds) were excluded from the analysis as the area provided each sub-flock was not provided. The majority of shed stocking densities clustered around 17 to 18 birds/m^2. The relationship between stocking density and percentage lameness culls was not significant (Fig 4).

3.1.2.4 The relationship between age of farm and lameness culls

Broiler sheds included in this analysis ranged in age from 3 to 40 years. Farm age was not a significant explanatory variable for the percentage of lameness culls.

3.1.2.5 The relationship between shed design and lameness culls

The design of the shed walls was significantly correlated (regression coefficient = 0.67) with the percentage lameness culls in flocks (p = 0.004). The median percentage lameness culls was higher in sheds with curtain walls than those with solid walls (Fig 5). The type of floor (earth or concrete) did not have a significant relationship to the percentage lameness culls of a flock (p = 0.130). All farms in this investigation raised birds on 100% litter therefore no comparison could be made between different types of litter systems. Most farms reported using either wood shavings or rice hulls as a litter substrate but this information was not given for individual flocks, and therefore no analysis was possible on the type of litter.

The average distance between feeder and drinker lines (shed width/number of feeder and drinker lines) had a near significant relationship with the percentage lameness culls (regression coefficient -1.03, p = 0.054). The percentage lameness culls compared to the average distance between feeder and drinker lines is shown in more detail in Figure 6. The sheds with the lower distances had higher median percentage lameness culls than the sheds with more space between the drinkers and feeders (0.94%, 1.43%, 0.62%, 0.5%, 0.42% and 0.7% for sheds with 1.41m, 1.45m, 1.51m, 1.57m, 1.62m and 1.68m respectively). However, a statistically significant difference in the percentage lameness culls was only apparent in flocks with an average distance of 1.45m when compared to the other groups.

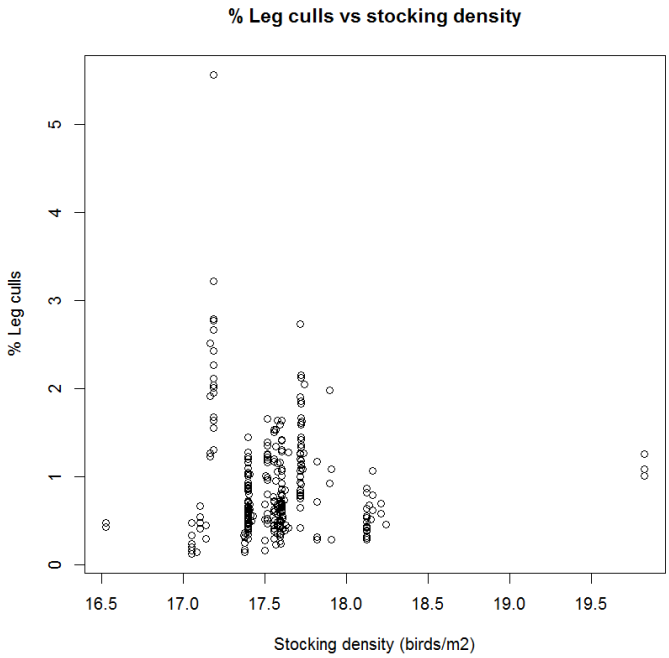

Figure 4. *The percentage lameness culls versus the average stocking density of flocks in the shed. The regression coefficient for stocking density was -0.07 once possible confounding variables such as the farm and shed were included in the model. The relationship was not statistically significant.*

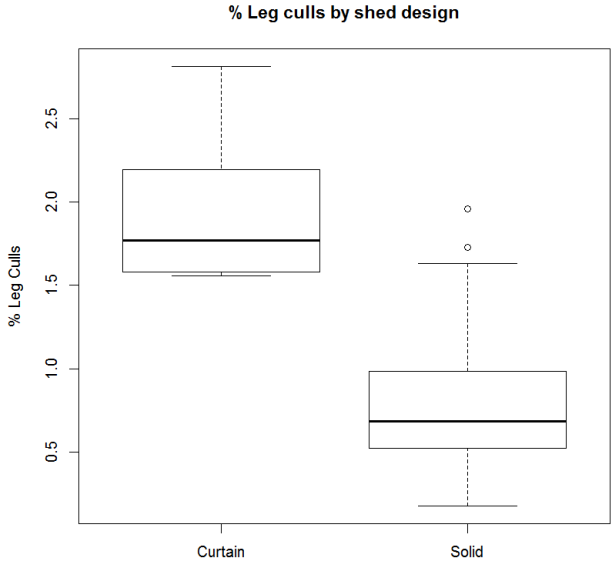

Figure 5. *Percentage leg culls by shed wall construction. The median lameness culls in curtain wall sheds was 1.7% while solid wall sheds had a median of 0.68% lame culls when averaged by shed. The difference in lameness culls between the two types of sheds was statistically significant in the mixed model.*

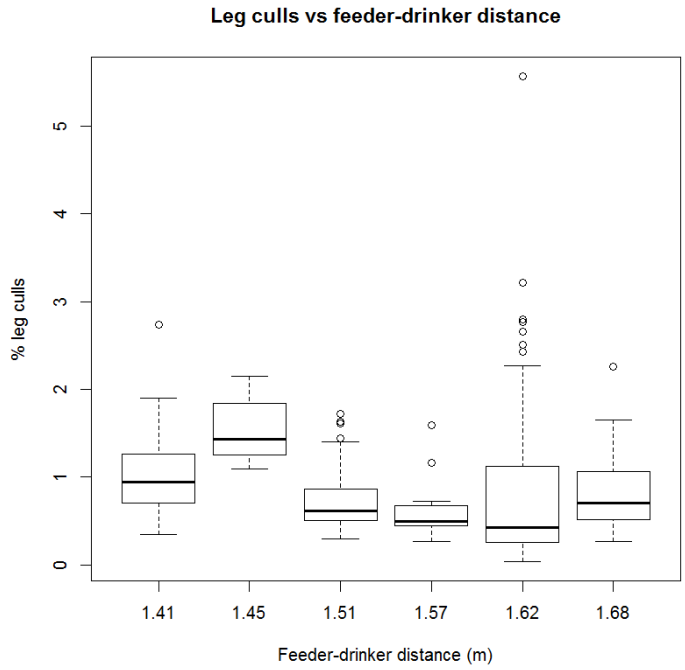

Figure 6. *Percentage lameness culls versus average feeder-drinker line distance. The smaller distances have higher median percentage lameness culls (1.41m – 0.94% , 1.45m – 1.4%), but only the sheds with 1.45m between drinker and feeder lines was significantly different to the other groups.*

3.2 Pathological and microbiological analysis of cull birds from commercial farms

3.2.1 Bacteriology

A total of 287 bacterial isolates were recovered from the tissues of 109 birds. The overall summary of isolated bacteria is shown in Table 3. *E. coli* was the most common bacteria isolated from the birds (66.2% of total), followed by *Staphylococcus* spp. (17%) and other Gram-positive organisms (16.8%). Of birds with positive bone cultures, 41% were positive for the same bacterium in multiple bones. Almost all the *E. coli* isolates (99%) from bones with inflammation were classified as avian pathogenic *E. coli* (APEC) (Table 4) although they belonged to a number of different serotypes (results not shown). Of the 154 *E. coli* isolates from bones, 71 isolates (46% of total) were positive for the all the virulence genes examined. In birds with *E. coli* isolated from multiple bones, the virulence gene distribution was identical at all sites in 63% of cases. A total of 41 staphylococcal isolates were recovered from bones and 6 of these isolates were categorised as coagulase positive *Staphylococcus spp.*

Table 3. Total bacteria isolated from all sites

Site	Total isolates	*E.coli*	Staph spp.	Others	Total
Left femur	63	40	11	12	276
Right femur	59	40	9	10	274
Left tibiotarsus	61	37	13	11	278
Right tibiotarsus	59	39	8	12	270
Yolk sac	9	8	0	1	68
Liver	10	7	3	0	60
Pericardium	3	3	0	0	4
Peritoneum	3	3	0	0	3
lungs	0	0	0	0	1
Air sacs	3	0	3	0	3
Right hock	6	5	0	1	11
Left hock	4	3	1	0	6
Right knee	2	1	0	1	3
Left knee	1	1	0	0	2
Right foot pad	2	1	1	0	2
Left foot pad	1	1	0	0	1
Skin wound	1	1	0	0	1

Table 4. Distribution of the virulence genes among the APEC isolates in the proximal end of the femur or tibia of the birds

Virulence gene pattern	No. isolates
iutA, iss, hlyF, omT and *iroN*	71
iutA, iss, hlyF and *omT*	26
iutA, iss, hlyF and *iroN*	33
iutA, iss and *omT*	2
iutA and *iss*	3
iss, hlyF and *omT*	1
iss, hlyF and *iroN*	9
omT and *iroN*	3
iroN only	6

3.2.1.1 Bacterial isolates from the proximal head of the femur or tibiotarsus bones

Due to the possibility of post-mortem bacterial colonization of the bone, cases of BCO in this study were defined as birds with evidence of inflammation within the proximal tibia or femur, rather than the presence of bacterial organisms alone (see section 2.2 for further details). Of the birds with positive bacterial culture and histological evidence of inflammation in the bone (n = 52), *E. coli* was recovered from 34 birds (65.3%), *Staphylococcus* spp. were isolated from 8 birds (11.5%), other bacteria species were isolated from 8 birds (15.3%) and a mixed population was recovered from the 4 birds (8%). There was a strong association between the presence of a positive bacteriological culture of the femur and the presence BCO (confirmed by histopathology, please see below) in the same leg. The odds of left-sided BCO for birds that had a positive bacteriological culture of the left femur was 17 (95% CI 4.5-108) times that of birds that had a negative bacteriological culture of the left femur (*z* test statistic 3.655; $p < 0.010$). The odds of right-sided BCO for birds that had a positive bacteriological culture of the right femur was 18 (95% CI 5.9-76) times that of birds that had a negative bacteriological culture of the right femur (*z* test statistic 4.522; $p < 0.010$).

Bacterial isolates from birds with histologically-confirmed BCO stratified by age are listed in Table 5. There were significantly higher numbers of *E. coli* isolates than *Staphylococcus* spp. present in all flocks at all time points (week 1: *E. coli,* 84% and *Staphylococcus* spp. 6.4%, $p < 0.0001$; week 4: *E. coli,* 68% and *Staphylococcus* spp. 6.4%,$p < 0.0004$; week 5: *E. coli,* 60% and *Staphylococcus* spp. 6.6%,$p < 0.0315$). Of birds with positive bone cultures, 55% were positive for the same bacterium in other non-bone tissues, but as cultures were only taken from other tissues when gross lesions were identified, the statistical correlation between bone and tissue isolates could not be assessed.

Table 5. Age distribution of bacterial isolates and histological detection inflammation in the proximal end of the femur or tibia of the birds

Age (Weeks)	Total number of isolates	Number of *E.coli* isolation	Number of Staph. isolation	Number of Other bacteria	Number of Mixed isolations	Total cases (No of birds)
1	77	65[a]	5[b]	6	1	27
4	31	21[a]	2[b]	7	1	17
5	15	9[a]	1[b]	3	2	8

Values marked with the same superscript in the same row are not significantly different ($p > 0.05$)

3.2.2 Histopathology

3.2.2.1 Histological assessment of lesions

Due to the possibility of post-mortem bacterial colonization of the bone, cases of BCO in this study were defined as birds with evidence of inflammation within the proximal tibia or femur, rather than the presence of bacterial organisms alone (Fig 7). With the presence of histological inflammatory lesions as the gold standard for diagnosis of BCO, visual detection of bacteria in the bone was found to have a good negative predictive value (95.6%) but a poor positive predictive value (61.7%). Of bones with evidence of BCO, 46.4% were femurs, and 53.6% were tibias. 29.3% of lesions were present within the growth plate, 25.7% were within the subchondral bone, and 45.0% were in both sites.

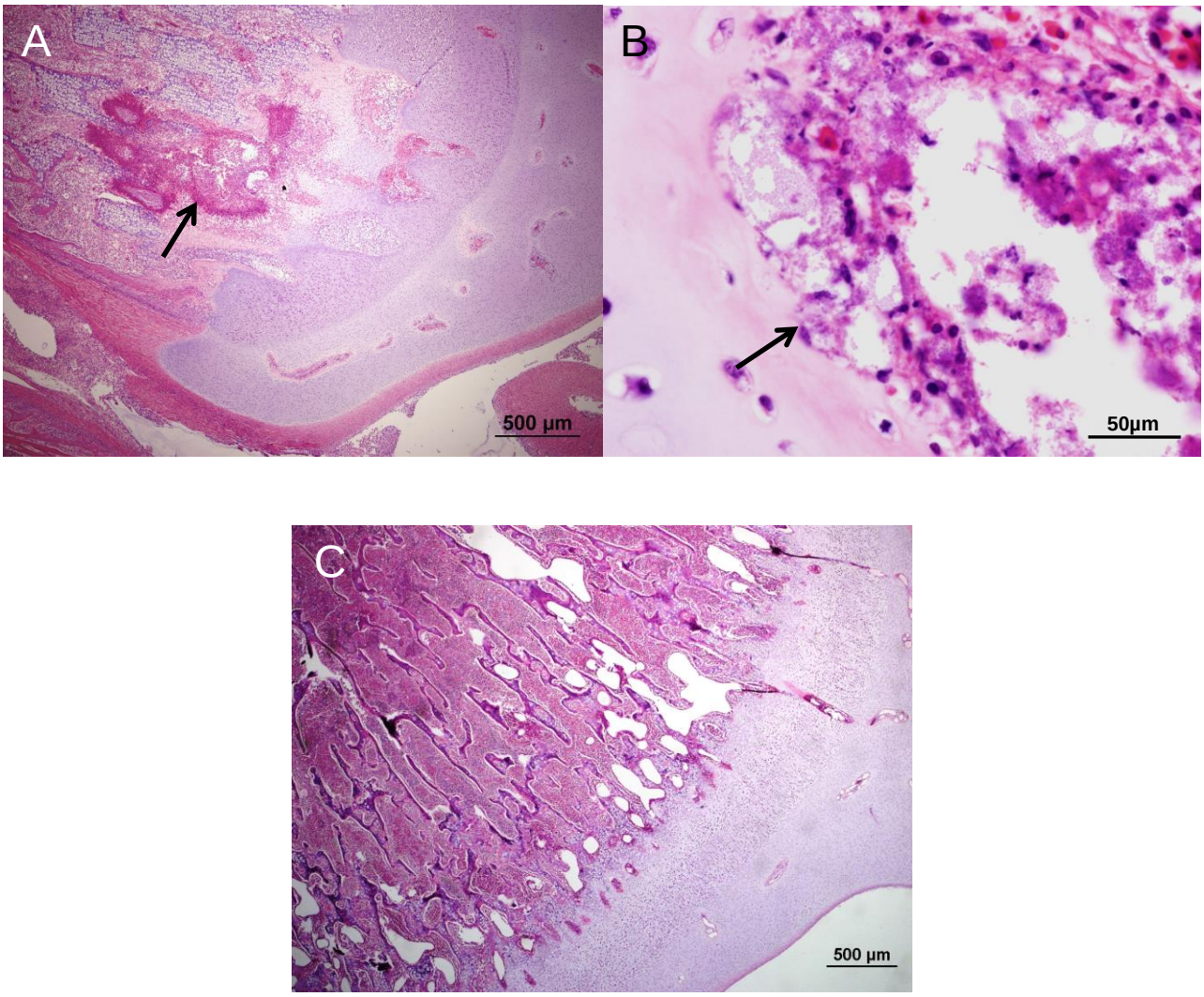

Figure 7. A. *An example of a typical BCO inflammation within the epiphyseal growth plate (arrow).* ***B.*** *Bacterial colonies arising within cartilage channel (arrow).* ***C.*** *Growth plate of normal control bird*

Degeneration of epiphyseal growth plate cartilage was defined as evidence of matrix eosinophilia (indicative of necrosis), or separation or clefting within the cartilage. Separation of zones of the epiphyseal growth plate was very common, but in many cases this appeared to be artefactual. For this study, only cases with additional evidence of remodelling, debris within the clefts or marginal matrix alterations at the site of separation were considered genuine (Fig 8). There was no significant variation in prevalence of cartilage degeneration with age (p = 0.08); however, there was a slight but significant increase in prevalence of degeneration in femurs compared to tibiotarsi (15.6% and 11.2% respectively, p = 0.032).

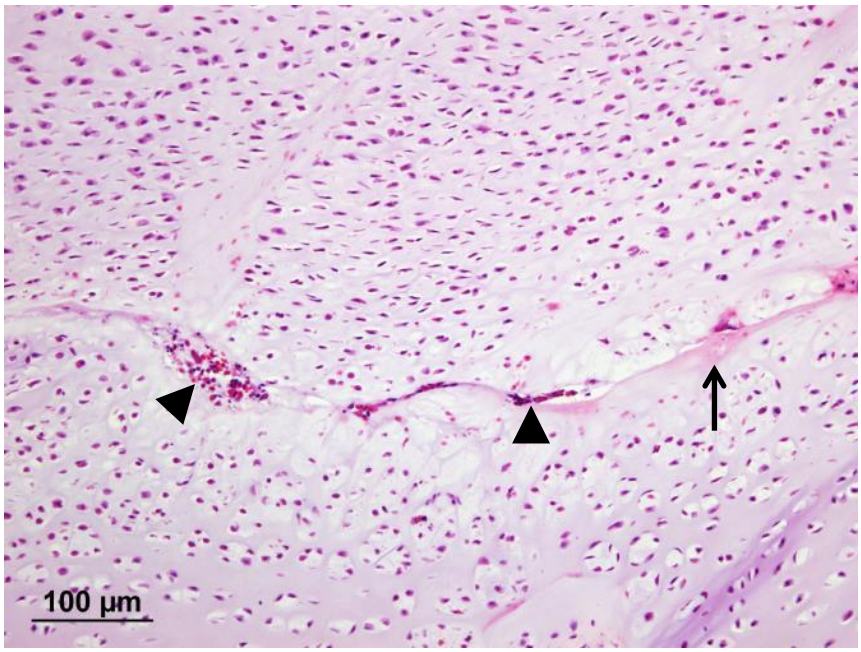

Figure 8. *Non-artefactual separation at the hypertrophic zone of the growth plate. Note evidence of remodelling at the margins of the cleft (eosinophilic matrix, arrow) and cellular debris within the cleft (arrowheads).*

Retained cartilage plugs - defined as persistence of non-remodelled growth plate cartilage extending into the diaphysis Fig 9 - were observed in significantly more tibiotarsi than femurs (18.1% of femurs and 23.4% of tibiotarsi, $p = 0.031$). Plugs predominantly occurred in birds under 2 weeks of age (49.2% of birds) and were rare in older birds (1.4% of birds aged between 2-4 weeks, 2.7% of birds over 4 weeks of age); this difference was statistically significant ($p < 0.0001$).

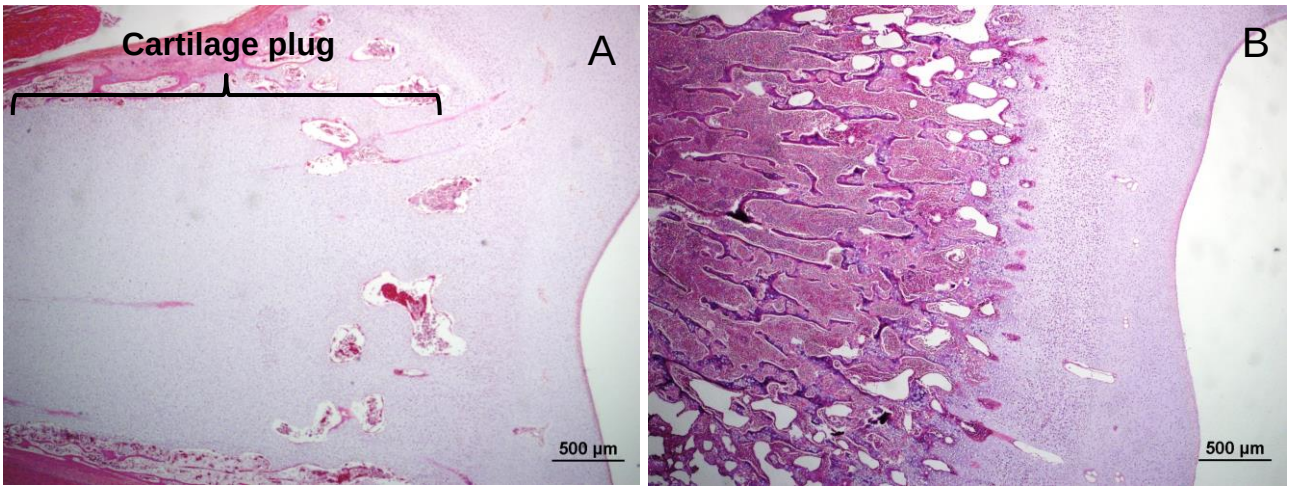

Figure 9. A. *Large plug of retained cartilage extending down the diaphysis as indicated.* ***B.*** *Control bird of same age with normal metaphyseal cartilage remodelling.*

Histological evidence of vascular degeneration within the growth plate was extremely common (identified in 43.6% of bones), but the degeneration typically displayed ordered progressive replacement by cartilage matrix without evidence of necrosis in areas supplied by the vessel. Early changes in degenerate vessels consisted of fibrin accumulation within cartilage channels, typically in association with pyknotic nuclear debris (Fig 10b). This initial degenerative change was followed by induction of chondrocytic metaplasia of mesenchymal cells within the channel (Fig 10c). Subsequently, these cells generated chondroid matrix which eventually effaced the cartilage channel (Fig 10d). There was a statistically significant increase in the number of birds displaying vascular degeneration at 4 and 5 weeks compared with birds < 2 weeks of age (23.8% of birds <2 weeks old versus 53.8% of birds 2-4 week old and 60.8% of birds 5 weeks old, $p < 0.0001$), and a higher prevalence of vascular degeneration was noted in femurs than tibiotarsi (48.9% of femurs versus 38.2% of tibiotarsi, $p = 0.025$).

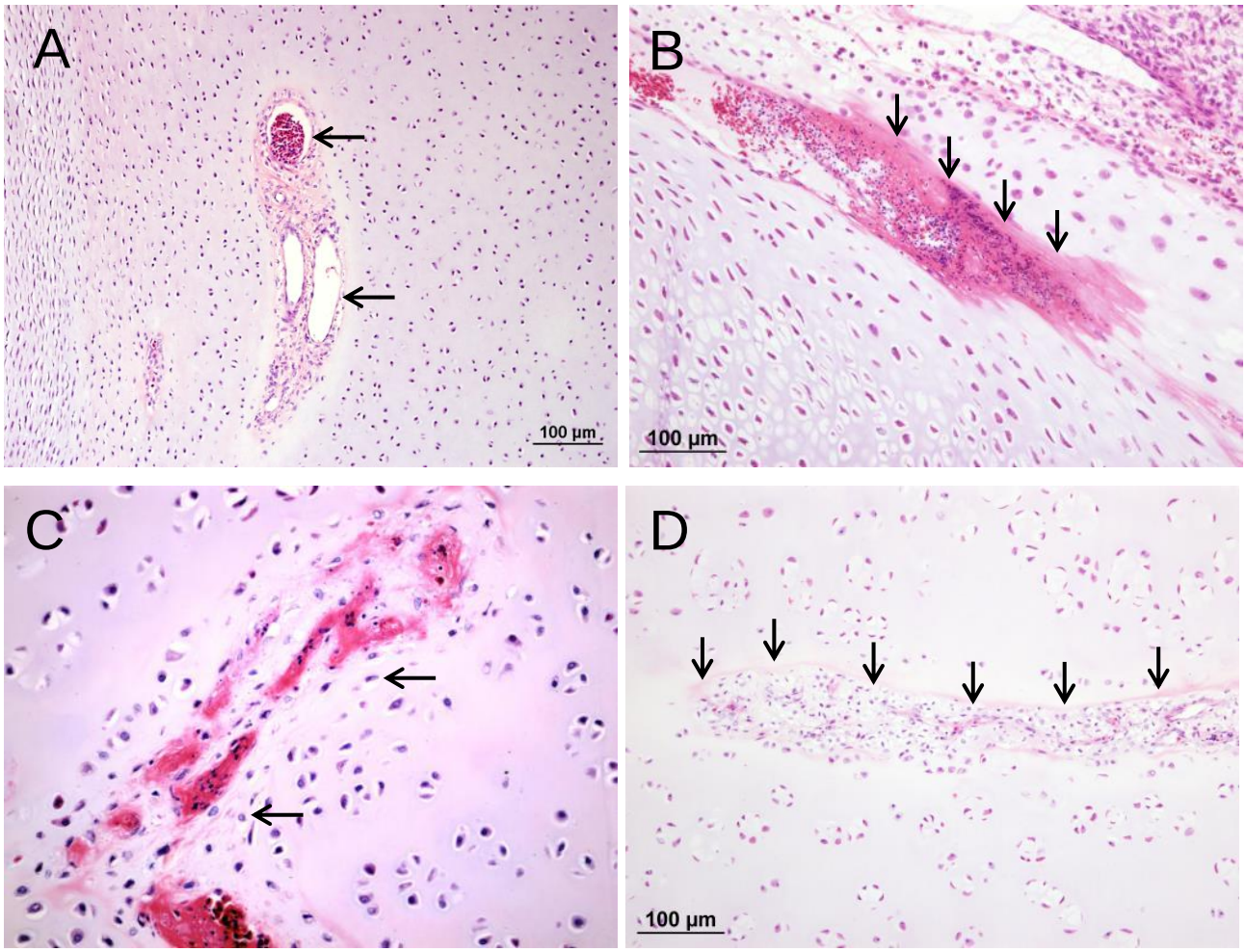

Figure 10 A. *Normal vascular channel containing patent blood vessels (arrows).* ***B.*** *Early stage vascular regression: cartilage channel displaying fibrin accumulation containing pyknotic nuclear debris (arrows).* ***C.*** *Mid-stage vascular regression. Chondrocytic metaplasia of mesenchymal cells (arrows) surrounding degenerate vessels.* ***D.*** *Late stage vascular regression. Effacement of channel by cartilage matrix (arrows).*

Lymphoid follicles within the bone marrow (Fig 11) were documented as a possible correlate for immunological stimulation. There was significant variation in prevalence of lymphoid follicles with bird age, with birds older than 2 weeks of age significantly more likely to display follicles (0.9%, 9.3% and 6.2% in birds <2 weeks, 2-4 weeks and >4 weeks, respectively, $p < 0.0001$), and distribution within bones was also uneven, with more follicles in the femur than tibia (6.5% versus 3.7%, $p = 0.033$).

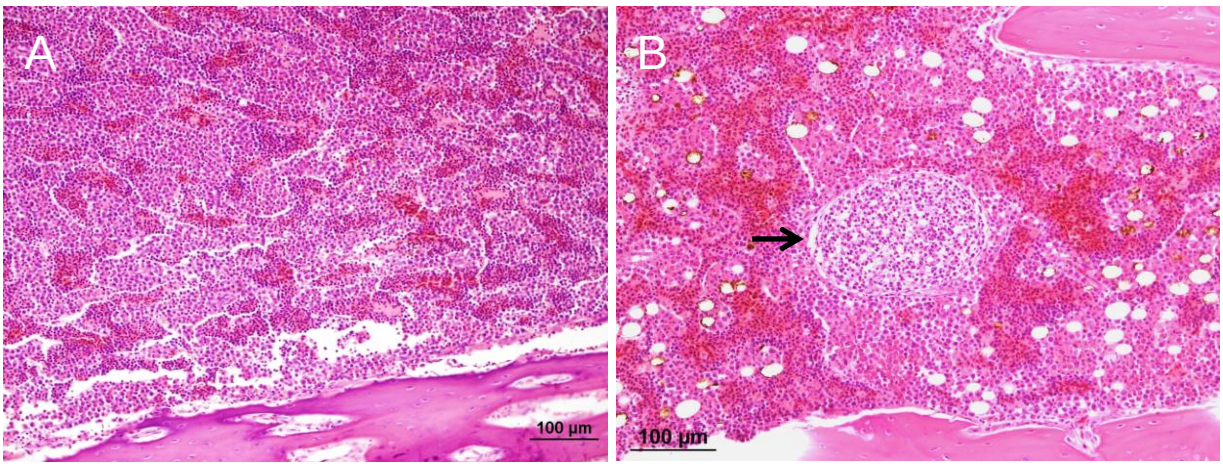

Figure 11. A. *Normal bone marrow.* ***B.*** *Bone marrow with follicular lymphoid hyperplasia (arrow).*

Synovitis was predominantly (76.9%) fibrinous, heterophilic or necrotizing (Fig 12), with the remainder being proliferative or lymphoplasmacytic. Synovitis was significantly more likely to occur in the stifle than the coxofemoral joints (9.2% versus 16.5%, $p < 0.0001$), but the prevalence of synovitis did not significantly vary with age.

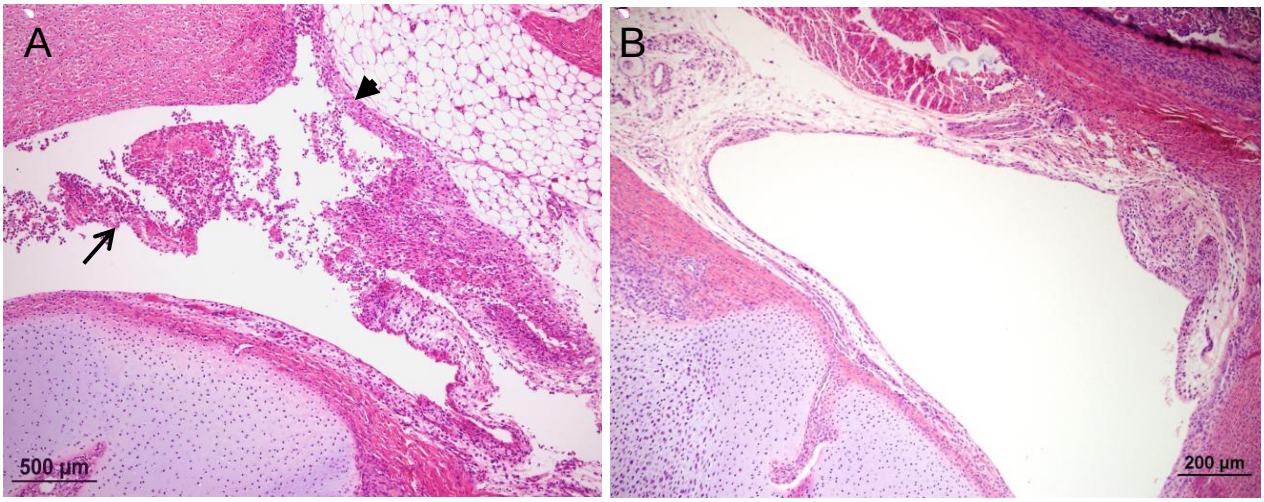

Figure 12. A. *Example of fibrinoheterophilic synovitis with exudate in joint (arrow) and thickened synovium (arrowhead).* ***B.*** *Normal control with thin synovium and no exudate*

Infection with coccidia was relatively mild in all cases, and none of the birds displayed evidence of protozoal invasion or inflammation extending beyond the mucosa (Fig 13). Similarly, where detected, other gastrointestinal lesions were only mild in nature, predominantly manifesting as increased numbers of crypts distended by debris (crypt microabscesses), sometimes associated with fibrosis (Fig 14**)**. Clinically significant ulcerative or necrotizing gastrointestinal disease was extremely rare (<5 cases). Prevalence of enteritis did not vary significantly with age.

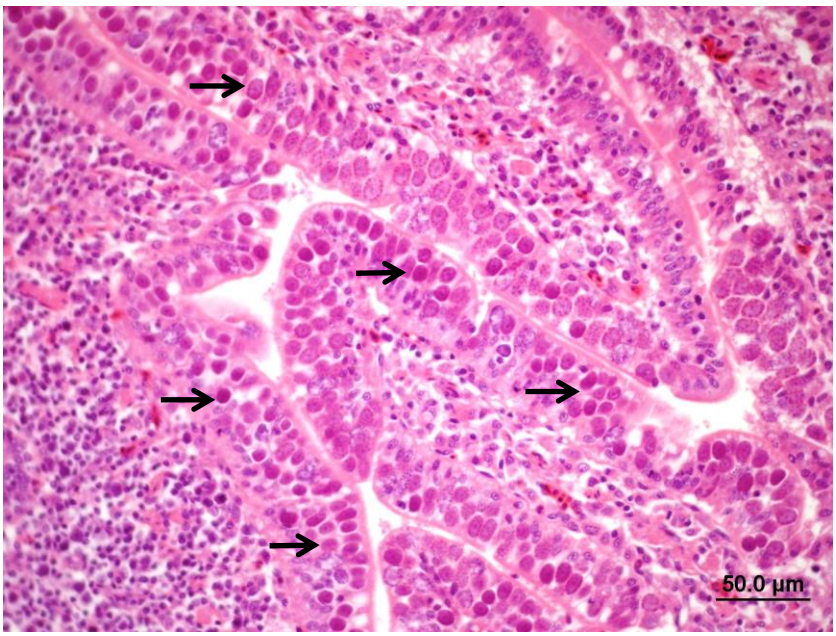

Figure 13. *Duodenal mucosa containing frequent coccidial oocysts and other life stages (arrows).*

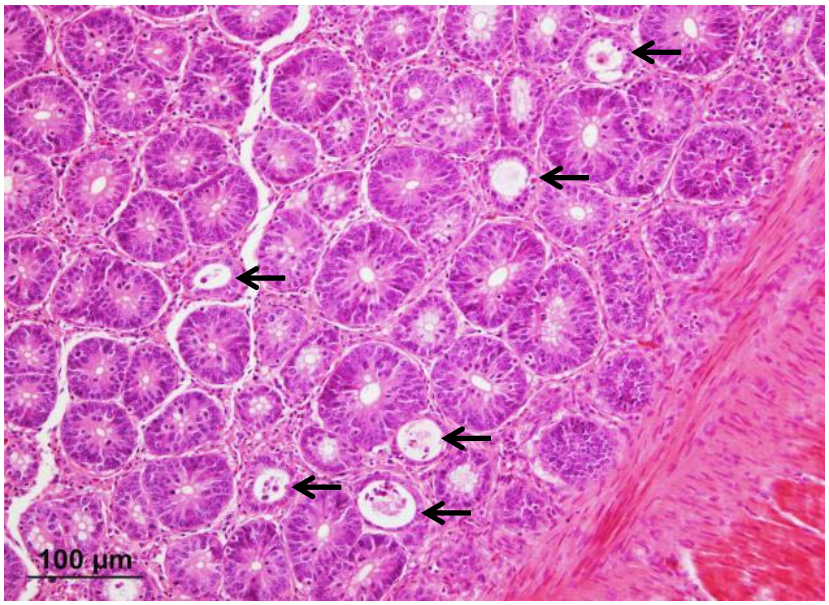

Figure 14. *Duodenal mucosa with increased numbers of distended crypts containing degenerate leukocyte debris (arrows).*

Hepatitis predominantly presented as multifocal areas of necrosis associated with a variable inflammatory cell infiltrate (Fig 15). There was no significant association between the prevalence of hepatitis and age.

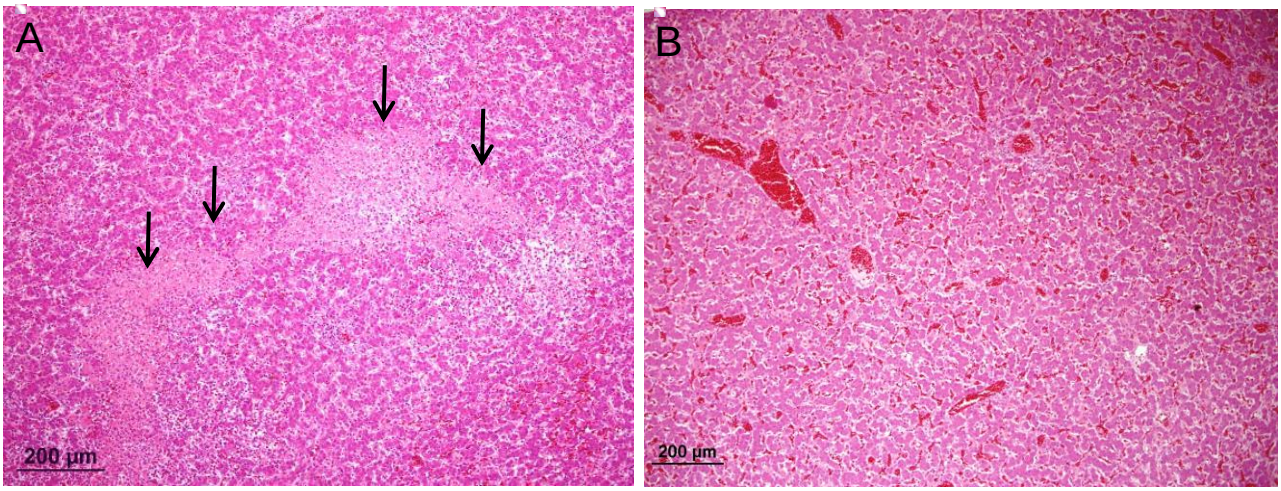

Figure 15. A. *Typical coalescing regions of parenchymal necrosis in a case of hepatitis (arrows).* ***B.*** *Normal control liver.*

Omphalitis and yolk sac infection were grouped together, defined as the gross observation of an unhealed or exudative navel, or histological evidence of inflammation within the yolk sac remnant (Fig 16). Expectedly, there was a statistically significant decrease in prevalence of omphalitis or yolk sac infection with age (44.7% of birds less than 2 weeks, versus 22.1% of birds 2-4 weeks old and 6.8% of birds over 4 weeks old, $p < 0.0001$).

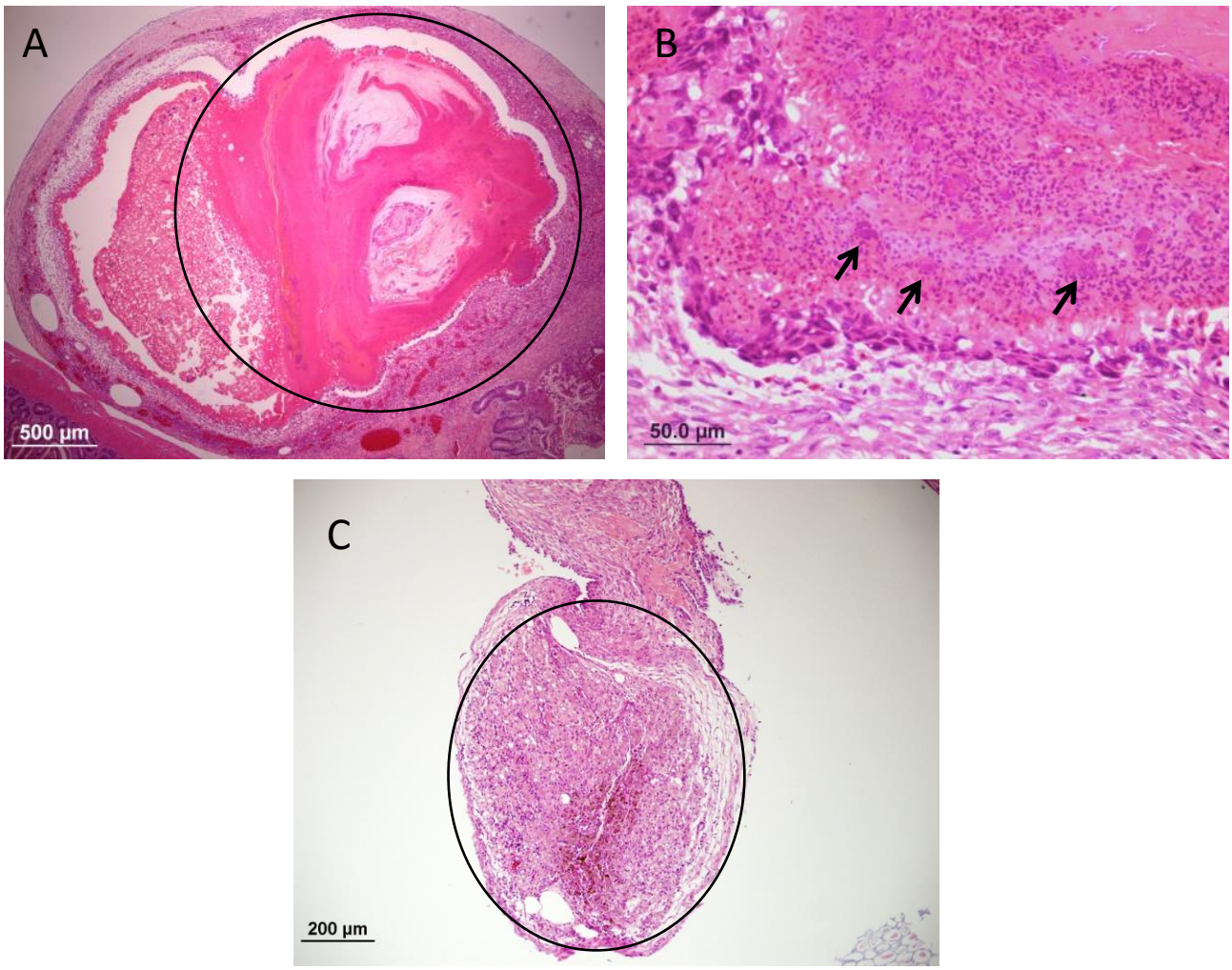

Fig 16 A. *Yolk sac remnant from 4 week old bird displaying accumulation of fibrinous exudate (circled).* ***B.*** *High magnification of (a) displaying degenerate heterophils and small bacterial colonies (arrows).* ***C.*** *Control yolk sac undergoing normal regression, with central cavity effaced by fibrous tissue and macrophages (circled).*

3.2.2.2 Prevalence of BCO

For this study, a case of BCO was defined as the presence of inflammation within the proximal femur or tibia. Based on this definition, the prevalence of BCO in the mortalities or culled birds examined is summarized in Tables 6 and 7 overleaf:

Table 6. Prevalence of histologically confirmed BCO in necropsied birds

	Mean % flock with BCO	Standard deviation	Range
All flocks	26.3%	17.3%	0-50%
Single time-point sampled flocks	27.4%	18.4%	0-50%
Flock by age			
0-2 weeks	28.4%	18.0%	0-50%
2-4 weeks	29.3%	17.7%	0-42.8%
5 weeks	22.1%	17.3%	0-36.4%

Table 7. Prevalence of histologically confirmed BCO at different ages in necropsied birds from longitudinally sampled flocks

	% culled birds in flock with BCO			
	Week 1	**Week 4**	**Week 5**	**Overall**
Flock 1	10.00%	42.86%	16.67%	21.7%
Flock 2	45.00%	0.00%	0.00%	30.0%
Flock 3	15.38%	42.86%	36.36%	31.6%
Flock 4	10.00%	5.56%	0.00%	3.60%
Flock 5	41.18%	25.00%	25.00%	34.50%
Overall	24.31%	23.26%	15.61%	24.28%

There was marked variation in the prevalence of BCO in culled/mortality birds between flocks, and even between ages in the same flocks. Overall mean BCO prevalence in culled/mortality birds remained remarkably similar between groups, with roughly a quarter of all birds displaying histological evidence of disease. The prevalence did not vary substantially with age, though there was a small non-significant decreasing trend in the 5 week old birds. Maximum flock culled/mortality prevalence was 50%, and BCO was not identified (0% prevalence) in 3 flocks sampled at single time-points, as well as in two of the longitudinally sampled flocks.

3.2.2.3 Association between BCO and other pathology

The co-prevalence of other diseases in birds with BCO is summarized in Table 8, while prevalence of lesions in longitudinally sampled flocks is presented in Table 9.

Almost 50% of birds with BCO displayed lesions in multiple bones. Birds with evidence of hepatitis were over 10 times more likely to have concurrent BCO than unaffected birds, and over 80% of birds with hepatitis had concurrent BCO. Birds with synovitis were also

greater than 10 times more likely to display BCO than those without BCO lesions. The presence of cartilage abnormalities either growth plate cartilage defects or retained cartilage plugs within the medulla was also strongly correlated with the occurrence of BCO. Non-significant association trends were noted with vascular channel regression (p = 0.0724), and concurrent respiratory disease (p = 0.0729), though the calculated correlation was slight.

No significant association was noted between BCO and omphalitis/yolk sac infections, skin scratches, tibial malformation (either within the same limb or the contralateral limb), presence of coccidia or enteritis, lymphoid hyperplasia within bone marrow, or marrow depletion (see Table 8). While no significant correlation was noted with serositis, there was a trend towards statistically significant association in serositis cases where *E. coli* was isolated (p = 0.064), in comparison with *Staphylococcus* (p = 0.3).

Table 8. Association between BCO and other lesions

Lesion	Unit assessed	Overall % affected	% with concurrent BCO	Δ prevalence with BCO (%)	*p* value	Odds ratio
BCO in multiple limbs	per bird	-	44.6%	-	**<0.0001**	12.89
Respiratory disease	per bird	6.6%	10.8%	4.3%	0.073	2.333
Bone marrow depletion	per bird	3.3%	2.5%	-0.8%	0.734	0.6605
Coccidia	per bird	46.0%	45.5%	-0.5%	0.946	0.9722
Enteritis	per bird	67.3%	69.7%	2.4%	0.826	1.173
Skin injuries	per bird	21.0%	21.4%	0.4%	0.877	1.038
Hepatitis	per bird	53.7%	81.0%	27.3%	**<0.0001**	10.38
Serositis	per bird	12.5%	12.1%	-0.5%	0.884	0.944
Serositis - E. coli	per bird	41.3%	51.3%	10.0%	0.064	3.158
Serositis - Staph	per bird	13.8%	25.0%	11.2%	0.3	3.167
Omphalitis/Yolk infection	per bird	25.5%	28.9%	3.4%	0.461	1.273
Tibial malformation	per limb	8.1%	10.2%	2.1%	0.4	1.364
Cartilage defects	per bone	13.4%	28.7%	15.3%	**<0.0001**	3.183
Retained cartilage plug	per bone	20.8%	31.6%	10.9%	**0.0008**	1.944
Growth plate vascular regression	per bone	43.6%	50.7%	7.1%	0.072	1.389
Bone marrow lymphoid hyperplasia	per bone	5.1%	2.2%	-2.9%	0.102	0.3873
Synovitis	per bone	12.8%	47.8%	35.0%	**<0.0001**	10.68

Table 9. Lesion prevalence in longitudinal studies by time point

	Lesion (% birds affected)																																				
	Resp disease			BM depletion			Coccidia			Enteritis			Skin injuries			Hepatitis			Serositis			Omphalitis			Tibial malform			Cartilage defects			Cartilage plug			Vascular degeneration			Lymphoid hyperplasia			Synovitis		
	Week			Week			Week			Week			Week			Week			Week			Week			Week			Week			Week			Week			Week			Week		
	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5	1	4	5
Farm 1	0	0	8	10	0	0	0	67	8	43	50	33	5	71	83	-	-	-	20	7	67	60	7	0	0	0	8	45	14	58	70	0	8	20	86	83	0	0	0	10	29	17
Farm 2	0	0	0	15	0	0	0	25	-	100	50	-	0	100	100	57	-	-	5	14	50	45	14	0	0	0	0	25	86	50	80	14	0	60	100	100	0	0	0	55	0	0
Farm 3	0	21	9	15	0	0	-	79	50	-	71	75	0	21	0	-	67	63	8	14	0	62	21	18	77	36	27	8	29	45	92	0	9	38	100	100	0	57	27	15	50	45
Farm 4	10	11	10	0	0	0	-	50	40	-	58	40	0	6	20	-	29	10	30	0.	10	10	22	20	50	39	30	0	0	0	100	6	20	80	100	100	0	28	10	80	22	20
Farm 5	17	0	0	6	0	25	-	-	-	-	-	-	0	100	100	-	-	-	24	0.	0	82	25	0	0	0	0	76	63	75	71	0	0	59	75	100	0	0	0	29	13	25

3.2.2.4 Correlation with other bird factors

BCO was identified in 26.6% of male birds and 32.86% of female birds; this difference was not statistically significant (p = 0.355, odds ratio 0.742). The association of BCO with bodyweight is summarized in Table 10.

Table 10. Correlation between BCO and bodyweight at different ages

	Mean weight without BCO (g)	Mean weight with BCO (g)	P value
Birds < 2 weeks old	107.4	75.3	**0.0021**
Birds 2-4 weeks old	961.2	822.2	**0.0495**
Birds 5 weeks old	1469	1339	0.3629

There was a significant correlation between BCO and lower bodyweight in birds under 5 weeks of age, but this association did not hold for 5 week old birds.

There was no statistically significant association between BCO and the hatchery of origin (p = 0.273, Table 11). This holds true even for exclusive assessment of birds <2 weeks of age (data not shown, p = 0.233).

Table 11. Prevalence of BCO by hatchery

Hatchery	Total % birds with BCO	Mean farm prevalence with BCO
Hatchery 1	24.10%	27.10%
Hatchery 2	30.90%	28.40%

3.2.3 Serology

3.2.3.1 Serological examination of the flocks showed elevated chicken infectious anaemia virus (CIAV) antibodies later in bird's life.

As viral immunosuppression has been proposed as risk factors for development of BCO, serological testing for CIAV and IBDV exposure was performed on all of the longitudinally sampled flocks. Reovirus antibody titres were also assessed, as the virus has been associated with musculoskeletal disease. Four out of five flocks used in this study had minimal CIAV antibodies in week 1 but had significantly elevated antibodies at weeks 4 and 5 of age (Table 12). Reovirus antibodies remained relatively low at all time points examined for all flocks in this study, though three out of five flocks showed a very mild increase in Reovirus antibodies in week 5. With IBDV antibodies, four out of five flocks had

moderate to high antibody titres in week 1 but showed significantly reduced antibody titres at weeks 4 and 5 of age.

When serological titres for all flocks were combined and analysed (Table 12), CIAV antibodies were found to be low in week 1 but significantly elevated in weeks 4 ($p < 0.0001$) and 5 ($p < 0.0001$) (Fig. 17). Reovirus antibodies were also low in week 1, showed no increase in week 4 and only a very mild but significant increase in week 5 ($p = 0.001$) (Fig. 18). In contrast, IBD antibodies were relatively strong in week 1, but significantly reduced in week 4 ($p = 0.000$) and then only mildly elevated in week 5 ($p = 0.040$) (Fig. 19).

There was no correlation between CIAV titre and inflammation in the bones, using either individual bird (correlation coefficient = 0.184) or flock average data (0.202). With the view that CIAV antibodies take few weeks to develop, this analysis was repeated only for older flocks (over 3 weeks), however correlation was still very poor (-0.025).

Table 12. Average (SD) of chicken infectious anaemia virus (CIAV), infectious bursal disease virus (IBDV) and Reovirus (ReoV) ELISA antibodies in broiler flocks examined in this study.

Flock	1wo	4wo	5wo
		CIAV	
1	0.21^{A} (0.24)	0.99^{B} (0.09)	0.67^{C} (0.28)
2	0.72^{A} (0.31)	1.03^{B} (0.10)	NT*
3	0.12^{A} (0.11)	1.48^{B} (0.20)	1.56^{B} (0.08)
4	0.18^{A} (0.19)	0.94^{B} (0.05)	0.97^{B} (0.22)
5	0.44^{A} (0.34)	0.10^{B} (0.10)	NT*
Overall	0.41^{A} (0.36)	1.06^{B} (0.21)	1.00^{B} (0.33)
		ReoV	
1	0.09^{AB} (0.10)	0.07^{A} (0.05)	0.17^{B} (0.13)
2	0.03^{A} (0.07)	0.03^{A} (0.04)	NT*
3	0.08^{A} (0.07)	0.06^{A} (0.03)	0.11^{A} (0.07)
4	0.06^{A} (0.03)	0.11^{A} (0.10)	0.26^{B} (0.16)
5	0.05^{A} (0.04)	0.01^{A} (0.01)	NT*
Overall	0.06^{A} (0.06)	0.09^{A} (0.10)	0.21^{B} (0.17)
		IBDV	
1	0.73^{A} (0.33)	0.59^{A} (0.27)	0.75^{A} (0.25)
2	0.75^{A} (0.31)	0.11^{B} (0.10)	NT*
3	0.88^{A} (0.43)	0.12^{B} (0.10)	0.17^{B} (0.16)
4	0.95^{A} (0.27)	0.25^{B} (0.13)	0.34^{B} (0.29)
5	0.59^{A} (0.27)	0.11^{B} (0.09)	NT*
Overall	0.76^{A} (0.34)	0.31^{B} (0.29)	0.66^{A} (0.39)

In each row for each organism, means with different superscript letters are statistically significant ($P \leq 0.5$) different.
**Not tested*

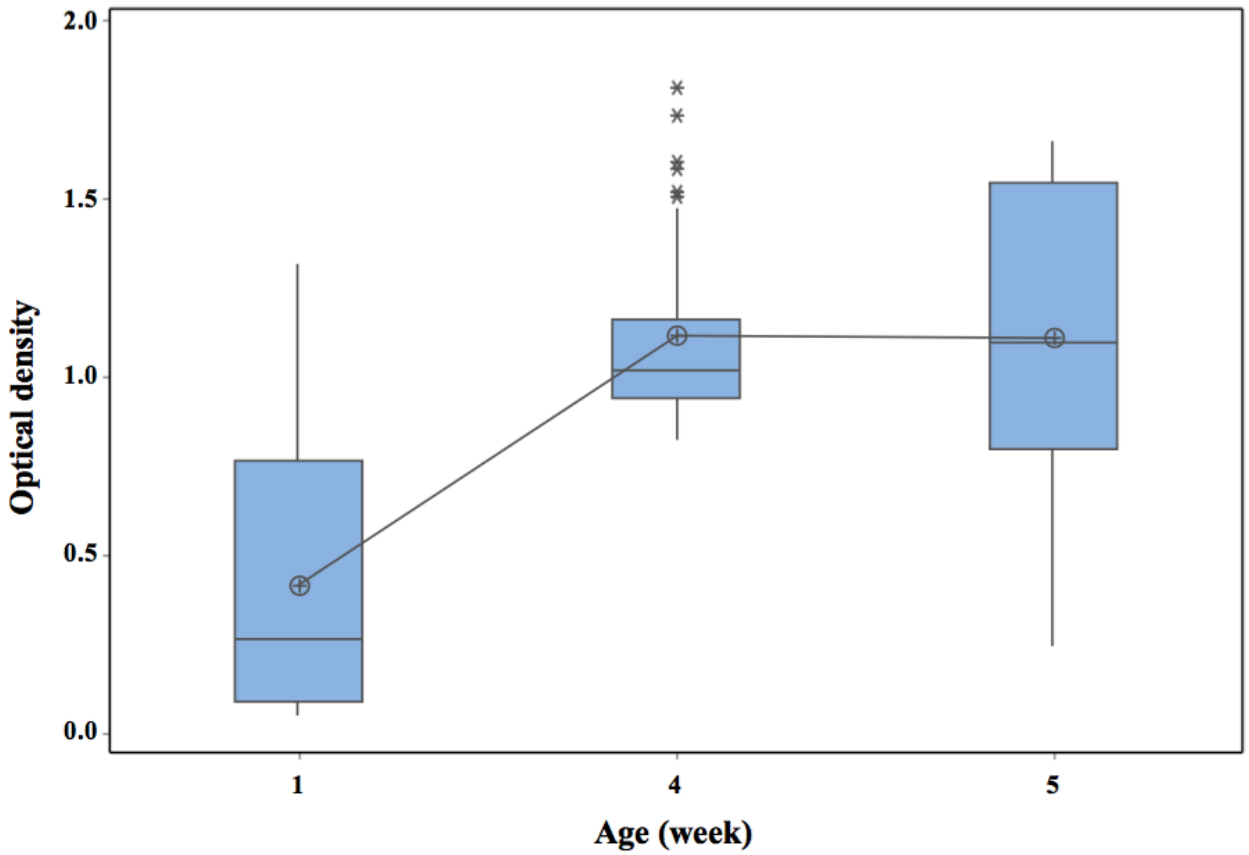

Figure 17. *Boxplot of CIAV ELISA optical densities at weeks 1, 4 and 5 of age. The box represents the middle 50% of the data. The line through the box represents the median. The lines (whiskers) extending from the box represent the upper and lower 25% of the data (excluding outliers). Outliers are represented by asterisks (*). The symbol on each plot represents the mean of the sample.*

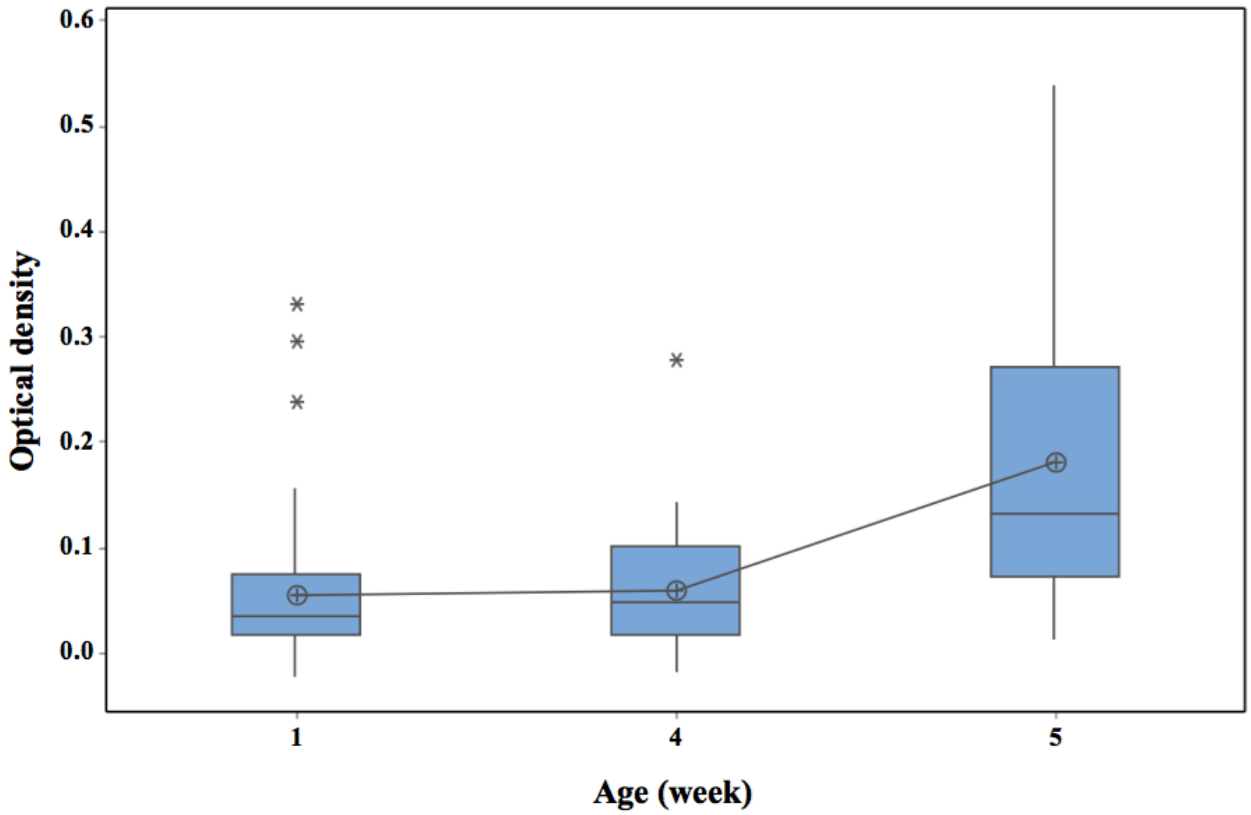

Figure 18. *Boxplot of Reovirus ELISA optical densities at weeks 1, 4 and 5 of age. The box represents the middle 50% of the data. The line through the box represents the median. The lines (whiskers) extending from the box represent the upper and lower 25% of the data (excluding outliers). Outliers are represented by asterisks (*). The symbol on each plot represents the mean of the sample.*

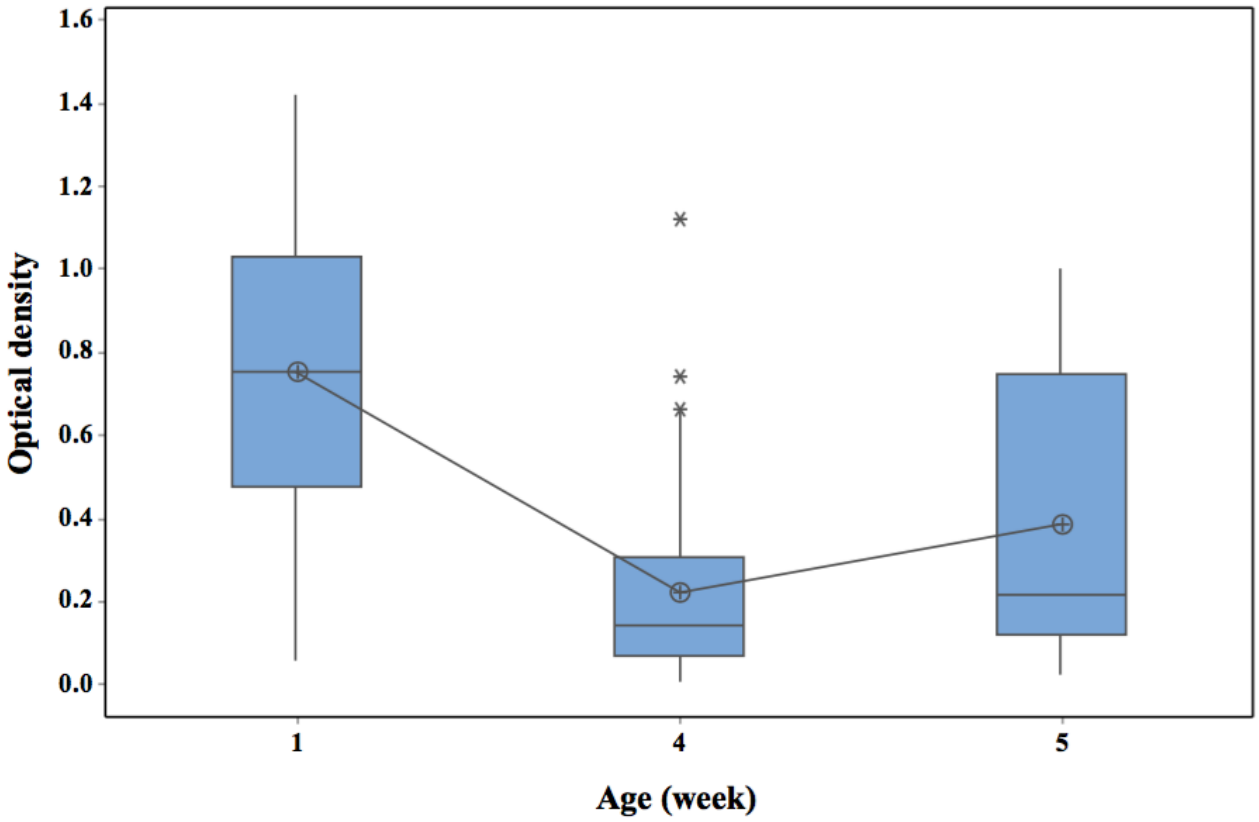

Figure 19toT. *Boxplot of IBDV ELISA optical densities at weeks 1, 4 and 5 of age. The box represents the middle 50% of the data. The line through the box represents the median. The lines (whiskers) extending from the box represent the upper and lower 25% of the data (excluding outliers). Outliers are represented by asterisks (*). The symbol on each plot represents the mean of the sample.*

4. Discussion of Results

4.1 Epidemiological survey into farm and flock risk factors associated with lameness culls

Two statistically significant correlations between the percentage lameness culls in the broiler flocks and the shed or flock characteristics were found in this study. The first was housing in solid versus curtain walls, and the second was the average distance between feeder and drinker lines. The shed wall design was considered unlikely to be a direct cause of lameness in the flocks, but may have been a reflection of unmeasured variables such as shed temperature variability, air flow or even the efficiency of the end of production clean out. Ineffective airflow or temperature variability at bird level could be a cause of stress in the young growing birds, which could result in increased susceptibility to infectious agents. Curtain wall sheds are typically older sheds and their design may not be optimized for efficient clean out (e.g. they may have exposed battens or unsealed joins between sheets of cladding). There may therefore be more carry-over of pathogens between batches of birds in these older sheds. In this analysis only 3 of the 18 farms had curtain walled sheds. Studies including more curtain wall sheds and shed design variables would be beneficial to determine which factors are truly related to the increase in lameness culls.

The distance between feeder and drinker lines was included in this investigation as a possible crude proxy for bird movement. Inactivity has been proposed as a risk factor for the development of BCO as leg circulation may be impaired in sedentary birds, potentially facilitating bacterial colonization of long bones. The percentage of lameness culls was higher in the sheds with the smaller distances, but only the lameness culls in the sheds with the second lowest feeder-drinker distance were significantly different to the sheds with larger distances. Only about 30cm existed between the largest and smallest distances and it could not be determined as to whether this was a true difference or a difference observed in this study due to other shed or flock factors. A better measure of the level of activity in the shed might be achieved through the use of technology such as cameras or motion sensors. While beyond the scope of the current study, comparison of the prevalence of BCO in free-range and indoor broiler flocks may also provide further indication of the significance

of bird activity in the development of the disease, but confounding environmental factors may make direct comparison between production systems difficult.

A small positive correlation existed between lameness culls and non-start culls. Several industry veterinarians have reported a problem with BCO in the first week of life of a broiler flock, and it is therefore possible that a problem in the hatchery or during placement and brooding could play a role in the disease. Hatchery and brooding problems could also result in an increased number of chicks which do not start feeding or drinking properly. In this study all flocks reported using whole shed brooding but no more details about the hatchery production or chick placement were available.

Lameness culls only provide approximate incidence of BCO as there are multiple causes of lameness in broiler chickens. Previous work has indicated femoral head necrosis was found to be present in 13.7% of culled or dead birds (McNamee & Smyth, 2000). Nevertheless, cull data is a useful correlate for femoral head necrosis, as RSPCA accredited farms are required to keep a running tally of the number of birds culled because of leg problems. The accuracy of the statistic might be able to be improved by providing producers some guidelines to differentiate a lame bird from a sick bird reluctant to walk.

4.2 Pathological and microbiological analysis of cull birds from commercial farms

4.2.1 Bacteriology

In this study a total of 325 cull birds from 20 different farms were necropsied, with swab samples taken from all the femurs and tibiotarsi, as well as from other sites where gross lesions were present. There was a strong correlation between the presence of chondronecrosis and osteomyelitis lesions and isolation of bacteria from bone, supporting the notion that that this process is ultimately due to bacterial infection (BCO). *E*. *coli* was the most frequently isolated bacterium from all the sites that were swabbed, including bones with BCO. Previous studies have most commonly associated BCO with *Staphylcoccus aureus* infection (McNamee, 1998; Wideman & Prisby, 2013), to the extent that this is the generally accepted perception on the matter (Andreasen, 2013). Only one paper has previously noted *E*. *coli* as the

main organism isolated from BCO-associated osteomyelitis (Dinev. I, 2009), similar to the findings of this study. The results of this study clearly indicate that, at least in Victorian flocks, *E. coli* is the major pathogen in BCO, and preventative measures should be focused on reducing exposure or increasing immunity to this organism. Moreover, while coagulase-positive staphylococcal species are typically considered to be more pathogenic than coagulase-negative species, in current study only 14% of staphylococci isolated from bones were coagulase-positive. While several species of *Staphylococcus* are potentially coagulase-positive in addition to *S. aureus* (*S. intermedius, S. pseudintermedius, S. delphini*, together with coagulase-variable *S. schleiferi subsp. coagulans, S. hyicus and S. lutrae* (Devriese *et al.*, 2005.) these results strongly demonstrate that *S. aureus* is a minor contributor to the pathogenesis of BCO in the Victorian broiler industry. Coagulase-negative staphylococci, accounting for the majority of *Staphylococcus* isolates in our study, have long been considered relatively apathogenic, but in human medicine they are increasingly recognized as pathogens in opportunistic infections.

E. coli is part of the commensal microbiota found in the intestines of the birds (Ewers *et al.*, 2009). However, APEC strains possess additional virulence genes, including the ColV plasmid-associated genes *omT*, *hlyF*, *iss*, *iutA*, and *iroN* (Johnson *et al.*, 2008a), that potentiate their pathogenicity in poultry (Johnson *et al.*, 2008a; Kaper *et al.*, 2004). Due to a lack of definitive classification criteria, there is currently no single reliable method for identification and characterisation of APEC strains due to their high diversity, however ColV plasmid-associated virulence genes have been proposed as potential markers for differentiation and identification (Rodriguez-Siek *et al.*, 2005). In this study, *E. coli* isolates were categorized as APEC if they were positive for any of the five ColV-associated virulence genes mentioned above. The majority of the *E. coli* isolates from the bones possessed all five genes, and the rest of the isolates carried at least one virulence gene. Some studies suggest that the APEC population may be divided into number of subpopulations based on their pathology and virulence factors, essentially classified as different pathotypes (Hussein *et al.*, 2013; Maturana *et al.*, 2011). However, in approximately one third of cases from this study, *E. coli* isolates from different sites in the same bird displayed different virulence genes, suggesting that the gene profile of APEC organisms may be quite fluid. Further work is required to determine if the APEC strains isolated in

this study display a specific tropism for bone, as lesions were located exclusively in bone in approximately half of the necropsy cases.

4.2.2 Histopathology

There was a marked variation in prevalence of BCO between individual flocks in this study, consistent with the sporadic and highly variable nature of the disease. Despite this, the mean prevalence of BCO was remarkably similar in all age groups and also overall, ranging between 22-29% of culled or dead birds. This supports the notion that BCO is a major cause of wastage in Australian commercial broiler birds, as reported anecdotally, and appears to be associated with approximately 1 in 4 culled or dead birds. These figures are nearly double those reported for UK broiler flocks, where 13.7% of dead or culled birds displayed histological evidence of BCO (McNamee & Smyth, 2000). Moreover, these numbers do not capture the number of subclinically affected birds, which could not be assessed due to the unavailability of clinically normal birds for full pathological examination, and so total prevalence in flocks is almost certainly higher than the figures provided.

The consistent prevalence across all age groups is another interesting finding, as previous studies have predominantly recognized BCO in older birds (Dinev. I, 2009; McNamee *et al.*, 1999a; Nairn & Watson, 1972). The reason for this variation from previous studies is unclear; it could reflect a focus on older birds in previous research, or it may represent an epidemiological peculiarity of Australian broiler flocks. Nevertheless, it is clear that BCO is a more significant disease process in younger birds than has previously been recognized, and many birds that have previously been classified as non-specific early mortalities may have been cases of unrecognized BCO. It is also important to note that BCO was more frequently identified within the proximal tibiotarsus than the femur. Previously the femoral head was considered the primary site of infection, as denoted by the outdated term “femoral head necrosis”. The findings of this study indicate that the tibiotarsus may be a more diagnostically useful site to assess for the presence of this condition in the field, and confirm that the term femoral head necrosis is not a suitable term for this disease.

Of all bone lesions, growth plate cartilage abnormalities were found to be strongly associated with the development of BCO. Separation, clefting or ischemia of the cartilage would appear to provide a suitable local environment for subsequent bacterial colonization. These findings account for the increased incidence of BCO observed with the wire-floor experimental model developed by Wideman (Wideman *et al.*, 2012). In this model, birds are thought to be more susceptible to BCO due to the degeneration of epiphyseal cartilage induced by increased shearing forces within the limbs. The wire flooring used in this model is unlikely to provide an accurate representation of the field environment, but it is quite possible that such lesions are induced at a lesser level by flooring issues in broiler sheds. It is interesting to note that there was no significant correlation between femoral head necrosis and tibiotarsal malformation. If sustained alteration to limb forces predisposed to BCO, a statistical association between these diseases would be expected. This lack of association instead suggests that cartilage defects are more likely induced by sudden or dynamic shifts in the forces applied to limbs, such as from slippage or uneven flooring. Based on this, ensuring well-maintained, non-slip, flat and level flooring, as well as avoiding unnecessarily sudden movement by birds (eg. slow and calm movement through poultry sheds) should aid in reducing the prevalence of BCO, but this hypothesis would need to be confirmed experimentally.

Retention of a cartilage plug within the tibial or femoral medulla was also strongly correlated with the occurrence of BCO. Cartilage plugs are avascular islands of retained cartilage which presumably provide a suitable environment for bacterial colonization, similar to sites of separation, clefting or ischemia within the epiphyseal growth plate. While some of these cartilage plugs were of sufficient size to warrant a diagnosis of clinical dyschondroplasia, the majority were smaller islands of retained cartilage consistent with subclinical manifestations of the disease. Nevertheless, based on this study it appears that even mild cartilage retention and delayed maturation can produce a suitable local environment for development of BCO, and so reducing the prevalence of dyschondroplastic lesions may provide the additional benefit of reduction in prevalence of BCO. Dyschondroplasia in broiler birds is a complex multifactorial disease, and development of the condition has been associated with a range of factors, including nutritional imbalances (calcium:phosphate ratio, hypovitaminosis D), bird breed and dietary mycotoxins

(Khan *et al.*, 2010; Leach & Monsonego-Ornan, 2007). Nutritional issues in particular are a factor that can be addressed readily on broiler farms, and so supplying a diet that minimizes dyschondroplasia (eg. cholecalciferol supplementation, optimizing dietary calcium and phosphate concentrations), may be cost-effective means of reducing the incidence of BCO.

Irrespective of the strong association between BCO and cartilage lesions, it is important to note that 25% of birds displayed inflammatory lesions exclusively within the subchondral bone, and so while lesions predominantly develop in the epiphyseal growth plate, changes within the cartilage are not necessary for the development of BCO.

Vascular degeneration within the growth plate has been proposed as a predisposing factor in the pathogenesis of BCO (Wideman & Prisby, 2012). The ordered regression of vessels and cartilage canals without evidence of ischemia that was observed in birds in this study is consistent with the process of chondrification; a physiological remodelling and reduction of the vascular supply to the cartilage during normal development. This age-related process is well-documented in horses and pigs (Ytrehus *et al.*, 2004), and though the change has previously been suggested to predispose to development of osteochrondrosis dissecans in these species, studies have failed to document an association. In the present study, there was a slight non-significant trend of correlation between BCO and vascular regression, and it is possible that chondrification sites may provide a suitable site for initiation of BCO in some instances. As chondrification is physiological, altering this process in order to prevent development BCO is likely impractical. However, further investigation into the impact of bird strain and growth rate on the chondrification process may aid in future selective breeding for resistance to BCO.

Synovitis was strongly associated with the presence of BCO, but to a large extent this correlation is likely to reflect local extension of inflammation through the articular surface. Almost half of bones with BCO displayed evidence of synovitis within the adjacent joint, and the majority of these were fibrinoheterophilic in character, consistent with a bacterial aetiology.

Nearly half of birds with BCO displayed lesions in multiple bones. The dispersed nature of the infection in these birds suggests either the presence of overwhelming bacteraemia in these birds or systemic impairment of host defence, rather than simply a suitable local environment. This is further supported by the strong correlation between the presence of hepatitis and BCO. In the majority of cases, the hepatitis manifested as coalescing foci of necrosis within the parenchyma, which is a typical finding in bacterial septicaemia.

Experimental models of BCO have induced the disease through both respiratory (McNamee *et al.*, 1999b) and gastrointestinal (Al-Rubaye *et al.*, 2015) bacterial exposure, but this study was not able to determine a common route of entry for bacteria involved in BCO under field conditions. Impairment of the intestinal mucosal barrier has been suggested as a suitable source of entry, particularly for potential enteric flora such as *E. coli*, but no significant association between the presence of enteritis or intestinal coccidia and BCO was noted in this study. However, the scarcity of severe enteric lesions in the sampled birds, particularly those with defects extending beyond the mucosa, means that the intestine cannot be entirely excluded as a potential source of bacteraemia. Nevertheless, it appears that the presence of enteritis is not a necessary condition for the development of BCO. Similarly, no statistical association was noted between the presence of BCO and omphalitis/yolk sac infection, and neither were associations noted for skin injuries or respiratory disease. Overall, these findings suggest that BCO may not result from bacteraemia caused by deficiencies in a particular mucosal or skin barrier, but instead is more likely to reflect opportunistic infection following low-level endemic bacteraemia. This is consistent with recent findings (Jiang *et al.*, 2015) which detected a range of bacterial organisms within the epiphyses of normal chickens via 16S rRNA sequencing, and also identified a range of bacteria (including proteobacteria) within the blood of broiler chickens (Mandal *et al.*, 2016). Rather than indicating a consistent location for bacterial entry into systemic circulation, these findings suggest that bacterial translocation and bacteraemia may be inevitable, and that local environmental or host immune factors are likely to determine the successful colonization of bacteria within the bone. If this is the case, improving immunological resistance to bacterial colonization though vaccination against common pathogens, or ensuring appropriate protection from other immunosuppressive disease may

prove to be effective in reducing the prevalence of the disease. Alternatively, shifting the microbiological flora towards non-pathogenic organisms, such as through the use of probiotics, is another approach that may limit development of BCO.

The presence of BCO was significantly associated with decreased bodyweight in younger birds. This is consistent with previously reported research (Emslie *et al.*, 1983), where decreased weight has been used as a predictor for the presence of BCO. It has previously been suggested that this decrease in bodyweight reflects reduced nutritional and water intake secondary to lameness. However, in this case the birds assessed were all culls or mortalities, and therefore illness is likely to have reduced food and water intake in all of the birds to some degree prior to death. It is therefore possible that this trend towards decreased weight is a genuine association rather than a secondary effect. Irrespective, these findings provide further confirmation that development of BCO is not directly correlated with heavier birds, and so restriction of weight gain is unlikely to reduce the prevalence of BCO. Differences in breed prevalence were unable to be reliably assessed in this study, as the vast majority of birds were Ross, with only 2 of 15 flocks composed of Cobb birds.

The lack of correlation with hatchery of origin suggests that bacterial contamination of the egg shell is not a significant cause of BCO. In the present study, hatchery 1 disinfected the eggs via fumigation, while hatchery 2 performed no sterilization of the eggs; hence, a difference in prevalence of BCO would be expected if egg sterilization was important in prevention of the disease. This finding is also consistent with the lack of association with omphalitis, which indicates bacterial infection in cases of BCO is unlikely to be obtained in the immediate post-hatching period. Based on these findings, it is more likely that pathogens involved in BCO colonize birds on the farm rather than the hatchery. However, this does not exclude the possibility that individual egg conditions such as incubation temperature may predispose to development of BCO.

4.2.3 Serology

For the farms in the present study, parent flocks were vaccinated for both IBDV and CIAV. The serum of sampled birds appeared to carry ample maternal antibodies to

IBDV and showed only a modest elevation of IBDV antibodies in weeks 4 and 5; therefore there was very little evidence of field IBDV infection early in chick's life. There was also no evidence of Reovirus maternal antibodies, nor indication of serious field Reovirus exposure later in chick's life. In contrast, it was apparent - based on the low titres in young birds and the subsequent titre increase in older birds - that chicks carry only minimal maternal antibodies against CIAV and they are usually exposed to a field challenge with CIAV early in life. CIAV infection has been used in BCO models to increase susceptibility to the condition (McNamee *et al.*, 1999b), and corticosteroid administration has also been demonstrated to increase the risk of BCO, presumably through immunosuppression (Wideman & Pevzner, 2012). However, despite these suggestions that CIAV infection may predispose to BCO, we were unable to demonstrate a correlation between CIAV titres and the presence of BCO lesions. It is possible that better indications of an association may be obtained through specific viral testing (PCR), rather than assessing for an association with immunity through antibody titres.

5. Implications and recommendations

Lameness is a major economic and welfare problem in commercial broilers and is often a result of bacterial chondronecrosis and osteomyelitis (BCO). This study investigated the epidemiology and aetiology of BCO amongst Victorian broiler flock in an attempt to understand epidemiology and pathogenesis of the disease in the Australian broiler flocks. We found that BCO is a common multifactorial disease, characterized by chondronecrosis with osteomyelitis in the proximal end of the femur and tibiotarsus.

Results of this study also revealed that BCO occurs throughout the life of the broiler flocks at an alarmingly high rate - approximately one quarter of the mortalities and culls. The condition also frequently occurs within the tibiotarsus, and so this site should be assessed for diagnostic purposes in addition to the femoral head.

BCO is an infectious process that appears to occur most commonly as a result of bacteraemia and haematological spread of bacterial pathogens - especially *E. coli* - to the bones. We observed that almost all *E. coli* isolated from cases of BCO are avian pathogenic *E. coli* (APEC), suggesting that preventative measures should be directed at this organism. An APEC vaccine should be developed and evaluated for its potential to control the condition. To do this, a reproducible BCO infection model must be developed for APEC, which could subsequently be used to evaluate the efficacy of *E. coli* vaccine candidates, as well as other preventative strategies in the control of BCO. The bone isolates identified in this study provide a valuable resource for future work, both in assessing for virulence factors associated with bone colonization, and for selecting optimal strains as potential vaccine candidates. Whole genome sequencing of these isolates will provide an invaluable resource for comparative genomics analysis and comparison with APEC strains isolated from other sites, in order to rationally select the optimal candidate for use in vaccine development and challenge models. Research investigating and characterizing the bacterial isolates obtained in this study will be continued following conclusion of this report, with further findings to be reported in scientific journal publications.

We found no evidence that BCO could be directly attributed to hatchery conditions (including whether eggs are fumigated or not), and instead it seems likely that the bacteria involved in development of BCO are derived from the grower environment. Moreover, we found no evidence to associate BCO with disease at potential bacterial entry sites (such as skin, gut and umbilicus), suggesting that the bacterial translocation is a sporadic and unpredictable process that can occur even when mucosal and dermal barriers are intact. Based on these findings, prevention of bacteraemia is likely to difficult, though probiotics may provide a way to alter bacterial flora and reduce the proportion of BCO pathogens entering circulation. Additional preventative measures should be aimed towards reducing predispositions for bone colonization (ie. cartilage defects) and improving the bird immunological defences against the identified pathogens.

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Glossary

Abbreviation	
FHN	Femoral head necrosis
BCO	Bacterial chondronecrosis and osteomyelitis
E. coli	*Escherichia coli*
PCR	Polymerase chain reaction
APEC	Avian pathogenic *Escherichia coli*
RSPCA	Royal Society for the Prevention of Cruelty to Animals
UPEC	Uropathogenic *Escherichia coli*
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
TMB	3,3',5,5'-Tetramethylbenzidine
CIAV	Chicken infectious anaemia virus
IBDV	Infectious bursal disease virus
Glossary	
Chondrodystrophy	Abnormal development of growth plate cartilage
Coccidia	Protozoal pathogens that predominantly infect the gastrointestinal tract
Diaphysis	The shaft or central part of a long bone
Eosinophilia	An increase in the number of eosinophils in the blood
Epiphyseal growth plate	Hyaline cartilage plate in the metaphysis at each end of a long bone. It is the part of a long bone where new bone growth takes place
Epiphysis	The end part of a long bone
Fibrinous inflammation	Inflammation with vascular leakage of fibrin
Granulomatous inflammation	Inflammation predominantly involving macrophages
Heterophils	The major leukocyte (white blood cell) of innate immunity in avian species, loosely analogous to neutrophils in mammalian species
Hepatitis	Inflammation of the liver
Hyperplasia	The enlargement of an organ or tissue caused by a cellular proliferation
Inflammation	An immune system response to an insult, such as injury or infection
Lameness culls	Birds culled due to lameness issues
Metaphysis	The transitional zone in a long bone between the terminal epiphysis and the diaphyseal shaft
Necrosis	Death of cells within living tissue
Non-start culls	Birds culled for poor development during the first few days of production
Omphalitis	Inflammation of the navel, identified in this study by a

	wetness, swelling or exudate at this site.
Osteochondrosis dissecans	A degenerative condition resulting in undermining and separation of superficial articular cartilage
Retained cartilage plug	Persistence of non-remodelled growth plate cartilage extending into the diaphysis
Serositis	Inflammation of the serosa of a body cavity (eg. coelome)
Subchondral bone	The bone underneath the epiphyseal growth plate
Synovitis	Inflammation of the joint lining
Yolk sac infection	Histological evidence of inflammation within the yolk sac remnant

Appendix 1.

Post mortem investigation

Farm	
Shed #	

Date	
Bird age	

History

Bird id:		Weight:		Sex:		Comments
Scratches:	None	Superficial Feet/Toes	Deep foot scratch/ pododermatitis	Superficial scratches to skin	Deep infected scratches to skin	
Other diseases	Unhealed navel	Omphalitis	Peritonitis	Airsacculitis	Arthritis	
	Bursal size		Thymus size			
Legs	Left Femur	Left Tibiotarsus	Right Femur	Right Tibiotarsus	Vertebrae	
Histo samples	Duodenum	Mid jejunum	Colon/Caecae	Liver		

Bird id:		Weight:		Sex:		Comments
Scratches:	None	Superficial Feet/Toes	Deep foot scratch/ pododermatitis	Superficial scratches to skin	Deep infected scratches to skin	
Other diseases	Unhealed navel	Omphalitis	Peritonitis	Airsacculitis	Arthritis	
	Bursal size		Thymus size			
Legs	Left Femur	Left Tibiotarsus	Right Femur	Right Tibiotarsus	Vertebrae	
Histo samples	Duodenum	Mid jejunum	Colon/Caecae	Liver		

Bird id:		Weight:		Sex:		Comments
Scratches:	None	Superficial Feet/Toes	Deep foot scratch/ pododermatitis	Superficial scratches to skin	Deep infected scratches to skin	
Other diseases	Unhealed navel	Omphalitis	Peritonitis	Airsacculitis	Arthritis	
	Bursal size		Thymus size			
Legs	Left Femur	Left Tibiotarsus	Right Femur	Right Tibiotarsus	Vertebrae	
Histo samples	Duodenum	Mid jejunum	Colon/Caecae	Liver		

Appendix 2

Bacteriology

Farm		Date	
Shed #		Bird age	

Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											
Bird ID:				**Site:**							
SBA:						**MAC:**					
Haemolysis:		Lactose:		Gram:		Shape:		Catalase:		Coagulase:	
Methyl Red:		VP:		Indole:		Citrate:		PGUA:		Oxidase:	
Urease:											

POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	An investigation into the epidemiology and pathogenesis of femoral head necrosis in broilers in Australia
Poultry CRC Sub-Project No.:	1.5.8
Researcher:	Andrew Stent
Organisation:	Faculty of Veterinary and Agricultural Sciences, The University of Melbourne
Phone:	+613 8001 2549
Fax:	
Email:	andrew.stent@unimelb.edu.au
Sub-Project Overview	An investigation of the pathogenesis and risk factors involved in the development of bacterial chondronecrosis and osteomyelitis (also known as “femoral head necrosis”) in broiler poultry
Background	Lameness is a major economic and welfare issue for poultry, and a large proportion of lameness in broiler poultry is believed to be related to bacterial chondronecrosis and osteomyelitis (BCO). The pathogenesis and underlying risk factors for development of this condition are poorly understood.
Research	An epidemiological and pathological survey of broiler farms to assess the prevalence of BCO and identify farm and bird factors related to the pathogenesis of the condition
Sub-Project Progress	The project determined that BCO is an infectious process that is predominantly caused by avian pathogenic *Escherichia coli* (APEC). The condition occurs frequently at all ages of bird, accounting for approximately 25% of broiler mortalities or culls.
Implications	The confirmation of an infectious aetiology and identification of APEC as the predominant pathogen highlights the need for preventative measures aimed at limiting exposure to the pathogen or altering bacterial flora (eg. probiotic usage), improving bird immunity (eg. vaccination) and reducing the incidence of predisposing cartilage injury.
Publications	