ABSTRACT

CHEN, LAURA R. Pathogenesis of Osteochondrosis in Chickens, a Critical Host-Factor for Infection with Pathogenic *Enterococcus cecorum*. (Under the direction of Drs. Luke Borst and Isabel Gimeno).

First recognized in 2002, enterococcal spondylitis (ES) is an economically significant cause of morbidity and mortality in broiler and broiler breeder chickens. There have been substantial advances in the understanding of the causative agent, *Enterococcus cecorum*; the clinical disease; the epidemiology; and parts of the pathogenesis of this bacterial infection, yet adequate and appropriate control methods remain elusive. As osteochondrosis (OC) dissecans lesions at the free thoracic vertebra (FTV) are a critical host-factor that predispose birds to ES, the goal of this thesis was to identify factors that contribute to OC in chickens in order to decrease host susceptibility or develop novel, non-antimicrobial control methods for ES. Following a review of ES with a focus on host factors that influence disease including OC of the FTV, we provide two studies aimed at identifying factors that contribute to OC prevalence and severity. In the first study, OC prevalence and severity in the FTV was compared among three modern commercial broiler strains and a 1950's meat-type chicken. Histologic lesion scores for OC of the FTV were compared with sex, age, strain, body weight, and incubation temperature profile to identify significant correlations. Overall, the prevalence of FTV OC was high in all strains with >80% samples containing an OC-spectrum lesion. No association was observed between mean OC score and broiler strain, incubation temperature profile, sex, age, or body weight. While a trend toward decreased OC lesion scores in female 1950's meat type chickens was observed, the absence of a clear strain correlation with mean OC score was a surprising finding given the strong support for OC heritability in mammalian species. This led to the subsequent hypothesis that evolutionarily more dissimilar chicken lineages would demonstrate a

strain correlation with OC prevalence and severity. In a subsequent study, FTV, proximal femoral, and proximal tibiotarsal OC prevalence and severity were examined in the red jungle fowl, two heritage breed chickens, and one modern commercial broiler chicken strain. Histologic lesion scores were compared among sex, body weight, and strain variables. Similar to the first study, no association was observed with mean OC score and sex within strains. However, there was a strain-wide effect with significantly decreased FTV lesion scores in red jungle fowl compared to all other breeds and significantly decreased prevalence and severity of femoral OC in all breeds compared to the modern broiler. Taken together, these findings indicate a potential heritable component for OC in chickens that is independent of growth rate. In addition, through our studies, we also observed changes in growth cartilage including multiple thrombosed cartilage canals which suggest ischemic injury may be the key step in OC pathogenesis in broilers, as it is in other veterinary species. These studies lay the foundation for future work into the genetic drivers and pathophysiologic mechanisms of OC in chickens that, if successful, would provide valuable information for the control of this important disease of broilers.

Pathogenesis of Osteochondrosis in Chickens, a Critical Host-Factor for Infection with Pathogenic *Enterococcus cecorum*

by
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DEDICATION

To my husband, Mark Widgren, who has supported all my career endeavors and made life much more enjoyable along the way.

BIOGRAPHY

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CHAPTER 1: A Review of *Enterococcus cecorum* Infection in Poultry

Jung A.*, L.R. Chen*, M.M. Suyemoto, H.J. Barnes, and L.B. Borst. A review of *Enterococcus cecorum* infection in poultry. Avian Dis. 62(3):261-271. 2018.

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Introduction

Enterococcus cecorum was initially described in 1983 as a benign constituent of the gut microbiota of adult poultry, particularly chickens (*Gallus gallus domesticus*) (23, 24). However, over the past 15 yr, pathogenic strains of *E. cecorum* have emerged as an important cause of skeletal disease in broiler and broiler breeder chickens (12, 44, 46). Outbreaks of pathogenic *E. cecorum* in commercial chickens were initially described in 2002 in Scotland (79) and the Netherlands (21). Multiple reports followed describing pathogenic *E. cecorum* outbreaks in Belgium (38), Canada (65), Germany (46), Hungary (52), Iran (70), Poland (69), South Africa (2), Switzerland (3), and the United States (61). With additional descriptions every year, pathogenic *E. cecorum* now appears to be regionally endemic worldwide.

The most striking feature of infection with pathogenic *E. cecorum* is paralysis due to an inflammatory mass that develops in the spinal column at the level of the free thoracic vertebra (FTV). Recognition of this spinal lesion has given rise to several disease names for pathogenic *E. cecorum* infection which include vertebral osteomyelitis, vertebral enterococcal osteomyelitis and arthritis, enterococcal spondylitis and, colloquially, “kinky-back.” It must be noted that kinky-back is also the common name for the developmental spinal anomaly,

spondylolisthesis. To avoid ambiguity, the authors prefer the use of the etiologic diagnosis, enterococcocal spondylitis (ES), when referring to this disease.

Significant progress has been made over the past decade in understanding ES in chickens. This first review of the current understanding of pathogenic *E. cecorum* infections in broiler and broiler breeder chickens focuses on the clinical features of disease, the bacteriologic and genetic characteristics of pathogenic *E. cecorum* strains, and the role of host factors in the pathogenesis of infection. Due to the significant role of *Enterococcus* spp. in nosocomial infections, the broader implications of *E. cecorum* infections in chickens on human health, including antimicrobial resistance, will be discussed.

Clinical and Pathological Features

Broiler flocks experiencing *E. cecorum* outbreaks have elevated mortality due to a combination of sepsis early in the growing period and dehydration and starvation of paralyzed birds late in the growing period (2, 12, 21, 38, 46, 61, 65, 70). Paralysis from infection of the FTV is the most striking feature of this disease, with affected birds exhibiting a classic sitting position with both legs extended cranially (Fig. 1.1a) (12). Daily and total morbidity and mortality are variable (38, 46, 49, 65, 70), with the highest overall morbidity and mortality reported at 35% and 15%, respectively (Table 1.1) (11). Flock mortality and cost of therapy represent only part of the economic impact for the farmer. Increased condemnation rates up to 9.75% at the processing plant can occur; most of the chickens are rejected due to scratching and dehydration, both of which are secondary consequences of paralysis caused by *E. cecorum* infection (3, 18, 45). As the onset of clinical disease occurs near the end of the growing period, some birds with mild or subclinical lameness are processed and enter the food supply (67).

Table 1.1 Onset of clinical signs and mortality in EC outbreaks in broiler flocks.

Onset clinical signs[A] and mortality (dph[B])	Age at slaughter	Overall mortality[C]	Reference
21-32	41-42	2-7%[D]	(18)
21	34	7.2%	(46)
28-42	61	4.5-5.5%	(12)
35-42	61	4.1-11.7%	(61)
14	35-45	3.1-8.1%	(49)

[A]Lameness and/or paralysis. [B]Days post hatch. [C]Including euthanized animals. [D]In this study only the mortality which was associated with the EC infection was given.

Septic Phase

In broilers, morbidity and mortality can be variable in the septic phase of the disease, which is often subclinical. For example, in longitudinal studies of *E. cecorum* outbreaks, the organism was recovered from yolk sacs or spleens of asymptomatic birds prior to day 14 in flocks that later developed skeletal lesions of *E. cecorum* at 4 wk of age (12,46). In broiler flocks though, slight to moderate increases in mortality due to sepsis have been reported around weeks 2–3 (46, 61, 65). Gross lesions include fibrinous pericarditis and perihepatitis and splenomegaly (Fig. 1.1b, c) from which *E. cecorum* can be recovered in pure culture (46,49). As the gross lesions of pathogenic *E. cecorum* septicemia are nearly identical to lesions observed in other systemic bacterial infections (most notably colibacillosis), culture is needed for an accurate diagnosis. The incidence of bacteremia within a broiler flock increases throughout the growing period, with pathogenic *E. cecorum* present in the spleen of over 80% of birds by week 6 (12). The steady increase in bacteremia over the life of the flock indicates that birds do not readily clear infection.

Figure 1.1 Clinical presentation and gross pathologic lesions of *E. cecorum*. (a) Prototypically, clinically affected flocks present after 5 weeks of age with hindlimb paresis to paralysis with a standard hock sitting to cranially extended hindlimb position. (b - c) Early mortality caused by enterococcal septicemia may also be identified, manifesting as (b) fibrinous pericarditis (*) and perihepatitis (arrow) with (c) splenomegaly (arrow). Femoral osteomyelitis (arrow) can occur later in the disease (d). The characteristic feature of enterococcal spondylitis is vertebral osteomyelitis (e) which always occurs at the free thoracic vertebra (arrows) and its articulations


A
B
C
D
E

Skeletal Infection

While the mortality that heralds the onset of the septic phase of *E. cecorum* infection is variable, it is consistently followed by a second, larger wave of mortality that is due to culling, dehydration, or starvation subsequent to paralysis because of spinal lesions (12, 61). Mortality in this second phase typically peaks at weeks 5–6 in broiler flocks and either at the same time or slightly later around week 13 in broiler breeders (2, 52, 65).

During this later phase of disease, lameness can be from spinal lesions or osteomyelitis of the proximal femur, which is often referred to as "femoral head necrosis" or "bacterial chondronecrosis and osteomyelitis" (Fig. 1.1d) (46, 49, 65). Pathogenic *E. cecorum* can be isolated in pure culture from femoral head lesions. A recent review thoroughly describes lesions observed with bacterial chondronecrosis and osteomyelitis (75). Synovitis and arthritis of the coxofemoral, stifle, and hock joints can also occur and are characterized by a yellow-opaque exudate filling and distention of the joint space with or without strands and clumps of fibrin (38, 49, 65). Osteomyelitis of the proximal femur, synovitis, and arthritis due to infection with *E. cecorum* are grossly similar to lesions caused by other infectious agents, and culture is needed to recognize these lesions as a component of an *E. cecorum* outbreak.

The most striking skeletal lesion in an *E. cecorum* infection is spondylitis and osteomyelitis at the FTV and its articulations with the notarium, synsacrum, or both (2, 7, 21, 38, 61, 65, 70, 79). On gross examination, articulations of the FTV and adjacent vertebral bodies are replaced by an inflammatory mass, which often has adhesions between the lung and spinal lesion (Fig. 1.1e) (7, 53, 61). Sagittal and transverse sections of the inflammatory mass reveal a central core of yellow, granular, caseous, necrotic material with gritty debris from bone and cartilage fragments mixed with hemorrhage, all of which is surrounded by a thick circumferential

granulation tissue or fibrous capsule (Fig. 1.2a). The expansile nature of the inflammatory mass is best seen in sagittal or transverse planes. The mass expands ventrally into the body cavity and dorsally causing stenosis and dorsoventral compression of the spinal cord. Spinal cord compression results in the characteristic symmetrical paresis and paralysis typical of ES.

Histologically, early mild lesions of the FTV are characterized by bacterial colonization of osteochondrosis dissecans lesions (see predisposing factors below) with progressive infiltration of heterophils and macrophages into degenerative, necrotic, and fragmented articular cartilage and underlying bone (12, 53, 61). Over time, lesions form a more organized heterophilic abscess with features of bony repair and remodeling along the periphery including reactive bone proliferation, granulation tissue deposition, and attempted isolation of the infection by a fibrous capsule with variably present lakes of cartilage matrix similar to callus formation observed in fracture repair (Fig. 1.2b–d). Vertebral bodies are most affected. As lesions progress they collapse, resulting in dorsal buckling of the spine (kyphosis). Inflammation rarely extends into or above the vertebral canal. Malacia with neuronal necrosis and Wallerian degeneration, and less frequently hemorrhage, occur in the overlying spinal cord. Lymphocytic cuffing is occasionally seen in the spinal cord. Very rarely, bacterial colonies can be observed in the spinal cord itself.

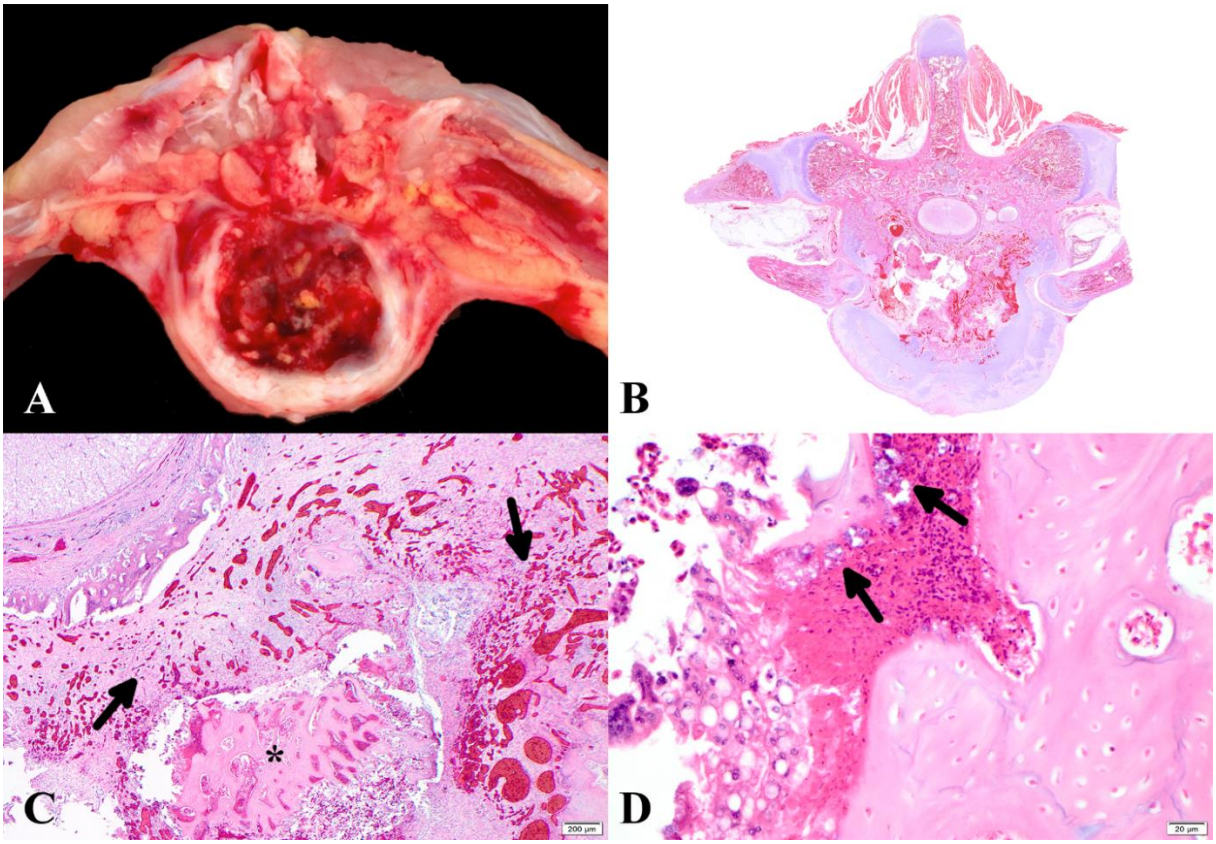

Figure 1.2 Gross and microscopic pathology of enterococcal spondylitis. (a) Cross section through a chicken free thoracic vertebra with chronic *E. cecorum* infection. (b) Sub-gross view of (a) following tissue fixation and standard H&E processing. (c) Microscopically, the vertebral body is replaced by necrotic bone (sequestrum) (asterisk), heterophilic and granulomatous inflammation, and granulation tissue (arrows) and variably mature fibrous stroma. (d) With higher magnification, intralesional colonies of cocci bacteria are scattered throughout the inflammation (arrows).

Infection in Other Avian Species

While outbreaks of *E. cecorum* causing osteomyelitis have not been identified in other poultry species, ducks (Anatidae) have been shown to be susceptible to experimental infection with pathogenic *E. cecorum* that mimics the septic phase (47, 55). In field infections, mortality occurred in the first 3 days posthatch in 2-wk-old conventional ducklings and in Pekin breeding

ducks (47, 55). Affected ducks showed generalized poor condition but, in contrast to the disease in broilers, no signs of lameness were observed. Mortality due to *E. cecorum* infection has also been identified in 2-wk-old Muscovy ducks (*Cairina moschata*; Jung, pers. obs.). Navel and yolk sac inflammation was identified in 1- to 3-day-old Pekin ducklings (*Anas platyrhynchos domesticus*). Two-week-old infected ducks showed pericarditis, hepatitis, and splenitis. Pekin breeding ducks with *E. cecorum* infection typically had oophoritis and ascites. *Enterococcus cecorum* was isolated from all lesions, often in pure culture, but sometimes together with *Escherichia coli* (55).

Enteroccocus cecorum infection in racing pigeons is characterized by depression, anorexia, and elevated mortality (14, 48). Pigeons that died of *E. cecorum* infection at necropsy were in good body condition but had enlarged, congested livers and ulcers in the gizzard (14, 48). *Enteroccocus cecorum* was isolated from heart, liver, spleen, crop, and jejunum (48) and from lung, liver, and crop (14).

Predisposing Factors in Disease Pathogenesis

The most important pathologic change in *E. cecorum*-infected broiler and broiler breeder chickens is inflammation of the sixth thoracic vertebra (T6), which is the only freely articulating vertebra in the thoracolumbar spine. Thoracic vertebrae 2 to 5 are fused, forming the notarium, and the seventh thoracic vertebra is fused with the synsacrum. There is some confusion about the numbering of the FTV. Some authors count only vertebrae with complete ribs as thoracic and therefore identify the free movable vertebra between the notarium and synsacrum as the fourth vertebra (T4) (75). Further clouding the situation is the fact that, occasionally, some birds have two articulating thoracic vertebra (e.g., T5 and T6) (author's observations). The notarium supports the cranial half of the bird and the synsacrum supports the caudal. The FTV in-between

is therefore exposed to repeated mechanical torsional and sheer stress, which creates microfractures in the cartilage of the vertebra (75).

In 2017, Borst *et al*. (12) identified osteochondrosis dissecans lesions in the epiphyseal cartilage of the FTV, the articulating epiphyseal cartilage of the notarium and synsacrum, or both, as a predisposing factor for ES in broiler chickens (12). Briefly, osteochondrosis (OC) is a defect in endochondral ossification in either the epiphyseal or physeal cartilage that results in foci of cartilage necrosis (osteochondrosis latens); cartilage retention into the metaphysis, diaphysis, or both (osteochondrosis manifesta); or clefting and flap formation of the cartilage (osteochondrosis dissecans) (83). Osteochondrosis spectrum lesions have been well described in chickens (16, 30, 31) as well as in multiple other animal species (57, 83). Despite its rampant presence in veterinary medicine as well as its role in decreased welfare and increased lameness, the pathogenesis of OC remains to be fully understood (57, 83). Regardless, in ES, only birds with osteochondrosis dissecans lesions (cartilage cleft in articular cartilage) and bacteremia develop lesions of ES (12). In 2017, Chen *et al*. (15) found that osteochondrosis dissecans lesions are common in modern broilers, with a similar prevalence in male Athens Canadian Random Bred (ACRB) control broilers (15). In modern broilers, there was no sex predilection for OC; however, female ACRB broilers had decreased histologic lesion scores for OC compared to modern broilers of either sex and to male ACRB broilers (15). In chickens, prevalence or severity of OC does not appear to be related to growth rate (15).

The role of immunosuppression, as well as other potential comorbidities, has yet to be described in the pathogenesis of *E. cecorum* infections. It has been hypothesized that damage to the intestinal barrier, due to other pathogenic bacteria (e.g., *E. coli* or parasites e.g. *Eimeria* spp.), increases the prevalence and the severity of ES outbreaks, but no correlation between intestinal

barrier injury and ES has been found to date (12). It is clear that in experimental *E. cecorum* infections, injury to the intestinal barrier is not required to develop septicemia or ES; however, further work is needed to understand the mechanisms of *E. cecorum* employs to escape the gut (12, 53)

Prevention and Treatment

Currently, there is no effective vaccine for preventing pathogenic *E. cecorum* infection. However, inactivated flock-specific vaccines can be applied in broiler breeder flocks, although no studies have investigated whether maternally derived antibodies can protect progeny from ES. Anecdotal reports of the effectiveness of prophylactic penicillin treatment administered in the first 2 wk of life for predicted *E. cecorum* outbreaks have not been validated. After diagnosis of ES, antibiotic treatment may be applied as soon as possible to prevent further progression of the disease; however, antimicrobial therapy is ineffective for paralyzed birds, which should be culled. The choice of the antimicrobial substance to be used should be based on resistograms. Penicillin derivatives are probably the most frequently used substances to combat ES. Different studies indicate the susceptibility of *E. cecorum* to penicillin whereas macrolide and tetracycline resistance is relatively common (see below).

Adjustments of lighting control programs to slow growth in the first weeks of life have also been used to blunt the impact of *E. cecorum* infection; however, no studies have been performed to validate the efficacy of this treatment.

Epidemiology

Despite the rapid global emergence of this pathogen, and several works on the subject, the mechanism by which pathogenic *E. cecorum* spreads within and among vertically integrated broiler production systems remains unclear. To identify vertical transmission, Kense and

Landman (49) compared genetic profiles between *E. cecorum* recovered from the ceca of breeder birds and pathogenic isolates from their offspring. Matching profiles were not identified, nor was pathogenic *E. cecorum* recovered from the hatchery environment. Likewise, Robbins *et al*. (61), using both genetic and metabolic profiling, were unable to detect matching profiles between commensal *E. cecorum* from broiler breeder parent flocks and pathogenic strains from infected progeny. Pathogenic *E. cecorum* was not recovered from the hatchery environment or from dead embryos in this study, despite the fact that chicks from that hatchery, placed on two separate farms, subsequently developed outbreaks caused by pathogenic *E. cecorum* strains with identical genetic and phenotypic profiles (61). The role of vertical transmission in the spread of pathogenic *E. cecorum* has been further undermined by the fact that experimentally infected broiler breeders apparently do not pass the bacterium into their eggs or embryos (73).

Regardless of the route pathogenic *E. cecorum* takes to reach a flock, horizontal transmission of pathogenic strains within the flock is rapid. In horizontal transmission, both fecal-oral and inhalation routes of infection have been postulated (12, 46). Bacteria from the genus *Enterococcus* are especially resistant to drying (68) and, although there are no data available for *E. cecorum* specifically, an infection via inhaled dust particles is theoretically possible (50). However, as commensal *E. cecorum* strains are part of the physiologic microbiota of chickens (24, 34), the intestinal tract is the most probable route of infection for pathogenic *E. cecorum* strains. Lesions of *E. cecorum* infection are observed after experimental oral inoculation (12, 53) and intravenous inoculation (53). Furthermore, in natural infection, genetically identical isolates can be cultured from the gastrointestinal tract prior to isolating them from spleen and skeletal lesions within the flock, supporting a primary gastrointestinal portal of entry (12). In a longitudinal study of natural infection with pathogenic *E. cecorum*, intestinal

colonization approached 60% of birds at week 1 and increased to 90% of birds by week 3 (12). In nonaffected broiler cycles, commensal *E. cecorum* was not detected before the third or fourth week posthatch (12, 45). These divergent colonization patterns may be explained by different colonization abilities of pathogenic and commensal strains. It is still unclear how pathogenic *E. cecorum* strains translocate through the intestinal epithelium and enter the circulatory system. Coinfections with intestinal pathogens were never detected in *E. cecorum* outbreaks (12, 18, 46) although, in theory, a coinfection could lead to epithelial lesions in the intestine, which may serve as a portal of entry for *E. cecorum*.

Once farms experience an outbreak of pathogenic *E. cecorum*, they can have multiple successive outbreaks of pathogenic *E. cecorum*, supporting the idea of a farm-associated environmental or biologic reservoir (11, 12, 49, 61). However, a biologic or environmental reservoir has yet to be identified. Multiple environmental niches within and around the affected houses have been investigated, but pathogenic *E. cecorum* strains have not been isolated from any of these places (12, 61). In unpublished observations, the authors have been able to culture pathogenic *E. cecorum* strains from fly homogenates (order *Diptera*, species unidentified) associated with experimentally infected birds. Therefore, flies associated with on-farm composting may be a possible reservoir for pathogenic *E. cecorum*; however, further work is needed. Darkling beetles (*Alphitobius diaperinus*) were also cultured from those same studies, but *E. cecorum* was not recovered (Borst L., unpubl. data).

Bacteriologic Properties of *E. cecorum*

Background and Culture Characteristics

Enterococcus cecorum was first described in 1983 as *Streptococcus cecorum*, with the isolates obtained from the intestinal tract of chickens (23). In 1989, *S. cecorum* was reclassified

to the *Enterococcus* genus based on 16S RNA sequencing (77). Since its initial description, *E. cecorum* has been identified as a commensal enteric bacterium with a wide host range that includes chickens, turkeys (*Meleagris gallopavo*), pigeons (*Columba livia domestica*), pigs (*Sus scrofa domesticus*), cattle (*Bos taurus*), horses (*Equus ferus caballus*), canaries (*Serinus canaria domestica*), and a mallard duck (*Anas platyrhynchos*) (8, 22, 62). In the intestinal tract of chickens, commensal *E. cecorum* is absent in 1-day-old chicks, comprises a minority of the enterococcal and streptococcal species of 3- to 4-wk-old chickens, and increases to become the predominate species of the enterococcal and streptococcal flora in 12-wk-old chickens (24). In contrast, pathogenic strains of *E. cecorum* are pioneer intestinal microbes appearing in the intestines of chicks as young as 7 days and then decreasing in prevalence as commensal *E. cecorum* becomes established (12).

An important phenomenon within the genus *Enterococcus* is the existence of species groups with similar phenotypic characteristics and close phylogenetic relationships (68). *Enterococcus cecorum*, together with *Enterococcus columbae*, forms the *Enterococcus cecorum* group. Based on 16S-rRNA gene sequences, this group forms a discrete branch of the phylogenetic tree, which is separated from all other *Enterococcus* species (68). Similar to other enterococcal species, *E. cecorum* is a Gram-positive coccus, nonspore-forming, facultative anaerobe. Growth requirements resemble those of streptococci, with strains requiring addition of 5% sheep blood to solid media and 5% CO_2 for optimal growth. Macroscopically, *E. cecorum* forms round, slightly mucoid, raised, cream to gray colonies with entire margins and slight α-hemolysis following overnight growth at 37 C with 5% CO_2 on trypticase soy agar with 5% sheep blood (28). Cultures grown at 42 C show increased α-hemolysis compared to those grown at 37 C. Growth is also reported on Columbia agar with 5% sheep blood, Columbia colistin

nalidixic acid (CNA) agar with 5% sheep blood, Edwards agar with 5% sheep blood, and weakly on Kanamycin Aesculin Azide agar (partial blackening) (22, 23, 25, 28). In contrast to other *Enterococcus* spp., *E. cecorum* fails to grow or grows poorly when cultured with modified esculin bile media, including BBL™ Enterococcosel broth and agar (67). Growth is also poor to absent on Slanetz and Bartley agar with tetrazolium chloride, Rogosa agar, lactose TTC agar with tergitol, and tellurite agar (23, 24, 28). Colony size is somewhat variable, with most isolates forming colonies 2–3 mm in diameter following overnight growth and with fewer strains distinctly forming colonies <1 mm (44). Phenotypically, *E. cecorum* is catalase negative, oxidase negative, yellow pigment negative, Lancefield group D negative, and nonmotile (24, 25, 28). Metabolic characterization has demonstrated variable capacity to metabolize mannitol with pathogenic isolates frequently auxotrophic for mannitol metabolism (13, 25, 28).

Laboratory identification of *E. cecorum*, particularly pathogenic strains, has been complicated. First, recovery of isolates from lesions can be difficult due to the presence of sample contamination, usually from fecal material during the necropsy evaluation. Common contaminates observed include coliforms and *Proteus* spp., which can obscure the slower-growing small colonies of *E. cecorum*. In fact, commensal *E. cecorum* from fecal material can contaminate lesion samples, which can be quite problematic for subsequent molecular epidemiologic investigations. To avoid sample contamination, a spray of 70%–95% ethanol over the lesion prior to incision with a sterile scalpel blade can significantly improve the ability to recover *E. cecorum* in pure culture. Further complicating the identification of *E. cecorum* is the fact that pathogenic isolates have not been used to inform the reference databases for automated identification systems. Occasionally, when identification systems (e.g., Vitek, API strips, Biolog, MALDI-TOF, etc.) fail to provide a species identification for *E. cecorum*, the algorithms used by

these techniques will provide an identification of *Enterococcus* spp. or *Streptococcus* spp., or will identify *E. cecorum* as a related enterococcus (e.g., *E. sulfureus*, *E. gallinarum*, etc.). However, once these databases are informed with profiles from pathogenic *E. cecorum*, they often function reliably. PCR and sequencing of the 16S ribosomal RNA gene has been reliable for identification of *E. cecorum*. A previously published species-specific multiplex PCR assay based on the *sodA* gene has been shown to be accurate for the speciation of *E. cecorum* (42). Recently, a highly specific quantitative real-time PCR was published which can be used for identification of *E. cecorum* and also for colonization studies (45). However, differentiation between pathogenic and commensal strains is not possible with this qPCR. Separation of pathogenic from commensal *E. cecorum* remains difficult. Pulsed-field gel electrophoresis (PFGE; discussed below) can identify common circulating pathotypes. Alternatively, PCR assays for conserved virulence genes (discussed below) can be used to identify pathogenic strains. However, to date, no rapid and accurate diagnostic test exists that specifically identifies pathogenic strains.

Emergence of Pathogenic Strains

Multiple reports have shown that pathogenic *E. cecorum* recovered from lesions are genetically similar to each other, as evidenced by dendrogram analysis of PFGE banding patterns, while commensal isolates originating from the gastrointestinal tract are genetically diverse (9, 11, 61). Even greater genetic similarity or clonality is reported within individual farm outbreaks as well as between outbreaks that seemingly share some connection (61, 76). Robbins *et al*. (61) identified >90% similarity via PFGE banding patterns between isolates from outbreaks on two separate farms that received chicks from the same hatchery. Similarly, Wijetunge *et al*. (76) identified clonality by PFGE banding patterns, RAPD-PCR, and ERIC-

PCR from pathogenic isolates on separate farms of the same company or from the same farm but in temporally distinct flocks. In a molecular epidemiologic investigation, Borst *et al*. (11) discovered clonal isolates were responsible for 26 epidemiologically distinct outbreaks across the southeastern United States which included all five states with the highest broiler production.

Currently, 20 full genome sequences of *E. cecorum* are available through the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/genome/genomes/12497). Four of these genomes represent the type strain ATCC 43198 (DSM 20682, NCDO 2674), leaving 13 published (13, 26, 29) and three unpublished sequences of *E. cecorum* field strains. All sequenced strains have been isolated from birds located in North America or Europe. The 20 *E. cecorum* genomes have a median total length of 2.33 megabases (Mb), 2156 protein coding sequences, and a median GC content of 36.4%. One study analyzed the genomic data in depth (13). Using whole genome sequencing, Borst *et al*. (10, 13) compared three commensal isolates to three lesion isolates that represented the most common genotypes recovered from 26 distinct outbreaks. Compared to commensal isolates, pathogenic *E. cecorum* isolates had increased GC content, decreased genome size, and a type IC CRISPR-cas system as opposed to the type IIA system found in commensal isolates (13). However, these differences were not detected when examining five pathogenic and two commensal strains from Poland (26, 29). In addition, phylogenic analysis of five genes commonly used to speciate enterococci revealed that all three pathogenic *E. cecorum* isolates cluster separately from commensal isolates. This finding indicates divergent evolution between commensal and pathogenic *E. cecorum* strains (13).

While pathogenic *E. cecorum* isolates tend to be more similar to each other than are commensal isolates, there are also reports of diversity among pathogenic *E. cecorum*. Using multiple genotyping methods on 30 isolates, Wijetunge *et al*. (76) found that, while there was

clustering (>80% similarity) on dendrogram analyses of pathogenic *E. cecorum*, multiple clusters were consistently present in all methods, supporting some degree of genetic diversity. Additionally, using 16S rRNA sequencing, MALDI-TOF MS analysis, serotyping, and fatty acid composition by gas chromatography, Jung *et al*. (44) did not identify clustering on dendrogram analyses of pathogenic *E. cecorum* that was distinct from the commensal isolates. It should be noted that strains analyzed in this work were acquired over a 20-yr period from multiple host species, which may have contributed to increased genetic diversity in this cohort of strains (44).

Virulence Assessment

As commensal and pathogenic *E. cecorum* occupy the gut niche simultaneously, and contamination of lesional cultures by commensal *E. cecorum* is possible, a reliable objective screen for virulence is needed for accurate categorization of *E. cecorum* as commensal or pathogenic. The chicken embryo lethality assay has been well studied and established as a means of discriminating between virulent and avirulent strains of *E. coli*, correlating well with other virulence assays (32, 33, 56, 80). Borst *et al*. (10) demonstrated the utility of the chicken embryo lethality assay to discriminate between commensal and pathogenic isolates of *E. cecorum*. Embryos infected with pathogenic isolates were found to have significantly lower survival compared to embryos infected with commensal isolates (10). Jung *et al*. (44) also recently confirmed the discriminatory capabilities of the assay with additional pathogenic and commensal chicken isolates. For this assay, 11-day-old embryos were inoculated with approximately 10^2 colony forming units into the allantoic cavity via a small hole drilled through the shell that was then sealed. For the following 5–7 days, eggs were candled daily to determine embryo viability (10, 44).

Virulence Determinants

Many virulence factors have been identified in *Enterococcus* spp. that cause nosocomial, opportunistic infections in humans. With the onset of chicken outbreaks of *E. cecorum*, multiple reports assessed the presence of known enterococcal virulence genes in pathogenic *E. cecorum* using PCR (Table 1.2) (28, 43, 44). Jackson *et al*. (43) evaluated both carcass rinsate and pathogenic isolates for aggregation protein gene (*agg*), extracellular metalloendopeptidase gene (*gelE*), cytolysin genes (*cylMBA*), adhesion genes (*efaAfs*, *efaAfm*, and *esp*), and genes that encode sex pheromones which aide in conjugation (*cpd*, *cob*, *cad*, and *ccf*) via PCR. Carriage of these virulence genes was uncommon in *E. cecorum* (3 out of 105) with the exception of the cell wall adhesion *efaAfm*, which was found in 104 of 105 isolates. No particular virulence gene was found to be enriched in pathogenic *E. cecorum* (43). Likewise, in 2017 Jung *et al*. (44) PCR-amplified known enterococcal virulence genes (*cylA*, *esp, asa1*, *hyl*, *gelE*, *efaAfm*, *efaAfs*, and *ccf*) in both commensal and pathogenic *E. cecorum* and also failed to identify conserved known virulence genes. In 2016, Dolka *et al*. (28) PCR-amplified pathogenic *E. cecorum* for seven virulence genes; *asa1*, *gelE*, *hyl*, *esp*, *cylA*, *efaA*, and *ace*. Of 82 isolates, only 32.9% harbored at least one of these virulence genes. Overall, virulence determinants previously described in enterococci do not appear to be conserved in pathogenic *E. cecorum*.

Table 1.2 *Enterococcus* virulence genes detected in *Enterococcus cecorum* isolates using PCR.

Virulence factor	Gene	Detected in number of pathogenic strains/number of analyzed pathogenic strains[A]	Detected in number of commensal strains/number of analyzed commensal strains[A]	References
Cytolysin activation	*cylA*	0/21; 2/148; 0/30	0/29; 1/75	(27, 43, 44)
Cytolysin transport	*cylB*	0/30	1/75	(43)
Cytolysin posttranslational modification	*cylM*	0/30	1/75	(43)
Enterococcal surface protein	*esp*	3/21; 0/148; 0/30	3/29; 0/75	(27, 43, 44)
Hyaluronidase	*hyl*	3/21; 0/148; 0/30	0/29; 0/75	(43, 44)
Aggregation substance of *E. faecium*	*asa1*	0/21; 19/148	4/29	(27, 44)
Aggregation substance of *E. faecalis*	*agg*	0/30	2/75	(43)
Gelatinase	*gelE*	3/21; 19/148; 0/30	5/29; 1/75	(27, 43, 44)
Cell wall adhesin of *E. faecium*	*efaAfm*	0/21; 30/30	0/29; 74/75	(43, 44)
Cell wall adhesin of *E. faecalis*	*efaAfs*	1/21; 10/148; 0/30	1/21; 1/75	(27, 43, 44)
Collagen-binding protein	*ace*	20/148	ND[B]	(27)
Sex pheromone	*ccf*	1/21; 0/30	3/29; 1/75	(43, 44)
Sex pheromone	*cad*	0/30	1/75	(44)
Sex pheromone	*cob*	0/30	1/75	(44)
Sex pheromone	*cpd*	0/30	1/75	(44)

[A]Results of different studies are separated by a semicolon. [B]Not done.

Borst *et al*. (13) expanded the search for virulence genes in 2015 by interrogating genomic sequences for predicted proteins that were enriched in pathogenic strains and shared homology with known virulence determinants. Using this approach, multiple putative virulence determinants conserved in pathogenic strains were identified. These determinants included a novel capsular polysaccharide locus homologous to that described in *Enterococcus faecium*, an enterococcal polysaccaraide antigen (*epa*) locus similar to that in *Enterococcus faecalis*, and a pilin locus that closely resembles the endocarditis and biofilm pilin (*ebp*) locus of *E. faecalis*, among others (13).

The putative capsule locus of *E. cecorum* contains an approximately 12-kb region which encodes several polysaccharide biosynthesis genes that are highly conserved among pathogenic strains but, in commensal strains, are variable (97%–20%) or entirely absent (13). A second rhamnose-containing polysaccharide cell wall component was discovered which is similar to the enterococcal polysaccharide antigen (*epa*) locus in *E. faecalis* (13). Both the novel capsule and *epa* loci encode polysaccharide surface modifications that are thought to confer resistance to phagocytosis in *E. faecium* and *E. faecalis*, respectively (4, 5, 58, 60, 71, 82). The polysaccharide produced by the *epa* locus is highly antigenic in *E. faecalis* (72, 81) and mediates translocation from the intestinal tract (84), biofilm formation, and tissue invasion. The arrangement of this gene cluster differs between pathogenic and commensal strains, and some genes are absent in the latter isolates. For example, *epa* is highly conserved in three pathogenic *E. cecorum* strains but absent in three commensal strains (13).

A 7-gene locus with homology to the enterococcal biofilm pilus (*ebp*) locus of *E. faecalis* and *E. faecium* was also identified by comparative genomic sequencing in pathogenic *E. cecorum* (13). The *ebp* locus of *E. faecalis* originally gained interest due to high immune reactivity in immune serum from convalescent patients (64). In both *E. faecalis* and *E. faecium*, deletion mutants in the *ebp* locus result in decreased bacterial adherence to collagen and attenuation in rodent models (54, 63).

Other putative virulence genes that were found to be highly conserved in pathogenic strains but showed lower identities or were absent in commensal isolates include genes for different cell-wall associated proteins, which may bind host tissue as collagen-binding proteins, fibronectin-binding proteins, lipoproteins, and proteins containing a cell wall targeting the

LPXTG domain (13). Additional work is required to further elucidate the function of these genes in *E. cecorum* and their role in virulence.

Lipids are important macromolecules that are found mainly in the cytoplasmatic membrane and storage granules of bacteria and the cell wall of Gram-negative bacterial species. Fatty acids are the main component of these lipids (44). Bacterial species can be characterized by their fatty acid profile. Dodecanoic acid (C12:0), tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), hexadecanoic acid (C16:0), heptadecanoic acid (C17:0), octadecanoic acid (C18:0), (11Z)-11-octadecenoic acid (C18:1 w7c), (9Z)-9-octadecenoic acid (C18:1 w9c), and (5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoic acid (C20:4 w6,9,12,15c) were detected as the major fatty acids in *E. cecorum* strains using gas chromatography (23, 44). No differences between pathogenic and commensal *E. cecorum* isolates were found concerning the fatty acid profiles (44).

Antimicrobial Resistance in E. cecorum

Most of the information about intrinsic antimicrobial resistance in enterococci is based on *E. faecalis* and *E. faecium*, the leading causes of enterococcal infection in humans (40, 51). However, there are differences regarding intrinsic resistance between these two species and *E. cecorum*. *Enterococcus faecalis* and *E. faecium* express low-affinity penicillin binding proteins (PBPs) that bind weakly to β-lactam antibiotics and, therefore, only partly disrupt bacterial cell wall synthesis (40). This results in minimum inhibitory concentrations (MICs) for penicillin of 2–8 μg/ml for *E. faecalis* and 8–16 μg/ml for *E. faecium* (40) while most *E. cecorum* isolates show MICs below 2 μg/ml (9, 11, 67, 78). It can be speculated that *E. cecorum* may produce different types of PBPs similar to those found in streptococci (78). *Enterococcus faecalis* and *E. faecium* are also intrinsically resistant to clinically achievable

concentrations of aminoglycosides, which precludes their use as single agents, but they may be used for the therapy of enterococcal infections in combination with penicillins in human medicine (40, 51). Based on combined data from several studies, the resistance patterns detected in *E. cecorum* isolates from poultry were streptomycin 17.7%, gentamicin 11.6%, neomycin 17.0%, and spectinomycin in 17.3% of the tested isolates (Table 1.3).

In comparison to other bacterial genera, enterococci are unusual in that they can absorb folic acid from the environment, bypassing the effects of sulfonamides (40). *Enterococcus cecorum* may also have this trait, as 98.8% and 93.5% of the tested isolates were resistant to sulfadimethoxine and sulfathiazole, respectively (Table 1.3). Intrinsic resistance to lincosamides has been reported for *E. faecalis* (40), and 58.4% of *E. cecorum* isolates from poultry were resistant to lincomycin (Table 1.3).

Macrolide resistance was found to be relatively higher in the poultry isolates of *E. cecorum* compared to *E. faecalis* and *E. faecium*, with erythromycin resistance reported at 70.5% and tylosin resistance at 49.8% (67). Acquired resistance to tetracycline, oxytetracycline, and doxycycline was found in 70.8%, 86.2%, and 70.6%, respectively, of *E. cecorum* strains from poultry (Table 1.3). Additionally, 38.9% of *E. cecorum* strains showed enrofloxacin resistance, which is also categorized as acquired resistance. The level of acquired resistance in *E. cecorum* isolates to different antibiotics may be associated with the use of these substances as therapeutic agents in poultry (28).

Special attention has been given to the rise of nosocomial infections in hospitalized human patients with vancomycin-resistant enterococci, predominantly *E. faecium* (6). In *E. cecorum* strains from poultry origin, vancomycin resistance was detected in only two isolates (1.3% of all tested strains; Table 1.3). Additionally, vancomycin resistance was detected

in one strain isolated from retail poultry meat (37). The origin of the vancomycin resistance gene was not known. Harada *et al*. (37) suspected an exchange of *vanA* between *Enterococcus* strains and stable chromosomal integration into the *E. cecorum* genome several years ago when avoparcin, a related antibiotic agent, was routinely used on Japanese poultry farms. Teicoplanin resistance in *E. cecorum* isolates from poultry origin was only tested in one study using the agar diffusion test, and 85.4% of Polish strains were resistant to this antibiotic (28). Quinupristin/dalfopristin resistance was found in 27.0% and linezolid resistance in 1.8% of *E. cecorum* strains from poultry sources. Rifampicin resistance was never tested in *E. cecorum* strains from poultry. Taken together, *E. cecorum* strains isolated from poultry show a relatively high level of resistance to different antimicrobials, which is a consistent property of bacteria from the genus *Enterococcus*. Nevertheless, *E. cecorum* shows some divergent patterns compared to *E. faecalis* and *E. faecium*.

When pathogenic *E. cecorum* were compared to commensal isolates, multiple reports have found greater resistance to antimicrobial agents in pathogenic *E. cecorum*, though with exceptions. Boerlin *et al*. (9) compared 50 pathogenic to 63 commensal isolates and found significantly higher MICs for enrofloxacin and gentamicin and significantly greater resistance to erythromycin and high-level streptomycin in pathogenic isolates. Similarly, Borst *et al*. (11) found consistent, increased resistance to tetracycline, oxytetracycline, and erythromycin in pathogenic *E. cecorum* compared to more-variable resistance in commensal isolates. While Dolka *et al*. (28) examined only pathogenic isolates, they found remarkably high levels of resistance, with one of the 82 isolates examined resistant to all 13 antimicrobials tested and 91% of isolates resistant to at least two antimicrobials.

Antimicrobial resistance in *E. cecorum* has important implications for human and animal health. Foremost, serious infection with *E. cecorum* has occasionally been reported in people. Case descriptions of disease include endocarditis, septicemia, peritonitis, and surgical site or urinary tract infections in people with significant comorbidities such as liver cirrhosis, malnutrition, and prolonged steroid treatment (17, 19, 32, 35, 41, 59, 74). To date, all reported *E. cecorum* isolates from humans have been susceptible to vancomycin (1, 17, 19, 41, 78). Human isolates were susceptible to β-lactam antibiotics and other antimicrobials commonly used in human medicine such as teicoplanin, rifampicin, quinupristin/dalfopristin, and linezolid (1, 17, 19, 41, 78).

The source of these human infections remains unknown, although Delauney *et al*. (19) suggested that food contamination may be a source, as food animals are colonized with *E. cecorum*. In 2017, Suyemoto *et al*. (67) demonstrated that culture methods for antimicrobial surveillance employed by the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS; https://www.cdc.gov/narms/index.html) in the United States select against pathogenic *E. cecorum*. Furthermore, multidrug-resistant pathogenic *E. cecorum* could be recovered from lesions in whole birds for sale at local grocery stores (67). However, a direct link between chicken and human infection with pathogenic *E. cecorum* has yet to be made. Even though *E. cecorum* is an infrequent cause of human disease, its role in the propagation and spread of antimicrobial resistance may not be as limited. *Enterococcus* spp. are notorious for frequent sharing of antimicrobial resistance and virulence genes via conjugative plasmids and transposons, not only among different species of *Enterococcus* but also among different genera (20, 36, 39). Therefore, pathogenic *E. cecorum*, which are also multidrug resistant, may be an important reservoir for antimicrobial resistance.

Table 1.3 Antimicrobial resistance of *Enterococcus cecorum* isolated from poultry

Antimicrobial class	Antimicrobial agent	References; number of resistant isolates/total number of isolates (% of resistant isolates)						Total % resistance
		Boerlin *et al.* (9)	Borst *et al.* (12)	Dolka *et al.* (29)	Jackson *et al.* (44)	Stepien *et al.* (67)	Suyemoto *et al.* (68)	
Aminocoumarins	Novobiocin	-[A]	70/260 (26.9%)	-	-	-	-	26.9%
Aminoglycosides	Gentamicin	27/73 (37.0%)	35/260 (13.5%)	1/82 (1.2%)	1/105 (1.0%)	-	0/32 (0.0%)	11.6%
	Kanamycin	-	-	-	14/105 (13.3%)	-	2/32 (3.7%)	11.7%
	Neomycin	-	44/260 (17.0%)	-	-	-	-	17.0%
	Spectinomycin	-	45/260 (17.3%)	-	-	-	-	17.3%
	Streptomycin	41/73 (56.2%)	32/260 (12.3%)	-	8/105 (7.6%)	-	2/32 (6.3%)	17.7%
β-lactams	Amoxicillin	-	78/260 (30.0%)	-	-	0/37 (0.0%)	-	26.3%
	Ampicillin	-	-	1/82 (1.2%)	-	-	-	1.2%
	Penicillin	2/73 (2.7%)	5/260 (1.9%)	0/82 (0.0%)	-	-	0/32 (0.0%)	1.6%
Glycopeptides	Teicoplanin	-	-	70/82 (85.4%)	-	-	-	85.4%
	Vancomycin	-	-	1/82 (1.2%)	-	1/37 (2.7%)	0/32 (0.0%)	1.3%
Glycylcyclines	Tigecycline	-	-	-	-	-	0/32 (0.0%)	0.0%
Lipopeptides	Daptomycin	-	-	-	-	-	0/32 (0.0%)	0.0%
Lincosamides	Lincomycin	-	-	-	60/105 (57.1%)	-	20/32 (62.5%)	58.4%
Macrolides	Erythromycin	65/73 (89.0%)	233/260 (89.6%)	38/82 (46.3%)	28/105 (26.7%)	-	25/32 (78.1%)	70.5%
	Tylosin	-	161/260 (61.9%)	-	15/105 (14.3%)	19/37 (51.4%)	21/32 (65.6%)	49.8%
Nitrofurans	Nitrofurantoin	-	-	0/82 (0.0%)	-	-	0/32 (0.0%)	0.0%
Oxazolidinones	Linezolid	-	-	0/82 (0.0%)	4/105 (3.8%)	-	0/32 (0.0%)	1.8%
Phenicols	Chloramphenicol	-	-	0/82 (0.0%)	1/105 (1.0%)	-	0/32 (0.0%)	0.5%

Table 1.3 (continued).

	Florfenicol	12/73 (16.4%)	53/260 (20.4%)	-	-	4/37 (10.8%)	-	18.6%
Quinolones	Ciprofloxacin	-	-	-	-	-	0/32 (0.0%)	0.0%
	Enrofloxacin	29/73 (39.7)	56/260 (21.5%)	71/82 (86.6%)	-	20/37 (54.1%)	-	38.9%
Streptogramins	Quinupristin/ Dalfopristin	-	-	-	28/105 (26.7%)	-	9/32 (28.1%)	27.0%
Sulfonamides	Sulfadimethoxine	-	257/260 (98.8%)	-	-	-	-	98.8%
	Sulfathiazole	-	243/260 (93.5%)	-	-	-	-	93.5%
Tetracyclines	Doxycycline	-	-	68/82 (82.9%)	-	16/37 (43.2%)	-	70.6%
	Oxytetracycline	-	224/260 (86.2%)	-	-	-	-	86.2%
	Tetracycline	72/73 (98.6%)	224/260 (86.2%)	5/82 (6.0%)	68/105 (64.7%)	-	22/32 (68.8%)	70.8%

[A]Not done.

Conclusion

Pathogenic *E. cecorum* with multiple drug resistance has rapidly emerged in global broiler production systems as a significant cause of mortality due to skeletal lesions and, less commonly, septicemic lesions. While pathogenic *E. cecorum* spread rapidly within a house, how this organism is spread throughout broiler production systems remains unclear, with vertical transmission suspected, but not proven. Current methods for prevention and treatment are limited. No effective vaccines exist for this organism and antimicrobial therapy is ineffective once clinical signs of paralysis appear within the flock. While progress has been made understanding disease progression and characterizing pathogenic isolates, knowledge gaps in the pathogenesis of enterococcal spondylitis still exist. For example, it remains unclear how this pathogen escapes the gut niche and evades the host immune system to set up persistent infections. However, study of the natural host pathogen relationship between pathogenic *E. cecorum* and broilers provides a valuable opportunity to identify novel virulence mechanisms of enterococci with potential impact for human and animal health.

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CHAPTER 2: Prevalence and Severity of Osteochondrosis of the Free Thoracic Vertebra in Three Modern Broiler Strains and the Athens Canadian Random Bred Control Broiler

Chen, L. R., Suyemoto, M.M., Sarsour, A.H., Cordova, H.A., Oviedo-Rondon, E.O., Barnes, H.J., & Borst, L.B. (2017). Prevalence and severity of osteochondrosis of the free thoracic vertebra in three modern broiler strains and the Athens Canadian Random Bred control broiler. *Avian Pathology*, 47, 152-160.

Introduction

Osteochondrosis (OCD) is defined as a disturbance of endochondral ossification that can affect both the epiphyseal and physeal cartilage in multiple animal species, including dogs, horses, pigs, and chickens (Ytrehus et al., 2007; Laverty & Girard, 2013; Olstad et al., 2015). OCD can present as focal or multifocal lesions that often occur at species-specific site predilections (Ytrehus et al., 2007).

Despite much work in mammalian species, the pathogenesis of OCD has not been clearly established. The underlying cause of OCD in mammals is accepted to be multifactorial, with stronger evidence supporting anatomic conformation and genetics and less support for higher growth rates and diet as contributing factors (Ytrehus et al., 2007). The two prevalent hypotheses for OCD development are either ischaemic injury due to defects in vascular supply to the cartilage or altered collagen type-II metabolism (Ytrehus et al., 2007; Laverty & Girard, 2013; Olstad et al., 2015).

Regardless of the exact pathogenesis, OCD occurs only in cartilage of weight-bearing articulations, e.g. epiphyseal and physeal cartilage, and manifests histologically as a spectrum of

lesions. Early, mild lesions, referred to as osteochondrosis latens, are identified as variably sized areas of pale eosinophilic staining articular hyaline cartilage with retention of cartilage architecture resembling coagulative necrosis. These lesions are thought to either become static or progress in severity to osteochondrosis manifesta, and ultimately, osteochondrosis dissecans. Osteochondrosis manifesta lesions are characterized by delayed or abnormal endochondral ossification, which results in regions of retained cartilage that do not become trabecular bone. In osteochondrosis dissecans, the most severe manifestation of OCD, clefts form within regions of devitalized articular cartilage and retained cartilage, which can result in free cartilaginous fragments within the joint space (Ytrehus et al., 2007). As the histologic manifestations of OCD vary in severity, so do the clinical manifestations of OCD lesions, which range from asymptomatic (Duff, 1988) to significant lameness that negatively impacts health and welfare (Ytrehus et al., 2007).

In chickens, OCD in the free thoracic vertebra (FTV) has primarily been described in work published in the 1970s and 1980s, though a few more recent descriptions do exist (Wise, 1971; Poulos et al., 1978; Rowland et al., 1980; McCaskey et al., 1982; Riddell et al., 1983; Duff, 1988, 1989a; Dämmrich & Heitmann, 1994; Dinev, 2013). Lesions described in the FTV and its articulations are consistent with manifestations of OCD; however, descriptive terminology is inconsistent in these foundational publications. Examples of terminology used to describe OCD spectrum lesions include eosinophilic streaks (Riddell et al., 1983); necrotic acellular seams (Duff, 1989a); acellular foci (Duff, 1989a); chondrolysis (Duff, 1989a); and chondrocyte death with matrix alterations (Duff, 1988).

Interest in OCD in broiler chickens has been renewed due to the discovery that osteochondrosis dissecans lesions in the FTV predispose to enterococcal spondylitis (ES) (Borst

et al., 2017). The characteristic clinical feature of ES is symmetrical leg paresis or paralysis due to necrosis and inflammation of the FTV caused by pathogenic *Enterococcus cecorum*. This disease, first described in 2002, continues to plague poultry-production systems worldwide (Devriese et al., 2002; Wood et al., 2002). In ES, genetically related strains of *E. cecorum* share putative virulence determinants and are capable of escaping the intestinal niche. Bacteraemia increases in prevalence in an infected flock throughout the first 5 weeks of life (Borst et al., 2014, 2015, 2017). Additional lesions identified during outbreaks of pathogenic *E. cecorum* infection include pericarditis, perihepatitis, and femoral head osteomyelitis (McGillveray et al., 2002; Stalker et al., 2010; Robbins et al., 2012; Jung & Rautenschlein, 2014). Recently, in both naturally and experimentally infected flocks, chickens without OCD or with either osteochondrosis latens or manifesta lesions in the FTV or its articulations were not found to develop ES. Only birds with osteochondrosis dissecans were susceptible to ES (Borst et al., 2017).

As OCD in mammals is considered to have a genetic component (Ytrehus et al., 2007), our first objective was to identify prevalence and severity of OCD lesions in three modern broiler lines and the Athens Canadian Random Bred (ACRB) control strain, which represents 1950s broiler genetics (Collins et al., 2014, 2016). Since incubation temperature profiles are known to impact musculoskeletal health (Oviedo-Rondon et al., 2009a, b), our second objective was to determine if incubation temperature profiles influenced the occurrence and severity of OCD. Our final objective was to determine the effects of sex, body weight, and age on OCD prevalence and severity. Our hypotheses were (1) modern strains would have increased prevalence and severity of OCD compared to ACRB chickens, (2) incubation profiles associated with poor

musculoskeletal development would positively correlate with increased OCD scores, and (3) body weight, age, and male sex would positively correlate with increased OCD scores.

Materials and methods

Experimental design

Experimental use of birds was approved by the Animal Care and Use Committee and conducted in compliance with the Guidelines for Care and Use of Laboratory Animals at North Carolina State University.

Treatment groups and bird numbers are provided in Table 1. Both sexes of four broiler chicken strains and two incubation profiles for a total of 16 treatments were evaluated for prevalence and severity of OCD in the FTV. For each of three modern commercial broiler chicken strains (A/A, A/B, and C/C), 1000 fertile eggs were obtained from parent broiler-breeder flocks, which had received similar nutrition and management. In addition, 1000 fertile eggs of ACRB chickens, a line representative of 1950s broiler chickens, were obtained from the University of Georgia (Athens, GA).

Table 2.1 Treatment groups in experimental design.

Group	Pens	N (per pen)	Strain	Incubation	Sex
1	5	12	A/A	L/H	M
2	5	12	A/A	L/H	F
3	5	12	A/A	S	M
4	5	12	A/A	S	F
5	5	12	A/B	L/H	M
6	5	12	A/B	L/H	F
7	5	12	A/B	S	M
8	5	12	A/B	S	F
9	5	12	C/C	L/H	M
10	5	12	C/C	L/H	F
11	5	12	C/C	S	M
12	5	12	C/C	S	F
13	5	12	ACRB	L/H	M
14	5	12	ACRB	L/H	F
15	5	12	ACRB	S	M
16	5	12	ACRB	S	F

Fertile eggs from all strains were randomly divided between two incubators programmed either with standard (S) or low-high (LH) incubation temperature profiles, as previously described (Da Costa et al., 2016). Two Chick Master G18 machines (Chick Master Incubator Company, Medina, OH, USA) were used for incubation, each with a relative humidity of 49% and an hourly turning angle of 45°. Eggshell temperature for five eggs per incubator was measured four times daily using a Thermistor TM99A (Cooper-Atkins Corporation, Middlefield, CT, USA) pipe probe to avoid repeated opening of the incubator; ambient air temperature was measured every five minutes.

The S temperature profile maintained optimal eggshell temperature at 37.8°C throughout the incubation period by modulating ambient temperature. In the hatcher for treatment S, ambient temperature was initially set at 36.9°C, decreasing to maintain an eggshell temperature no greater than 37.9°C and then decreasing slowly to 34.7°C at the time chicks were removed. For the LH temperature profile, ambient temperature was set to 37.4°C without modulation based on eggshell temperature, resulting in eggshell temperatures below optimum (36.9°C) during the first 72 hours of incubation and eggshell temperatures higher than optimum (39°C) following the 16th day of incubation. Temperatures were decreased to 36.9°C for the final 24 hours in the hatcher to maintain hatchability.

On the day of hatch, chicks were weighed, separated by sex using feather or vent sexing, individually tagged, and placed into 80 floor pens with 12 birds per pen (n = 960). There were five pen replicates (60 birds) for each of the 16 broiler line/sex/incubation profile combinations (Table 1). Birds were raised on six inches of pine shaving litter covering concrete floors. Birds were fed *ad libitum* with diets formulated to meet nutritional requirements containing corn, soybean meal, and distiller's dried grains with solubles, milled into a crumble for the starter diet

(days 0–14) and pellets for the grower (days 15–35) and finisher (days 36 to end of study) diets. Nicarbazin (1 lb/ton) was added to the starter and grower diets to control *Eimeria* spp.

Sample collection

At 2, 4, 6, and 8 weeks of age, 10 birds were sampled for each broiler line/sex/incubation temperature combination by randomly selecting two birds from each of the five replicate pens. As there were 16 broiler line/sex/incubation temperature conditions, 160 birds were evaluated at each sampling time point. At each time point, birds were euthanized by cervical dislocation, weighed, and the FTV and its articulations with the notarium and synsacrum were collected into 10% neutral buffered formalin.

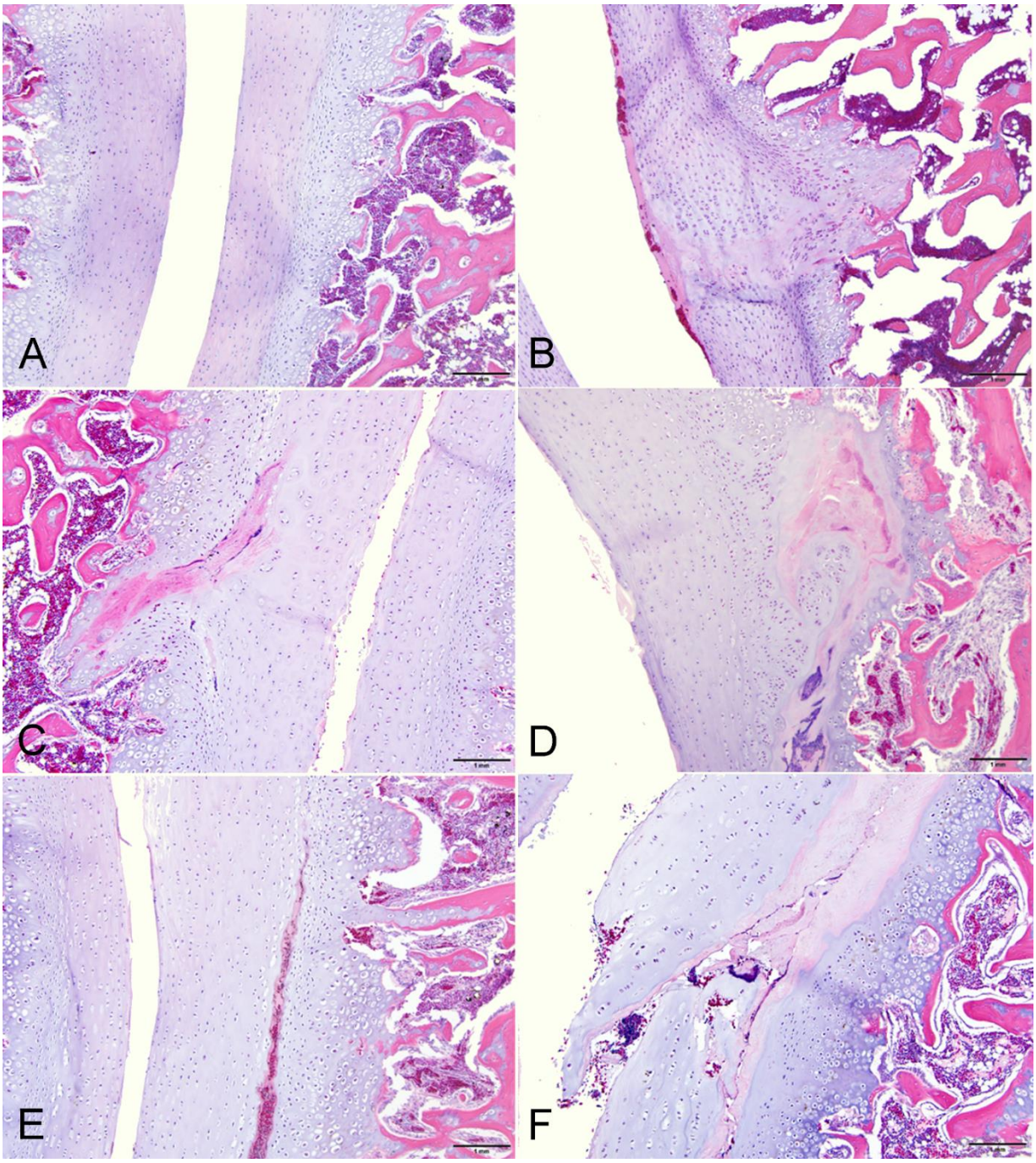
Tissue processing

Formalin-fixed tissue samples were transferred to 5% neutral buffered formalin containing 10% formic acid for 24–48 hours (depending on the age of the bird), trimmed in the sagittal plane to capture the spinal canal, and processed into paraffin wax blocks. Samples were then cut into 3- to 5-µm-thick sections and stained using routine procedures for haematoxylin and eosin.

Histomorphologic scoring

Scoring OCD lesions in the FTV and articulating cartilages of the notarium and synsacrum was performed as previously described (Borst et al., 2017). OCD lesions were scored as follows: 0 = normal; 1 = osteochondrosis latens; 2 = osteochondrosis manifesta; 3 = osteochondrosis dissecans; and 4 = osteochondrosis dissecans with clefting and flap formation in the articular cartilage. All articulating surfaces were scored; the highest score of all surfaces being the final score (Figure 1). Samples were excluded if adequate representation of the FTV and its articulations was not present in the sections. A total of 544 sections were scored for OCD.

Figure 2.1. Histology of normal and OCD-affected vertebral articular cartilage. (a) Normal articular cartilage. (b) Osteochondrosis manifesta (severity score 2) lesion with a focus of retained articular cartilage (arrows) into the metaphyseal region replacing primary spongiosa. Intermixed areas of degenerative (*) and viable chondrocytes and matrix are present. (c–e) Osteochondrosis dissecans (severity score 3) lesions with a linear (c, e) to irregularly shaped (d) focus of necrotic cartilage with cleft formation (arrows). Retention of cartilage into the metaphyseal region (c–d) and accumulation of erythrocytes (e) is variably present. (f) Osteochondrosis dissecans lesion with clefting and flap formation (arrows) through the margin of the articular cartilage (severity score 4) with accumulation of erythrocytes, thrombocytes, and fibrin along the flap.


A
B
C
D
E
F

Statistical analyses

Mean OCD scores by sex were compared among broiler strains and between incubation temperature profiles using the non-parametric Kruskal–Wallis test with pairwise *post hoc* comparisons performed using a Mann–Whitney U test. In addition to comparing mean OCD scores among groups, a categorical approach was also employed in which birds were categorized as having an OCD score ≥3 or an OCD score ≤2 and comparisons among breed/sex groups were performed using a Fisher's exact test. This categorical approach was performed because the OCD score ≥3 has been shown to have biologic relevance in predisposing broilers to pathogenic *E. cecorum*.

Average body weights by sex and strain were compared among broiler strains at weeks 2, 4, 6, and 8 using a one-way analysis of variance with pairwise *post hoc* comparisons using the Tukey–Kramer method. Correlation of OCD score and body weight by strain was determined by calculating Spearman's rho (r). In all cases, significance was established to be $P < 0.05$.

Results

OCD scoring

Score 1: Osteochondrosis latens lesions were characterized by linear to irregularly shaped foci of articular cartilage necrosis. In areas of necrosis, extracellular matrix was pale, hypereosinophilic, and contained necrotic chondrocytes with pyknotic to karyolytic nuclei and shrunken, hypereosinophilic cytoplasm. Score 2: Osteochondrosis manifesta lesions contained foci of variably necrotic to viable cartilage into the metaphysis and diaphysis, replacing primary and secondary spongiosa. When viable, cartilage was composed of disorganized, hypertrophied chondrocytes (Figure 2.1(b)). Frequently, histologic sections captured changes indicating progression from score 2 (manifesta) to score 3 (dissecans) lesions (Figure 2.1(c–e)). When

changes consistent with multiple scores were present in the same section, the highest score was recorded. Score 3: Sections had linear foci of necrotic cartilage and clefting consistent with osteochondrosis dissecans. Clefts in the cartilage were either empty or contained mild to moderate numbers of erythrocytes, thrombocytes, and fibrin. Score 4: Osteochondrosis dissecans lesions (Figure 2.1(f)) were similar to score 3 lesions but had clefts that extended through a margin of the cartilage, resulting in flap formation.

Prevalence and severity of OCD

Overall, OCD spectrum lesions were observed in 461 of 544 (84.7%) spinal sections. OCD score 1 (osteochondrosis latens) was observed in 77 (14.2%) sections; score 2 (osteochondrosis manifesta) in 36 (6.6%) sections; score 3 (osteochondrosis dissecans) in 302 (55.5%) sections; and score 4 (osteochondrosis dissecans with flap formation) in 46 (8.5%) sections (Table 2.2). Previously, OCD scores ≥3 were shown to be clinically relevant because they predispose to infections with pathogenic *E. cecorum*; overall 348 sections had OCD scores ≥3 (64.0%). Numbers of birds with OCD scores ≥3 per strain/sex combination are provided in Table 2.2.

Table 2.2 Prevalence of osteochondrosis score by strain and sex.

			OCD Score										
Strain	**Sex**	**N**	**0**		**1**		**2**		**3**		**4**		**Total OCD Score ≥3**
A/A	F	68	15	22.1%	8	11.8%	2	2.9%	31	45.6%	12	17.6%	43 (63.2%)[ab]
	M	68	6	8.8%	11	16.2%	6	8.8%	42	61.8%	3	4.4%	45 (66.2%)[ab]
A/B	F	66	5	7.6%	9	13.6%	4	6.1%	35	53.0%	13	19.7%	48 (72.7%)[a]
	M	71	4	5.6%	10	14.1%	6	8.5%	48	67.6%	3	4.2%	51 (71.8%)[a]
C/C	F	71	12	16.9%	9	12.7%	5	7.0%	40	56.3%	5	7.0%	45 (63.3%)[ab]
	M	66	9	13.6%	14	21.2%	2	3.0%	38	57.6%	3	4.5%	41 (62.1%)[ab]
ACRB	F	67	20	29.9%	9	13.4%	5	7.5%	31	46.3%	2	3.0%	33 (49.3%)[b]
	M	67	12	17.9%	7	10.4%	6	9.0%	37	55.2%	5	7.5%	42 (62.7%)[ab]

[ab] Items that do not share superscript letters are significantly different ($p<0.05$).

Incubation temperature profile

Mean OCD scores were compared between chickens incubated under optimal temperature conditions (S) and those incubated under sub-optimal temperature conditions (LH) within time points and sex. Overall, there were no significant differences observed between OCD scores from birds exposed to the two incubation profiles (Figure 2.2). Rarely, individual age/sex/strain combinations at individual time points had significantly different OCD scores ($P < 0.05$); however, these findings were inconsistent and did not form a recognizable trend (Appendix A).

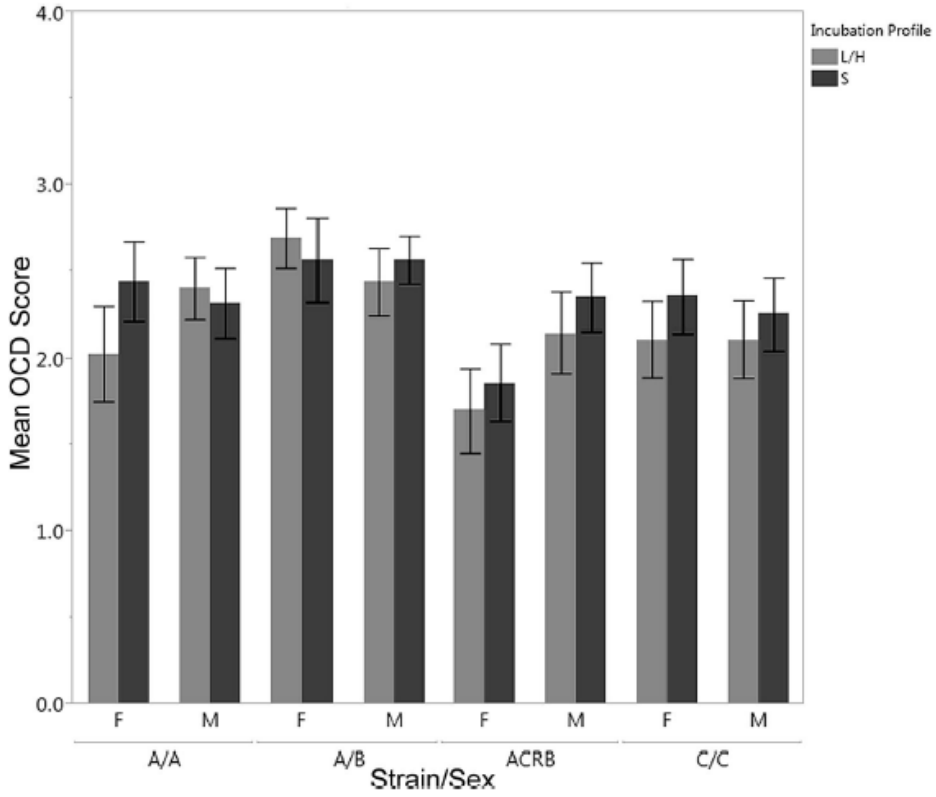


Figure 2.2 Mean OCD score of each incubation temperature profile within strain/sex combinations. Significant differences in mean OCD scores were not observed in any strain/sex combinations between birds incubated at optimal (S) and sub-optimal (L/H) temperature conditions.

Body weight

As expected, ACRB male and female birds had significantly lower body weights ($P < 0.05$) than all other treatment groups. Final average body weights of ACRB birds were approximately four to five times less than modern broiler strains. By week 8, there were no significant differences among any of the body weights of modern commercial broiler strain males and females. However, when compared to each other, there were significant differences in average body weights between males and females of modern broiler lines (Table 2.3).

Table 2.3 Body weight by strain per week.

			Week							
			2		4		6		8	
Strain	**Sex**	**N**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
A/A	F	68	506.9 ^{ab}	10.1	1588.6 ^{cd}	32.5	2950.0 ^{bc}	56.5	4161.7 ^{b}	64.1
	M	68	545.7 ^{a}	7.8	1756.3 ^{ab}	43.0	3439.7 ^{a}	77.7	4635.7 ^{a}	159.0
A/B	F	66	528.4 ^{a}	11.2	1689.5 ^{bc}	28.7	3095.8 ^{b}	68.4	4144.4 ^{b}	62.1
	M	71	531.9 ^{a}	9.1	1838.7 ^{a}	27.5	3649.5 ^{a}	57.3	4831.4 ^{a}	173.5
C/C	F	71	473.5 ^{b}	10.0	1482.4 ^{d}	28.7	2847.6 ^{c}	48.6	3893.4 ^{b}	78.8
	M	66	506.6 ^{ab}	10.6	1759.7 ^{ab}	31.7	3478.8 ^{a}	62.8	5073.0 ^{a}	130.9
ACRB	F	67	141.7 ^{c}	5.5	329.3 ^{e}	9.4	598.6 ^{d}	19.8	883.4 ^{c}	21.6
	M	67	149.0 ^{c}	6.9	394.1 ^{e}	10.7	718.9 ^{d}	36.1	1081.0 ^{c}	21.5

SEM – Standard error of the mean

^{abcd} Items that do not share superscript letters are significantly different ($p<0.05$).

To assess the correlation of OCD score with body weight, scatter plots of body weights categorized by mean OCD score for each time point were generated (Figure 3) and correlation coefficients were calculated. No correlation between body weight and OCD scores was detected at any time point except C/C males at 2 weeks (significant but weak positive correlation $r^2 = 0.40$) and ACRB females at 4 weeks (significant but weak negative correlation $r^2 = 0.22$) (Appendix B). Overall, no consistent trends correlating body weight to OCD score were identified.

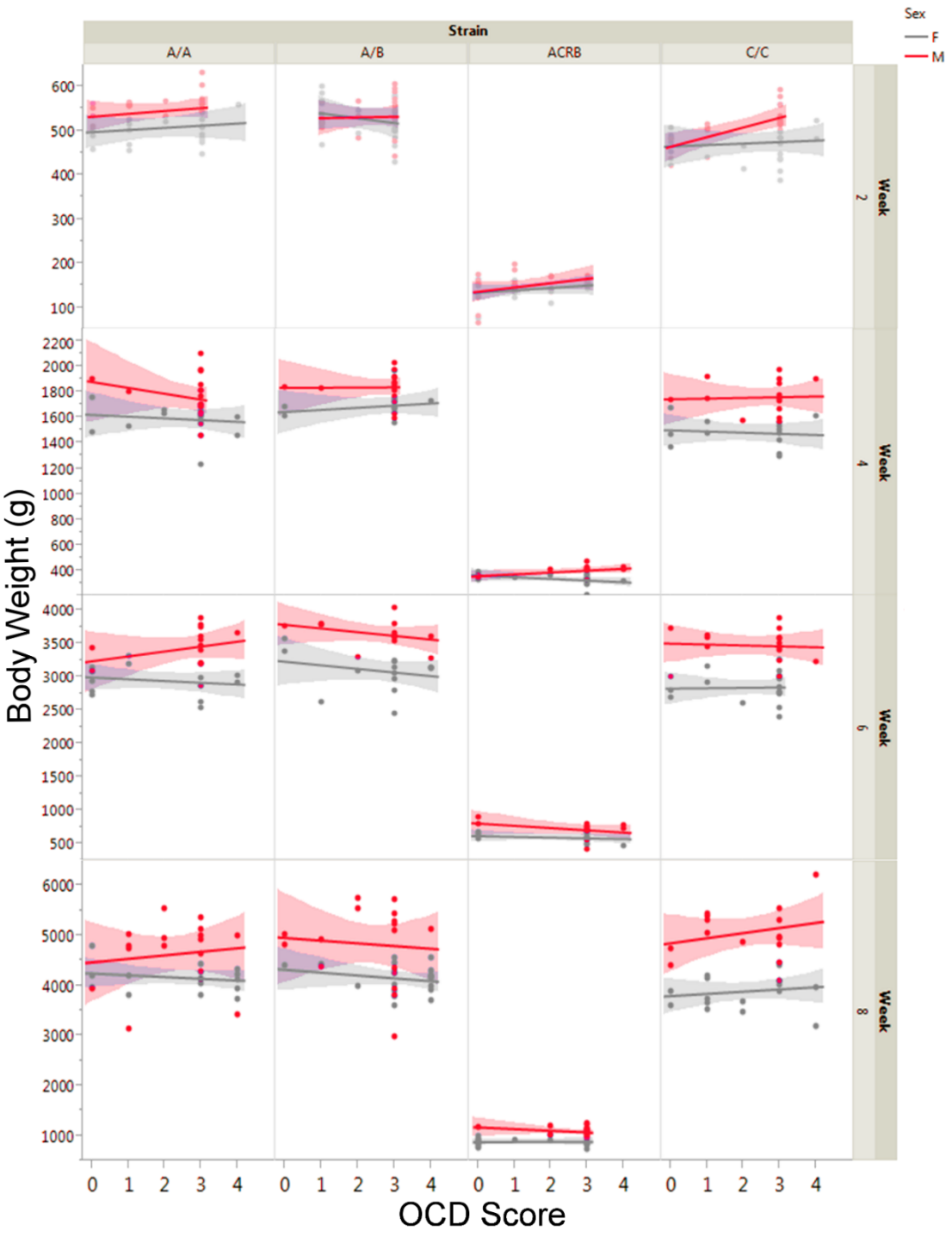


Figure 2.3 Scatter plot of body weights based on mean OCD score within sex/strain combinations at each time point. No consistent, significant correlations between body weights and OCD scores were identified (see results for additional details).

Breed and sex

There were no significant differences in the means of OCD scores among male chickens of all broiler lines examined. Within each strain, there were no significant differences in the means of OCD scores between males and females. When comparing mean OCD scores, the only significant difference was females in the ACRB had significantly lower OCD scores compared to A/B strain females (Figure 2.4).

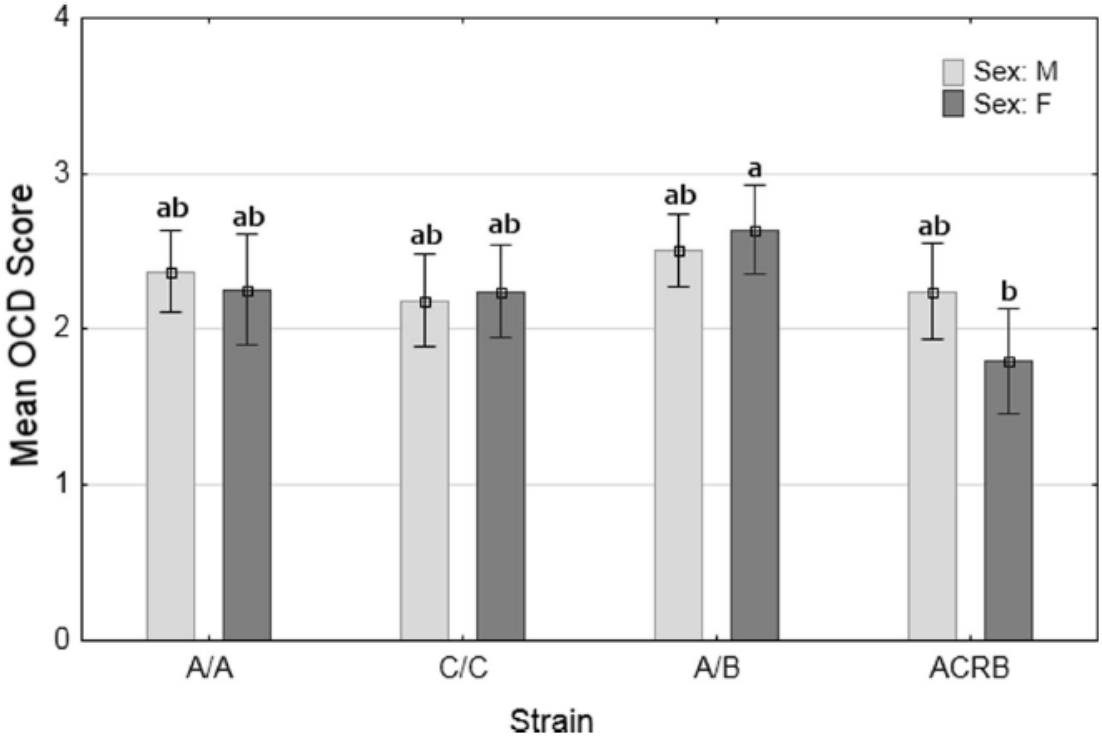


Figure 2.4 Mean OCD score of each strain/sex combination. No significant differences were identified among males of each strain. No significant differences occurred between male and female chickens within each strain. ACRB female chickens had significantly lower mean OCD scores compared to A/B female chickens, with no differences among females of the other strains.

In a second analysis, OCD scores were categorized using OCD score of 3 as a cut-off and compared among sex/strain combinations. As with the comparisons of OCD score means, there were no significant differences among ACRB males, modern broiler strain males, and modern

broiler strain females. Likewise, a significant difference between female ACRB and females of the A/B strain was observed as before. However, with this analysis, A/B males were also found to have significantly more birds with OCD score ≥3 than ACRB females.

Discussion

In this study, a histomorphological scoring method was used to compare prevalence of OCD in the FTV among three modern broiler strains and the ACRB control strain and to observe any correlations of incubation profile, sex, and body weight with OCD severity. The pathogenesis of OCD in the FTV of chickens has not been thoroughly studied, but based on both primary poultry research as well as comparative pathology, correlations with these parameters were expected. While ACRB females had significantly lower OCD scores than one modern broiler cross, overall there were no clear correlations of OCD in the FTV with any of the parameters.

A high prevalence of OCD in the FTV occurred in all three modern commercial broiler strains as well as the ACRB strain at all ages. OCD spectrum lesions were present in 84.7% of birds and greater than 60% of birds had an OCD severity score of 3 or greater. These findings are consistent with our prior study that identified a modern commercial broiler strain housed in similar conditions with 89% prevalence of OCD spectrum lesions and 76% prevalence of OCD severity score of 3 or greater (Borst et al., 2017). In a prior field case study, a significant increase in OCD severity score of the FTV in a commercial broiler flock was observed during the first 4 weeks of life (Borst et al., 2017). A similar progression of lesions was not identified in this study, with no statistical difference among OCD severity scores at any of the sampling weeks. This difference may be a consequence of decreased frequency of early sampling. In our prior study, chickens were sampled four times in the first 4 weeks compared with just twice in this

study. Additionally, birds in this study were housed in pens, on litter covering a concrete floor. In the prior study, birds were housed on litter over dirt at a commercial broiler farm. As such, environmental factors in litter management and flooring that vary among production systems may be responsible for differences in the progression of OCD lesions in the two studies.

Breed associations with OCD in other species exist (Goedgebuure, Häni, van der Valk, & van der Wal, 1980; Slater et al., 1991), and breed associations with other cartilaginous diseases in chickens are reported (Reiland et al., 1978). Genetic markers associated with OCD have been identified in several species using genome-wide association studies (Andersson-Eklund et al., 2000; Christensen et al., 2010; Rangkasenee et al., 2013; Bates et al., 2014). Therefore, we hypothesized that there would be a strain correlation with OCD in the FTV. Surprisingly, no strain-wise associations with OCD severity were observed among modern broilers and male ACRB birds. One modern strain cross A/B did demonstrate significantly more severe OCD compared to the female ACRB. However, even the ACRB females had high rates of OCD with ~49% having histologic OCD scores ≥3 and there were no differences between ACRB females and either sex of any of the remaining genetic lines. Our findings fit with previous descriptions of OCD in the FTV of broiler chickens performed in the 1980s (Duff, 1988, 1989a, 1989b) and by evaluating the ACRB broiler chicken, we were able to demonstrate that any potential genetic drivers of OCD were already bred into broilers in the 1950s. It is unclear how far back ancestrally this lesion is present, though it would be interesting to determine and may provide additional information on genes important in the pathogenesis of OCD in the FTV.

Associations between sex and OCD in multiple species have been inconsistent (Slater et al., 1991; Sandgren et al., 1993; Jørgensen, 2003). Associations between sex and OCD in the FTV have not been examined in poultry species. In this study, there was no consistent correlation

between sex and OCD severity. This is particularly surprising as male chickens had significantly greater body weights compared to female chickens; however, body weights also did not correlate with OCD severity. Similarly, correlations between body weight and OCD in multiple species are inconsistent (Goedgebuure et al., 1980; Carlson et al., 1988; Slater et al., 1991; Ytrehus et al., 2004a, 2004b).

As previously mentioned, there was no correlation between body weight and OCD severity in this study. The most surprising aspect of this finding was the lack of difference in OCD severity scores in ACRB chickens compared to modern commercial broiler chickens even though the body weight of ACRB chickens was less than 25% of the body weight of the modern commercial broiler lines at 8 weeks. This supports the idea that OCD is not a body-weight-dependent disease; however, additional work needs to be done to see if the clinical manifestation of lameness from OCD lesions might be correlated with body weight.

Incubation temperature profiles have wide-reaching impacts on chicken physiology. Within the past decade, researchers have identified a significant negative impact on leg health in chickens incubated in sub-optimal incubation temperature conditions as seen with multi-stage incubation (Oviedo-Rondon et al., 2009a, b; Da Costa et al., 2016). Lesions correlated with suboptimal incubation temperature profiles include crooked toes, decreased relative weights of femurs and shanks, increased incidence of twisted legs, and increased (poorer) gait scores (Oviedo-Rondon et al., 2009a). Despite expecting a correlation between incubation temperature profiles and OCD of the FTV, none was found. As sub-optimal incubation profiles frequently result in leg health abnormalities, correlations between OCD and incubation temperature profiles may exist in bones of the legs.

In summary, OCD in the FTV of multiple modern broiler chicken strains as well as the ACRB strain is highly prevalent and despite a significant difference in OCD severity between ACRB females and one modern broiler cross; overall we observed no significant correlation with age, sex, body weight, strain, or incubation temperature profile. The high prevalence of OCD in modern broiler chickens, and its role as a predisposing lesion to ES, indicates that OCD is an important disease in broilers. OCD has also been implicated in the pathogenesis of other causes of broiler lameness, including femoral head necrosis, tibial dyschondroplasia, and bacterial chondritis and osteomyelitis, which negatively impact welfare and livability (Wideman, 2016). Despite progress made with broiler genetics over the last century, it appears that OCD remains a common underlying condition in broilers. This study indicates that OCD plays a significant role in musculoskeletal health of broiler chickens and establishes a basis for understanding the pathogenesis of OCD. Given the central role OCD appears to play in broiler lameness, further work on this disease is needed. The prevalence of OCD in heritage breeds or meat-type chickens housed in non-conventional production systems remains unknown. Future studies are warranted to assess other potential contributing factors to OCD as well as the distribution of OCD lesions throughout the chicken skeleton.

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CHAPTER 3: Prevalence and Severity of Osteochondrosis in the Red Jungle Fowl, Two Heritage Breed Chickens, and One Commercial Broiler Strain

Introduction

Osteochondrosis (OC) is a well described skeletal disease and cause of lameness in multiple animal species, including humans, dogs, horses, pigs, and chickens (Ytrehus et al., 2007; Olstad et al., 2015; Craig et al., 2016). Osteochondrosis is characterized by a focal failure of endochondral ossification and has three progressive manifestations in the articular-epiphyseal cartilage complex (Yrehus et al., 2007; Craig et al., 2016). The earliest observable manifestation of OC, called OC latens, is a focal region of epiphyseal growth cartilage necrosis. This earliest lesion can progress to a region of retained necrotic epiphyseal growth cartilage that extends into subchondral bone termed OC manifesta. The fulminant OC lesion, OC dissecans, has a characteristic cleft formation in the necrotic epiphyseal growth cartilage (Yrehus et al., 2007; Craig et al., 2016). While it is possible for these lesions to develop in any articular-epiphyseal cartilage, each species has their own unique site predilections (Yrehus et al., 2007; Craig et al., 2016).

Much of the research on chicken OC was published in the latter half of the 20th century (Poulos et al., 1978; Reiland et al., 1978; Rowland et al., 1980; Riddell et al., 1983; Duff, 1988; Duff, 1989a, 1989b) but recently there has been renewed interest in the disease. This interest stems from identification of free thoracic vertebral (FTV) OC dissecans in broiler chickens predisposing to enterococcal spondylitis (Borst et al., 2017; Jung et al., 2018). Enterococcal spondylitis is an important disease of broiler chickens where FTV osteomyelitis caused by pathogenic strains of *Enterococcus cecorum* leads to mortality that can approach 20% (Robbins

et al., 2012; Borst et al., 2017; Jung et al., 2018). *E. cecorum* is a normal gut commensal bacterial organism of chickens that typically appears in the intestinal microbiota around 3 weeks of age and grows to be a dominant member by 12 weeks of age (Devriese et al., 1991). However, pathogenic strains are capable of early colonization of the gut in the first 3 weeks of life which is then quickly followed by bacterial septicemia, and ultimately flock outbreaks of bacterial osteomyelitis of the FTV (Borst et al., 2017; Jung et al., 2018). Also contributing to greater interest in OC and related to the above disease is the hypothesis by some researchers that the pathogenesis of bacterial chondronecrosis and osteomyelitis, an important skeletal disease affecting the hips of broiler chickens, includes osteochondrosis as the key factor for host susceptibility (Wideman 2016; Wideman & Prisby 2016). In our studies, birds that lacked OC dissecans lesions did not develop bacterial osteomyelitis, which indicates that there may be the potential for control these important causes of broiler lameness and livability challenges without the use of antibiotics if the pathogenesis of OC could be elucidated.

Presently, the understanding of OC pathogenesis in chickens is in its infancy with most of the literature on chicken OC descriptive in nature (Duff, 1988; Duff, 1989a; Duff, 1989b; Reiland et al., 1978; Rowland et al., 1980; Riddell et al., 1983). In mammalian species, there has been more in-depth investigative work into the pathogenesis of OC. In those species, there is stronger support for heritability, anatomic conformation, and microtrauma contributing to OC formation with the key step being compromise of the vascular supply and ischemic injury to the cartilage (Carlson et al., 1995; Ytrehus et al., 2004a, 2007; Olstad et al., 2015; Craig et al., 2016). Recently, Chen et al. (2017) analyzed three modern commercial broiler strains and a 1950's meat type chicken in a longitudinal study to identify factors that correlated with increased prevalence and severity of FTV OC. In broilers, no significant correlations were observed

between FTV OC and sex, body weight (i.e. growth rate), incubation temperature conditions, and broiler chicken strain (Chen et al., 2017). The findings that sex, body weight, and incubation temperature conditions did not significantly correlate with FTV OC was not unexpected as there is either an absence of or inconsistent literature support for these correlations (Goedgebuure et al., 1980; Carlson et al., 1988; Slater et al., 1991; Sandgren et al., 1993; Jørgensen, 2003; Ytrehus et al., 2004b, 2004c). While a trend toward decreased OC in female birds in the 1950's broiler strain was observed, the finding of similar OC severity among strains was unexpected given the support for a heritable component in other species (Ytrehus et al., 2007; Stattin et al., 2010; Storskrubb et al., 2010; Rangkasenee et al., 2013; Craig et al., 2016). We hypothesized that the genetic drivers of OC may have been bred into the broiler line early and then propagated over time.

Therefore, the first objective of this study was to compare prevalence and severity of OC lesions in more dissimilar chicken strains (Sharma et al., 2001; Eltanany and Distl, 2010). To this end, we selected the red jungle fowl which is phenotypically similar to the presumed progenitor of modern domestic chickens and the two heritage breed chickens which were crossed to generate the modern broiler strain (Dark Cornish and White Plymouth Rock) to compare to a modern commercial broiler strain. Sites examined for OC lesions included the FTV as well as the proximal tibiotarsus and proximal femur. Our second objective was to re-evaluate parameters demonstrated to lack a correlation with OC in chickens previously (Chen et al., 2017), including sex and bodyweight. Our hypotheses in this study were that (1) there would be a strain differences in OC severity and (2) there would be no correlation of sex or bodyweight with OC severity.

Materials and methods

Experimental design

Experimental use of birds was approved by the Animal Care and Use Committee and conducted in compliance with the Guidelines for Care and Use of Laboratory Animals at North Carolina State University.

Twenty-five, 1-day-old chicks from the four different chicken strains were obtained from three different sources. The Dark Cornish (DC) and White Plymouth Rock (WR) breeds were obtained from a mail-to-order online hatchery (Murray McMurray, Webster City, Iowa, USA). The red jungle fowl (JF) breed chickens were obtained from a separate mail-to-order online hatchery (Cackle Hatchery, Lebanon MO, USA). The modern commercial broiler chickens were obtained from a local commercial hatchery (North Carolina, USA).

Upon arrival, all chicks were immediately placed in a large floor pen containing six inches of pine shaving litter overlying concrete floors. Temperature and lighting conditions were provided to meet physiologic needs over the rearing period. Birds were fed an *ad libitum* diet containing corn, soybean meal, and distillers dried grains with solubles formulated to meet nutritional requirements. Diets were milled into a crumble for the starter diet (0 – 14 days) and then pelleted thereafter. Birds were monitored at least once daily, with any bird unable to adequately obtain feed and/or water euthanized by cervical dislocation.

Sample collection and processing

At 5 weeks of age, all birds were euthanized by cervical dislocation and weighed. Immediately, the free thoracic vertebrae and its articulations with the notarium and synsacrum; both proximal femurs in their acetabula; and both proximal tibiotarsi were collected into 10% neutral buffered formalin (NBF).

Following adequate fixation in 10% NBF, tissue samples were transferred to 5% NBF containing 10% formic acid for 24 – 48 hours (depending on the size of the sample). Subsequently, the FTV and proximal tibiotarsus samples were sectioned in the sagittal plane and the proximal femur samples were sectioned in the frontal plane, with the plane chosen to maximize the amount of visible surface area of the articular and growth plate cartilage. Samples were then processed into paraffin wax blocks; cut into 3 – 5 um thick sections; and stained using routine procedures for hematoxylin and eosin.

Histomorphologic scoring

Scoring of the OC lesions was performed as previously described (Borst et al., 2017). Briefly, the scoring system is as follows: 0 = normal; 1 = osteochondrosis latens; 2 = osteochondrosis manifesta; 3 = osteochondrosis dissecans; and 4 = osteochondrosis dissecans with clefting and flap formation in the articular cartilage. If multiple lesions were present in one sample, the most severe lesion (i.e. highest score) was recorded. For the proximal tibiotarsus and proximal femur samples, the average score of the left and right samples examined for each bird was used as the score for that anatomic location. Samples were excluded from analysis if the amount of articular and growth cartilage were inadequate in the tissue sections.

Statistical analyses

Mean OC scores, representative of OC severity, were compared between sexes within chicken strains using the non-parametric Kruskal-Wallis test. Mean OC scores were compared among chicken strains using the non-parametric Kruskal-Wallis test with *post hoc* pairwise comparisons performed using a Dunn's test with Bonferroni's correction on the p-value. Prevalence of OC, with birds categorized as either having or lacking OC, was compared using Fisher's exact test.

Average body weights by strain were compared using a one-way analysis of variance with pairwise *post hoc* comparisons using the Tukey-Kramer method. Correlation of OC score and body weight among all strains and within each strain were determined by calculating Spearman's rho (ρ). In all cases, significance was established to be p-value < 0.05.

Results

Tissue evaluation and OC scoring

Histologic characteristics of the OC spectrum lesions were similar to those previously described (Chen et al. 2017). In the proximal femur and proximal tibiotarsus were prominent cartilage canal profiles. While the vessels within the cartilage canals did not overlap in appearance with OC spectrum lesions, when captured tangentially, the bordering cartilage appeared paucicellular and granular with altered tinctorial quality similar to necrotic cartilage matrix in OC lesions. In order to prevent overinterpretation of tangential sections of vessel profiles in the proximal femur and proximal tibiotarsus, foci of cartilage demonstrating features of degeneration that overlap with OC lesions were ignored if 1) they were associated either proximally or distally with a vessel profile and 2) were perpendicularly oriented to the articular surface.

In addition to the OC spectrum lesions observed, in only MB birds, multiple sections of the proximal femur and proximal tibiotarsus had uncommon, scattered, thrombosed and necrotic cartilage canals. These cartilage canals were characterized by replacement of the vessel profile with a coagulum of thrombocytes, fibrin and smaller amounts of necrotic cellular and nuclear debris with loss of endothelial cell profiles.

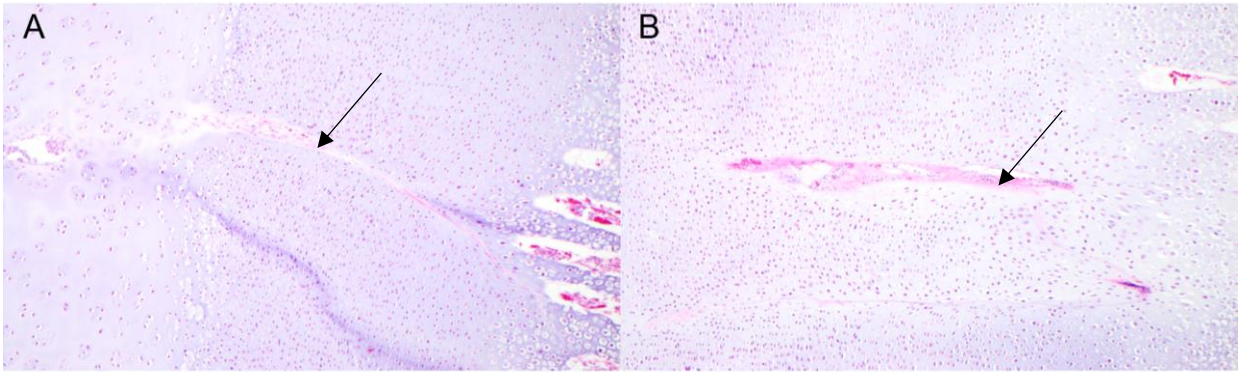

Figure 3.1 Penetrating vessel profiles in the epiphyseal cartilage. In (a) there is a normal vessel profile (arrow) oriented perpendicular to the articular surface and traversing from the resting zone, through the zone of proliferation, and in section nearly anastomosing with a penetrating metaphyseal vessel profile. In (b), rather than endothelial definition with a vessel lumen in the cartilage canal (arrow), there is a thrombus filling the cartilage canal characterized by eosinophilic fibrinous material, thrombocytes, and necrotic cellular and nuclear debris.

Prevalence and Severity of OC by strain

OC prevalence and severity differed among the strains and among anatomic sites (Table 3.1, Figure 3.2). Consistent with previous work, the prevalence of OC of the FTV was observed to be quite high among the strains with observed prevalence ranging from 70.83 to 95.83%. While there was a trend toward decreased OC prevalence in the FTV of JF, no statistically significant (p-value 0.0776) differences among strains was observed (Table 3.1). However, significant differences in OC severity was observed among strains (p-value = 0.033), with the JF having a significantly lower mean OC score compared to the mean OC score of the MB (p-value 0.0359) (Figure 3.2). In the proximal femur, the MB had significantly higher prevalence (p-value <0.0001) of OC with 75% of birds affected compared to 0 – 3.85% in other strains (Table 3.1). Proximal femur OC severity in the MB was also significantly higher (p-value <0.0001) compared to all other strains (Figure 3.2). In the proximal tibiotarsus, there was overall low

prevalence and severity of OC lesions and no statistically significant differences were observed among strains.

Table 3.1 Prevalence of osteochondrosis by chicken strain.

	OC Prevalence (# samples examined)		
Strain	**FTV**	**PF**	**PTT**
DC	84% (25)[a]	0.00% (24) [a]	0.00% (25) [a]
JF	70.83% (24)[a]	0.00% (25) [a]	8.00% (25) [a]
MB	95.83% (24) [a]	75% (24)	16.67% (24) [a]
WR	92.31% (26) [a]	3.85% (26) [a]	3.85% (26) [a]

[a] items that do not share a letter within a column are significantly different (p-value <0.05)

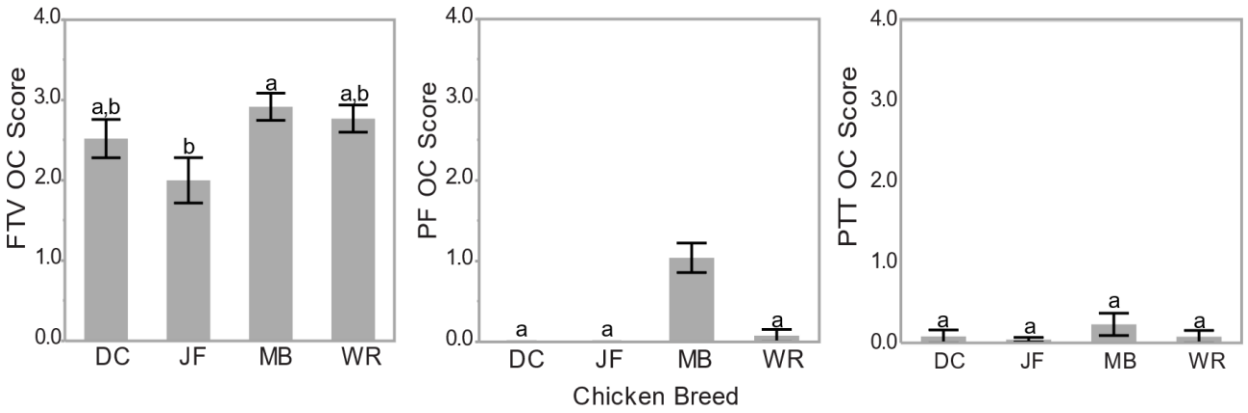

Figure 3.2 Mean OC score of each strain at FTV, proximal femur (PF), and proximal tibiotarsus (PTT). Significant differences in mean OC scores were observed in the FTV and PF. In the FTV, the JF had a significantly lower mean OC score compared to the OC score of the MB. In the PF, the MB had a significantly higher mean OC score compared to all other strains. Bars that do not share superscripts are significantly different.

Body weight

Unsurprisingly, there were statistically significant differences in body weight among the strains (p-value < 0.0001) (Figure 3.3). At 5 weeks of age, the JF had a significantly lower mean body weight (263.64 g) compared to all other strains and the MB had a significantly higher mean

body weight at 5 weeks of age (2632.23 g) compared to all other strains. There were no significant differences in mean body weight between the DC (480.29 g) and the WR (474.50 g).

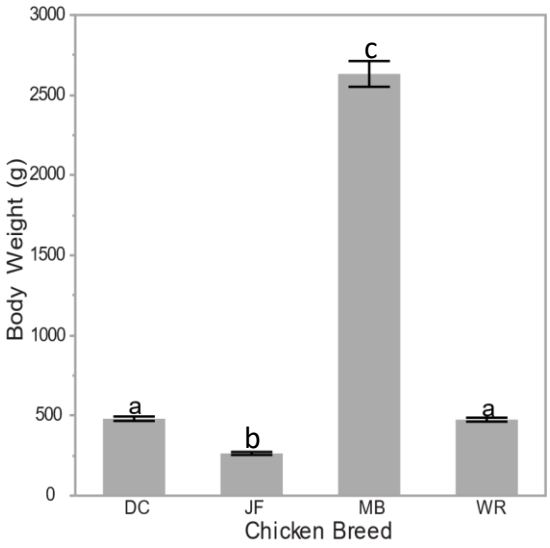

Figure 3.3 Mean body weight at 5 weeks of age for each strain. The JF had a significantly lower mean body weight compared to all other strains while the MB had a significantly higher mean body weight compared to all other strains. Bars that do not share superscripts are significantly different.

When examined within each strain, there were no significant correlations of OC score at any of the anatomic sites with body weight (Appendix C). When examined among all chickens as a single cohort, there was a strong positive correlation of body weight with proximal femur OC score ($\rho = 0.6069$, p-value <0.0001) (Figure 3.4). Free thoracic vertebral and proximal tibiotarsal OC scores did not significantly correlate with body weight among all chickens as a single cohort.

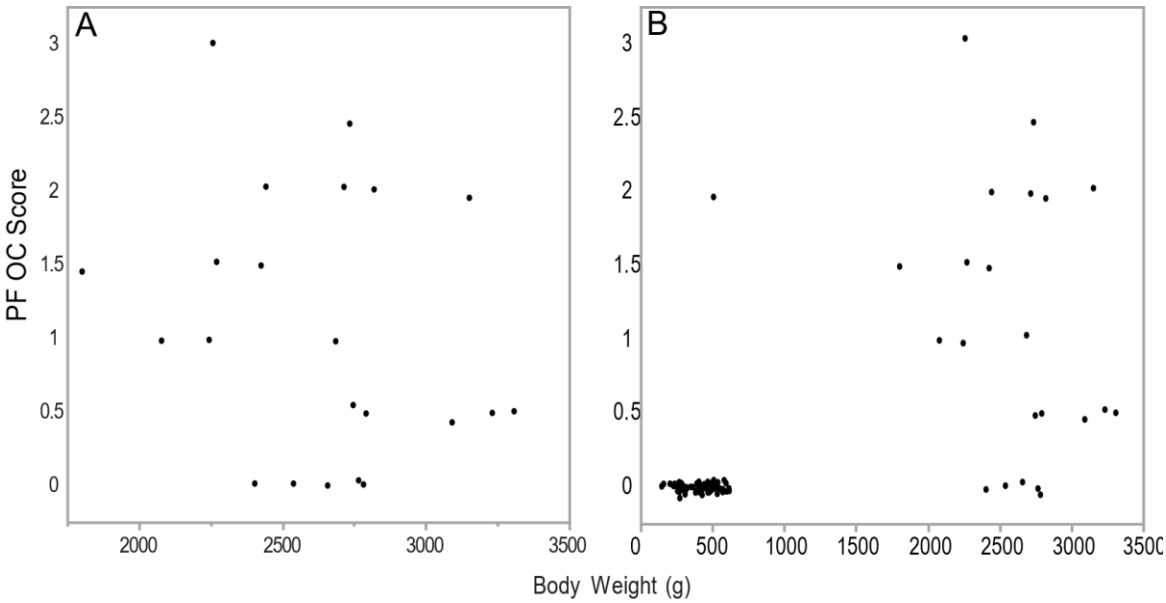

Figure 3.4 Scatterplot of body weight based on proximal femur (PF) OC score of (a) MB chickens only and (b) all chicken strains. When the correlation coefficient of PF OC score with body weight is calculated within each chicken strain, there is no significant correlation, as can be observed in MB strain in (a). When the correlation coefficient of PF OC score with body is calculated among all chickens as a single cohort, there is a significant strong positive correlation score ($\rho = 0.6069$, p-value <0.0001) of PF OC score with body weight.

Sex

There were no significant differences in mean OC score between males and females within strains at any of the anatomic sites examined (Figure 3.5).

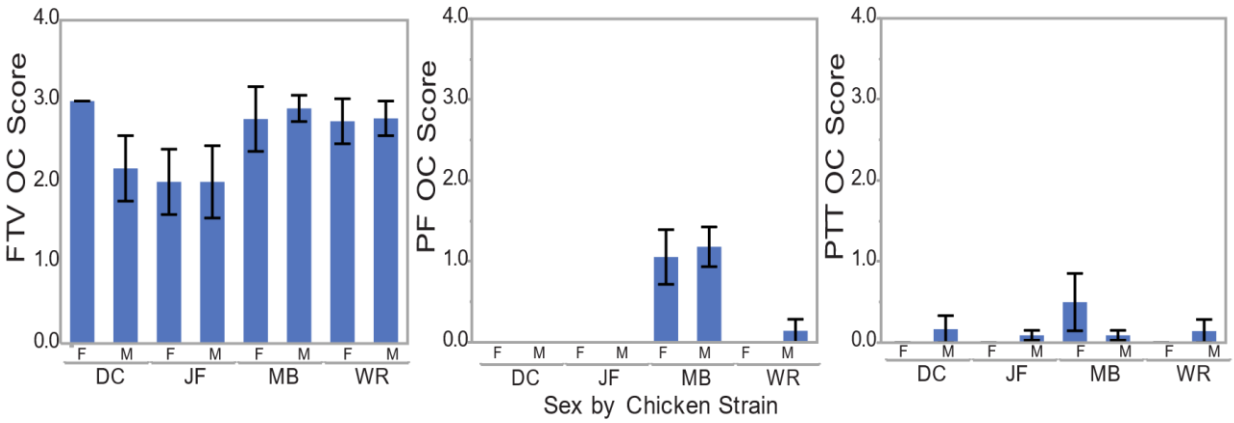

Figure 3.5 Mean OC score of male and female chickens compared within each strain at FTV proximal femur (PF), and proximal tibiotarsus (PTT). There were no significant differences in mean OC score between male and females within any strain.

Discussion

There are few previously published studies examining contributing factors and/or pathogenic mechanisms in chicken OC. In this study, we evaluated 4 strains of birds for OC at several anatomic locations including the free thoracic vertebra and proximal aspects of the femurs and tibiotarsi using a histologic scoring system. OC scores were used to assess the roles of strain, sex, and body weight in predisposing individuals to OC.

In a prior study, no significant differences in OC scores were noted among modern broiler strains and the 1950's broiler strain (Chen et al., 2017). However, in this study comparing heritage and ancient breeds to modern broilers, we observed a significant correlation between mean OC score and prevalence, albeit not constantly across all anatomic sites. For example, in the FTV, the MB had mean OC scores that were statistically indistinguishable from the heritage breeds and significantly higher to only JF scores. In contrast, OC scores form the proximal femur of MB were significantly higher compared to all other strains. These findings together suggest a potential heritable component of OC in chickens, which would be consistent with OC in other

species in which there has been demonstrable heritability and the hypothesis that the previous absence of strain correlation due to the high degree of relatedness of previously examined strains (Distl, 2013; Bates et al., 2014; Chen et al., 2017).

In humans there are multiple reports of a high prevalence of OC across generations within single families and uncommon identification of a specific gene mutation associated with familial inheritance (Mubarak et al., 1979; Stattin et al., 2010). In pigs and horses alike, there has not been identification of a single gene associated with OC but there has been support of a heritable component by both familial lineage and molecular genomic analyses (quantitative trait loci and single nucleotide polymorphisms) with the OC phenotype (Andersson-Eklund et al., 2000; Storskrubb et al., 2010; Laenoi et al., 2011; Aasmundstad et al., 2013; Distl 2013; Aasmundstad et al., 2014; Hilla & Distl 2014; Lykkjen et al., 2014; McCoy et al., 2018). Heritability estimates are variable among studies but generally range between 0.1 to 0.5 (Lundeheim 1987; Andersson-Eklund et al., 2000; Distl 2013). For example, Storskrubb et al. (2010) examined bone strength, meat percentage, and osteochondrosis in a total of 790 pigs of two different breeds with each pig having known familial lineage. Among these pigs, the authors calculated a heritability estimate for distal femoral OC to be 0.26, consistent with moderate heritability (Skorskrubb et al., 2010). Genomic analyses across studies do not necessarily identify the same quantitative trait loci or single nucleotide polymorphisms but tend to overlap in candidate gene function with extracellular matrix proteins among the functions often identified (Lykkjen, et al., 2010; Bates et al., 2013; Rangkasnee et al., 2013). Neither family lineage nor genomic analyses studies examining OC in chickens are described in the literature but are warranted to further assess for a genetic component.

Potentially complicating assessment for heritability of OC in chickens, particularly with the strains used in this study, are strain differences in growth rate (Mignon-Grasteau et al., 1999). Most studies do not favor growth rate as a cause of OC (Woodard et al., 1987; Uhlhorn et al., 1995; Ytrehus et al., 2004b, 2007; Craig et al., 2016) though it has historically been hypothesized to have a role and there are occasional reports with a positive correlation between growth rate and OC (Lundeheim 1987; Ytrehus et al., 2007). In this study, the DC and WR did not significantly differ in mean body weight at 5 weeks of age but the MB had significantly higher mean body weights and the JF had significantly lower mean body weights compared to all other strains. While no correlation of OC with body weight was identified at any anatomic location when assessed within strains, there was a significant, strong positive correlation of OC with body weight in the proximal femur when all birds were assessed as one cohort. However, this finding overlaps with the fact that MB birds had the highest mean body weight as well as the highest prevalence and severity of proximal femur OC confounding clear distinction of a correlation between OC and growth rate versus OC and chicken strain.

An association of sex with OC severity across species is also inconsistent in the literature (Slater et al., 1991; Sandgren et al., 1993; Jørgensen 2003). Chen et al. (2017) did not find a correlation between sex and OC in chickens previously and this holds true in this study, further supporting that a sex-association with OC is considered less likely.

While assessment for cartilage canal and vascular lesions in the epiphyseal growth cartilage was not an aim of this study, incidentally identified during the histologic scoring process were multiple necrotic cartilage canals in the proximal tibiotarsus and proximal femur in the MB, albeit without consistent association with OC lesions. In multiple animal species, the key step in OC pathogenesis is a failure in the vascular supply to the cartilage (Carlson et al.,

1995; Ytrehus et al., 2004a, 2007; Olstad et al., 2015). Though it was beyond the scope of this paper, further assessment of OC with vascular injury in chickens is warranted to better understand the relevance of this lesion with OC.

In summary, this study demonstrated a correlation of OC prevalence and severity in a subset of the anatomic sites examined in four chicken strains and suggests a potential heritable component of OC in chickens. It builds the foundation for future work on OC heritability in chickens, with the understanding that heritability will need to be distinguished from a correlation with other heritable traits, notably including growth rate. This study also incidentally identified cartilage canal necrosis in the MB strain, which warrants further investigation considering the central role of ischemic injury to OC pathogenesis.

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CHAPTER 4: Conclusions and Future Directions

Osteochondrosis (OC) is a major animal health and welfare concern for several veterinary species. In OC, necrosis of growth cartilage and subsequent abnormal endochondral ossification can lead to lameness, pain, and decreased performance. For the broiler chicken, lameness contributes to mortality, as the lame bird is unable to compete for resources. To date, the majority of pathogenesis research for OC has been performed in horses and pigs. However, renewed interest in chicken OC stems from the emergence of enterococcal spondylitis (ES) as a significant cause of morbidity and mortality in chickens and the identification of a strong correlation between OC dissecans and enterococcal spondylitis. Additionally, some researchers hypothesize that OC is the initiating event of bacterial chondronecrosis and osteomyelitis (femoral head necrosis). Taken collectively, these skeletal conditions are responsible for a significant proportion of livability issues in broiler flocks. Therefore, the goal of this thesis was to identify factors that contribute to OC in chickens in order to detect potential mechanisms to decrease host susceptibility or develop novel, non-antimicrobial control methods for ES.

In two separate studies, multiple chicken strains were histologically evaluated and scored for osteochondrosis spectrum lesions. The major findings of a longitudinal study of free thoracic vertebral (FTV) OC in three modern broiler lines and the 1950's broiler strain were that OC lesions 1) were very common (~80% prevalence); 2) occurred with the same frequency and severity in the modern broiler strains and the 1950's broiler strain; and 3) were not correlated with sex, growth rate, or incubation profile. In a follow-up study comparing two heritage breeds, the red jungle fowl, and a modern broiler strain, we found that breed did play a significant role in the prevalence and severity of OC. In this study, mean OC scores of the FTV were significantly decreased in the red jungle fowl compared to the modern broiler strain and the modern broiler

had significantly more frequent and higher mean OC scores in the proximal femur. These findings together support a potential heritable component of OC pathogenesis in chickens, which has been described in other veterinary species but not previously in chickens. In addition, we identified multiple necrotic cartilage canals in the proximal femur of the modern commercial broiler strains, suggesting that ischemic injury to growth cartilage is a key step in OC pathogenesis in chicken as it is in other species.

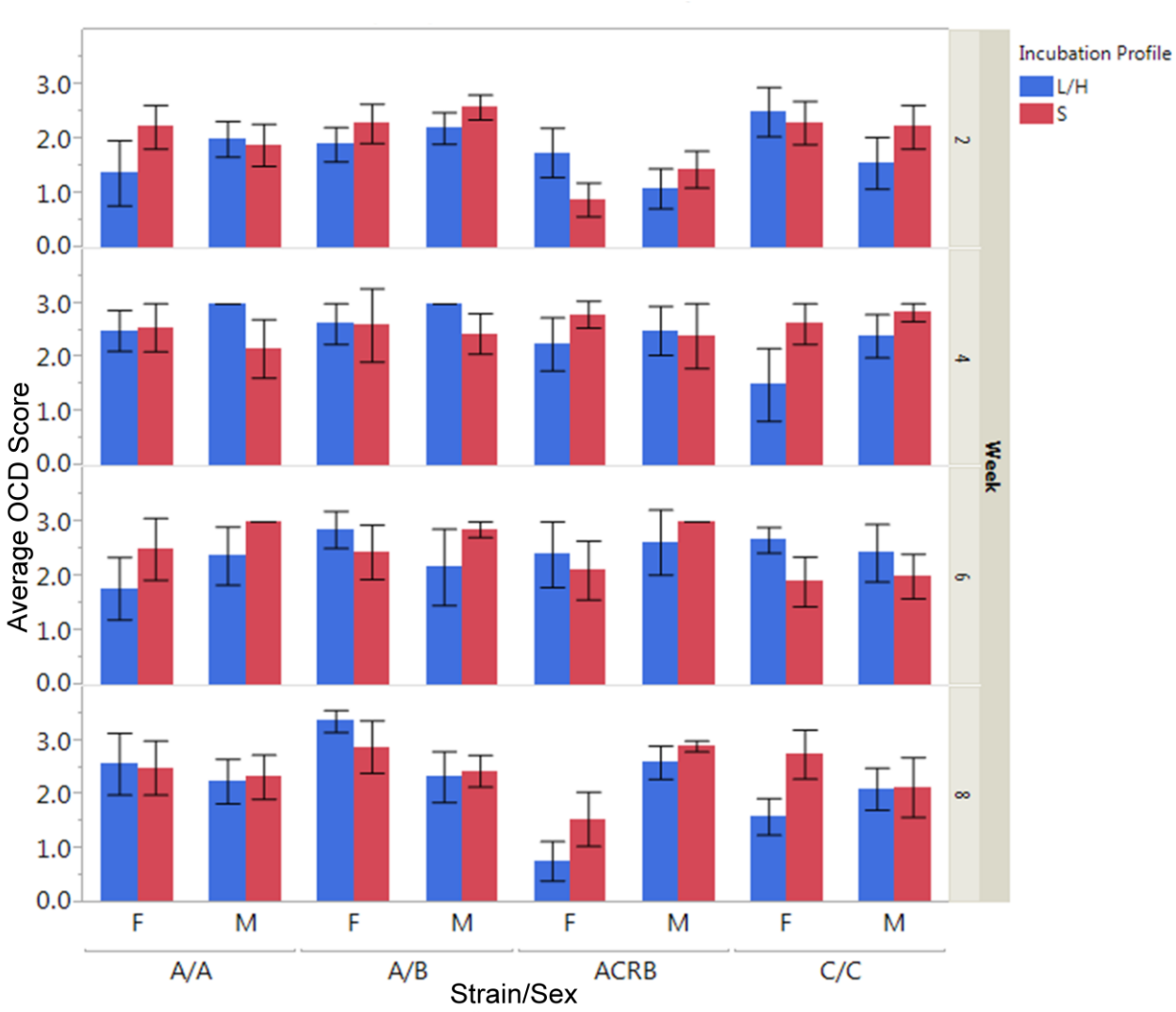
There are two natural paths of research that follow the results of this thesis. The first is to follow up with more direct measurements of OC heritability in chickens. In other veterinary species, support for a heritable component has included a combination of OC phenotype evaluation in the context of familial lineage data and correlations of the OC phenotype with molecular genetic data including both single nucleotide polymorphisms and quantitative trait loci. All of these methodologies are worthy of consideration depending on the availability of data and resources. If genetic drivers or markers for OC could be discovered in chickens, selection for birds resistant to OC could potentially be performed with enormous benefit to animal health and welfare. The second path of research is to further elucidate the cause of cartilage canal necrosis and its relationship with OC. Prevalence of these lesions; associations with other lesions including OC; and associations with other production parameters and environmental conditions are warranted prior to further endeavors to elucidate a causal role with OC. Gene expression studies could be performed on these regions and compared to adjacent normal cartilage to identify deranged pathways involved in lesion development.

In conclusion, this work sheds light on the host factors that do and do not contribute to host susceptibility to OC in broilers. This work lays the foundation for future studies which, if

successful, could provide the key information needed to develop strategies targeting the elimination of this important disease of broilers.

APPENDICES

Appendix A



Average OCD scores are compared between incubation temperature conditions within sex and strain at each collection time point. Rarely, there were significant differences between individual strain/sex/age combinations but there was no consistent trend.

Appendix B

Pair of Variables	Strain	Sex	Week	Valid N	Spearman R	t(N-2)	p-value
Aggregate Results Spearman Rank Order Correlations (Final data set) MD pairwise deleted Marked correlations are significant at p <.05000							
OCD & BW	A/A	M	2	20	0.144404	0.619146	0.543578
OCD & BW	A/A	F	2	17	0.153930	0.603361	0.555283
OCD & BW	C/C	F	2	20	0.134913	0.577670	0.570643
OCD & BW	C/C	M	2	18	0.634789	3.286144	0.004653
OCD & BW	A/B	F	2	17	-0.203585	-0.805348	0.433200
OCD & BW	A/B	M	2	22	0.022483	0.100574	0.920890
OCD & BW	ACRB	F	2	17	0.199533	0.788648	0.442605
OCD & BW	ACRB	M	2	20	0.260298	1.143781	0.267698
OCD & BW	A/A	F	4	17	-0.173382	-0.681833	0.505737
OCD & BW	A/A	M	4	16	-0.253098	-0.978879	0.344253
OCD & BW	C/C	F	4	14	0.041658	0.144434	0.887555
OCD & BW	C/C	M	4	16	0.123506	0.465683	0.648604
OCD & BW	A/B	F	4	13	0.301718	1.049601	0.316415
OCD & BW	A/B	M	4	18	0.066242	0.265549	0.793977
OCD & BW	ACRB	F	4	18	-0.468437	-2.12083	0.049911
OCD & BW	ACRB	M	4	15	0.445127	1.792280	0.096379
OCD & BW	A/A	M	6	14	0.367763	1.369975	0.195782
OCD & BW	A/A	F	6	15	-0.119174	-0.432773	0.672273
OCD & BW	C/C	F	6	19	0.069332	0.286553	0.777918
OCD & BW	C/C	M	6	16	-0.223569	-0.858242	0.405213
OCD & BW	A/B	F	6	16	-0.183941	-0.700190	0.495281
OCD & BW	A/B	M	6	13	-0.410496	-1.49306	0.163539
OCD & BW	ACRB	F	6	14	-0.270449	-0.973128	0.349698
OCD & BW	ACRB	M	6	12	-0.299598	-0.993027	0.344117
OCD & BW	A/A	M	8	17	0.137234	0.536580	0.599426
OCD & BW	A/A	F	8	17	-0.132435	-0.517475	0.612371
OCD & BW	C/C	F	8	18	0.228286	0.937910	0.362234
OCD & BW	C/C	M	8	16	0.269536	1.047270	0.312720
OCD & BW	A/B	F	8	20	-0.146271	-0.627322	0.538325
OCD & BW	A/B	M	8	18	-0.034109	-0.136515	0.893117
OCD & BW	ACRB	F	8	18	-0.025693	-0.102806	0.919395
OCD & BW	ACRB	M	8	20	-0.288133	-1.27658	0.217974

Spearman's rank correlation coefficient between mean OCD score and body weight was calculated for each age/strain/sex combination. Statistically significant coefficients are red.

Appendix C

Spearman Rank Correlation Coefficient						
	FTV		**PF**		**PTT**	
Strain	**ρ**	**p-value**	**ρ**	**p-value**	**ρ**	**p-value**
DC	-0.11	0.62	N/A	N/A	0.24	0.26
JF	-0.06	0.76	N/A	N/A	0.25	0.24
MB	-0.35	0.11	-0.20	0.37	-0.14	0.55
WR	-0.22	0.27	0.20	0.33	-0.17	0.40

Spearman's rank correlation coefficient between mean OC score and body weight by strain calculated for each anatomic location (free thoracic vertebra [FTV], proximal femur [PF}, and proximal tibiotarsus [PTT]). No statistically significant correlations were observed.