EFFECT OF 25-HYDROXYCHOLECALCIFEROL SUPPLEMENTATION ON BROILER CHICKEN SKELETAL MUSCLE GROWTH AND DEVELOPMENT

By

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ABSTRACT

To determine the effect of 25-hydroxycholecalciferol (25OHD3) on broiler chicken skeletal muscle growth and development, day-old male Ross 708 broiler chickens (Aviagen Group, Huntsville, AL; n = 150) were randomly assigned to one of two corn and soybean meal-based diets. The control diet (CTL) was formulated to contain 5,000 IU D_3/kg of diet, while the experimental diet (25OHD3) contained 2,240 IU vitamin D_3/kg diet + 69 µg 25OHD3/kg diet. Chickens were housed in Start Grow cage units (Alternative Design Manufacturing and Supply, Siloam Springs, AR) and allowed *ad libitum* access to feed and water. At the end of each seven-day period, ten birds from each treatment were harvested. Two hours prior to harvest, birds were injected intraperitoneally with 5'-bromo-2'deoxyuridine (BrdU) to facilitate labeling of mitotically active cells. Prior to harvest, blood was collected from each bird. The left *Pectoralis major* (PM) and *Biceps femoris* (BF) muscles were removed and weighed to determine muscle yields, while the right PM and BF were processed for cyrohistological determination of skeletal muscle fiber cross-sectional area and enumeration of Myf-5+ satellite cells, and mitotically active cells. Average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (G:F) were determined weekly. Circulating 25OHD3 concentrations were greater in 25OHD3-fed birds on d 7, 14, 21, 28, 35, 42, and 49 when compared to CTL ($P < 0.001$). No differences among treatment groups were observed in ADG, ADFI, G:F, PM and BF muscle mass, or the number of mitotic active cells. However, broiler chickens fed 25OHD3 tended to have greater Myf-5+ satellite cell density ($P = 0.09$) on d 14, greater total nuclear density ($P = 0.05$) on d 28, and greater

PM muscle fiber cross-sectional area ($P = 0.09$) on d 49 compared to their CTL counterparts. These results suggest that feeding 25OHD3 in place of vitamin D_3 may impact broiler chicken skeletal muscle growth in a satellite cell-mediated manner.

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LIST OF ABBREVIATIONS

1αOHase	1-α-hydroxylase
1,25OHD3	1,25-dihydroxycholecalciferol
25OHD3	25-hyroxycholecalciferol
ADFI	Average daily feed intake
ADG	Average daily gain
BF	*Biceps femoris*
BrdU	5'-bromo-2'-deoxyuridine
CTL	Control
D3	Vitamin D3, cholecalciferol
G:F	Gain to feed ratio
PM	*Pectoralis major*
VDR	Vitamin D receptor

CHAPTER I

INTRODUCTION

Without a doubt, the poultry industry represents the greatest improvement in animal agriculture in the last 50 years. Thanks to superior genetics, nutrition, and management, the modern broiler is five times heavier, nearly fifty percent more feed efficient, and costs the same per pound as a broiler chicken in 1957 (Havenstein et al., 2003). It is estimated that between seven and twenty three percent of the increase in broiler chicken body weight since 1957 can be attributed to improvements in nutrition (Havenstein et al., 2003, Havenstein et al., 1994). Among one of the most influencial strides in poultry nutrition was the addition of vitamin D to poultry diets. Vitamin D was first introduced into poultry diets when chickens began to be housed indoors during the winter months to supplement for the lack of natural sunlight and to prevent rickets in growing birds (Ewing, 1947). Today, broilers grow at such fast rates that vitamin D is absolutely essential to prevent structural problems caused by insufficient bone mineralization. However, the addition of vitamin D to poultry diets has also brought welcome and unforeseen added benefits; including improved immune function and embryonic growth and development (Aslam et al., 1998, Chou et al., 2009, Stevens et al., 1984b)

The connection between vitamin D metabolites, and poultry skeletal muscle growth and development has been established by several studies (Angel et al., 2005; Bar et al., 2003; Chou et al., 2009; Fritts and Waldroup, 2003; Yarger et al., 1995a; Yarger et al., 1995b). However, the mechanism by which vitamin D and its metabolites influence skeletal muscle growth remains unclear.

Research and innovation have been the driving forces in the phenomenal improvements in the poultry industry over the last 50 years. In order to ensure improvements in the future, it is imperative that potential improvements in poultry nutrition continue to be researched and their effects on the growing birds continually be elucidated in order to maintain the industry's precedence in producing a profitable, efficient product that meets the needs of an increasingly busy and convenience oriented consumer base. Therefore, the objective of this study was to determine the effect of supplemental 25-hydroxyvitamin D3 on broiler chicken growth performance, performance, vitamin D status, and skeletal muscle growth and development.

CHAPTER II

REVIEW OF LITERATURE

In 2010, the United States broiler industry produced 16.7 billion kg of meat for a total of 45 billion retail dollars (USDA, 2011). As such a major component of the consumer meat supply, it is important to evaluate every opportunity to develop a higher quality, more affordable, efficiently produced product. Studies investigating the effect of vitamin D and its metabolites on broiler chickens indicate that vitamin D may play a role in skeletal muscle growth performance. Therefore, this review contains a brief discussion of the poultry industry and the impact of vitamin D and its metabolites on broiler chicken production.

THE POULTRY INDUSTRY

The poultry industry includes the production of chicken, turkey, goose and duck. The industry is dominated by multi-billion dollar vertically integrated chicken companies. These companies contract with individual farmers to grow birds under strict company established guidelines. The chicken companies can be categorized into three main sections: primary breeders, broiler producers and egg producers. The former two segments pertain to this paper and are therefore described below. The later will not be discussed, although 25-hydroxycholecalciferol (25OHD3) has also been suggest to have significant implications in improvements of egg production (IRS, 2002).

Primary breeder companies are the first step in poultry production and provide the majority of the genetics in the broiler industry. They are heavily invested in research and genetic engineering and go to great lengths to constantly improve their genetics in order

to provide broiler companies with faster growing, more feed efficient birds. Eggs produced from primary breeder flocks are often hatched and sold as breeder chicks for large-scale chicken production. Thus, primary breeders produce the grandparent stock of the broiler industry (IRS, 2002).

Most major chicken producers purchase chicks from a primary producer, which are raised in pullet houses until approximately 20 weeks of age. At this time hens are moved to breeder houses where they begin a 40-week breeding cycle. By the fourth week of this cycle, hens are producing one egg every other day. At the end of the laying cycle, breeders are typically harvested. It is possible to allow the birds to undergo a molt and second laying cycle, although decreased egg production rarely justifies the cost of a second cycle (IRS, 2002).

Eggs collected during the laying cycle are delivered to hatcheries where they undergo a 21-day incubation. Upon hatching they are placed in grow-out facilities, an open spanning building typically 122 m by 12 m. Feed, water, medication and temperature are all highly regulated in order to maximize feed efficiency and growth (IRS, 2002).

Three types of broiler flocks are grown: mixed sex, all male and all female. Mixed flocks are typically grown to approximately 1.7 to 2.0 kg. Female flocks are raised when a small finished weight is desired as the females tend to outperform their male counterparts in early stages of growth. Male flocks are raised when a larger finished weight is desired due to their increased growth potential (IRS, 2002).

Today's fully integrated broiler production system is much more efficient than the industry was 50 years ago. Thanks to the foresight of important scientists in the poultry

industry, the 1957 broiler stain has been preserved. Today, it is a very useful tool in measuring the genetic improvement in the broiler industry. The present day broiler is able to grow approximately three times as fast and consume half the feed when compared to its 1957 counterpart when placed on diets representative of the time (Havenstein et al., 2003).

VITAMIN D

Rickets is a diseases caused by insufficient calcium deposition in the bones of growing children or animas. The calcium deficiency causes softening of the bones which can lead to fractures and structural deformities (Feldman et al., 2011). With the industrial revolution in the 1800s, came an increased incidence of rickets, which spurred the search for a dietary cure for the disease and ultimately resulted in the discovery of vitamin D (Feldman, 2011). Although the discovery of vitamin D was not made for many years, several foods were known to prevent the disease. Cod liver oil was first prescribed as a treatment for rickets in 1824 (McCollum, 1957). In 1918, Mellanby induced the development of rickets in dogs which could be corrected by adding butterfat or cod liver oil to their diet (Mellanby, 1918). Although first assumed to be vitamin A, it was soon discovered that the prevention of rickets was caused by a novel substance, which was named vitamin D (McCollum et al., 1921). Several researchers observed that sunshine was just as effective at preventing rickets as dietary supplements (Chick et al., 1922; Hess and Unger, 1921). Thus, vitamin D became known as the "sunshine vitamin."

Over time, two forms of the vitamin were discovered. Vitamin D2, known as ergocalciferol, is synthesized by plants and vitamin D3 (D3), known as cholecalciferol, is synthesized by animals (Wolf, 2004). Vitamin D3 is a fat soluble vitamin that can be

obtained in the diet as previously mentioned, or synthesized in the skin through the photolysis of 7-dehyrocholesterol by UV light (Ceglia, 2009; DeLuca, 2008; Feldman, 2011).

Once vitamin D3 enters the blood stream, it is transported to the liver where nearly all of it is hydroxylated to 25OHD3 (Ceglia, 2009; DeLuca, 2008). 25-hydroxycholecalciferol is the circulating form of vitamin D and is a the most common indicator of vitamin D status in poultry (Soares et al., 1995). It is also considered the storage form of the vitamin and is found in much higher concentration in the body than the active hormone 1,25-dihydroxyvitamin D3 (1,25OHD3).

From the liver, 25OHD3 is transported to the kidney where it undergoes 1α-hydroxylation to form the active steroid-derived hormone 1,25OHD3 (Ceglia, 2009; DeLuca, 2008; Feldman et al., 2011). This reaction takes place in the inner mitochondrial membrane by cytochrome P450 enzymes (Angel et al., 2005; Fritts and Waldroup, 2003) possibly with the aid of a colocalized 1α-hydroxylase (1αOHase) associated protein (Chou et al., 2009b). In mammals, CYP27B1 is the cytochrome responsible for 1-alpha-hydroxylation activity (Hewison et al., 2000), however the specific P450 cytochrome in poultry has not yet been elucidated.

1,25-dihydroxycholecalciferol can elicit a transcriptional effect on tissues throughout the body, including skeletal muscle, and its production is therefore highly regulated (Capiati et al., 1999; de Boland et al., 1994; Simpson et al., 1985). Once in the blood stream, 1,25OHD3 diffuses through the cell membrane and binds to vitamin D receptor (VDR) in the cytoplasm. The VDR is part of the type II steroid nuclear transcription

family that contains classic steroid receptor structural motifs including a ligand-binding domain, nuclear localization sequence and highly conserved DNA binding domain. The activated ligand-receptor complex heterodimerizes with retinoid X receptor and is translocated to the nucleus where it binds to vitamin D response elements on target genes to regulate transcription (Rochel et al., 2001).

Traditionally, vitamin D is known for its role in calcium homeostasis and bone mineralization. In the small intestine, vitamin D increases calcium absorption by facilitating several calcium transport pathways. 1,25-dihydroxycholecalciferol stimulates the ATP-dependent calcium pumps, the opening of calcium channels and increases the transcription of calcium binding protein to facilitate calcium movement across the cell (Dusso et al., 2005). In the skeleton, vitamin D deficiency results in rickets in growing children or animals, and osteomalacia in the mature adult. Not only is 1,25OHD3 necessary to absorb the calcium needed for bone mineralization, but is also necessary for proper osteogenesis and osteoclastogenesis (Dusso et al., 2005; Panda et al., 2004). A negative feedback pathway also exists between 1,25OHD3 and parathyroid hormone (Feldman et al., 2011). Thus, vitamin D is instrumental in maintaining a tight regulation on calcium homeostasis.

More recently, vitamin D has also been associated with a myriad of other physiological functions including cell growth and differentiation, immunity, reproduction and skeletal muscle development (Stevens et al., 1984b; Yarger et al., 1995; Chou et al. 2009).

IMPLICATIONS OF VITAMIN D3 IN THE POULTRY INDUSTRY

Vitamin D changed the poultry industry when it was discovered that it could prevent rickets. Prior to this, chickens could not thrive in the winter due to lack of sunlight, making the industry seasonal and expensive. The fortification of poultry feeds with vitamin D allowed the broiler industry to operate year round, develop industrialized facilities and significantly lower production costs (Ewing, 1947). Today, vitamin D is an absolutely vital component of the growing broiler's diet, allowing it to absorb calcium in the gut. The modern broiler grows at such a fast rate that they are constantly challenged with absorbing enough calcium to meet the demands of their growing skeleton. For this reason, significant research dollars have been spent trying to optimize the use of vitamin D in the broiler industry in a manner that most efficiently support maximum growth and meat yield.

Much of the previous research conducted posed the question: which vitamin D metabolite is the safest and most effective supplement for poultry diets? Researchers found that the active metabolite, 1,25OHD3 was more effective at preventing rickets, tibial dyschondroplasia and was two to four times more biologically active than vitamin D3 (Boris et al., 1977; McNutt and Haussler, 1972; Norman and Wong, 1972). However, 1,25OHD3 caused a significant decrease in gain to feed ratio and had a much lower toxicity level compared to either 25OHD3 or D3 (Edwards, 1989). For these reasons, the industry shifted away from incorporating 1,25OHD3 into poultry feeds at an early stage, and focused on the differences between vitamin D3 and 25OHD3.

Between the mid 1970s and the present day, several other benefits of vitamin D metabolites have been elucidated using the poultry model. Among the most notable are

the increases in performance and skeletal muscle development, immunity and reproduction (Stevens et al., 1984b; Yarger et al., 1995; Chou et al. 2009)

It is not surprising that vitamin D would cause an increase in performance and skeletal muscle development as a secondary effect caused by increased bone mineralization and growth. Yet, VDR-null mice develop muscle weakness and atrophy that can be observed *independent* of low calcium or phosphorus levels (Endo et al., 2003). The target tissues of vitamin D are traditionally considered to be the kidney and intestine; however, multiple studies have shown that nearly all nucleated tissue specifically binds 25OHD3 (Dueland, 1983; Haddad and Stamp, 1974). In 1975, Birge and Haddad demonstrated that 25OHD3 repletion of vitamin D-deficient muscle caused a change in muscle metabolism leading to an increase in ATP content and protein production (Haddad and Birge, 1975). In addition, myopathic diseases such as osteomalacic myopathies have been linked to vitamin D deficiencies (Boland, 1986). This suggests that vitamin D may play a more direct role in muscle growth than previously thought.

In the poultry industry, muscle is considered a prime target for vitamin D (Boland et al., 1995) although, there is considerable debate over which vitamin D metabolite is more beneficial to muscle growthperformance. 25-hydroxycholecalciferol has been shown to have a higher binding affinity in the blood and skeletal muscle compared to D3. Moreover, the binding affinity for 25OHD3 is higher in skeletal muscle than serum, suggesting the active recruitment of 25OHD3 from the blood into the muscle (Boland, 1986; Boland et al., 1985; Haddad and Birge, 1975). The high binding affinity of skeletal muscle remains unexplained. In a large scale study, Yarger et al. (1995a; 1995b) reported an increase in body weight and *Pectoralis major* meat yield in chickens fed 25OHD3

compared to those fed D3. The authors postulated that the difference may, in part, be due greater intestinal absorption capacity for 25OHD3 compared to D3 (Bar et al., 1980). Fritts and Waldroup (2003), who conducted a similar study comparing the effects of D3 to 25OHD3 at various concentrations in the feed, later confirmed these findings. The study reported an increase in performance and body weight at d 21 and d 42 for broilers fed 25OHD3. However, the authors conceded that the majority of the differences observed between D3 and 25OHD3 were seen at low levels of dietary inclusion, and that little difference was observed when the birds were fed vitamin D dietary concentration comparable to industry practice at the time the study was conducted.

However, several studies have not observed a performance difference in vitamin D metabolites. In 2009, Chou et. al. reported no weight gain or feed efficiency differences between D3 and 25OHD3. Their findings agree with several other studies (Angel et al., 2005; Bar et al., 2003; Edwards, 1989). Nevertheless, the 2009 study reported a decreased small intestine weight and improved villus length to crypt depth ratio, suggesting that 25OHD3 may promote more efficient nutrient absorption, likely resulting in a lower maintenance requirement of the small intestine and perhaps more nutrients available for growth (Chou et al., 2009).

Interestingly, the *in vitro* studies conducted on chick myoblasts have not agreed directly with the results of live poultry studies. Many studies implicate 1,25OHD3 in the inhibition of cellular growth (Artaza et al., 2010; Boland, 1986; Dusso et al., 2005). The hormone has been shown to closely regulate cell proliferation and differentiation in other cell types including leukemia and hematopietic stem cells, chondrocytes and cardiac myocytes. It is thought that 1,25OHD3 represses genes involved in the cellular cycle, for

example the proto-oncogenes *c-myc, c-fos* and *c-jun* (Boland et al., 1995; Nibbelink et al., 2007). For this reason, vitamin D metabolites including both 1,25OHD3 and 25OHD3 have shown promising results in their potential as cancer therapy drugs (Barreto et al., 2000; Kunakornsawat et al., 2004; Nibbelink et al., 2007). However, because cultured myoblast experiments implicate vitamin D as growth inhibiting and live poultry studies indicate that vitamin D promotes growth and skeletal muscle development, it is clear that much more research in the field is necessary in order to fully elucidate the true mechanism of vitamin D.

Apart from its role in growth and skeletal muscle development, vitamin D has also been implicated in the immune response and in reproduction. In a 2009 study conducted by Chou et al., which was discussed previously, 25OHD3 tended to decrease serum IgG and IgA concentrations in healthy birds when compared to D3. This suggests that 25OHD3 may allow the bird to be more selective in nutrient partitioning than D3. Moreover, Aslam et al. found that vitamin D deficiency depresses cell-mediated immunity without affecting humoral immuntity (Aslam et al., 1998).

Vitamin D can also have serious implications to reproduction and embryonic development. It is well established that increasing vitamin D status in hens results in a greater number of eggs produced, that are heavier and have thicker shells compared to hens on a low vitamin D diet (Stevens et al., 1984b). Maternal vitamin D status is also directly related to 1αOHase activity in the kidneys of poults. Poults of mothers fed diets low in vitamin D do not establish 1αOHase activity comparable to their high vitamin D counterparts until 8-18 days of age. Poults of low vitamin D mothers also exhibit lower

bone ash, smaller body weighs and a higher incidence of developing rickets compared to poults of high vitamin D mothers (Stevens et al., 1984a).

MYOGENESIS AND THE SATELLITE CELL

Satellite cells play a primary role in postnatal muscle growth. First observed by Alexander Mauro, satellite cells were appropriately named for their position just outside the muscle fiber cell membrane but inside the basal lamina (Mauro, 1961). Chickens have been used as a model of satellite cell related muscle development since the cells were first described in 1961.

Prenatally, the process of muscle development, myogenesis, can be characterized by four major phases; determination of cells to myogenic lineage, proliferation of muscle precursor cells, differentiation and subsequent fusion into myofibers, and final maturation of the muscle tissue. In the embryonic phase, somites migrate to sites of future muscle formation. This layer of cells is called the myotome. The commitment of somites to the myogenic lineage is referred to as determination, and is triggered by the expression of transcription factors MyoD and Myf-5. These muscle precursor cells, called myoblasts, undergo multiple rounds of proliferation. A high myoblast cell density triggers the expression of MRF4 and myogenin by the myoblast population, which causes differentiation into myotubes (Gerrard and Grant, 2006). The myoblasts first stop dividing and begin to orient themselves in a somewhat linear fashion. In a chick embryo, the first alignment of myoblasts is observed on day nine of incubation in the hind limbs and day 12 of incubation in the breast (Stojanović et al., 2009). Cell membranes of aligned myoblasts fuse to form primary myotubes. Once fused together, nuclei within these cells are terminally differentiated and can never re-enter the cell cycle. Primary

myotube formation is followed a second wave of myogenesis in the fetal phase, resulting in secondary myotube formation (Gerrard and Grant, 2006).

In the resulting mature muscle, only satellite cells remain undifferentiated and unfused with existing myotubes. By the time of hatching, the muscle is fully developed. Post-hatch skeletal muscle growth occurs exclusively through an increase in myofiber size with no increase in myofiber number (Remignon et al., 1995). By fusing with an existing myotube, the satellite cell donates its nucleus to the myofiber, leading to increased nuclear content and greater potential for protein production (Lipton and Schultz, 1979; Schultz and Lipton, 1982). Therefore, the satellite cell, as the only mitotically active muscle cell found in post-hatch chicks, is therefore very important in hypertrophic growth.

MYOGENIC REGULATORY FACTORS

The transcription factors discussed above are, as a group, referred to as myogenic regulatory transcription factors (MRF). They are members of the basic helix-loop-helix superfamily and are important regulators of muscle development. The MRF: Myf-5, MyoD are responsible for the determination of muscle precursor cells to the myogenic cell lineage (Megeney et al., 1996; Shibata et al., 2006). Myogenin and MRF4 are responsible for the differentiation of myoblasts into mature, multinucleated myofibers (Megeney et al., 1996; Shibata et al., 2006).

Myostatin is a transcription factor that negatively regulates skeletal muscle growth and is known to decrease satellite cell activation (McCroskery et al., 2003). Mice lacking the

Myostatin gene grow to be two to three times heavier and have marked increase in muscle mass when compared to their wild type counterparts (McPherron and Lee, 1997).

SATELLITE CELL TRANSRIPTIONAL SIGNATURE

As knowledge of transcription factors and their influence on myogenesis has progressed, they have become instrumental in the identification of satellite cells for research purposes. By identifying transcription factors that are uniquely expressed in satellite cells, those transcription factors can be used to identify and characterize the satellite cell population both *in vitro* and *in vivo* (Yablonka-Reuveni et al., 2007).

Satellite cells could first only be identified using electron microscopy (Hawke and Garry, 2001; Mauro, 1961). Later, the satellite cell could be identified by its position underneath the myofiber basal lamina, with the assistance of antibodies against cell surface and basement membrane proteins (Charge and Rudnicki, 2004; Kuang et al., 2007; Zammit et al., 2006).

A breakthrough came in 2000, when a novel paper introduced the paired box (Pax) transcription factor, Pax7 (Seale et al., 2000). Satellite cells that expressed Pax7 were found to express the transcription factor uniformly throughout the life of the cell and produce daughter cells who also express Pax7 (Yablonka-Reuveni et al., 2007). This allowed for the detection of satellite cells using immunofluorescence staining. The Pax7 protein is a member of a large Pax family of transcription factors that are involved in cell type and organ determination during embryogenesis. Although a large population of satellite cells express Pax7, satellite cells have been detected in the early postnatal period of Pax7-null mice, suggesting that cells can survive without expressing Pax7

constitutively (Yablonka-Reuveni et al., 2007). The role of Pax7 in postnatal muscle is still to be determined. Another paired box transcription factor, Pax3 is also expressed by satellite cells but is specific to certain muscle types such as the diagram (Day et al., 2007; Relaix et al., 2006).

Satellite cells can also express Myf-5, which has been used for cell identification. As discussed previously, Myf-5 is an MRF involved in the determination of cells to the myogenic lineage. While it was originally believed that satellite cells did not express MRF, more recent studies have shown that the Myf-5 gene is active in satellite cells. However, there appears to be a small population of satellite cells that do not express Myf-5 (Beauchamp et al., 2000; Zammit et al., 2006).

MyoD expression is low in quiescent cells, but is rapidly up regulated at satellite cell activation and during proliferation. Once cells begin differentiation, they express myogenin similar to embryonic myogenesis (Yablonka-Reuveni et al., 2007). Satellite cell populations have been characterized by any number and combination of the markers discussed above.

CONCLUSION

Vitamin D is an important component of healthy metabolic function in the chicken. Traditionally, vitamin D has been associated with bone mineralization and calcium homeostasis. It has been particularly influential in the prevention of rickets and tibial dyschondroplasia in the growing broiler. Aside from its traditional roles, vitamin D shows exciting promise in other areas of physiological function including immunity, embryonic development as well as skeletal muscle growth and development. Further

investigation will be necessary to clarify the role played by vitamin D at its metabolites in the development and growth of skeletal muscle.

CHAPTER III

EFFECT OF 25-HYDROXYCHOLECALCIFEROL SUPPLEMENTATION ON BROILER CHICKEN SKELETAL MUSCLE GROWTH AND DEVELOPMENT

INTRODUCTION

The connection between vitamin D metabolites, and poultry skeletal muscle growth and development has been established by several studies (Angel et al., 2005; Bar et al., 2003; Chou et al., 2009; Fritts and Waldroup, 2003; Yarger et al., 1995a; Yarger et al., 1995b). Traditionally, vitamin D is known for its role in calcium homeostasis and bone mineralization. In the small intestine, vitamin D increases calcium absorption (Dusso et al., 2005). Yarger et al. (1995b) demonstrated an increase in breast meat yield in nine out of ten trials when birds were fed 25OHD3; the singularly hydroxylated vitamin D metabolite. The importance of vitamin D metabolites on skeletal muscle was strikingly apparent in VDR-null mice, which exhibit deregulation of myogenic regulatory factors resulting in a 20% decrease in skeletal muscle fiber diameter (Endo et al., 2003). Still, the mechanism by which vitamin D influences skeletal muscle in poultry remains unclear. Post-hatch skeletal muscle growth occurs exclusively through hypertrophy of pre-existing muscle fibers. Previous work has suggested that the satellite cell population is the only remaining source of muscle stem cells that retain the ability to proliferate and fuse with existing postnatal myofibers. Therefore, the satellite cell is the only means of increasing the nuclear content of the muscle fibers and thus increases the potential for protein accretion (Yablonka-Reuveni et al., 2007). It is therefore plausible that the satellite cell may be involved in the mechanism by which vitamin D influences skeletal muscle growth. Thus, our objective was to determine the effect of 25OHD3 on broiler chicken

growth performance, vitamin D status, satellite cell activity, as well as PM and BF meat yield, nuclear density and muscle fiber cross-sectional area.

MATERIALS AND METHODS

The Texas Tech University Animal Care and Use Committee reviewed and approved all animal procedures used in this study.

Birds and Housing

Day-old male Ross 708 broiler chicks (Aivagen Group, Huntsville, AL; n = 150) were transported from a commercial hatchery in Waco, TX to the Texas Tech University Animal and Food Sciences Animal Facility, Lubbock, TX within 24 hours of hatching. Chicks were randomly assigned to one of twelve poultry Start Grow cage units (Alternative Design Manufacturing and Supply, Siloam Springs, AR ; n = 12 or 13 per cage). Cage dimensions were 610 m wide by 610 cm deep by 380 cm tall and provided 372,100 cm^2 of floor space. Each cage was equipped with one nipple waterer and one feed tray. During the first week of the experiment, cage temperatures were maintained at 32.3°C and gradually decreased by 2.5°C each week until they reached an ambient temperature of 21.2°C where they were maintained for the duration of the study. Birds were housed with 23 h light per day and were allowed *ad libitum* access to food and water for the entire study.

Dietary Treatments

Upon arrival birds were randomly assigned to one of two corn and soybean meal-based dietary treatments. Vitamin D was provided at equal concentrations as vitamin D3 or a combination of D3 and 25OHD3. The control diet contained 5,000 IU D_3/ kg diet,

whereas the experimental diet contained 2,240 IU D_3/ kg diet + 69 µg of 25OHD3/ kg diet (ROVIMIX, Hy•D, DSM Nutritional Products Ltd, Basel, Switzerland). From d 0 to 14 of the experiment birds were fed a starter diet in mash form (Table 3.1) and from d 14 to 49 were fed a pelleted grower diet (Table 3.2). Both diets were formulated to meet or exceed the nutrient requirements of the NRC (1994) recommendations for broiler chickens at these stages of life. A sub-sample of birds (ten per treatment) was harvested each week for seven weeks (equivalent to d 7, 14, 21, 28, 35 42 and 49 of the study). After the d 42 harvest, birds from the original 12 cages (CTL n = 6 cage; 25OHD3 n = 6 cages) were combined to 9 cages (CTL n = 5 cages ; 25OHD3 n = 4 cages) to maintain equal bird density among treatments.

Growth Performance

Growth performance was monitored throughout the study. Average daily feed intake (ADFI) was measured daily on a per cage basis. Individual body weights were measured on all birds weekly to determine average daily gain (ADG). Gain to feed ratio (G:F) was determined on a per bird basis using the individual bird ADG and the mean cage ADFI. Average daily gain, ADFI and G:F were calculated for every harvest period as well as for the starter (d 0 to 1 and grower (d 14 to 49) period.

BrdU Injection and Harvest

At the end of each seven-day period and two h prior to harvest, birds were injected intraperitoneally with an aqueous solution containing 5'-bromo-2'-deoxyuridine (BrdU), a mitotic S-phase marker, at 100 µg/ g of body weight as described previously (Halevy et al., 2000). Two h post-injection, blood was collected and the birds were then euthanized by asphyxiation with CO_2 followed immediately by cervical dislocation. The left

Pectoralis major (PM) and *Biceps femoris* (BF) muscles were removed and weighed, while the right PM and BF were processed for cyrohistological analysis described below.

Analysis of Circulating 25-hydroxycholecalciferol Concentrations

Prior to harvest, blood was collected from each bird and used to determine circulating 25OHD3 concentrations. Blood was centrifuged (1,000 x *g*) for 15 min at 20°C. Serum was removed and stored at -80°C. Serum 25OHD3 concentrations were determined by Heartland Assays (Ames, IA) using a previously described radioimmunoassay (Hollis et al., 1993). Assays conducted by this laboratory have a lower detectable limit for 25OHD3 of 2.5 ng/ mL.

Cryohistology

Samples collected from the right PM and BF were frozen in Optimal Cutting Temperature compound (OCT; VWR International, West Chester, PA) in liquid nitrogen-cooled molds. Embedded muscle samples were stored at -80°C. Samples were stored at -20°C for 24 to 48 hours prior to cryohistological analysis.

Muscle samples were cryosectioned using a Lecia CM 1950 cryomicrotome. Ten micron-thick serial cross-sections were taken from each muscle and mounted on positively charged glass slides (Superfrost Plus; VWR International). Slides were stored at -20°C prior to immunofluorescence staining (IF).

Immunofluorescence Staining

Cryosections were fixed and stained as previously described (Day et al., 2009) with slight modifications. Briefly, cryosections were fixed in 4% paraformaldehyde (4% PFA; USB, Clevaland, OH) for 10 min at RT followed by a 10 min PBS (Invitrogen, Grand

Island, NY) rinse. Sections were then incubated in 5% Triton X-100 (Fisher Scientific, Fairlawn, NJ) to permeablize the cell membranes, followed by a 10 min incubation in 4 N hydrochloric acid (Fisher Scientific, Fairlawn, NJ) to facilitate the binding of anti-BrdU antibodies to the BrdU incorporated into the DNA of mitotically active cells. Slides were then incubated with 10% horse serum (HS; Invitrogen, Grand Island, NY), 2% bovine serum albumin (BSA; MP Biomedical, Solon, OH), in 0.2% Trition-X100 in PBS for 30 min at RT to block non-specific antigen binding. Next, sections were incubated in primary antibody overnight (15 h) at 4°C followed by a 30 minute incubation in secondary antibody. Primary and secondary antibodies are described in detail in the following section. Slides were rinsed with PBS three times for 5 min each directly following both primary and secondary incubations to remove any residual antibody. Cryosections were incubated in 1 µg per mL 4',6-diamidino-2-phenylindole (DAPI; Fisher Scientific, Rockforl, IL) for 1 min and rinsed with PBS twice. Slides were then coversliped using Aqua Mount mounting media (Lerner Laboratories, Pittsburg, PA) and thin glass cover slips (VWR Micro Cover Glass; VWR International), and left to dry at 4°C overnight. All cryosections were analyzed within 48 h of IF staining.

Primary and Secondary Antibodies

Primary antibodies used for the IF staining of PM and BF muscles were rabbit polyclonal anti-C terminus human Myf-5 (1:00 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-BrdU hybridoma cell supernatant (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA). Primary antibodies were detected using the secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG heavy and light chain (dilution 1:1000), and Alexa Fluor 546-conugated goat anti-mouse

IgG1 (dilution 1:1000), respectively. Satellite cells were identified using Myf-5 and mitotically active cells were identified using BrdU.

Imaging

Slides were imaged using an inverted fluorescence microscope (Nikon Eclipse, T*i*-E, Nikon Instruments Inc., Mellville, NY) with a UV light source (Nikon Intensilight, C-HGFIE, Nikon Instruments Inc.) using a 20X working distance magnification. Images were captured by a CoolSnap ES^2 monochrome camera and artificially colored by NIS Elements Imaging software (Basic Research, 3.10; Nikon Instruments Inc.) Five random images were taken from each muscle of each bird. Every image was taken at 200x magnification and contained 0.1485 mm^2 area (0.45 mm X 0.33 mm). Within an image, the cross-sectional area was measured on each muscle fiber. DAPI is a fluorescent DNA stain that was used to determine total nuclear density. Myf-5+ satellite cells, BrdU+ mitotically active cells, and mitotically active satellite cells (Myf-5+ and BrdU+) were also identified.

Statistical Analysis

Statistical analysis was performed using the MIXED procedures of PC SAS, version 9.2 (SAS Inst., Inc., Cary, NC). Treatment was the fixed effect and bird was included as the random effect when analyzing nuclear densities, Myf-5+, BrdU+ or Myf-5+ and BrdU+ cell densities. Bird served as the experimental unit and the Kenward-Roger adjustment was used to correct the degrees of freedom. Means were separated using the PDIFF option and were declared significant when $P \leq 0.05$. Differences among treatment means were considered a trend when $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

While there has been a considerable research conducted on the effect of 25OHD3 on the broiler chicken (Angel et al., 2005; Bar et al., 2003; Bar et al., 1980; Chou et al., 2009; Coto, 2010; Edwards, 1989; Fritts and Waldroup, 2003; Yarger et al., 1995a; Yarger et al., 1995b), it remains unclear whether 25OHD3 supplementation directly enhances skeletal muscle growth through a satellite cell-mediated mechanism. Studies conducted in poultry suggest that 25OHD3 may impact breast meat yield (Yarger et al., 1995b). Satellite cells have the ability to produce differentiating progeny that fuse with existing muscle fibers (Yablonka-Reuveni et al., 2007). Postnatally, this is the only way to increase the DNA content within an existing muscle fiber, thereby increasing the fiber's potential for muscle hypertrophy. Therefore, it is possible that the difference observed in broiler chicken breast meat yield in 25OHD3-supplemented broilers could be the direct effect of satellite cell-mediated muscle hypertrophy. Thus, we conducted an experiment to determine whether the replacement of 2,760 of the total 5,000 IU of D3 with 25OHD3 had a direct effect on circulating 25OHD3 concentration, performance characteristics, Myf-5+ satellite cells, and skeletal muscle hypertrophy in a commercially available broiler chicken line.

Circulating Serum 25-hydroxycholecalciferol Concentration

Circulating serum 25OHD3 concentration was measured before treatments were applied (CTL, n = 5; 25OHD3, n = 5) and weekly for seven weeks thereafter (CTL, n = 10 birds per week; 25OHD3, n = 10 birds per week). Concentration of 25OHD3 did not differ between treatment groups on d 0 (CTL = 19.7 ng per mL; 25OHD3 = 19.5 ng per mL; $P = 0.97$; Figure 1). However, broiler chickens fed 25OHD3 had significantly

greater serum concentrations of 25OHD3 at d 7, 14, 21, 28, 35, 42 and 49 ($P < 0.001$) when compared to chickens fed the CTL diet (Figure 1). These results are consistent with previous work feeding 25OHD3 to broiler chickens (Horst et al., 1981; Hughes et al., 1977; Yarger et al., 1995b). The mechanism by which 25OHD3 supplementation causes such dramatic increases in serum 25OHD3 can, in part, be attributed to more efficient absorption of 25OHD3 in the upper small intestine (Bar et al., 1980) and an increased half-life compared to D3 (Stamp et al., 1977).

Growth Performance

Average daily gain, ADFI and G:F were evaluated to determine the effect of 25OHD3 on broiler chicken growth performance. No significant differences were found in ADG or G:F between the CTL and 25OHD3 fed broiler chickens (Table 3.3). Control birds had a significantly higher feed intake over the second and sixth weeks of the trial (d 7 to 14 and d 35 to 42, respectively,) while the 25OHD3-fed birds had a significantly higher feed intake over the third and fourth weeks of the trial (d 14 to 21 and d 21 to 28, respectively). However, over the entire seven-week trial, there were no significant differences in ADFI between CTL and 25OHD3 fed broilers ($P = 0.31$). These results therefore indicate that 25OHD3 does not effect growth performance of broiler chickens and are in agreement with the previous works of Roberson (1999), Bar et al. (2003), Angel et al. (2005), and Chou et al. (2009). In contrast, other studies have reported an increase in BW and feed efficiency as a result of feeding 25OHD3 (Fritts and Waldroup, 2003; Yarger et al., 1995b). Although, in the study conducted by Fritts and Waldroup six different levels of both D3 and 25OHD3 ranging from 125 IU to 4,000 IU were fed to broiler chickens. They reported a significant increase in BW of 25OHD3-fed birds at low

supplementation levels, but no difference in BW at the supplementation level used in this study or typical industry vitamin D3 levels.

According to the performance objectives published by Aviagen (2012) for the Ross 708 broiler line, the birds in this study performed above expected during the first two weeks of the study. Broiler chicks are expected to have an ADG of 19.4 g and 21.7 g for the first and second weeks of life, respectively. Regardless of treatment, chicks in this study gained an average of 26.4 and 35.0 g per day from d 0 to 7 and d 7 to 14, respectively. Performance objectives were below the expected values for two weeks following d 14 of the study, when the birds were switched from a mashed starter diet to a pelleted grower diet. During the third and fourth week of the study the expected ADG for the 708 strain was 62.4 and 82.3 g, respectively. The birds in this study gained 20.0 and 37.7 g daily during weeks three and four, respectively. The decrease in ADG was most likely due to the switch from a mashed to a pelleted feed. By the fifth week of the study the birds performed as expected when compared to the published ADG. Average daily gains were lower than expected over the sixth and seventh week of the trial. The birds were expected to gain an average of 101.1 and 100.1 g per day during the sixth and seventh weeks of like. The birds in this trial gained an average of 67.5 and 73.5 g per day during the sixth and seventh week of the trial regardless of treatment. The decrease in ADG over the sixth and seventh weeks of the trial compared to the expected ADG could have been due to being housed in cages as opposed to being on the floor. Although birds performed below expected during some time periods of this study, the trends were similar for both treatments.

Pectoralis major and Biceps femoris Muscle Developmental Characteristics

Several factors were evaluated to determine the effect of 25OHD3 on muscle development within the PM and BF including satellite cell density, overall mitotic activity and mitotic activity of Myf-5+ satellite cells, nuclear density, fiber cross-sectional area, and muscle weight.

Myf-5 was used in this study to mark a subpopulation of satellite cells. There was a tendency ($P = 0.09$) for the PM of 25OHD3-fed birds to have a higher Myf-5+ satellite cell density when compared to the PM of CTL-fed birds. No other differences were observed at any time point during the study in either the PM of BF. However, it is well established that satellite cells express a variety of transcription factors unique to their cell lineage (Beauchamp et al., 2000; Relaix et al., 2006; Zammit et al., 2006). Therefore, data from this study can only be used to draw conclusions from the Myf-5+ satellite cell population and may not be indicative of the satellite cell population as a whole. Nevertheless, these results indicate that 25OHD3 supplementation may impact PM development in a satellite cell-mediated manner, but did not appear to have to same effect on the BF.

BrdU was used to mark cells traversing the S-phase of mitosis. There were no treatment differences observed in BrdU+ cell density (Table 3.4, 3.5). Little is known about the potential of 25OHD3 to affect mitotic activity *in vivo*. Primary cultures supplemented with 25OHD3 show a decrease in proliferation in human and dog epithelial cells (Barreto et al., 2000; Kunakornsawat et al., 2004). Previous literature indicates that other vitamin D metabolites exhibit a similar effect on broiler chicken satellite cells in culture (Boland et al., 1985; de Boland et al., 1994). However, increased cellular mitotic

activity could explain increases in breast meat yield seen in 25OHD3 fed broiler chickens reported previously (Soares et al., 1995; Yarger et al., 1995b).

Cells which are positive for both Myf-5 and BrdU indicate the Myf-5+ satellite cell subpopulation which is mitotically active. Differences in mitotically active satellite cells would indicate an increased potential for muscle hypertrophy. However, there were no differences observed in this study between treatments at any time point (Table 3.4, 3.5).

There were no significant differences in PM or BF weights between treatments at any time points (Table 3.6). This is in contrast to previous work showing that 25OHD3 supplementation results in an increase in breast meat yield (Yarger et al., 1995b). Similarly, there were no significant differences in nuclear densities between treatments on d 7, 14, 21, 35, 42, or 49. However, on d 28 the 25OHD3-fed birds exhibited a higher nuclear density in the PM when compared to CTL birds (CTL = 2,009 cells per mm^2; 25OHD3 = 2,251 cells per mm^2, $P = 0.05$). On d 49, 25OHd3-fed birds tended ($P = 0.09$) to have larger muscle fiber cross-sectional areas in the PM when compared to CTL-fed birds. Although the numerical increase in muscle fiber cross-sectional area did not result in an increase in breast muscle weights in this study at any time point (Table 3.6), an increase in fiber area could, in part, explain the increase in breast meat yield observed previously (Yarger et al., 1995b). No other treatment differences were observed in muscle cross-sectional area at any time point.

Overall, the results of this study indicate that supplementing broiler chickens with 25OHD3 does not result in increased performance when compared to dietary vitamin D supplementation alone. However, there was a tendency for the PM of 25OHD3 fed birds

to have more Myf-5+ satellite cells at d 14, followed by a significantly greater nuclear density at d 28 and a trend toward larger muscle fiber cross-sectional areas at d 49. Taken together, these data indicate that 25OHD3 may impact muscle development in a satellite cell-mediated manner. No differences in PM or BF muscle weights observed in this experiment. However, the impact of 25OHD3 we observed on the Myf-5+ satellite cell population combined with the increase in muscle fiber cross-sectional area does provide evidence toward the mechanism by which the increase in PM yield observed by Yarger et al. (1995b) occurred.

CHAPTER IV

CONCLUSION

Vitamin D has been a staple supplement in the broiler diet since the commercialization of the poultry industry. There has been continual debate concerning which vitamin D metabolite is the most beneficial to the commercial producer. In terms of muscle growth and development in the broiler chicken, some studies have indicated increased muscle capacity and growth performance in broiler chickens fed 25OHD3 (Fritts and Waldroup, 2003; Yarger et al., 1995a; Yarger et al., 1995b) while other studies have found no difference between vitamin D metabolites (Angel et al., 2005; Bar et al., 2003; Chou et al., 2009). Still, the amount of information regarding the mechanism by which 25OHD3 influences muscle growth and development remains limited. Understanding the satellite cell's role in the muscle growth of 25OHD3 supplemented broilers compared to unsupplemented ones is important in understanding the mechanism by which 25OHD3 influences muscle growth.

In terms of growth performance, we did not observe any differences as a result of replacing nearly half of the D3 in the diet with 25OHD3. Nor were there any differences in PM or BF muscle weights, the total number of proliferative Myf-5+ satellite cells, or mitotically active cells.

However, broiler chickens supplemented with 25OHD3 tended to more Myf-5+ satellite cells at d 14, greater nuclear density at d 28 and a larger muscle fiber cross-sectional area in the PM on d 49 of the study. This may explain the differences in breast meat yield observed in pervious studies (Yarger et al., 1995b). However, no differences in meat yield from either muscle were observed among treatment groups in this study.

While these results provide evidence for the increase in PM yield observed by Yarger et al. (1995b), other researchers have also failed to observe a significant increase in PM muscle weight effect when 25OHD3 is included in the diet (Angel et al., 2005; Bar et al., 2003; Chou et al., 2009).

Further research in large scale commercial settings is necessary to determine if 25OHD3 supplementation can elicit a cellular response under industry conditions and if this response has an impact on muscle yields in the broiler chicken. Ultimately, this will determine if 25OHD3 is economically beneficial to the broiler chicken industry.

Table 3.1. Composition of broiler chicken starter diet (as-fed basis)[1]

	Treatment	
Item	CTL[2]	25OHD3[3]
Ingredient, %		
Corn	56.6	56.6
Soybean meal	33.5	33.5
Porcine meat and bone meal	4.0	4.0
Soybeal oil	2.9	2.9
Dicalcium phosphate	1.2	1.2
Limestone	0.6	0.6
Sodium chloride	0.5	0.5
DL-methionine	0.3	0.3
Choline chloride	0.1	0.1
Mineral premix	0.1	0.1
Vitamin premix	0.1	0.1
Lysine hydrochloride	0.1	0.1
Threonine	0.1	0.1
Calculated values		
Vitamin D, IU/kg of diet	5,000	2,240
25OHD3, μg/kg diet	0	69
Ca, %	1.0	1.0
P, %	0.5	0.5
ME, Mcal/kg of diet	3,050	3,050
CP, %	22.3	22.3
Lysine, %	1.4	1.4
Methionine, %	0.7	0.7
Tryptophan, %	0.3	0.3

[1] Birds were allowed *ad libitum* access to their assigned diet.

[2]Control-fed (CTL) broiler chickens (n = 10 per harvest period)

[2]25-hydroxycolecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

Table 3.2. Composition of broiler chicken starter diet (as-fed basis)[1]

	Treatment	
Item	CTL[2]	25OHD3[3]
Ingredient, %		
Corn	64.0	64.0
Soybean meal	25.9	25.9
Porcine meat and bone meal	5.0	5.0
Soybean oil	2.8	2.8
Dicalcium phosphate	0.8	0.8
Limestone	0.4	0.4
Sodium chloride	0.4	0.4
DL-methionine	0.3	0.3
Choline chloride	0.1	0.1
Mineral premix	0.1	0.1
Vitamin premix	0.2	0.2
Lysine hydrochloride	0.1	0.1
Threonine	0.1	0.1
Calculated values		
Vitamin D, IU/kg of diet	5,000	2,240
25-hydroxycholecalciferol, µg/kg	0	69
Ca, %	0.9	0.9
P, %	0.4	0.4
ME, Mcal/kg of diet	3,130	3,130
CP, %	19.9	19.9
Lysine, %	1.2	1.2
Methionine, %	0.6	0.6
Tryptophan, %	0.2	0.2

[1] Birds were allowed *ad libitum* access to their assigned diet.

[2]Control-fed (CTL) broiler chickens (n = 10 per harvest period)

[2]25-hydroxycolecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

Table 3.3. Effect of feeding 25-hydroxycholecalciferol on broiler chicken average daily gain (ADG), feed intake (ADFI) and feed efficiency (G:F)

Item	CTL[1]	25OHD3[2]	SEM	*P*-value
d 0 to 7[3]				
ADG, g	26.3	26.4	0.32	0.82
ADFI, g	34.8	35.1	0.18	0.19
G:F	0.757	0.754	0.01	0.84
d 7 to 14[4]				
ADG, g	49.9	48.7	0.86	0.29
ADFI, g	65.5	63.3	0.38	< 0.0001
G:F	0.762	0.769	0.01	0.72
d 14 to 21[5]				
ADG, g	13.7	16.3	2.24	0.38
ADFI, g	111.7	116.6	0.57	< 0.0001
G:F	0.123	0.140	0.02	0.48
d 21 to 28[6]				
ADG, g	17.0	20.7	2.79	0.32
ADFI, g	137.2	140.7	0.90	0.01
G:F	0.125	0.146	0.02	0.39
d 28 to 35[7]				
ADG, g	93.7	92.1	3.58	0.75
ADFI, g	165.30	168.6	1.30	0.06
G:F	0.566	0.547	0.02	0.50
d 35 to 42[8]				
ADG, g	73.5	61.5	5.31	0.11
ADFI, g	96.8	84.3	3.16	0.01
G:F	0.765	0.735	0.07	0.75
d 42 to 49[9]				
ADG, g	74.9	72.1	9.84	0.83
ADFI, g	258.8	267.7	9.67	0.50
G:F	0.295	0.273	0.04	0.70
d 0 to 14[10]				
ADG, g	38.2	37.6	0.55	0.41
ADFI, g	49.0	48.0	0.26	0.01
G:F	0.779	0.783	0.01	0.77
d 14 to 49[11]				
ADG, g	72.2	66.6	3.22	0.24
ADFI, g	117.8	114.3	3.18	0.44
G:F	0.614	0.584	0.03	0.40
d 0 to 49[11]				
ADG, g	72.2	66.6	3.22	0.24
ADFI, g	116.0	113.3	1.82	0.31
G:F	0.622	0.588	0.03	0.37

[1]Control-fed (CTL) broiler chickens

[2]25-hydroxycholecalciferol-fed (25OHD3) broiler chickens

[3]CTL n = 75; 25OHD3 n = 75

[4] CTL n = 65; 25OHD3 n = 63

[5] CTL n = 55; 25OHD3 n = 51

[6] CTL n = 45; 25OHD3 n = 40

[7] CTL n = 35; 25OHD3 n = 29

[8] CTL n = 23; 25OHD3 n = 20

[9] CTL n = 13; 25OHD3 n = 10

[10] CTL n = 65; 25OHD3 n = 62

[11] CTL n = 10; 25OHD3 n = 10

Table 3.4. Effect of feeding 25-hydroxycholecalciferol on Myf-5+ satellite cell density, mitotic activity, and satellite cell mitotic activity in broiler chicken *Pectoralis major* muscle

Item	CTL[1]	25OHD3[2]	SEM	*P*-value
d 7				
Myf-5+ per mm^2	1,209	1,270	224	0.85
BrdU+ per mm^2	179	163	23	0.31
Myf-5+;BrdU+ per mm^2	42	46	8	0.75
d 14				
Myf-5+ per mm^2	66	184	47	0.09
BrdU+ per mm^2	89	87	123	0.89
Myf-5+;BrdU+ per mm^2	3	7	2	0.50
d 21				
Myf-5+ per mm^2	219	164	50	0.45
BrdU+ per mm^2	42	44	7	0.82
Myf-5+;BrdU+ per mm^2	6	5	1	0.50
d 28				
Myf-5+ per mm^2	142	185	47	0.52
BrdU+ per mm^2	35	30	9	0.71
Myf-5+;BrdU+ per mm^2	6	13	5	0.34
d 35				
Myf-5+ per mm^2	120	113	30	0.87
BrdU+ per mm^2	18	13	3	0.23
Myf-5+;BrdU+ per mm^2	3	3	1	0.75
d 42				
Myf-5+ per mm^2	28	29	5	0.94
BrdU+ per mm^2	66	115	37	0.36
Myf-5+;BrdU+ per mm^2	3	2	1	0.44
d 49				
Myf-5+ per mm^2	192	137	51	0.40
BrdU+ per mm^2	211	140	73	0.50
Myf-5+;BrdU+ per mm^2	46	21	18	0.33

[1]Control-fed (CTL) broiler chickens (n = 10 per harvest period)
[2]25-hydroxycolecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

Table 3.5. Effect of feeding 25-hydroxycholecalciferol on Myf-5+ satellite cell density, mitotic activity, and satellite cell mitotic activity in broiler chicken *Biceps femoris* muscle

Item	CTL[1]	25OHD3[2]	SEM	*P*-value
d 7				
Myf-5+ per mm^2	1,015	1,151	178	0.59
BrdU+ per mm^2	126	131	18	0.84
Myf-5+;BrdU+ per mm^2	39	40	9	0.95
d 14				
Myf-5+ per mm^2	130	183	40	0.36
BrdU+ per mm^2	55	58	12	0.87
Myf-5+;BrdU+ per mm^2	6	5	2	0.58
d 21				
Myf-5+ per mm^2	116	53	47	0.35
BrdU+ per mm^2	269	224	123	0.80
Myf-5+;BrdU+ per mm^2	64	9	43	0.38
d 28				
Myf-5+ per mm^2	106	117	32	0.81
BrdU+ per mm^2	21	26	6	0.49
Myf-5+;BrdU+ per mm^2	4	5	1	0.82
d 35				
Myf-5+ per mm^2	134	100	29	0.42
BrdU+ per mm^2	88	117	29	0.49
Myf-5+;BrdU+ per mm^2	13	25	7	0.22
d 42				
Myf-5+ per mm^2	110	84	23	0.43
BrdU+ per mm^2	63	34	18	0.26
Myf-5+;BrdU+ per mm^2	7	6	2	0.94
d 49				
Myf-5+ per mm^2	118	97	49	0.76
BrdU+ per mm^2	201	175	70	0.79
Myf-5+;BrdU+ per mm^2	56	44	32	0.78

[1]Control-fed (CTL) broiler chickens (n = 10 per harvest period)

[2]25-hydroxycholecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

Table 3.6. Effect of feeding 25OHD3 on broiler chicken *Pectoralis major* muscle weight, muscle fiber cross-sectional area and nuclear density

Item	CTL[1]	25OHD3[2]	SEM	*P*-value
d 7				
Nuclear density, mm^2	3,959	3,940	58	0.93
Cross-sectional area, μm^2	854.33	858.63	374.77	0.99
Muscle weight, g	7.7	8.0	0.4	0.58
d 14				
Nuclear density, mm^2	2,832	2,762	158	0.75
Cross-sectional area, μm^2	1,474.70	1,476.26	96.48	0.99
Muscle weight, g	26.9	29.5	1.6	0.26
d 21				
Nuclear density, mm^2	2,334	2,446	76	0.31
Cross-sectional area, μm^2	2,368.02	2,568.24	150.66	0.35
Muscle weight, g	60.5	64.1	2.8	0.37
d 28				
Nuclear density, mm^2	2,009	2,251	85	0.05
Cross-sectional area, μm^2	3,667.98	3,695.18	216.20	0.93
Muscle weight, g	103.6	104.8	5.8	0.88
d 35				
Nuclear density, mm^2	1,987	1,951	101	0.82
Cross-sectional area, μm^2	4,679.88	4,722.59	286.82	0.92
Muscle weight, g	168.6	177.5	6.7	0.37
d 42				
Nuclear density, mm^2	1,736	1,883	112	0.36
Cross-sectional area, μm^2	5,634.88	5,733.94	439.82	0.88
Muscle weight, g	223.4	231.8	11.3	0.60
d 49				
Nuclear density, mm^2	1,799	1,708	109	0.56
Cross-sectional area, μm^2	6,172.54	7,077.43	362.93	0.09
Muscle weight, g	305.3	282.8	16.6	0.48

[1]Control-fed (CTL) broiler chickens (n = 10 per harvest period)

[2]25-hydroxycholecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

Table 3.7. Effect of feeding 25-hydroxycholecalciferol on broiler chicken *Biceps femoris* muscle weight, muscle fiber cross-sectional area and nuclear density

Item	CTL[1]	25OHD3	SEM	*P*-value
d 7				
Nuclear density, mm^2	3,549	3,696	167	0.54
Cross-sectional area, μm^2	526.85	736.21	154.29	0.33
Muscle weight, g	1.8	1.7	0.2	0.61
d 14				
Nuclear density, mm^2	3,176	3,249	211	0.80
Cross-sectional area, μm^2	1,416.82	1,172.13	120.33	0.17
Muscle weight, g	3.4	3.6	0.2	0.62
d 21				
Nuclear density, mm^2	2,236	2,278	63	0.64
Cross-sectional area, μm^2	1,943.89	2,201.89	111.80	0.11
Muscle weight, g	6.7	6.9	0.4	0.71
d 28				
Nuclear density, mm^2	2,086	2216	72	0.22
Cross-sectional area, μm^2	2,122.96	2,232.21	98.05	0.43
Muscle weight, g	10.8	12.2	0.9	0.26
d 35				
Nuclear density, mm^2	1,955	1,894	45	0.35
Cross-sectional area, μm^2	3,240.67	3,250.60	177.59	0.97
Muscle weight, g	20.8	21.5	1.9	0.79
d 42				
Nuclear density, mm^2	1,764	1,756	68	0.93
Cross-sectional area, μm^2	3,736.28	3,927.66	259.22	0.61
Muscle weight, g	21.5	25.9	1.9	0.79
d 49				
Nuclear density, mm^2	1,824	1,818	101	0.97
Cross-sectional area, μm^2	4,019.90	3,849.34	213.98	0.58
Muscle weight, g	35.3	29.3	2.3	0.11

[1]Control-fed (CTL) broiler chickens (n = 10 per harvest period)

[2]25-hydroxycholecalciferol-fed (25OHD3) broiler chickens (n = 10 per harvest period)

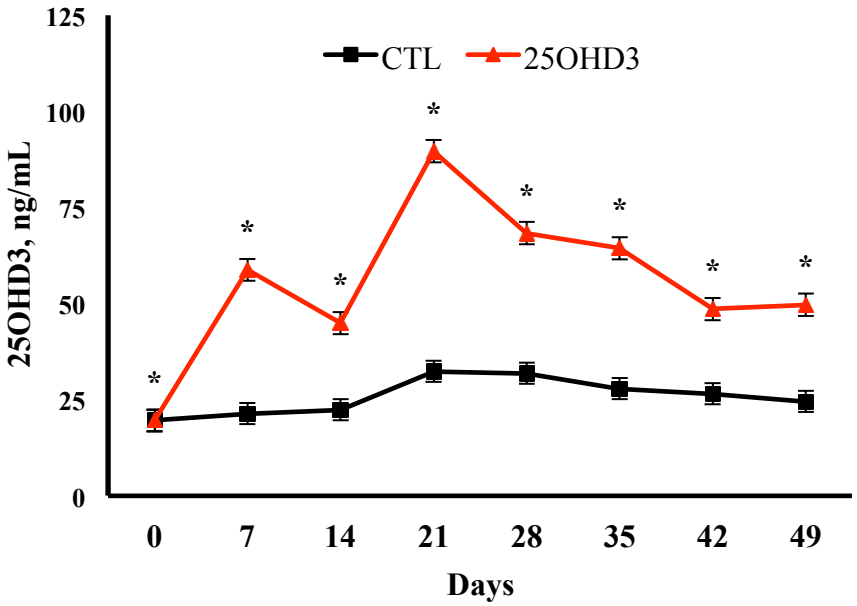

Figure 3.1 Circulating concentrations of serum 25-hydroxycholecalciferol (25OHD3) concentrations from control (CTL) and 25OHD3-fed broiler chickens from d 0 to 49 (n = 10 chickens per treatment per harvest period). *Indicates treatment means within a day which are significant ($P < 0.001$).

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