# EFFECTS OF GENETIC SELECTION FOR GROWTH PERFORMANCE ON BROILER CALCIUM AND PHOSPHORUS METABOLISM DURING EMBRYONIC AND EARLY POST-HATCH DEVELOPMENT

by

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(Under the Direction of LAURA E. ELLESTAD)

## ABSTRACT

Genetic selection for improved growth performance of commercial broilers is associated with an increase in leg disorders. Bone health is determined by calcium and phosphorus metabolism through regulatory pathways in kidney, intestine, and liver. Therefore, this study sought to define effects of genetic selection on systems regulating homeostasis of these minerals. Gene expression of physiological regulators and transporters was measured in kidney, jejunum, and liver and factors associated with bone health were determined during embryonic and early post-hatch development in legacy (Athens Canadian Random Bred) and modern (Cobb 500) broilers. Gene expression associated with enzymatic conversion of vitamin $D_3$, and hormonal regulation of calcium and phosphorus was found to be diminished in Cobb. This suggests that genetic selection has had an impact on regulation of calcium and phosphorus metabolism in broilers, potentially leading to impairment of bone health that contributes to increased incidence of leg disorders in commercial broilers.

INDEX WORDS: Vitamin $D_3$, calcium, phosphorus, transporters, bone health, broilers

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# DEDICATION

I dedicate this thesis to my beloved wife, Rahab Alejandra Garcia Mejia; my mother, Luz Mila Benavides; my father, Alfonso Arango; my sister, Maria del Mar Sanchez; my brother, Alfonso Alejandro Arango; my cousin, Miguel Angel Zapata; my brother-in-law, Daniel Sanchez; and my niece, Abib Valentina Sanchez. Thank you for inspiring me to keep moving forward, for your support in difficult times, and your unconditional love.

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# CHAPTER 1

# INTRODUCTION

Modern broilers, or meat-type birds, have achieved increased growth rate, higher meat yield, and improved feed utilization over the last 60 years, with about 90% of this due to genetic selection and the last 10% due to dietary improvements (Havenstein et al., 2003b). In the past, genetic selection programs have focused primarily on the commercial traits listed above, and other relevant parameters such as bone health that could lead to a decrease in animal welfare were ignored (Richard, 1998b; Su et al., 1999). However, companies are now focusing on improvement for welfare traits also (Neeteson et al., 2016). Bone matrix is formed mainly by a combination of calcium (Ca) and phosphorus (P) during the mineralization process. Thus, a possible inefficiency in physiological regulation of Ca and P metabolism could lead fast-growing bird strains, such as modern commercial broilers, to have bone abnormalities like tibial dyschondroplasia, deformed bones, and lameness (Veum, 2010).

Vitamin $D_3$ is important for bone health due to its function in many physiological processes that lead to bone mineralization such as intestinal absorption and kidney reabsorption of Ca and P, increasing their availability for skeletal tissue (Rennie et al., 1996). Dietary vitamin $D_3$ biological activation occurs in three steps (Bikle et al., 2008; Galliera et al., 2018). The first hydroxylation step occurs in the liver by 25-hydroxylase (CYP2R1), converting it to $25(OH)D_3$ (Bikle, 2018; Cheng et al., 2004; Cheng et al., 2003a). Subsequent hydroxylation occurs in kidney and is performed by 1α-hydroxylase (CYP27B1), which transforms $25(OH)D_3$ into its most biologically active form, $1,25(OH)_2D_3$ (Bikle, 2014). Although this gene has been identified in mammals and

fish (Cali et al., 1991; Chun et al., 2014), it has not been identified in any bird species. Both $25(OH)D_3$ and $1,25(OH)_2D_3$ can be further metabolized, mainly in the kidney, by 24-hydroxylase (*CYP24A1*), effectively inactivating these metabolites (Dusso et al., 2011).

The Ca and P regulatory system might have become stressed to accommodate the increased Ca and P needs for fast-growing broilers. Therefore, components of this regulatory system could be markedly influenced by genetic selection for rate of growth, and as a consequence increasing the incidence of leg disorders, especially in broilers growing to their maximal genetic potential. A slow-growing strain of birds that represent broilers prior to intensive genetic selection that can be used as a genetic control are the Athens Canadian Random Bred (ACRB) that are representative of broilers from the mid-1950's (Collins et al., 2016). Therefore, a comparison between this legacy bird strain and a modern commercial broiler line (Cobb 500) was made to identify the affected enzymatic and hormonal regulatory pathways influencing vitamin $D_3$, Ca, and P metabolism.

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# CHAPTER 2

# LITERATURE REVIEW

## Genetic selection in broilers

The broiler (meat-type) chicken industry plays an important role in human nutrition, with a great increase in chicken meat consumption from the 20$^{th}$ century through present times (Scanes, 2007). This increase could be because the efficiency of poultry production has created a noticeable gap between prices for broiler-derived meat ($1.781/lb) and red meat derived from cattle ($4.786/lb) and pork ($3.258/lb), as is evident in pricing changes in the United States over the last 20 years (USDA, 2020). These lower prices have been achieved as a result of a poultry industry effort focused on increasing body weight gain and feed conversion ratio (FCR; g feed intake/g body weight gain), attaining growth rates increased by over 400% and FCR reduced by almost 50% between 1950 and 2005 (Zuidhof et al., 2014). The main reason for this productive enhancement is associated with the improvement of technologies used in the industry, with genetic selection and breeding techniques responsible for 80-90% of these advances (Havenstein et al., 2003a; Havenstein et al., 1994).

Commercial selection programs have been used to produce high-yielding broiler strains. During early breeding programs, selection was based on phenotypic selection, which focused on the bird's performance parameters. However, this method was not applicable for low heritability traits such as disease resistance (Saxena et al., 2018). In response to this, a system using the combination of the individual's performance and one from its closest relative (parents, siblings, or progeny) was developed, called the selection index (Hazel, 1943). Furthermore, correction of

known non-genetic differences on the estimation of breeding values was achieved by mixed equations, though this only accounted for close relatives. In 1976, an algorithm was made that allowed development of a pedigree matrix, giving the possibility to include information on all relatives (Henderson, 1976; Quaas, 1976).

A new era of poultry breeding occurred with the discovery of DNA, which carries genetic information that will determine the phenotype parameters of the birds. Quantitative trait loci (QTL) allowed the discovery of regions that were associated with commercial traits, and this information could be used for marker-assisted selection (MAS) (Briles et al., 1977). Later, the chicken genomic sequence revealed differences at single nucleotides [single nucleotide polymorphisms (SNPs)], and over 600,000 SNPs were identified (Powell et al., 2012). This large amount of data allowed the use of the full length of the genome to estimate breeding values, instead of focusing on a few large QTLs, thus improving breeding value accuracy, allowing a greater possibility to select for sex-limited and hard-to-measure traits, and shortening generation intervals (Meuwissen, 2001).

Different approaches for commercial trait improvement used by breeding companies has achieved increased growth rate, leading to decreased age at slaughter, and reduced FCR, reflecting less feed intake per unit growth (Havenstein et al., 2003a). As consumer demand increased for breast meat, its value rose and there was a need to have birds with optimized breast yield. This led to selection for higher breast yield, which achieved a genetic gain of 277% every generation without altering body weight, FCR, or fertility (Schmidt et al., 2006). Furthermore, meat quality is also important for the consumer, and this is associated with a meat parameter that is highly heritable, final meat pH, which is also highly related to other quality parameters such as color, water-holding capacity, and decreased incidence of pale and exudative meat (Le Bihan-Duval et

al., 2001). In sum, over the past 60 years, broiler genetic selection programs have led to increased production efficiency by improving growth rate, feed utilization, and meat quality.

The narrowed focus of early selection programs for a small number of economically beneficial traits risked compromising traits that do not have a commercial interest, leading to increased concerns about animal welfare (Richard, 1998a). Intensification of poultry production, due in part to a growing human population with a demand for animal protein sources, has pushed genetic selection programs to deliver birds with higher growth rates, improved feed efficiency, and increased meat yield, potentially causing a further predisposition towards comprised welfare (Decuypere et al., 2010). Fast growth rates and heavy body weights in birds are directly related to specific diseases, such as cardiovascular disease, skin lesions, and skeletal disorders (Bessei, 2006; Bradshaw et al., 2002; Knowles et al., 2008). Improvements in diet, health management, and environmental control have been made to try to soothe the negative impacts of genetic selection; however, several welfare issues related to the disorders listed above still exist in poultry production systems.

Modern broilers have a high muscle-to-bone ratio and caloric intake, leading to an increased rate of cardiovascular diseases and subsequent ascites (Kalmar et al., 2013). This is one of the most common problems caused by high growth rates leading to functional hypoxia, due to the increased oxygen requirement for muscle growth causing ascites. Selection for ascites resistance managed to diminish the incidence of this issue from 43.6% to 6.4% in male broilers after two generations while avoiding affecting weigh gain (Balog, 2003). Many efforts have been made to decrease the incidence of ascites by selective breeding, but cardiovascular diseases are still a problem in commercial broiler production, and it is thought that cardiac arrhythmias have an incidence of 27% in fast-growing birds (Zhang et al., 2018). Another identified heart disease is

cardiac insufficiency that can lead to ascites and sudden death syndrome. This could have several causes, though growth rate seems to represent the main contributor to this problem (Bessei, 2006). A comparison made between fast-growing, and heritage slow-growing broilers showed evidence of the genetic implications that intensive commercial programs have on the incidence of cardiac dysfunction in modern broilers, where modern broilers had an increased cell apoptosis and inhibited cell cycle due to lipid accumulation, oxidative stress and endoplasmic reticulum stress. The authors presented as the solution setting specific trait improvements for healthier cardiac function without altering the productive parameters (Zhang et al., 2018).

A low locomotor activity means that the bird will spend more time sitting and laying in moist litter, which could cause skin lesions mainly on the feet, hocks, and breast area (Haslam et al., 2007). Different studies showed that fast-growing broilers could be more susceptible to deeper footpad lesions, with up to 88% of the flock showing signs. This decreases both animal welfare and market value of poultry products (Allain et al., 2009). Improving traits related to skin health could be achievable without negative impacts on body weight in proper selection programs (Ask, 2010; Kjaer et al., 2006).

Modern broilers are more susceptible to specific pathologies such as bacterial chondronecrosis with osteomyelitis and non-infectious skeletal disorders like tibial dyschondroplasia and varus or valgus leg deformities. It has been demonstrated that broiler crosses that grew faster at early ages showed a stronger predisposition to develop bacterial chondronecrosis with osteomyelitis when compared to slower-growing birds (Wideman Jr et al., 2013; Wideman Jr et al., 2014). As birds affected by bacterial chondronecrosis with osteomyelitis showed differential expression of several genes when compared to normal birds, this suggests a possible focus for improvement in genetic selection programs (Petry et al., 2018).

High incidence of tibial dyschondroplasia and other bone deformities was found across four fast-growing broilers crosses while in a single population of slower-growing birds showed lower tibial dyschondroplasia scores when compared to faster-growing birds. As a result, the authors concluded that slowing down bird growth would help proper development of the skeleton in these animals (Kestin et al., 1999; Shim et al., 2012). However, it has been demonstrated a correlation between body weight and leg health traits; therefore, it should be possible to focus breeding programs on improving bone health to achieve stronger skeletal tissue in fast-growing birds without compromising growth performance parameters needed for efficient production (Kapell et al., 2012; Rekaya et al., 2013).

A major problem with musculoskeletal disorders in modern broilers is that these conditions reduce access to feed and water and do not allow birds to express their normal behavior (Weeks et al., 2000). Low locomotor activity can cause further deterioration of the animal's skeletal system (Bessei, 2006; Bizeray et al., 2000), which in modern broilers could be more noticeable due to their reduced ability to walk because of leg weakness (Wilhelmsson et al., 2019). Using a six-point scoring system for walking ability (Kestin et al., 1992), where '0' is normal locomotion and '6' is complete inability to walk, many researchers have found an average of 75-90% of broilers with a walking score higher than 0 (Kestin et al., 1992). Also, it has been reported that birds with a walking score over '3' could be between 15-55% of an entire flock, which has animal welfare implications (Sanotra et al., 2001). Another study showed that slow-growing birds had better walking scores compared to commercial broilers; however, when the effect of live weight was removed, most of the differences in walking scores disappeared, suggesting that the problem with the walking ability of commercial broilers was due to their heavy body weight's impact on bones and joints (Kestin et al., 2001). Furthermore, use of a suspended harness mechanism to reduce the

bird's body weight by 50% enhanced the locomotor activity in modern broilers (Rutten et al., 2002). Aside from heavy body weight, intensive selection in modern broilers has altered body composition, and pectoral hypertrophy leads to whole body mass with an unbalanced gravity center (Caplen et al., 2012), so the overall body conformation could be another cause for the decrease in locomotor activity.

Genetic selection has improved production performance in meat-type chickens, producing birds that can yield 3 pounds of weight in 30 days, and that have a FCR ratio close to 1.5. However, fast-growing birds have become more susceptible to bone health issues due to their heavy weight, leading to bone deformities, decreased locomotor ability, increased mortality, and decreased performance.

**Bone development**

On embryonic ages, an appendicular skeleton is formed from hyaline cartilage (Gilbert, 1997). Endochondral ossification gets initiated *in ovo*, however the main ossification occurs after hatch when the growth plate in bone uses this process to replace hyaline cartilage formed at the epiphyseal plate (Bain et al., 1993; Marks Jr et al., 1988). This ossification occurs by the deposition of $Ca^{2+}$ and $P_i$ on a collagen matrix, forming hydroxyapatite which is more than 85% of the bone mass. Commercial broilers reach target weight before reaching the stage of remodeling characteristic of adult vertebrates; therefore, their bones do not develop to full maturity (Roberson et al., 2005).

Endochondral ossification involves a sequence of events that starts with proliferation of chondrocytes at the proximal edge of the growth plate. After cells reach maturity, they secrete substances of hyaline cartilage, such as sulfate proteoglycans and type II collagen that form the fibers on which the proteoglycans are contained (Brighton, 1984; Tselepis et al., 1996).

Chondrocytes gets encapsulated in an extracellular matrix without blood vessels, which contains growth factors, cytokines, prostaglandins, and small molecular weight proteoglycans (Reddi, 2000; Wu et al., 1997). The distal end of the growth plate is where chondrocytes enlarge and go through final differentiation (Wu et al., 2002), and their secretory products change to include type X and type II collagen and other proteins such as annexin and fibronectin. Furthermore, high levels of alkaline phosphatase activity are noticeable in this zone, and this activity is associated with bone mineralization. Chondrocytes also synthesize type I collagen, which is a protein produced by osteoblasts (Kirsch et al., 1997).

Chondrocytes initiate mineralization by concentrating $Ca^{2+}$ in their mitochondria when their supply of glycogen runs out and the cell shifts into anaerobic metabolism (Brighton, 1984; Kirsch et al., 1997). These cells start expelling mitochondrial matrix vesicles with several proteins and a complex of $Ca^{2+}$ and $P_i$, which serve as a nucleation site for calcification during endochondral ossification. Annexins contained in the matrix vesicles increase the $Ca^{2+}$ influx and help to anchor them to the extracellular matrix by binding type II and X collagen (Wu et al., 1991). Inside the vesicle, ionized zinc ($Zn^{2+}$) and copper ($Cu^{2+}$) help initiate the crystallization of hydroxyapatite, causing the vesicle to rupture and the beginning of hydroxyapatite deposition along the type I collagen fibers synthesized by mature chondrocytes (Arsenault, 1988; Kirsch et al., 1997).

Invasion of the cartilage model by the vascular system allows delivery of osteoclasts, large multinucleated cells responsible for mineral deposition to the bone matrix, to the interior of the model. This occurs simultaneously with the initial mineralization steps at the distal edge of the growth plate. Transport of osteoblasts to the cartilage model initiates the actual process of bone formation (Gilbert, 1997). Collagenase-3 enhances degeneration of the cartilage matrix, leading increased vascularization that can bring more osteoblasts to the area that bind the mineralizing

cartilage matrix. This begins the process of formation of osteoid, or the extracellular matrix for bone mineralization. Osteoblasts on the osteoid secrete type I collagen, giving the structure where the hydroxyapatite crystals are deposited and grow (Orth, 1999; Wu et al., 1991). Following initial extracellular matrix and mineral deposition, alignment and crosslinking of collagen in the extracellular matrix increases bone breaking strength (Rath et al., 1999).

**Important musculoskeletal problems in broilers**

Among the costliest disorders for poultry production are skeletal issues because they are chronic, cause a reduction in feed intake, and lead to frequent condemnation of birds at processing. Bone abnormalities can be initiated by degenerative, nutritional, environmental, infectious, or developmental causes and are often related to more than one of these problems, resulting in skeletal lesions that cause abnormalities in bone remodeling (Bain et al., 1993)

As mentioned above, higher growth rates of broilers can cause a predisposition for these birds to exhibit many skeletal defects. Their rapid growth rate generates bone that have a thick growth plate with inadequate strength underneath it, resulting in tendons and bones without sufficient strength to support the heavy weight of these animals (Richards, 2005), and several non-infectious causes of bone health disorders in modern broilers are related to rapid growth. These include tibial dyschondroplasia, varus-valgus deformations, rotated tibias, rupture of the gastrocnemius tendon, and epiphyseal separation (Whitehead et al., 2003).

*Tibial dyschondroplasia*

Tibial dyschondroplasia is a multi-factorial disease, but the primary causes appear to be rapid growth, deficiency of growth factors, high anion:cation ratio in feed, and inherited susceptibility from breeding programs (Leach et al., 1994; Rath et al., 2000; Reddi, 2000). It is a growth plate abnormality that often occurs in commercial broilers, causing an abnormal mass

under the growth plate when the proliferating avascular growth plate fails to be transformed into hypertrophied cartilage that will be replaced by bone at the lower edge of the growth plate (Farquharson et al., 2000). When these lesions are large, the bones get weak and allow for deformations due to the tension provoked by the gastrocnemius muscle. If the bending is within a few degrees, the bird will lose the ability to walk with the affected leg. Fractures can easily occur during processing or spontaneously, or birds can develop necrosis around the cartilage plug, facilitating fractures and severe lameness (Richard, 1998a).

*Varus-valgus deformations*

Varus-valgus deformations, or twisted legs, are the most frequent cause of lameness in commercial broilers, being responsible for over 60% of skeletal diseases. It is a tibiotarsus lateral deformity, deviating the metatarsus and displacing the gastrocnemius tendon. This produces bow-legged (varus) or knock-kneed (valgus) birds, and begins around day 10, exerting a significant deformity until after day 21. Ligaments on the intertarsal joint become stretched, loosening this joint, and if this causes a severe deformity, it can cause spontaneous fractures (Richards, 1984). This disease can be related to over-nutrition, rapid growth, and poor management that provoke continuous bone growth or uneven tendon tension for fast-growing bones (Classen, 1992; Richards, 1984).

*Rotated tibias*

Usually, a rotated tibia is noticeable in only one leg, leading to a straddle-legged posture caused by an outward torsion of the tibiotarsus and resulting in one foot pointing laterally. The hock joint or the gastrocnemius position is not affected. It can affect up to 10% of the flock, and even though it is not a condition caused by rapid growth, it can be associated with poor management in hatching trays, shipping boxes, the brooding area, or cages (Crespo, 2013).

*Rupture of the gastrocnemius tendon*

Rupturing of the gastrocnemius tendon is a common problem in heavy broilers that have inadequate tensile strength in their tendons. Usually, a rupture of the tendon occurs above the hock caused by the tension in it. It is frequently a bilateral rupture, due to a first rupture in a tendon of one leg that puts further stress on the healthy leg. The healthy leg will then present the condition with time (Richard, 1998a).

*Epiphyseal separation*

Diaphyses of long bones are covered by a cartilage capsule that is thicker in fast-growing chickens compared to slow-growing birds, affecting its attachment to the bone. In many necropsies, this cartilage can be separated easily from the bone and frequently this cartilage rupture causes breakage of the femur head (Richard, 1998a). Management issues can increase the predisposition of this cartilage to rupture and the femur head to break when heavy birds are mishandled. Picking the bird up from one leg, a dislocation of the hip joint can also occur, leading to fracture of the femur head and causing hemorrhages (Mitchell et al., 1986). The thickened cartilage in the joint can also diminish the blood flow, causing ischemic necrosis in the bone diaphysis (Packialakshmi et al., 2015). It is expected that these types of injuries will affect flock production by reducing feed and water intake, ultimately decreasing body weight gain.

*Economic impact of leg disorders in broiler meat production*

Birds affected by any of the conditions listed above will have difficulties exerting normal behavior and accessing food and water, directly affecting animal production efficiency and welfare (Butterworth et al., 2002; Weeks et al., 2000). Furthermore, skeletal abnormalities that impair normal gait of the bird can also lead to an increase in mortality and the necessity to cull birds due to poor welfare conditions. It is expected that approximately 1% of flock mortality is due to leg

disorders. There is also a loss in production at processing, due to increased carcass condemnation, reduced meat yield, and reduced breast and leg meat quality. It is observed that 2.1% of the birds received in the slaughterhouse are negatively affected by leg disorders, causing estimated economic losses caused by these conditions between 80 and 120 million dollars (Morris, 1993).

**Bone illness prevention**

Leg disorders have a negative economic and welfare impact on broiler production, so there is a necessity to prevent bone health issues in the farms by assessing different strategies to mitigate these consequences. Different strategies in use are described below.

*Feed intake restrictions*

Incidence of several bone abnormalities can be prevented by feed restriction to reduce rate of gain, thereby increasing the body ash content to improve the growth rate:bone growth ratio (Pattison, 1992). However, this approach must be done carefully since it is economically impractical if the time to reach commercial weigh is delayed and the production cycle is disrupted (Riddell et al., 1983). Restricting feed intake during the second week of life has been most effective for reducing leg disorders without affecting the time to reach market weigh due to compensatory growth after the restriction (Robinson et al., 1992). Furthermore, reducing specific parameters in feed could also be beneficial for preventing bone illness. When metabolizable energy was reduced in the feed, it decreased the incidence of leg disorders (Haye et al., 1978; Hulan et al., 1987; Robinson et al., 1992) and time to reach market weight was not affected (Griffiths et al., 1977).

*Lighting schedules*

Historically, commercial broiler production attempted to maximize feed intake and therefore growth rate by applying a continuous lighting program where birds were exposed to 23 to 24 hours of light (Classen, 1992). Current primary breeder recommendations include an increase

in the duration of the dark period to 4 – 6 h after about 5 days of age (Aviagen, 2014; Cobb-Vantress, 2012). Another approach is to apply an intermittent lighting schedule, which could not have negative effects on body weights though may improve bone health and lower incidences of leg disorders, due to an increased activity of broilers during the light hours and enhanced use of nutrients in the dark hours (Buyse et al., 1996; Renden et al., 1991; Wilson et al., 1984). Intermittent lighting programs have been shown to decrease leg disorders incidence and mortality (Liboni et al., 2013). Thus, better lighting program management could improve leg health within a flock without affecting productive parameters.

*Improvement of selection programs*

As mentioned previously, leg abnormalities are highly related to fast growth rates in broilers achieved by genetic selection programs that were initially focused only on commercial traits (Havenstein et al., 1994) . The lack of focus on other important traits, such as leg health, has increase the incidence of leg disorders. However, reduction in the incidence of leg disorders can be made by focusing selection programs against them. It was demonstrated that random selective breeding for 11 generations diminished the incidence of leg issues, even when standard conditions, such as normal lighting programs, flock density, and diet were employed (Kestin et al., 1992). Furthermore, a study focused on behavioral activity showed that selection of young chicks that exhibited more mobile activity could improve the later activity of these birds and decrease the incidence of leg disorders (Bizeray et al., 2000). All this together suggests that using selection programs could be a viable strategy to ameliorate bone health in emerging modern commercial broiler strains.

*Nutritional factors to improve bone health*

Poultry nutrition has a great influence on bone health because deficiencies and sometimes excesses are a main cause of problems in bone tissue. Normal intestinal absorption is crucial to maintain homeostasis in tissues; therefore, anything affecting to this process will impact nutrient availability for tissues. For bone, this can translate to effects for its health. To guarantee correct animal nutrition, minimal requirement values have been reviewed, and for leg health different vitamin and mineral needs have been assessed (NRC, 1994). However, these requirements might not reflect daily conditions in production farms, and nutritionists could decide to use higher levels of vitamins and minerals to supply the physiologic demand of the bird.

Calcium and phosphorus requirements

Bone tissue is comprised mainly of hydroxyapatite, a combination of Ca and P in an approximate ratio of 2.2:1. Requirements for these two minerals depend on bird age, and a Ca:P ratio of 2.2:1 is generally thought to be necessary. However, on early post-hatch ages modern broilers' Ca:P ratio requirement seems to be greater to achieve normal growth plate morphology and lower the bone remodeling process during the first two weeks post-hatch, , which lowers bone matrix Ca (Williams Solomon et al., 2000; Williams Waddington et al., 2000). Optimal availability of Ca and P is crucial for the bone remodeling process; therefore, requirements will be higher when this is happening. Ca required for bone calcification is higher than that for animal growth and maintenance, though P requirements seem to be the same for bone mineralization and other physiological processes (Bar et al., 2003).

Vitamin $D_3$ requirements

Vitamin $D_3$ needs UV light to be synthetized in the skin, however bird houses do not have a natural source of light that is required to produce vitamin $D_3$ in the skin. Therefore, the

requirements must be covered through the diet, but diet raw ingredients contain low amounts of this vitamin (Atencio et al., 2005). Therefore, this deficiency must be covered by adding supplements into the feeding programs. Furthermore, faster growth rate could increase requirements needed for maintenance and bone formation, which can increase the necessity for higher inclusion of Ca and P in feeding programs that in turn will influence the requirements for vitamin $D_3$ (Waldenstedt, 2006). Synthetic forms of vitamin $D_3$ are supplied within broiler diets due to its low availability in conventional feed ingredients. The vitamin $D_3$ recommendation is 3,000 - 5,000 IU/kg during the first 3 week of age to allow bone mineralization during this time (NRC, 1994), although supplementation in practice is much higher and reaches a range between 8000-10000 IU/kg feed when normal levels of Ca and P are provided in commercial diets (Whitehead et al., 2004). However, vitamin $D_3$ requirements might be affected by dietary factors such as Ca and P inclusion in feed, housing conditions, metabolic disorders and stress conditions (Yarger et al., 1995).

High levels of vitamin $D_3$ have been used to eradicate any type of rickets within broiler flocks (Elliot et al., 1997; Ledwaba et al., 2003). The influence of P in the diet on dietary vitamin $D_3$ levels appears to be more than that for Ca, since lower levels of P in the diet required higher levels of vitamin $D_3$ to achieve maximum growth and bone ash content. Therefore, low levels of dietary P could be activating responses to increase vitamin $D_3$ activation and further absorption and resorption form jejunum and kidney, respectively. However, when Ca inclusion was slightly lower than the requirement, adding increased quantities of vitamin $D_3$ in the diet did not show any impact on growth performance or bone ash (Baker et al., 1998).

Metabolites of vitamin $D_3$ such as $25(OH)D_3$ or $1,25(OH)_2D_3$ have higher biological activity and can be used in diets to prevent leg disorders (Mitchell et al., 1986; Rennie et al., 1996).

In feed with deficient Ca and P levels, addition of either $25(OH)D_3$ or $1,25(OH)_2D_3$ helped to alleviate tibial dyschondroplasia and rickets. However, these metabolites have to be treated carefully since $1,25(OH)_2D_3$ can depress growth when the addition of Ca in the feed reaches 10,000 IU (Roberson et al., 1994; Thorp et al., 1993). Therefore, a use of vitamin $D_3$ or its metabolites could be an advisable approach to improve bone health in fast-growing commercial birds.

In conclusion, different approaches can be used to improve leg disorders in broilers. However, as these problems are usually caused by multiple factors, it is important that a combination of several strategies be used to achieve an improvement in leg health within broiler flocks.

## Hormonal systems regulating Ca and P homeostasis

*Vitamin $D_3$*

Vitamin $D_3$ is the main vitamin involved in regulating Ca and P homeostasis throughout the body. It stimulates expression of genes related to absorption of these minerals in the gastrointestinal tract and reabsorption of them in the kidney, as well as regulates osteoclast differentiation and function during the bone remodeling process (St-Arnaud, 2008). This vitamin can be either synthetized in the skin or through diet supplementation (Holick et al., 1977). To be used by the bird's body, it must be metabolized in liver and kidney by hydroxylase enzymes. After activated, it needs to bind its receptor to exert its genomic functions and enhance absorption and reabsorption in jejunum and kidney, respectively (Norman et al., 1992).

To exert its functions in target tissues, vitamin $D_3$ must be biologically activated in different organs. Vitamin $D_3$ binding protein transports dietary vitamin $D_3$ through circulation, and it is either stored in adipose tissue or further metabolized in liver and kidney (Holick, 1981). In liver and kidney, vitamin $D_3$ gets activated by two sequential hydroxylation steps. The first

hydroxylation at carbon 25 by 25-hydroxylase [Cytochrome P450 Family 2 Subfamily R Member 1 (CYP2R1)] occurs in the liver and results in the formation of 25(OH)$D_3$ (Cheng et al., 2003b). This initial metabolite has a higher bioactivity compared to vitamin $D_3$, since its addition to feed can result in reduced incidence of tibial dyschondroplasia (Ledwaba et al., 2003). The second hydroxylation occurs mainly in the kidney and is mediated by 1α-hydroxylase [Cytochrome P450 family 27 subfamily B member 1 (CYP27B1)], which converts 25(OH)$D_3$ to its most biologically active form, 1,25$(OH)_2D_3$ (Fraser et al., 1970). This is the main hormonal form of vitamin $D_3$ that controls Ca and P homeostasis by increasing bone resorption, stimulating intestinal absorption, and enhancing kidney reabsorption of these minerals, and its production is enhanced by parathyroid hormone (PTH) produced in the parathyroid glands (Pike et al., 2007). Even though the 1α-hydroxylase gene (*CYP27B1*) has been identified in mammals and fish (Cali et al., 1991; Chun et al., 2014), it has not been identified in any bird species including chickens. Low plasma $Ca^{2+}$ concentrations stimulate PTH production, which enhances the expression and activity of 1α-hydroxylase. This leads to an increase in circulating 1,25$(OH)_2D_3$, which in turn enhances $Ca^{2+}$ uptake in jejunum and kidney. However, when $Ca^{2+}$ concentrations are in the normal physiological range, 1,25$(OH)_2D_3$ gets inactivated in the kidney by 24-hydroxylase [Cytochrome P450 family 24 subfamily A member 1 (CYP24A1)] that hydroxylates carbon 24 in 1,25$(OH)_2D_3$ and in 25(OH)$D_3$ to produce 1,24,25$(OH)_3D_3$ and 24,25$(OH)_3D_3$, respectively. Its expression and activity is induced by 1,25$(OH)_2D_3$, PTH, or calcitonin (CALC) (Murayama et al., 1998).

To exert its biological functions, 1,25$(OH)_2D_3$ binds to the vitamin $D_3$ receptor (VDR), a nuclear receptor that acts as a ligand-activated transcription factor (Brown et al., 1999). VDR controls gene transcription by a series of steps that are related to vitamin D structure. 1.25$(OH)_2D_3$ binds VDR at the ligand-binding domain (LBD), leading to heterodimerization with retinoid-X-

receptors (RXRs). Together, VDR/RXR bind to vitamin $D_3$ response elements (VDREs) through their DNA binding domain (DBD) and recruit other proteins to regulate transcription (Brown et al., 1999).

$1,25(OH)_2D_3$ binds VDR through its 1α-hydroxyl group at the LBD, which has a higher affinity for $1,25(OH)_2D_3$ than for other metabolites (Mellon et al., 1979). When bound to $1,25(OH)_2D_3$, VDR undergoes a three-dimensional structural change and moves from the cytoplasm to the nucleus (Barsony et al., 1992; Racz et al., 1999). The transactivating function of VDR depends on another conformational change when it forms a heterodimer with RXRs (Sulcová et al., 1997). The RXRs identified in chicken are RXRA and RXRG (Eager, 1993; Seleiro et al., 1995). When the VDR-RXR heterodimer binds the promoter region of $1,25(OH)_2D_3$-responsive genes at their VDREs due to high affinity of these regions for the DBD of VDR, another conformational change occurs that leads to recruitment of additional transcription factors to the targeted genes and an increase or decrease in expression (Pike et al., 2007). These promoter regions are present in important genes related to Ca and P homeostasis, such as *CYP24A1*, calbindin 9k (*CALB1-9k*) and 28k (*CALB1-28k*), Ca ion channels, and *PTH* (Liu et al., 1996; Lu et al., 2000; Pike et al., 2007). Thus, the complex formed by $1,25(OH)_2D_3$-VDR-RXR is responsible for the specific actions of vitamin $D_3$ in classic vitamin $D_3$ responsive tissues.

*Parathyroid hormone*

Low plasma $Ca^{2+}$ concentration stimulates synthesis of PTH in the chief cells of the parathyroid glands. PTH increases plasma $Ca^{2+}$ levels. It indirectly stimulates intestinal Ca absorption by enhancing metabolic conversion of vitamin $D_3$ to its biologically active form, $1,25(OH)_2D_3$, and directly increases $Ca^{2+}$ absorption in the convoluted tubule and collecting duct. PTH also stimulates osteoclast activation in bone tissue (Friedman et al., 1996; Hoenderop et al.,

1999; Hoenderop et al., 2005; Schröder et al., 2006b). In birds with hypocalcemia, the activity of PTH is increased and in birds with high $Ca^{2+}$ concentrations in blood, PTH activity is decreased (Bar et al., 1972; Feinblatt, 1975). Bioactive vitamin $D_3$ has an inhibitory effect on PTH gene transcription via VDREs present in the PTH promoter region (Liu et al., 1996).

PTH exert its functions through its G-coupled receptors (GPCRs) on specific target tissues that control Ca and P homeostasis (e.g., bone, kidney, and intestine), where its activation triggers intracellular pathways such as activation of protein kinase A (PKA) and subsequent accumulation of cAMP, and activation of phospholipase C that stimulates protein kinase (PKC) and intracellular $Ca^{2+}$ release (Abou-Samra et al., 1989; Zuscik et al., 2002). The most common receptors for PTH are PTH1R and PTH2R in mammals. PTH1R and PTH3R are present in the chicken genome, whereas PTH2R has not been identified in these animals (Bhattacharya et al., 2011; Lagerström et al., 2006).

*Calcitonin*

CALC produced by C cells in the thyroid gland has an opposite function compared to PTH, and it decreases $Ca^{2+}$ concentration by inhibiting bone resorption (Hoff et al., 2002). A further role of CALC is to regulate bone formation, since CALC gene knock-out mice showed an increased bone mass and growth as compared to wild-type animals (Hoff et al., 2002). In addition, Ca feed levels exert effects on the expression of CALC in broilers, where a Ca-deficient diet lowered plasma CALC concentration (Eliam et al., 1988). CALC actions are produced by its high affinity receptor (CALCR), another GPCR that also signals through PKA and PKC pathways (Pondel, 2000). In bone, it will interfere with osteoclast motility and produce a gradual retraction of osteoclasts; it also inhibits the release of acid phosphatase and the expression of carbonic anhydrase that act to solubilize hydroxyapatite and release Ca and P (Chambers et al., 1991; Zaidi

et al., 1994; Zheng et al., 1994). Furthermore, CALC in kidney will increase urine $Ca^{2+}$ and $P_i$ excretion due to an inhibition of renal tubular resorption, decreasing Ca in plasma (Pondel, 2000).

*Calcium sensing receptor*

The parathyroid chief cells that produce PTH in response to changes in plasma Ca concentration exert a Ca sensing function through expression of Ca-sensing receptor (CASR) that detects variations in plasma Ca concentration. When these levels are high, it stimulates intracellular Ca-dependent proteases inactivate PTH in these cells (Gardella, 2010). CASR is also found in other tissues that regulate Ca uptake, such as kidney and intestine (Diaz et al., 1997). Its levels are influenced by dietary vitamin $D_3$ and Ca, and chickens fed low levels of vitamin $D_3$ exhibited a reduced Ca concentration and lower expression of CASR in duodenum (Hoenderop et al., 1999). Conversely, when vitamin $D_3$ levels were optimal or when PTH was injected, maximum expression of CASR was detected with higher levels of Ca in plasma (Yarden et al., 2000).

**Ca transport**

Transport of Ca across the apical cell membrane in mammals is facilitated by two transporters that are members of the transient receptor potential cation channel subfamily V (TRPV5 and TRPV6) (Montell, 2001). However, in chickens the gene that codes for TRPV5 has not been identified (Flores-Aldama et al., 2019; Proszkowiec-Weglarz et al., 2013). These channels have high selectivity for Ca, preventing Ca overload in the cell by limiting the entry rate through transcellular transport (Hoenderop et al., 2004). In mammalian intestine, TRPV6 is highly expressed, especially in duodenum and is the primary intestinal $Ca^{2+}$ transporter (Peng et al., 2001). However, the expression of this gene in broiler intestines is controversial, since this gene was not detected in duodenum (Proszkowiec-Weglarz et al., 2013) or jejunum (Wu et al., 2021). Once Ca is in the cell, it gets bound to the cytoplasmic chaperone CALB1-28k and translocated from the

apical to the basolateral membrane in intestinal or renal cells (Gross et al., 1990; Nemere et al., 1991). *CALB1-28k* expression is regulated by the presence of vitamin $D_3$, as was demonstrated when birds fed low-Ca diets with a normal inclusion of vitamin $D_3$ had increased *CALB1-28k* expression, while birds fed a diet deficient in vitamin $D_3$ struggled to adapt to these deficiencies and showed decreased expression of *CALB1-28k* (Spencer et al., 1978). Furthermore, fast-growing birds showed higher expression of *CALB1-28k* in the intestine compared to slow-growing strains (Hurwitz et al., 1995). Ca gets delivered to basolateral membrane pumps, such as plasma membrane calcium-transporting ATPase 1 (PMCA1) that is the main transporter expressed in broiler intestine and kidney (Davis et al., 1987; Melancon et al., 1970; Qin et al., 1993; Quinn et al., 2007). Its expression was increased with the addition of vitamin $D_3$ or $1,25(OH)_2D_3$ to the diet when compared to deficient diets (Bindels et al., 1992; Cai et al., 1993a; Van Baal et al., 1996). In contrast sodium/calcium exchanger 1 (NCX1) expression is increased as a response to Ca deficient diets in intestine (Centeno et al., 2004; Hoenderop et al., 2005) and is one of the main transporters of Ca in the basolateral membrane of renal tissue (Bindels et al., 1992; Van Baal et al., 1996).

**P transport**

Functions of the sodium-dependent inorganic phosphate transporters 1 and 2 ($P_i$t-1 and $P_i$t-2) are better understood in mammals, and their locations in these animals are the intestine, kidney, and parathyroid glands. These transporters are regulated by dietary $P_i$ abundance in both tissues increasing their expression when $P_i$ plasma concentration are low (Breusegem et al., 2009; Giral et al., 2009; Villa-Bellosta et al., 2009). Both $P_i$t-1 and $P_i$t-2 can have regulatory functions in parathyroid glands and intestinal tissues, respectively, in response to dietary $P_i$ concentrations, showing higher expression when dietary $P_i$ is low in these tissues. In the parathyroid glands, $P_i$t-1 can act as a $P_i$ sensor and activate PTH production when $P_i$ blood levels are high to diminish

absorption of $P_i$ from the intestine. Furthermore, in the intestine $P_i$ absorption depends on available dietary $P_i$ and $1,25(OH)_2D_3$, increasing $P_i$t-2 expression and further activity when vitamin $D_3$ deficient animals were treated with $1,25(OH)_2D_3$ (Collins et al., 2004; Katai Tanaka et al., 1999). Dietary $P_i$ also helps to regulate the expression of $P_i$t-2 in the intestine, since a switch from high- to low-$P_i$ increased the expression of $P_i$t-2 on the brush border membrane and a switch from low- to high-$P_i$ downregulated the expression (Villa-Bellosta et al., 2009).

Sodium-dependent phosphate co-transporter types IIa and IIb ($NaP_i$-IIa and $NaP_i$-IIb) play major roles in P absorption from the intestine and reabsorption in the kidney. In mammals, 70 to 80% of kidney P transport is conducted through $NaP_i$-IIa, (Tenenhouse, 2005). Expression and function of $NaP_i$-IIa in the intestine is highly regulated by PTH and dietary $P_i$ levels (Kempson et al., 1995; Pfister et al., 1998; Tenenhouse, 2005). $NaP_i$-IIa goes through retrieval by endocytosis stimulated by PTH and high dietary $P_i$, decreasing its function in the intestinal brush border membrane of the intestine. Conversely, an increase in the $P_i$ uptake is going to happen as a response to low dietary $P_i$ levels by a recruitment of this transporter to the apical membrane of the intestine (Pfister et al., 1998). When plasma $P_i$ levels were increased in laying hens, *$NaP_i$-IIa* mRNA expression was reduced in kidney, decreasing potential for $P_i$ absorption (Huber et al., 2006). Furthermore, avian kidneys can either re-absorb or secrete $P_i$ in kidney tubules, regulated by PTH (Bijvoet et al., 1977; Clark et al., 1976).

$NaP_i$-IIb is a major Na-dependent $P_i$ transporter in jejunum regulated by $1,25(OH)_2D_3$ and $P_i$ levels in the feed in mammals (Katai Miyamoto et al., 1999), and PTH can indirectly affect its expression through stimulation of $1,25(OH)_2D_3$ synthesis (Murer et al., 2001). The relationship between both $NaP_i$-IIa and $NaP_i$-IIb has been noted. In mammals, when $NaP_i$-IIb is downregulated in intestinal tissue, $NaP_i$-IIa in renal tissue will get upregulated and vice versa (Reining et al., 2010;

Sabbagh et al., 2009). Further, increased expression of $NaP_i$-IIb was observed in jejunum and ileum when a diet low in $P_i$ was given to the birds (Olukosi et al., 2011).

**Tissue-specific regulation of Ca and P homeostasis**

Target tissues where vitamin $D_3$ acts to regulate $Ca^{2+}$ and $P_i$ homeostasis are intestine, kidney, bone, and parathyroid gland (Pike et al., 2007). Its specific functions in these tissues will be described below.

*Intestine*

Dietary $Ca^{2+}$ and $P_i$ uptake is highly influenced by the action of $1,25(OH)_2D_3$-bound VDR in the intestine (Panda et al., 2004), and it can be saturable (Van Cromphaut et al., 2001). Intestinal $Ca^{2+}$ absorption can occur either passively, utilizing a differential chemical gradient to move $Ca^{2+}$ through the spaces between cells without influence of vitamin $D_3$ (Bronner, 1998; Bronner et al., 1999; Bronner et al., 1986; Buckley et al., 1980; Rogers et al., 1995) or actively, following three vitamin $D_3$-dependent sequential steps. In mammals, transcellular $Ca^{2+}$ absorption occurs by the stimulation of *TRPV6* expression in the brush border membrane by $1,25(OH)_2D_3$ (Song et al., 2003; Wasserman, 2005). Once $Ca^{2+}$ is inside the intestinal cell, it needs to be transported to the basolateral membrane, which is done by CALB1-28k whose expression, similar to TRPV6, gets increased by low dietary $Ca^{2+}$ concentration and $1,25(OH)_2D_3$ (Peng et al., 2003; Song et al., 2003). Furthermore, CALB1-28k can also act as a buffer against cytotoxic levels of $Ca^{2+}$ inside the cell by sequestration of $Ca^{2+}$ (Lambers et al., 2006). The last step of the transport is the extrusion of $Ca^{2+}$ through the basolateral membrane of the cell, as mentioned above, where $1,25(OH)_2D_3$ stimulates the intestinal plasma pump PMCA1 (Cai et al., 1993b). In response to low $Ca^{2+}$ levels in plasma, expression levels of *NCX1* in intestine also get enhanced, even though its main site of action is renal tissue (Bindels et al., 1992; Centeno et al., 2004; Van Baal et al., 1996).

*Kidney*

Kidney Ca and P homeostasis occurs in a similar process to that in the intestine. Vitamin $D_3$ can control its own homeostasis because $1,25(OH)_2D_3$ suppresses 1α-hydroxylase activity and enhances 24-hydroxylase activity, ultimately leading to reduced $1,25(OH)_2D_3$ and higher levels of inactive catabolites [$1,24,25(OH)_3D_3$ or $24,25(OH)_2D_3$] (Liu et al., 1998). Renal $Ca^{2+}$ transport in the distal convoluted tubules is mainly an active process that gets increased by $1,25(OH)_2D_3$, dietary Ca, and PTH. $1,25(OH)_2D_3$ enhances $Ca^{2+}$ reabsorption from the luminal membrane of the distal convoluted tubules, increasing expression of TRPV6 (Norman et al., 1992). Production of CALB1-28k that collects cytosolic $Ca^{2+}$ and moves it to the basolateral membrane is also increased by $1,25(OH)_2D_3$. In distal tubular cells, PMCA1 is upregulated by low $Ca^{2+}$ levels in the blood and also because of the presence of $1,25(OH)_2D_3$ (Borke et al., 1989; Gross et al., 1990).

*Bone*

The main function of $1,25(OH)_2D_3$ is to maintain blood $Ca^{2+}$ concentrations at a certain level so it can exert its normal physiological functions, and this can be done by absorption from the diet, reabsorption in the kidney, or $Ca^{2+}$ mobilization from the bone (Holick, 1999). Bone acts as a Ca and P reservoir, where $1,25(OH)_2D_3$ in association with VDR can start the bone remodeling process to resorb $Ca^{2+}$ from bone and move it to the blood stream. This process starts with induction of the cell membrane surface expression of receptor activator of nuclear factor kappa-B ligand (*RANKL*) and a decrease in expression of osteoprotegerin (*OPG*), which inhibits osteoclastogenesis by binding to and inhibiting RANKL. These changes are induced by $1,25(OH)_2D_3$ acting on osteoblasts, which allows osteoclasts to differentiate, mature, and get activated. The presence of macrophage colony-stimulating factor (M-CSF) enhances binding between osteoblast RANKL and RANK in osteoclast precursors to promote osteoclast

differentiation and development (Horwood et al., 1998; Kearns et al., 2008; Simonet et al., 1997; Yasuda et al., 1998). Furthermore, $1,25(OH)_2D_3$ may increase expression of integrin $\alpha_V\beta_3$ for osteoclast adhesion to the bone surface for bone resorption (Mimura et al., 1994). In osteocytes, which are cells contained in the calcified matrix because they are derived from osteoblasts surrounded by their products, $1,25(OH)_2D_3$ does not seem to have a recognized function; however, osteocytes can secrete dentin matrix protein 1 (DMP-1) and fibroblast growth factor 23 (FGF23) that can suppress renal $1,25(OH)_2D_3$ production by inhibiting 1α-hydroxylase activity (Feng et al., 2006; Shimada et al., 2004).

*Parathyroid gland*

The vitamin $D_3$ system is considered a regulator of parathyroid function. It directly inhibits PTH synthesis and parathyroid cell growth, as well as indirectly affects parathyroid VDR, which will be increased by elevations in plasma $Ca^{2+}$ (Brown et al., 1995). Furthermore, the $1,25(OH)_2D_3$-VDR complex induces the expression of *CASR* that regulates parathyroid gland response to different levels of plasma $Ca^{2+}$. This function suggests that vitamin $D_3$ has a cooperative effect with Ca to control PTH synthesis (Brown et al., 1996).

## Rationale and objectives

Genetic selection programs are responsible for the improvement in growth rate, meat yield, feed efficiency, and time to reach commercial weight in modern broilers (Leyva-Jimenez et al., 2019). The main focus of genetic selection programs over the past century was improving commercial traits (Richard, 1998b; Su et al., 1999); however, breeding companies are now showing interest in improvement of welfare parameters, such as leg health (Neeteson et al., 2016). Bird genotype has a significant relationship with bone composition, since modern broilers that

have a faster growth rate at earlier ages can show a predisposition to suffer bone illnesses (Wideman Jr et al., 2014).

Bone tissue is comprised of a combination of Ca and P and is regulated by different physiological pathways that enhance absorption, reabsorption, and resorption of these minerals from intestine, kidney, and bone (Holick, 1999). An imbalance in Ca and P plays a crucial role in leg health and mobility disorders. Therefore, a better comprehension of the different physiological pathways that regulate Ca and P homeostasis and deposition in bones may allow further improvements in genetic selection for commercial broiler lines or lead to development of alternative strategies to mitigate the negative impact of rapid growth on skeletal health. Thus, this research seeks to elucidate the mechanisms regulating Ca and P metabolism that could have been affected by genetic selection of commercial broilers.

We hypothesize that genetic selection of commercial broilers has affected physiological regulation of Ca and P, leading to an alteration in Ca and P transport, absorption, and resorption, and contributing to an increase in the incidence of leg disorders in these birds. Therefore, the objectives of this research were to:

1. Determine the influence of genetic selection on expression of genes involved in the regulation of Ca and P metabolism during late embryonic and early post-hatch development.
2. Identify the effect of genetic selection on factors influencing bone strength during late embryonic and early post-hatch development.

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# CHAPTER 3

## DEVELOPMENTAL EXPRESSION OF GENES RELATED TO CALCIUM AND PHOSPHORUS METABOLISM IN LEGACY AND MODERN BROILERS

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**ABSTRACT**

Bone is comprised of calcium (Ca) and phosphorus (P), and the mineralization process is regulated by absorption and reabsorption of these minerals in the jejunum and kidney, respectively, which is controlled by vitamin $D_3$ action. This study sought to evaluate if Ca and P metabolism pathways have been affected by genetic selection. To this end, expression of enzymes involved in controlling bioactivity of vitamin $D_3$, receptors for hormones regulating Ca and P homeostasis, and chaperones and transporters for Ca and P were measured in kidney, jejunum, and liver of legacy [Athens Canadian Random Bred (ACRB)] and modern (Cobb 500) male broilers between embryonic day (E) 14 and post-hatch day (D) 13. Circulating vitamin $D_3$ metabolites and bone properties were also determined. Expression of vitamin $D_3$ 24-hydroxylase (*CYP24A1*) in kidney approached a significant line-by-age effect (P=0.0705), with higher expression on E18 for ACRB followed by upregulation in Cobb birds on D3 and D10. In jejunum, ACRB had higher expression of 25-hydroxylase (*CYP2R1*) on D1 and D7. In plasma, there were no line-by-age effects for any metabolite, though $25(OH)D_3$ showed a line effect where it was higher for ACRB birds. $24,25(OH)_2D_3$ also showed a line effect, being higher in Cobb birds. Vitamin $D_3$ receptor and its transcriptional partner retinoid-x-receptor alpha showed line-by-age effects in kidney, being higher in ACRB on D7, and D7 and D10, respectively. Parathyroid hormone (PTH) enhances Ca and P uptake in intestine and kidney, and PTH receptor 1 showed a line-by-age effect in jejunum. ACRB had higher levels on E16, and Cobb had higher levels on D1 and D3. Plasma Ca is also regulated by calcitonin (CALC), and plasma Ca levels are detected by calcium sensing receptor (CASR). Calcitonin receptor (CALCR) showed interactive effects in kidney and jejunum, where ACRB had higher expression on E16, D7, and D10 in kidney and on D1 and D13 in jejunum. CASR was higher in jejunum of ACRB on almost all post-hatch days. In liver, a line-by-age interactive effect

was detected for sodium-calcium exchanger 1, being higher in Cobb on E16 and at all post-hatch ages, while plasma membrane Ca ATPase mRNA expression was higher for Cobb during embryonic development and several post-hatch ages. In kidney, inorganic phosphate transporter 2 (*$P_i$T-2*) showed a line-by-age effect, being higher in Cobb during late embryonic development and early post-hatch. *$P_i$T-1* showed an interactive effect in jejunum, where its mRNA expression was higher in ACRB birds on most post-hatch days. These results indicate that Ca and P metabolism may have been influenced by genetic selection and could contribute to skeletal weakness observed in modern broilers.

## INTRODUCTION

A direct relationship exists between the fast growth rate of modern commercial broilers and several bone diseases, including tibial dyschondroplasia, varus-valgus deformities, rotated tibias, and epiphyseal separation (Bradshaw et al., 2002; Knowles et al., 2008). Bone tissue is formed by hydroxyapatite, which is comprised of Ca and P in a collagen matrix. As growth rates have increased over the past several decades due to genetic selection programs, a sufficient nutritional supply and the ability to absorb and utilize these minerals and maintain physiological concentrations for bone remodeling and other physiological functions is needed (Williams Waddington et al., 2000). Regulation of Ca and P concentrations within a physiological range occurs due to feedback mechanisms in intestine, kidney, and bone. Molecules such as the active form of vitamin $D_3$, parathyroid hormone (PTH), and calcitonin (CALC) exert their function through their different receptors in each tissue, regulating Ca and P transporter and chaperone expression in target tissues (Veum, 2010).

When circulating Ca levels are low, vitamin $D_3$ gets transformed to $25(OH)D_3$ by 25-hydroxylase (CYP2R1) in liver. Next, in kidney 1-α hydroxylase (CYP27B1) forms $1,25(OH)_2D_3$. This active form binds to the vitamin D receptor (VDR), which heterodimerizes with retinoid-X-receptors alpha and gamma (RXRA and RXRG) to produce vitamin $D_3$ genomic responses (Bettoun et al., 2003). When $1,25(OH)_2D_3$ binds the VDR-RXR complex, this then binds to vitamin D response elements (VDREs) in vitamin D-responsive genes to regulate expression of Ca and P transporters in intestine and kidney, and facilitates bone resorption (Ebihara et al., 1996). This serves to increase Ca and P concentrations in the blood (Boyle et al., 1972; Suda et al., 2003; Yamamoto et al., 1984). To stop its function, vitamin $D_3$ is converted by 24-hydroxylase (CYP24A1) to $1,24,25(OH)_3D_3$ and $24,25(OH)_2D_3$, which are biologically inactive.

PTH, CALC, and Ca sensing receptor (CASR) also regulate physiological concentrations of Ca and P (Veum, 2010). PTH has two functions in the kidney, where it increases Ca reabsorption in the renal tubules and enhances production of 1α-hydroxylase, which increases the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ that can stimulate intestinal Ca absorption (Forster et al., 2006; Schröder et al., 2006a). $1,25(OH)_2D_3$ exerts negative feedback over PTH expression in chickens, when the ligand-bound VDR/RXR complex binds to VDREs in the PTH gene promoter and blocks transcription (Liu et al., 1996). As an additional control, when plasma Ca is elevated, CASR in parathyroid cells stimulates activation of proteases that cleave and inactivate PTH (Gardella, 2010). Likewise, CALC is secreted in response to high plasma Ca concentrations and inhibits bone resorption to lower Ca plasma levels and counteract effects of $1,25(OH)_2D_3$ and PTH (Matsuda et al., 2006).

Vitamin $D_3$ increases the expression of Ca transporters in intestine and kidney. In mammals, the main intestinal apical Ca transporter is TRPV6; however, this gene has not been detected in the intestine of broilers (Peng et al., 2001; Proszkowiec-Weglarz et al., 2013; Wu et al., 2021). $1,25(OH)_2D_3$ also stimulates the expression of calbindin-28k (*CALB1-28k*), which transports Ca to the basolateral membrane and delivers it to basolateral plasma membrane pumps (Gross et al., 1990; Nemere et al., 1991). In the basolateral membrane, plasma membrane calcium-transporting ATPase 1 (PMCA1) and sodium/calcium exchanger 1 (NCX1) move Ca from the cell cytoplasm to the blood stream to maintain Ca at physiological levels (Bindels et al., 1992; Cai et al., 1993b; Centeno et al., 2004; Hoenderop et al., 2005).

Sodium-dependent inorganic phosphate transporters 1 and 2 ($P_i$t-1 and $P_i$t-2) mediate $P_i$ absorption. *$P_i$t-2* expression is increased when dietary $P_i$ levels were decreased and in the presence of higher dietary of 1,25(OH)2D3. $P_i$t-1 has also been theorized to serve as P-sensing molecule,

working to reduce plasma $P_i$ by increasing PTH expression in the parathyroid gland when plasma $P_i$ is high (Collins et al., 2004; Katai Miyamoto et al., 1999). Furthermore, sodium-dependent phosphate co-transporter types IIa and IIb ($NaP_i$-IIa and $NaP_i$-IIb) in the intestine are highly regulated by $1,25(OH)_2D_3$ and dietary $P_i$, and indirectly regulated by PTH because this increases plasma levels of $1,25(OH)_2D_3$. A relationship was noted between tissue-specific expression of these two transporters, where a decrease of $NaP_i$-IIb in the intestine will produce an upregulation of $NaP_i$-IIa in kidney and vice-versa (Murer et al., 2001; Reining et al., 2010; Sabbagh et al., 2009).

An impairment in the expression of any of these regulators could affect downstream events that are supposed to maintain Ca and P homeostasis, therefore leading to inefficient utilization of these minerals that could affect bone mineralization. Furthermore, it has been observed that fast-growing birds have more porous cortical bone, less bone mineralization, and an imbalance in the molar Ca:P ratio above the expected range in cortical bone compared to slow-growing birds (Morris, 1993). Therefore, a legacy bird line [Athens Canadian Random Bred (ACRB)], which is a genetic control line that represents birds from the mid-1950's and that have not been further altered by genetic selection (Collins et al., 2016; Vaccaro et al., 2021), and a modern commercial broiler line (Cobb 500) were compared to determine if components of physiological systems regulating Ca and P metabolism or bone mineral content have been affected by genetic selection.

## MATERIALS AND METHODS

### *Animals and tissue collection*

An experiment was conducted in which liver, jejunum, and kidney were collected from male ACRB and Cobb 500 broilers between embryonic (E) day 14 and post-hatch day (D) 13. Birds of both lines were incubated under the same conditions (60% humidity and 99 ºF), hatched, and raised concurrently. Only male birds were used in this study to avoid sex-specific effects on

vitamin $D_3$ metabolism, as it is known that in females higher levels of estrogens can influence vitamin $D_3$ metabolism in kidney (Pike et al., 1978). From E18 to D13, males were sexed looking directly into the gonads of each specimen. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Fertile eggs from ACRB and Cobb 500 broiler lines were co-incubated under standard conditions (37.5°C, 55% relative humidity, rotation every 2– 3 h), with the day that the eggs were set defined as E0 and the day of hatch as D0. On D0, birds from each line were allocated into 5 pens within the same battery (28 birds/pen and given free access to water and a standard commercial-type starter diet (24% crude protein, 2.5% crude fat, 4.5% crude fiber, 1.35% lysine, 1.0% Ca and 0.55% P). At each sampling age, 2 – 3 birds of each line were randomly selected from each pen. Birds were weighed, euthanized by cervical dislocation, and tissues were collected on E14, E16, E18, E20, D0, D1, D3, D5, D7, D10, and D13. On E14 and E16, up to 24 embryos were used for sample collection so that 6 – 8 males could be identified for analysis by molecular sexing. From E18 onwards, tissues were only collected from up to 8 male birds that were visually identified by gonadal appearance. On embryonic ages, right and left kidneys were collected, whilst on post-hatch ages only the right kidney was sampled. The right lobe of the liver and a middle section of the jejunum (defined as beginning at the distal end of the duodenal loop and ending at Meckel's diverticulum) were collected on all ages. Tissues were snap frozen in liquid nitrogen prior to storage at -80°C for later RNA extraction and gene expression analysis. On E14 and E16, a second piece of the left liver lobe was collected on ice prior to storage at -20°C for subsequent genomic DNA (gDNA) extraction and molecular sexing. Blood was collected into heparinized tubes by cardiac puncture on each post-hatch age, and samples were stored on ice until centrifugation at 1,500 x g for 10 minutes at 4°C to isolate plasma. Plasma was stored at -20°C

prior to analysis of circulating vitamin $D_3$ metabolite levels. Left and right tibiae were collected from each bird and stored at -20$^{o}$C until used for determination of bone parameters as described below.

*Molecular sexing of embryos*

To determine embryo sex on E14 and E16, gDNA was isolated using the QIAamp Fast DNA Tissue kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Sex was determined by PCR amplification of the chromo-helicase DNA binding protein 1 gene, using 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') primers (Fridolfsson et al., 1999; Griffiths, 2000). Reaction contained a mixture of GoTaq Green DNA polymerase, 0.4 µM of each forward and reverse primer mentioned above, and 50 ng of gDNA template. The PCR conditions were 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. After PCR was concluded, agarose gel electrophoresis was conducted on the products to determine the number of bands produced, where two bands determined female samples and a single band determined male samples (Figure 1).

*Reverse transcription-quantitative PCR*

Gene expression was conducted for 8 replicate male birds from each line for kidney (n=8) and 6 replicate male birds for jejunum and liver (n=6). All samples were processed for mRNA expression by reverse transcription-quantitative PCR (RT-qPCR) using protocols established in our laboratory (Ellestad et al., 2011; Payne et al., 2019; Vaccaro et al., 2021). Total RNA was isolated with the RNeasy Mini kit (Qiagen) utilizing the manufacturer's protocol for jejunum and kidney, and a modified version of the protocol for lipid-rich tissues as described below was used for liver. Liver tissue samples were mechanically homogenized in 1 mL Qiazol reagent (Qiagen)

for 30 sec and then samples were incubated at room temperature for 5 min. After the incubation period, 200 µL chloroform was added to the samples, followed by manual shaking for 15 sec. An additional incubation was done at room temperature for 3 min, and the samples were centrifuged for 15 min at 12,000 x g. After this, 700 µL of 70% ethanol was added to the supernatant of each sample before the remainder of the isolation was carried out according to the manufacturer's instructions. Total RNA quantification was done using a Nanodrop ND1000 spectrophotometer (Thermofisher, Waltham, MA), and the integrity was evaluated via agarose gel electrophoresis.

Total RNA (1 µg) was converted to cDNA using M-MuLV reverse transcriptase (200 U; New England Biolabs), oligo-dT primers [5 µM; Integrated DNA Technologies (IDT), Coralville, IA], and random hexamer primers [5 µM] (ThermoFisher). A pool (1 µg) generated from equivalent amounts of all samples was used to prepare a negative control reaction without reverse transcriptase enzyme (no RT) that helped to detect gDNA contamination in the samples. Each cDNA sample was diluted 5-fold prior to analysis by qPCR, and these were diluted an additional 10-fold for detection of 18s ribosomal RNA (18S) as a housekeeping gene in kidney and liver. Intron-spanning primers (Table 1) from IDT were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The following parameters were used: melting temperature between 58-60°C, 40-60% GC content, 18-30 nucleotides in length, and amplicon length of 100-150 base pairs.

Quantitative PCR was done using 96-well plates in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Duplicate reactions (10 µL) were run containing 2 µL of each diluted cDNA sample, 5 µL 2X PowerUp SYBR Green Master Mix (ThermoFisher), and 400 nM each primer. After analysis, calculations were performed using the mean Ct value from these duplicate reactions. Cycling was performed with the following conditions: 95°C for 5 min,

followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec, followed by a disassociation curve analysis to ensure amplification of a single product. Target transcript levels were normalized to mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in jejunum and *18S* rRNA in liver and kidney. Data were transformed and normalized using the equations $\Delta Ct = Ct_{target} - Ct_{reference}$ and $2^{-\Delta Ct}$ (Ellestad et al., 2009; Ellestad et al., 2013; Ellestad et al., 2015; Payne et al., 2019; Vaccaro et al., 2021). Within each gene, line-by-age interactive means are expressed relative to the line with the highest mRNA level across all ages, and main effect line or age means are expressed relative to the line or age with the highest mRNA level. In all cases, the line-by-age, line, or age value with the highest expression level is 100%.

*Vitamin $D_3$ metabolite determination*

Once blood samples were collected, they were centrifuged at 1,500 x *g* and 4°C for 5 min and the resulting plasma was stored at -20°C until analysis. To determine vitamin $D_3$ metabolite levels in plasma, liquid chromatography-tandem mass spectrometry (Heartland Assays, Ames, IA) was done to sample with lower limits of detection (LOD) and quantification (LOQ) for each analyte as follows: $25(OH)D_3$ – 0.5 ng/mL (LOD) and 1.5 ng/mL (LOQ); $24,25(OH)_2D_3$ – 0.1 ng/mL (LOD) and 0.3 ng/mL (LOQ); and $1,25(OH)_2D_3$ – 5.0 pg/mL (LOD) and 10 pg/mL (LOQ). This analysis was conducted for 3 male birds of each line in D3, D7, and D10 (n=3).

*Bone parameters*

Bone ash and bone concentration were determined in the left tibia from each bird using a modified version of methods reported by Kim et al. (2004). Briefly, cartilage and flesh were carefully removed from bones with forceps and scissors. Then, bone volume was calculated by distilled water displacement with the assumption that the specific gravity of distilled water is 1 $g/cm^3$ at 24°C. Bone was then dried at 85°C for 24 h in a forced air oven. Fat was extracted from

dried bones using hexane in a refluxed Soxhlet apparatus for 36 h at 70°C. The fat-free bones were then dried an additional time in a forced air oven at 85°C for 24 h and weighed. Bones were subsequently ashed at 600°C overnight, and the ash weighed the next day. Bone ash (%) was determined by dividing the ash weight by the fat-free dry weight and multiplying by 100, and bone density ($g/cm^3$) was calculated by dividing the ash weight by the volume of the bone.

*Statistical analysis*

Body weight, mRNA expression (ΔCt), bone ash (%) using a log10 transformation, and bone density ($g/cm^3$) were analyzed in JMP version 16 using a two-way ANOVA, with the line-by-age interaction, line, and age as model effects. Whenever the ANOVA indicated a statistically significant interactive or main effect, a multiple means comparison was performed using the test of least significant difference. All differences were considered significant at $P \leq 0.05$. Whenever the interactive effects were not statistically significant, significantly different main effect means are presented.

**RESULTS**

*Growth performance*

Body weight was measured for ACRB and Cobb 500 birds at each age where tissue samples were collected. A significant line-by-age effect was observed, where Cobb birds were significantly heavier than ACRB after D3, with the largest difference at D13 when Cobb birds were 2-fold heavier (Figure 2; $P \leq 0.05$). As expected, modern commercial broilers were significantly heavier than legacy broilers post-hatch.

*Gene expression for enzymatic conversion of vitamin $D_3$*

To understand how selective breeding may have influenced vitamin $D_3$ metabolism, expression levels of two enzymes that are responsible for initial activation of vitamin $D_3$ and

inactivation of its biologically active metabolites were analyzed in kidney, jejunum, and liver. Even though 1α-hydroxylase (*CYP27B1*) has been identified in mammals and fish (Cali et al., 1991; Chun et al., 2014), its transcript has not been identified in chickens, therefore it was not analyzed. There were no significant line-by-age or line effects for *CYP2R1* mRNA in kidney tissue (Figure 3A, Table 2; P>0.05); however, a significant age main effect was observed for this gene (Table 3; P≤0.05). It decreased towards D0 and had a subtle but not statistically significant increase after hatch. Additionally, *CYP24A1* approached a significant line-by-age effect in kidney (Figure 3B; P=0.07), where ACRB had higher expression on E18, and Cobb had a higher expression on D3 and D10 (P≤0.05). The main effect of line was significantly different, with Cobb birds having higher kidney expression of *CYP24A1* than ACRB birds (Table 2; P≤0.05), and there was also a main effect of age where levels decreased between E14 and D0 and increased again after hatch through D13 (Table 3; P≤0.05).

In the jejunum, a significant line-by-age interaction was found for *CYP2R1*, where ACRB had higher expression on D1 and D7 (Figure 4A; P≤0.05). Conversely, *CYP24A1* expression did not show a significant line-by-age effect (Figure 4B; P>0.05), though it approached a significant main effect of line in jejunum being higher overall in ACRB (Table 2; P=0.0714). It also showed a main effect of age, being higher on embryonic ages and decreasing steadily after hatch (Table 3; P≤0.05).

In liver, a significant line-by-age effect for *CYP2R1* was not observed (Figure 5; P>0.05), though it exhibited significant main effects of line and age. Overall, levels of *CYP2R1* mRNA were higher in liver of Cobb broilers (Table 2; P≤0.05), and when the age effect was analyzed the mRNA expression decreased in both lines between E14 and E20, after which its expression began to increase again to reach maximum levels on D5 (Table 3; P≤0.05). Meanwhile, *CYP24A1*

expression was undetected in liver, consistent with previous observations in chicken (Shanmugasundaram et al., 2012).

*Levels of vitamin $D_3$ in circulation*

To assess functionality of the enzymes described above, circulating levels of vitamin $D_3$ metabolites were measured in plasma, and even though mRNA expression of *CYP27B1* could not be evaluated, the metabolite produced by that enzyme was measured [1,25$(OH)_2D_3$]. Circulating 25$(OH)D_3$ did not show a line-by-age interactive effect or a main effect of age (Figure 6; $P>0.05$); however, it did approach a significant main effect of line, where ACRB birds had higher plasma concentrations of this metabolite (Table 2; $P=0.0770$). Furthermore, 1,25$(OH)_2D_3$ did not show any line-by-age effect (Figure 6B; $P>0.05$), or main effects of line or age (Table 3; $P>0.05$). When 24,25$(OH)_2D_3$ was assessed, it did not show a line-by-age effect (Figure 6C; $P>0.05$) or an age effect (Table 3; $P>0.05$), but a line effect was observed for this metabolite, where Cobb birds had a higher overall concentration compared to ACRBs (Table 2; $P\leq0.05$).

*Gene expression for vitamin $D_3$ action*

To exert its functions, bioactive vitamin $D_3$ interacts with VDR, which binds VDREs in target genes with its heterodimeric transcriptional partners that include RXRA and RXRG. In kidney, *VDR* approached a significant line-by-age effect (Figure 7A; $P=0.07$), with ACRB birds having a higher expression on D7 ($P\leq0.05$). Furthermore, it did show a significant line effect and was higher for ACRB birds (Table 4; $P\leq0.05$). It also had an age main effect, where its expression decreased after hatch until an upregulation on D13 (Table 5; $P\leq0.05$). Expression of *RXRA* exhibited an interactive effect in kidney, where levels were higher in ACRB birds on D7 and D10 (Fig. 7B; Table 4; $P\leq0.05$). *RXRG*, another heterodimeric transcriptional partner of *VDR*, was not detected in this tissue.

In jejunum, expression levels of VDR, RXRA, or RXRG did not show any significant line-by-age interactive effects (Figure 8; P>0.05); however, both *RXRA* and *RXRG* showed a significant main effect of line, and all three genes exhibited a significant age main effect. For both *RXRA* and *RXRG,* ACRB exhibited higher levels in jejunum when the main effects of line were calculated (Table 4; P≤0.05). Expression of *VDR* steadily increased with age in this tissue, so the lowest levels were observed on embryonic ages (Table 5; P≤0.05). Meanwhile, *RXRA* had its lowest jejunal expression on D3 and D5 (Table 5; P≤0.05), and *RXRG* showed an opposite main effect of age to *VDR* expression in jejunum, where its expression decreased from embryonic through post-hatch ages, exhibiting the highest levels on E14 and the lowest levels after D3 (Table 5; P≤0.05).

The expression of *VDR* in the liver did not show a line-by-age effect (Figure 9A; P>0.05); however, main effects of line and age were detected. For the line main effect, Cobb birds showed higher liver *VDR* gene expression overall (Table 4; P≤0.05). For the age main effect, expression of *VDR* steadily decreased from E14 towards E20 and remained low thereafter (Table 5; P≤0.05). Furthermore, *RXRA* had a significant line-by-age interaction, where Cobb liver expressed higher levels than ACRB liver on D7, D10, and D13 (Figure 9B; P≤0.05). *RXRG* mRNA levels did not show line-by-age interaction (Figure 9C; P>0.05) or main line effects (Table 4; P>0.05), but an age main effect was observed. Its expression decreased from E14 to E20 and increased to intermediate levels at hatch, where it was maintained until D13 (Table 5; P≤0.05).

*Gene expression for hormonal regulation of Ca and P homeostasis*

Ca and P physiological levels are also regulated by PTH acting through its receptors (PTH1R and PTH3R) in target tissues. It elevates their levels in blood by directly increasing Ca and P reabsorption in kidney and facilitating jejunum absorption of these minerals indirectly by enhancing conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ in the kidney. CALC acts through its receptor

(CALCR) to oppose these effects, primarily reducing circulating Ca and P by inhibiting bone resorption. A line-by-age effect was not present in kidney when *PTH1R* mRNA expression was evaluated (Figure 10A; P>0.05); however, main effects of line and age were detected, where ACRB had higher expression compared to Cobb (Table 4; P≤0.05). Furthermore, when the main age effect was assessed, its expression decreased at E18, increased towards D3, and decreased again until D10 (Table 5, P≤0.05). A line-by-age interactive effect for *CALCR* gene expression was exhibited in kidney, with higher mRNA levels in ACRB birds on E16, D7, and D10 (Fig 10B; P≤0.05). Kidney expression of *PTH3R* was not detected.

In jejunum, *PTH1R* mRNA expression showed a line-by-age effect, where its expression was higher for ACRB birds on D1 and D3 (Figure11A; P≤0.05). Gene expression for *CALCR* approached a significant line-by-age effect (Figure 11B; P=0.0878), where it exhibited a higher expression in ACRB birds on D1 and D3 (P≤0.05). Furthermore, it also approached a main effect of line, where ACRB birds had higher expression (Table 4; P=0.0528). A main effect of age was observed, where the highest expression was shown on embryonic ages and then it steadily decreased on post-hatch ages (Table 5; P≤0.05). Similar to kidney, *PTH3R* mRNA was not detectable in jejunum.

In liver, *PTH1R* mRNA expression approached a significant line-by-age effect (Figure 12A; P=0.0717), where ACRB had higher expression on D3 (P≤0.05). No line main effect was detected in liver for *PTH1R* mRNA expression (Table 4; P>0.05). A main age effect was detected in liver for *PTH1R*, where the highest expression was detected on E16 and it decreased towards D0, remaining constant through all post-hatch ages (Table 5; P≤0.05). Furthermore, *PTH3R* also approached a line-by-age effect (Figure 12B; P=0.0616), where Cobb birds showed higher expression of this gene on D1 and D10 (P≤0.05). No line main effect was detected in liver for

*PTH3R* mRNA expression (Table 4; P>0.05). Furthermore, *PTH3R* showed main age effect, exhibiting the highest expression on D10 and D13 (Table 5, P≤0.05). Expression of *CALCR* was not detected in liver of either line at any age.

*Gene expression for Ca transport*

To maintain normal Ca levels in plasma, flux of this mineral must occur in different target tissues by membrane transporters, chaperones, and Ca sensors. In kidney, expression of *TRPV6* did not show significant line-by-age (Figure 13A; P>0.05) or main line effects (Table 6; P>0.05), though it did exhibit a main age effect. Its mRNA expression decreased through embryonic ages and was lowest on E20, and it increased again after hatch (Table 7; P≤0.05). For expression of *CALB1-28k* in kidney, a significant line-by-age interaction was observed, and Cobb birds had a higher expression on E16, while ACRB exhibited higher mRNA levels on E20 (Figure 13B; P≤0.05). Kidney *NCX1* did not show significant line-by-age (Figure 13C; P>0.05) or main line effects (Table 6; P>0.05) but did show a significant main effect of age similar to that of *TRPV6*, with a decrease to its lowest levels on E20 then increasing again after D0 (Table 7; P≤0.05). *PMCA1* mRNA expression did not exhibit any significant line-by-age, line, or age effects in kidney (Figure 13D; Tables 6 and 7; P>0.05). Expression of CASR mRNA in kidney did exhibit a significant line-by-age effect, where its mRNA expression was higher in Cobb birds on D0 (Figure 13E; P≤0.05).

In jejunum, *TRPV6* mRNA was not detected, consistent with other literature investigating intestinal expression of this gene in chickens (Proszkowiec-Weglarz et al., 2013; Wu et al., 2021). Gene expression for *CALB1-28k* approached a significant line-by-age effect (Figure 14A; P=0.0619). It tended to be higher in Cobb birds on E16 (P=0.058) and was significantly higher in Cobb D1 (P≤0.05) and higher in ACRB on E20 (P≤0.05). Furthermore, it also approached a

significant line effect (Table 6; P=0.0702), being higher in Cobb birds overall. A main effect of age was exhibited for *CALB1-28k* mRNA expression, increasing with age and showing the highest expression on D10 (Table 7; P≤0.05). *NCX1* mRNA expression did not show significant line-by-age interaction (Figure 14B; P>0.05) or main line effects (Table 6; P>0.05), though it did show a main effect of age and exhibited the highest expression on E18, with levels decreasing steadily on post-hatch ages (Table 7; P≤0.05). *PMCA1* mRNA levels also did not show a significant line-by-age interaction (Figure 14C; P>0.05), but they approached a main line effect, being higher for Cobb birds (Table 6; P=0.071). In addition, it also showed a main age effect, with a progressive increase with age and the highest mRNA expression for *PMCA1* in jejunum on D13 (Table 7; P≤0.05). Jejunum CASR expression showed a significant line-by-age effect, where ACRB birds exhibited higher mRNA expression levels on all post-hatch ages except for D0 and D5 (Figure 14D; P≤0.05).

In liver tissue, *NCX1* mRNA showed a line-by-age interactive effect, where Cobb birds had higher expression on E16 and all post-hatch days except on D0 (Figure 15A; P≤0.05). A line-by-age interactive effect was also detected for *PMCA1* in liver, and its expression was higher in Cobb birds on E14, E20, D1, D5, and D10 (Figure 15B; P≤0.05). On D0 ACRB mRNA expression was higher (Figure 15B; P≤0.05). Similar to jejunum, mRNA expression of *TRPV6* was not detected in liver, and neither was expression of *CALB-28k* or *CASR*.

*Gene expression for P transport*

Expression levels of P transporters were evaluated in these tissues, due to the importance of this mineral in the balance of good bone health. In kidney, while *$P_iT$-1* mRNA expression did not show a significant line-by-age interaction (Figure 16A; P>0.05), it did exhibit a significant main effect of line and ACRB birds had higher expression of this gene overall (Table 8; P≤0.05).

Developmental *$P_iT$-1* expression approached a significant main effect of age (Table 9; P=0.0654), and *PiT-2* exhibited a significant line-by-age effect in kidney, where Cobb had a higher expression on E16 and from E20 to D7 (Figure 16B; P≤0.05). The *$NaP_i$-IIa* and *$NaP_i$-IIb* phosphate transporters were not detected in kidney tissue from birds of either line.

Jejunum mRNA expression of *PiT-1* had a significant line-by-age effect, where expression was higher in ACRB after hatch at all timepoints except D0 and D5 (Figure 17A; P≤0.05). *$P_iT$-2* did not show a significant line-by-age effect (Figure 17B; P>0.05); however, it did exhibit main effects of both line and age. Overall mRNA expression of *Pit-2* was higher in ACRB birds (Table 8; P≤0.05). Furthermore, when the age effect was analyzed, it showed a steady increase towards D7, where it reached its highest expression, and the lowest expression was shown on E16 (Table 9; P≤0.05). *$NaP_i$-IIb* in jejunum did not exhibit a significant line-by-age effect (Figure 17C; P>0.05), though significant main effects of both line and age were observed. *$NaP_i$-IIb* mRNA expression was higher in Cobb birds (Table 8; P≤0.05), and when the age effect was evaluated, expression of *$NaP_i$-IIb* increased between E14 and D0 (Table 9; P≤0.05), and levels remained elevated without differences after hatch. *$NaP_i$-IIa* was not detected in jejunum tissue from birds of either line.

In liver, *$P_iT$-2* mRNA expression did not show significant line-by-age (Figure 18A; P>0.05) or line effects (Table 8; P>0.05). It did exhibit a main effect of age, transiently decreasing around hatch and showing its lowest levels between E20 and D3 (Table 9; P≤0.05). Likewise, *$NaP_i$-IIa mRNA* did not show significant line-by-age (Figure 18B; P>0.05) or main line effects (Table 8; P>0.05) but did exhibit an age main effect. Its expression also decreased towards hatch, where it was at its lowest expression on E20 through D7, and then increased again through D13 (Table 9; P≤0.05). Both *$P_it$-1 and $NaP_i$-IIb* mRNA expression was not detected in liver tissue.

*Tibia ash content*

In order to determine if observed differences in gene expression between the lines impacted skeletal integrity, tibial ash percentage and ash concentration were determined. There were no significant line-by-age interactions (Figure 19A; $P>0.05$) or main effects of line for bone ash (Table 10; $P>0.05$), but main effects of age were detected for this parameter. For tibial ash %, levels were dynamic and tended to be lower during embryonic development, increasing numerically and significantly to highest levels on D13 (Table 11; $P\leq0.05$). No line-by-age interaction was observed for tibial ash concentration (Figure 19B; $P>0.05$). When the line main effect was analyzed, ACRB birds had higher ash concentration (Table 10; $P\leq0.05$), and an age main effect for tibial ash concentration was also observed and reached its highest levels on D10 (Table 11; $P\leq0.05$).

**DISCUSSION**

Broiler growth has increased for over more than six decades due to genetic selection programs that have mainly focused on parameters of economic importance for producers, such as growth rate, feed efficiency, and meat yield (Leyva-Jimenez et al., 2019). However, modern commercial broilers have been shown to have a higher incidence of leg health problems, so primary breeding companies have started focusing genetic selection efforts on the improvement of traits related to bone health (Bradshaw et al., 2002; Knowles et al., 2008; Neeteson et al., 2016). Therefore, it is important to determine if the prevalence of leg disorders could be due to an impairment of the physiological regulation of Ca and P metabolism by assessing effects of intensive genetic selection on these systems. This information could improve selection efforts by identifying genetic markers related to these systems, as well as provide fundamental information leading to development of novel nutritional or management strategies that reduce the incidence of

leg disorders. Ultimately, successful efforts could both improve animal welfare as well as economic profits for producers in the broiler industry. This study determined the influence of genetic selection on genes related to Ca and P metabolism and bone mineral content in legacy and modern broilers during late embryonic and early post-hatch development.

Regulation of Ca and P metabolism is carried out primarily by biologically active vitamin $D_3$, PTH, and CALC in bone, kidney, and small intestine (Veum, 2010). Vitamin $D_3$ activation involves sequential hydroxylation reactions at the 25 and 1α positions in liver and kidney, respectively (Dusso et al., 2005), and this hydroxylation process is regulated, in part, by PTH, CALC, and CASR (Brenza et al., 1998; Zhong et al., 2009). Inactivation of vitamin $D_3$ metabolites depends on hydroxylation at the 24 position to stop its effects in target tissues (Nemere, 1999). Therefore, we investigated gene expression for enzymes carrying out these processes in kidney, jejunum, and liver of legacy ACRB and modern Cobb broilers on late embryonic and early post-hatch ages. While the first hydroxylation step that occurs in liver by CYP2R1 is responsible for most circulating 25(OH)$D_3$ (Zhu et al., 2013), this enzyme was detected in both lines in kidney and jejunum as well. Of the three tissues, a line-by-age interaction was only detected in jejunum, where levels of *CYP2R1* were higher in ACRB on two ages within the first week post-hatch. It has been suggested that even though 25(OH)$D_3$ does not directly elevate Ca uptake in intestine, its presence facilitates this function by 1,25$(OH)_2D_3$ due to local conversion (Heaney et al., 2003). The fact that expression of *CYP2R1* was increased in legacy broilers might suggest more effective local vitamin $D_3$ metabolism in this line. In liver, the primary site where CYP2R1 acts, there was a main effect of line, and this was expressed overall in Cobb at higher levels. This could reflect the presence of lower 25(OH)$D_3$ in circulation in modern birds, which was observed in our findings where legacy broilers had higher plasma concentrations of this molecule. The enhanced expression

of *CYP2R1* in liver could be in a compensatory manner to the low levels in plasma mentioned above. Furthermore, an increase in *CYP2R1* expression was observed in liver as the birds got older, which could suggest an increased ability to utilize dietary vitamin $D_3$ as birds develop. In addition, *CYP2R1*'s higher expression in kidney denoted its importance on embryonic ages in this time as well (Ameenuddin et al., 1982; Narbaitz et al., 1989). In kidney, ACRB birds presented higher expression of the catabolic enzyme 24-hydroxylase during embryonic ages, and Cobb birds had higher levels during post-hatch ages. This could suggest that increased activation of vitamin $D_3$ is occurring during crucial embryonic ages of bone formation in ACRB, leading to higher expression of 24-hydroxylase as a negative feedback process exerted by $1,25(OH)_2D_3$ (Dusso et al., 2005). Conversely, higher levels in Cobb birds after hatch on D3 and D10 might mean delayed development of this negative feedback system or increased catabolism of bioactive vitamin $D_3$ during important periods of mineralization leading to stronger bones.

Overall, there was a main effect of line for expression of *PTH1R* in kidney, and it was higher in ACRB birds, which could lead to enhanced production of 1α-hydroxylase and an improved activation of vitamin $D_3$. There was a line-by-age interaction for kidney expression of *CALCR*, and ACRB birds showed an increased expression of this gene on E16, D7, and D10. This suggests that ACRB birds might have reduced circulating CALC, necessitating increased levels of *CALCR* to maintain tissue sensitivity to this hormone.

In intestine, the common dogma is that PTH indirectly enhances Ca transport by increasing $1,25(OH)_2D_3$ production by kidney; however, it seems to induce Ca transport in the intestine as well (Nemere et al., 1986). In jejunum, *PTH1R* was highly expressed in Cobb birds on E16, but on post-hatch days it was higher in ACRB on D1 and D3. This could be more beneficial for dietary Ca transport by ACRB in this tissue. Both kidney and jejunum had no detectable levels of *PTH3R*

mRNA expression because it is believed that it might be involved in the development of non-skeletal tissues instead of classical Ca-regulating functions of PTH (Pinheiro et al., 2012). Furthermore, jejunum Ca absorption might be more efficiently regulated in ACRB birds, since mRNA levels of *CALCR* and *CASR* were higher in ACRB. This could allow improved Ca-sensing and response to dietary and circulating Ca levels.

Biologically active vitamin $D_3$ can exert its functions through VDR, which acts together with its heterodimeric partners RXRA and RXRG (Bettoun et al., 2003). Once $1,25(OH)_2D_3$ gets bound to the VDR-RXR complex, it activates specific pathways that increase Ca and P metabolism in target tissues (Brown et al., 1999). In our study, kidney *VDR* and *RXRA* showed a line-by-age effect, being higher for ACRB birds on D7 and on D7 and D10, respectively. On early post-hatch ages, starting around D7 (Sanchez-Rodriguez et al., 2019), broilers have a bone structure comprised of woven bone with labile carbonate that makes it more soluble, and the Ca:P ratio is higher than that in older birds (Rodriguez-Navarro et al., 2018). The ionic substitutions in this tissue to form a stronger structure demand more mineral availability at younger ages (Rey et al., 2009). Our results showed an increase in expression of *VDR* and *RXRA* in ACRB birds early post-hatch, potentially indicating increased kidney sensitivity to $1,25(OH)_2D_3$ on ages where these changes are starting to happen. This suggests that ACRB birds could have a better response to the increased demand of these minerals at this time.

In jejunum, *VDR* and both of its heterodimeric partners showed a significant age main effect. *VDR* expression levels seemed to get higher after hatch, perhaps in response to exogenous feed, while *RXRG* mRNA was decreased after hatch. Though *RXRA* did demonstrate a significant age effect, its levels did not change as much, and it remained relatively constant. Together, this suggests that RXRG might be an important transcriptional partner for VDR in jejunum during

embryonic development and RXRA may play a larger role in this tissue after hatch. Furthermore, that both *RXRA* and *RXRG* had higher expression in ACRB broilers in jejunum suggests that they may be more responsive to $1,25(OH)_2D_3$ signaling, since they are necessary for VDR action (Bettoun et al., 2003).

Once $1,25(OH)_2D_3$ -VDR-RXR complex reaches the VDREs, it can alter gene transcription of things like Ca and P transporters in kidney and intestine (DeLuca, 2004). In kidney, Ca reabsorption starts with luminal intake of Ca from kidney tubules by TRPV6, which will move it into the cytoplasm where CALB1-28k will transport it to the basolateral membrane where PMCA1 or NCX1 will move it to the blood stream (Lieben et al., 2011). Ca transport gene expression in kidney showed similar patterns for all the transporters except *PMCA1*, in which no interactive or main effects were detected. This system is regulated by vitamin $D_3$, PTH, and CALC; therefore, a decrease in the mRNA expression of *CYP2R1*, accompanied by the decreased expression of *PTH1R* in kidney and jejunum of Cobb birds, could have influenced the expression patterns of Ca transporters in these tissues.

In jejunum, consistent with other literature investigating expression of this transporter in chicken intestine (Proszkowiec-Weglarz et al., 2013; Wu et al., 2021), *TRPV6* was not detected. This could mean that a different transporter is moving Ca from the intestinal lumen into the cytoplasm. It seems that in this tissue, different basolateral membrane pumps might function at different ages. *NCX1* expression was higher on embryonic ages, and *PMCA1* expression was higher on post-hatch days, suggesting that these transporters play different roles during different developmental stages in chicken skeletal development.

Liver is a highly metabolic tissue, and its functions depend on a regulated intracellular and extracellular Ca balance, which can be controlled by expression of different membrane transporters

such as PMCA1 and NCX1 that remove Ca from the cytosol (Brini et al., 2011; Meldolesi et al., 1998; Zylińska et al., 2000). Higher expression levels of these transporters were observed in Cobb birds on several post-hatch ages, suggesting that fast-growing birds requires more liver Ca so that it can exert its metabolic functions since basal metabolic rate increases with age and weight (Kuenzel et al., 1977).

Ca and P have an intricate relationship to maintain a normal bone mineralization process through their balanced deposition in the bone matrix in form of hydroxyapatite, which gives the bone its strength (Berner et al., 1988). In kidney, P transport by $P_i$T-2 is regulated by the presence of P (Villa-Bellosta et al., 2009). ACRBs had a higher expression of *$P_i$T-1* mRNA in this tissue, and its expression increased with age. Conversely, *$P_i$T-2* had a higher expression in Cobb kidney between late embryonic development and the first week of hatch. Differences in expression of these P transporters between the two lines suggests that this process may have been impacted by genetic selection and that each line could use different transporters for P reabsorption in kidneys.

In jejunum, most of the P intake that occurs is mainly mediated by $NaP_i$-IIb and its functions are enhanced when dietary P is low (Xu et al., 2002). Furthermore, $P_i$T-1 and $P_i$T-2 may have some P transport and regulation functions and are also influenced by dietary P (Hu et al., 2018). In jejunum, *$NaP_i$-IIb* showed a main effect of line, being higher for Cobb birds; *$P_i$T-1* expression levels exhibited a line-by-age effect and were higher for ACRB birds on post-hatch ages. *$P_i$T-2* showed a main line effect, being higher in ACRB. Cobb birds could have an enhance P intake due to a higher expression of *$NaP_i$-IIb* in jejunum; however, a study suggested that *$NaP_i$-IIb* might not be the only P transporter in broiler intestine (Olukosi et al., 2013), and enhanced intestinal expression of *$P_i$T-1* and *$P_i$T-2* in ACRB birds might suggest that these transporters are functioning in this tissue as well.

In liver, transporters can move P from the bile for its use in the hepatocyte metabolic functions (Frei et al., 2005). No line-by-age or main line effects were detected for *$P_iT$-2* and *$NaP_i$-IIb* expression, but they did exhibit similar developmental expression patterns, where levels decreased towards hatch and steadily increased afterwards. This pattern could be due to the beginning of feed consumption. As bile secretion in duodenum becomes necessary, this could decrease P availability in the gallbladder and lead to increased expression of *$P_iT$-2* and *$NaP_i$-IIb* to compensate for these changes.

Bone mineral density is regulated by Ca and P intake in target organs (Ryan et al., 2015). A higher availability of the 25(OH)$D_3$ metabolite in ACRB birds and increased expression of *VDR*, *RXRA*, and *RXRG* in different tissues could enhance the levels and activity of some Ca and P transporters and lead to improved cytoplasmic transport and a faster passage into the cell. This could increase Ca and P availability for bone mineralization in legacy broilers, ultimately contributing to the higher ash concentration observed in these birds which could translate to improved conformation in ACRB over modern broilers.

Together, these findings indicate that several pathways related to Ca and P homeostasis may have been altered in modern broilers. Metabolism of vitamin $D_3$ could have been affected, where its activation might be impaired in kidney due to an increase in the inactivation enzyme 24-hydroxylase and lower 25 hydroxylation in Cobb birds. Furthermore, kidney of ACRB broilers seem to have a higher genomic response to 1,25$(OH)_2D_3$ due to increased expression of *VDR* and *RXR* on ages where mineral flux could be more demanding. Our results suggest that genetic selection programs focused primarily on economic traits related to production efficiency could have contributed to an alteration of mechanisms that regulate Ca and P homeostasis in modern broilers. These findings could be beneficial for primary breeders that are looking to improve their

genetic stock, as well as provide information for improving dietary or management strategies that could reduce the incidence of leg problems in commercial broilers.

**Table 1**. Primers used for reverse transcription-quantitative PCR.

| Gene symbol | Forward primer (5'-3') | Reverse primer (5'-3') | Transcript ID[1] |
|---|---|---|---|
| Enzymatic conversion of vitamin $D_3$ | | | |
| *CYP2R1* | GGACAGCAATGGACAGTTTG | AGGAAAACGCAGGTGAAATC | 09745 |
| *CYP24A1* | CCATTTGGCATTGGGAAG | CATCCGTTGCTACAAGTTGG | 75763 |
| Hormonal regulation of Ca and P metabolism | | | |
| *VDR* | GGGCTTAAAGAAGCTGAACC | CGGATGTAGGTCTGGAGGAT | 71682 |
| *RXRA* | ACTGCCGCTACCAGAAGTGT | GACTCCACCTCGTTCTCGTT | 59924 |
| *RXRG* | GAAGCCTACACGAAGCAGAA | CCGATCAGCTTGAAGAAGAA | 49224 |
| *PTH1R* | CCAAGCTACGGGAAACAAAT | ATGGCATAGCCATGAAAACA | 08796 |
| *PTH3R* | ATGCCCTACACCGAAGTCTC | CCCATTGCAAAAGCAGTAGA | 78021 |
| *CALCR* | GCAGTTGCAAGAGCCAAATA | AGCTTTGTCACCAACACTCG | 15478 |
| Ca transport | | | |
| *TRPV6* | TATGCTGGAACGAAAACTGC | TTGTGCTTGTTGGGATCAAT | 23779 |
| *CALB1-28* | AAGCAGATTGAAGACTCAAAGC | CTGGCCAGTTCAGTAAGCTC | 74265 |
| *NCX1* | TCACTGCAGTCGTGTTTGTG | AAGAAAACGTTCACGGCATT | 13920 |
| *PMCA1* | TTAATGCCCGGAAAATTCAC | TCCACCAAACTGCACGATAA | 80355 |
| *CASR* | CTGCTTCGAGTGTGTGGACT | GATGCAGGATGTGTGGTTCT | 55986 |
| P transport | | | |
| *$P_i$t-1* | TATCCTCCTCATTTCGGCGG | CTCTTCTCCATCAGCGGACT | 22329 |
| *$P_i$t-2* | CCATCCCCGTGTACCTTATG | AGACATGGCCATCACTCCTC | 51992 |
| *Na$P_i$-IIb* | GTGACCGCAGTGAGTTCAAA | CAGGTACCCACTCACCACCT | 58978 |
| *Na$P_i$-IIb* | AAAGTGACGTGGACCATG | GAGACCGATGGCAAGATCAG | 23222 |
| Housekeeping genes | | | |
| *GAPDH* | AGCCATTCCTCCACCTTTGAT | AGTCCACAACACGGTTGCTGTAT | 23323 |
| *18 S*[2] | AGCCTGCGGCTTAATTTGAC | CAACTAAGAACGGCCATGCA | |

[1] Transcript identification from Ensembl chicken genome assembly GRCg6a (*https://www.ensembl.org/Gallus_gallus/Info/Index*) preceded by ENSALGT0000000.

[2] This sequence is not on the assembled chicken genome and primers were designed using the sequence in GenBank (accession number AF173612).

**Table 2.** Means[1] (±SEM) and ANOVA P-values of the line main effect for genes related to enzymatic conversion of vitamin $D_3$ and vitamin $D_3$ metabolites in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | | ACRB | Cobb | P-value |
|---|---|---|---|---|
| Kidney (%)[2] | | | | |
| | *CYP2R1* | 94.3±7.3 | 100±8.2 | 0.5640 |
| | *CYP24A1* | 75.7±12.0[b] | 100±9.5[a] | 0.0293 |
| Jejunum (%)[2] | | | | |
| | *CYP24A1* | 100±8.0 | 78.7±7.1 | 0.0714 |
| Liver (%)[2] | | | | |
| | *CYP2R1* | 75.5±4.5[b] | 100±7.8[a] | <0.0001 |
| Plasma[3] | | | | |
| 25(OH)$D_3$ (ng/mL) | | 16.6±1.83 | 11.6±1.89 | 0.0770 |
| 1,25$(OH)_2D_3$ (pg/mL) | | 76.4±12.8 | 49.9±8.7 | 0.1204 |
| 24,25$(OH)_2D_3$ (ng/mL) | | 0.8±0.15[b] | 1.7±0.27[a] | 0.0367 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 14 and post-hatch day 13 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (equal to 100%).
[3]Circulating vitamin $D_3$ metabolites are expressed as an absolute concentration.
[a,b] Values within each parameter that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 3.** Means[1] (±SEM) and ANOVA P-values of the age main effect for genes related to enzymatic conversion of vitamin $D_3$ and vitamin $D_3$ metabolites in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney (%)[2] | | | | | | | | | | | | |
| *CYP2R1* | 99.8±16.6$^{a}$ | 100±10.4$^{a}$ | 58.3±4.2$^{ab}$ | 58.9±9.8$^{ab}$ | 40.1±5.5$^{b}$ | 40.4±20.1$^{b}$ | 56.0±5.0$^{ab}$ | 70.2±12.4$^{ab}$ | 40.56±5.78$^{b}$ | 40.6±7.0$^{b}$ | 58.3±11.5$^{ab}$ | <.0001 |
| *CYP24A1* | 78.1±14.2$^{a}$ | 100±10.2$^{a}$ | 55.1±15.3$^{ab}$ | 41.7±11.9$^{ab}$ | 30.8±8.1$^{b}$ | 40.4±20.1$^{b}$ | 45.8±7.55$^{ab}$ | 55.9±13.2$^{ab}$ | 48.2±12.6$^{b}$ | 41.4±9.1$^{b}$ | 95.7±23.6$^{ab}$ | 0.0055 |
| Jejunum (%)[2] | | | | | | | | | | | | |
| *CYP24A1* | 48.8±5.2$^{a}$ | 100±54.5$^{a}$ | 40.3±3.6$^{ab}$ | 27.3±3.8$^{abc}$ | 13.2±1.1$^{cd}$ | 16.9±4.9$^{bcd}$ | 16.2±6.2$^{cd}$ | 8.2±0.804$^{d}$ | 12.4±3.0$^{cd}$ | 11.5±6.3$^{d}$ | 9.3±2.2$^{d}$ | <.0001 |
| Liver (%)[2] | | | | | | | | | | | | |
| *CYP2R1* | 59.3±5.1$^{abc}$ | 42.2±4.9$^{cd}$ | 45.7±4.9$^{c}$ | 26.8±3.2$^{d}$ | 59.4±11.1$^{bc}$ | 77.6±8.6$^{ab}$ | 88.1±7.4$^{a}$ | 100±13.6$^{a}$ | 90.0±5.8$^{a}$ | 80.7±6.1$^{ab}$ | 88.8±9.9$^{a}$ | <.0001 |
| Plasma[3] | | | | | | | | | | | | |
| $25(OH)D_3$ | | | | | | | 15.1±0.6 | | 11.4±2.3 | 15.7±3.6 | | 0.3613 |
| $1,25(OH)_2D_3$ | | | | | | | 56.9±16.2 | | 59.1±6.5 | 78.5±17.4 | | 0.4103 |
| $24,25(OH)_2D_3$ | | | | | | | 1.2±0.1 | | 1.3±0.4 | 1.3±0.4 | | 0.9487 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic (E) and post-hatch (D) day.

[2]Data within each gene are expressed relative to the age with the highest mRNA level (equal to 100%).

[3]Circulating vitamin $D_3$ metabolites are expressed as an absolute concentration. This analysis was only done for post-hatch days D3, D7 and D10.

[a-d] Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 4.** Means[1] (±SEM) and ANOVA P-values of the line main effect for genes related to hormonal regulation of Ca and P metabolism in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | ACRB | Cobb | P-value |
|---|---|---|---|
| Kidney (%)[2] | | | |
| *VDR* | 100±6.7[a] | 87.7±9.3[b] | 0.0437 |
| *PTH1R* | 100±8.9[a] | 83.3±9.7[b] | 0.0105 |
| Jejunum (%)[2] | | | |
| *VDR* | 96.1±5.4 | 100±5.9 | 0.4935 |
| *RXRA* | 100±9.0[a] | 85.7±6.2[b] | 0.0138 |
| *RXRG* | 100±4.7[a] | 74.9±9.7[b] | 0.0312 |
| *CALCR* | 100±28.0 | 80.7±23.1 | 0.0528 |
| Liver (%)[2] | | | |
| *VDR* | 77.1±8.0[a] | 100±8.6[b] | <0.0001 |
| *RXRG* | 94.4±9.9 | 100±9.6 | 0.3599 |
| *PTH1R* | 99.9±9.4 | 100±11.0 | 0.9079 |
| *PTH3R* | 96.3±9.1[a] | 100±8.8[b] | 0.4107 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 14 and post-hatch day 13 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (equal to 100%).
[a,b] Values within each gene that do not share a common letter are significantly different (P≤0.05).

**Table 5.** Means[1] (±SEM) and ANOVA P-values of the age main effect for genes related to different hormonal regulation of Ca and P metabolism in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney (%)[2] | | | | | | | | | | | | |
| *VDR* | 85.3±3.7$^{a}$ | 90.9±11.2$^{a}$ | 100±6.9$^{a}$ | 82.7±6.5$^{a}$ | 83.5±5.5$^{a}$ | 62.7±3.6$^{ab}$ | 76.1±6.9$^{a}$ | 59.8±10.2$^{ab}$ | 52.1±10.5$^{b}$ | 43.1±8.3$^{b}$ | 71.3±14.1$^{ab}$ | <.0001 |
| *PTH1R* | 100±12.2$^{a}$ | 87.8±12.4$^{ab}$ | 51.6±6.4$^{ab}$ | 58.6±7.8$^{ab}$ | 66.6±6.0$^{ab}$ | 70.8±8.5$^{ab}$ | 93.4±7.3$^{a}$ | 71.6±11.7$^{ab}$ | 58.9±14.1$^{b}$ | 55.0±8.9$^{ab}$ | 68.8±10.8$^{ab}$ | 0.0021 |
| Jejunum (%)[2] | | | | | | | | | | | | |
| *VDR* | 6.1±0.9$^{g}$ | 10.3±1.6$^{f}$ | 22.5±2.3$^{e}$ | 35.4±3.2$^{d}$ | 80.6±9.5$^{abc}$ | 80.9±10.7$^{abc}$ | 56.2±4.9$^{c}$ | 63.8±6.3$^{bc}$ | 93.9±11.9$^{ab}$ | 95.8±7.2$^{ab}$ | 100±6.3$^{a}$ | <.0001 |
| *RXRA* | 75.3±4.6$^{abc}$ | 71.9±9.5$^{bc}$ | 82.5±9.8$^{abc}$ | 90.0±6.2$^{ab}$ | 100±11.6$^{a}$ | 80.5±8.3$^{abc}$ | 64.6±7.7$^{c}$ | 69.9±10.0$^{c}$ | 94.9±12.4$^{a}$ | 91.1±8.8$^{ab}$ | 90.2± 7.8$^{ab}$ | 0.0364 |
| *RXRG* | 100±26.1$^{a}$ | 65.8±25.1$^{ab}$ | 60.9±16.2$^{ab}$ | 79.6±27.9$^{ab}$ | 27.6±4.1$^{bc}$ | 23.2±3.7$^{bcd}$ | 17.4±6.5$^{cde}$ | 8.4±2.3$^{e}$ | 6.6±1.7$^{e}$ | 7.1±1.8$^{e}$ | 10.6±3.7$^{de}$ | <.0001 |
| *CALCR* | 100±32.7$^{a}$ | 61.2±18.8$^{a}$ | 51.4±13.2$^{a}$ | 53.1±11.2$^{a}$ | 15.8±4.4$^{b}$ | 6.7±1.9$^{c}$ | 2.7±0.9$^{d}$ | 2.1±1.1$^{de}$ | 1.3±0.3$^{de}$ | 1.7±0.5$^{de}$ | 2.00±0.9$^{e}$ | <.0001 |
| Liver (%)[2] | | | | | | | | | | | | |
| *VDR* | 100±12.3$^{a}$ | 76.7±4.4$^{a}$ | 65.3±7.1$^{ab}$ | 46.2±6.7$^{bc}$ | 37.1±5.7$^{c}$ | 40.3±3.3$^{c}$ | 37.8±2.5$^{c}$ | 36.1±4.8$^{c}$ | 37.8±3.8$^{c}$ | 40.1±8.0$^{c}$ | 42.2± 6.7$^{c}$ | <.0001 |
| *RXRG* | 100±9.1$^{a}$ | 63.6±8.6$^{ab}$ | 58.6±9.8$^{b}$ | 34.7±10.7$^{c}$ | 55.1±4.8$^{b}$ | 71.9±5.6$^{ab}$ | 53.1±2.7$^{b}$ | 51.2±6.1$^{b}$ | 44.6±3.9$^{bc}$ | 45.8±3.6$^{b}$ | 53.1±11.1$^{b}$ | <.0001 |
| *PTH1R* | 56.1±14.5$^{a}$ | 100±43.9$^{abc}$ | 63.4±18.1$^{ab}$ | 54.8±18.9$^{bcd}$ | 16.3±4.0$^{d}$ | 12.1±2.5$^{bcd}$ | 11.7±4.0$^{abcd}$ | 7.3±2.0$^{cd}$ | 8.7±1.8$^{bcd}$ | 8.6±2.1$^{d}$ | 6.6±1.4$^{bcd}$ | <.0001 |
| *PTH3R* | 66.6±5.7$^{abcd}$ | 61.4±6.7$^{cd}$ | 63.6±9.1$^{cd}$ | 61.4±6.8$^{cd}$ | 50.0±5.7$^{d}$ | 51.5±2.9$^{d}$ | 67.3±4.3$^{abcd}$ | 66.8±8.6$^{bcd}$ | 79.0±5.4$^{abc}$ | 96.7±12.8$^{a}$ | 100±17.7$^{ab}$ | <.0001 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic (E) and post-hatch (D) day.

[2]Data within each gene are expressed relative to the age with the highest mRNA level (equal to 100%).

$^{a-e}$ Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 6.** Means[1] (±SEM) and ANOVA P-values of the line main effect for genes involved in Ca transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | ACRB | Cobb | P-value |
|---|---|---|---|
| Kidney (%)[2] | | | |
| *TRPV6* | 100±3.8 | 98.2±12.9 | 0.5111 |
| *NCX1* | 100±8.8 | 97.8±9.5 | 0.2754 |
| *PMCA1* | 100±14.7 | 97.5±14.7 | 0.6441 |
| Jejunum (%)[2] | | | |
| *CALB1-28k* | 66.5±8.5 | 100±17.2 | 0.0702 |
| *NCX1* | 100±5.7 | 86.7±10.8 | 0.1958 |
| *PMCA1* | 93.2±8.9 | 100±6.53 | 0.0710 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 14 and post-hatch day 13 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (equal to 100%).

**Table 7.** Means[1] (±SEM) and ANOVA P-values of the age main effect for genes involved in Ca transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney (%)[2] | | | | | | | | | | | | |
| *TRPV6* | 100±10.2$^{a}$ | 67.6±15.4$^{abc}$ | 31.6±5.5$^{cd}$ | 14.8±1.1$^{d}$ | 36.4±4.8$^{bc}$ | 38.0±2.2$^{bc}$ | 65.7±8.2$^{ab}$ | 64.3±9.7$^{abc}$ | 61.2±7.0$^{abc}$ | 54.7±6.1$^{abc}$ | 75.2±12.3$^{ab}$ | <.0001 |
| *NCX1* | 100±16.0$^{a}$ | 51.1±10.2$^{ab}$ | 35.9±6.9$^{b}$ | 19.5±2.6$^{c}$ | 52.3±5.2$^{ab}$ | 58.1±8.3$^{ab}$ | 83.5±12.0$^{a}$ | 58.6±13.8$^{ab}$ | 62.9±9.1$^{ab}$ | 48.2± 5.6$^{ab}$ | 67.2±20.3$^{ab}$ | <.0001 |
| *PMCA1* | 100±18.0$^{a}$ | 89.4±16.0$^{a}$ | 93.1±13.6$^{a}$ | 85.6±15.3$^{a}$ | 82.7±14.0$^{a}$ | 75.3±12.3$^{a}$ | 88.2±14.4$^{a}$ | 84.0±17.3$^{a}$ | 80.5±14.0$^{a}$ | 70.8±7.7$^{a}$ | 76.2±15.2$^{a}$ | 0.3071 |
| Jejunum (%)[2] | | | | | | | | | | | | |
| *CALB1-28k* | 0.1±0.02$^{c}$ | 0.2±0.1$^{c}$ | 0.02±0.01$^{d}$ | 0.2±0.06$^{c}$ | 15.4±2.1$^{b}$ | 30.6±5.04$^{ab}$ | 36.5±4.35$^{ab}$ | 83.1±11.8$^{a}$ | 65.9±10.7$^{ab}$ | 100±12.5$^{a}$ | 96.1±32.8$^{ab}$ | <.0001 |
| *NCX1* | 77.8±8.0$^{ab}$ | 85.8±8.1$^{a}$ | 100±8.0$^{a}$ | 86.2±3.4$^{ab}$ | 49.7±3.2$^{abc}$ | 37.7±3.7$^{abc}$ | 33.4±9.5$^{bc}$ | 24.6±2.5$^{c}$ | 23.7±4.3$^{c}$ | 20.4±3.5$^{c}$ | 24.0 ± 8.6$^{c}$ | <.0001 |
| *PMCA1* | 9.3±0.8$^{f}$ | 11.2±0.8$^{f}$ | 17.1±2.2$^{e}$ | 28.3±2.1$^{d}$ | 70.3±4.1$^{ab}$ | 64.5±8.5$^{bc}$ | 50.0±6.5$^{c}$ | 71.4±11.4$^{bc}$ | 81.8±8.0$^{ab}$ | 86.7±9.7$^{ab}$ | 100±10.2$^{a}$ | <.0001 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic (E) and post-hatch (D) day.

[2]Data within each gene are expressed relative to the age with the highest mRNA level (equal to 100%).

$^{a-d}$ Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 8.** Means[1] (±SEM) and ANOVA P-values of the line main effect for genes involved in P transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | ACRB | Cobb | P-value |
|---|---|---|---|
| Kidney (%)[2] | | | |
| *$P_iT$-1* | 100±12.1[a] | 83.6±18.1[b] | 0.0101 |
| Jejunum (%)[2] | | | |
| *PiT-2* | 100±16.4[a] | 66.5±8.3[b] | 0.0097 |
| *$NaP_i$-IIb* | 63.8±9.6[a] | 100±12.1[b] | 0.0132 |
| Liver (%)[2] | | | |
| *$P_iT$-2* | 100±5.7 | 96.7±4.5 | 0.5804 |
| *$NaP_i$-IIb* | 100±4.8 | 96.7±5.4 | 0.5305 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 14 and post-hatch day 13 for each line.
[2]Data within each gene are expressed relative to the line with the highest mRNA level (equal to 100%).
[a,b] Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 9.** Means[1] (±SEM) and ANOVA P-values of the age main effect for genes involved in P transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney (%)[2] | | | | | | | | | | | | |
| *$P_iT$-I* | 36.4±5.9 | 24.5±3.6 | 18.1±4.4 | 61.2±14.4 | 47.9± 6.9 | 44.2±9.4 | 49.0±9.4 | 46.1±11.3 | 55.3±9.1 | 62.6±30.0 | 100±56.6 | 0.0654 |
| Jejunum (%)[2] | | | | | | | | | | | | |
| *$P_iT$-2* | 65.3±8.1$^{e}$ | 64.2±7.0$^{e}$ | 76.3±15.2$^{cde}$ | 80.4±15.0$^{abcde}$ | 78.1±15.1$^{abcde}$ | 72.7±7.7$^{bcde}$ | 78.7±6.7$^{e}$ | 98.6±6.8$^{de}$ | 100±8.2$^{abc}$ | 90.3±6.4$^{ab}$ | 86.8±12.6$^{abcd}$ | <.0001 |
| *$NaP_i$-IIb* | 7.2±1.3$^{d}$ | 9.2±1.9$^{cd}$ | 18.7±4.5$^{bcd}$ | 36.3±8.4$^{abc}$ | 100±25.6$^{a}$ | 48.2±13.7$^{ab}$ | 43.6±7.6$^{ab}$ | 64.7±13.4$^{a}$ | 81.1±22.2$^{a}$ | 54.6±13.7$^{ab}$ | 54.0±6.8$^{ab}$ | <.0001 |
| Liver (%)[2] | | | | | | | | | | | | |
| *$P_iT$-2* | 100±8.7$^{a}$ | 89.0±4.8$^{ab}$ | 74.2±6.3$^{abc}$ | 61.0±8.6$^{bcd}$ | 48.8 ±7.9$^{d}$ | 44.0±5.2$^{d}$ | 51.6±5.9$^{cd}$ | 60.6±4.5$^{bcd}$ | 65.5±5.5$^{abcd}$ | 77.0±8.6$^{abc}$ | 82.8±6.4$^{ab}$ | <.0001 |
| *$NaP_i$-IIa* | 100±4.8$^{a}$ | 80.3±6.8$^{ab}$ | 67.0±7.2$^{abc}$ | 55.1±6.4$^{bcd}$ | 45.0±7.1$^{d}$ | 39.4±3.8$^{d}$ | 49.6±7.3$^{cd}$ | 55.1±3.8$^{bcd}$ | 58.7±4.0$^{bcd}$ | 68.9±8.2$^{abc}$ | 70.2±6.7$^{abc}$ | <.0001 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic (E) and post-hatch (D) day.

[2]Data within each gene are expressed relative to the age with the highest mRNA level (equal to 100%).

$^{a-d}$ Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 10.** Means[1] (±SEM) and ANOVA P-values of the line main effect for bone ash (%) and ash concentration ($g/cm^3$) in tibia of male ACRB and Cobb broilers during late embryonic and early post-hatch development in tibia bone.

| | ACRB | Cobb | P-value |
|---|---|---|---|
| Bone ash[2] (%) | 32±1.23 | 31±0.57 | 0.2542 |
| Ash concentration[3] ($g/cm^3$) | 0.11±0.05[a] | 0.09±0.004[b] | 0.0020 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated between embryonic day 14 and post-hatch day 13 for each line.
[2]Data for bone ash was calculated as ash weight divided by fat-free dry weight multiplied by 100.
[3]Data for ash concentration was calculated by dividing ash weight by volume of the bone.
[a,b] Values that do not share a common letter are significantly different ($P \leq 0.05$).

**Table 11.** Means[1] (±SEM) and ANOVA P-values of the age main effect for bone ash and ash concentration in tibia of male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bone ash[2] (%) | 30±3.6$^{d}$ | 27±0.90$^{cd}$ | 33±1.5$^{abc}$ | 32±1.5$^{abcd}$ | 28±0.60$^{bcd}$ | 28±0.70$^{bcd}$ | 31±0.40$^{abcd}$ | 34±0.30$^{abc}$ | 35±0.50$^{ab}$ | 36±0.50$^{ab}$ | 38±0.50$^{a}$ | <.0001 |
| Ash concentration[3] (g/cm$^{3}$) | 0.09±0.01$^{b}$ | 0.07±0.01$^{b}$ | 0.10±0.01$^{ab}$ | 0.09± 0.01$^{b}$ | 0.08±0.01$^{b}$ | 0.09± 0.01$^{b}$ | 0.10 ±0.01$^{ab}$ | 0.11±0.01$^{ab}$ | 0.12±0.01$^{ab}$ | 0.14±0.02$^{a}$ | 0.14±0.01$^{a}$ | <.0001 |

[1]Means are only presented for data where a significant line-by-age interaction was not present and were calculated across both lines at each embryonic (E) and post-hatch (D) day.
[2]Data for bone ash was calculated as ash weight divided by fat-free dry weight multiplied by 100.
[3]Data for ash concentration was calculated by dividing the ash weight by the volume of the bone.
$^{a-d}$ Values that do not share a common letter are significantly different ($P \leq 0.05$).

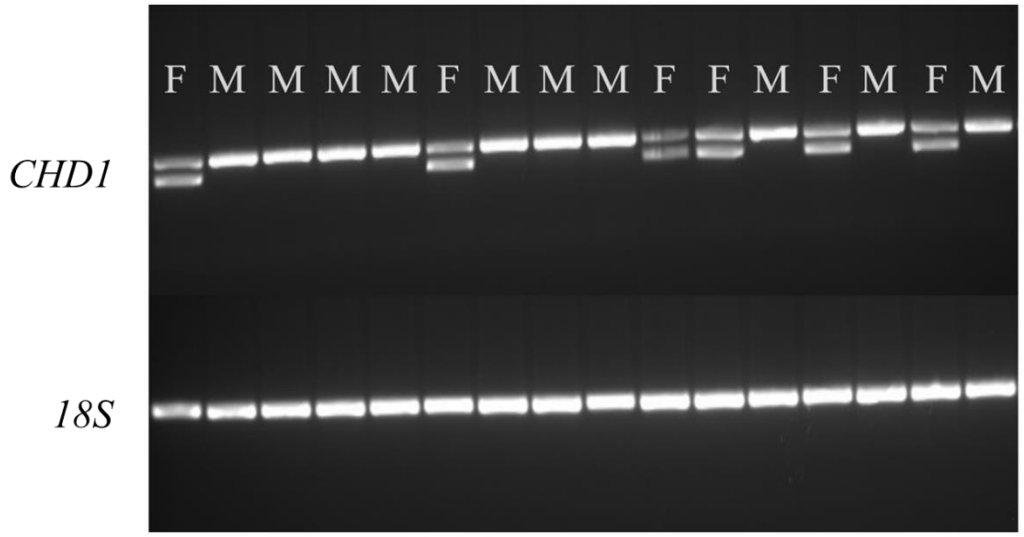


**Figure 1.** Agarose gel electrophoresis of representative PCR products resulting from molecular sexing of embryonic day 14 broilers. The upper bands are the chromodomain helicase DNA-binding protein 1 gene (*CDH1*), and the lower bands are the 18S rRNA gene. Products with two bands for *CHD1* are female samples and those with one band are male samples.

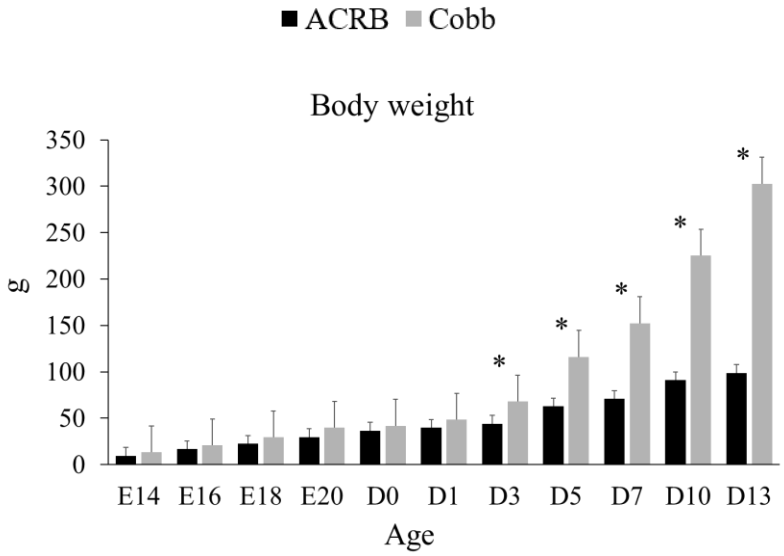


**Figure 2**. Body weight (g) of ACRB and Cobb male broilers on embryonic day (E) 14, E16, E18 and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 (n=6). A significant line-by-age interaction was detected (P=0.0001) and the presence of an asterisk (∗) indicates a significant difference in body weight between lines at indicated ages (P≤0.05).

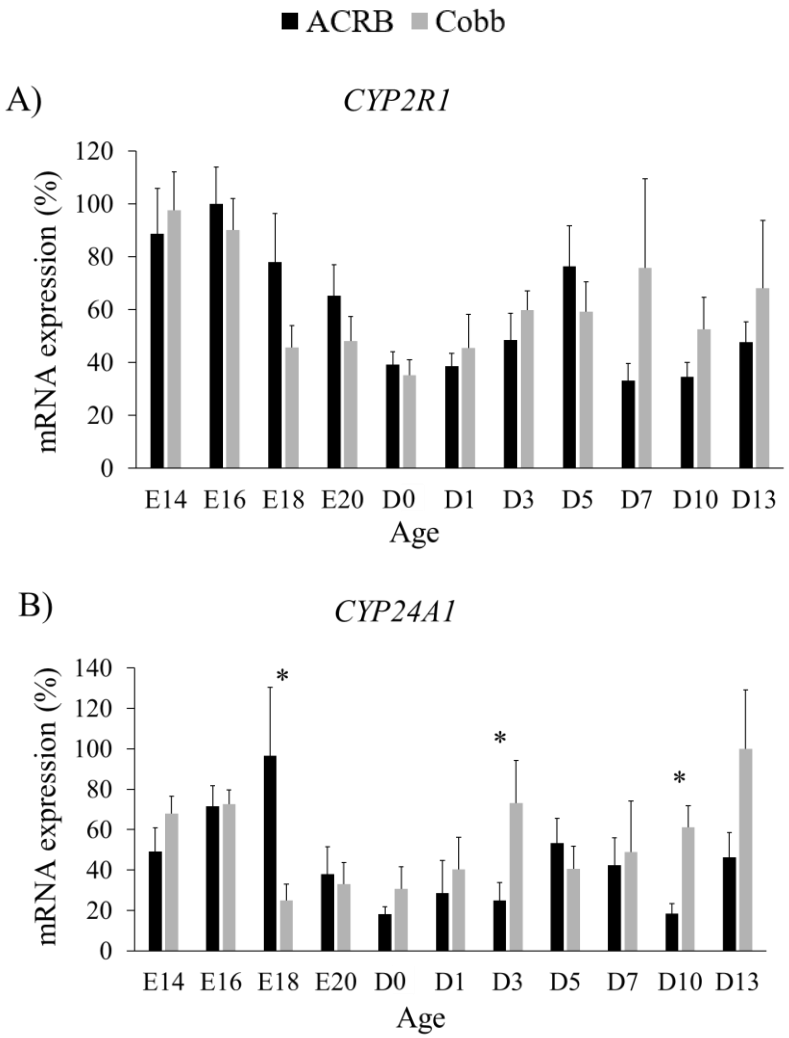


**Figure 3.** Relative mRNA expression of enzymatic regulators of vitamin $D_3$ metabolism in kidney of ACRB and Cobb male broilers. (A) *CYP2R1* and (B*) CYP24A1* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=8). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), no significant line-by-age effects (P=0.1448) were detected for *CYP2R1*. In (B), the line-by-age interaction approached significance (P=0.0705), and an asterisk (*) indicates significant differences between the lines at that age (P≤0.05). For both genes, main effect means for line and age are presented in Tables 2 and 3, respectively.

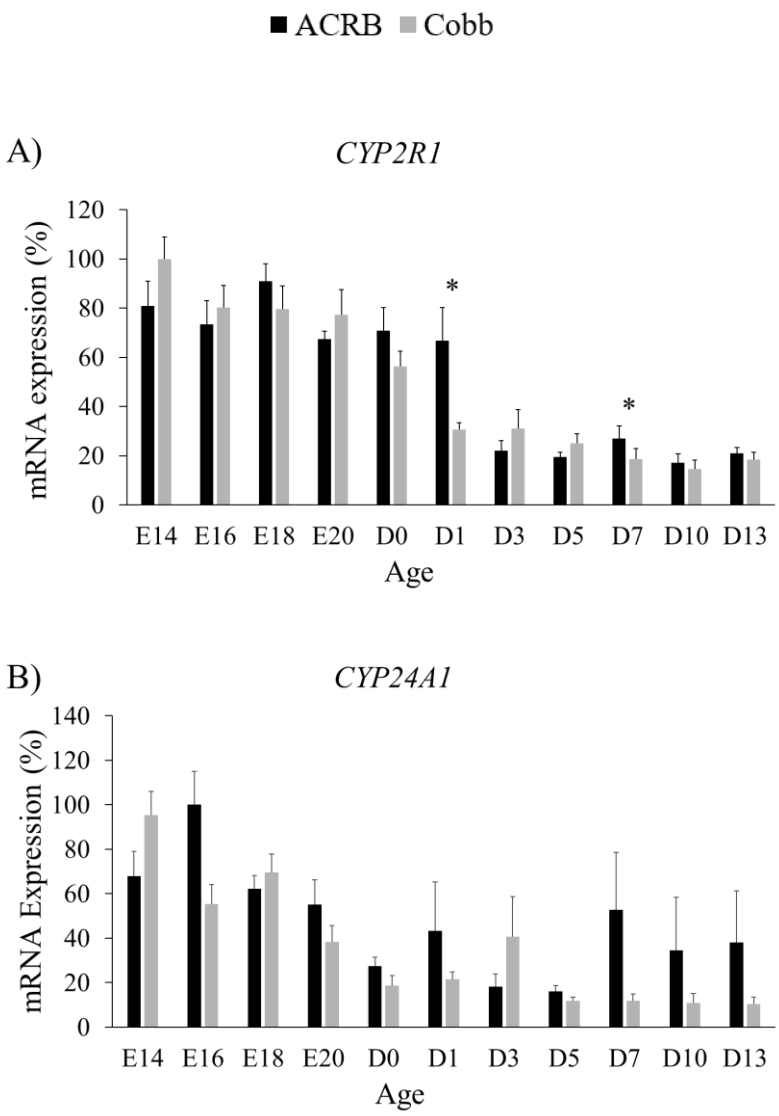


**Figure 4.** Relative mRNA expression of enzymatic regulators of vitamin $D_3$ metabolism in jejunum of ACRB and Cobb male broilers. (A) *CYP2R1* and (B) *CYP24A1* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *GAPDH* mRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), a significant line-by-age effect was detected for *CYP2R1* (P=0.0017), and an asterisk (*) indicates significant differences between the lines at that age (P≤0.05). In (B) a significant line-by-age effect was not detected for *CYP24A1* (P=0.3072). For both genes, main effect means for line and age are presented in Tables 2 and 3, respectively.

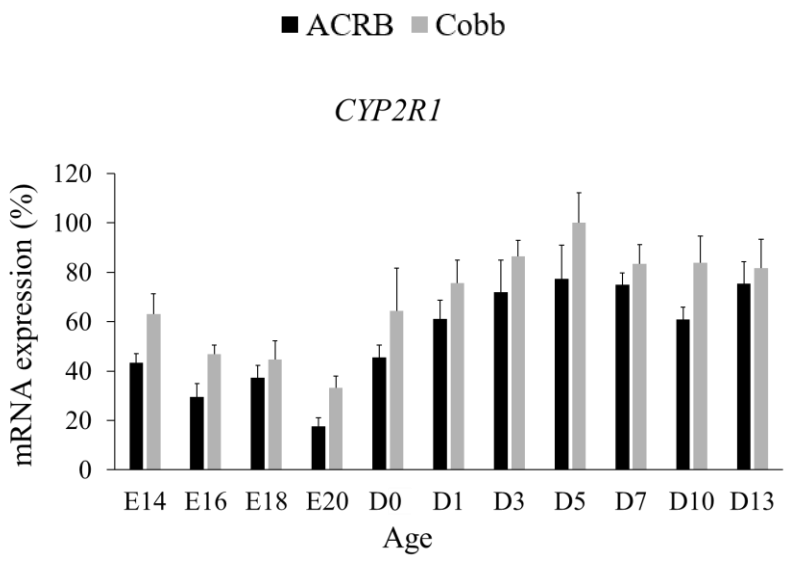


**Figure 5.** Relative mRNA expression of enzymatic regulators of vitamin $D_3$ metabolism in liver of ACRB and Cobb male broilers. *CYP2R1* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR and normalized to *18S* rRNA (n=6). The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). A significant line-by-age effect was not detected (P=0.5476). For both genes, main effect means for line and age are presented in Tables 2 and 3, respectively. *CYP24A1* mRNA was not detected in liver.

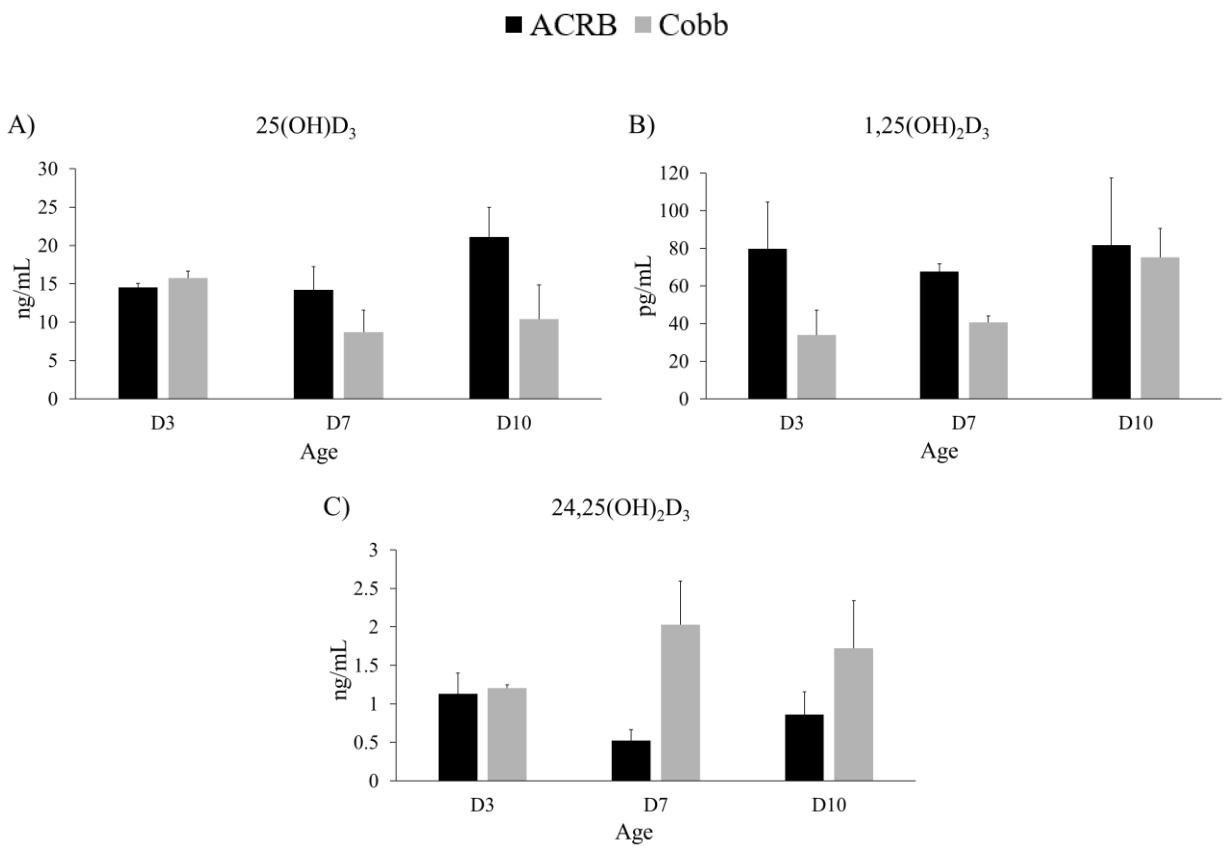


**Figure 6.** Plasma concentration of vitamin $D_3$ metabolites in ACRB and Cobb male broilers. (A) $25(OH)D_3$, (B) $1,25(OH)_2D_3$, and (C) $24,25(OH)_2D_3$ concentrations were measured on post-hatch day (D) 3, D7, and D10 using liquid chromatography-tandem mass spectrometry (n=3). No significant line-by-age effects were detected for any of the metabolites [$25(OH)D_3$, P=0.2043; $1,25(OH)_2D_3$, P=0.6062; $24,25(OH)_2D_3$, P=0.2675], and main effect means for line and age are shown in Tables 2 and 3, respectively.

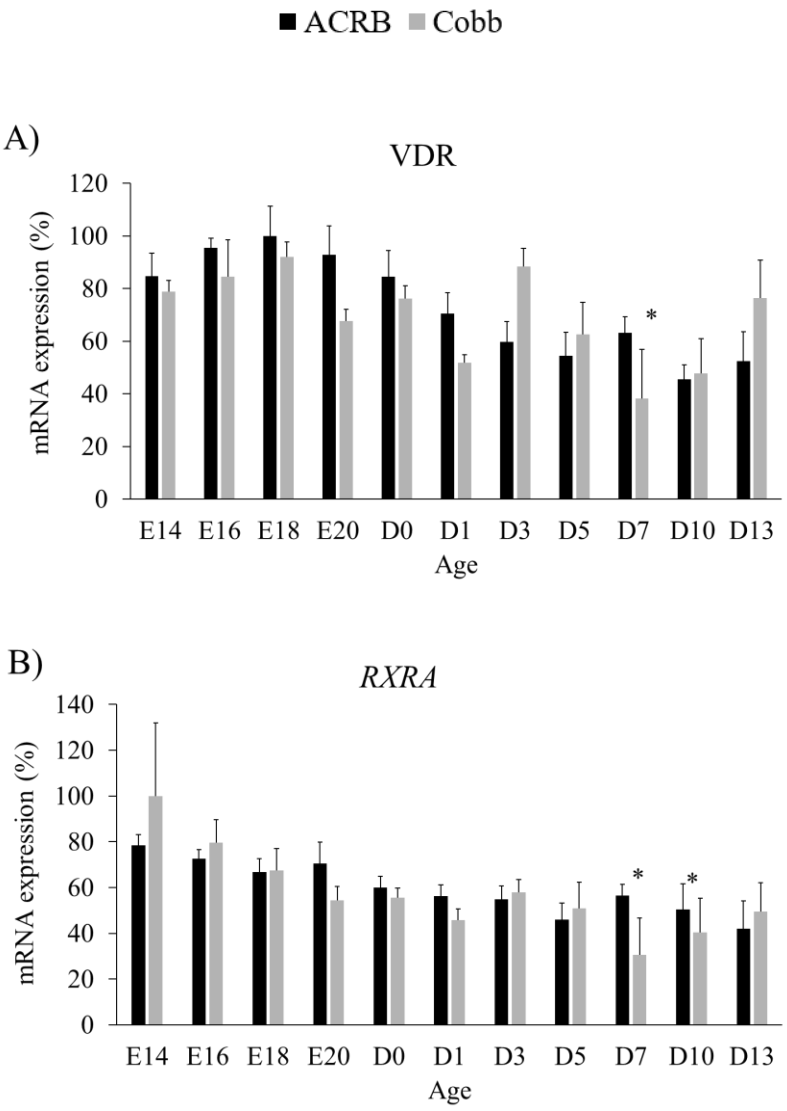


**Figure 7.** Relative mRNA expression of transcriptional regulators of vitamin $D_3$ action in kidney of ACRB and Cobb male broilers. (A) *VDR* and (B) *RXRA* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=8). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), the line-by-age effect approached significance for *VDR* (P=0.0705), and an asterisk (*) indicates significant differences between the lines at that age (P≤0.05). Main effect means for line and age are shown in Tables 4 and 5, respectively. In (B), a significant line-by-age interaction was observed for *RXRA* (P=0.0196), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). *RXRG* mRNA was not detected in kidney.

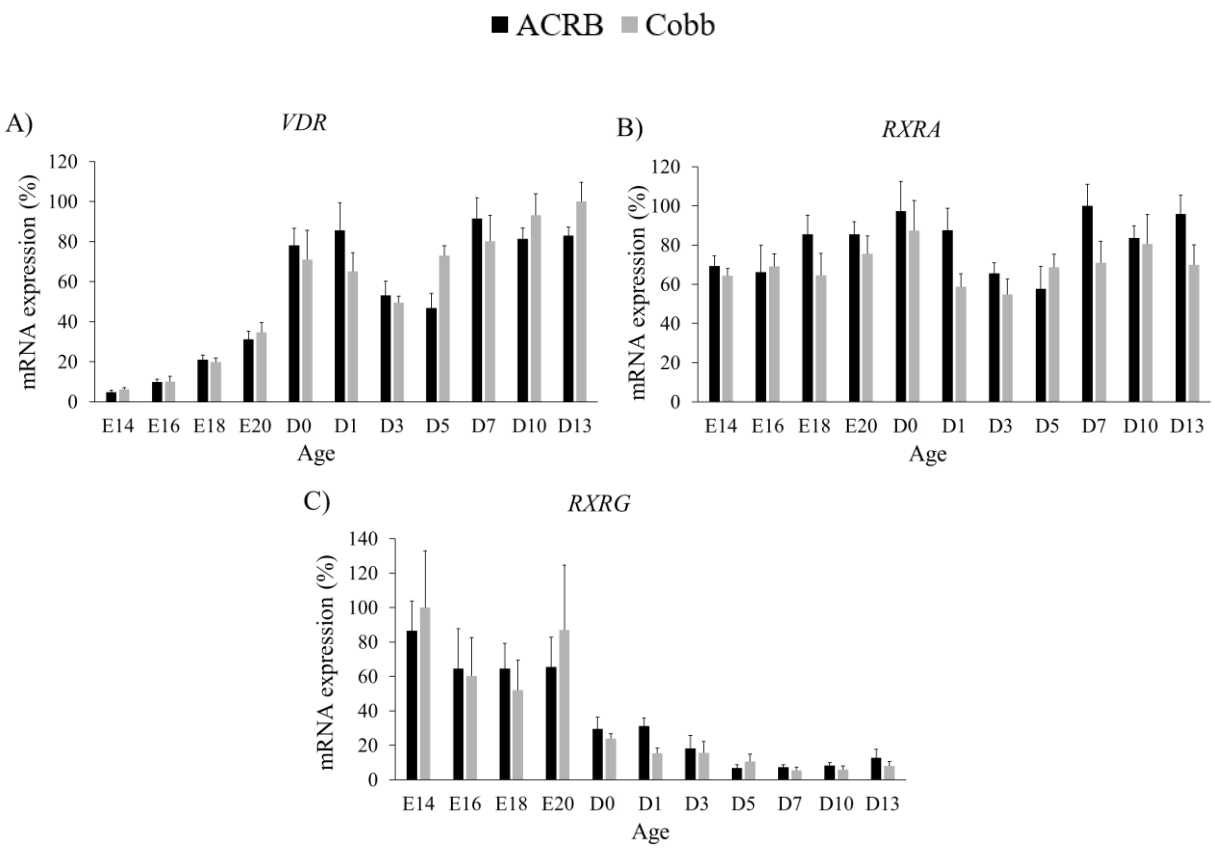


**Figure 8.** Relative mRNA expression of transcriptional regulators of vitamin $D_3$ action in jejunum of ACRB and Cobb male broilers. (A) *VDR*, (B) *RXRA*, and (C) *RXRG* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *GAPDH* mRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age effects were detected for any of the genes (*VDR*, P=0.2481; *RXRA*, P=0.2083; *RXRG*, P=0.9359), and main effect means for line and age are presented in Tables 4 and 5, respectively.

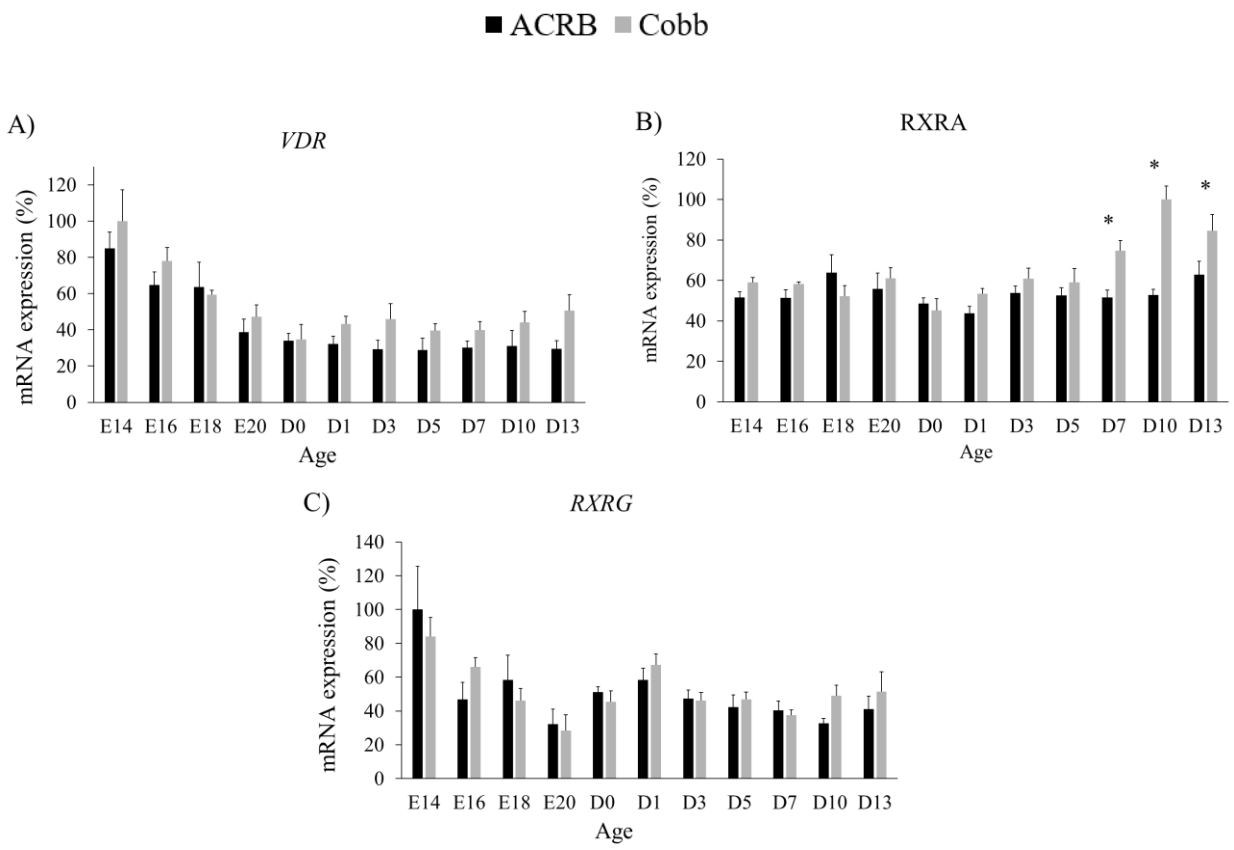


**Figure 9.** Relative mRNA expression of transcriptional regulators of vitamin $D_3$ action in liver of ACRB and Cobb male broilers. (A) *VDR*, (B) *RXRA*, and (C) *RXRG* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A) and (C), no significant line-by-age effects were detected for *VDR* (P=0.4590) or *RXRG* (P=0.4223), and main effect means of line and age are presented in Tables 4 and 5, respectively. In (B), a significant line-by-age interaction was detected for *RXRA* (P=0.0001), and an asterisk (*) indicates significant differences between lines at those ages (P≤0.05).

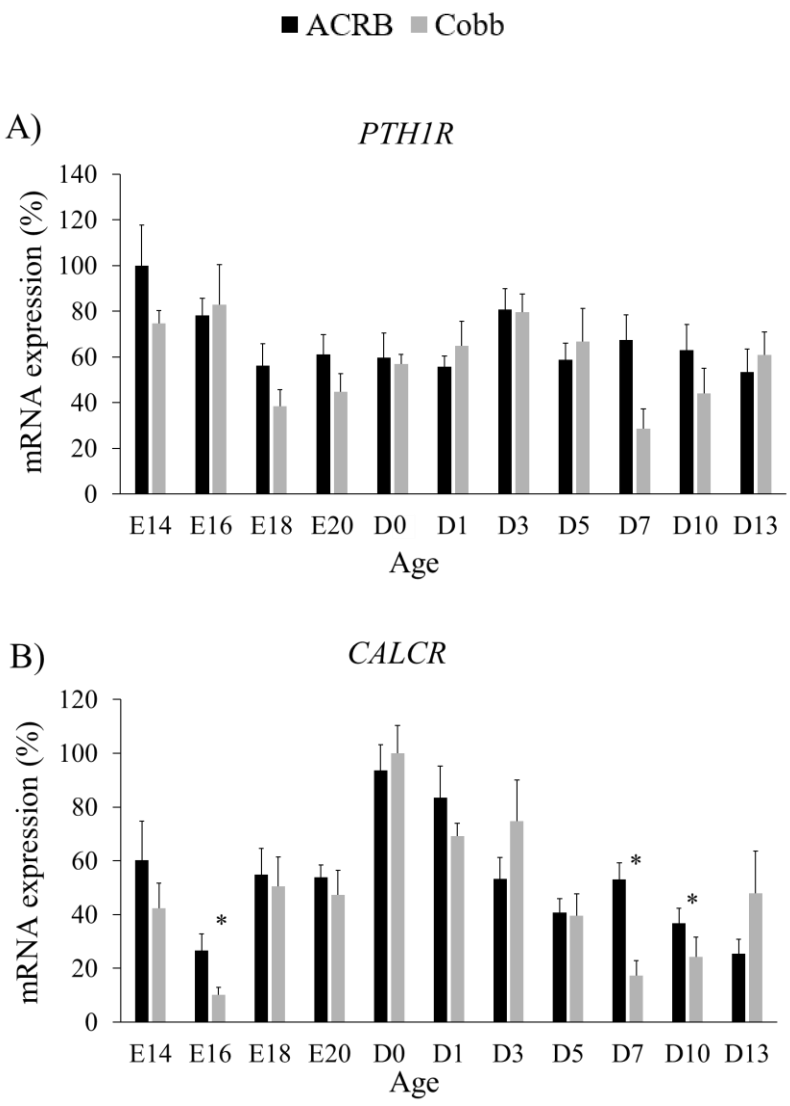


**Figure 10.** Relative mRNA expression of hormonal regulation of Ca and P in kidney of ACRB and Cobb male broilers. (A) *PTH1R* and (B) *CALCR* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR. Target genes were normalized to *18S* rRNA (n=8). The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), no significant line-by-age effect was detected for *PTH1R* (P=0.2873), and main effect means for line and age are presented in Tables 4 and 5, respectively. In (B), a significant line-by-age interaction was detected for *CALCR* (P=0.0136), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). *PTH3R* was not detected in kidney.

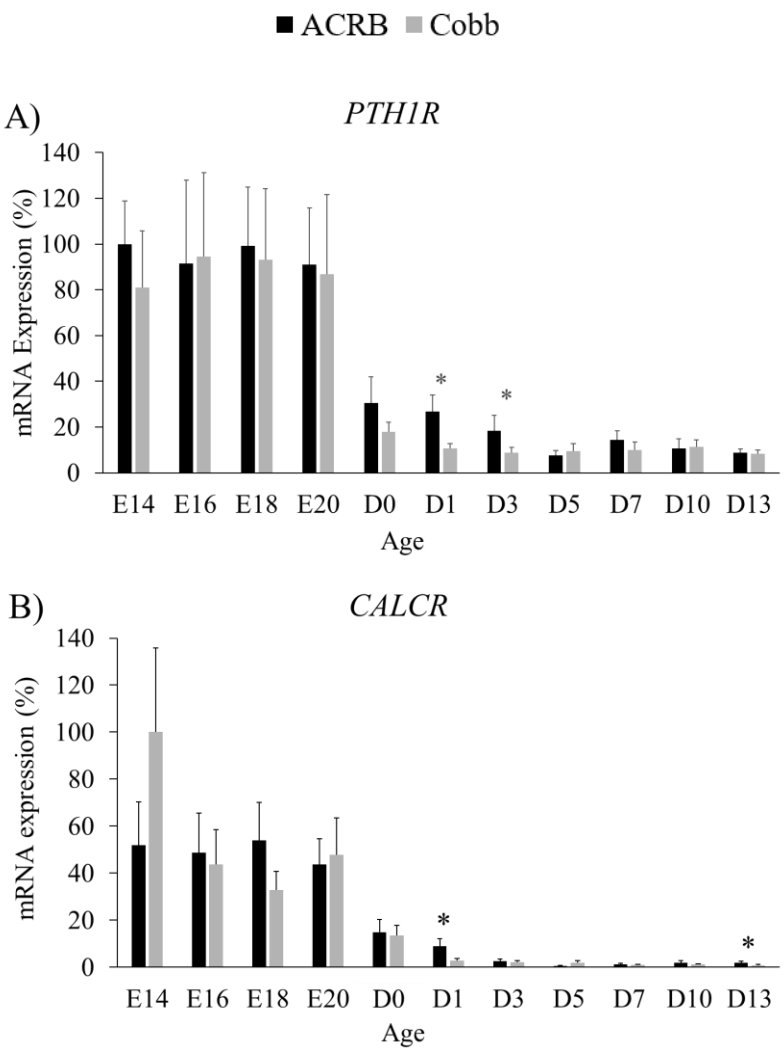


**Figure 11.** Relative mRNA expression of hormonal regulation of Ca and P in jejunum of ACRB and Cobb male broilers. (A) *PTH1R* and (B) *CALCR* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *GAPDH* mRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), a significant line-by-age effect was detected for *PTH1R* (P=0.0272), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). In (B), the line-by-age effect approached significance for *CALCR* (P=0.0878), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). Main effect means for line and age are presented in Tables 4 and 5, respectively. *PTH3R* was not detected in jejunum.

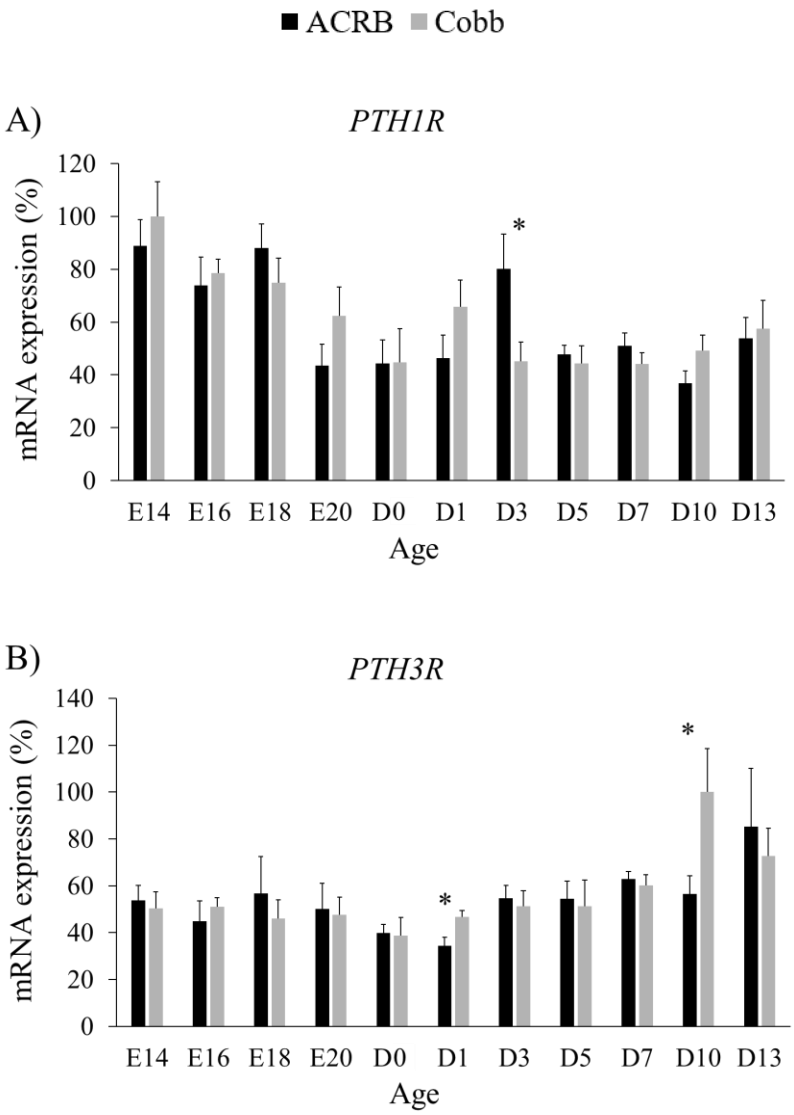


**Figure 12.** Relative mRNA expression of hormonal regulation of Ca and P in liver of ACRB and Cobb male broilers. (A) *PTH1R* and (B) *PTH3R* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). The line-by-age interaction approached significance for *PTH1R* (P=0.0717) and *PTH3R* (P=0.0616), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). Main effect means for line and age are presented in Tables 4 and 5, respectively. *CALCR* was not detected in liver.

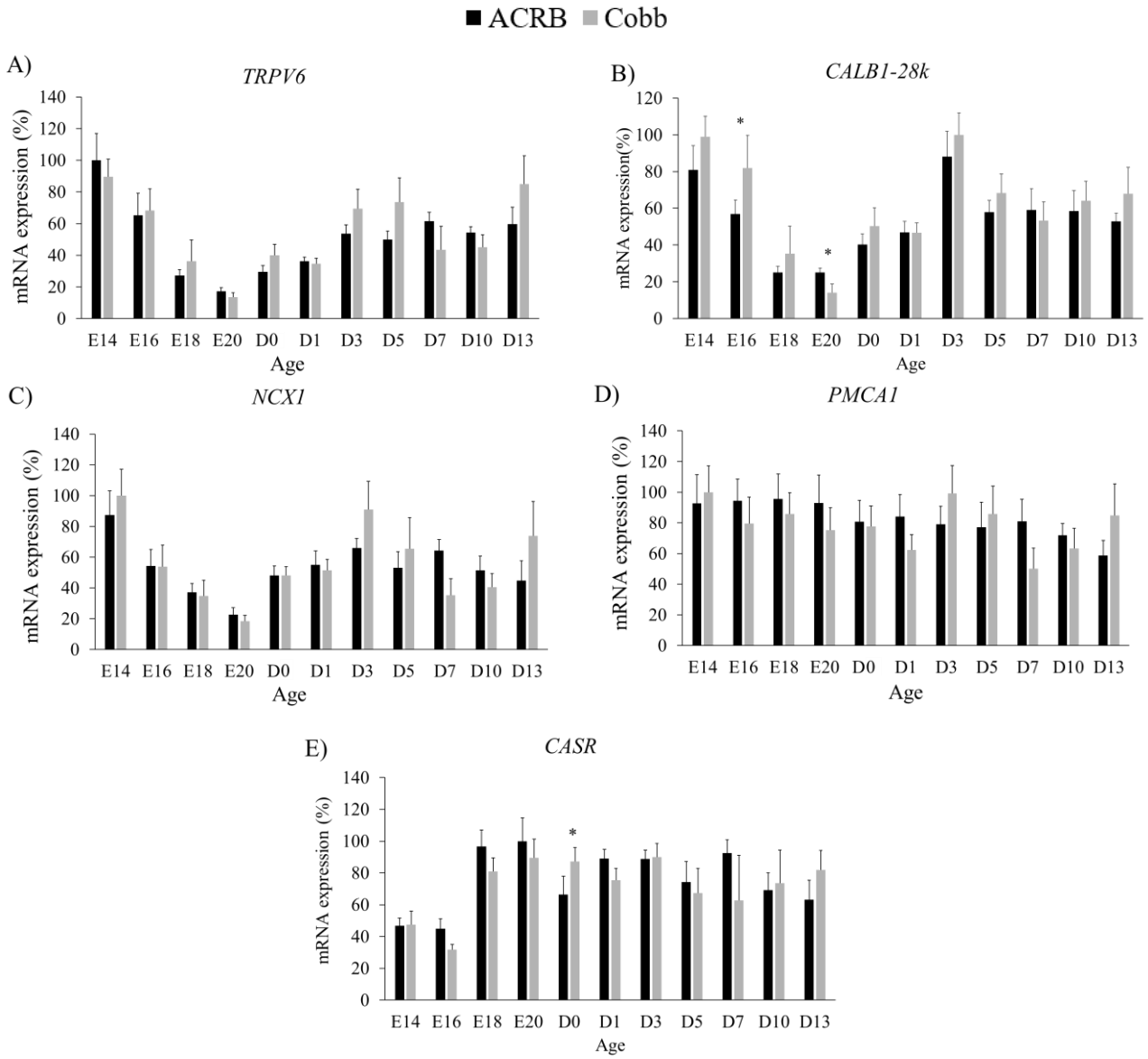


**Figure 13.** Relative mRNA expression of genes involved in Ca transport in kidney of ACRB and Cobb male broilers. (A) *TRPV6*, (B) *CALB1-28k*, (C) *NCX1*, (D) *PMCA1*, and (E) *CASR* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=8). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), (C), and (D), no significant line-by-age effects were detected for *TRPV6* (P=0.6274), *NCX1* (P=0.6839), or *PMCA* (P=0.1358), and main effect means for line and age are presented in Tables 6 and 7, respectively. In (B) and (E), significant line-by-age interactions were detected for *CALB1-28k* (P=0.0126) and *CASR* (P=0.0226), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05).

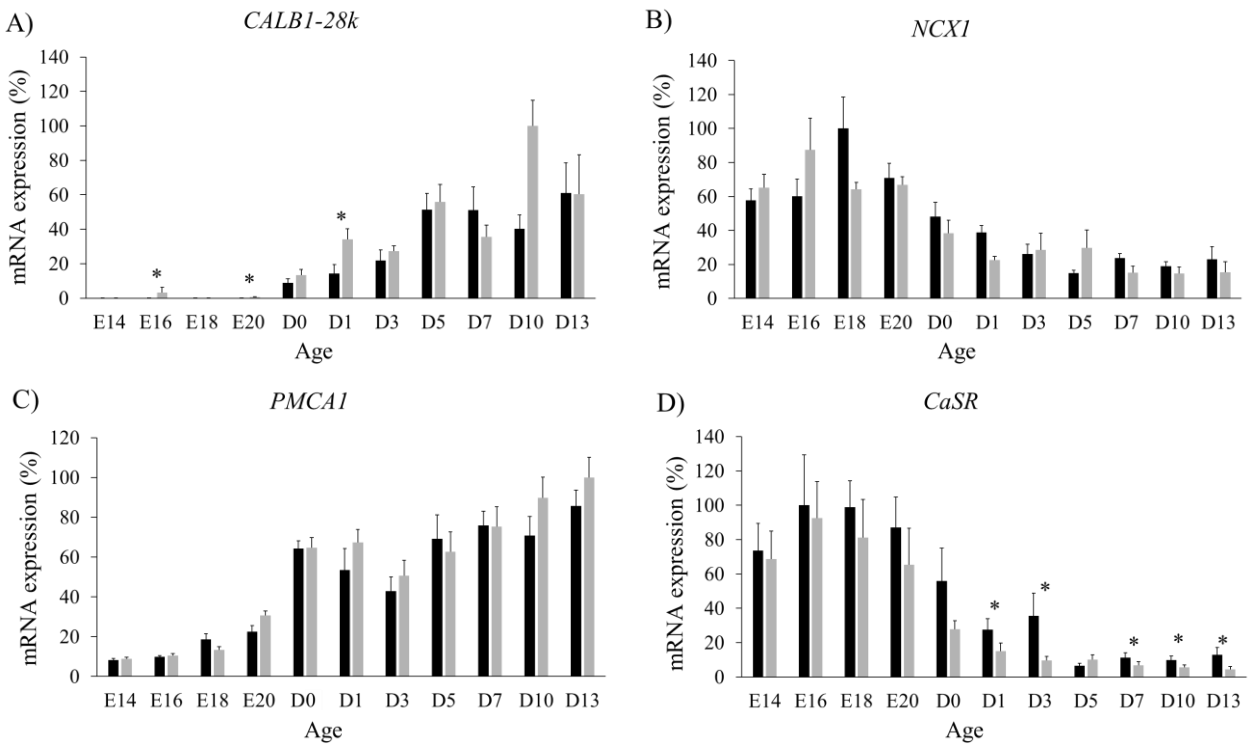


**Figure 14.** Relative mRNA expression of genes involved in Ca transport in jejunum of ACRB and Cobb male broilers. (A) *CALB1-28k*, (B) *NCX1*, (C) *PMCA1*, and (D) *CASR* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *GAPDH* mRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), the line-by-age interaction approached significance for *CALB1-28k* (P=0.0619), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). In (B) and (C), no significant line-by-age effects were detected for *NCX1* (P=0.4022) or *PMCA1* (P=0.1243), and main effect means for line and age are presented in Tables 6 and 7, respectively. In (D), a significant line-by-age interaction was detected for *CASR* (P=0.0045), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). *TRPV6* was not detected in jejunum.

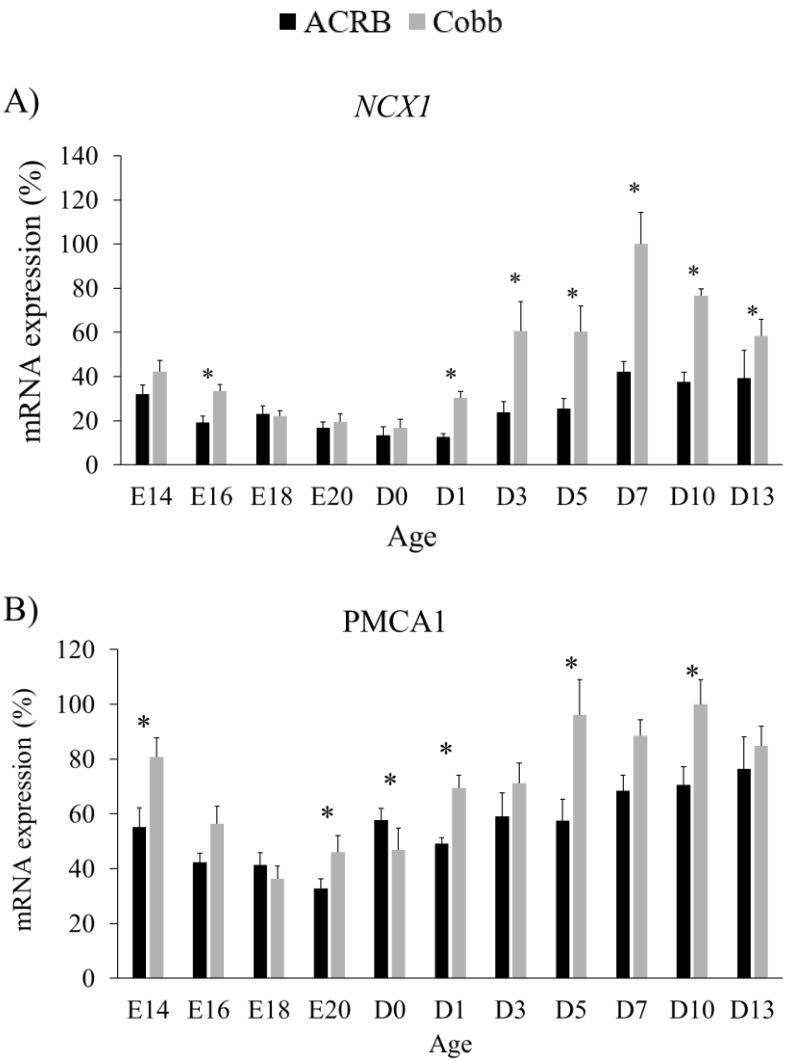


**Figure 15.** Relative mRNA expression of genes involved in Ca transport in liver of ACRB and Cobb male broilers. (A) *NCX1* and (B) *PMCA1* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *18S* rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). Significant line-by-age interactions were detected for both genes (*NCX1*, P=0.0184; *PMCA1*, P=0.0070), and an asterisk (*) indicates significant differences between the lines at those ages (P≤0.05). *TRPV6*, *CALB1-28k*, and *CASR* were not detected in liver.

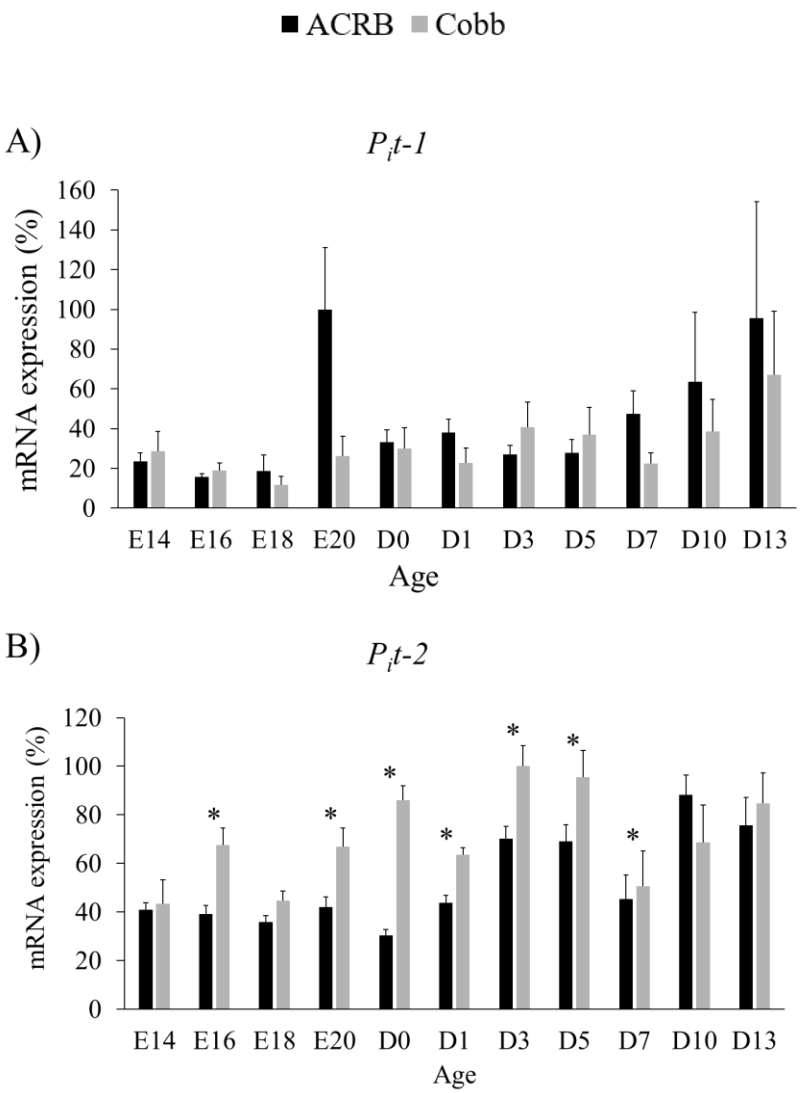


**Figure 16.** Relative mRNA expression of genes involved in P transport in kidney of ACRB and Cobb male broilers. (A) *$P_i$T-1* and (B) *$P_i$T-2* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=8). Target genes were normalized to *18S* rRNA. The data (mean + SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), no significant line-by-age effect was detected for *$P_i$T-1* (P=0.336), and main effect means for line and age are presented in Tables 8 and 9, respectively. In (B), a significant line-by-age interaction was detected for *$P_i$T-2* (P<0.0001), and an asterisk (*) indicates significant differences between the lines at those age (P≤0.05). *$NaP_i$-IIa* and *$NaP_i$-IIb* were not detected in kidney.

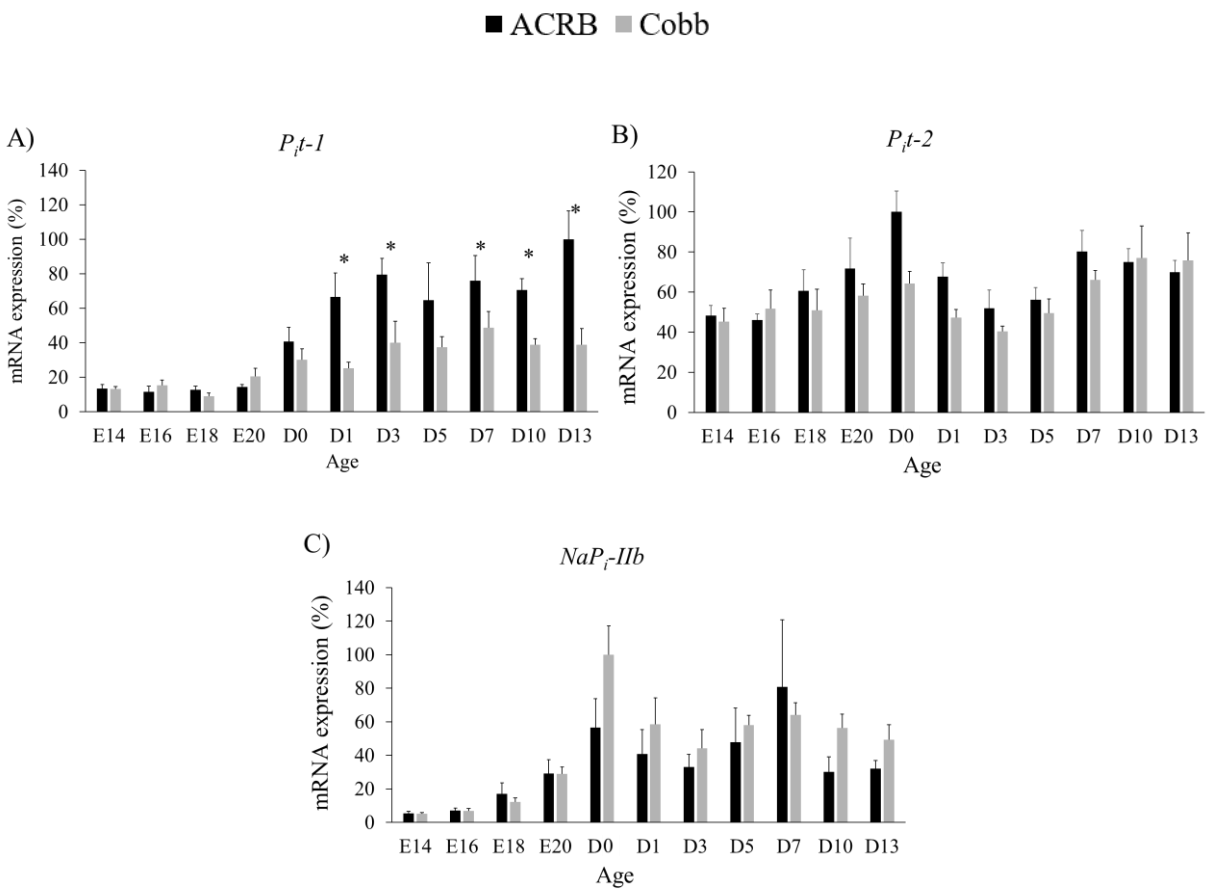


**Figure 17.** Relative mRNA expression of genes involved in P transport in jejunum of ACRB and Cobb male broilers. (A) *$P_i$T-1*, (B) *$P_i$T-2*, and (C) *$NaP_i$-IIb* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to *GAPDH* mRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). In (A), a significant line-by-age effect was detected for *$P_i$T-1* ($P<0.0001$), and an asterisk (*) indicates significant differences between the lines at those ages ($P\leq0.05$). In (B) and (C), no significant line-by-age effects were detected for *$P_i$T-2* ($P=0.6999$) or *$NaP_i$-IIb* ($P=0.8995$), and main effect means for line and age are presented in Tables 8 and 9, respectively. *$NaP_i$-IIa* was not detected in jejunum.

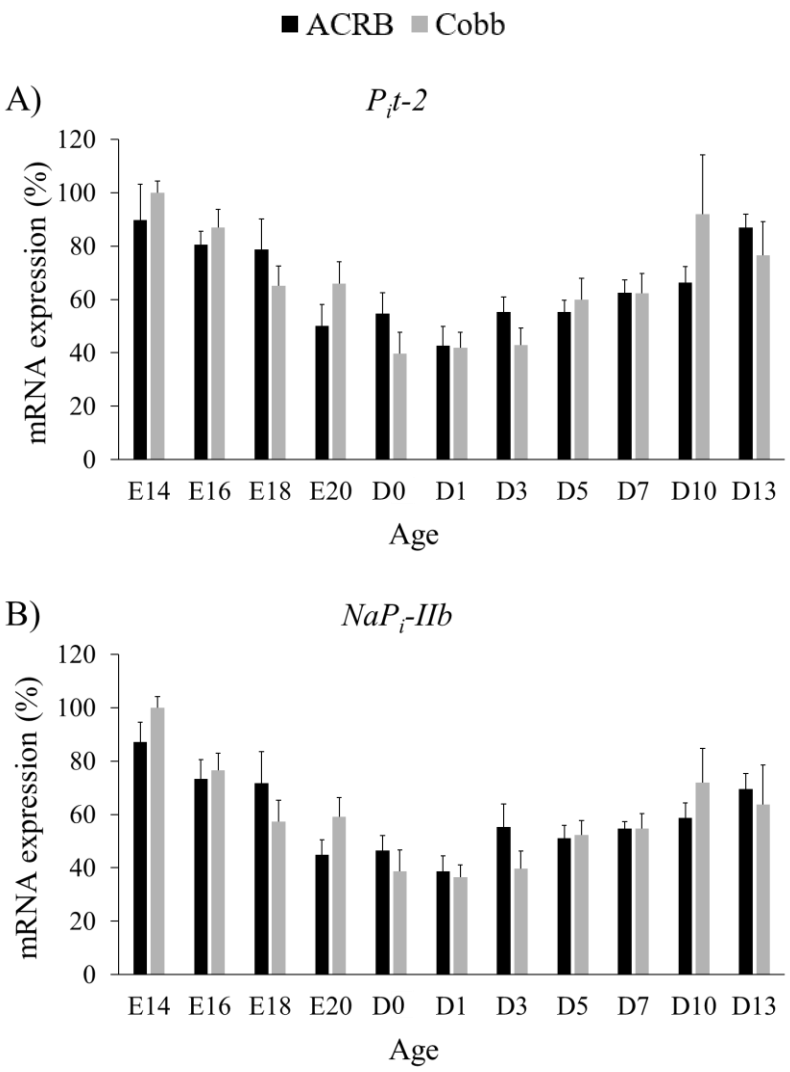


**Figure 18.** Relative mRNA expression of genes involved in P transport in liver of ACRB and Cobb male broilers. (A) *$P_i$T-2* and (B) *$NaP_i$-IIb* mRNA levels were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 using RT-qPCR (n=6). Target genes were normalized to 18S rRNA. The data (mean+SEM) are expressed relative to the line and age with the highest expression level (equivalent to 100%). No significant line-by-age effects were detected for *$P_i$T-2* (P=0.2071) or *$NaP_i$-IIb* (P<0.3271), and main effect means for line and age are presented in Tables 8 and 9, respectively. *$P_i$T-1* and *$NaP_i$-IIa* were not detected in liver.

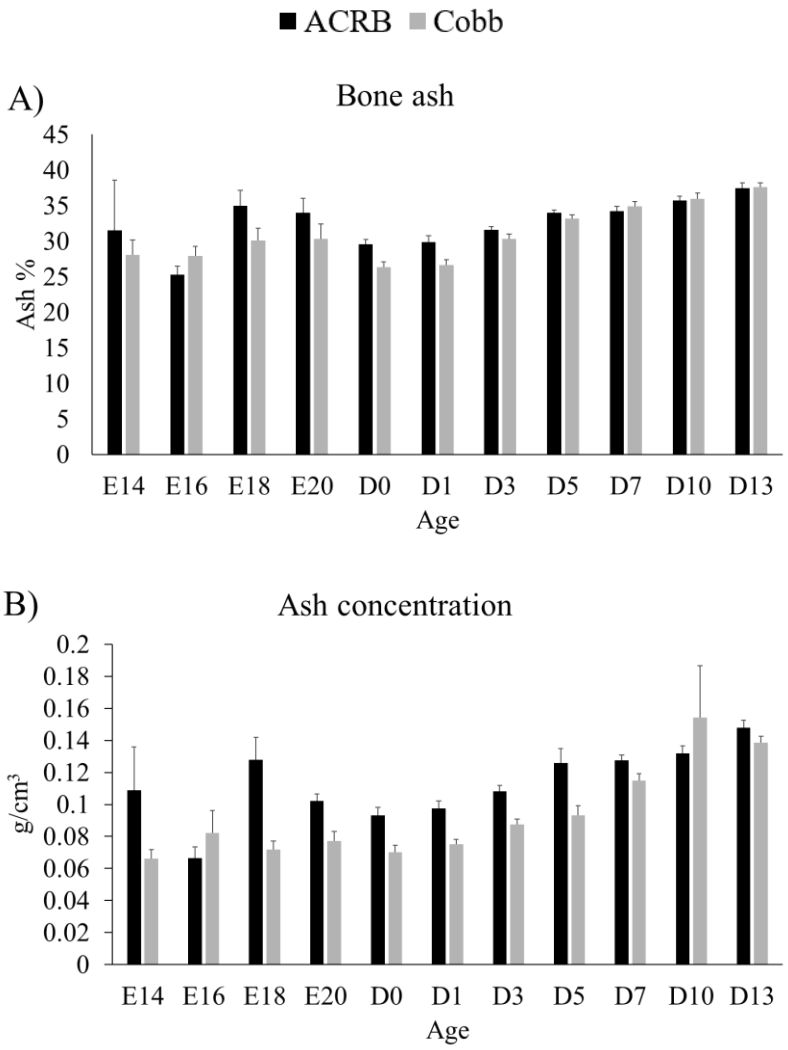


**Figure 19.** Bone measurements in tibia of ACRB and Cobb male broilers. (A) Bone ash (%) and (B) ash concentration (g/cm$^3$) were measured on embryonic day (E) 14, E16, E18, and E20 and post-hatch day (D) D0 (day of hatch), D1, D3, D5, D7, D10, and D13 (n=6). No significant line-by-age effects were detected for bone ash (P=0.8648) or ash concentration (P=0.1324), and main effect means for line and age are presented in Tables 10 and 11, respectively.

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# CHAPTER 4

# CONCLUSIONS

Broiler genetic selection programs have started to pay attention to other traits besides just ones that are economically significant for production. Breeding companies have realized that welfare traits that improve bone health and well-being of birds and allow them to exert their normal behavior are both ethically correct and can also help producers avoid economic losses. A common welfare issue has been determined to be leg disorders in fast growing broilers, which causes tremendous economic losses to producers due to increased mortality, decreased growth performance, and carcass condemnations at processing. This increased interest in welfare traits has led to improvements in animal nutrition, management, environmental enrichment, and genetic selection. For improvements related to nutrition and genetic selection, it is important to understand the underlying physiology of Ca and P metabolism that could have been altered in the search for a more productive bird. Therefore, an understanding on how pathways that regulate Ca an P have been affected by selection programs plays an essential role in improving bone health in commercial broilers, and ultimately production efficiency.

Improvements in skeletal health could be further achieved through genetic selection focused on leg health traits; however, to achieve this goal it is necessary to understand how affected the Ca and P mechanisms in modern birds are. Therefore, in this study, how pathways potentially influencing leg health could have been altered by genetic selection was determined. A comparison of physiological systems related to metabolism of Ca and P was made between legacy ACRB birds representative of broilers prior to intensive genetic selection and modern Cobb 500 broilers. Our

findings suggest that modern commercial broilers could have altered pathways regulating enzymatic conversion of vitamin $D_3$. They exhibited downregulated expression of 25-hydroxylase in jejunum and kidney, suggesting conversion at the local level of target tissues might be impaired. Furthermore, in kidney, the inactivating enzyme for vitamin $D_3$ had higher expression in modern broilers post-hatch, which might increase the clearance rate of $1,25(OH)_2D_3$. This was observable in post-hatch plasma concentrations of these metabolites, where $25(OH)D_3$ was higher in ACRB birds and $24,25(OH)D_3$ was higher in Cobb birds. Legacy birds seem to have a better response to variations of Ca through detection of plasma Ca levels via CALCR in kidney and CASR in jejunum. This could help them respond faster and take compensatory actions to improve their response to variations in Ca and P concentrations in the body.

Together, these findings suggest that modern broilers have some pathways related to bone health that have been altered by intensive genetic selection focusing only on commercial traits. The altered pathways regulate Ca and P homeostasis, and these minerals are the main components of bone tissue. Therefore, alterations in these pathways could decrease leg health and impair animal welfare. Leg health is a trait that in the long run improves broiler economic production. Therefore, breeding companies should continue to focus their future genetic selection efforts on the improvement of bone structure, as well as the usual commercial parameters. Future studies should focus on evaluating these pathways not only at the mRNA expression level, but at the protein level, as well as utilizing novel *in vitro* and *in vivo* models to help elucidate how molecular mechanisms regulate vitamin $D_3$, Ca, and P metabolism in chickens.

## APPENDIX

**Table A1.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to enzymatic conversion of vitamin $D_3$ and in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *CYP2R1* | | | | | | | | | | | | |
| | ACRB | 88.7±17.1 | 100±13.9 | 78.0±18.4 | 65.2±11.9 | 39.2±4.8 | 36.7±4.8 | 48.4±10.1 | 76.4±15.4 | 33.1±6.5 | 34.5±5.4 | 47.8±7.6 | 0.1448 |
| | COBB | 97.6±14.5 | 90.0±12.0 | 45.7±8.1 | 48.0±9.4 | 35.1±6.0 | 45.5±12.7 | 59.8±7.3 | 59.2±11.3 | 75.7±33.7 | 52.6±12.1 | 68.1±25.7 | |
| | *CYP24A1* | | | | | | | | | | | | |
| | ACRB | 49.1±11.8 | 71.6±10.1 | 95.6±33.8[a] | 37.9±13.6 | 18.2±3.6 | 28.5±16.3 | 24.8±9.0[b] | 53.2±12.2 | 42.4±13.6 | 18.6±4.8[b] | 46.2±12.4 | 0.0705 |
| | COBB | 67.8±8.6 | 72.7±7.0 | 24.8±8.1[b] | 33.0±10.7 | 30.6±10.8 | 40.27±15.6 | 73.2±20.9[a] | 40.5±11.3 | 48.9±25.3 | 61.2±10.5[a] | 100±29.2 | |
| Jejunum | *CYP2R1* | | | | | | | | | | | | |
| | ACRB | 80.9±10.1 | 73.3±9.7 | 91.0±7.0 | 67.3±3.2 | 70.9±9.4 | 66.7±13.6[a] | 22.1±4.1 | 19.6±2.0 | 27.0±5.0[a] | 17.2±3.6 | 20.9±2.5 | 0.0042 |
| | COBB | 100±8.9 | 80.3±8.9 | 79.6±9.4 | 77.2±10.2 | 56.2±6.2 | 30.6±2.9[b] | 31.1±7.7 | 24.9±3.9 | 18.7±4.3[b] | 14.5±3.6 | 18.5±3.1 | |
| | *CYP24A1* | | | | | | | | | | | | |
| | ACRB | 9.6±1.6 | 14.1±2.1 | 8.8±0.8 | 7.8±1.6 | 3.9±0.6 | 6.1±3.1 | 2.6±3.1 | 2.3±0.4 | 7.4±3.6 | 4.9±3.4 | 5.4±3.3 | 0.309 |
| | COBB | 13.5±1.5 | 100±91.6 | 9.9±1.2 | 5.4±1.1 | 2.7±0.6 | 3.1±0.5 | 5.8±2.5 | 1.7±0.3 | 1.7±0.4 | 1.5±0.6 | 105±0.4 | |
| Liver | *CYP2R1* | | | | | | | | | | | | |
| | ACRB | 43.4±3.6 | 29.6±5.4 | 37.3±4.9 | 17.7±3.3 | 45.5±5.1 | 61.2±7.5 | 72.0±12.8 | 77.4±13.6 | 74.9±4.7 | 60.8±5.1 | 75.4±8.9 | 0.6257 |
| | COBB | 63.1±8.2 | 46.8±3.6 | 44.7±7.6 | 33.2±4.8 | 64.4±17.3 | 75.6±9.3 | 86.4±6.5 | 100±12.2 | 83.3±7.9 | 83.9±10.9 | 81.7±11.7 | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table A2.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for plasma vitamin $D_3$ metabolites in male ACRB and Cobb broilers during early post-hatch development.

| Metabolite | | D3 | D7 | D10 | P-value |
|---|---|---|---|---|---|
| 25(OH)$D_3$ (ng/ml) | | | | | |
| | ACRB | 14.5±0.6 | 14.2±3.0 | 21.1±3.9 | 0.2043 |
| | COBB | 15.8±0.9 | 8.7±2.9 | 10.4±4.5 | |
| 1,25$(OH)_2D_3$ (pg/ml) | | | | | |
| | ACRB | 79.8±24.9 | 67.6±4.0 | 81.8±35.6 | 0.6062 |
| | COBB | 34.0±13.2 | 40.6±3.6 | 75.2±15.4 | |
| 24,25$(OH)_2D_3$ (ng/ml) | | | | | |
| | ACRB | 1.1±0.3 | 0.5±0.1 | 0.9±0.3 | 0.2675 |
| | COBB | 1.2±0.1 | 2.0±0.6 | 1.7±0.6 | |

[1]Means were calculated across both lines at post-hatch day (D) 3, D7, and D10.
[2]Circulating vitamin $D_3$ metabolites are expressed as an absolute concentration.

**Table A3.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to genomic response of vitamin $D_3$ in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *VDR* | | | | | | | | | | | | |
| | ACRB | 84.7±8.7 | 95.4±3.6 | 100±11.2 | 92.8±11 | 84.6±9.9 | 70.5±8.0 | 59.7±7.7 | 54.4±9.0 | 63.1±6.2[a] | 45.5±5.5 | 52.5±11.1 | 0.0708 |
| | COBB | 78.9±4.1 | 84.4±14.0 | 92.1±5.6 | 67.5±4.7 | 76.1±5.0 | 51.9±3.0 | 88.3±7.0 | 62.6±12.2 | 38.1±18.7[b] | 47.7±13.2 | 76.4±14.4 | |
| | *RXRA* | | | | | | | | | | | | |
| | ACRB | 78.5±4.6 | 72.5±4.0 | 66.7±6.0 | 70.5±9.4 | 59.9±4.9 | 56.2±5.0 | 54.7±5.9 | 45.9±7.3 | 56.5±4.9[a] | 50.4±11.2[a] | 42.1±12.1 | 0.0196 |
| | COBB | 100±31.9 | 79.7±10.0 | 67.4±9.6 | 54.4±6.0 | 55.5±4.2 | 45.8±4.9 | 57.8±5.7 | 50.8±11.4 | 30.7±16.0[b] | 40.3±15.1[b] | 49.6±12.5 | |
| Jejunum | *VDR* | | | | | | | | | | | | |
| | ACRB | 4.9±0.9 | 10.0±1.4 | 21.0±2.3 | 31.1±4.2 | 78.1±8.5 | 85.7±13.7 | 53.1±7.1 | 46.8±7.4 | 91.5±10.5 | 81.5±5.4 | 82.9±4.4 | 0.3233 |
| | COBB | 6.2±0.9 | 10.1±2.6 | 19.8±2.1 | 34.6±4.9 | 71.0±14.7 | 65.2±9.2 | 49.5±3.2 | 73.1±4.9 | 80.2±12.9 | 93.2±10.7 | 100±9.7 | |
| | *RXRA* | | | | | | | | | | | | |
| | ACRB | 69.4±5.1 | 66.3±13.6 | 85.5±9.8 | 85.6±6.3 | 97.3±15.2 | 87.6±11.1 | 65.6±5.4 | 57.8±11.3 | 100±11.0 | 83.7±6.2 | 95.9±9.6 | 0.2360 |
| | COBB | 64.2±3.8 | 69.2±6.4 | 64.6±11.2 | 75.6±9.0 | 87.3±15.5 | 58.7±6.6 | 54.8±8.0 | 68.7±6.6 | 71.0±11.0 | 80.4±15.2 | 69.9±10.1 | |
| | *RXRG* | | | | | | | | | | | | |
| | ACRB | 86.6±17.3 | 64.6±23.1 | 64.5±14.7 | 65.6±17.1 | 29.5±6.8 | 31.2±4.7 | 18.1±7.6 | 6.7±2.1 | 7.2±1.4 | 8.2±1.6 | 12.9±4.9 | 0.9422 |
| | COBB | 100±32.8 | 60.2±22.3 | 52.1±17.3 | 86.9±37.7 | 23.8±2.9 | 15.3±3.1 | 15.5±6.6 | 10.5±4.3 | 5.5±1.9 | 5.8±2.3 | 7.9±2.6 | |
| Liver | *VDR* | | | | | | | | | | | | |
| | ACRB | 85.0±8.8 | 64.7±7.2 | 63.6±13.6 | 38.8±7.0 | 34.0±4.0 | 32.2±4.4 | 29.3±5.0 | 28.8±6.6 | 30.2±3.7 | 31.2±8.3 | 29.5±4.5 | 0.5153 |
| | COBB | 100±17.3 | 78.0±7.3 | 59.3±2.7 | 47.2±6.6 | 34.8±8.2 | 43.3±4.1 | 45.8±8.5 | 39.6±3.8 | 39.9±4.7 | 44.1±6.1 | 50.6±8.9 | |
| | *RXRA* | | | | | | | | | | | | |
| | ACRB | 51.6±2.8 | 51.3±4.2 | 63.9±8.7 | 55.9±7.8 | 48.5±2.8 | 43.7±3.5 | 53.7±3.6 | 53.7±3.8 | 51.7±3.6[b] | 52.7±2.8[b] | 62.8±6.7[b] | 0.0024 |
| | COBB | 59.0±2.4 | 58.3±0.9 | 52.1±5.2 | 61.1±5.2 | 45.1±5.9 | 53.5±2.5 | 60.9±5.2 | 59.1±6.9 | 74.6±5.1[a] | 100±6.7[a] | 84.6±7.9[a] | |
| | *RXRG* | | | | | | | | | | | | |
| | ACRB | 100±25.5 | 46.9±10.0 | 58.4±14.6 | 32.2±9.0 | 51.2±3.0 | 58.4±7.0 | 47.3±5.1 | 42.3±7.2 | 40.4±5.4 | 32.6±3.0 | 41.0±7.8 | 0.6477 |
| | COBB | 84.1±11.2 | 65.9±5.6 | 46.2±7.3 | 28.3±9.4 | 45.3±6.5 | 67.3±6.3 | 46.2±4.8 | 46.8±4.4 | 37.4±3.3 | 49.0±6.2 | 51.5±11.7 | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table A4.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to hormonal regulation of vitamin $D_3$ in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *PTH1R* | | | | | | | | | | | | |
| | ACRB | 100±17.8 | 78.2±7.4 | 56.2±9.7 | 61.1±8.5 | 59.8±10.5 | 55.7±4.8 | 80.6±9.3 | 58.6±7.3 | 67.4±11.1 | 63.1±11.3 | 53.3±10.2 | 0.2873 |
| | COBB | 74.6±5.7 | 82.8±17.6 | 38.4±7.4 | 44.8±7.9 | 56.8±4.3 | 64.7±10.8 | 79.6±7.9 | 66.7±14.5 | 28.6±8.7 | 44.1±10.9 | 60.9±10.2 | |
| | *CALCR* | | | | | | | | | | | | |
| | ACRB | 60.3±14.4 | 26.6±6.3$^{a}$ | 54.9±9.7 | 53.9±4.6 | 93.7±9.6 | 83.5±11.8 | 53.2±8.1 | 40.7±5.2 | 53.0±6.2$^{a}$ | 36.8±5.5$^{a}$ | 25.5±5.2 | 0.0136 |
| | COBB | 42.3±9.4 | 10.1±2.8$^{b}$ | 50.5±10.9 | 47.4±9.1 | 100±10.4 | 69.1±4.9 | 74.7±15.4 | 39.5±8.3 | 17.3±5.4$^{b}$ | 24.2±7.4$^{b}$ | 47.9±15.8 | |
| Jejunum | *PTH1R* | | | | | | | | | | | | |
| | ACRB | 23.5±4.4 | 21.5±8.6$^{b}$ | 23.3±6.0 | 21.4±5.8 | 7.2±2.7 | 6.3±1.7$^{a}$ | 4.3±1.6$^{a}$ | 1.8±0.5 | 3.4±0.9 | 2.5±0.9 | 2.1±0.4 | 0.0272 |
| | COBB | 19.1±5.8 | 100±77.3$^{a}$ | 21.9±7.4 | 20.4±8.2 | 4.2±1.0 | 2.5±0.5$^{b}$ | 2.0±0.6$^{b}$ | 2.2±0.8 | 2.4±0.8 | 2.7±0.7 | 1.9±0.4 | |
| | *CALCR* | | | | | | | | | | | | |
| | ACRB | 51.9±18.6 | 48.8±16.8 | 54.0±16.0 | 43.7±10.9 | 14.9±5.5 | 9.0±3.1$^{a}$ | 2.6±1.0 | 0.6±0.1 | 1.2±0.5 | 1.8±1.1 | 2.0±0.8$^{a}$ | 0.0878 |
| | COBB | 100±35.9 | 43.8±14.8 | 32.9±7.9 | 47.9±15.6 | 13.6±4.3 | 2.8±0.9$^{b}$ | 2.1±0.8 | 1.9±0.9 | 1.0±0.2 | 1.2±0.3 | 0.9±0.5$^{b}$ | |
| Liver | *PTH1R* | | | | | | | | | | | | |
| | ACRB | 88.9±9.9 | 74.0±10.7 | 88.1±9.0 | 43.6±8.1 | 44.3±9.1 | 46.3±8.8 | 80.1±13.1$^{a}$ | 47.8±3.5 | 51.0±4.8 | 36.8±4.6 | 53.8±7.8 | 0.0616 |
| | COBB | 100±13.2 | 78.5±5.2 | 74.9±9.2 | 62.3±11.0 | 44.8±12.8 | 65.7±10.2 | 45.1±7.3$^{b}$ | 44.3±6.8 | 44.1±4.4 | 49.1±6.0 | 57.5±10.6 | |
| | *PTH3R* | | | | | | | | | | | | |
| | ACRB | 53.7±6.4 | 44.9±8.7 | 56.8±15.8 | 50.0±11.0 | 39.8±3.6 | 34.3±3.7$^{b}$ | 54.6±5.5 | 54.4±7.6 | 63.0±3.2 | 56.5±7.7$^{b}$ | 85.3±24.7 | 0.6477 |
| | COBB | 50.3±7.1 | 51.0±4.0 | 46.0±8.0 | 47.7±7.4 | 38.6±7.8 | 46.7±2.6$^{a}$ | 51.3±6.6 | 51.2±11.4 | 60.2±4.6 | 100±18.5$^{s}$ | 72.6±11.9 | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different ($P \leq 0.05$).

**Table A5.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to Ca transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *TRPV6* | | | | | | | | | | | | |
| | ACRB | 100±16.8 | 65.2±14.0 | 27.3±3.7 | 17.2±2.5 | 29.6±4.0 | 36.4±2.4 | 53.7±5.5 | 49.8±5.3 | 61.5±5.6 | 54.2±3.9 | 59.74±10.7 | 0.6405 |
| | COBB | 89.6±11.2 | 68.2±13.7 | 36.3±13.3 | 13.6±2.8 | 39.9±7.1 | 34.6±3.6 | 69.4±12.3 | 73.7±15.2 | 43.6±14.8 | 45.1±7.9 | 84.9±17.9 | |
| | *NCX1* | | | | | | | | | | | | |
| | ACRB | 87.4±15.7 | 54.2±10.7 | 37.3±5.7 | 22.5±4.7 | 48.2±6.2 | 55.0±9.0 | 65.9±6.3 | 53.1±10.4 | 64.3±7.2 | 51.5±9.3 | 44.8±12.9 | 0.7194 |
| | COBB | 100±17.0 | 53.9±14.1 | 34.8±10.3 | 18.4±3.9 | 48.0±5.8 | 51.4±7.2 | 90.9±18.3 | 65.5±20.1 | 35.4±10.5 | 40.4±8.9 | 73.8±22.4 | |
| | *PMCA1* | | | | | | | | | | | | |
| | ACRB | 92.8±18.7 | 94.4±14.1 | 95.7±16.2 | 92.9±18.3 | 80.8±13.8 | 84.0±14.5 | 79.1±11.7 | 77.0±16.4 | 80.9±14.4 | 72.0±7.7 | 58.6±10.0 | 0.1358 |
| | COBB | 100±17.1 | 79.5±17.2 | 85.7±13.9 | 75.3±14.5 | 77.7±13.4 | 62.2±10.3 | 99.2±18.2 | 85.7±18.4 | 50.1±13.5 | 63.2±13.1 | 84.8±20.6 | |
| Jejunum | *NCX1* | | | | | | | | | | | | |
| | ACRB | 57.8±6.5 | 60.2±10.1 | 100±18.5 | 70.9±8.6 | 48.1±8.5 | 38.8±4.2 | 26.1±5.8 | 15.0±1.6 | 23.8±2.5 | 19.0±2.6 | 23.1±7.3 | 0.4011 |
| | COBB | 65.2±8.0 | 87.5±18.6 | 64.3±3.9 | 66.8±4.8 | 38.4±7.6 | 22.6±2.1 | 28.5±9.9 | 29.7±10.5 | 15.1±3.9 | 14.8±3.8 | 15.4±6.1 | |
| | *PMCA1* | | | | | | | | | | | | |
| | ACRB | 8.2±0.8 | 9.9±0.5 | 18.5±2.9 | 22.6±3.0 | 64.2±4.0 | 53.5±10.8 | 43.0±7.0 | 69.2±12.1 | 75.9±7.1 | 70.8±9.7 | 85.8±7.8 | 0.2189 |
| | COBB | 8.8±0.8 | 10.6±0.9 | 13.3±1.6 | 30.6±2.4 | 64.6±5.1 | 67.3±6.6 | 50.7±7.7 | 62.7±9.9 | 75.4±9.8 | 89.8±10.2 | 100±10.2 | |
| Liver | *NCX1* | | | | | | | | | | | | |
| | ACRB | 32.1±4.1 | 19.2±3.0$^{b}$ | 23.2±3.6 | 16.8±2.6 | 13.5±3.8 | 12.7±1.4$^{b}$ | 23.9±4.7$^{a}$ | 25.6±4.4$^{a}$ | 42.3±4.4$^{a}$ | 37.6±4.4$^{a}$ | 39.2±12.7$^{a}$ | 0.0184 |
| | COBB | 42.3±4.9 | 33.4±2.9$^{a}$ | 22.1±2.5 | 19.5±3.5 | 16.8±3.9 | 30.4±2.9$^{a}$ | 60.5±13.5$^{b}$ | 60.3±11.6$^{b}$ | 100±14.3$^{b}$ | 76.6±3.2$^{b}$ | 58.4±7.6$^{b}$ | |
| | *PMCA1* | | | | | | | | | | | | |
| | ACRB | 55.2±6.9 | 42.4±3.2 | 41.5±4.4 | 32.8±3.6$^{b}$ | 57.8±4.3$^{a}$ | 49.2±2.1$^{b}$ | 59.2±8.5 | 57.5±7.9$^{b}$ | 68.4±6.7 | 70.5±6.7$^{b}$ | 76.4±11.7 | 0.0070 |
| | COBB | 80.8±6.9 | 56.4±6.4 | 36.3±4.8 | 46.1±6.1$^{a}$ | 46.8±8.1$^{b}$ | 69.4±4.7$^{a}$ | 71.3±7.3 | 96.1±12.9$^{a}$ | 88.4±6.0 | 100±9.1$^{a}$ | 84.9±7.1 | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different (P≤0.05).

**Table A6.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to Ca transport regulators in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *CALB1-28k* | | | | | | | | | | | | | |
| | | ACRB | 81.0±13.1 | 57.0±7.5$^{b}$ | 25.0±3.4 | 17.6±3.4$^{a}$ | 40.2±5.9 | 46.9±6.0 | 63.3±6.7 | 58.0±6.4 | 59.1±11.5 | 58.5±11.1 | 45.4±6.4 | 0.0127 |
| | | COBB | 99.0±11.2 | 81.9±17.9$^{a}$ | 35.2±15.1 | 14.1±4.7$^{b}$ | 50.3±10.0 | 46.7±5.4 | 100±11.8 | 68.4±10.3 | 53.3±10.2 | 64.0±10.8 | 67.9±14.4 | |
| | *CASR* | | | | | | | | | | | | | |
| | | ACRB | 46.7±5.1 | 44.8±6.4 | 96.7±10.4 | 100±14.7 | 66.3±11.6$^{b}$ | 89.1±5.8 | 88.7±5.6 | 74.3±12.8 | 92.5±8.2 | 69.2±10.9 | 63.1±12.3 | 0.0225 |
| | | COBB | 47.4±8.6 | 31.7±3.3 | 81.0±8.3 | 89.6±11.7 | 87.1±8.9$^{a}$ | 75.5±7.3 | 89.9±8.6 | 67.3±15.5 | 62.6±28.5 | 73.6±20.7 | 81.9±12.3 | |
| Jejunum | *CALB1-28k* | | | | | | | | | | | | | |
| | | ACRB | 0.1±0.0 | 0.1±0.0$^{b}$ | 0.0±0.0 | 0.1±0.0$^{b}$ | 8.8±2.3 | 14.4±5.1$^{b}$ | 21.8±6.2 | 51.5±9.4 | 51.2±13.5 | 40.3±8.0 | 61.1±17.4 | 0.0658 |
| | | COBB | 0.0±0.0 | 3.2±3.2$^{a}$ | 0.1±.01 | 0.6±0.2$^{a}$ | 13.5±3.1 | 34.1±6.2$^{a}$ | 27.4±2.9 | 55.8±10.2 | 35.6±6.9 | 100±15.0 | 60.4±22.9 | |
| | *CASR* | | | | | | | | | | | | | |
| | | ACRB | 73.5±15.9 | 100±29.3 | 98.9±15.5 | 87.1±17.9 | 56.0±19.1 | 27.5±6.4$^{a}$ | 35.6±13.2$^{a}$ | 6.6±1.5 | 11.2±3.0$^{a}$ | 9.9±2.4$^{a}$ | 12.9±4.2$^{a}$ | 0.0061 |
| | | COBB | 68.7±16.3 | 92.5±92.5 | 81.1±22.3 | 65.4±21.3 | 27.7±5.1 | 15.1±4.7$^{b}$ | 9.7±2.4$^{b}$ | 10.1±2.7 | 6.8±2.2$^{b}$ | 5.7±1.2$^{b}$ | 4.5±1.4$^{b}$ | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different (P≤0.05).

**Table A7.** Means[1] (±SEM) and ANOVA P-values of the line-by-age interactive effect for genes related to P transport in male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | Gene | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kidney | *$P_i$t-1* | | | | | | | | | | | | |
| | ACRB | 23.6±4.3 | 15.6±1.8 | 18.7±8.2 | 100±31.1 | 33.3±6.1 | 37.9±6.8 | 27.1±4.4 | 27.9±6.7 | 47.4±11.7 | 63.7±34.9 | 95.6±58.6 | 0.3361 |
| | COBB | 28.5±10.0 | 19.0±3.7 | 11.7±4.2 | 26.3±9.8 | 30.0±10.5 | 22.7±7.5 | 40.8±12.5 | 36.9±13.8 | 22.4±5.4 | 38.5±16.2 | 67.0±32.0 | |
| | *$P_i$t-2* | | | | | | | | | | | | |
| | ACRB | 40.9±2.9 | 39.1±3.6[b] | 35.9±2.5 | 42.0±4.3[b] | 30.4±2.4[b] | 43.9±3.0[b] | 70.1±5.2[b] | 69.0±7.0[b] | 45.4±9.7[b] | 88.1±8.1 | 75.8±11.4 | <0.0001 |
| | COBB | 43.3±10.0 | 67.6±7.0[a] | 44.7±4.0 | 66.8±7.8[a] | 86.0±6.0[a] | 63.5±2.9[a] | 100±8.5[a] | 95.6±10.9[a] | 50.6±14.5[a] | 68.6±15.5 | 84.7±12.5 | |
| Jejunum | *$P_i$t-1* | | | | | | | | | | | | |
| | ACRB | 13.5±2.3 | 11.6±3.2 | 12.7±2.2 | 14.4±1.4 | 40.8±8.0 | 66.5±14.0[a] | 79.5±9.3[a] | 64.7±21.5 | 75.9±14.6[a] | 70.6±6.6[a] | 100±16.4[a] | <0.0001 |
| | COBB | 13.1±1.6 | 15.3±2.9 | 8.8±1.8 | 20.5±4.8 | 30.0±6.5 | 25.1±3.7[b] | 40.0±12.5[b] | 37.5±6.0 | 48.8±9.3[b] | 38.8±3.6[b] | 38.8±9.3[b] | |
| | *$P_i$t-2* | | | | | | | | | | | | |
| | ACRB | 48.2±5.2 | 46.0±3.2 | 60.6±10.6 | 71.8±15.1 | 100±10.3 | 67.7±6.8 | 52.0±9.1 | 56.2±6.1 | 80.2±10.6 | 75.0±6.7 | 69.9±5.8 | 0.6999 |
| | COBB | 45.3±6.5 | 51.8±9.2 | 51.0±10.4 | 58.1±6.0 | 64.2±6.2 | 47.2±4.1 | 40.4±2.6 | 49.5±7.1 | 66.0±4.7 | 77.0±15.9 | 75.9±13.6 | |
| | *$NaP_i$-IIb* | | | | | | | | | | | | |
| | ACRB | 5.4±1.2 | 7.1±1.4 | 17.1±6.5 | 29.0±8.3 | 56.6±17.2 | 40.7±14.6 | 33.0±7.6 | 47.8±20.3 | 80.7±40.0 | 30.0±9.0 | 32.0±5.0 | 0.8977 |
| | COBB | 5.0±0.8 | 6.7±1.5 | 12.1±2.5 | 28.9±4.1 | 100±17.0 | 58.5±15.8 | 44.2±11.1 | 57.9±5.8 | 63.9±7.5 | 56.3±8.3 | 49.3±8.9 | |
| Liver | *$P_i$t-2* | | | | | | | | | | | | |
| | ACRB | 89.8±13.3 | 80.6±5.0 | 78.7±11.4 | 50.0±8.1 | 54.7±7.7 | 42.7±7.1 | 55.4±5.5 | 55.3±4.4 | 62.6±4.8 | 66.3±6.0 | 86.9±5.1 | 0.2071 |
| | COBB | 100±4.4 | 87.0±6.8 | 65.0±7.5 | 65.8±8.3 | 39.6±8.1 | 41.9±5.7 | 42.9±6.4 | 59.8±8.1 | 62.3±7.3 | 91.9±22.4 | 76.6±12.6 | |
| | *$NaP_i$-IIb* | | | | | | | | | | | | |
| | ACRB | 5.0±0.8 | 6.7±1.5 | 12.1±2.5 | 28.9±4.1 | 100±17.0 | 58.5±15.8 | 44.2±11.1 | 57.9±5.8 | 63.9±7.5 | 56.3±8.3 | 49.3±8.9 | 0.8977 |
| | COBB | 100±4.1 | 76.5±6.3 | 57.3±7.9 | 59.2±7.1 | 38.8±7.9 | 36.5±4.9 | 39.8±6.5 | 52.3±5.4 | 54.8±5.5 | 72.0±12.8 | 63.7±14.7 | |

[1]Data within each gene on indicated embryonic (E) and post-hatch (D) days are expressed relative to the line and age with the highest mRNA level (equal to 100%).

[a,b] Values within each gene that do not share a common letter are significantly different (P≤0.05).

**Table A8.** Means[1] (±SEM), and ANOVA P-values of the line-by-age interactive effect for bone ash, ash concentration, bone ash weight, and fat-free dry weight (FFDW) in tibia of male ACRB and Cobb broilers during late embryonic and early post-hatch development.

| | E14 | E16 | E18 | E20 | D0 | D1 | D3 | D5 | D7 | D10 | D13 | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bone ash (%) | | | | | | | | | | | | |
| ACRB | 31.6±7.0 | 25.3±1.2 | 35.0±2.2 | 34.0±2.0 | 29.6±0.7 | 29.9±0.9 | 31.6±0.4 | 34.0±0.4 | 34.3±0.7 | 35.7±0.6 | 37.4±0.7 | 0.8648 |
| COBB | 28.1±2.1 | 27.9±1.4 | 30.1±1.7 | 30.3±2.1 | 26.4±0.7 | 26.6±0.8 | 30.3±0.7 | 33.2±0.5 | 34.9±0.7 | 36.0±0.8 | 37.6±0.6 | |
| Ash concentration ($g/cm^3$) | | | | | | | | | | | | |
| ACRB | 0.11±0.03 | 0.07±0.01 | 0.13±0.01 | 0.10±0.0 | 0.09±0.0 | 0.10±0.0 | 0.11±0.0 | 0.13±0.01 | 0.13±0.0 | 0.13±0.0 | 0.15±0.0 | 0.1324 |
| COBB | 0.07±0.01 | 0.08±0.01 | 0.07±0.01 | 0.08±0.01 | 0.07±0.0 | 0.08±0.0 | 0.09±0.0 | 0.09±0.01 | 0.12±0.0 | 0.15±0.03 | 0.14±0.0 | |
| Bone ash weigh (g) | | | | | | | | | | | | |
| ACRB | 0.002±0.0003 | 0.004±0.0004 | 0.01±0.0008 | 0.014±0.001 | 0.02±0.0005 | 0.02±0.0007 | 0.03±0.002 | 0.05±0.002[b] | 0.06±0.003[b] | 0.09±0.008[b] | 0.1±0.009[b] | 0.0001 |
| COBB | 0.002±0.0003 | 0.0044±0.0002 | 0.01±0.0005 | 0.02±0.0008 | 0.02±0.0004 | 0.02±0.001 | 0.04±0.002 | 0.07±0.005[a] | 0.12±0.008[a] | 0.21±0.01[a] | 0.30±0.02[a] | |
| FFDW (g) | | | | | | | | | | | | |
| ACRB | 0.006±0.0003 | 0.02±0.001 | 0.03±0.003 | 0.04±0.005 | 0.07±0.001 | 0.08±0.002 | 0.10±0.005 | 0.14±0.007[b] | 0.18±0.009[b] | 0.24±0.02[b] | 0.28±0.02[a] | 0.0001 |
| COBB | 0.007±0.001 | 0.02±0.001 | 0.4±0.002 | 0.06±0.005 | 0.08±0.003 | 0.09±0.003 | 0.13±0.006 | 0.21±0.02[a] | 0.33±0.02[a] | 0.60±0.03[a] | 0.78±0.06[b] | |

[1]Data were determined on indicated embryonic (E) and post-hatch (D) days.
[2]Values for bone ash were calculated as ash weight divided by fat-free dry weight multiplied by 100.
[3]Values for ash concentration were calculated by dividing ash weight by volume of the bone.