Abstract

WARREN, MATTHEW FRANCIS. Evaluation of Dietary Vitamin D₃ on Physiology and Vitamin D Metabolism in Chicken (Gallus gallus domesticus) Model. (Under the direction of Dr. Kimberly A. Livingston).

Dietary cholecalciferol (vitamin D₃) is important for calcium absorption for bone mineralization; however, vitamin D also has other metabolic functions relating to physiology. Vitamin D₃ is a fat-soluble vitamin that is stored in adipose tissue and other tissues such as the kidneys. Considering there are different vitamin D₃ metabolites that circulate through bloodstream and are functionally important for vitamin D metabolism, vitamin D can be considered a hormone. Vitamin D fortification in food, supplementation, and synthetics are three ways that humans can increase vitamin D intake without increasing sunlight exposure. Knowledge pertaining to vitamin D toxicity as a result of vitamin D supplementation is not well known. In poultry, vitamin D is nutritionally important for bone structure, eggshell quality and is also deposited in the egg yolk. Vitamin D’s nutritional function was also discovered using poultry as a model where vitamin D deficiency was observed to cause rickets in chicks. Examining how extreme dosage of vitamin D or synthetic vitamin D influence physiologic status in poultry has powerful implications to elucidate potential effects that need to be taken into considerations for human therapeutic treatments. My dissertation compares the dietary effects of vitamin D on various factors that influence physiological status in chicken, Gallus gallus domesticus.

First, I provide an overview of vitamin D metabolism and how it impacts an animal through specific minerals like calcium. I also describe vitamin D pathways and how those pathways influence physiological status. Second, I examine how
supplementation with a synthetic form of vitamin D₃, 1-α-hydroxycholecalciferol (1α), along with different levels of calcium inclusion, affects plasma vitamin D levels and genes influenced by vitamin D in starter and grower diets of broiler chicks. I observed that supplementing 1α on top of dietary vitamin D₃ in starter phase diets causes an increase in ionized blood calcium in broilers as calcium inclusion levels increase. However, 1α supplementation in grower phase had no effect on ionized blood calcium and cause a decrease in plasma 25-hydroxycholecalciferol (25-OH-D₃) concentration as calcium inclusion levels increase. Third, I explore production performance in aged laying hens fed super-doses of vitamin D₃. Hens fed diets with extreme levels of vitamin D₃ did not exhibit negative responses: their egg production was similar to hens given nutritionally sufficient levels of vitamin D₃. Dietary vitamin D super-doses did not affect eggshell strength or eggshell calcium which suggests that physiological costs for eggshell production are relatively static. However, when hens are vitamin D deficient which leads to brittle eggshells as previous research has observed because of poor dietary calcium absorption. Finally, I also examine vitamin D status in aged laying hens fed super-doses of vitamin D₃ and how their vitamin D metabolism and physiology was affected. Feeding extreme levels of vitamin D₃ to aged laying hens did not affect their bone calcium levels; however, calcium composition was relatively higher in the humerus bone compared to the tibia, regardless of dietary vitamin D₃ levels. Plasma vitamin D₃ and 25-OH-D₃ concentration increased relative to dietary vitamin D₃ intake. Plasma 24,25-dihydroxycholecalciferol (inactive vitamin D metabolite) concentration also increased, but at a much slower rate and exhibited an asymptotic relationship at dietary vitamin D₃ super-dose levels. This asymptotic relationship represents a novel finding:
there is a limit to converting circulating vitamin D levels, a fact which has important implications, namely that extreme dosages of vitamin D intake, albeit for improving bone health, can potentially have a risk of vitamin D toxicity because of limitations of in the animals’ ability to excrete excess vitamin D. My research expresses importance of dietary vitamin D₃ mechanisms with vitamin D metabolism and physiology and how future researchers can further elucidate these mechanisms to improve human and animal health.
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Evaluation of Dietary Vitamin D$_3$ on Physiology and Vitamin D Metabolism in Chicken
(*Gallus gallus domesticus*) Model

by
Matthew Francis Warren

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APPROVED BY:

__________________________                                  ___________________________
Kimberly A. Livingston     Peter R. Ferket
Chair of Advisory Committee

__________________________                                  ___________________________
Matthew D. Koci      Gavin C. Conant
Biography

Matthew F. Warren was from Lemoore, California as the youngest of two full siblings and one half-sibling. Being from a military family, Matthew was involved in Navy Junior Reserve Officer Training Corps (N.J.R.O.T.C.) all four years at his high school, Lemoore High School. He graduated from Lemoore High School in 2008 and completed the N.J.R.O.T.C. program with the ranking of Cadet Lieutenant Commander and as a Unit Department Head: Administrative Officer. Wanting to learn more about biology, Matthew attended a local community college, West Hills College Lemoore which awakened his passion for physiology, specifically, immunology. He graduated from West Hills three years later in May 2011 with two associates, Biology, and Liberal Arts-Math and Sciences. He transferred to the University of California-Davis (UC Davis) as an Animal Science major with a specialization in Avian Sciences.

An extremely positive experience thanks to the welcoming environment, people and wonderful professors, UC Davis awakened Matthew’s confidence where he developed a career interest in academia, to be a professor someday just like how his professors at UC Davis inspired him. Matthew conducted research in avian immunology as an undergraduate under Dr. Kirk C. Klasing and Dr. Annie J. King at UC Davis and was heavily involved with Minorities in Agriculture, Natural Resources and Related Sciences (MANRRS). Matthew graduated with his B.S. in June 2014 and traveled far to Auburn University to continue his education as a M.S. student in Biological Sciences under Dr. Wendy R. Hood.

Transitioning from working with birds to rodents, Matthew was exposed to a magnificent cohort and peers while at Auburn University that made the home-far-away-
from-home an everlasting experience from his first teaching experiences to hanging out with colleagues at the bars to talk about science. Matthew’s M.S. research was examining how dietary protein intake affected fecal and milk microbiota in rats. Matthew’s M.S. research introduced him to bioinformatics and thanks to Dr. Hood, developed his powerful interest in maternal effect on offspring development. Matthew completed his M.S. in August 2016 and started his Ph.D. in Nutrition at North Carolina State University a few weeks later. Starting with Dr. Peter Ferket, Matthew transitioned to Dr. Kimberly Livingston’s lab group to focus on comparative nutrition because Dr. Livingston was a former Ph.D. student of Dr. Klasing from UC Davis. Later during his Ph.D. program, Matthew learned about the importance of peer mentoring while at North Carolina State University which was one of the most significant experiences for him as a Ph.D. student. Matthew’s ultimate career goal is to be a professor at a university so he can help students unlock their true potential just his undergraduate professors did for him.
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My academic journey has been filled with magnificent lifelong experiences and not just because I studied abroad one summer as an undergraduate! I would like to thank my family starting with my parents, Charlie and Celia Warren, my siblings, Charles, Amy, and Virginia; my cousin Renzo Dampitan, and my best friends and also family to me, Jeremy and Josh Bowman (all those hours of playing video games will never be forgotten) and their mother Joan. They were all there for me during my years before I discovered my scientific interests and they all had the greatest pleasure watching me metamorphosize into the scientist I am today. I also would like to thank other best friends and family to me, Layth Bukhari, whom I met during my final year as an undergraduate at UC Davis; and Michael Lewis, my roommate when I transferred to UC Davis; both of them were tremendous help with contributing to my rich undergraduate experiences with all the outings and life talks. I also want to say a special thank you to my mentee and good friend, Catherine Lopez for being patient with me and really giving me a powerful experience with being a mentor to her over the years since we met when she was a freshman and I was a graduating senior at UC Davis. I also want to thank all the wonderful people I met during my time at UC Davis, CA&ES staff like Lili Bynes, students, faculty, etc.; all of your support was significant with getting me this far!

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as a graduate student, I did not know what to expect, aside from a whole new adventure
about 3,000 miles away from home! Dr. Hood was and still is one of the most influential
people I met in my life because she taught me so much about being critical in biology
and questioning to find out the truth. Dr. Hood teaching me how to collect milk from rats
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developed tremendous respect for mothers and how significant maternal effect is with
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Introduction

Vitamin D is a fat-soluble vitamin that also acts as a hormone to exert biological effects in animals with the most significant effect being the regulation of bone health (1). Cholecalciferol (vitamin D₃) is biogenically synthesized on skin exposed to sunlight, which has led to vitamin D being known as the ‘sunshine vitamin’ because its requirement can be met via sunlight exposure or from diet (2). Animals require vitamin D to trigger signaling cascades to lead to expression of proteins involved with intestinal calcium and phosphorus absorption (3, 4). Without vitamin D, neonatal animals will exhibit signs of rickets while mature animals will display osteomalacia or calcium deficiency (2, 5). Dietary sources of vitamin D are sought by animals to ensure vitamin D requirements are met in cases where sunlight exposure is limited, which is particularly important for domesticated animals housed indoors.

Our understanding of the biological significance of vitamin D₃ has expanded tremendously over the last seven decades since its discovery, with focus of characterization being on improving bone health (6). Studies explored how vitamin D affected how much dietary calcium and phosphorus were absorbed, leading to vitamin D’s biological relevance to bone health (4, 7). A tremendous discovery was how 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃) would bind to vitamin D receptor (VDR) and how VDR acted as a transcription factor for driving transcription of genes related to calcium and phosphorus absorption (8, 9). Although a plethora of vitamin D₃ research has been conducted, there is a lack of exploration of the effects of nontraditional sources of vitamin D, such as synthetic forms, and how they impact growth in young animals. There is also a gap of knowledge on how extremely high dietary levels can
affect elderly animals with regards to their bone health. There is value to empirical studies in helping to understand the impacts of dietary vitamin D₃ from a comparative nutrition perspective, allowing researchers to elucidate new outlooks with advancing our understanding of vitamin D’s significance with physiological systems.

Many vitamin D metabolites are biogenically synthesized in order to enact biological signaling in animals (10). There are also biochemically relevant synthetic forms of vitamin D that have been used. Primarily used as a supplement for renal cancer patients, 1-α-hydroxycholecalciferol (1α) has been valuable for helping humans maintain vitamin D status with and hence bone health in the presence of an inability to synthesize 1,25-(OH)₂-D₃ (11). The kidney is the primary organ in which 1,25-(OH)₂-D₃ is synthesized; it also is responsible for regulating synthesis and degradation of vitamin D (12) which can be an issue for 1α which bypasses this regulation. Although there were some studies in poultry that conclude there is no potential toxic effects of calcification due to 1α supplementation (13, 14), there is need to explore how 1α can impact growing animals with regards to calcium absorption and homeostatic balance to ensure there is no complications with growth.

Vitamin D supplementation is commonly utilized to maintain bone health in humans and animals throughout every life-stage. Traditionally, vitamin D supplementation is incorporated into many food sources for humans such as fortified milk, dairy products such as yogurt, or bread and other grain-products (15). Some supplementation can be taken in supplement form which is recommended for humans are greater risk of vitamin D deficiency. Animal diets are formulated to meet their vitamin D requirements with either rich natural sources of vitamin D or supplemented
with crystalline vitamin D to meet demands. Vitamin D toxicity is rarer, as is common for fat-soluble vitamins and unlike others such as vitamin A (16, 17). However, there is greater risk with high levels of 25-hydroxycholecalciferol supplementation (18). There is significance with examining how dietary super-doses of vitamin D can impact bone health and vitamin D metabolism in older animals to compare how such impacts can be related to humans, specifically older women in peri- and post-menopausal stages of life.

My aim through these studies is to explore dietary impacts of vitamin D₃ on vitamin D metabolism with emphasis on physiology using chicken (Gallus gallus domesticus) as the experimental model. I start with an overview of vitamin D metabolism and how poultry are a valuable model with furthering vitamin D research. This review also explores areas of high interest with vitamin D metabolism in humans and how the poultry model can be valuable with understanding mechanisms such as vitamin D toxicity. My first study addresses how 1α supplementation with differing levels of calcium inclusion impacts physiological biomarkers in growing broiler chicks. Maximizing growth potential is an important nutritional goal in animal agriculture and broiler chickens are significant because of the shorter time needed to grow them to market weight as compared to other meat yielding animals. Supplementing 1α in broiler chick diets has potential to improve calcium absorption for bone mineralization with hopes of reducing bone issues as the chicks age (13). However, 1α bypasses a negative feedback mechanism with vitamin D metabolism and can potentially cause hypercalcemia or soft-tissue calcification. Therefore, this study examines calcium inclusion impacts with 1α supplementation to determine 1α’s mechanistic implications with dietary calcium. The working hypothesis is: chicks fed diets with high level of
dietary calcium with 1α supplementation will be hypercalcemic because of 1α’s quicker response with metabolism compared to vitamin D₃. I test this hypothesis with broiler chicks at the two earliest phases of feeding to ascertain 1α’s effect on the chicks’ blood chemistry and gene expression of calcium binding genes from intestine. My second study explores how dietary super-doses of vitamin D₃ affects aged laying hens in production. Laying hens are important as a model for ascertaining bone mineralization and turnover because, like mammals, hens have a high physiological cost of calcium utilization due to their egg production (19). Examining how super-doses of dietary vitamin D₃ affects egg production of aged laying hens has implications with improving hens’ health by improving bone mineralization and maintaining optimal eggshell quality. Also, there have been a few studies that observed that hens fed dietary vitamin D₃ supplementation laid eggs with increased vitamin D₃ content in their egg yolks (20, 21), leading to value-added eggs for consumers needing food sources with greater vitamin D content. Lastly, using the same hens, I also explore how dietary super-doses of vitamin D₃ impacts vitamin D metabolism from a physiological viewpoint with analyses of gene expression of multiple vitamin D hydroxylases. Characterizing vitamin D super-dose effects on vitamin D metabolism has significance for women who take vitamin D supplements because there may be potential toxic effects associated with extreme vitamin D supplementation even though it would take an exorbitant amount to reach such levels. Furthermore, exploration of these vitamin D mechanisms in aged hens can lead to better understanding of vitamin D’s effect in geriatric animals and if supplementation is effective for improving health versus other options like using a more active vitamin D metabolite like 25-hydroxycholecalciferol.


Chapter 1

Implications of vitamin D in poultry nutrition with regards for humans and perspectives for the future

Manuscript in preparation with co-authors Peter R. Ferket and Kimberly A. Livingston to submit to Current Developments in Nutrition

Abstract

Addressing vitamin D insufficiency in humans requires better understanding of how excessive intakes can impact vitamin D metabolism. Supplements are a primary means for increasing vitamin D intake, but without a clear consensus on what constitutes vitamin D sufficiency, there is also risk of toxicity with taking supplements. Research with vitamin D supplementation using poultry can be expanded from production parameters to examining nutritional indicators such as vitamin D status. This review evaluates vitamin D metabolism and then discusses how poultry can be beneficial for advancing nutrition for humans. Chickens grown for meat are sensitive to vitamin D effects on bone because of their rapid growth rate. Laying hens fed vitamin D enriched diets can produce eggs with higher vitamin D content in egg yolk, which can be a good source of vitamin D for consumers of these eggs. Better understanding of vitamin D receptor in poultry will be significant for connecting vitamin D effects to physiological results observed in poultry. Future research with elucidating vitamin D toxicity can examine excessive supplementation-levels in diets for poultry to help ascertain how vitamin D status is affected by excessive dietary intake. Expanding
vitamin D-related research in poultry to include more nutritional aims in vitamin D status has great implications with developing better strategies to improve human health.

**Introduction**

Globally, many humans are at risk of vitamin D insufficiency because they lack sufficient sunlight exposure or dietary intake of the vitamin (1, 2). Vitamin D has significance as a nutrient because of its metabolic impacts with increasing calcium (Ca) absorption in animals (3). Vitamin D is also a fascinating nutrient because its most active form acts as a hormone to exert its biological effect (4). Traditionally associated with bones, vitamin D’s effects on the body are broad from immune function to reproduction (5). Although vitamin D research is one of the most funded in US (6), there are gaps in knowledge with understanding vitamin D in relation to excessive intakes in humans that could explored with animal models.

Domesticated poultry species like chicken (*Gallus gallus*) or turkey (*Meleagris gallopavo*) are becoming increasingly important as a food source for addressing world population demands (7). Chickens have been a powerful model for research because of their quick generation time and because they are easy to house large numbers, have sensitivity to light in their feeding behavior and hormonal regulation (8, 9), and are widely used for testing feed additive effects because of their quick response time that can be measured by growth performance (10-12). Rodents and swine are mammalian models that have similar digestive tracts to humans, but there are factors in which poultry are superior for vitamin D-related research. Rats exhibit a preference to ergocalciferol (vitamin D$_2$) when fed both D$_2$ and cholecalciferol (D$_3$) because they were...
observed to circulate higher serum concentrations of 25-OH-D$_2$ compared to 25-OH-D$_3$ (13). Swine have a simple hindgut like humans, but it takes about 6 months to reach sexual maturity, a problem which is important when comparing nutritional impacts at adult stage (14). By contrast, chickens are an excellent model for studying vitamin D$_3$ because they reach sexual maturity in a shorter time period and their preference to use D$_3$ over D$_2$ has implications for examining pharmacological effects. Poultry provide a dimension of not only being important for research for understanding metabolic mechanisms, but they are a food source via meat and egg production. Laying hens are frequently used as an ovarian cancer model (15, 16) and in examining geriatric effects (17).

The first half of this review focuses on an overview of vitamin D mechanisms with regards to vitamin D metabolism and metabolites and Ca metabolism and how vitamin D affects Ca in mammals and birds. The second half of this review surveys how vitamin D research in the poultry model can be a means for understanding vitamin D insufficiency in humans and managing it. Vitamin D toxicity is an area of research that has been experimentally observed in poultry to help understand how vitamin D supplementation needs to be carefully administered to avoid potential complications. We also discuss the significance of incorporating the vitamin D receptor (VDR) into future poultry vitamin D research that focuses on vitamin D mechanisms. The last section of this review is dedicated to describing how future research with vitamin D in poultry can help address high-impact areas of human nutrition.
Figure 1. Comparison of vitamin D precursors and their nutritionally relevant forms vitamin \( D_3 \) and \( D_2 \). In animals, when ultraviolet B rays or sunlight hits 7-dehydrocholesterol (7-DHC) on skin, then 7-DHC will undergo multiple reactions and be converted to cholecalciferol (vitamin \( D_3 \)). In fungi and microalgae, ergosterol undergoes the same pathway as 7-DHC to become ergocalciferol (vitamin \( D_2 \)).

Vitamin D metabolites and mechanisms

There are two major classes of vitamin D which are further subdivided into various forms that exert hormonal and physiological effects or inactive forms that are excreted.

Cholecalciferol (\( D_3 \)) is a major class of vitamin D and is synthesized de novo by animals (18, 19) (Figure 1). Ergocalciferol (\( D_2 \)) is another major class of vitamin D that is primarily synthesized by microalgae and fungi (20, 21). Animals can use both \( D_2 \) and \( D_3 \), but \( D_3 \) has been reported to have higher binding affinity to VDR (22). Vitamin D has
intact A, B, and D steroid rings because of photolysis of B ring of 7-dehydrocholesterol (7-DHC; pro-vitamin D₃) when compared to generic steroids (23). The 7-DHC’s structure allows the A ring to have the conformational capacity to undergo interconversion between two chair confirmations. D₃’s structure comprises of a saturated eight-carbon side chain, which is metabolically produced by photolysis of 7-DHC on skin surface exposed to ultraviolet irradiation (24). This review will focus on D₃ because of its bioavailability and involvement in humans and chickens.

Vitamin D₃ synthesis in animals is a quick process dependent on exposure to ultraviolet B light (UVB, 290-315 nm) against skin (1). Vitamin D synthesis begins when cholesterol is converted to 7-dehydrocholesterol (7-DHC; pro-vitamin D₃) in skin (Figure 2). UVB interacts with 7-DHC by inducing electrolytic ring opening because of light absorption of B ring’s 5,7-diene, which converts 7-DHC to pre-vitamin D₃. Pre-vitamin D₃ can photochemically convert to lumisterol, tachysterol, or D₃ by thermal isomerization. D₃ is a major product of thermal isomerization because it requires the least energy for thermal rearrangement. D₃ on skin that is exposed too long to sunlight will be degraded to 5,6-trans-vitamin D₃ which has no calcemic effects like lumisterol or tachysterol (25) Thermal rearrangement gives pre-vitamin D₃ and D₃ a state of equilibrium and reversibility; although this equilibrium favors D₃. In vivo synthesis in humans converts 10-15% of available 7-DHC to D₃ (24). Physical “sun-screen” properties of skin, such as clothing or darker skin, can reduce yield; environmental factors such as time of day, season, and latitude also affect yield (19, 26). The half-life of D₃ was measured as approximately 4 to 5 days (27, 28) in human serum. Vitamin D₃ can subsequently convert into different metabolite forms as illustrated in (Figure 3).
Figure 2. Biochemical reactions of 7-dehydrocholesterol (7-DHC) that leads to synthesis of vitamin D$_3$ and potential non-calcemic metabolites. When ultraviolet B (UVB) rays or sunlight hits 7-dehydrocholesterol (7-DHC) on skin, then 7-DHC is converted to pre-vitamin D$_3$ which is then converted to D$_3$, lumisterol-3, or tachysterol-3 by thermal isomerization. D$_3$ in skin can be converted to 5,6-trans-vitamin D$_3$ by UVB if it does not go into circulation.
**25-Hydroxylation**

Most vitamin D is taken up by liver and is hydroxylated at side chain C-25 to yield 25-hydroxycholecalciferol (25-OH-D₃). 25-OH-D₃ is the major circulating form of vitamin D, and it is synthesized in the liver of mammals (29-31) and the liver and kidneys of birds (32, 33). Hydroxylation of C-25 is facilitated by the enzyme 25-hydroxylase, which consists of at least six cytochrome P-450-dependent mixed-function oxygenases of varying binding affinities and capacities (34). The vitamin D binding protein (DBP) is a protein that binds to circulating 25-OH-D₃ in blood which causes 25-OH-D₃ to be a major circulating form of vitamin D. Circulating levels of 25-OH-D₃ along with DBP are valuable for vitamin D status with determining vitamin D deficiency or toxicity (35, 36). There is no consensus on what circulating level of 25-OH-D₃ is normal in humans, but it appears to be around 32-100 ng/mL with intoxication beginning above 150 ng/mL (1, 37). Adult chicken 25-OH-D₃ concentration was measured to be about 25.3 ng/mL and adult turkey was 18.2 ng/mL (38). Although 25-hydroxylase and 25-hydroxylation is predominant in liver, there has been studies that observed 25-hydroxylase expression in small intestine (33, 39). The half-life of 25-OH-D₃ is much greater than D₃ and is at least 18 d (40, 41)
Figure 3. Metabolic pathway of vitamin D₃ to its subsequent metabolite forms. Vitamin D₃ in circulation goes to the liver to be converted to 25-hydroxycholecalciferol (25-OH-D₃). 25-OH-D₃ can be further hydroxylated to either 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃) or 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃). When 1,25-(OH)₂-D₃ binds to vitamin D receptor (VDR), then biological effects are exerted through gene transcription. 1,25-(OH)₂-D₃ can also be further hydroxylated by 24-hydroxylase to 1,24,25-trihydroxycholecalciferol undergoes a series of reactions to ultimately become calcitroic acid, a water-soluble metabolite that is safely excreted in urine.
**1-Hydroxylation**

A further hydroxylation step of 25-OH-D₃ happens at the 1-C position to yield 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃). The kidney is the location of 1α-hydroxylase and it is where 1-hydroxylation of vitamin D occurs. This is also why vitamin D is considered a hormone because 25-OH-D₃ has to be transported in circulation to the kidney for further hydroxylation (4, 42). Most 1-hydroxylation occurs in kidney, but there are extrarenal tissue such as kidney and lymph nodes that express 1α-hydroxylase to convert 25-OH-D₃ to 1,25-(OH)₂-D₃ to denote its possible function as a modulator for vitamin D activities in those tissues (43, 44). 1,25-(OH)₂-D₃ has a fast turnover rate in serum of about 24 hr (45-47). There is also strict regulation of 1,25-(OH)₂-D₃ concentration (48), making 1,25-(OH)₂-D₃ a poor biomarker for vitamin D status (49).

Considering 1,25-(OH)₂-D₃ is the vitamin D form that exerts biological effect as a ligand for vitamin D receptor (VDR), 1α-hydroxylase activity is tightly regulated to avoid homeostatic imbalance (50). 1α-hydroxylase is primarily regulated by parathyroid hormone (PTH), FGF23, Ca, phosphate, and 1,25-(OH)₂-D₃ (51-54). Administering PTH stimulates renal production of 1,25-(OH)₂-D₃ (55, 56), though this PTH effect can be mirrored using cyclic adenosine monophosphate (cAMP), which indicates that a part of PTH’s effect on 1,25-(OH)₂-D₃ production is mediated by adenylate cyclase (51). However, it is not clear on how PTH mediates 1α-hydroxylase gene expression (57).
24-Hydroxylation

There is a hydroxylation step that adds a hydroxyl group to the C-24 position via 24-hydroxylase (58). Studies with rats denoted that 24-hydroxylase activity occurs in kidney and intestine (55, 59). It was also noted that the intestinal 24-hydroxylase $K_m$ value is similar to kidney 24-hydroxylase and with 1,25-(OH)$_2$-D$_3$, and it was speculated that intestinal 24-hydroxylase’s role is to initiate degradation of 1,25-(OH)$_2$-D$_3$ (55). In poultry, 24-hydroxylase expression was highest in kidney, then thymus and bursa, then intestine, lower in various muscle tissues, and little to no expression in liver (60).

Hydroxylation of C-24 can occur with 25-OH-D$_3$ or with 1,25-(OH)$_2$-D$_3$, which results in 24,25-dihydroxycholecalciferol (24,25-(OH)$_2$-D$_3$) and 1,24,25-trihydroxycholecalciferol, respectively (61, 62). 24,25-(OH)$_2$-D$_3$ is considered an inactive form of vitamin D because studies have shown that 24,25-(OH)$_2$-D$_3$ inhibits signaling cascades involved with Ca and P absorption which also inhibits bone mineralization (63). An in vitro study reported how rat kidney 24-hydroxylase expression increased with absence of PTH (64). Infants diagnosed with idiopathic infantile hypercalcemia had severe hypercalcemia and also have a genetic mutation of 24-hydroxylase (65). Hypercalcemia is caused by unregulated Ca absorption as an effect of increased sensitivity to vitamin D without 24-hydroxylase. Reduced 24,25-(OH)$_2$-D serum concentration can also be indicative of chronic kidney disease because of 24,25-(OH)$_2$-D’s negative correlation with increasing PTH concentrations (66). Half-life of serum 24,25-(OH)$_2$-D$_3$ may not be as long as 25-OH-D$_3$, but it can last at least 15 d (67, 68).
1-Alpha-hydroxycholecalciferol

First synthesized and described in 1973 (69), 1-alpha-hydroxycholecalciferol (1α) is a synthetic form of vitamin D₃ that has similar efficacy as 1,25-(OH)₂-D₃, but has an added benefit of being cheaper to synthesize for dietary uses (70). It is assumed that the 1-α-hydroxyl group on 1α is a structurally required for vitamin D’s hormonal activity (70). 1α is readily used in humans with renal cancer because of vitamin D deficiency caused by lack of 1α-hydroxylase activity to synthesize 1,25-(OH)₂-D₃ in kidneys (71, 72). However, 1α-hydroxylase is a critical regulatory enzyme that prevents overproduction of 1,25-(OH)₂-D₃ because 24-hydroxylase works with to regulate serum 1,25-(OH)₂-D₃ levels (73, 74). Therefore, it is possible that 1α can result in hypercalcemia because of its mode of action to avoid the critical regulation necessary to maintain homeostatic levels of 1,25-(OH)₂-D₃ and bypass the negative feedback mechanism caused by 1α-hydroxylase activity (70). It is not clear on what 1α’s half-life is in serum, but its biological activity from intravenous administration can last about 12-16 h (70, 75).

Vitamin D absorption, transportation, excretion, and tissue distribution

Vitamin D absorption occurs in the small intestine via passive diffusion with micelle solubilization and cholesterol transporters (76-78). As a fat-soluble vitamin, vitamin D is dependent on fats and bile salts to be absorbed; otherwise, its absorption is greatly decreased similar to how lipids are metabolized (79). Pharmacological doses of D₃ administered to rats expressed greatest rates of absorption more in the proximal part of small intestine than the distal part without any signs of saturation (80). There is a
difference in dietary vitamin D absorption efficacy between different animals, with young chicks and turkey poults having about 84% intestinal absorption of D₃ (81) and almost 20% absorptions in humans (82), and 42% in rats (83). The high level of absorption in chicks and poults also highlights why they are good models for vitamin D research. They are highly susceptible to vitamin D deficiency due to malabsorption caused by infectious or non-infectious factors. Vitamin D also lacks an active transport mechanism unlike vitamin K₁ (84) or A (85) which suggests that absorption could lead to toxic levels if there is excessive intake (80). Chickens are able to absorb dietary vitamin D₃; however, their capacity to absorb vitamin D₂ has been observed to be about 10x less than D₃ (86) is caused by faster clearance rate as a result of competition for affinity binding to DBP (87). Once absorbed, dietary vitamin D will go into lymphatic circulation in chylomicra. Polar metabolite forms of vitamin D (25-OH-D₃; 1,25-(OH)₂-D₃) are transported by DBP (35).

Translocation of de novo synthesized vitamin D from skin is facilitated by DBP (88). Almost all circulating vitamin D is bound to protein (89). DBP is in the same gene family as albumin and in mammals it is a glycosylated, cysteine-rich, α-globulin of 458 amino acids with a molecular weight of 55-58 kDa (90). Chickens have two DBPs, 54 and 60 kDa, both of which preferentially bind to 25-OH-D₃ over 25-OH-D₂ which denotes why D₂ has lower bioavailability for chickens (91). Well-nourished humans have DBP binding about 88% of 25-OH-D₃ in serum with a much greater affinity than 1,25-(OH)₂-D₃, signifying DBP’s binding affinity is dependent on stochiometric aspects of vitamin D which is different based on hydroxyl grouping (92). DBP is also maintained at a significant concentration in plasma that greatly exceeds concentration of 25-OH-D₃ (93).
DBP is also not affected by age, sex, or vitamin D status and is constant, suggesting that a low percentage of DBP (~5%) in circulation is bound to vitamin D and DBP likely has other metabolic functions (89). Plasma DBP has short turnover (93) (1-3 d) compared to 25-OH-D$_3$ with at least 18 d (41). There are alleles of DBP polymorphisms that are inversely related to circulating 25-OH-D$_3$ comparable to vitamin D intake (94).

Vitamin D$_3$ is found in mammalian liver, but the liver will contain high levels of 25-OH-D$_3$ because the liver is a transient organ for hydroxylating vitamin D$_3$ to 25-OH-D$_3$ (95, 96). Vitamin D is found in multiple tissues, specifically in adipose tissues (97, 98), brain (99). Plasma vitamin D is mostly 25-OH-D$_3$ and then 24,25-(OH)$_2$-D$_3$ (38). Kidneys contain great levels of 25-OH-D$_3$ which makes them potential organs that can be subjected to calcification from vitamin D toxicity (100). Variable levels of distribution of vitamin D and its multiple metabolite forms denotes differences in tissue lipid content and DBP associated with tissues.

When 25-OH-D$_3$ blood concentration needs to be decreased, the body can convert 25-OH-D$_3$ to 24,25-(OH)$_2$-D$_3$ (62). The 24-hydroxylation generates biologically inactive forms of vitamin D that are bound to bile and excreted in feces (101). Ultimately, this pathway results in a water-soluble molecule called calcitroic acid that will be safely excreted (101). Esvelt and De Luca (102) observed calcitroic acid having much lower binding affinity to VDR from chick intestines with similar binding affinity to 24,25-(OH)$_2$-D$_3$ as compared to 1,25-(OH)$_2$-D$_3$ which indicates both calcitroic acid and 24,25-(OH)$_2$-D$_3$ were inactive vitamin D forms.

Kidneys are the only tissues that express 1α-hydroxylase, 24-hydroxylase, and VDR, which are all important for regulating vitamin D metabolism (59, 64, 103).
CYP24A1 expression is upregulated when 1,25-(OH)\(_2\)-D\(_3\) binds to VDR; therefore, 24-hydroxylase expression is regulated by 1,25-(OH)\(_2\)-D\(_3\) activity (73). 24-hydroxylation activity of 25-OH-D\(_3\) will lead to its decreased concentration and reduces 1,25-(OH)\(_2\)-D\(_3\) concentration. 24-hydroxylase-null mice without 24-hydroxylase activity are unable to convert 1,25-(OH)\(_2\)-D\(_3\) to its 24-hydroxylated forms (63). The mice had 49% mortality rate at weaning and impaired bone mineralization (104). Even though 24-hydroxylase is tightly regulated by 1,25-(OH)\(_2\)-D\(_3\), it is important to maintain Ca homeostasis by preventing hypercalcemia (105).

**Vitamin D interaction with calcium storage, absorption, and excretion**

Ca is most abundant mineral in body with 99% of body’s Ca stored in bones and teeth. Bone Ca bound to phosphorus (P) is important for bone structure and has implications on homeostasis. Lactating mammals deposit Ca in milk for neonatal development, whereas in avian species, Ca along with P are major components of eggshells that is mostly comprised of calcium carbonate. Eggshells obtains Ca from hens’ medullary bone because medullary bone is an avian-specific trait in which Ca:P buildup occurs in bone to be mobilized for eggshell production (106, 107). About 2-3 g of Ca are used to form eggshells almost daily and it comprises about 10% of laying hen’s total Ca (108). Homeostatic Ca balance is maintained through intestinal Ca absorption, Ca excretion, and bone mineralization (109). Ca is mobilized through blood and bone. Blood Ca can circulate as two forms: ionized Ca that is free and protein-bound Ca that is bound to plasma calcium-binding protein vitellogenin and albumin (110). Ionized blood Ca is hormonally regulated and is available for any necessary
physiologically processes (111). Bone Ca is where most Ca in hens is stored (109). Bone stores Ca and P as hydroxyapatite crystals which comprise bone matrix and gives bones and teeth their hard composure (112).

Ca absorption in small intestine involves transcellular and paracellular mechanisms. In mammalian and avian species, paracellular transport is a saturable pathway that accounts for the most Ca absorption under high Ca intake (113-116). Paracellular transport quantitatively occurs in ileum because of its length to facilitate Ca absorption (117). Paracellular transport is also vitamin D-independent; however, unlike transcellular transport, most research on paracellular transport was with mammalian models (116, 118, 119). Further understanding of paracellular transport of Ca in avian species has value for improving poultry nutrition via feed composition to optimize Ca absorption through paracellular transportation.

In mammalian and avian species, transcellular transport, predominant in the duodenum and upper jejunum, is the vitamin D-dependent active transport pathway that occurs by three steps (120, 121): 1) Ca passively diffuses across the epithelial membrane by using the transient receptor potential vanilloid channel type 5 or 6 (TRPV5, TRPV6); 2) Ca is bound to the protein calbindin D_9k (intestinal tissues of avian species contain calbindin D_28k(122)) and transported across enterocytes; and 3) Ca is extruded across basolateral membrane and into circulation by Ca-ATPase (plasma membrane calcium ATPase isoform 1 [PMCA1]) pump, but there is also a Na-Ca exchanger-1 (NCX1) that accounts for about 20% of Ca extrusion. It should also be noted that this final step involves moving Ca against a tremendous concentration gradient (10,000-fold Ca differential concentration). As intracellular Ca levels are about
$10^{-7}$ mol/L in comparison to extracellular Ca ($10^{-4}$ mol/L) (120), an energy-dependent pump on the basolateral membrane is necessary to move Ca out of enterocytes (123).

Ca is absorbed in its ionized form which means it has to be freed from insoluble salts in food or dietary supplements (124). The stomach’s acidic pH will dissolve Ca salts, but Ca ions can form complexes with other nutrients or components, such as phytates, and become insoluble (125). Growing animals have greater Ca absorption meet the demand for bone growth and mineralization; whereas adult animals in non-reproductive status will have less demand for Ca absorption because the main reservoir for Ca flux will be bone that is under constant turnover (126). When hens begin laying eggs, they have greater demand for Ca absorption because Ca will be allocated towards eggshell formation (127).

In mammals and birds, the TRPV6 gene is regarded as a gatekeeper for Ca absorption to maintain Ca homeostasis (128-130). In mammals, studies have reported TRPV6 is expressed and functionally active in multiple tissues such as the intestine, skin, bones, and kidneys; any tissues that exhibit high Ca mobilization express TRPV6 (131, 132). TRPV6 is also important for Ca transport between mammalian mother and fetus by being expressed in placental yolk sac along with calbindin D$_{9k}$ (133). In chickens, TRPV6 is expressed in intestine, kidney, and eggshell gland (129, 134); which are all related to Ca absorption, resorption, and eggshell formation, respectively. During oviposition, TRPV6 along with calbindin D$_{28k}$ have increased expression and protein concentrations in egg shell gland in which transcellular transport is inferred for moving Ca from blood to intestinal lumen for eggshell formation (134).
Excreting Ca occurs mostly in urine in mammals and birds (110, 135-137).

Circulating Ca in mammalian kidney is almost completely reabsorbed (~99%) with about 60% reabsorbed in proximal convoluted tubule, 20% at loop of Henle, 10% at distal convoluted tubule, and 5% by collecting ducts (138). Paracellular absorption via passive diffusion of Ca is coupled to sodium (Na) and water reabsorption and accounts for about 80% of reabsorbed Ca in proximal convoluted tubule (139). Transcellular absorption of Ca using active transport mechanisms accounts for about 10-15% reabsorption in proximal convoluted tubule (140). Vitamin D and PTH both increase Ca reabsorption (141, 142). Avian kidneys functions like mammalian kidneys with Ca excretion and PTH stimulation being responsible for almost all filtered Ca to be resorbed (143).

There are dietary factors that affect Ca reabsorption and excretion. For example, a person consuming a high protein diet will have reduced reabsorption capacity for Ca (144, 145). Higher protein intake causes hypercalciuria as a result of acid-production load from dietary protein. Reducing dietary protein intake has been recommended to improve Ca balance for bone health (146). If Na excretion increases from dietary intake, then Ca excretion will increase because of its coupled relationship in kidney reabsorption (147, 148). Caffeine elicits a strong diuretic response to inhibit water reabsorption, which also reduces Na and Ca reabsorption because of their relationship to water (149, 150). PTH increases Ca reabsorption in kidneys by causing increased expression of tight-junction proteins, Ca channel proteins, and active transporters (142, 151, 152). PTH’s antagonist, calcitonin, causes increased Ca and Na excretion (153). Dietary influence on Ca reabsorption makes diet an important factor to ensure Ca homeostasis is maintained.
Addressing vitamin D insufficiency in humans by understanding vitamin D metabolism in poultry

Value of dietary vitamin D₃ with broiler chicken production

It has been recently speculated that vitamin D deficiency in humans will be a global issue for addressing health because of implications related to osteomalacia (154-156). There have been clinical studies that examined different aspects of vitamin D metabolism in humans by examining how D₃ supplementation affected vitamin D status and immune function in pregnant women, D₃ supplementation in overweight and obese African Americans, obese adults and sunlight exposure, D₃ supplementation in young people being treated with tenofovir disoproxil fumarate for HIV (157-161). One drawback with clinical trials is controlling variation with vitamin D intake, whether it is caused by sunlight exposure between participants or their diet, outside of the experimental study, can affect vitamin D status (157); a secondary drawback is participants failing to adhere to their daily supplementation as part of the study design (158). Clinical trials are powerful for ascertaining treatment effects in humans and complements animal studies that can validate effects in controlled environments.

Chickens have been thoroughly used for vitamin D-related studies to elucidate specific impacts of vitamin D intake with physiology and metabolism and study designs can control for sunlight exposure or monitoring feed intake. Growth performance in broiler chickens (grown for meat) is a quantitative measure that ascertains nutrient effects for examining metabolic relationships. Despite having low utilization of D₂, there are significant implications for researching vitamin D effects using chickens. The poultry industry has interest with reducing tibial dyschondroplasia in fast-growing broilers. Tibial
dyschondroplasia occurs in fast growing avian species and is a lesion in which the growth plate of tibia head is avascular and is not mineralized causing bowing of the tibiotarsus and lameness of the bird (162, 163). Increasing vitamin D with increased dietary Ca has been observed to reduce incidence and severity of tibial dyschondroplasia in broilers in young chickens (164-167). There are studies that examined vitamin D₃ supplementation with other nutrients: strontium reduced body weight gain (168); Zn interfered with Ca absorption, but improved immunocompetence (169); adding D₃ to diets with increased P and microbial phytase improved P and Ca utilization (170).

25-OH-D₃ is used as a vitamin D status indicator in humans, and clinical trials that normally administer D₃ or 25-OH-D₃ as intravenous, intramuscular, or oral doses to examine how vitamin D status is affected (171-176). Exploring dietary intake of vitamin D with other nutrients can cause variation, but it will also help address vitamin D insufficiency with regards to how food sources can affect vitamin D status. Many studies examining dietary D₃ effects in broilers have repeatedly shown improved bone health (165, 167, 177-179). Chicken feed can be supplemented with 25-OH-D₃ and has been very effective with reducing tibial dyschondroplasia compared to broilers only fed D₃ (180, 181). Studies involving 1,25-(OH)₂-D₃ being fed to broilers observed increase in bone ash and plasma Ca and more effective with reducing incidence of rickets (182-184). 1α has been observed to have better bioefficacy than 25-OH-D₃ when used as an additive with broiler feed (185) and it is about 8x more effective than D₃ (186). It has also been suggested that 1α’s high bioavailability in a synthetic mixed micelle with
human intestinal cells (Caco-2) that 1α can be used to treat severe vitamin D deficiency (187).

*Effects of dietary vitamin D₃ with laying hens in production*

Laying hens are a stellar model to use with characterizing vitamin D effects because when they are in production, they have greater Ca requirements and bone turnover (127). There was improved bone structure in laying hens that were fed vitamin D₃, especially with increased dietary Ca (188); however, Ca intake is more important for egg production and quality (189-191). While many studies involving vitamin D₃ with laying hens focused on egg quality, one study explored how vitamin D affected hen ovary during follicle development (192). Enriching laying hen diets with specific vitamins has direct impact on vitamin content in egg yolk.

The nutrient quality of eggs from laying hens as powerful value for addressing nutrient deficiencies, especially for lipid-related nutrients (193). Fatty acid profiles of egg yolk are directly related to carotenoids and diet quality of a hen (194, 195). When hens were fed layer-breeder diets with supplemented vitamin A, the vitamin A concentration in egg yolk was greatly increased (196). Increasing levels of vitamin E in a laying hen diet also increases vitamin E content in egg yolk (197). It should be noted that competitive antagonism for intestinal absorption can occur between fat-soluble vitamins when dietary supplemental levels are provided to hens (198, 199). However, there are possibilities of creating specific value-added eggs to address specific vitamin needs by feeding hens with a particular vitamin in their diet (193).
Vitamin D content of egg yolk can reach a level that will meet daily vitamin D demands if a hen is fed diets with high levels of vitamin D₃ (200). Although there has been some research that examined value-added eggs when hens were fed diets with high levels of dietary vitamin D₃ (200-202), there are implications for exploring feasibility of value-added eggs that can be a food supply to address areas of nutrient demands in locations that are geographically poor in sunlight and have little access to other dairy products or fortified foods. There are positive implications for exploring how vitamin D-enriched eggs can improve nutritional status of at-risk populations such as the elderly.

**Vitamin D toxicity and deficiency in humans and poultry**

Even though vitamin D is stored in tissues, vitamin D deficiency can occur with specific conditions. An animal consuming a vitamin D-poor diet and not exposed to sunlight will eventually express signs of deficiency such as rickets for young, growing animals, increased risk of bone fractures, or muscle weakness (203-205). Elderly men and women are most susceptible to vitamin D deficiency (206, 207); however, children and young adults can also be at risk of vitamin D deficiency if they have a vitamin D-poor diet or are in geographical locations with poor sunlight exposure (208-210). Vitamin D deficiency can also occur if there is genetic defect with VDR (211). A review on VDR-null mice explains how significant VDR is for homeostasis (212). Lack of signaling from 1,25-(OH)₂-D binding to VDR also affects various genes responsible for cell proliferation and may influence cancer incidence (213-215).

Vitamin D toxicity is rare but excessive intake of vitamin D from supplements is a means to reach intoxication (1). Holick (1) noted how humans taking daily vitamin D
doses greater than 50,000 IU/d increase their serum 25-OH-D₃ concentration to 150 ng/mL which is associated with hypercalcemia and hyperphosphatemia. Laying hens fed 15,000 IU D₃/kg of feed for 48 wks were observed to not have any histopathological issues with their liver, kidney, heart, and brain (201). Plasma vitamin D was not measured in those laying hens, but vitamin D content in egg yolk of those hens were increased relative to dietary vitamin D₃. Broiler chicks are reported to have high tolerance to excess dietary D₃ because chicks fed 1,250 µg D₃/kg of feed (50,000 IU D₃/kg of feed) had similar body weight gain, feed intake, and tibia ash as chicks fed 2.5 µg/kg D₃ (216). Based on what Baker et al. (216) observed compared to Morrissey et al. (100) observed with chicks, feeding high levels of D₃ is nowhere as toxic as 25-OH-D₃.

There was a clinical trial during winter in which men were given daily oral doses of 5000 IU D₃ for about 20 weeks which led to an increase in serum 25-OH-D₃ concentration to maintain vitamin D status (172). When taken into consideration with what was observed with rats and chickens, excessive vitamin D₃ intake may not be as toxic as suspected, especially with regulatory feedback mechanisms.

Increasing intake of 25-OH-D₃ may be more effective for eliciting vitamin D pathways for increasing Ca absorption, but there are risks associated with toxicity. Vitamin D intoxication caused emaciation and deaths of chicks fed 100 mg 25-OH-D₃ diets (100). In young chicks, toxicity can cause lesions and mineralization in kidney and fragile bones (100). In rats, excessive levels of dietary 25-OH-D₃ led to a linear decrease in body weight and rats given 4600 nmol/d of 25-OH-D₃ had grayish-white kidney coloration as a sign of calcification (217). Laying hens fed 825 µg 25-OH-D₃ / kg of feed (10x) exhibited toxic effects as hens had a decrease in body weight, reduction in
feed efficiency, decreased eggshell thickness, egg production, and egg quality
parameters such as egg and albumen weights (218). Broiler chicks fed 10 times basal
level of 25-OH-D₃ (690 µg) per kg of feed exhibited renal calcification (219), but renal
calcification may be dependent on dietary Ca (100). In a clinical study with adult men
and women given single oral doses of 25-OH-D₃ (5 or 10 µg per kg), serum 25-OH-D₃
increased over a course of 4 h and then dropped to near baseline after a week (220).
25-OH-D₂ may be a safer form of vitamin D supplementation because it is not likely to
be as potent and toxic (221). Dietary supplemental 25-OH-D₃ dosages should be taken
with extra care because of its potency and potential cause of pathologies.

**Vitamin D receptor research in poultry has great potential to elucidate vitamin D metabolism**

VDR is a transcription factor which 1,25-(OH)₂-D₃ binds to and exerts vitamin D’s
activity through gene expression (222). When 1,25-(OH)₂-D₃ binds to VDR, a
conformational change transforms VDR to interact with other factors in gene
transcription (223). VDR is classified as a nuclear transcription factor which requires a
specific ligand (1,25-(OH)₂-D) and heterodimerizes with retinoid X receptor (RXR)(224).
Found in many animals with some form of calcified skeleton (212) and lamprey (225);
VDR’s evolutionary origins are still unclear. One of VDR’s primary functions is regulating
Ca and phosphate homeostasis for bone mineralization as was characterized in VDR
null mice (226, 227). Genetic defects in VDR cause vitamin D-dependent rickets type II
(211) because Ca absorption for bone mineralization is heavily reduced as VDR is
necessary for expressing proteins that facilitate Ca absorption. In mammals, VDR mutations can lead to baldness because of a corepressor that binds to VDR (228, 229).

There is a lack of the research into the VDR in poultry that will be necessary for illustrating vitamin D mechanisms. However, the chicken genome has been sequenced (230, 231), so VDR has been annotated and there is some research that explored VDR as a biological candidate gene for improving production (growth rate for broilers, egg production and egg quality for layer hens) (232-234). To our knowledge, there are currently no studies that explored VDR and bone mineralization in poultry model yet. Therefore, further characterizing VDR in chickens has significance for researchers and poultry industry. Future research with examining vitamin D using the chicken model should go a step further with examining how VDR expression is influenced because such conditions can provide greater insights into vitamin D metabolism.

Hypothesis testing for vitamin D studies in chickens can increase their impact by considering VDR levels. VDR has been identified to be present in almost all tissues in mammalian models, but does the same principle apply to avian model which has a distinctly different kidney structure (235) that is also very efficient? Birds evolved to mobilize Ca quickly from their bones for eggshell formation and this Ca may also be resorbed from kidneys, which is also a site for VDR (236, 237). If chickens were fed exorbitant levels of 25-OH-D₃ and were at risk of renal calcification, then VDR expression with 1α-hydroxylase should be greatly increased to explain that effect. However, the alternative hypothesis would be explained by a decrease in 24-hydroxylase which can also explain why renal calcification would be possible because the chicken had higher Ca absorption without feedback inhibition. By understanding
how VDR is affected by experimental treatments in chickens, future researchers will be able to draw conclusions that can connect biological effects of vitamin D that can be translated towards human nutrition to identify ways to address vitamin D insufficiency while reducing risk of vitamin D toxicity.

**Future research with vitamin D in poultry can help address high-impact areas of human nutrition**

Better understanding of vitamin D impacts will be necessary for improving human health and devising strategies to address vitamin D insufficiency. A recent review covered how 25-OH-D accumulates in skeletal muscle cells to be utilized during winter months (238). Broiler chickens would be valuable for illustrating this mechanism because they can be housed in controlled environments to prevent UVB exposure, fed dietary 25-OH-D$_3$ during their growing phases, and examine 25-OH-D$_3$ concentration in plasma and muscle tissue.

Exploring consumer aspects of vitamin D-enriched eggs will be important for trying to address vitamin D insufficiency because if the vitamin D-enriched eggs lose a lot of their content from cooking, then its value is lost. Therefore, it will be necessary to quantifying how different processing methods affect vitamin D content in egg yolk, especially in vitamin D-enriched eggs. Geriatric age humans are at greatest risk of osteopathies and vitamin D deficiency and are given high supplemental doses of vitamin D to address such issues (239, 240). If vitamin D metabolic activity is decreased because of age, then such supplemental doses may have potential negative effects that should be examined. Aged laying hens can be fed extraordinary dietary levels of vitamin
D and tissues can be collected to observe how their vitamin D metabolism was influenced by such experimental diets; therefore, providing an understanding if high supplemental levels are potentially harmful or if the hens can cope with such levels and regulate their homeostasis with benefit of improved bone health.

**Conclusions**

Addressing global vitamin D insufficiency cannot be solely dependent on fortification methods or increasing sunlight exposure. Further understanding of vitamin D mechanisms relative to its intake will be essential for elucidating how effective supplementation is with addressing nutritional needs. Studies involving dietary intake of vitamin D with chickens provide a foundational base that we can build upon for developing critical questions to address undiscovered ways we can improve vitamin D intake and/or metabolism. Prospective research with chickens and vitamin D should incorporate more molecular techniques to explore physiological dimensions of how the birds are affected so gaps in chicken nutrition can be compared to humans with targeting and developing preventative strategies to improve vitamin D status.
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Chapter 2

Efficacy of 1-α-Hydroxycholecalciferol supplementation in young broiler feed suggests reducing calcium levels at grower phase

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Abstract

Increasing biopotency of cholecalciferol (D₃) from vitamin sources is essential in the poultry industry to meet nutritional demands and counter stressors. D₃ exhibits hormonal traits and is responsible for calcium (Ca) absorption. 1-α-hydroxycholecalciferol (1α) is a synthetic form of D₃ that has equal efficacy on broilers and is cheaper to synthesize than 1,25-dihydroxycholecalciferol (active form of D₃). However, 1α bypasses a critical regulatory point, the kidney, and may consequently lead to toxicity levels of Ca via Ca absorption. This study examined 1α supplementation in broiler diets with different Ca inclusion levels to determine if 1α at higher Ca levels caused Ca toxicity at starter and grower phases with Ross 708 male broiler chicks. In Experiment 1 (1-15 d of age), chicks were assigned to one of ten treatment starter diets with five levels of Ca inclusion (0.80, 0.95, 1.10, 1.25, and 1.40%) with or without 1α supplementation (5 µg 1α /kg in feed) and 8 replicate cages per treatment. In Experiment 2, chicks were fed common starter diet until 16 d of age, then they were assigned to one of eight treatment diets with four levels of Ca inclusion (0.54, 0.76,
0.98, or 1.20%) with or without 1α supplementation (5 µg 1α /kg in feed). At the end of both experiments, blood was collected from broilers to determine blood chemistry, including concentrations of vitamin D metabolites. Intestinal tissues were also collected to examine gene expression. In Experiment 1, broilers not fed 1α exhibited a quadratic effect in ionized blood Ca (iCa) as dietary Ca inclusion levels increased; 1α-fed broilers displayed an increase in iCa as Ca inclusion levels increased (p = 0.0002). For Experiment 2, 1α-fed broilers displayed a decrease in 25-hydroxycholecalciferol plasma concentration as dietary Ca inclusion levels increased (p = 0.035); also, increasing Ca inclusion in diets increased expression of duodenal sodium-phosphate cotransporter type II b (NPTIIb, p = 0.03). Our findings imply inclusion of 1α was beneficial because 1α enhanced Ca absorption during starter phase; however, to avoid potential Ca toxicity or antagonism while using 1α during grower phase, there should be consideration with reducing dietary Ca levels.
Introduction

Increasing biopotency of cholecalciferol (D₃) from vitamin sources is essential in the poultry industry to meet nutritional demands while accounting for environmental stressors. D₃ is necessary for accommodating fast growth of broilers by increasing absorption of calcium (Ca) and its deposition into bones (1, 2). The biopotency of a nutrient can be enhanced by utilizing synthetic forms of the nutrient (3), increasing bioavailability to accommodate a greater response if the nutrient’s metabolic effect is dose dependent (4), or adding supplemental enzymes to increase efficacy of the nutrient of interest (5). Supplemental enzymes can be costly and their effectiveness can vary based on nutrient load and feed processing (6). Synthetic forms of a nutrient could have unintended effects and require further testing (7, 8), but some are viable economical solution for the poultry industry (9).

Previous studies reported inclusion of 1-α-hydroxycholecalciferol (1α), a synthetic analog of vitamin D₃, improved Ca absorption in growing broiler chicks over D₃ alone and showed 1α having equal efficacy to 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃). 1α is cheaper to synthesize and supply in diets as compared to 1,25-(OH)₂-D₃ (3, 10). 1α’s structure is similar to 1,25-(OH)₂-D₃, but only the 1-alpha carbon is hydroxylated instead of both the 1-alpha and 25 carbon. 1α has greater biopotency over D₃ because it is quickly hydroxylated in the liver to its active form, 1,25-(OH)₂-D₃, and consequently bypasses the hydroxylation step occurring in the kidney. In contrast, D₃ requires two hydroxylation steps, first in the liver to form 25-hydroxycholecalciferol (25-OH-D₃) which is further hydroxylated in kidney to 1,25-(OH)₂-D₃. However, 1α bypasses the critical regulatory hydroxylation by 1α hydroxylase in kidney (10, 11), 25-OH-D₃ levels might
significantly increase to result in excessive Ca absorption leading to hypercalcemia (12). Although levels of ionized blood Ca (iCa) toxicity in broilers is not established, Hurwitz, Plavnik (13) fed fast-growing chicks diets ranging from 0.4 – 2.0% Ca with 0.7% P diets and observed a weight loss in fast growing chicks.

Ca has an important relationship with phosphorus (P), because, together, they comprise a major part of bone structure (14). In the form of limestone or calcium carbonate, Ca is an inexpensive ingredient and is used as a carrier for many other feed ingredients including mineral premixes and drugs (15). Ca’s counterpart, P, is one of the more expensive feed ingredients which limits the amount incorporated into diets. This relationship can result in varying Ca:P ratio from 1:1 to 2.6:1 in weight (2). Studies have reported that increasing dietary Ca levels reduced incidence of tibial dyschondroplasia and therefore, improved animal health, welfare, and economic value (2, 16, 17). Also, an elevated dietary Ca:P ratio of 2.6:1 does not appear to negatively affect tibial growth plate morphology at two weeks of age (18).

Although studies have been done on 1α and how it influences vitamin D status in broilers (3, 19), no studies to date have examined how 1α affects Ca absorption and vitamin D status when broilers are fed differing levels of dietary Ca. This study explores how 1α impacts vitamin D status in broiler chickens when they are fed diets with different levels of Ca in starter and grower phase.
Methods

Two experiments were conducted to analyze effects of 1α supplementation and increasing levels of Ca inclusion on blood chemistry of starter and grower phases of broilers. All animal protocols (# 014-113 for Experiment 1 and # 17-125-A for Experiment 2) were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Experiment 1 – 1α supplementation at starter phase

Birds and housing

Four-hundred and eighty one-day-old Ross 708 chicks were hatched at North Carolina State University’s Chicken Education Unit in Raleigh, NC. Chicks were housed in Petersime battery cages with six birds per cage and eight replicate cages per treatment. The experimental design was a completely randomized design with or without 1α supplementation (Alpha D₃; (Premex, Antioquia, Colombia) at 5 µg/kg of feed and five levels of Ca inclusion which were added on top of basal diet (Table 1) (20). For this study, broilers from dietary treatments with 1α are noted as D₃+1α; broilers not fed 1α are noted as D₃. Ca inclusion levels were 0.80, 0.95, 1.10, 1.25, and 1.40% with 0.50% available P in all diets and birds were fed ad libitum. Lighting program was set for 23:1 L:D hours for first 7 days and last 8 days was of experiment was set to 17:7. Room temperature was set to be adjusted daily to ensure thermoneutral temperatures as birds grew. Blood was collected at 15 d from two birds per cage. At 17 d, all remaining birds were culled. A total of 16 plasma samples per treatment, D₃ and D₃+1α, from broilers
given diets at 0.95% Ca had their plasma sent (no pooling) to Heartland Assays (Ames, IA) for analysis of vitamin D metabolites by LC-MS/MS.
Table 1. Ingredient composition and calculated nutrient content of starter basal diet (1-17 days of age) for Ross-708 broilers (From ref 20).

<table>
<thead>
<tr>
<th>Ingredient Name</th>
<th>% Nutrient</th>
<th>Nutrient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>53.25</td>
<td>Dry matter</td>
<td>88.55</td>
</tr>
<tr>
<td>Soybean meal, 46% CP</td>
<td>30.94</td>
<td>Moisture</td>
<td>11.45</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>5.00</td>
<td>Crude protein</td>
<td>22.71</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>0.00</td>
<td>Calcium</td>
<td>0.30</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>4.20</td>
<td>Total phosphorous</td>
<td>0.38</td>
</tr>
<tr>
<td>Celite</td>
<td>1.00</td>
<td>Nonphytate phosphorous</td>
<td>0.18</td>
</tr>
<tr>
<td>Filler (Limestone+CaHPO₄+Sand)</td>
<td>3.15</td>
<td>Phytate phosphorous</td>
<td>0.24</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.29</td>
<td>Total methionine</td>
<td>0.67</td>
</tr>
<tr>
<td>DL-Methionine, 99%</td>
<td>0.30</td>
<td>Total cysteine</td>
<td>0.36</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.31</td>
<td>Total lysine</td>
<td>1.39</td>
</tr>
<tr>
<td>Dicalcium phosphate (CaHPO₄)¹</td>
<td>0.27</td>
<td>Total tryptophan</td>
<td>0.25</td>
</tr>
<tr>
<td>Mineral premix²</td>
<td>0.20</td>
<td>Total threonine</td>
<td>0.98</td>
</tr>
<tr>
<td>Limestone Cerne Pure Cal 12-4³</td>
<td>0.17</td>
<td>Total isoleucine</td>
<td>0.94</td>
</tr>
<tr>
<td>L-Lysine-HCl, 78.8%</td>
<td>0.38</td>
<td>Total valine</td>
<td>1.05</td>
</tr>
<tr>
<td>Choline chloride, 60% choline</td>
<td>0.18</td>
<td>Total leucine</td>
<td>2.13</td>
</tr>
<tr>
<td>L-Threonine, 98%</td>
<td>0.15</td>
<td>Total arginine</td>
<td>1.40</td>
</tr>
<tr>
<td>Selenium premix</td>
<td>0.05</td>
<td>Total Sulphur amino acids</td>
<td>1.04</td>
</tr>
<tr>
<td>Vitamin premix⁴</td>
<td>0.10</td>
<td>Total glycine</td>
<td>0.88</td>
</tr>
<tr>
<td>Anticoccidial⁵</td>
<td>0.05</td>
<td>Sodium</td>
<td>0.22</td>
</tr>
<tr>
<td>Natuphos E®</td>
<td>0.01</td>
<td>Potassium</td>
<td>0.85</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>Chloride</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible lysine</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible methionine</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible cysteine</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible total Sulphur amino acids</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible threonine</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible tryptophan</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible isoleucine</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible leucine</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible valine</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestible arginine</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metabolizable Energy, kcal/kg</td>
<td>3,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dietary electrolyte balance, mEq/100 g</td>
<td>254</td>
</tr>
</tbody>
</table>

¹ Dicalcium phosphate contains 19.79% calcium, 17.91% phosphorus, and 17.73% available phosphorus.

² Trace minerals provided per kg of premix: 60 g manganese (Mn SO4); 60 g zinc (ZnSO4); 40 g iron (FeSO4); 5 g copper (CuSO4); 1.25 g iodine [Ca(IO3)2].

³ Limestone (Cerne Pure Cal 12-4) contains 39.467% calcium.

⁴ Vitamins provided per kg of premix: 13,227,513 IU vitamin A; 3,968,253 IU vitamin D3; 66,137 IU vitamin E; 39.6 mg vitamin B12; 13,227 mg riboflavin; 110,229 mg niacin; 22,045 mg d-pantothenic acid; 3,968 mg menadione; 2,204 mg folic acid; 7,936 mg vitamin B6; 3,968 mg thiamine; 253.5 mg biotin.

⁵ Coban® 90 (Monensin), Elanco Animal Health, Greenfield, IN, at 500 g/ton in the starter and grower diets.

⁶ Natuphos E® (500 FTU/kg, 50 g/ton FTU)
Experiment 2 – 1α supplementation at grower phase

Birds and housing

Nine-hundred and sixty Ross 708 chicks were hatched at North Carolina State University’s Chicken Education Unit in Raleigh, NC. Chicks were housed in 40 floor pens with 24 birds per pen. All chicks were fed a common starter diet (Table 2 and 3) (20) until 17 d of age. It should be noted that all starter diets for these chicks had 1α supplementation (5 µg/kg of feed). Like the prior experiment, broilers from dietary treatments with 1α are noted as D₃+1α; broilers not fed 1α are noted as D₃. At 17 d of age, birds were switched to grower diet and assigned to one of eight treatment groups with four levels of Ca inclusion (added on top of basal diet; 0.54, 0.76, 0.98, or 1.20% of diet) and with or without 1α supplementation (5 µg/kg of feed) with five replicate pens per treatment. All diets contained 0.50% available P. At 35 d of age, blood was collected from two birds per pen, euthanized, and duodenal and jejunal tissue were collected. Duodenal tissue was washed with saline and stored in RNAlater at -20°C. Jejunal tissue was washed with saline and stored in 104% formalin for histology. Plasma from each treatment was pooled using 4 birds per housing row into 1 pooled sample, for a total of 3 pooled reps per treatment and sent to Heartland Assays (Ames, IA) for analysis of vitamin D metabolites.
Table 2. Ingredient composition of starter diet and grower basal diets for Ross-708 male broilers (From ref 20).

<table>
<thead>
<tr>
<th>Ingredient Name</th>
<th>Starter (1-16 d) (%)</th>
<th>Grower (17-35 d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>55.84</td>
<td>57.59</td>
</tr>
<tr>
<td>Soybean Meal, 46% CP</td>
<td>31.70</td>
<td>26.89</td>
</tr>
<tr>
<td>Corn gluten Meal</td>
<td>4.90</td>
<td>5.00</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>3.12</td>
<td>4.85</td>
</tr>
<tr>
<td>Dicalcium phosphate (CaHPO₄)³</td>
<td>1.37</td>
<td>0.31</td>
</tr>
<tr>
<td>Limestone Cerne Pure Cal 12-40²</td>
<td>1.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.36</td>
<td>0.23</td>
</tr>
<tr>
<td>L-Lysine-HCl, 78.8%</td>
<td>0.36</td>
<td>0.31</td>
</tr>
<tr>
<td>DL-Methionine, 99%</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>Mineral premix³</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Choline chloride, 60%</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>Vitamin premix⁴</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Anticoccidial⁵</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Selenium premix</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Sand</td>
<td>0.01</td>
<td>3.60</td>
</tr>
<tr>
<td>Celite</td>
<td>-</td>
<td>1.00</td>
</tr>
<tr>
<td>Limestone dicalcium base</td>
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</tr>
<tr>
<td>1α(OH)D₃</td>
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</tr>
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<td>Natuphos E®⁶</td>
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<td>0.005</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

¹Dicalcium phosphate contains 19.79% calcium, 17.9091% phosphorus and 17.73% available phosphorus (99%).
²Limestone (Cerne Pure Cal 12-4) contains 39.01% calcium.
³Trace minerals provided per kg of premix: 60 g manganese (Mn SO₄); 60 g zinc (ZnSO₄); 40 g iron (FeSO₄); 5 g copper (CuSO₄); 1.25 g iodine [Ca(IO₃)₂].
⁴Vitamins provided per kg of premix: 13,227,513 IU vitamin A; 3,968,253 IU vitamin D₃; 66,137 IU vitamin E; 39.6 mg vitamin B12; 13,227 mg riboflavin; 110,229 mg niacin; 22,045 mg d-pantothenic acid; 3,968 mg menadione; 2,204 mg folic acid; 7,936 mg vitamin B6; 3,968 mg thiamine; 253.5 mg biotin.
⁵Coban® 90 (Monensin), Elanco Animal Health, Greenfield, IN, at 500 g/ton in the starter and grower diets.
⁶Natuphos E® (500 FTU/kg, 50 g/ton FTU)
Table 3. Nutritional content of basal starter and grower diets for Ross-708 male broilers (From ref 20).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Starter (1-16 d)</th>
<th>Grower (17-35 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>23.14</td>
<td>20.94</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.87</td>
<td>0.24</td>
</tr>
<tr>
<td>Total phosphorous</td>
<td>0.58</td>
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</tr>
<tr>
<td>Non phytate phosphorous</td>
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<td>0.18</td>
</tr>
<tr>
<td>Phytate phosphorous</td>
<td>0.24</td>
<td>0.22</td>
</tr>
<tr>
<td>Total methionine</td>
<td>0.65</td>
<td>0.59</td>
</tr>
<tr>
<td>Total cysteine</td>
<td>0.38</td>
<td>0.35</td>
</tr>
<tr>
<td>Total lysine</td>
<td>1.41</td>
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</tr>
<tr>
<td>Total tryptophan</td>
<td>0.27</td>
<td>0.24</td>
</tr>
<tr>
<td>Total threonine</td>
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<td>0.86</td>
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<tr>
<td>Total isoleucine</td>
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<td>0.87</td>
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<tr>
<td>Total valine</td>
<td>1.09</td>
<td>0.98</td>
</tr>
<tr>
<td>Total leucine</td>
<td>2.15</td>
<td>2.00</td>
</tr>
<tr>
<td>Total arginine</td>
<td>1.43</td>
<td>1.27</td>
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<tr>
<td>Total sulphur amino acids</td>
<td>1.03</td>
<td>0.93</td>
</tr>
<tr>
<td>Total glycine</td>
<td>0.90</td>
<td>0.81</td>
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<tr>
<td>Digestible lysine</td>
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<td>1.12</td>
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<tr>
<td>Digestible methionine</td>
<td>0.63</td>
<td>0.56</td>
</tr>
<tr>
<td>Digestible cysteine</td>
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<td>0.29</td>
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<tr>
<td>Digestible total sulphur amino acids</td>
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<td>Digestible threonine</td>
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<td>0.75</td>
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<tr>
<td>Digestible tryptophan</td>
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<td>Digestible, isoleucine</td>
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<td>0.79</td>
</tr>
<tr>
<td>Digestible leucine</td>
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<td>1.85</td>
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<td>Digestible valine</td>
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<td>0.87</td>
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<td>Digestible arginine</td>
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<td>1.18</td>
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<td>Sodium</td>
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<td>Potassium</td>
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<td>0.275</td>
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<tr>
<td>Dietary Electrolyte Balance, mEq/kg</td>
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Blood collection and blood chemistry

Blood was collected into BD Vacutainer lithium heparin tubes (Franklin Lakes, NJ) from two birds per cage/pen. Blood chemistry parameters and iCa were determined using an i-STAT™ blood analyzer (Abaxis, Union City, CA) using CG8+ cartridges (Abaxis, Union City, CA) and the remaining blood was spun down and plasma was collected and stored at -80°C to determine vitamin D metabolite levels. All plasma samples were sent to Heartland Assays (Ames, IA) for measuring D₃, 25-OH-D₃, and 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃, inactive form of D₃).

RNA extraction and qPCR

Total mRNA was extracted from cecal tonsils and duodenal tissue using Qiagen’s RNeasy Mini Kit (Germantown, MD). Extracted RNA was diluted and normalized to ~200 ng/µL and reverse transcribed to complementary DNA (cDNA) using Applied Biosystems’ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA) and protocol to make a 20 µL working solution. Cycling procedure for reverse transcription started with 25°C for 10 min, 37°C for 120 min, 85°C for 5 mins, then held at 5°C indefinitely until storage or use.

Genes expressed for qPCR were vitamin D receptor (VDR), mucin 2 (MUC2), calbindin D28k (CALB), sodium-phosphate cotransporter type IIb (NPTIIb), occludin (OCLN), and glyceraldehyde (GAPDH) as housekeeping gene (Table 4). qPCR was conducted using PowerUP SYBR Master Mix (Life Technologies, Grand Island, NY) using Applied Biosystems protocol to make a 20 µL working solution and using Applied Biosystems StepOnePlus Real-Time PCR System (Carlsbad, CA). Cycling procedure
started with 95°C for 10 min then 40 cycles of 95°C for 15 s for denaturing and 15 s at 60°C for annealing. All samples were run in triplicates. For statistics: Experiment 1 – starter diet: D$_3$ with 0.95% Ca inclusion was set as control for comparing relative expression between treatments. Experiment 2 – grower diet: D$_3$ with 0.76% Ca inclusion was set as control.

Table 4. Primer sequences for quantitative real-time PCR (qPCR).

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Histology

Light microscopy (40x magnification) was used for morphometric analysis of histological serial sections of jejuna prepared using standard hematoxylin and eosin staining to examine if dietary treatments affected gut morphology. Villus height, crypt depth and villus width were measured using image analyzer AmScope version 3.7 (Irvine, CA). Ten measurements for villus surface area ($\mu$m$^2$) and villus height/crypt depth were made per experimental unit (bird).
Data Analysis

All data are reported as means ± standard error means. Statistical analyses were conducted using general linear model using the following model:

\[ Y_{ijk} = \mu + \beta_1(Ca)_i + \beta_2(Vit. D)_j + \beta_3(Ca \times Vit. D)_{ij} + \varepsilon_{ijk} \]

where \( Y_{ijk} \) is individual observation; \( \mu \) is experimental mean; \( Ca \) is Ca inclusion effect of \( i^{th} \) level; \( Vit. D \) is effect of \( 1\alpha \) supplementation of \( j^{th} \) level; and \( \varepsilon_{ijk} \) is error. \( \beta \)'s are slopes for each predictor. This model was used to compare differences in blood chemistry concentrations, vitamin D metabolite plasma concentrations, and relative gene expression using SAS 9.4®. Tukey-Kramer test was used for multiple comparisons for differences between and within treatment groups for blood chemistry, vitamin D metabolites, and mRNA relative expression. All mRNA relative expressions were normalized using \( 2^{-\Delta\Delta CT} \) (21) with GAPDH as a housekeeping gene control. Statistical significance was established at \( p < 0.05 \) and statistical trends were noted when \( 0.05 < p \leq 0.10 \).

Results

Experiment 1 – starter diet

1α efficacy dependent on dietary calcium inclusion levels with ionized blood calcium (iCa) concentration

In terms of blood chemistry, only iCa exhibited any differences between 0.80% and 1.10% Ca inclusion (Figure 1). For D₃+1α broilers, iCa increased as dietary Ca
increased (1.46 mmol/L iCa at 1.40% Ca inclusion). Broilers from D3 treatments exhibited a quadratic effect with increasing iCa from 1.29 mmol/L to peak of 1.47 mmol/L when dietary Ca increased from 0.80% to 1.10% Ca inclusion. Once dietary Ca went over 1.10%, iCa decreased (interaction: F = 4.02, degrees of freedom (df) = 4, p = 0.006). This interaction was driven by Ca inclusion (p = 0.009), not 1α supplementation (p = 0.66).

Figure 1. Ionized blood calcium of 15 d broiler chickens fed different levels of calcium with or without 1-alpha-hydroxycholecalciferol supplementation (D3+1α; D3 respectively). Line graphs show means ± standard error means (n=8). Interaction effect observed between calcium inclusion and 1α supplementation (General linear model (GLM), * p < 0.05).
**1α supplementation did not affect plasma vitamin D₃ levels at 0.95% Ca inclusion**

At 0.95% Ca inclusion, 1α supplementation did not affect plasma concentrations of 24,25-(OH)₂-D₃ or 25-OH-D₃. A statistical trend was observed with D₃+1α broilers having a higher D₃ concentration compared to D₃ broilers (data not shown; p = 0.07).

**Calcium inclusion levels, not 1α supplementation, affected MUC2, OCLN, or VDR gene expression in cecal tonsils**

1α had no effect on relative expression for MUC2, OCLN, or VDR genes in cecal tonsils. MUC2 and OCLN expression decreased as Ca inclusion level increased in diets compared to control (Ca-inclusion: F = 12.7, df = 4, p < 0.0001; Ca-inclusion: F = 9.4, df = 4, p < 0.0001, respectively; **Figure 2A-B**). A decrease in relative expression with VDR also occurred as Ca inclusion increased (Ca-inclusion: F = 17.3, df = 4, p < 0.0001; **Figure 2C**). Although not statistically different, an interesting observation was that D₃+1α broilers fed diet with 0.80% Ca inclusion had increased VDR expression compared to D₃ broilers.
Figure 2. Gene expression in cecal tonsil tissue of 15 d broiler chickens fed different levels of calcium with or without 1-alpha-hydroxycholecalciferol supplementation (D$_3$+1α; D$_3$ respectively). A. Occludin (OCLN) B. Mucin 2 (MUC2) C. Vitamin D receptor (VDR). Cecal tonsil tissue was analyzed using qPCR normalized against glyceraldehyde phosphate dehydrogenase (GAPDH; housekeeping gene) expression (n = 5). Bars with common letters are not statistically different from each other (General linear models (GLM), p < 0.05).
Experiment 2 – grower diet

**Specific blood chemistry parameters are affected by Ca inclusion or 1α supplementation**

1α had no effect on 35 d broilers’ iCa concentration. As dietary Ca inclusion increased, iCa concentration increased (Ca-inclusion: $F = 5.7$, $df = 3$, $p = 0.003$; **Figure 3A**). D₃+1α broilers had higher blood bicarbonate concentration (Vit. D: $F = 4.5$, $df = 1$, $p = 0.041$) and a trend was observed with bicarbonate concentration with increasing Ca inclusion levels (Ca-inclusion: $F = 2.4$, $df = 3$; $p = 0.08$; **Figure 3B**). Base excess of extracellular fluid (BEecf) is when hydrogen ions diffuse into red blood cells which causes plasma alkalinity to rise as a result as a base excess (22). The statistical model for BEecf initially exhibited a statistical trend with both vitamin D and Ca-inclusion. However, the model expresses a statistical difference when only vitamin D is in the model which is reported in this study. BEecf concentration was increased in D₃+1α broilers fed 0.54% Ca inclusion (Vit. D: $F = 5.11$, $df = 1$, $p = 0.03$; **Figure 3C**).

**Plasma vitamin D₃ metabolite concentration is affected by 1α supplementation and calcium inclusion**

No difference was noted among dietary treatments with 24,25-(OH)₂-D₃ plasma concentration (overall: $F = 1.66$, $df = 7$, $16$, $p = 0.19$; **Figure 4A**). For D₃+1α broilers, 25-OH-D₃ plasma concentration decreased as dietary Ca inclusion level increased (interaction: $F = 3.80$, $df = 3$, $p = 0.035$; **Figure 4B**) although pen location contributed to this effect ($F = 6.59$, $df = 1$, $p = 0.01$). This interaction for 25-OH-D₃ concentration was highly influenced by 1α supplementation (Vit. D: $F = 35.2$, $df = 1$, $p < 0.0001$) compared
to Ca inclusion (F = 1.53, df = 3, p = 0.25). Broilers fed 1α had a linear decrease in plasma D₃ as calcium inclusion levels increased, except broilers from 0.76% Ca treatment had the highest D₃ concentration (interaction: F = 0.02, df = 3; p = 0.02; Figure 4C).
Figure 3. Selected blood chemistry of 35 d broiler chickens fed different levels of calcium with or without 1-alpha-hydroxycholecalciferol supplementation (D3+1α; D3 respectively). A. Ionized blood calcium B. Base excess of extracellular fluid (BEecf) C. Blood bicarbonate (HCO3). Line graphs show means ± standard error means (n=5). Calcium inclusion effect observed for ionized blood calcium; 1α supplementation effect with BEecf. (General linear models (GLM), p < 0.05).
Figure 4. Vitamin D metabolite plasma concentrations of 35 d broiler chickens fed different levels of calcium with or without 1-alpha-hydroxycholecalciferol supplementation (D₃+1α; D₃ respectively). A) 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃) B) 25-hydroxycholecalciferol (25-OH-D₃) C) Cholecalciferol (Vitamin D₃) D) Comparison of relative concentration between each vitamin D₃ metabolite between D₃ and D₃+1α groups; diagonal patterned bars denote D₃ and solid bars denote D₃+1α. Interaction between calcium inclusion and 1α supplementation with 25-OH-D₃ and vitamin D₃. Line graphs show means ± standard error means (n=3; General linear models (GLM), * p < 0.05; *** p ≤ 0.0001).
Higher calcium inclusion levels influenced duodenal CALB, VDR, and NPTIIb gene expression

No Ca inclusion effects or 1α supplementation effects were observed for CALB (p = 0.52; Figure 5A). Broilers in D₃+1α treatments had decreased relative expression of MUC2 compared to control treatment (Vit. D: F = 10.4, df = 1, p = 0.002; Figure 5B). A trend was denoted for VDR expression for 1α supplementation (Vit. D: F = 3.12, df = 1, p = 0.084) and Ca inclusion (F = 2.71, df = 3, p = 0.055) with a slight increase in expression with D₃ broilers. D₃ broilers expressed an increase in NPTIIb expression compared to D₃+1α broilers as dietary Ca increased (interaction: F = 3.33, df = 3, p = 0.03, Figure 5D).

1α supplementation and calcium inclusion level did not affect height/crypt depth ratio of jejunal villi

Feeding 1α to broilers had no impact on surface area or height/crypt depth ratio of villi (data not shown; p = 0.45, p = 0.86; respectively). Similarly, Ca inclusion levels did not affect either parameter (p = 0.40, p = 0.34; respectively).
Figure 5. Gene expression in duodenal tissue of 35 d broiler chickens fed different levels of calcium with or without 1-alpha-hydroxycholecalciferol supplementation (D$_3$+1α; D$_3$ respectively). A. Calbindin d28k (CALB) B. Mucin 2 (MUC2) C. Vitamin D receptor (VDR) D. Sodium-phosphate cotransporter type II b (NPTIIb). Duodenal tissue was analyzed using qPCR normalized against glyceraldehyde phosphate dehydrogenase (GAPDH; housekeeping gene) expression (n = 5). (General linear models (GLM), * p < 0.05).
Discussion

Our study indicated that including 1α in young broiler diets can improve their blood iCa status during starter phase with increased Ca inclusion. 1α supplementation at grower phase can also cause a decrease in plasma vitamin D metabolites in young broilers. 1α supplementation seems to exhibit greater efficacy during the starter phase compared to the grower phase of broiler diets in its alteration of blood iCa. However, broilers fed grower diets with 1α supplementation had a decrease in plasma 25-OH-D₃ concentration as Ca inclusion increased. An interesting effect of 1α supplementation on blood chemistry was that it increased BEecf concentration during the grower phase. Increased BEecf concentration indicated broilers were metabolically exceeding their capacity to maintain acid-base balance in body, resulting in more alkali blood, an effect previously described by Mongin (23). We argue this effect is caused by excess dietary Ca, as an electrolyte when present in tissues, (24) has biological implications for potentially using 1α in diets with lower Ca levels for grower phase; otherwise, these birds are under some form of duress to reduce BEecf to homeostatic balance.

Blood Ca is tightly regulated for various reasons including the need to control Ca distribution into tissues and to maintain blood pH (25). Levels of iCa in our two experiments were similar in range to those studies that reported plasma Ca concentrations from young broilers of similar ages (19, 26, 27), indicating no unusual physiological levels to denote Ca deficiency or toxicity. For broilers from the starter diet experiment, at 1.10% Ca inclusion, D₃+1α broilers had decreased iCa as compared to D₃ broilers; this exhibits 1α’s increased efficacy on Ca utilization because D₃+1α broilers most likely absorbed more Ca for bone metabolism than D₃ broilers having a much
higher blood iCa concentration. Han et al. (19) fed broiler chicks different levels of 1α and reported a linear effect with increasing 1α also increased plasma Ca concentration; although their broilers had a higher average concentration than our birds by almost 1 mmol/L with their 5 µg/kg 1α supplementation with 0.25% Ca diet. However, their plasma Ca was collected at 21 d whereas our study collected iCa at 15 d. Further investigation with an even lower dietary Ca inclusion level may help fit a better regression line for this relationship with 1α’s efficacy on Ca absorption into bone. 

Examining hydroxylase expression in kidney and liver tissue could determine whether pathological issues are developing due to Ca intake. There would be greater expression of 25-hydroxylase in liver with regards to 1.40% Ca inclusion level of D3+1α broilers having a higher iCa concentration than their counterparts without 1α supplementation. If this case were to occur, then histopathological examination of kidneys and liver would verify soft-tissue calcification. 

During the grower phase, increasing dietary Ca inclusion levels caused an increase in blood Ca concentration, regardless of 1α supplementation. Our result is in agreement with Sebastian et al. (28) who also report how increasing dietary Ca inclusion levels led to increased blood Ca concentration. Increased blood Ca concentration indicates Ca requirements are met in these chickens and Ca is most likely being excreted or stored in bone which was observed with various strains of broilers because of how tightly regulated blood Ca is (26, 28). 1α’s impact on increasing blood bicarbonate concentration is probably caused by a shift in acid-base balance. We did not observe any changes in pH or CO2 blood concentration; however, we speculate bicarbonate’s increase without any change to CO2 implies these broilers may be under
physiological stress with alkalosis. Metabolic alkalosis can be caused by chloride depletion, but can also be caused by hypercalcemia because hypercalcemia increases bicarbonate resorption (29). Therefore, it is possible that 1α, with addition of dietary vitamin D, could have caused greater Ca absorption that led to greater bicarbonate being resorbed. There is also the possibility that at time of blood sampling, the broilers may have metabolically compensated partial pressure of CO₂ to maintain pH in reaction to increased bicarbonate levels (30). A caveat of our study is that we did not examine blood chloride levels which could potentially explain the BEecf increase. Future studies should explore the mechanism with Ca inclusion in broilers on why blood bicarbonate and BEecf increased even though pH did not change.

There were no differences in vitamin D₃ metabolite blood concentrations in broiler chicks fed starter diets with or without 1α supplementation at 0.95% Ca inclusion. 1α may not be affecting vitamin D pathways considering there was no difference in ionized blood Ca concentration at this level. Based on data from the National Research Council (31), 0.95% Ca inclusion is closest to starter phase minimum requirements for broilers. We may find a difference in vitamin D metabolite blood concentrations in broilers fed 1.10% Ca because of how drastically Ca concentration differed between D₃ and D₃+1α broilers.

However, 1α’s effects are more pronounced when broilers are older and fed a grower diet. An inverse relationship of vitamin D₃ plasma concentration implies how 1α’s influence in vitamin D metabolism is dependent on dietary Ca inclusion (Figure 4C). This relationship is also connected to 25-OH-D₃ because 1α is likely exerting an effect to reduce vitamin D₃ because of 1α’s efficacy. We believe D₃ is being excreted in feces,
but we are unable to find any poultry-related studies that report vitamin D in excreta. A clinical study discussed how 25-OH-D₃ was intravenously administered to patients and positively correlated to increased 25-OH-D₃ in urine (32). It is important to note that broilers grown through the grower phase for this study were fed common starter diets with 1α supplementation. When these broilers were sampled, broilers not fed 1α in grower phase had a period of 18 d since they were given 1α supplementation. However, this may not impact our findings because in plasma, 1,25-(OH)₂-D₃ has a half-life of about 10 – 20 h and 25-OH-D₃ is about 15 d (12). Considering broilers not fed 1α had consistent plasma levels of 25-OH-D₃, it is unlikely that 1α they were fed from starter diet was remaining in their bodies because it would have been converted to 1,25-(OH)₂-D₃ and excreted or used up before blood was collected.

Excess 25-OH-D₃ in chickens is converted to 24,25-(OH)₂-D₃ by 24-hydroxylase in kidneys (33). 24,25-(OH)₂-D₃ is an inactive vitamin D form that is excreted, but this form is not the end-product of this pathway, 24,25-(OH)₂-D₃ goes through a series of conversions and ends up becoming calcitroic acid, a water-soluble molecule that is readily excreted (34). Although no difference in 24,25-(OH)₂-D₃ plasma concentration was observed, we speculate calcitroic acid concentration would be higher in 1α-fed broilers because 1α cannot be converted to 24,25-(OH)₂-D₃. 1α would likely be converted to 1,25-(OH)₂-D₃ and then undergo multiple conversions to become calcitroic acid. We constructed a hypothetical model to compare how dietary D₃ and 1α would be converted to calcitroic acid to be excreted (Figure 6).
Figure 6. Hypothetical model on comparing how dietary vitamin D$_3$ and 1-$\alpha$-hydroxycholecalciferol (1$\alpha$) are converted to water-soluble calcitroic acid to be excreted. Black arrows denote vitamin D$_3$’s pathway to calcitroic acid and orange arrows denote 1$\alpha$’s pathway. Dashed arrows signify 24-hydroxylation step. Multiple arrows between 1,24,25-(OH)$_3$-D$_3$ and calcitroic acid denote number of conversion steps. 25-OH-D$_3$ is 25-hydroxycholecalciferol; 24,25-(OH)$_2$-D$_3$ is 24,25-dihydroxycholecalciferol; 1,25-(OH)$_2$-D$_3$ is 1,25-dihydroxycholecalciferol; and 1,24,25-(OH)$_3$-D$_3$ is 1,24,25-trihydroxycholecalciferol.
1α-fed broilers exhibiting a linear drop in 25-OH-D₃ plasma concentration is a consequence of 1α being converted to 1,25-(OH)₂-D₃ which will reduce the amount of vitamin D₃ being converted into 25-OH-D₃ due to a negative feedback loop (35). As dietary Ca inclusion increased in 1α-fed broilers, 25-OH-D₃ concentration likely dropped because of 1α’s effects on Ca absorption. Purifying fecal content to measure vitamin D may provide insights to how much D₃ was excreted relative to how much was fed.

Decreased expression in OCLN and MUC2 as dietary Ca inclusion levels increased indicates Ca in cecal tonsils could be causing a decrease in gene expression to accommodate increased Ca in that region of gastrointestinal tract. OCLN is a tight junction protein which functions to ensure cell-to-cell integrity is maintained to prevent pathogens from passing paracellularly and reaching basal membrane and blood vessels (36). It is unclear what biological relevance this effect may have with gene expression because cecal tonsils are in distal gut and most Ca absorption occurs in duodenum and jejunum where transporters are in greater abundance (37). The fact that OCLN’s highest expression difference was only 0.2 more than starter diet control (D₃, 0.95% Ca) means relative expression did not change much with addition of 1α in diets with 0.80% Ca inclusion, suggesting that higher levels of dietary Ca can reduce OCLN expression. Juvenile pigs also exhibited a similar phenomenon in their jejunum when they were fed diets with high levels of Ca (38). An in vitro study observed that local increases in intracellular Ca can disrupt tight junction formation by preventing cytoskeletal stabilization (39). Therefore, it is possible that increased dietary Ca inclusion with our broilers decreased OCLN expression in their cecal tonsils.
Compared to control for starter diets, broilers fed 1α at 0.80% Ca inclusion had increased expression of MUC2. This observation is interesting because Ca has been reported to be important for the matrix structure of mucin by acting as a shield against negatively charged glycans (40). MUC2’s biological significance with cecal tonsils is to form a protective layer for protecting the cecal tonsils from pathogens (41, 42). Relative to iCa of 0.80% Ca-fed broilers, 1α could be eliciting a response in which decreased Ca is reaching cecal tonsils which could be driving expression of MUC2 to ensure absorbed Ca is used to make mucin at cecal tonsils for protection. We could not express TRPV6 (43) from cecal tonsils, but its significance as a Ca channel in gut could highlight MUC2’s expression. TRPV6 is channel protein that absorbs Ca in intestine (44). Hoenderop et al. (43) identified this protein and reported how its expression is dependent on 1,25-(OH)2-D3 presence along as it colocalizes with CALB.

For broilers sampled during grower phase, broilers fed 0.54% Ca without 1α had a 0.1 increase of CALB compared to control (D3, 0.76% Ca inclusion): this observation could signify that broilers were trying to bind dietary Ca for transport and absorption. Our results were similar to a different study by Li, Yuan (45) in which low Ca upregulated CALB expression. Although our results indicated MUC2 was statistically lower in D3+1α broilers, relative expression was only decreased by about 0.5 compared to D3-fed broilers which indicates no change in MUC2 expression. This finding is indicative that these broilers can be considered healthy, an inference supported by the FITC-D data which denoted no difference in jejunal morphology.

VDR is responsible for signal transduction of Ca absorption genes when 1,25-(OH)2-D3 binds to it (46). VDR expression is highest in D3+1α and 0.80% Ca inclusion
level fed broilers, indicating 1α supplementation may have influenced expression to accommodate for greater levels of vitamin D. To our knowledge as of this study, no information is known about VDR expression in cecal tonsils and how it may be influenced. For VDR expression in duodenum of 35 d broilers, even though it was not statistically different, a trending increase of VDR expression as dietary Ca inclusion increased for broilers not fed 1α indicates dietary Ca’s regulatory role with increasing VDR expression. VDR’s role as a signal transducer for gene expression for proteins (TRPV6, CALB) explains why vitamin D is important for dietary Ca absorption (47).

NPTIIb is an important cotransporter protein that requires sodium to move P in its phosphate form into cells from intestinal lumen (48). Increasing Ca inclusion led to an increase in duodenal NPTIIb expression in 35 d D₃ broilers was likely caused by P imbalance. Excess dietary Ca compared to P can cause Ca to bind to P and form insoluble tricalcium phosphate (49). Our results were similar to Li et al. (45), in which increased dietary Ca levels led to increased NPTIIb expression in duodenum. Li et al. also noted how high dietary Ca could cause a decrease in available P which triggers the increased expression of NPTIIb in duodenum. A proposed model of the inverse relationship between CALB and NPTIIb expression relative to Ca inclusion levels is denoted for broilers not fed 1α (Figure 7). Further characterization of this relationship can elucidate the need to reevaluate nutrient interrelationships in animal production. There may be unintended impacts of using excessive nutrients as a safety net for meeting nutrient requirements because potential deficiencies can be caused by nutrient antagonism, but these consequences can be detected by examining genes related to absorption such as NPTIIb and CALB.
Figure 7. Inverse relationship between duodenal calbindin d28k (CALB) and sodium-phosphate cotransporter type IIb (NPTIIb) in broiler chickens relative to calcium availability. As duodenal calcium concentration increases, then CALB expression decreases and NPTIIb expression increases to potentially maximize phosphate absorption because of the potential excessive calcium binding to phosphorus to form tricalcium phosphate and making phosphorus unavailable.
Our work highlights 1α supplementation with certain levels of Ca inclusion can impact blood Ca concentration and affect vitamin D metabolites concentration. Our findings exhibit that 1α can improve Ca utilization in young broilers with an implication for reducing dietary Ca in their diets. We suggest 1α should be supplemented in broiler diets for the starter phase and either removed for grower phase or provided with reduced dietary Ca levels. 1α’s potential to reduce dietary Ca without any negative impacts on growth performance signifies importance of synthetic nutrients for improving animal production while reducing potential environmental impact from excreted excess nutrients. Future research should explore how much 1α supplementation will cause vitamin D toxicity in growing animals to characterize an animal’s regulatory limits of removing vitamin D with consideration of 1α's bypassing of negative feedback regulation.
Acknowledgments

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References


Chapter 3

Dietary vitamin D₃ super-doses does not affect egg production in aged laying hens

Supplement of manuscript (Chapter 4) in preparation to be submitted to Journal of Nutrition

Abstract

Vitamin D is important for regulating calcium (Ca) metabolism and improves skeletal health of egg production hens. Laying hens fed a super-dose of vitamin D can produce eggs providing up to 1,000 IU of vitamin D to combat deficiency in humans. However, little is known about how super dosage levels of dietary vitamin D impacts laying hens physiologically. Our study investigated if super-dosage levels of vitamin D₃ affected egg production in laying hens. Forty-eight 68-week old HyLine Brown laying hens were assigned to six dietary treatments with vitamin D₃ supplementation levels ranging from 400 IU/kg to 36,000 IU/kg for eleven weeks. Diets with vitamin D₃ super-doses had no effect on egg production, eggshell quality, ileal digesta Ca or P, or bone Ca and P. Our findings suggest that dietary vitamin D₃ super-doses does not negatively impact egg production. Functional significance of reduced dietary Ca and P should be further explored with vitamin D₃ super-doses for potential situations where available P is severely limited.
**Introduction**

Dietary vitamin D supplementation is used to improve bone health of laying hens because of vitamin D’s effect on calcium (Ca) mobilization between blood, tissues, and eggshell production (1-3). Many commercial layer farms supplement vitamin D beyond NRC requirements to ensure hens meet vitamin D requirements and are able to lay eggs with eggshells with desirable parameters for consumers. Egg production require a pool of Ca that is derived from medullary bone which is a reservoir birds use for Ca (4, 5). However, laying hens have leg issues because of bone weakness from rigors of Ca mobilization (6). Hormones regulate ionized Ca or unbound Ca to maintain a constant range in blood and facilitate Ca movement between bone, other tissues, and the Ca used for eggshell formation (4, 6). Increasing dietary Ca is inexpensive, but there is a limit to how much Ca can be added before dietary P levels need to be increased to maintain a 2:1 Ca:P (7): a concomitant P increase is more challenging as P has environmental implications to account for (8).

Producers can reduce hens’ bone issues by increasing dietary Ca and phosphorus (P), raising free-range hens which are able to move around and build bone strength (9, 10), or feeding hens more vitamin D₃ in their diets (11). Eggs from free-range hens have consumer support because of welfare implications; however, free-range hens require more space, and moreover the eggs are exposed to cross-contamination with contact from feces, and have increased labor costs associated with their operations. Supplementing vitamin D in hens diet has positive impacts on bone health (11) and results in improvements to hens’ health have economic value to farmers. A few studies examined how super-doses of vitamin D improved vitamin D
deposition in egg yolk for consumers, but it is not known how hens are physiologically affected and if older birds still benefit from super-doses of vitamin D with egg production.

Little is known about how super dosage levels of vitamin D affects laying hen physiological status and how Ca mobilization is influenced. Therefore, our study explored how different levels of vitamin D meet NRC guidelines, but below commercial standard, and super-dose levels affect egg production, eggshell parameters, and Ca mobilization in older laying hens.

**Methods**

**Birds and housing**

Forty-eight 68-week old HyLine Brown laying hens were housed at North Carolina State University, Raleigh, NC. Hens were individually housed in cages between two two-level battery cages with eight birds per treatment. Experimental design was a randomized complete block design with six levels of dietary vitamin D supplementation blocked by cage level. Vitamin D supplementation levels were 400, 800, 7,400, 14,000, 20,000, and 36,000 IU/kg of feed (Tables 1 and 2). Birds were fed *ad libitum* along with water. North Carolina State University’s Institutional Animal Care and Use Committee approved all methods for this study, protocol ID number: 18-093-A.
Table 1. Ingredient composition of the experimental basal diet for aged HyLine Brown laying hens.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>63.40</td>
</tr>
<tr>
<td>Soybean meal, 46% CP</td>
<td>20.60</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>9.20</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>2.33</td>
</tr>
<tr>
<td>Dicalcium phosphate¹</td>
<td>1.95</td>
</tr>
<tr>
<td>L-Lysine-HCl, 78.8%</td>
<td>1.01</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.57</td>
</tr>
<tr>
<td>Vitamin premix²</td>
<td>0.25</td>
</tr>
<tr>
<td>Mineral premix³</td>
<td>0.20</td>
</tr>
<tr>
<td>Choline chloride, 60% choline</td>
<td>0.20</td>
</tr>
<tr>
<td>DL-Methionine, 99%</td>
<td>0.14</td>
</tr>
<tr>
<td>Salt</td>
<td>0.10</td>
</tr>
<tr>
<td>Selenium premix</td>
<td>0.05</td>
</tr>
</tbody>
</table>

¹Dicalcium phosphate contains 19.79% calcium, 17.91% phosphorus, and 17.73% available phosphorus.

Table 2. Nutrient content of experimental diet for aged HyLine Brown laying hens.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>90.46</td>
</tr>
<tr>
<td>Moisture</td>
<td>9.54</td>
</tr>
<tr>
<td>Crude protein</td>
<td>16.00</td>
</tr>
<tr>
<td>Crude fat</td>
<td>4.98</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>1.81</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.00</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.67</td>
</tr>
<tr>
<td>Ash</td>
<td>12.44</td>
</tr>
<tr>
<td>Available phosphorus</td>
<td>0.46</td>
</tr>
<tr>
<td>Methionine+Cysteine</td>
<td>0.64</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.57</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.40</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.26</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.57</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.19</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.60</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.72</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.43</td>
</tr>
<tr>
<td>Valine</td>
<td>0.83</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.43</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.83</td>
</tr>
<tr>
<td>Metabolizable Energy, kcal/kg</td>
<td>2,900</td>
</tr>
<tr>
<td>Dietary electrolyte balance, mEq/100 g</td>
<td>202</td>
</tr>
</tbody>
</table>

¹Dicalcium phosphate contains 19.79% calcium, 17.91% phosphorus, and 17.73% available phosphorus.

²Provided as milligrams per kilogram of diet: Thiamin HCL, 1.8; Riboflavin, 3.6; Ca pantothenate, 10; Niacin, 25; Pyridoxine HCL, 3; Folacin, 0.55; Biotin, 0.15; B12, 0.01; Vitamin A 1500 IU/g, 6; Vitamin D3 400 IU/g, 1; Vitamin E 10 IU/g, 40; Vitamin K, 0.55; Ethoxyquin, 125.

³Trace minerals provided per kg of premix: 60 g manganese (Mn SO4); 60 g zinc (ZnSO4); 40 g iron (FeSO4); 5 g copper (CuSO4); 1.25 g iodine [Ca(IO3)2].
Sample collection

Eggs were collected every morning and stored at 7°C for egg quality analyses. Birds were individually weighed weekly for eleven weeks and on week 0, 3, 6, and 10. Twenty-four birds were bled to have their blood chemistry analyzed via i-STAT Blood Analyzer (12). Feces were collected from individual birds the day before end of experiment. All birds were sacrificed by cervical dislocation and tissue samples were collected from 43 birds. Samples collected were right humerus and tibia and ileal digesta; they were stored at -20°C for ash content.

Egg quality

Three eggs from each bird per week were used for measuring eggshell strength, eggshell elasticity, and eggshell thickness. Eggshell strength and elasticity were measured using compression with a texture analyzer (TA-XT2, Texture Technologies Corp., Scarsdale, NY) using an acrylic disk to apply force to egg. Eggs were placed with equatorial part of egg on a holding apparatus for compression. Eggs were placed in this fashion because equatorial ends of eggs are observed to be weakest point of egg because of topological feature compared to polar ends (13). Peak force was measured in g in which how much force in grams was required to fracture eggshell. Elasticity is how much shell integrity deforms before fracture and was measured in mm.

Egg contents were removed and eggs were washed with warm water and dried for at least 48 h prior to measuring shell thickness. Shell thickness was measured using a micrometer and measuring two pieces of a shell at random locations of equatorial region. Measurements were to a range difference of 0 - 0.02 mm.
Calcium and phosphorus content of various sites

Eggshells from week 0, 3, 4, 6, and 9 were washed again with warm water to remove shell membrane and dried for 48 h. Dried eggshells were pre-weighed and dried at 68°C for 72 h using dry oven (Blue M, Atlanta, GA) and weighed again. Eggshells were crushed into fine powder and subjected to digestion to measure Ca composition of eggshells. Feces and ileal digesta were also subjected to same steps as eggshells. Dried samples were weighted placed in a muffle furnace at 500°C overnight to ash samples. Ashed samples were dehydrated in using 2 mL of distilled water to wet samples and 4 mL of 6 N hydrochloric acid was added and mixed sample was heated. Heated sample in solution was poured into volumetric flask and deionized water was added. Flask was inverted 12 times to mix solution and solution was filtered using #40 filter paper into 15 mL centrifuge tubes for analysis.

Bones were wrapped in petroleum ether moistened cheesecloth and placed in a dessicator for 72 h to extract fat from bones. Fat-extracted bones were pre-weighed and dried for 24 h at 100°C to evaporate petroleum ether residues. Fat- and moisture-free bones were weighed and ashed using same methods as eggshell, feces, and ileal digesta for Ca and P composition.

Statistical Analyses

All data shown as means ± SEM. Statistical analyses were conducted using SAS 9.4®. ANOVA was used to examine differences with egg production for each week of study and Tukey-Kramer test was used for multiple comparisons for differences between and within treatment groups for vitamin D level and if cage level had an impact.
on egg production and overall feed intake. Repeated measures ANOVA was used to examine differences in body weight over study duration, eggshell strength, elasticity, and thickness between treatment groups over time with birds grouped by treatment as a covariate. Statistical significance was established at $p < 0.05$.

Results

**Vitamin D<sub>3</sub> super-doses did not affect hens’ body weight**

Dietary super-doses of vitamin D<sub>3</sub> did not affect body weight of these laying hens ($p = 0.08$; Table 3), though all of the hens exhibited a small decrease in body weight overtime ($F = 7.07$, $df = 9, 428$, $p < 0.0001$). There was an interaction overtime with an increase in feed intake over time with hens from 7,400 and 36,000 IU D<sub>3</sub> treatments having highest feed intake ($F = 5.45$, $df = 45, 413$, $p < 0.0001$; Table 3); however, there is likely noise with feed intake because of overestimation caused by feed spillage from hens to overcompensate how much feed the hens consumed.
Table 3. Body weight and feed intake of 78 wk old laying hens fed diets with different levels of vitamin D$_3$ over an eleven-week period. Means with standard errors are given and p-values were generated by repeated measures Analysis of Variance (ANOVA). Statistical significance established when p < 0.05.

<table>
<thead>
<tr>
<th>Treatment (IU Vitamin D$_3$/kg of feed)</th>
<th>Body Weight (kg)</th>
<th>Feed Intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>2.13±0.09</td>
<td>128.6±4.9</td>
</tr>
<tr>
<td>800</td>
<td>2.32±0.07</td>
<td>134.3±3.5</td>
</tr>
<tr>
<td>7,400</td>
<td>2.36±0.05</td>
<td>167.4±3.6</td>
</tr>
<tr>
<td>14,000</td>
<td>2.21±0.10</td>
<td>137.3±7.3</td>
</tr>
<tr>
<td>20,000</td>
<td>2.18±0.08</td>
<td>140.5±8.8</td>
</tr>
<tr>
<td>36,000</td>
<td>2.12±0.05</td>
<td>171.3±4.4</td>
</tr>
</tbody>
</table>

Dietary Vitamin D Level 0.08 <0.0001
Week <0.0001 <0.0001

Vit D x Week 0.19 <0.0001

Egg production and eggshell quality were not influenced by dietary vitamin D$_3$

A dietary effect was observed for egg production for the entire study duration (F = 2.52, df = 5, p = 0.047; Table 4) with hens from 14,000 IU D$_3$ treatment group having highest production rate of 86.7%. Eggshell strength and eggshell thickness were not affected by dietary vitamin D$_3$ levels (p = 0.19, p = 0.72, respectively). There was a trending interaction between dietary vitamin D$_3$ level over time in which there was a decrease in eggshell elasticity (p = 0.07).
Table 4. Egg production and eggshell quality measurements of 78 wk old laying hens fed diets with different levels of vitamin D₃ over an eleven-week period. Means with standard errors are given and p-values were generated by repeated measures Analysis of Variance (ANOVA); egg production p-value was determined by one-way ANOVA with Tukey’s post-hoc test. Statistical significance established when p < 0.05.

<table>
<thead>
<tr>
<th>Treatment (IU Vitamin D₃/kg of feed)</th>
<th>Egg Production (%)</th>
<th>Shell Strength (kg)</th>
<th>Shell Thickness (mm)</th>
<th>Shell Elasticity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>77.4±1.9</td>
<td>4.51±0.19</td>
<td>0.374±0.010</td>
<td>0.209±0.003</td>
</tr>
<tr>
<td>800</td>
<td>86.8±1.6</td>
<td>4.51±0.10</td>
<td>0.371±0.006</td>
<td>0.222±0.006</td>
</tr>
<tr>
<td>7,400</td>
<td>80.4±3.4</td>
<td>4.58±0.17</td>
<td>0.372±0.010</td>
<td>0.221±0.004</td>
</tr>
<tr>
<td>14,000</td>
<td>86.7±2.6</td>
<td>4.13±0.11</td>
<td>0.360±0.010</td>
<td>0.205±0.007</td>
</tr>
<tr>
<td>20,000</td>
<td>79.4±3.9</td>
<td>4.29±0.12</td>
<td>0.366±0.009</td>
<td>0.208±0.006</td>
</tr>
<tr>
<td>36,000</td>
<td>85.0±1.7</td>
<td>4.36±0.14</td>
<td>0.377±0.008</td>
<td>0.207±0.007</td>
</tr>
</tbody>
</table>

Dietary Vitamin D Level | 0.05 | 0.19 | 0.72 | 0.044 |
Week | ND | 0.01 | 0.29 | <0.0001 |
Vit D x Week | ND | 0.10 | 0.10 | 0.072 |

ND = Not determined
Table 5. Ionized blood calcium of 78 wk old laying hens fed diets with different levels of vitamin D₃ over an eleven-week period. Means with standard errors are given and p-values were generated by repeated measures ANOVA. Statistical significance established when p < 0.05.

<table>
<thead>
<tr>
<th>Treatment (IU Vitamin D₃/kg of feed)</th>
<th>Week 0</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Week 10</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>1.84±0.05</td>
<td>1.59±0.08</td>
<td>1.68±0.05</td>
<td>1.58±0.04</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>1.59±0.02</td>
<td>1.62±0.07</td>
<td>1.69±0.09</td>
<td>1.68±0.10</td>
<td></td>
</tr>
<tr>
<td>7,400</td>
<td>1.82±0.07</td>
<td>1.55±0.04</td>
<td>1.61±0.04</td>
<td>1.55±0.06</td>
<td></td>
</tr>
<tr>
<td>14,000</td>
<td>1.65±0.03</td>
<td>1.53±0.02</td>
<td>1.48±0.05</td>
<td>1.48±0.07</td>
<td></td>
</tr>
<tr>
<td>20,000</td>
<td>1.61±0.09</td>
<td>1.59±0.09</td>
<td>1.51±0.02</td>
<td>1.63±0.03</td>
<td></td>
</tr>
<tr>
<td>36,000</td>
<td>1.63±0.06</td>
<td>1.46±0.03</td>
<td>1.54±0.08</td>
<td>1.53±0.06</td>
<td></td>
</tr>
</tbody>
</table>

Dietary Vitamin D Level

<table>
<thead>
<tr>
<th></th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week</td>
<td>0.65</td>
</tr>
<tr>
<td>Vit D x Week</td>
<td>0.69</td>
</tr>
</tbody>
</table>
Ionized blood calcium is affected by dietary vitamin D₃ levels

There was no temporal effect on ionized blood Ca (p = 0.65). With week 0 considered as a covariate, there was a strong dietary effect on ionized blood Ca (p = 0.002, Table 5), but it is unclear on how dietary levels of D₃ influenced ionized blood Ca concentration because their levels are both high and low across standard D₃ hens and super-dose D₃ hens.

Fecal calcium is affected by dietary vitamin D₃ levels, not ileal digesta or eggshell calcium or phosphorus

No difference in eggshell Ca and P were observed (p = 0.64 for both). No dietary effect was observed for ileal digesta Ca or P. A dietary effect was observed with fecal Ca with hens fed 14,000 IU D₃ diet had 10.6±0.83% Ca in their feces, whereas all other treatments were 8-9%, with exception of 36,000 IU D₃ fed hens which had 7.36±0.43% fecal Ca (p = 0.03, Table 6). There was no dietary effect on fecal P (p = 0.76).
Table 6. Calcium (Ca) and phosphorus (P) composition of eggshell, ileal digesta, and feces of 78 wk old laying hens fed diets with different levels of vitamin D₃ over an eleven-week period. Means with standard errors are given and p-values were generated by one-way Analysis of Variance (ANOVA) with dietary vitamin D as the factor. Tukey’s post-hoc test was conducted for multiple comparisons. Statistical significance established when p < 0.05.

<table>
<thead>
<tr>
<th>Treatment (IU Vitamin D₃/kg of feed)</th>
<th>Eggshell Ca (%)</th>
<th>Ileal Digesta Ca (%)</th>
<th>Fecal Ca (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>38.1±2.2</td>
<td>3.32±0.84</td>
<td>8.71±0.74</td>
</tr>
<tr>
<td>800</td>
<td>40.4±0.70</td>
<td>3.94±0.63</td>
<td>8.10±0.55</td>
</tr>
<tr>
<td>7,400</td>
<td>38.8±1.08</td>
<td>2.51±0.37</td>
<td>9.20±0.30</td>
</tr>
<tr>
<td>14,000</td>
<td>40.5±0.81</td>
<td>3.76±1.39</td>
<td>10.6±0.83</td>
</tr>
<tr>
<td>20,000</td>
<td>38.8±1.47</td>
<td>3.05±1.14</td>
<td>8.70±0.13</td>
</tr>
<tr>
<td>36,000</td>
<td>40.7±1.13</td>
<td>2.79±0.37</td>
<td>7.36±0.43</td>
</tr>
</tbody>
</table>

| Diet                                | 0.64           | 0.74                 | 0.03         |

1 For fecal Ca %, fecal P was used as a predictor and vice versa. Values without a common superscript letter are statistically different. ND = Not determined
**Humerus have more calcium and phosphorus than tibia**

No dietary effects of vitamin D supplementation were observed bone Ca or P (p = 0.79, p = 0.63, respectively). Humerus bones have more Ca and P than tibia bones (p = 0.020, p = 0.015, respectively; **Figure 1**).

**Figure 1.** Percent by weight of bone calcium and phosphorus from 78 wk HyLine Brown laying hens fed different levels of dietary D₃. **A)** Tibia and humerus calcium. **B)** Tibia and humerus phosphorus. Blue squares with lines denote tibia and orange triangles with dashed lines denote humerus (n = 7). (General linear models (GLM), p < 0.05).
Discussion

Vitamin D supplementation is important for facilitating Ca absorption and increasing bone mineralization (14). Laying hens are highly susceptible to osteoporosis if they are fed diets deficient in vitamin D or calcium (15). Our study reveals that egg production and bone composition of aged laying hens are not affected by super-doses of dietary vitamin D₃. Like other studies (16, 17), vitamin D supplementation did not affect eggshell quality. No vitamin D-related effect to bone Ca or P denotes that these hens may not be as sensitive to vitamin D supplementation as younger hens with Ca utilization (18).

Body weight has been repeatedly shown to influence egg weight with lighter hens producing lighter eggs (19). In this study, although body weight in these hens decreased slightly (Table 3), this effect was likely geriatric related as their tibia and humerus bones showed no differences between hens fed differing levels of dietary vitamin D₃. Feed intake for this study increased over time, but this was likely an overestimation because feed was frequently spilled from feeders as the hens fed. Feeders were checked daily and were refilled to almost full which likely contributed to feed spillage; however, there was a week where feed given was lower than normal which likely reduced the hens’ intake for that week. Many studies reported that laying hens consume about 100 g of feed per day (10, 20-22). This study had then hens’ feed intake averaging about 150 g per day. Hens fed dietary super-doses of vitamin D₃ consumed similar levels of feed as non-super-dose fed hens.

Egg production is an important trait for laying hens as a production animal. Laying hens have been intensively bred to yield higher egg production rates over a
production cycle to maximize feed efficiency (23). Dietary Ca and P are two minerals that can affect eggshell quality with extreme levels of available phosphorus reducing eggshell quality (24). Egg production is dependent on energy intake (25) and is apparently unaffected by low Ca intake (7, 24). In this study, hens fed exorbitant levels of D₃ had similar egg production rates as hens fed D₃ levels closer to nutritional requirements. Although one experimental dietary treatment had a statistical increase compared to the rest (7,400 IU D₃, 86.7% egg production), hens from the rest of the treatments were about 3-4% lower in production. Eggshell strength and thickness were not affected by D₃ in this study. Eggshell strength and thickness are affected by dietary Ca fed to hens, with the form factor of Ca being important (26). Low dietary Ca level is associated with reduced eggshell strength and thickness (27). We observed no difference in eggshell strength and thickness. Vitamin D₃ is essential for Ca mobilization in which increasing dietary Ca with vitamin D₃ leads to reduced number of crack eggs with no difference in eggshell thickness (16). Elasticity is characterized as egg deformation is how prone an egg is to breakage upon impact (28, 29). Although not statistically different, eggshell elasticity from eggs in this study decreased overtime, and it should be noted these eggs were stored at 7°C for at least a month before being analyzed. Storage time and hen age have been reported to reduce eggshell quality (30). Even though there were no differences in ileal digesta and eggshell Ca, there was an unusual difference in fecal Ca (Table 6). Hens fed 14,000 IU D₃ had highest fecal Ca and hens fed 20,000 and 36,000 IU D₃ had lower fecal Ca. Although it could be related to oviposition and time of day with relation to serum Ca because of how Ca is mobilized (31). A possible reason why there was no dietary effect on fecal P is likely because
there was no phytase included in the diets. One study with broiler chicks reported increased phytate P utilization when chicks were fed 1,25-(OH)$_2$-D$_3$ (32).

Bones are an important Ca pool for egg production for laying hens (4). Ca is released from bone as a result of osteoclastic activity via actions of parathyroid hormone and enters blood circulation (4). Medullary bone is where Ca is used for forming eggshells and is under constant turnover (33). In this study, there was an observed dietary effect in ionized blood Ca, but a caveat of this finding may be caused by when the hen last laid an egg. Circulating Ca levels are dependent on the timing when an egg is laid (34). It is important to note that ionized blood Ca of these hens was considered a normal range as measured by i-STAT Blood Analyzer (35). Dietary vitamin D intake is important for Ca absorption, but high levels of D$_3$ beyond a hen’s requirement does not affect its bone Ca or P. Younger hens or pullets were reported to have increased bone mineral density when they were fed higher levels of D$_3$ (17). Increased dietary Ca can increase bone Ca, but it does little to aged laying hens (10).

One important aspect of this study highlights is that extreme levels of dietary vitamin D$_3$ may not be valuable for egg producers for egg yield. The aged laying hens in this study maintained a high production rate relative to similar aged hens from other studies (10, 36). Even though egg quality parameters, outside of eggshell, were not measured, egg yolk is one component of the egg that will be affected by vitamin D intake which has implications of value-added eggs that are vitamin D-enriched (11, 17). Another important avenue that can elucidate aged laying hen performance is altering dietary Ca levels. Hens fed a Ca-poor diet may benefit from vitamin D$_3$ super-doses to improve Ca utilization for egg production. This proposal has value to egg producers who
may look into reducing dietary P, struggle with incorporating P into diets, or are unable to access/add microbial phytase (37), but want to ensure their hens lay good quality eggs. Our findings were negative in terms of observing differences with egg production, but we can confirm that aged laying hens fed dietary super-doses of D₃ are not negatively affected either. Future research should evaluate laying hens fed super-dose levels of D₃, but with Ca- and P-poor diets to illustrate vitamin D₃ super-dosage efficacy for bone mobilization.

**Conclusion**

Our work illustrates dietary vitamin D₃ super-doses does not affect older laying hens’ egg production or eggshell quality. Future work should evaluate mechanisms of D₃ super-doses with Ca- or P-poor diets.
References


Chapter 4

Super-doses of dietary vitamin D$_3$ intake in aged laying hens illustrates limitation of 24,25-dihydroxycholecalciferol conversion and importance of vitamin D$_3$ deposition into egg yolk

Manuscript in preparation with coauthors Pete M. Pitman and Kimberly A. Livingston to submit to the Journal of Nutrition

Abstract

**Background:** Elderly humans take vitamin D supplements to reduce the risk of vitamin D deficiency and osteoporosis. However, it is unclear how dietary super-dose (10,000x greater than requirement) can affect vitamin D status in aged animals. Aged laying hens can be compared to women in peri- or postmenopausal stages of life because their bone health is physiologically taxed from egg production and they are highly susceptible to osteoporosis.

**Objective:** We investigated dietary super-dose impacts of cholecalciferol (vitamin D$_3$) on vitamin D status in aged laying hens in production.

**Methods:** Forty-eight 68-week old HyLine Brown laying hens were individually housed in cages with eight hens per dietary treatment for eleven weeks. Hens were randomly assorted between six levels of dietary vitamin D$_3$ supplementation and fed *ad libitum*. Supplementation levels were 400 (recommended dosage for hens), 800, 7,400, 14,000, 20,000, and 36,000 IU (international unit) D$_3$/kg of feed. At end of study, all hens were euthanized to collect blood, liver, and kidney. Gene expression of hydroxylase genes
were measured from liver and kidney samples. Plasma and egg yolk vitamin D metabolites were also measured.

**Results:** Increasing dietary vitamin D$_3$ led to increased plasma vitamin D$_3$, 25-hydroxycholecalciferol (25-OH-D$_3$), and 24,25-dihydroxycholecalciferol concentration (p < 0.0001 for all 3 metabolites). Super-dose fed hens had decreased kidney 24-hydroxylase expression (p = 0.0006).

**Conclusions:** Plasma and egg yolk vitamin D levels increased relative to dietary intake. There are implications of potential vitamin D toxicity risk with dietary vitamin D$_3$ super-doses, but that risk is lower in laying hens because they can remove excess D$_3$ through egg production. Increased vitamin D$_3$ intake at super-doses likely reduces 24-hydroxylase expression; therefore, limiting 24-hydroxylation activity. Combined with 25-OH-D$_3$ half-life, plasma 25-OH-D$_3$ can potentially increase to toxic levels.
Introduction

Considering that vitamin D deficiency is directly linked to osteoporosis, increasing vitamin D intake through diet or supplements is a means to reduce risk and prevent vitamin D deficiency (1, 2). Older women in menopausal or post-menopausal stages of life contend with increased demands of nutrient absorption with onset of hormonal changes that are an effect of menopause (3-5). Reduction of physiological turnover activity as an added effect of aging is another demand which makes vitamin D deficiency more prevalent for older women (6). A recent and intriguing study reported how prevalent levels of vitamin D deficiency was in middle-aged and elderly people from Lanzhou population in China despite this population being regularly exposed to sunlight; however, other factors such as smoking were correlated to contributing to vitamin D deficiency (7). Culmination of these nutrient demands is what leads to increased susceptibility of osteoporosis and underlying potential health risks associated with bone vulnerability (8).

Older people tend to take vitamin D supplements to increase or maintain vitamin D levels (9). While vitamin D toxicity is uncommon, vitamin D supplements and overfortified foods are the only known means of reaching intoxication levels (10, 11). Adult men in winter months were given daily doses of D3 for about 20 wks and their serum 25-hydroxycholecalciferol (25-OH-D3) increased, but their 25-OH-D3 concentration was in a safe range (12). There was also a study that examined how small doses of 25-OH-D3 would affect adults and it increased their serum 25-OH-D3 concentration quickly, but half-life of 25-OH-D3 was long (~22 d) which signified that 25-OH-D3 can remain in circulation and build up to toxic levels (13). Young laying hens fed
Dietary vitamin D supplementation is important for laying hens in production because their bone health is physiologically taxed from egg production (14-16). Laying hens in commercial farms are fed diets with supplemented vitamin D beyond NRC requirements to ensure hens are able to lay eggs and maintain adequate Ca absorption for eggshell formation and importantly, bone mineralization (17). There are a few studies which investigated high vitamin D dosages impacts on laying hen production and a few metabolic indicators (18-20), but there are no studies that examined how super-doses of vitamin D affect gene expression and how affected vitamin D-related genes correlate to vitamin D metabolism. Understanding how gene expression and metabolic indicators such as vitamin D metabolite levels in blood are affected by super-doses of dietary vitamin D in aged laying hens has implications for exploring if similar effects occur in older women and justify increasing vitamin D fortification levels in food if there is little risk of toxicity.

This study examines effects of dietary vitamin D\textsubscript{3} super-doses on plasma and egg yolk vitamin D metabolites and relative gene expression of vitamin D related genes in aged laying hens in production. We fed hens diets containing 400, 800, 7,400, 14,000, 20,000, and 36,000 IU D\textsubscript{3}/kg of feed to ascertain vitamin D supplementation impacts. Hens consuming diets with vitamin D\textsubscript{3} greater than 10,000 IU/kg are expected
to have increased 24,25-dihydroxycholecalciferol (24,25-(OH)2-D3) because 24,25-(OH)2-D3 is an inactive form to indicate they reached vitamin D saturation. Hens consuming super-doses of D3 should also lay eggs with increased vitamin D3 content because they would deposit excess D3 into the egg (19). This hypothesis would be supported from experimental data that would indicate a similar level of gene expression of hydroxylase genes to accommodate for vitamin D metabolism.

Methods

Birds and housing

Forty-eight 68-week old HyLine Brown laying hens were housed at North Carolina State University, Raleigh, NC. Hens were individually housed in cages between two two-level battery cages with eight birds per treatment. Experimental design was a randomized complete block design with six levels of dietary vitamin D supplementation blocked by cage level. Vitamin D supplementation levels were formulated to be 250, 500, 1,500, 15,000, 30,000, and 60,000 IU D3/kg of feed, but the analyzed vitamin D3 in feed (and dietary treatments in this study are referred as) were 400, 800, 7,400, 14,000, 20,000, and 36,000 IU/kg of feed (Tables 1 and 2). Birds were fed ad libitum along with water. North Carolina State University’s Institutional Animal Care and Use Committee approved all methods for this study, protocol ID number: 18-093-A.
### Table 1. Ingredient composition of the experimental basal diet for aged HyLine Brown laying hens.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>63.40</td>
</tr>
<tr>
<td>Soybean meal, 46% CP</td>
<td>20.60</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>9.20</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>2.33</td>
</tr>
<tr>
<td>Dicalcium phosphate(^1)</td>
<td>1.95</td>
</tr>
<tr>
<td>L-Lysine-HCl, 78.8%</td>
<td>1.01</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.57</td>
</tr>
<tr>
<td>Vitamin premix(^2)</td>
<td>0.25</td>
</tr>
<tr>
<td>Mineral premix(^3)</td>
<td>0.20</td>
</tr>
<tr>
<td>Choline chloride, 60% choline</td>
<td>0.20</td>
</tr>
<tr>
<td>DL-Methionine, 99%</td>
<td>0.14</td>
</tr>
<tr>
<td>Salt</td>
<td>0.10</td>
</tr>
<tr>
<td>Selenium premix</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^1\)Dicalcium phosphate contains 19.79% calcium, 17.91% phosphorus, and 17.73% available phosphorus.
\(^2\)Provided as milligrams per kilogram of diet: Thiamin HCL, 1.8; Riboflavin, 3.6; Ca pantothenate, 10; Niacin, 25; Pyridoxine HCL, 3; Folacin, 0.55; Biotin, 0.15; B12, 0.01; Vitamin A 1500 IU/g, 6; Vitamin D3 400 IU/g, 1; Vitamin E 10 IU/g, 40; Vitamin K, 0.55; Ethoxyquin, 125.
\(^3\)Trace minerals provided per kg of premix: 60 g manganese (Mn SO4); 60 g zinc (ZnSO4); 40 g iron (FeSO4); 5 g copper (CuSO4); 1.25 g iodine [Ca(IO3)2].

### Table 2. Nutrient content of experimental diet for aged HyLine Brown laying hens.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>90.46</td>
</tr>
<tr>
<td>Moisture</td>
<td>9.54</td>
</tr>
<tr>
<td>Crude protein</td>
<td>16.00</td>
</tr>
<tr>
<td>Crude fat</td>
<td>4.98</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>1.81</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.00</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.67</td>
</tr>
<tr>
<td>Ash</td>
<td>12.44</td>
</tr>
<tr>
<td>Available phosphorus</td>
<td>0.46</td>
</tr>
<tr>
<td>Methionine+Cysteine</td>
<td>0.64</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.57</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.40</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.26</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.57</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.19</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.60</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.72</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.43</td>
</tr>
<tr>
<td>Valine</td>
<td>0.83</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.43</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.83</td>
</tr>
<tr>
<td>Metabolizable Energy, kcal/kg</td>
<td>2,900</td>
</tr>
<tr>
<td>Dietary electrolyte balance, mEq/100 g</td>
<td>202</td>
</tr>
</tbody>
</table>
**Sample collection**

Eggs were collected every morning and stored at 7°C for egg quality analyses. Egg yolk was collected from each week from one egg per bird. Eggs were cracked open in a dim-lighted room to reduce photodegradative impacts of light on vitamin D in yolk. Yolk was separated from albumen and placed in a small plastic container wrapped in aluminum foil and stored at 4°C until they were freeze-dried using a freeze-dryer (FreeZone 6 Liter Benchtop Freeze Dry System, Labconco, Kansas City, MO). All 48 birds were bled to have their plasma vitamin D metabolites measured. All birds were sacrificed by cervical dislocation and tissue samples were collected from 43 birds (minimum of seven birds per treatment) due to time constraints. Liver and kidney were collected and stored in RNAlater at -20°C until RNA extraction for workflow for quantitative real-time PCR (qPCR).

**RNA extraction and qPCR**

Total mRNA was extracted from cecal tonsils and duodenal tissue using Qiagen’s RNeasy Mini Kit (Germantown, MD). Extracted RNA was diluted and normalized to ~200 ng/µL for liver and 60 ng/µL for kidney, and reverse transcribed to complementary DNA (cDNA) using Applied Biosystems’ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA) and their recommended steps to make a 20 µL working solution. Cycling procedure for reverse transcription started with 25°C for 10 min, 37°C for 120 min, 85°C for 5 mins, then held at 5°C indefinitely until storage or use.
Genes amplified for qPCR were vitamin D receptor (VDR), 1α-hydroxylase, 25-hydroxylase, 24-hydroxylase, and glyceraldehyde (GAPDH) as housekeeping gene (Table 3). qPCR was conducted using PowerUP SYBR Master Mix (Life Technologies, Grand Island, NY) using Applied Biosystems recommended steps to make a 20 µL working solution and using Applied Biosystems StepOnePlus Real-Time PCR System (Carlsbad, CA). Cycling procedure started with 95°C for 10 min then 40 cycles of 95°C for 15 s for denaturing and 15 s at 60°C for annealing. All samples were run in triplicates.

**Table 3.** Primer sequences for quantitative real-time PCR (qPCR).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>Forward</td>
<td>TGCCTCCAGTCTGGCATCTC</td>
<td>297</td>
<td>NM_205098.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTGATTTTGCAGTCCCCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1α-OHase</td>
<td>Forward</td>
<td>ATGATTGGCGTCCCCCTTCAG</td>
<td>177</td>
<td>XM_422077.4</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCACGCTTTCACTCACACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-OHase</td>
<td>Forward</td>
<td>GCTGTCACTGGGATTTTTGC</td>
<td>160</td>
<td>NM_001277354.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAACCGAAAGGCCAACAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-OHase</td>
<td>Forward</td>
<td>AAACCCTGGAAGCCTATCG</td>
<td>133</td>
<td>NM_204979.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAGTTTCACCACCTCCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>TGTTGTGACCTGACCTGCC</td>
<td>291</td>
<td>NM_204305.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGGCTCActCCTTGGATGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Vitamin D metabolites**

Plasma from specific-fed hens from 400, 800, 14,000, and 36,000 IU D₃ groups were respectively pooled and sent to Heartland Assays (Ames, IA) for measuring D₃, 25-OH-D₃, and 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃, inactive form of D₃) using LC/MS/MS. Freeze-dried egg yolk from same hens were pooled like plasma, but only samples from 400, 14,000, and 36,000 IU D₃ groups were analyzed for D₃ and 25-OH-D₃ from Heartland Assays.

**Statistical Analysis**

Statistical analyses were conducted using general linear model using dietary dose as a factor. SAS 9.4® was utilized for all statistical tests and Tukey-Kramer test was used for multiple comparisons for differences between dietary treatments. All mRNA relative expression was normalized using 2⁻^{ΔΔT} with GAPDH as housekeeper gene. Statistical significance was established at p < 0.05.

**Results**

*Dietary super-dose levels of vitamin D₃ increased plasma and egg yolk vitamin D₃ metabolite concentrations*

Plasma vitamin D₃ was below the readable range for 400 and 800 IU treatments and those samples were considered zero and included in statistics to account for model building. All vitamin D metabolite data exhibited heteroskedasticity and were transformed using natural logarithm function. Transformed data exhibited linear and homoscedastic relationship and were used for statistics.
A significant increase in plasma concentration of D₃, 25-OH-D₃, and 24,25-(OH)₂-D₃ was observed for hens fed dietary super-dose levels of vitamin D₃ (\( D₃: F = 27.2, r^2 = 0.86, df = 2,9, p = 0.0002; 25\text{-}OH\text{-}D₃: F = 495.1, r^2 = 0.99, df = 4,15, p < 0.0001; 24\text{-}25\text{-}OH\text{-}D₃: F = 239.4, r^2 = 0.98, df = 4,15, p < 0.0001 \)). Although plasma D₃ concentration could not be read for 400 and 800 IU treatments, D₃ concentration had a strong positive correlation with 25-OH-D₃ and 24,25-(OH)₂-D₃ concentrations (\( r = 0.95, p < 0.0001; r = 0.92, p < 0.0001 \); respectively), and they both had similar concentration values as dietary vitamin D₃ increased with both metabolites having ~85 ng/mL at 36,000 IU treatment (Figures 1A and 1B). Rate of increase for plasma 24,25-(OH)₂-D₃ concentration relative to dietary treatment compared to the other two metabolites was a lot lower with 17.4 ng/mL at 36,000 IU (Figure 1C). The ratio of 24,25-(OH)₂-D₃ to 25-OH-D₃ increased as dietary vitamin D₃ levels increased (\( F = 18.8, r^2 = 0.83, df = 4,15, p < 0.0001 \)); however, there was no difference in the ratio between the 3 super-dose levels. The percentage ratio of 24,25-(OH)₂-D₃ to 25-OH-D₃ ranges from 8.7% to 20.5% with all three measured super-dose hens having a similar ratio of about 20% (Figure 1D).

Egg yolk D₃ also increased drastically as hens’ dietary D₃ intake increased (\( F = 537.8, df = 2,9, p < 0.0001 \); Figures 2A and 2B). Egg yolk 25-OH-D₃ also significantly increased in concentration as hens’ dietary D₃ increased (\( F = 44.8, df = 2,9, p < 0.0001 \)), but the rate of increase was much lower compared to egg yolk D₃ (D₃ ranged from 8.55 – 1009.9 ng/g; 25-OH-D₃ ranged from 3.48 – 51.6 ng/g). Yolk D₃ was greatly positively correlated with plasma D₃ and yolk 25-OH-D₃ (\( r = 0.90, p = 0.002; r = 0.96, p < 0.0001 \); respectively).
Figure 1. Vitamin D₃ metabolite plasma concentrations of 78 wk HyLine Brown laying hens fed different levels of dietary D₃. A) Cholecalciferol (Vitamin D₃) B) 25-hydroxycholecalciferol (25-OH-D₃) C) 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃) D) Ratio of 24,25-(OH)₂-D₃/25-OH-D₃ presented as a percentage. Blue squares denote standard NRC range D₃ levels in diet and orange squares denote super-dose levels of D₃ in diet (n = 4). Squares with common letters are not statistically different from each other (General linear models (GLM), p < 0.0001).
Figure 2. Egg yolk vitamin D₃ metabolite concentrations from 78 wk HyLine Brown laying hens fed different levels of dietary D₃. A) Cholecalciferol (Vitamin D₃) B) 25-hydroxycholecalciferol (25-OH-D₃). Blue squares denote standard NRC range D₃ levels in diet and orange squares denote super-dose levels of D₃ in diet (n = 4). Squares with common letters are not statistically different from each other (General linear models (GLM), p < 0.0001).
**Dietary vitamin D₃ does not affect liver and kidney vitamin D receptor expression**

There was no statistical dietary effect on VDR expression in the liver or kidneys (p = 0.17, p = 0.32; respectively) (Figures 3A and 3B).

**Figure 3.** Relative gene expression of vitamin D receptor (VDR) in liver and kidney of 78 wk HyLine Brown laying hens fed different levels of dietary D₃. A) Liver VDR B) Kidney VDR. Tissues were analyzed using qPCR normalized against glyceraldehyde phosphate dehydrogenase (GAPDH; housekeeping gene) expression. Blue bars denote standard NRC range D₃ levels in diet and orange bars denote super-dose levels of D₃ in diet (n = 5). Control treatment is 400 IU D₃. Bars with common letters are not statistically different from each other (General linear models (GLM), p < 0.05).
Dietary super-doses of D₃ decreased kidney 24-hydroxylase expression

The relative expression of 24-hydroxylase in the kidneys was lower in hens fed super-dose levels of D₃ (F = 8.42, df = 5, r² = 0.73, p = 0.0006) with 400 and 800 IU hens having about 1.32 expression and super-dose hens having 0.80 - 1.00 expression (Figure 4A). There was no dietary effect on 25-hydroxylase expression in liver (p = 0.95; Figure 4B). Also, no differences were observed with 1α-hydroxylase expression (p = 0.81; Figure 4C).

Figure 4. Relative gene expression of vitamin D hydroxylases in liver and kidney of 78 wk HyLine Brown laying hens fed different levels of dietary D₃. A) Kidney 24-hydroxylase (24-OHase) B) Liver 25-hydroxylase (25-OHase) C) Kidney 1α-hydroxylase (1α-OHase). Tissues were analyzed using qPCR normalized against glyceraldehyde phosphate dehydrogenase (GAPDH; housekeeping gene) expression. Blue bars denote standard NRC range D₃ levels in diet and orange bars denote super-dose levels of D₃ in diet (n = 5). Control treatment is 400 IU D₃. Bars with common letters are not statistically different from each other (General linear models (GLM), p < 0.05).
Discussion

Our results suggest that dietary super-doses of D3 greatly increase plasma and egg yolk D3 levels. Plasma vitamin D3 was increased to indicate these birds absorbed the vitamin D from their diets. However, the inactive vitamin D3 metabolite, 24,25-(OH)2-D3 is increased at a much lower rate than D3 and 25-OH-D3. In addition, egg yolk D3 drastically increased with yolk 25-OH-D3 having a smaller rate of increase. Therefore, we speculate that maximal 24-hydroxylation activity was achieved and these hens deposited excess D3 in egg yolks to avoid vitamin D toxicity.

Vitamin D, regardless of its biogenic or dietary origins, is transported and circulated in blood which is why vitamin D is also considered a hormone (16). Circulating vitamin D3 is transported to liver to have a hydroxyl group added to C-25 by 25-hydroxylase which results in 25-OH-D3. 25-OH-D3 moves to kidney to have a further hydroxyl group added at C-1 by 1α-hydroxylase to become 1,25-dihydroxycholecalciferol (1,25-(OH)2-D3) which is the most active form of vitamin D3 that exerts vitamin D’s biological effects via binding to VDR. However, there are regulatory pathways for 25-OH-D3 that can occur in kidney with 24-hydroxylase adding a hydroxyl group to C-24 to result in 24,25-(OH)2-D3 (16). 24,25-(OH)2-D3 is considered an inactive form of vitamin D that is excreted as a means for the body to remove excess vitamin D from circulation (21).

There is plethora of research regarding how vitamin D supplementation impacts plasma vitamin D concentrations from humans and other animals (9, 22-26). The studies that examined dietary super-doses of vitamin D in laying hens had similar findings with increase in vitamin D in egg yolk (19, 20); therefore, the novelty of our
study is examining how dietary vitamin D super-dosage affects plasma vitamin D concentration. Our hens’ plasma vitamin D₃ metabolites concentrations were influenced by their dietary vitamin D supplementation. Even though plasma D₃ concentration could not be measured in hens fed 400 and 800 IU D₃, super-dose fed hens had exponential increase in D₃ concentration with 36,000 IU D₃ fed hens having an average of 85 ng/mL D₃. Considering the experimental design was to observe effects of exponential increase of dietary vitamin D₃, this finding confirms what other studies have observed with dietary vitamin D or supplementation levels are correlated to relative increase in plasma D₃ concentration. An interesting aspect of this finding is that the slope became steeper between the three super-dose treatments which can indicate that these birds could not have reached vitamin D toxicity because D₃ concentration would have shown an asymptotic relationship. These hens were fed these diets for ten weeks and no discernable changes in egg production. A study in which laying hens were fed extremely high levels of D₃ also had no changes in egg production, except there were egg qualities like shell quality were reduced (27). One important consideration we should have investigated was kidney histology of these birds because soft-tissue calcification or renal kidney failure occurs with animals having vitamin D toxicity (28, 29). However, Mattila et al. (19) examined histological samples of younger hens fed greater dietary vitamin D₃ levels than our study and they did not observe any pathological issues with their hens’ kidneys, leading them to conclude that vitamin D toxicity had not occurred. One important finding in Mattila et al.’s study was that egg yolk vitamin D content increased relative to the dietary vitamin D fed to the hens. This observation is significant
because hens have an extra reservoir to remove excess vitamin D from their bodies by depositing it in egg yolk.

Our findings agree with Mattila et al. (19) and a recent study that examined even greater levels of dietary D₃ in laying hens throughout their production cycle (20) that high levels of dietary D₃ will increase D₃ content in yolk. One novel finding in our study was that 25-OH-D₃ in yolk increased with increased dietary D₃ even though it was much lower than D₃. Another study by Mattila et al. (18) reported how 25-OH-D₃ increased as dietary D₃ levels increased in hen feed. Although 25-OH-D₃ is more potent source for vitamin D activity, more research is needed to explore transfer rate of 25-OH-D₃ from hens fed crystalline 25-OH-D₃ in comparison to hens fed D₃ because our findings indicate that D₃ is deposited more readily into yolk versus 25-OH-D₃. However, one assumption is that hens fed 25-OH-D₃ will have regulatory feedback mechanisms act to reduce plasma 25-OH-D₃ levels so it is possible for that excess 25-OH-D₃ to be transferred to egg yolk instead of D₃ as observed with hens from this study.

Plasma 25-OH-D₃ concentration is important as a vitamin D status indicator (30, 31). Although plasma 25-OH-D₃ concentration ranges have not been fully characterized in laying hens, vitamin D status can still be ascertained by observing 25-OH-D₃ concentration relative to D₃ and 24,25-(OH)₂-D₃. In our study, the 400 and 800 IU D₃ treatments were formulated to meet NRC requirements for laying hens, whereas the 14,000, 20,000, and 36,000 IU D₃ treatments were dietary super-dose treatments for D₃. Higher dietary vitamin D₃ levels led to increasing plasma concentrations of vitamin D₃ and 25-OH-D₃ (Figures 1A and 1B). The fascinating aspect of this data was how large the range for 25-OH-D₃ was because it was 7.15 ng/mL 25-OH-D₃ for 400 IU hens to
85.2 ng/mL 25-OH-D₃ for 36,000 IU hens. Yolk 25-OH-D₃ levels were similar to plasma, 3.48 ng/g 25-OH-D₃ for 400 IU hens to 51.6 ng/g 25-OH-D₃ for 36,000 IU hens. This data implies that supplementing laying hen diets with 25-OH-D₃ may not be as effective for increasing egg yolk vitamin D content versus feeding hens exorbitant levels of D₃.

Vitamin D hydroxylases are part of a cytochrome P450 family that regulate vitamin D metabolism (21). The liver is a major site of 25-hydroxylation via 25-hydroxylase in liver mitochondria and microsomes (32). There are extrahepatic sources of 25-hydroxylase like kidney (33) and intestine (34); however, it is not clear how extrahepatic sources contribute physiologically to vitamin D metabolism (32). Human CYP2R1 is important for 25-hydroxylation in liver (35) and CYP2J3 was identified as an important enzyme for 25-hydroxylation in rat liver microsomes (36). To our knowledge, there is only one paper that described avian cytochrome P450 family in chicken liver (37). We generated primers from Genbank for chicken CYP2R1 (25-hydroxylase) based on RefSeq (Accession # XM_004941395.3) to examine how dietary super-doses of vitamin D₃ would affect liver 25-hydroxylase expression in aged laying hens. There was no difference in liver 25-hydroxylase expression which could signify it is not under metabolic control. Studies with rats and humans showed that increased plasma 25-OH-D₃ concentration is related to dietary vitamin D₃ intake (34, 38). Half-life of plasma 25-OH-D₃ is at least 18 d (39), so considering what was observed with hens from our study, increased 25-OH-D₃ concentration would not be caused by increased 25-hydroxylase expression, but 25-OH-D₃ is not metabolically regulated like 1,25-(OH)₂-D₃ so 25-hydroxylase activity is continuous to lead to 25-OH-D₃ accumulation. No differences in VDR expression in liver or kidney leads to speculation that D₃ may not affect its
regulation. VDR in humans decreases with age in muscle tissues so age may affect VDR expression (40).

Prior research characterized 24,25-(OH)₂-D₃’s function as an inactive vitamin D metabolite that is excreted from an animal’s body and 24,25-(OH)₂-D₃’s biological significance is to reduce 25-OH-D₃’s plasma concentration (41). Hydroxylation of C-24 is significant because it can also occur with 1,25-(OH)₂-D₃ to lead to 1,24,25-trihydroxylcholecalciferol which is also excreted (42). To our current knowledge, there is no research on 24,25-(OH)₂-D₃ plasma concentration in laying hens. Comparable to plasma 25-OH-D₃, 24,25-(OH)₂-D₃ in hens used in this study increased as their dietary D₃ intake increased. However, unlike plasma D₃ and 25-OH-D₃, there was a great difference in the rate of increase with 24,25-(OH)₂-D₃ having a tiny increase overall. Plasma 24,25-(OH)₂-D₃ in hens fed 14,000, 20,000, and 36,000 IU/kg D₃ was 6.8, 11.1, and 17.4 ng/mL, respectively which does not exhibit the same exponent increase like D₃ and 25-OH-D₃. This finding has strong implications to indicate these hens likely did not reach vitamin D toxicity because there would be a greater concentration of 24,25-(OH)₂-D₃ to reflect the need to reduce 25-OH-D₃ concentration. But considering the rate of increase for 24,25-(OH)₂-D₃ relative to D₃ and 25-OH-D₃ at super-dosage levels is about 20%. It is also possible that 24-hydroxylation activity reached its maximum because an asymptote was achieved at 14,000 IU. This can also imply why these hens’ D₃ and 25-OH-D₃ concentrations continued to increase because their intake greatly exceeded their excretion. However, kidney expression of 24-hydroxylase decreased in hens in this study that were fed high levels of dietary vitamin D₃. A study with rats reported how kidney 24-hydroxylase expression was reduced by 1,25-(OH)₂-D₃ administration (43).
The decreased 24-hydroxylase expression in hens fed super-dose levels of D₃ is possibly linked to increased levels of 1,25-(OH)₂-D₃. Another possibility is the long half-life of plasma 24,25-(OH)₂-D₃ (over 15 d (44)) caused 24,25-(OH)₂-D₃ to build up concentration in super-dose fed hens, as it was not converted to calcitroic acid, a watersoluble end product of vitamin D catabolism that is excreted (45).

There were a few limitations with this study that were realized when data was collected. Egg yolk was stored in a refrigerator for about a year before it was freeze dried. When compared to Mattila et al. (19) and Wen et al. (20) results, our findings potentially highlight minimal D₃ degradation which could indicate how stable D₃ is when it is stored in cold, dark conditions. Examining fatty acid profile would yield useful data because fatty acid composition would have changed because of diet (46) and over the long time period since the egg was laid. Egg yolk lipid profile may have protected the vitamin D content within egg yolk which could explain why D₃ levels were unaffected.

Accounting for excessive yolk D₃ levels, we speculate that hens fed super-doses of D₃ will aim to convert their 25-OH-D₃ to 24,25-(OH)₂-D₃ to avoid vitamin D toxicity, but 24-hydroxylation reached its maximal activity rate and plasma vitamin D concentration increases. Hens would shunt excess D₃ and 25-OH-D₃ to egg yolk as a consequence of increasing plasma vitamin D concentration. This rationale is further validated because these hens can only transfer so much D₃ to yolk and is why super-dose fed hens had higher levels of plasma D₃ even though they transferred a lot of D₃ to egg yolk. Our novel finding with plasma 24-OH-D₃ concentration has important implications for geriatric humans who take supplements. Except for lactating women who can transfer excess vitamin D to milk, there is a possibility that older people taking high levels of
vitamin D supplementation than intended could be at risk of vitamin D toxicity. To our knowledge, there are no studies that explored 24,25-(OH)2-D mechanisms when vitamin D is administered in great doses. Future research should elucidate vitamin D mechanisms with great levels of intake and how 24-hydroxylation is influenced.

Our study exhibits that feeding aged laying hens, super-doses of dietary D₃ increased their plasma and egg yolk D₃ metabolite concentrations. Importantly, there is a possible metabolic limit of 24-hydroxylation to remove excess circulating D₃. Investigating 24-hydroxylation mechanisms will be important to understanding vitamin D supplementation impacts in geriatric animals for improving bone health.
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Chapter 5

Conclusions and future directions

Vitamin D’s significance for preventing and healing rickets established its significance as an important nutrient for homeostasis (1). Biologically connected to calcium (Ca) absorption, vitamin D can be biogenically made on skin that is exposed to sunlight. Research on dietary impacts of vitamin D has been at the forefront of vitamin D-related research because of global vitamin D insufficiency being connected with potential issues in bone health in humans (2). Chickens have been used in studies to assess vitamin D supplementation effects (3, 4) and potential toxicity (5).

My dissertation explored how dietary vitamin D3 affected vitamin D metabolism in chickens. The first chapter provided a review of vitamin D metabolism and how chickens can be used for addressing specific questions pertaining to vitamin D mechanisms. Learning how specific mechanisms work in chickens would provide possible groundwork for comparative research with humans. Chapter 2 examined how 1-alpha-hydroxycholecalciferol (1α) affected broiler chicks fed diets with differing dietary Ca and their vitamin D metabolites with regards to metabolism. With Chapter 3, aged laying hens were fed super-doses of vitamin D3 to explore how egg production and eggshell quality were affected. Using the same hens from Chapter 3, Chapter 4 focused on investigating how dietary super-doses of vitamin D3 affected vitamin D metabolism of aged laying hens.

Based on literature pertaining to vitamin D, Chapter 1 demonstrated how chickens can be valuable for assessing dietary effects of vitamin D. With vitamin D insufficiency being an important factor for understanding means to improve vitamin D
status and intake, chickens would provide excellent results with or without sunlight exposure. A review described how skeletal muscles can store 25-hydroxycholecalciferol (25-OH-D₃) as a functional storage for winter months (6). Broiler chickens would be valuable for evaluating how this mechanism works in relation to muscle mass because broilers grow rapidly with their muscle growth being the primary factor; therefore, measuring how much 25-OH-D₃ is stored in skeletal muscle is a possible question that can be experimentally tested. Considering geriatric-age humans are given supplemental doses of vitamin D to reduce risk of vitamin D deficiency and osteoporosis, there is risk of negative effects from supplementation. Aged laying hens can be fed experimental diets to provide a means for illustrating risk of negative effects from vitamin D supplementation. Future research pertaining to vitamin D metabolism should target some of the proposed research questions because understanding 25-OH-D₃ storage capacity in skeletal muscles has significance with addressing vitamin D insufficiency in areas with long winters. If negative risks with vitamin D supplementation are identified in aged laying hens, then future research can explore value of less potent forms of vitamin D, such as D₂, and if it poses similar risks in chickens and humans.

In Chapter 2, chicks were fed diets with or without 1α supplementation with varying levels of dietary Ca to assess if 1α can potentially cause Ca toxicity in young broiler chicks. As a synthetic form of vitamin D, 1α is equal in effectiveness as vitamin D₃, but is cheaper to synthesize (7). During starter phase (1 -17 d), chicks fed 1α had an increase in ionized blood Ca as dietary Ca levels increased (p = 0.0002). However, 1α supplementation had no effect on ionized blood Ca in chicks fed 1α during grower phase (16 – 35 d; p = 0.23). Chicks fed a grower diet with 1α supplementation had a
decrease in plasma 25-OH-D₃ concentration as dietary Ca levels increased (p = 0.035). These findings exhibit that 1α can alter ionized blood concentration during starter phase, but is ineffective during grower phase. Also, 1α supplementation causes a decrease in plasma 25-OH-D₃, likely as a result of 1α being converted to 1,25-dihydroxycholecalciferol, the hormonally active form of vitamin D₃, which also regulates 25-OH-D₃ conversion through inhibition. 1α supplementation has value when fed to chicks during starter phase; however, its value is diminished during grower phase. This also indicates that higher levels of dietary Ca can be potentially toxic if 1α effect on Ca absorption is unchecked. Future research needs to elucidate how vitamin D metabolism is affected by 1α supplementation to establish how potential Ca toxicity can occur should 1α bypass regulatory mechanisms.

Chapter 3 explored super-doses effects of dietary vitamin D₃ on egg production in aged laying hens. Laying hens need vitamin D to facilitate Ca absorption necessary for eggshell formation (8). Super-doses of dietary vitamin D₃ did not affect eggshell quality, egg production, and Ca and P of ileal digesta and tibia and humerus bones. This suggests that eggshell quality and production is not affected by high levels of dietary vitamin D₃. However, future work should focus on if dietary Ca in conjunction with super-doses of dietary D₃ may affect eggshell quality and production. If super-doses of dietary D₃ can improve Ca absorption in low Ca diets for aged laying hens, then there is potential application for characterizing how effective vitamin D is in aged animals in production.

Using the same hens as Chapter 3, vitamin D metabolism was investigated in Chapter 4. Vitamin D deficiency is linked to osteoporosis (9) and increasing vitamin D
intake can help prevent vitamin D deficiency. This chapter focused on how super-doses of dietary vitamin D₃ would affect vitamin D metabolites and vitamin D hydroxylase expression in aged laying hens in production. Hens fed dietary super-doses of dietary D₃ had a drastic increase in plasma vitamin D₃, 25-OH-D₃, and 24,25-dihydroxycholecalciferol (24,25-(OH)₂-D₃) concentration (p < 0.0001 for all 3 metabolites). Egg yolk D₃ and 25-OH-D₃ also increased relative to dietary vitamin D₃ (p < 0.0001 for both metabolites). Only 24-hydroxylase in kidneys was affected by dietary D₃ intake. Hens fed super-doses of D₃ had downregulation of kidney 24-hydroxylase (p = 0.0006). A significant implication for this chapter was how dietary D₃ super-doses fed to aged laying hens led to downregulated kidney 24-hydroxylase. This enzyme is important because it adds a hydroxyl group to C-24 to convert vitamin D metabolites to inactive forms (10). A potential consequence of this downregulation illustrates a possible limit of 24-hydroxylation to remove excessive levels of circulating vitamin D. Future research should focus on elucidating why dietary super-dose levels of D₃ caused a decrease in 24-hydroxylase expression because it has implications on vitamin D metabolism for older people taking more supplementation than intended with having a possibility of vitamin D toxicity.

My dissertation highlighted three major findings with vitamin D metabolism. First, how 1α supplementation in growing broilers is effective with Ca absorption and can affect circulating vitamin D metabolite levels. Second, feeding aged laying hens super-dose levels of dietary D₃ does not affect eggshell quality and egg production. Third, dietary super-doses of D₃ caused 24-hydroxylase expression to decrease. With consideration of all these major findings, future researchers should explore dietary
influences on 24-hydroxylase. As a regulatory enzyme, 24-hydroxylase is responsible for controlling excessive levels of vitamin D to ensure an animal does not reach toxic levels. If regulatory aspects of 24-hydroxylase expression are understood, then the possibility of increasing vitamin D supplementation in humans can be ascertained without risk of toxicity to ensure vitamin D metabolism and its underlying effects with improving bone mineralization can help address vitamin D insufficiency in older populations.
References


