Protein Turnover in Broiler, Layers, and Broiler Breeders

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Protein Turnover in Broiler, Layers, and Broiler Breeders
ABSTRACT

Protein turnover in skeletal tissue for broiler breeders has shown to increase at sexual maturity and then decline with increased egg production. Regarding broiler chickens the industry is facing two situations that need to be clarified: the appearance of white stripes in birds over 56 days of age and the positive effect in breast meat yield that supplementation of 25OH-D3 is causing. A series of studies were conducted to determine the effect of sexual maturity in protein turnover in broiler breeders and laying hens and to understand the effect of 25OHD3 in breast meat yield in broiler chickens and why white striping occurs in birds older than 56 days of age. In the first trial, a 4x6 factorial study was conducted to determine the effect of sexual maturity on protein turnover in broiler breeder pure line (4 lines x 6 ages). Results showed that as the birds enter sexual maturity the degradation rate significantly increased and then it decreased as they pass peak egg production, suggesting that they might be relying on their muscle protein reserves for egg formation. In the second trial, a 4x10 factorial study was conducted to evaluate the effect of four different programs and sexual maturity in broiler breeder pullets and hens. Results showed no difference regarding protein turnover between feeding programs. Fractional breakdown rate showed the same pattern as the first trial. In the third trial, a study was conducted to evaluate the effect of sexual maturity on protein turnover in laying hens. Results showed that also in this birds, as they reach peak egg production, the fractional breakdown rate significantly increased. In the fourth trial, a study was conducted to track labeled leucine from the breast of the hens to the egg. Results showed, that hens are using their skeletal muscle for egg formation, since we were able to find labeled leucine in the egg. The last two trials were conducted in broiler chickens to understand the white striping issue and how 25OHD3 enhances muscle growth. We were able to conclude, that birds with severe white striping have a higher protein fractional breakdown rate
than the birds with no white stripes. Results regarding 25OHD3 showed that this supplement is enhancing muscle growth through the mTOR pathway.
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DEDICATION

To my Dad.
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INTRODUCTION

The net result of the protein synthesis and breakdown is the accretion of the body protein. The dynamic nature of protein metabolism has been known for 60 years thanks to the pioneering work of Schoeheimer and others (Shoeheimer et. al., 1939). Using stable isotopes of amino acids, they demonstrated that proteins continually were being broken down and resynthesized. In addition they reported that different organs have different rates of protein synthesis. The dynamic process by which body proteins are continually synthesized and broken down is protein turnover.

Studies on the growth of body protein have been a major area of research. The main reason for this is that, for example, up to 20-25% of the muscle protein can be broken down per day early in the life of humans and farm animals. This rate slows with age to 1-2 % day -1 in adults. Rates of synthesis and breakdown are influenced not only by age, but by plane of nutrition, stress, disease, hormones, exercise, and inactivity.

Research in protein turnover in broiler chickens has been studied for a while. However, it is still unknown what happens regarding protein turnover in broiler breeders, and how it changes with age, sexual maturity, laying period, and feeding programs. It is also still under research how gene expression of genes related to protein synthesis and degradation (IGF-1, ampk, antrogin-1, MURF1, and Cathepsin B) changes due to changes in protein turnover. These genes have already been sequenced for chickens (Dupont et al., 1998; Heck et al., 2003; Bigot et al., 2003; Tosca et al., 2006 and Tesseraud et al., 2007). Also, it is still not well understood how protein turnover changes in laying hens regarding sexual maturity, and throughout the production period.

On the other hand, regarding broiler chickens the industry is facing two situations that need to be clarified: the appearance of white stripes in birds over 56 days of age and the positive effect in
breast meat yield that supplementation of 25OH-D3 is causing. White striping is the white striation occasionally observed parallel to the direction of muscle fibers in broiler breast fillets and thighs at the processing plant. Broiler breast fillets can be categorized as normal (NORM), moderate (MOD), or severe (SEV) based on the degree of white striping. Histologically, SEV fillets are characterized by the highest degree of degeneration of muscle fibers along with fibrosis and lipidosis when compared with NORM (Kuttappan et al., 2013 a, b). It is known that white striping could be a potential reason for the rejection of raw breast fillets in the market (Kuttappan et al., 2012). However, it is still not clear what the origin of the problem is, or what is happening in the breast meat that make this white stripes to appear.

Since its commercialization, the use of supplemental 25(OH) D3, the recommended metabolite for commercial poultry use, has proven effective in improving growth performances. It has been shown, that compared to vitamin D3, dietary supplementation with 25(OH)D3 improves body weight gain and feed efficiency in broiler chickens (Yarger et al., 1995a,b). 25(OH) D3 has also been reported to improve breast muscle yield in broiler chickens (Yarger et al., 1995a) however the underlying molecular mechanisms for this improvement are still unknown.

The objectives of this study were: 1) To determine the effect of sexual maturity on protein turnover in breeders of different pure lines 2) To determine the effect of four different feeding programs: skip a day, normal feeding, under feeding and over feeding on protein turnover in parent stock breeders as pullets and during and after sexual maturity. 3) To determine the effect of sexual maturity on protein turnover in laying hens. 4) To determine the amino acid movement from the muscle to the egg. 5) To understand the mechanism by which 25OHD3 enhances muscle growth in broilers. 6) To understand why white striping occurs in broilers over 56 days of age. In order to accomplish all of the objectives six trials were carried out: pure lines trial,
feeding program trial, laying hens’ trial, amino acid movement trial, 25OHD3 trial, and white striping trial. In order to accomplish these objectives, six trials were carried out.
I. Literature Review
PROTEIN BIOCHEMISTRY

The two most critical nutrients beside water are energy and protein. Protein and its constituent amino acids are the building blocks for all tissues and critical in several metabolic pathways. They function as neurotransmitters, antioxidants, and shuttlers in intermediary metabolism. Protein may also serve as an energy source. Nutritionists do not solely consider crude protein requirements for dietary formulations, as amino acid requirements are primarily considered. The needs of amino acids for a specific function plus the rates of endogenous loss and utilization determine amino acid requirements. In growing birds, the amino acid requirements are largely dependent on protein synthesis and muscle accretion. In egg-laying birds, requirements are dependent on protein synthesis for egg formation with requirements for accretion being minimal.

Proteins can be classified as two types: globular or fibrous. Globular proteins include albumins, prolamines, and histones, among others. Fibrous proteins include collagens, elastins, and keratins. Proteins can also be conjugated to other groups such as in the lipoproteins, glycoproteins, mucoproteins, and nucleoproteins. Amino acids that can be readily synthesized from precursors are described as dispensible, whereas those that can only be obtained via the diet are termed non-dispensible. In the chicken, ten amino acids are considered essential (Table 1), although depending on the stage of life this number may be higher. During periods of rapid growth, the rate at which an amino acid precursor cannot keep up with the rate at which it is being utilized. For example, glycine and serine become essential during growth or during periods of high protein intake. Glycine is critical to uric acid synthesis. The glycine and serine pair is also example of an amino acid that can be synthesized from its essential precursor. These pairs include: methionine and cysteine, and phenylalanine and tyrosine. Cysteine can be derived from methionine, and tyrosine from phenylalanine.
PROTEIN SYNTHESIS: TRANSCRIPTI0N AND TRANSLATION

During transcription (Figure 1) a mRNA chain is generated, with one strand of the DNA double helix in the genome as a template. This strand is called the template strand. Transcription can be divided into 3 stages: initiation, elongation, and termination, each regulated by a large number of proteins such as transcription factors and coactivators that ensure that the correct gene is transcribed.

Transcription occurs in the cell nucleus, where the DNA is held. The DNA structure of the cell is made up of two helixes made up of sugar and phosphate held together by hydrogen bonds between the bases of opposite strands. The sugar and the phosphate in each strand are joined together by stronger phosphodiester covalent bonds. The DNA is "unzipped" (disruption of hydrogen bonds between different single strands) by the enzyme helicase, leaving the single nucleotide chain open to be copied. RNA polymerase reads the DNA strand from 3-prime (3') end to the 5-prime (5') end, while it synthesizes a single strand of messenger RNA in the 5'-to-3' direction. The general RNA structure is very similar to the DNA structure, but in RNA the nucleotide uracil takes the place that thymine occupies in DNA. The single strand of mRNA leaves the nucleus through nuclear pores, and migrates into the cytoplasm.

The first product of transcription differs in prokaryotic cells from that of eukaryotic cells, as in prokaryotic cells the product is mRNA, which needs no post-transcriptional modification, whereas, in eukaryotic cells, the first product is called primary transcript, that needs post-transcriptional modification (capping with 7-methyl-guanosine, tailing with a poly A tail) to give hnRNA (heterophil nuclear RNA). hnRNA then undergoes splicing of introns (noncoding parts of the gene) via spliceosomes to produce the final mRNA.
The synthesis of proteins from RNA is known as translation. Translation occurs in the cytoplasm, where the ribosomes are located (Figure 2). Ribosomes are made of a small and large subunit that surround the mRNA. In translation, messenger RNA (mRNA) is decoded to produce a specific polypeptide according to the rules specified by the trinucleotide genetic code. This uses a mRNA sequence as a template to guide the synthesis of a chain of amino acids that form a protein. Translation proceeds in four phases: activation, initiation, elongation, and termination (all describing the growth of the amino acid chain, or polypeptide that is the product of translation).

In activation, the correct amino acid (AA) is joined to the correct transfer RNA (tRNA). While this is not, in the technical sense, a step in translation, it is required for translation to proceed. The AA is joined by its carboxyl group to the 3' OH of the tRNA by an ester bond. When the tRNA has an amino acid linked to it, it is termed "charged." Initiation involves the small subunit of the ribosome binding to 5' end of mRNA with the help of initiation factors (IF), other proteins that assist the process. Elongation occurs when the next aminoacyl-tRNA (charged tRNA) in line binds to the ribosome along with GTP and an elongation factor. Termination of the polypeptide happens when the A site of the ribosome faces a stop codon (UAA, UAG, or UGA). When this happens, no tRNA can recognize it, but releasing factor can recognize nonsense codons and causes the release of the polypeptide chain. The capacity of disabling or inhibiting translation in protein biosynthesis is used by some antibiotics such as anisomycin, cycloheximide, chloramphenicol, tetracycline, streptomycin, erythromycin, puromycin, etc.
SKELETAL MUSCLE STRUCTURE AND PROTEIN TURNOVER

The organization and nature of the proteins in skeletal muscle has important effects on how they are turned over metabolically. Skeletal muscle has 3 classes of proteins based on their solubility; the myofibrillar class is the major class, and because the myofibrillar proteins are assembled into the myofibrillar structure in striated muscle, they present a special challenge to metabolic turnover of muscle proteins. Based on recent advances in the understanding of intracellular protein turnover, it now seems that the myofibrillar proteins must first be disassembled from the myofibril before they can be degraded and turned over. It is unclear how this dissociation occurs; it may involve the release of a group of easily releasable myofilaments, or it may involve direct exchange of myofibrillar proteins with their counterparts in the cell cytoplasm, or both of these mechanisms may be used (Goll, et al. 2008).

Classes of Muscle Proteins

Skeletal muscle contains 3 groups of protein when classified by solubility and their location in the muscle tissue. Sarcoplasmic proteins constitute ~30 to 35% of total protein in muscle tissue by weight. These are the cytoplasmic proteins that are soluble in low (<0.2 M)-salt solutions and comprise all the glycolytic enzymes, enzymes involved in metabolic pathways, etc. This class of muscle proteins contains at least several hundred different polypeptides and is located entirely intracellular. Myofibrillar proteins constitute 55 to 60% of total protein in muscle tissue by weight. These are the proteins that constitute the myofibril or contractile structure in skeletal muscle. Although some myofibrillar proteins such as α-actinin and CapZ are soluble at low ionic strength after they have been extracted from the myofibril and separated from their normal binding partners, high ionic strengths (0.3 M) are required for their initial extraction. Myosin and
actin are the 2 main proteins in this group, although more than 15 other proteins are also present in the myofibrillar structure. The myofibrillar proteins are located entirely intracellular. Stroma proteins constitute 10 to 15% of total protein in muscle tissue and are defined as those proteins that are insoluble in an aqueous solvent at neutral pH (Goll et al., 2008). Many of the proteins in this group are extracellular. Collagen and extracellular matrix proteins are the main proteins in this group, although some membrane proteins may also be included, because they are not soluble in the absence of detergents. The myofibrillar proteins are not only the largest class of skeletal muscle proteins but also are responsible for the contractile properties of muscle and for most of the functional and culinary properties of muscle and meat. Thus, studies on muscle growth and muscle protein turnover need to focus on the myofibrillar proteins.

**Myofibrillar Protein Turnover**

Myofibrils are unique to striated muscle and also present a special situation for metabolic turnover. The contractile function of myofibrils requires that the myofibrillar structure extend continuously from one end of the muscle cell to the other. Thus, turnover of myofibrillar proteins must be accomplished without disrupting this continuous structure. It was proposed over 35 yrs. ago (Dayton et al., 1975) that myofibrillar proteins can be turned over by releasing filaments from the surface of the myofibril, leaving a myofibril with a diameter that is smaller by 1 layer of myofilaments. This mechanism is consistent with the observations that atrophying muscle in different muscular dystrophies, after denervation or during fasting, has smaller diameter myofibrils than unaffected muscle (Badalamente and Stracher, 2000). Dayton et al. (1975) proposed that the calpains, which had just been isolated and purified from skeletal muscle (Dayton et al., 1976a,b), were responsible for release of myofilaments from the surface of myofibrils, because the calpains cleave many of those proteins that are involved in keeping
myofilaments attached to the myofibril. The calpains rapidly cleave titin and nebulin at the point where these 2 polypeptides enter the Z disk (Goll et al., 2003). These cleavages, together with cleavage of desmin and filamin, which encircle the myofibril at the Z disk and tether it to the sarcolemma, would release α-actinin (Goll et al., 1991), the principal Z disk protein, from the myofibril. Release of α-actinin would result in release of thin filaments from the surface of the myofibril. The calpains also degrade M proteins, tropomyosin and troponin, albeit at slower rates than the degradation of titin and nebulin (Goll et al., 1992, 1999). Cleavage of the M proteins, together with the cleavage of titin, severs the attachments of the thick filament to the myofibril, and in the presence of ATP to dissociate myosin crossbridge binding to thin filaments, the thick filaments would be released from the myofibril. Calpain cleavage of tropomyosin and troponin would facilitate disassembly of the thin filament to G-actin monomers, and calpain cleavage of C-protein would facilitate disassembly of the thick filament to myosin monomers, making both actin and myosin susceptible to degradation by the proteasome. The calpains do not cleave proteins to AA but rather make a few selective cleavages leaving large fragments. Because the calpains cleave those proteins that are involved in maintaining the myofibrillar proteins in the myofibrillar structure, they seem to be ideally suited for catalyzing the first step in myofibrillar protein turnover. Although a role of the calpains in myofibril disassembly as a first step in their metabolic turnover has been widely accepted, there has been little experimental evidence to directly support this role.
Proteolytic System in Muscle

*The Lysosomal System*

Proteases in this system (cathepsins) are located inside lysosomal structures and have acidic pH optima ranging from 3.5 up to 6.5. Because of their low pH optima, cathepsins are not active at the pH of cell cytoplasm (cathepsin B with a pH optimum of 6.0 to 6.5 may have some activity), so any role of cathepsins in muscle protein turnover would have to occur inside lysosomes. Moreover, cells including muscle cells contain cystatin, a potent inhibitor of Cys proteases such as cathepsin B, and L. Because of their low pH optima and cystatin, cathepsins would not be active in cell cytoplasm (Goll et al., 2008). Myofibrils, which are 0.5 to 3.0 µm in diameter, are too large to be engulfed by lysosomes (which would result in severing the myofibril and loss of function). Finally, normal skeletal muscle cells contain very few lysosomes, especially in comparison with organs like the liver or the spleen. Thus, it seems unlikely that lysosomal proteases are involved in metabolic turnover of myofibrillar proteins (Wildenthal et al., 1980; Lowell et al., 1986), although they may be responsible for necrotic degradation, especially during times of macrophage invasion of cells (Furuno and Goldberg, 1986; Lowell et al., 1986; Lecker et al., 1999). The primary role of lysosomal cathepsins is degradation of extracellular proteins that have been taken up via pinocytosis of receptor-mediated endocytosis and then transported by a series of vesicles to the lysosome, where they are degraded at the acidic pH in lysosomes.

*The Caspase System*

The caspases are responsible for degradation of proteins during apoptosis. The caspases are Cys proteases, as are the calpains and some of the cathepsins, but they do not require Ca2+ for
activity, as do the calpains. It is not known at the present time whether the caspases can efficiently degrade myofibrils. Because the caspases are activated by the events that initiate apoptosis, it seems unlikely that they have significant activity in normal-functioning muscle cells, although they may become activated during periods of muscle wasting. Although Du et al. (2004, 2005) have shown that purified, activated caspase-3 can degrade actin to a 14-kDa fragment and have suggested that caspase-3 acts upstream from the proteasome in turnover of myofibrillar proteins, it is not clear how degradation of actin would contribute to disassembly of a myofibril while retaining its functionality. The actin degradation reported by Du and coworkers was limited, detected by Western blotting, and it seems unlikely that normal muscle cells (i.e., not apoptotic) would activate sufficient caspase-3 to contribute to metabolic turnover of myofibrillar proteins. Thus, it is unlikely that the caspases have an important role in metabolic turnover of myofibrillar proteins.

The Calpain System

The calpain system includes 14 different members, plus calpastatin, in those mammals that have been studied carefully (Goll et al., 2003). Skeletal muscle contains significant amounts of the 2 ubiquitous well characterized calpains, the micromolar Ca2+, requiring Ca2+-dependent protease (µ-calpain), and the millimolar Ca2+, requiring Ca2+-dependent protease (m-calpain), and their specific inhibitor, calpastatin.

The Proteasome

The proteasome has a major role in intracellular protein degradation in all cells, including muscle cells. The unique properties of the proteasome indicate that it could not degrade intact myofibrils, and several studies have shown that it has no effect on intact myofibrils in in vitro
systems. Thus, of the 4 major protease systems in skeletal muscle, the available evidence indicates that only 2, the calpain system and the proteasome, have a major role in metabolic turnover of myofibrillar proteins.

**Ubiquitination: Targeting a Protein for Degradation**

Ubiquitination of polypeptides and degradation of the ubiquitinated polypeptides by the 26S proteasome has been studied extensively. In the ubiquitination pathway, polypeptides that have been designated for destruction, are first labeled by attachment through an isopeptide bond to ubiquitin. Attachment involves the C-terminal end of ubiquitin and usually occurs through an ε-amino group on the selected protein; it may also occur through the N-terminal AA of the selected protein, but the N-terminal amino group of many proteins (including most myofibrillar proteins) is blocked by acetylation, etc., precluding attachment to this group in these proteins.

Ubiquitination is done in a series of enzymatic steps. The first enzyme, called E1, activates the ubiquitin molecule in an ATP-dependent reaction to produce a high-energy E1-S ubiquitin complex (S designates the selected protein). The activated ubiquitin is transferred to 1 of a family of enzymes, the E2 enzymes (Figure 2) called the ubiquitin carrier proteins or ubiquitinconjugating enzymes. The E2 complex then interacts with 1 of a large family of proteins, the E3 proteins, called ubiquitin ligases; the ubiquitin is transferred to the E3 enzyme, the E3-ubiquitin conjugate selects a doomed protein and transfers the ubiquitin to the selected protein; the E2-E3-ubiquitin-protein is then recognized by the proteasome (Figure 3). To be recognized by the proteasome, the targeted protein must be tagged with a tail containing at least 4 ubiquitin molecules (Figure 3). The ubiquitin molecules that are added after the initial ubiquitin all use the same E1, E2, and E3 series of enzymes and attach the second (third, etc.)
ubiquitin to the ε-amino group of Lys48 of the first (second, third, etc.) ubiquitin. A single E1 enzyme can transfer the ubiquitin to any 1 of several E2 enzymes, which, in turn, can transfer their ubiquitin to any 1 of many E3 enzymes (Figure 3). This hierarchical arrangement evidently allows for specificity in designating proteins for degradation, but the mechanism underlying this specificity is not yet understood. The E3 ligases seem to have a tissue specificity; that is, there seem to be ubiquitin ligases specific for muscle protein degradation, specific for metabolic enzyme degradation, etc. The 19S regulatory particle recognizes and binds the ubiquitinated polypeptide. By action of its ATPases, it unfolds the polyubiquitinated molecule, and by action of its deubiquitinating enzymes, removes the ubiquitins from the unfolded polypeptide so they can be recycled. The proteasome has no subsite specificity for degrading a polypeptide such as trypsin or chymotrypsin; rather, the degree of cleavage seems to depend on the time that the polypeptide remains in the interior of the proteasome. Degradation is processive, with each polypeptide being degraded before degradation of a second polypeptide begins. The products of proteasome degradation are peptides ranging from 3 to 23 AA (most are 6 to 10 AA) with an average of ~8 AA. These peptides are then degraded to AA by the different di- and tripeptidases in the cell. Thus, degradation by the proteasome is irreversible, and once a polypeptide enters the proteasome, a new polypeptide will need to be produced by translation if the function of the degraded polypeptide is to be restored (Goll et al., 2008).

The Proteasome and Muscle Protein Turnover

It is estimated that 80 to 90% of all proteins in a cell are ultimately degraded via the proteasome pathway, and it is not surprising therefore that numerous reports have implicated the proteasome in muscle protein turnover. Many studies have reported that proteasome activity or expression of proteasome subunits or ubiquitinating enzymes is elevated during muscle atrophy (Lecker et al.,
Two recent studies found that the mRNA encoding 2 E3 ligases were significantly upregulated specifically during muscle atrophy induced by unweighting, denervation, or immobilization (rat muscle; Bodine et al., 2001) or during fasting, uremia, or streptozotocin-induced diabetes (mouse model; Gomes et al., 2001). The E3 ligases upregulated by unweighting, denervation, or immobilization were identified as MuRF1, for muscle ring finger, and MAFbx, for muscle atrophy F-box. The upregulation was specific for skeletal muscle and occurred in all 3 models of muscle wasting (Bodine et al., 2001). The gene whose expression was upregulated 7- to 9-fold by fasting was named atrogin (Gomes et al., 2001); sequence analysis showed that its sequence was 96% homologous to the rat MAFbx gene, suggesting that atrogin is the mouse homologue of rat MAFbx and that the 2 studies identified the same gene in 2 different species and 2 different models of muscle wasting. The ubiquitin ligases identified by Bodine and Gomes were expressed in skeletal and cardiac muscle but not in liver, brain, spleen, pancreas, placenta, or testis, indicating that they were E3 ligases specific for muscle protein degradation. Since these 2 reports, other studies have found that expression of MuRF1 and MAFbx E3 ligases were upregulated in a rat model of muscle wasting induced by sepsis. It was found that MuRF1 binds to titin at the Zdisk and M-line regions of the titin molecule. Titin is one of those proteins that is rapidly degraded by the calpains (leaving a fragment of ~500 kDa and several smaller fragments) and whose degradation at the Z-disk and M-line concentrations would be needed for dissociation of the thick and thin filaments from the surface of the myofibril. Location of MuRF1 at these regions of titin would position it for immediate ubiquitination and proteasomal degradation of any titin polypeptide if it were released by the calpains. It is clear that muscle protein degradation has an important role in enhancing both the rate and efficiency of skeletal muscle growth in domestic animals. The myofibrillar protein fraction constitutes over
50% of the protein in skeletal muscle, and it presents a unique challenge for turnover of its protein constituents. The proteasome undeniably is responsible for much of the intracellular protein degradation that occurs in muscle and other cells. It seems likely that the proteasome is directly responsible for degradation of the sarcoplasmic proteins that can be ubiquitinated and presented to the 26S proteasome. The myofibrillar proteins, however, cannot be directly degraded by the proteasome, because neither the myofibril itself (0.5 to 3.0 µm in diameter) nor thick and thin filaments (14 to 15 nm and 8 nm in diameter, respectively) could enter the central chamber of proteasome where the catalytic residues reside. The opening to this central chamber is ∼1.2 to 1.5 nm, and in eukaryotic cells, it is blocked by the N-termini of some of the α subunit polypeptides. Indeed, many studies have found that intact myofibrils are not degraded when they are incubated with a proteolytically active proteasome (Koohmaraie, 1992; Solomon and Goldberg, 1996). Solomon and Goldberg (1996) found that although the proteasome degraded myosin and actin, it had no effect on intact myofibrils, and they concluded that the rate-limiting step in degradation of myofibrillar proteins was their release from the myofibril. A subsequent study also found that the myosin heavy chain was degraded by the proteasome, but only after its disassembly from the myofibril, a process that was not associated with proteasome activity. Neither myofibrils nor thick and thin filaments can be degraded by the proteasome, because they cannot enter the central catalytic chamber of the proteasome, whether they are ubiquitinated or not. The calpains do not degrade proteins to AA, so measuring release of free AA to estimate rate of protein turnover cannot be used to determine whether the calpains are contributing to this turnover. The calpains do not degrade sarcoplasmic proteins (Tan et al., 1988; Smith and Dodd, 2007), so turnover of sarcoplasmic proteins is not caused by the calpains but is likely due to proteasomal activity. The myofibrillar proteins must be dissociated from the myofibril before
they can be degraded downstream to AA by the proteasome and cellular peptidases. Both the proteasome and the calpains are present in cells, including skeletal muscle cells, in large excess (Goll et al., 2003). Thus, it is unclear whether increased calpain or proteasome activity in in vitro assays would have any direct effect on the rate of myofibrillar protein turnover in vivo. Increased ubiquitination or a change in how activity of the calpains is regulated (e.g., calpastatin concentrations) may have greater significance to in vivo activity. Measuring mRNA concentrations for proteolytic enzymes does not necessarily reflect the activities of these enzymes (Wang et al., 1998). Less than 50% of the enzymes in cells have their activity regulated at the transcriptional concentration. Both the proteasome and the calpains are phosphorylated, and phosphorylation affects their activity. Measurements of protein concentrations and enzyme activities are more directly related to intracellular enzyme activity than message concentrations.

It should be noted that a large majority of the studies relating the calpains and especially the proteasome to muscle protein degradation involved studies of muscle that was atrophying because of denervation, unweighting, or other treatment to induce rapid muscle protein turnover. It is possible that the mechanism used to turnover muscle proteins under these conditions differs from that used to turnover muscle proteins during muscle growth. The rates at which myofibrillar proteins are released from myofibrils, whether it be via exchange or by calpain cleavage, will control their availability to the next downstream protease (likely the proteasome or possibly, lysosomal cathepsins in some instances). And, release of proteins from the myofibril will involve an equilibrium that is given by the rate of their association back onto the myofibril divided by the rate of their dissociation.

Thus, intracellular protein turnover has been maintained through evolution and selection, and knockout models that eliminate such turnover are invariably embryonically lethal.
The myofibrillar proteins are the major protein fraction in skeletal muscle, and alterations in the rates at which they are turned over would have significant effects on the rate of skeletal muscle growth.

DETERMINATION OF PROTEIN TURNOVER IN ANIMALS

The net result of the protein synthesis and breakdown is the accretion of the body protein. The dynamic nature of protein metabolism has been known for 60 years thanks to the pioneering work of Schoeheimer and others (Shoeheimer et. al., 1939). Using stable isotopes of amino acids, they demonstrated that proteins continually were being broken down and resynthesized. In addition they reported that different organs have different rates of protein synthesis. The dynamic process by which body proteins are continually synthesized and broken down is protein turnover.

Studies on the growth of body protein have been a major area of research. The main reason for this is that, for example, up to 20-25% of the muscle protein can be broken down per day early in the life of humans and farm animals. This rate slows with age to 1-2 % day -1 in adults. Rates of synthesis and breakdown are influenced not only by age, but by plane of nutrition, stress, disease, hormones, exercise, and inactivity.

Definitions

Synthesis:

The conversion of amino acids into proteins by the protein synthetic apparatus in the cytoplasm in the cell.

Breakdown:
The proteolysis of the polypeptides by different proteinases within both the cytoplasmic and lysosomal compartments of the cell.

Growth:

The net accumulation of protein that occurs when the rate of synthesis is greater than the rate of breakdown. The term growth is often used interchangeably with accretion or net synthesis.

Wasting:

The loss of protein that occurs when the rate of breakdown is greater than the rate of synthesis.

Turnover:

A general term that involves both synthesis and breakdown.

**Indirect Measurement of Whole Body Protein Turnover**

*Constant infusion of (15N) glycine (end-product method)*

There is a metabolic pool of N into which amino acids enter from the diet (I) and from the breakdown (B) of the body protein. N in the form of amino acids is synthesized into proteins or can be excreted into urine. The (15N) glycine model is based on the following assumptions: a) the metabolic pool of N remains constant during tracer infusions, b) (15N) glycine is not recycled, c) exogenous 15N is metabolized in a similar manner to endogenous and exogenous N, d) synthesis and excretion are the major pathways of N disposal, and e) amino acids from
breakdown and the diet are handle in the same way (Picou and Taylor-Roberts, 1969; Waterlow et. al., 1978).

The method involves the administration of (15N) glycine (intravenously or orally) at a continuous rate until a plateau in the 15N enrichment is achieved. This usually takes 20-40 h. Urine samples are taken at the plateau. The enrichment of (15N) urea is then determined. At steady state (when the rate of amino acids entering the metabolic pool is equal to the rate at which they leave), the equation for the model is:

\[ Q = I + B = S + E \]

The amino acid flux (Q) is equal to the infusion rate (F) divided by the enrichment of urea or ammonia (d), \( Q = \frac{F}{d} \). Therefore, synthesis (S) = \( Q - E \), where E is the total excretion and breakdown (B) = \( Q - I \), where I is intake (Waterlow et. al., 1978)

The disadvantage of this method is the time of infusion.

**Constant infusion of (13C) leucine**

Leucine was selected as the essential amino acid of choice because it is readily available cheaply in a pure form (L-leucine). In addition, when leucine is isotopically labeled as (1-13C) leucine or (1-14C) leucine, the label is completely removed as CO2 (Rathmacher, 2000).

The original model proposed by Waterlow (1978) is as follows: Labelled (13 C) leucine is infused in a constant way into the blood stream until an isotopic steady state is reached \( \in \)
plasma. The measurements taken are the dilution of the tracer by unlabeled leucine and the rate of labeled leucine CO2 excretion in breath. The dilution of the tracer defines the rate of appearance of leucine in plasma. The labeled CO2 excretion divided by the leucine tracer infusion rate defines the oxidation rate (C). The breakdown rate (B) in the post absorptive rate is equal to Q (leucine flux, leucine infusion/isotopic enrichment), and synthesis (S) = Q – C.

One of the disadvantages of this method is that the leucine tracer is infused into and sampled from blood, but leucine protein metabolism occurs within the cell. The other one is that leucine is transaminated inside cells to KIC (alpha-ketoisotocaproate). The KIC may suffer one of three fates: it may be decarboxylated, reaminaded to leucine or it may leave the cell. Because KIC is only found in the cell from leucine transamination, plasma KIC reflects the intracellular KIC enrichment and can be used as an index of intracellular leucine tracer enrichment. This approach expands the single-pool model into a four pool model (Shwenk et al., 1985). The calculations are made by substituting the leucine isotopic enrichment for the KIC enrichment. Where Breakdown (B) = F/ (1-13C) KIC. Enrichment, oxidation (C) = 13CO2/ (1-13C) KIC, enrichment; synthesis(S) = B – C.

The major disadvantage is that leucine has been reported to have a role in the control of protein synthesis in muscle (Buse & Reid, 1975; Fulks et. al., 1975) and can cause changes in nitrogen balance in starving man (Sherwin, 1978).

**Measurement of Tissue Protein Metabolism in Vivo**

In vivo, measurements of muscle protein turnover have utilized either pulse injection (Garlick et al., 1980) or continuous infusions (Pain & Garlick, 1974) of labeled amino acids, combined with
measurement of the tracer content of tissue protein from biopsy or necropsy specimens (Barret et al., 1987).

**Constant Infusion**

In this method, a labeled amino acid tracer is given by a constant infusion (with or without a prime) at a rate sufficient to achieve an enrichment of 5-10% of the tracer amino acid. The infusion continues for 4-12 h depending on the tissue or protein of interest. The enrichment of the free amino acid remains constant for a substantial portion of the infusion period, thus the kinetics of the tissue protein labeling are simple and linear. At the end of the infusion, tissue samples are taken and rapidly frozen until they can be processed further. The enrichment of the precursor amino acid and the enrichment of the labeled amino acid in the isolated tissue or protein are determined (Garlick, 1969).

The fractional synthetic rate (FSR) can be calculated by the following equation:

\[
\text{FSR} = \frac{(E_1 - E_0)}{(E_p \times (t_1 - t_0)) \times 100}
\]

Where \(E_0, E_1 \ldots E_4\) is the enrichment (tracer/tracee) of the tracer amino acid in the tissue protein at different times and \(E_p\) is the average precursor enrichment during the same time period that the tissue protein is being labeled.

An advantage of this method is that it is applicable to the measurement of proteins with a slow turnover. In addition, whole body protein turnover may be measured at the same time so that a relationship between whole-body protein turnover and tissue protein synthesis may be determined.
Flooding dose

The flooding dose technique was developed to overcome the limitations of the true precursor enrichment for the calculation of protein synthesis by the constant infusion method. The aim is to flood the free amino acid pools, thereby eliminating the difference between the intracellular and extracellular (entire precursors pool) free amino acid enrichments. This is accomplished by administering the tracer with a large bolus of trace. After the ‘flooding dose’, a biopsy of the tissue is taken and the enrichment determined. The FSR of tissue protein is determined using the following formula:

$$\text{FSR} = \frac{(eB+ - eB0)}{\int_0^t eA \, dt}$$

Where $eB+ - eB0$ is the increase in the isotopic enrichment over time $t$ and $\int_0^t eA \, dt$ is the area under the curve of the precursor enrichment versus time (Rathmacher, 2000).

The advantages of this method are the improved resolution of the precursor enrichment, and the shorter periods of measurement than constant infusion, for example: 10 min. flood versus 6 h infusion in rats; and 1-2 h flood versus 4-20 h infusion in humans.

Flooding dose using L- (4-3H) phenylalanine in rats

In this method the rats are infused with 150 µmol of L- (4-3H) phenylalanine in 1 ml of 0.9% NaCl per 100 body weight. The animals are decapitated 10 min after the start of the injection, and the appropriate muscles are rapidly dissected free under ice-cold NaCl and frozen in liquid nitrogen. The specific radioactivity of both the phenylalanine in the ‘flooded’ intracellular pool (SA) and covalently bound in protein (SB) is determined as described by Garlick et.al (1980).
The fractional synthesis rate of synthesis, $K_s$, as a percentage of the protein mass synthesized per day, is calculated as follow:

$$K_s = \frac{SB}{SA \times t} \times 100,$$

where $SA$ and $SB$ are the specific radioactivities of phenylalanine in the precursor pool and protein respectively, and $t$ is the time in days. Muscle growth is determined as the net accumulation of the protein over a maximum of 3 days either immediately before, or spanning, the time points at which protein synthesis is measured.

**Measure of skeletal muscle breakdown**

3-methylhistidin is formed by methylation of histidine and is only in action and myosin. As a consequence of actin and myosin breakdown, 3-MeH is liberated into the amino acid pool of blood plasma (Harris, 1981 and Young et al., 1978).

Skeletal muscle breakdown can be measure using 3-methylhistidine (3-MeH) because of the following reasons: a) the 3-MeH is present exclusively in muscle protein and at a constant amount, b) 3-MeH released after protein degradation is neither re-utilized for protein synthesis nor metabolized, and c) free 3-MeH is rapidly and quantitatively excreted in the urine (Ballard et al., 1983).

3-MeH can be determined in a muscle sample, and the total rate of the muscle protein breakdown in grams of muscle protein per day per kg of body weight can be readily obtained as: $\mu$mol of 3MeH day$^{-1}$ kg$^{-1}$ / $\mu$mol of 3-MeH/g of muscle protein (Ballard et al., 1983).
FEEDING PROGRAMS FOR BREEDER PULLETS AND BREEDERS

Breeder pullet feeding program

Pullets must be managed so as to achieve the desired uniform weight at time of photostimulation, which is usually around 22 – 24 weeks of age. Growth and uniformity are influenced by feeding program and to a lesser extent, feed formulation. Within reason, it is possible to achieve the desired weight for age when using diets with a vast range of nutrient specification (Table 2).

Nutrient intake is largely controlled by the degree of feed restriction. For example, it is theoretically possible to grow pullets on diets with energy concentrations ranging from 2600-3100 kcal ME/kg. In practice, diet energy concentration is usually within the range of 2750-2950 kcal ME/kg, although for diets necessarily formulated outside of this range, energy intake can be controlled by adjusting feed intake. (Leeson and Summers, 2005). It is usually more difficult to maintain uniformity with high-energy diets, since this necessarily implies much smaller quantities of feed being distributed at any one time.

Each commercial strain is going to have characteristic patterns of growth and these can be used to dictate feeding program. These strains will have an ‘optimum’ mature weight which is around 2.2 kg for pullets at 22 weeks of age. (Leeson and Summers, 2005)

Some type of physical feed restriction is used to control breeder growth. The traditional system has been skip-a-day where, as its name implies, birds are fed only on alternate days.

With skip-a-day, birds are given these quantities of feed only every other day. The concept behind this program is that with every other day feeding, birds are offered a considerable quantity of feed and this is easier to distribute so that even the smallest most timid bird can get a chance to eat. The usual alternative to skip-a-day feeding is feeding restricted quantities every day. There is a trend towards every day feeding since it is more efficient and with good
management and supervision, good uniformity can be achieved. Improved efficiency results from birds utilizing feed directly each day, rather than there being the inherent inefficiency of skip-a-day fed birds having to utilize stored energy for maintenance on the off-feed day. (Leeson and Summers, 2005. Cobb Management Guide, 2008)

**Breeder hen feeding program**

Adult breeders must be continued on some type of restricted feeding program with different nutrient specifications than pullets (Table 3). After 22 weeks of age, regardless of rearing program, all birds should be fed on a daily basis. Once birds have peaked in egg production, it is necessary to reduce feed intake. There is often confusion and concern as to how much and how quickly feed should be removed, and this is somewhat surprising, since the same basic rules used pre-peak also apply at this time. This means that birds should be fed according to egg production, body weight and clean-up time. After peak production, feed clean-up time often starts to increase, and this is an indication of birds being overfed. The main problem we are trying to prevent at this time is obesity. If feed is not withdrawn after peak, then because egg production is declining, proportionally more feed will be used for growth. After peak therefore, body weight becomes perhaps the most important variable used in manipulating feed allocation. It is still important for birds to gain some weight, since loss of weight is indicative of too severe a reduction in feed allowance. Peak production is determined when daily egg production has not increased for five consecutive days. Under normal circumstances feed can be reduced by a minimum of 1 g/week after peak production to depletion. This reduction should be made each week until the peak production feed amount has been reduced 14%. The feed reduction rate may be made faster or slower based on daily records of

FEEDING PROGRAMS FOR LAYER PULLETS AND LAYER HENS

Layer pullet feeding program

Laying pullets should be started and maintained on a feed program that provides all the known required nutrients for growth and sexual development. The objective is to be certain that the pullet reaches the target body weight during each week of growth. Uniformity of body weight is also critical to achieving the goals of efficient and high production. The rations used must be adequate to achieve the targeted body weights and uniformity under normal environmental conditions. Body weight measurements of the pullets should be taken beginning at 4 weeks of age and taken every week thereafter to peak production. Then measurements can be made every other week until peak egg mass is achieved. The recommended ration for the first six weeks is a starter ration. If at the end of six weeks of age, the birds are at the target body weight (410 grams or 0.90 lbs.), the ration can be changed to a grower ration. The grower ration is designed to be fed from 7 to 10 weeks of age. If the flock has continued to grow normally and has reached target body weights, with good uniformity, the flock should be moved to a developer I ration at 11 weeks of age. The Developer I ration is designed to allow for the rapid body weight growth that occurs at this age. At 16 weeks of age change birds to Developer II ration if body weight is on target. This diet has higher calcium concentrations that will allow for the development of the medullary bone that acts as a reservoir for calcium for eggshell formation. All pullet feeds should be fed ad libitum or without restrictions. (Bovans Brown Management Guide, 2012). Nutrient specification during these period of time should be found in table 4.
Laying hen feeding program

The laying hen should be allowed to consume feed ad libitum until the flock reaches its maximum egg mass output or the desired case weight has been achieved. Feed troughs should allow for access to feed throughout the morning and evening hours. No harm is done when the flock is allowed to clean the troughs during the middle part of the day. Body weights are an excellent tool to help determine if feed consumption for the flock is adequate for production and growth. Body weights should continue to increase, although very slowly, throughout the laying cycle. Decreasing body weights should be viewed as an indication that nutrient intake has not been adequate and egg production may soon suffer. Many nutrition programs successfully utilize the reduction of protein and amino acid density of the diets with increased environmental temperature later in the life of a flock to control egg size and maximize profits (Bovans Brown Management Guide, 2012). Nutrient specification during these period of time should be found in table 5.

MUSCULAR PROTEIN DEGRADATION IN ANIMALS DURING PRODUCTION

It is already know that the nutrients demands for any animal in production are extremely high. Unfortunately the nutrient content we provide them in their diets is not always enough, and they are forced to use their body reserves (mainly muscle) to keep up with production.

Lactation imposes a unique challenge to mammalian protein metabolism, especially for species such as the pig, which support high growth rate in their 10-12 offspring. This state represents a
physiological maximum in protein anabolism, with high rates of net protein export to the mammary gland (Clowes, et al., 2005). Because of the high rate of net protein export from the dam in the form of milk, lactation can represent a physiological maximum in protein mobilization in the dam, as dietary intakes are often inadequate. Lactation thus represents a highly orchestrated physiological state featuring intense milk protein anabolism and net catabolism of body reserves, especially skeletal muscle. Clowes et al. (2005) using a biopsy technique to collect triceps muscle samples in late gestation and midlactation and within 3 h of weaning from sows were able to find that muscle RNA concentrations decreased 10-15% between late gestation and midlactation in all treatments. Muscle mRNA concentrations of several key elements of the ATP-ubiquitin proteasome-dependent proteolytic pathway in muscle increased as lactation progressed. The 1.2-kb 14-kDa E2 transcript showed the most marked increase. This transcript more than doubled between late gestation and midlactation in all treatments. Furthermore, in all treatments, 2.6-kb ubiquitin transcript expression increased by 40-60% over lactation. Also, in agreement with this findings, most muscle free amino acids concentrations increased by ~ 30% between late gestation and midlactation.

In a study conducted by Overton and coworkers (1998) in lactating cows they found that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then decline until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Potential utilization of amino acids for gluconeogenesis is greater during the first 21 d of lactation than at other times during the productive cycle.

Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. In accordance to this study Blum et al.
(1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition (Figure 3).

In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both groups of animals using the phenylalanine approach, they found that absolute rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group.

In a study conducted in pregnant and non-pregnant women Flitch and King (1987) found that normal pregnant women excreted significantly more urinary 3-methylhistidine than did non-pregnant women.

On the other hand Duggleby and Jackson (2001) found in pregnant women that protein synthesis and breakdown both significantly increased between mid and late pregnancy, by 15% and 17% respectively. Also, in mid and late pregnancy, mothers with a greater lean body mass has higher rates of protein turnover.
Table 1: Essential and non-essential amino acids in the chicken.

<table>
<thead>
<tr>
<th>Essential</th>
<th>Non-essential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Lysine</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Histidine</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Leucine</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Valine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Methionine</td>
<td>Serine*</td>
</tr>
<tr>
<td>Threonine</td>
<td>Tyrosine*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Cysteine*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Proline*</td>
</tr>
</tbody>
</table>

*Semi-essential
Figure 1: Transcription and Translation

1. Transcription

DNA → RNA polymerase → mRNA → tRNA → amino acids → proteins

2. Translation

mRNA → polypeptide chain → Ribosome → proteins

Protein synthesis

Figure 2: The ubiquitin-proteasome pathway of proteolysis.

Source: Mitch and Goldberg, 1996.
Table 2: Diet specifications for broiler breeder pullets

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>Starter 0 – 4</th>
<th>Grower 4 – 12</th>
<th>Developer 12 – 22</th>
<th>Prebreeder 20 – 22</th>
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</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>18.5</td>
<td>17.0</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Metabolizable Energy (kcal/kg)</td>
<td>2850</td>
<td>2850</td>
<td>2850</td>
<td>2850</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.95</td>
<td>0.92</td>
<td>0.89</td>
<td>2.25</td>
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<tr>
<td>Available Phosphorus (%)</td>
<td>0.45</td>
<td>0.40</td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.20</td>
<td>0.19</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.42</td>
<td>0.35</td>
<td>0.32</td>
<td>0.37</td>
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<tr>
<td>Methionine + Cystine (%)</td>
<td>0.80</td>
<td>0.72</td>
<td>0.65</td>
<td>0.64</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>1.00</td>
<td>0.90</td>
<td>0.80</td>
<td>0.77</td>
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<tr>
<td>Threonine (%)</td>
<td>0.72</td>
<td>0.67</td>
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<td>0.58</td>
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<tr>
<td>Tryptophan (%)</td>
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<td>0.18</td>
<td>0.16</td>
<td>0.15</td>
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<tr>
<td>Arginine (%)</td>
<td>1.15</td>
<td>1.00</td>
<td>0.86</td>
<td>0.80</td>
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<tr>
<td>Valine (%)</td>
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<td>0.70</td>
<td>0.65</td>
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<tr>
<td>Leucine (%)</td>
<td>0.90</td>
<td>0.85</td>
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<tr>
<td>Isoleucine (%)</td>
<td>0.70</td>
<td>0.60</td>
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<tr>
<td>Histidine (%)</td>
<td>0.20</td>
<td>0.18</td>
<td>0.29</td>
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<tr>
<td>Phenylalanine (%)</td>
<td>0.65</td>
<td>0.60</td>
<td>0.53</td>
<td>0.49</td>
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<tr>
<td>Vitamins (per kg of diet)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A (I.U.)</td>
<td>8000</td>
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<td></td>
</tr>
<tr>
<td>Vitamin D$_3$ (I.U.)</td>
<td>3000</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vitamin E (I.U.)</td>
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<td></td>
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<tr>
<td>Vitamin K (I.U.)</td>
<td>3</td>
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<tr>
<td>Thiamin (mg)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>10</td>
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<td></td>
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<tr>
<td>Pantothenic acid (mg)</td>
<td>12</td>
<td></td>
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<tr>
<td>Folic acid (mg)</td>
<td>0.75</td>
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<tr>
<td>Biotin (µg)</td>
<td>100</td>
<td></td>
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<tr>
<td>Niacin (mg)</td>
<td>40</td>
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<tr>
<td>Choline (mg)</td>
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<tr>
<td>Vitamin B$_6_2$ (µg)</td>
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<tr>
<td>Trace minerals (per kg of diet)</td>
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</tr>
<tr>
<td>Manganese (mg)</td>
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<td>Iron (mg)</td>
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<tr>
<td>Copper (mg)</td>
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<tr>
<td>Zinc (mg)</td>
<td>60</td>
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<tr>
<td>Iodine (mg)</td>
<td>0.5</td>
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<tr>
<td>Selenium (mg)</td>
<td>0.3</td>
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</tr>
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Source: Leeson and Summers, 2005.
Table 3: Diet specifications for adult broiler breeders

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>Phase 1 22 – 34</th>
<th>Phase 2 34 – 54</th>
<th>Phase 3 54 – 64</th>
<th>Male 22 – 64</th>
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<tbody>
<tr>
<td></td>
<td>Crude Protein (%)</td>
<td>Metabolizable Energy (kcal/kg)</td>
<td>Calcium (%)</td>
<td>Available Phosphorus (%)</td>
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<tr>
<td></td>
<td>16.0</td>
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<td></td>
<td>15.0</td>
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<td>3.20</td>
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<td></td>
<td>14.0</td>
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<td>0.34</td>
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<tr>
<td></td>
<td>12.0</td>
<td>2750</td>
<td>0.75</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamins (per kg of diet)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Vitamin A (I. U.)</td>
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</tr>
<tr>
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<td>3000</td>
</tr>
<tr>
<td>Vitamin E (I. U.)</td>
<td>75</td>
</tr>
<tr>
<td>Vitamin K (I. U.)</td>
<td>3</td>
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<tr>
<td>Thiamin (mg)</td>
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</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>10</td>
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<tr>
<td>Pyridoxine (mg)</td>
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<tr>
<td>Pantothenic acid (mg)</td>
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<tr>
<td>Folic acid (mg)</td>
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<tr>
<td>Biotin (µg)</td>
<td>100</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>40</td>
</tr>
<tr>
<td>Choline (mg)</td>
<td>500</td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg)</td>
<td>15</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace minerals (per kg of diet)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Manganese (mg)</td>
<td>90</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>30</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>12</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>100</td>
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<tr>
<td>Iodine (mg)</td>
<td>0.5</td>
</tr>
<tr>
<td>Selenium (mg)</td>
<td>0.3</td>
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</tbody>
</table>

Source: Leeson and Summers, 2005.
Table 4: Diet specifications for laying pullets

<table>
<thead>
<tr>
<th></th>
<th>Starter (0-6 weeks)</th>
<th>Grower (7-10 weeks)</th>
<th>Developer I (11-15 weeks)</th>
<th>Developer II* (16-17 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein (%)</td>
<td>20.00</td>
<td>18.00</td>
<td>15.50</td>
<td>14.75</td>
</tr>
<tr>
<td>ME (Kcal/lb.)</td>
<td>1360</td>
<td>1330</td>
<td>1290</td>
<td>1280</td>
</tr>
<tr>
<td>ME (kcal/kg)</td>
<td>2980</td>
<td>2940</td>
<td>2840</td>
<td>2820</td>
</tr>
<tr>
<td>Linoleic Acid %</td>
<td>1.3</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Methionine %</td>
<td>0.45</td>
<td>0.40</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>M + C %</td>
<td>0.80</td>
<td>0.72</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Lysine %</td>
<td>1.10</td>
<td>1.00</td>
<td>0.85</td>
<td>0.80</td>
</tr>
<tr>
<td>Arginine %</td>
<td>1.20</td>
<td>1.10</td>
<td>0.97</td>
<td>0.95</td>
</tr>
<tr>
<td>Tryptophan %</td>
<td>0.21</td>
<td>0.19</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Threonine %</td>
<td>0.75</td>
<td>0.70</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td>Calcium %</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>2.25</td>
</tr>
<tr>
<td>Av. Phosphorus %</td>
<td>0.50</td>
<td>0.50</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Sodium (%)</td>
<td>0.18</td>
<td>0.17</td>
<td>0.17</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Note: The Developer II diet should be discontinued and a layer ration used at the onset of production.

Table 5: Diet specifications for adult laying hens

<table>
<thead>
<tr>
<th>RATION</th>
<th>PEAKING DIET</th>
<th>PHASE II</th>
<th>PHASE III</th>
<th>PHASE IV</th>
<th>PHASE V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Range</td>
<td>18-30 Weeks</td>
<td>31-45 Weeks</td>
<td>46-55 Weeks</td>
<td>56-65 Weeks</td>
<td>65+ Weeks</td>
</tr>
<tr>
<td>Feed Cons. Range</td>
<td>&lt;25 lbs/100 &lt;114 grams/bird</td>
<td>25 lbs/100 114 grams/bird</td>
<td>25 lbs/100 114 grams/bird</td>
<td>25 lbs/100 114 grams/bird</td>
<td>25 lbs/100 114 grams/bird</td>
</tr>
<tr>
<td>Egg Prod. Range</td>
<td>&gt;95%</td>
<td>95-99%</td>
<td>92-99%</td>
<td>88-85%</td>
<td>&lt;85%</td>
</tr>
<tr>
<td>ME (kcal/lb.)</td>
<td>1280</td>
<td>1280</td>
<td>1270</td>
<td>1260</td>
<td>1255</td>
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<tr>
<td>ME (kcal/kg.)</td>
<td>2816</td>
<td>2816</td>
<td>2794</td>
<td>2772</td>
<td>2761</td>
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<tr>
<td>% CP</td>
<td>17.50</td>
<td>16.90</td>
<td>16.50</td>
<td>15.90</td>
<td>15.30</td>
</tr>
<tr>
<td>Calcium %</td>
<td>4.00</td>
<td>4.00</td>
<td>4.10</td>
<td>4.20</td>
<td>4.30</td>
</tr>
<tr>
<td>Av. Phosphorus %</td>
<td>0.45</td>
<td>0.42</td>
<td>0.40</td>
<td>0.38</td>
<td>0.36</td>
</tr>
<tr>
<td>Sodium %</td>
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<td>0.19</td>
<td>0.18</td>
<td>0.18</td>
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</tr>
<tr>
<td>Methionine %</td>
<td>0.437</td>
<td>0.409</td>
<td>0.387</td>
<td>0.373</td>
<td>0.357</td>
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<tr>
<td>TSAA %</td>
<td>0.716</td>
<td>0.677</td>
<td>0.659</td>
<td>0.632</td>
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<tr>
<td>Lysine %</td>
<td>0.88</td>
<td>0.84</td>
<td>0.79</td>
<td>0.75</td>
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<tr>
<td>Tryptophan %</td>
<td>0.21</td>
<td>0.20</td>
<td>0.20</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td>Linoleic Acid %</td>
<td>1.40</td>
<td>1.30</td>
<td>1.25</td>
<td>1.15</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Figure 3: Concentrations of 3-methylhistidine in blood plasma before and after parturition in 13 cows.

Figure 1. Concentrations of 3-methylhistidine (3-MH) in blood plasma before and after parturition in 13 cows. Significance of difference from prepur- ent means: *P<.05, **P<.01, ***P<.001, l = Liter.

Source: Blum et al., 1985.
REFERENCES


Waterlow, J.C., P.J. Garlick, and D.J. Millward (1978) Protein turnover in Mammalian Tissues and in the Whole body. Elsevier North-Holland, Amsterdam.

II. The effect of sexual maturity and egg production on protein turnover in broiler breeder pure lines
ABSTRACT Protein turnover in skeletal tissue for broiler breeders has been shown to increase at sexual maturity and then decline with increased egg production (Ekmay et al., 2012). The authors believe the increased protein degradation rate at sexual maturity is to provide amino acids for egg production. The objective of the present study was to evaluate protein turnover in four broiler breeder pure lines during sexual maturity transition and throughout production. Protein turnover was determined in four broiler breeders’ pure lines (Line A, Line B, Line C and Line D) at 22, 27, 32, 37, 44 and 50 weeks of age. A complete randomized design with 4 treatments and 6 time periods (four lines, 6 ages) was performed. There were 5 replicates per treatment/time period and each hen represented a replicate. Analysis of variance was performed using JMP software. Five hens at each age were given an intravenous flooding-dose of 15N-Phe (150 mM, 40% APE) at 10 ml/kg. After 10 min, birds were slaughtered and the breast and leg muscle excised and frozen in liquid nitrogen. Excreta was also collected and frozen. The acid-soluble fraction (2% perchloric acid) containing free amino acids from muscle was separated from the protein precipitate. The ratio of 15N:14N of each fraction was determined via GCMS. Quantification of 3-methylhistidine in both muscle and excreta was also determined via GCMS. All birds were scanned for body composition before sampling. No statistical interaction was found for the breast fractional synthesis rates (FSR) between lines and ages. However a significant age effect was found. Breast FSR significantly decreased from week 24 (first egg) to week 30 (peak egg production) (9.4%/d and 3.22%/day, respectively; P. value < 0.05). There was a significant age effect on fractional breakdown rate (FBR) in breast muscle. The FBR in breast skeletal tissue significantly increased from 24 wk. of age (first egg) to week 30 (peak egg production) and remained the same at week 37 (21.23%/day, 26.79%/day and 28.47%/day, respectively; P. value < 0.0001), then it decreased significantly at week 44 (16.64%/day; P.
value < 0.0001) and again at week 50 (6.39 %/day; P. value <0.0001). There were no significantly differences for leg FSR rate between lines and ages. There was a significant age effect on leg FBR. This variable statistically increased from week 30 to week 37 (20.18 %/day and 24.34 %/day, respectively; P. value < 0.0001) and then it significantly decreased at week 44 (11.2 %/day) and again at week 50 (5.46 %/day; P. value < 0.0001). The FBR in leg muscle peaks at a lower rate at wk. 30 and does not show a plateau from 30 wk. to 37 wk. There is a large increase in FBR during the transition for the pullet to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in lean mass body content during this period of time. Broiler breeders may rely on skeletal muscle tissue as a source of nutrients for egg production.

Key words: Fractional breakdown rate, fractional synthesis rate, protein turnover, 15N phenylalanine, GC-MS.
INTRODUCTION

The net result of the protein synthesis and breakdown is the accretion of the body protein. The dynamic nature of protein metabolism has been known for 60 years thanks to the pioneering work of Schoeheimer and others (Schoeheimer et. al., 1939). Using stable isotopes of amino acids, they demonstrated that proteins continually were being broken down and resynthesized. In addition they reported that different organs have different rates of protein synthesis. The dynamic process by which body proteins are continually synthesized and broken down is protein turnover.

Studies on the growth of body protein have been a major area of research. The main reason for this is that, for example, up to 20-25% of the muscle protein can be broken down per day early in the life of humans and farm animals. This rate slows with age to 1-2 % day -1 in adults. Rates of synthesis and breakdown are influenced not only by age, but by plane of nutrition, stress, disease, hormones, exercise, and inactivity.

Since the first indication by Schoenheimer (1939) for the dynamic state of body protein, it is well established that intracellular proteins are subjected to substantial synthesis and degradation processes. As a result, accretion of protein in the whole body of the animals is the reection of small differences between synthesis and degradation rates (Muramatsu, 1990). Because protein accretion, hence overall growth at early stages or egg, wool and milk production at maturity, is one of the major concerns in livestock husbandry, therefore the interest in the regulation of these processes has increased in the recent years.

Protein turnover in broiler breeders has been studied by Ekmay et al. (2012). The researchers showed that protein degradation rate of breast meat increased at sexual maturity and then declined with additional egg production. However this phenomena is still under research.
whereas in mammals it has been largely studied. Overton and coworkers (1998) found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition. In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

The objective of the present study was to determine the effect of sexual maturity on protein turnover in breeders of different pure lines.

MATERIALS AND METHODS

Stock and Management

Four flocks (three hundred birds/flock) of Cobb Pure lines broiler breeder hens from lines: 12, 35, 58, and 74 of 20 weeks of age were reared. A total of one thousand and two hundred birds were used for this trial. The Cobb Breeder Management Guide (Cobb-Vantress, 2008) was used as a reference for all management conditions. Birds were reared in a production house and
individually caged. Cages (47 cm high, 30.5 cm wide, and 47 cm deep) were each equipped with an individual feeder and nipple drinker. All birds were weight every 5 weeks and egg production was recorded daily. All breeders were put on an everyday feeding system. The energy concentration utilized was 450 kcal, and the protein intake was 22 g/d at peak intake.

Experimental diet is shown in table 1. Each line represented a treatment and a total of 300 20 weeks old birds were used per treatment.

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee (protocol # 13002).

**Experimental Procedure**

*Infusion procedure*

A total of 4 birds per line (four infused, one control) were infused with a 40% APE solution of 15N PHEN at the following ages: 22, 27, 32, 37, 44, and 52 weeks of age with a dose of 10 ml/kg BW of 15N phe. Each bird was culled after 10 minutes of infusion. One sample of the breast muscle (*pectoralis major*) was taken, put in a label sample bag, and the bag was saved in liquid N. The same was done with the leg muscle (*gastrocnemius muscle*). A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the samples were stored at -20 C, until sample analysis.
**Body composition**

All birds were scanned for body composition using a DEXA equipment as previously described (Salas et al., 2012).

**Sample Processing**

The acid-soluble fraction containing free amino acids was removed by addition of 2% (w/v) perchloric acid. After homogenization, samples were centrifuged at 3,000 x g and the supernatant, containing free amino acids, removed. The protein precipitate was washed three times with 2% perchloric acid before being hydrolyzed in 6N HCl. The supernatant and precipitate, respectively, was then run through an ion-exchange column packed with Dowex 50WX8-200. Phenylalanine and 3-methylhistidine (3-MH) were eluted with 2ml of 4N NH4OH and 1ml of nanopure H2O into a new vial and dried under vacuum. The tert-butyldimethylsilyl (tBDMS) derivative was formed by addition of 800 µl of C2CH3CN-MTBSTFA (1:1) and incubation at 110 °C for 120 min. Excreta was processed without the removal of the acid-soluble fraction.

**GC/MS analysis**

Analysis of the protein precipitate of breast and legs samples and free amino acids was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature was 150°C and increased 50°C/min to 200°C, after which temperature was increased 20° C/min to 270° C and was held for 5.5 min. The mass spectrometer was operated under EI and SIM modes. The 394, and 395 m/z fragments, representing the M, and M+1
fragments of phenylalanine, was monitored. Standard solutions (in 0.1M HCl) were prepared from the same phenylalanine stock to validate APE linearity.

3-MH was determined on the same mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature will be 110°C and held for 0.65 min, then temperature was increased 30°C/min to 250°C, and held for 10 min. The mass spectrometer was operated under EI and SIM modes. The 238 m/z fragment of 3-MH was monitored.

Fractional synthesis rate was calculated as:

\[ K_s = \frac{APE_b}{APE_f} \times \frac{1}{t} \times 100 \]

Where \( APE_b \) = 15N atom percent excess (relative to natural abundance) of phenylalanine in protein

\( APE_f = 15N \) atom percent excess of free phenylalanine in tissues, assumed as the precursor pool

\( t = \) time [d].

Fractional degradation rate was calculated as:

\[ k_d = \frac{3-MH \text{ daily excretion}}{3-MH \text{ muscle pool}} \times 100 \]
EXPERIMENTAL DESIGN

The experimental design for this trial was a CRD with 4 treatments and 6 time periods (four lines, six ages) each time will have five replications.

The model to be used will be the following:

\[ Y_{ijk} = u + A + B + AB + e \]

Where:

\( u \) = general media

\( A \) = bird line

\( B \) = age of the bird

\( AB \) = interaction line x age

\( e \) = random error

Analysis of variance was performed using JMP software, and means were separated by Tukey test when p Value < 0.05.
RESULTS

Fractional synthesis and breakdown rate: breast muscle

Broiler breeder breast muscular fractional synthesis and breakdown rate by line and age are shown in Table 2 and Figures 1 and 2. No statistical interaction was found for the breast fractional synthesis rates (FSR) and fractional breakdown rate (FBR) between lines and ages. However, a significant age effect was found. FSR significantly decreased from week 24 (first egg) to week 30 (peak egg production) (9.4 %/d and 3.22 %/day, respectively; P. value < 0.05). A contrast was performed for FSR from line A and C, and it was significant. Line A showed significantly higher FSR than line C at 22 and 24 weeks of age (10.85 vs 4.79 and 12.31 vs 6.22, respectively; P. Value = 0.026 and 0.028, respectively). There was a significant age effect on fractional breakdown rate (FBR). The FBR in breast skeletal tissue significantly increased from 24 wk. of age (first egg) to week 30 (peak egg production) and remained the same at week 37 (21.23 %/day, 26.79 %/day and 28.47 %/day, respectively; P. value < 0.0001), then it decreased significantly at week 44 (16.64 %/day; P. value < 0.0001) and again at week 50 (6.39 %/day; P. value < 0.0001).

Fractional synthesis and breakdown rate: leg muscle

Broiler breeder leg muscular fractional synthesis and breakdown rate by line and age are shown in Table 3 and Figures 3 and 4. No statistical differences were found for the leg muscle fractional synthesis rates (FSR) between lines and ages. There was a significant age effect on fractional breakdown rate (FBR). The FBR in leg skeletal tissue significantly increased from 30 wk. of age (peak egg production) to week 37 (20.18 %/day and 24.34 %/day, respectively; P. value <
0.0001), then it decreased significantly at week 44 (11.2 %/day; P. value < 0.0001) and again at week 50 (5.46 %/day; P. value <0.0001).

**Body composition**

Broiler breeder body composition by line and age are shown in Figures 5 and 6 and tables 4 and 5. There was an interaction between age and line on fat mass as a percentage of total mass. Line C showed significantly higher fat percentage at 30 and 37 week of age. (27.41 % and 27.92%, respectively; P. value < 0.001). There was also an interaction between age and line on lean mass as percentage of total mass. Line C showed significantly lower lean percentage at 30 and 37 week of age. (62.62 % and 61.88%, respectively; P. value < 0.001). There was only an age effect regarding total mass. This variable significantly increased with age.

**DISCUSSION**

Results of the present study show that muscular protein fractional synthesis rate from broiler breeders significantly decreased as they approached peak egg production and the degradation rate significantly increased during the same period of time. After egg production slowed down, muscular fractional synthesis rate tended to increase whereas the breakdown rate decreased significantly. The fact that no difference were found between lines might suggest that this phenomena it is more related to the fact that the bird is in a production phase than the line itself. These results are in agreement with what was reported by Manangi and Coon (2006). These authors reported an increase in fractional degradation rates from 22 to 26 wk. The authors suggested that there is increased mobilization of skeletal muscle for egg formation. The rise in FBR in the present study as hens enter sexual maturity suggests that this may be the case. Salas
et al. (2013) conducted a trial where a group of pullets were fed a daily 15 mg dose of U-13C-glucose 10 d prior to sexual maturity continuing through 10 d following first egg. The authors were able to conclude that the U-13C-glucose is being utilized for yolk fatty acid synthesis in a higher concentration at the beginning of the production period. This indicates that glucose is needed for yolk fatty acid synthesis. The fact that more FBR is seen in the present study might suggest that birds are degrading protein for gluconeogenesis. The results in the present study are also in agreement with Overton and coworkers (1998) who found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition, which is in agreement with the present results.

In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

Lactation imposes a unique challenge to mammalian protein metabolism, especially for species such as the pig, which support high growth rate in their 10-12 offspring. This state represents a
physiological maximum in protein anabolism, with high rates of net protein export to the mammary gland (Clowes, et al., 2005). Because of the high rate of net protein export from the dam in the form of milk, lactation can represent a physiological maximum in protein mobilization in the dam, as dietary intakes are often inadequate. This statement can be also applied for a broiler breeder hen when is facing peak egg production as they are feed restricted being fed only every 24 hours. Clowes et al. (2005) using a biopsy technique to collect triceps muscle samples in late gestation and midlactation and within 3 h of weaning from sows were able to find that muscle RNA concentrations decreased 10-15% between late gestation and midlactation in all treatments. Muscle mRNA concentrations of several key elements of the ATP-ubiquitin proteasome-dependent proteolytic pathway in muscle increased as lactation progressed indicating muscular protein degradation was taking place. The 1.2-kb 14-kDa E2 transcript showed the most marked increase. This transcript more than doubled between late gestation and midlactation in all treatments. Furthermore, in all treatments, 2.6-kb ubiquitin transcript expression increased by 40-60% over lactation. Also, in agreement with these findings, most muscle free amino acids concentrations increased by ~ 30% between late gestation and midlactation. This later study is also in agreement with our findings regarding muscular fractional degradation rate.

As it can be seen in Figures 3 and 4 the FBR in leg muscle peaks at a lower rate at wk. 30 and does not show a plateau from 30 wk. to 37 wk. Which might suggest that birds are relying more on breast muscle than on leg muscle as a source of protein for egg formation. FSR decreases as the birds enter to egg production whereas FBR increases, suggesting again the use of muscular protein to produce eggs. The dramatic increased in FBR might be due to the feed restriction these birds are facing. Boonsinchai et al. (2014) conducted a study in broiler breeders where they were
testing six different feeding programs. The programs consisted in regular breeder diets with or without feed withdrawal after peak egg production and increasing feed intake at 32, 35 or 40 weeks of age. The authors found that maintaining and increasing feed after peak enhanced persistency of lay. They were able to conclude that either maintaining or increasing feed after peak resulted in higher body weigh which is corresponding to bigger eggs and hatched chicks. Also, feed withdrawal after peak (as commercial practice) does not provide enough energy for breeder hens to produce eggs. These results support our findings that there was more FBR. Since the hens in the present trial were subjected to feed withdrawal and no extra feed was added at any point of the laying period, the data clearly show how much muscular protein the hens were degrading. The results from Boonsinchai et al. (2014) study indirectly imply that the birds facing no feed withdrawal, but feed addition while laying eggs might have not been as restricted as the hens in the present study and that is why the persistency was enhanced. The birds in that study may not have been forced to use their body muscular reserves to produce eggs. Unfortunately, no protein turnover has been measured in hens that are not subjected to feed withdrawal or feed addition; it may be that these hens may not degrade as much as the ones in the present study. In the present study we found that there is a decrease in lean mass as a percentage of total mass, and this decreased was significant for line C. Line C is the best egg producer among all lines (data provided by Cobb-Vantress), and that might be the reason why this line is showing that response. Furthermore, the same line showed significantly higher fat mass as a percentage of total mass, and again, this might be because this line is the best egg producer and since it is not putting on lean mass, will put on fat instead. In general for the four lines, fat mass increases after peak egg production and lean mass decreases for the same period of time which supports our FBR findings. These results are in agreement with Salas et al. (2010) who were able to see a
similar trend in broiler breeders when evaluated six different diets with different energy concentrations. Figure 7 shows the pooled breast muscular FSR, FBR, lean mass and egg production. This graph shows us that as egg production increases, FSR decreases, FBR increases and lean mass (grams) does not either increase or decrease, whereas when egg production decreases (week 37), FSR increases, FBR decreases and lean mass increases.

Together our results and the one found in other species such as mammals that are also feed restricted (during pregnancy) suggest that the dramatic increased in muscular fractional degradation rate is a phenomena related more to a production state and a feed restriction issue than as to the specie itself and it might be triggered by the fact that the animals are not getting the nutrients they need to produce either eggs or milk.

There is a large increase in FBR during the transition for the pullet to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in lean mass body content during this period of time. Broiler breeders may rely on skeletal muscle tissue as a source of nutrients for egg production. Further studies need to be conducted in broiler breeders using different feeding programs involving frequency of feeding and evaluating muscular protein degradation to better understand why these birds have such a dramatic degradation rate during production. The answer might be a balance between feed frequency and quantity to optimize nutrient utilization for production without increasing maintenance requirements.
Table 1: Experimental diet and Nutritional Content

<table>
<thead>
<tr>
<th>INGRIDIENT, %</th>
<th>Nutrient, %</th>
</tr>
</thead>
<tbody>
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<td>Corn</td>
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</tr>
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</tr>
<tr>
<td>Fat</td>
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<td>Limestone</td>
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<td>dicalcium phosphate</td>
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<td>Salt</td>
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<td>Alimet</td>
<td>0.19</td>
</tr>
<tr>
<td>Choline</td>
<td>0.10</td>
</tr>
<tr>
<td>vitamin premix</td>
<td>0.07</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>0.1</td>
</tr>
<tr>
<td>mineral premix</td>
<td>0.075</td>
</tr>
<tr>
<td>TOTAL %</td>
<td>100</td>
</tr>
</tbody>
</table>

Calculated:
| ME, Kcal/kg | 2860 |
| Crude Protein | 15.5 |
| Dig. Lysine | 0.76 |
| Dig. Meth & Cyst | 0.67 |
| Dig. Methionine | 0.42 |
| Dig. Threonine | 0.52 |
| Dig. Tryptophan | 0.16 |
| Dig. Arginine | 0.95 |
| Phosphorus-Non | 0.41 |
| Sodium | 0.2 |

Analyzed:
| Crude Protein | 15.1 |
| Crude Fat | 4.83 |
| Ash | 10.27 |
| Calcium, ppm | 33664 |

Table 2: Broiler breeder breast muscle fractional synthesis and breakdown rate by age

<table>
<thead>
<tr>
<th>Age, wk.</th>
<th>FSR %/d</th>
<th>FBR %/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>7.92&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>18.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>27</td>
<td>9.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>33</td>
<td>5.63&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>26.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>37</td>
<td>3.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>44</td>
<td>3.97&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>16.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>5.88&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>6.39&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Concentrations not connected by same letter are significantly different.

**Figure 1:** Broiler breeder breast muscle fractional synthesis rate by age and line

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>22</th>
<th>24</th>
<th>30</th>
<th>37</th>
<th>44</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line a</td>
<td>10.85</td>
<td>12.31</td>
<td>4.62</td>
<td>2.91</td>
<td>4.05</td>
<td>7.36</td>
</tr>
<tr>
<td>Line b</td>
<td>8.13</td>
<td>10</td>
<td>5.99</td>
<td>3.35</td>
<td>4.1</td>
<td>4.91</td>
</tr>
<tr>
<td>Line c</td>
<td>4.79</td>
<td>6.22</td>
<td>5.44</td>
<td>3.03</td>
<td>3.28</td>
<td>4.29</td>
</tr>
<tr>
<td>Line d</td>
<td>7.91</td>
<td>9.05</td>
<td>6.47</td>
<td>3.59</td>
<td>3.44</td>
<td>5.32</td>
</tr>
</tbody>
</table>
**Figure 2:** Broiler breeder breast muscle fractional breakdown rate by age and line

<table>
<thead>
<tr>
<th>Age/Weeks</th>
<th>22</th>
<th>24</th>
<th>30</th>
<th>37</th>
<th>44</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line a</td>
<td>16.92</td>
<td>20.05</td>
<td>28.96</td>
<td>28.36</td>
<td>17.84</td>
<td>8.14</td>
</tr>
<tr>
<td>Line b</td>
<td>16.21</td>
<td>21.62</td>
<td>28.7</td>
<td>28.71</td>
<td>15.92</td>
<td>4.74</td>
</tr>
<tr>
<td>Line c</td>
<td>15.69</td>
<td>19.64</td>
<td>28.63</td>
<td>29.08</td>
<td>14.45</td>
<td>4.84</td>
</tr>
<tr>
<td>Line d</td>
<td>18.23</td>
<td>20.46</td>
<td>26.05</td>
<td>26.72</td>
<td>17.26</td>
<td>6.72</td>
</tr>
</tbody>
</table>

*age, weeks*
**Figure 3: Broiler breeder leg muscle fractional synthesis rate by age and line**

![Graph showing the fractional synthesis rate by age and line for broiler breeders.]

**Table 3: Broiler breeder leg muscle fractional breakdown rate by age**

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>FBR %/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>18.25(^b)</td>
</tr>
<tr>
<td>30</td>
<td>20.18(^b)</td>
</tr>
<tr>
<td>37</td>
<td>24.34(^a)</td>
</tr>
<tr>
<td>44</td>
<td>11.2(^c)</td>
</tr>
<tr>
<td>50</td>
<td>5.46(^d)</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.
P. Value < 0.0001, SEM: 1.21
Figure 4: Broiler breeder leg muscle fractional breakdown rate by age and line

![Graph showing leg muscle fractional breakdown rate by age and line]

Table 4: Fat mass (%) by line and age

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>Line a</th>
<th>Line b</th>
<th>Line c</th>
<th>Line d</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>16.6</td>
<td>13.72</td>
<td>18.22</td>
<td>16.28</td>
</tr>
<tr>
<td>24</td>
<td>16.65</td>
<td>14.94</td>
<td>16.43</td>
<td>12.93</td>
</tr>
<tr>
<td>30</td>
<td>15.68</td>
<td>18.91</td>
<td>27.41</td>
<td>11.63</td>
</tr>
<tr>
<td>37</td>
<td>17.95</td>
<td>13.54</td>
<td>27.92</td>
<td>16.31</td>
</tr>
<tr>
<td>44</td>
<td>18.99</td>
<td>8.98</td>
<td>14.53</td>
<td>15.36</td>
</tr>
<tr>
<td>50</td>
<td>20.21</td>
<td>12.95</td>
<td>20.87</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different. P. value < 0.001, SEM: 1.58
Figure 5: Fat mass (%) by line and age
Table 5: Lean mass (%) by line and age

<table>
<thead>
<tr>
<th>age, weeks</th>
<th>Line a</th>
<th>Line b</th>
<th>Line c</th>
<th>Line d</th>
</tr>
</thead>
<tbody>
<tr>
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<td>24</td>
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<td>$81.1_{abc}$</td>
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<td>30</td>
<td>$80.08_{abc}$</td>
<td>$75.37_{bc}$</td>
<td>$62.62^d$</td>
<td>$85.67^a_{ab}$</td>
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<tr>
<td>37</td>
<td>$76.78_{bc}$</td>
<td>$83.04_{abc}$</td>
<td>$61.88^d$</td>
<td>$79.16_{abc}$</td>
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<tr>
<td>44</td>
<td>$75.19_{bc}$</td>
<td>$89.5^a$</td>
<td>$81.69_{abc}$</td>
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<td>50</td>
<td>$73.31_{cd}$</td>
<td>$83.96_{abc}$</td>
<td>$72.33_{cd}$</td>
<td>$78.64_{abc}$</td>
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</tbody>
</table>

Concentrations not connected by same letter are significantly different. P. value < 0.001, SEM: 2.29
Figure 6: Lean mass (%) by line and age
Figure 7: Pooled FSR, FBR, egg production, lean mass
REFERENCES


Hitamoto, K., T. Muramatsu, and J. Okumura. (1990). Protein synthesis in tissues and in the


Scherwin, R.S. (1978) J. Clin. Invest. 61, 1471-1481


Waterlow, J.C., P.J. Garlick, and D.J. Millward (1978) Protein turnover in Mammalian Tissues and in the Whole body. Elsevier North-Holland, Amsterdam.

III. The effect of four different feeding programs from rearing period to sexual maturity on protein turnover in broiler breeder parent stock
ABSTRACT A study was conducted to evaluate the effect of four different feeding programs on muscular protein turnover in pullet parent stock (PS) broiler breeders. The four feeding programs based on body weight curves utilized for the study were as follows: Everyday feeding, skip a day feeding (Cobb Standard body weigh curve), under feeding (body weigh curve 20% under) and over feeding (body weigh curve 20% over). Each pullet feeding program (Treatment) consisted of 150 day old pullets and were provided the different feeding programs from 4 wk. to 20 wk. of age. Protein turnover was determined in PS pullets/breeders at 6, 10, 12, 16, 21, 25, 31, 37, 46, and 66 weeks of age. A complete randomized design was used with 4 treatments and 10 time periods (four feeding programs, 10 ages), with five replications, and each pullet represented a replicate. Analysis of variance was performed using JMP software. Five pullets/breeders at each age were given an intravenous flooding-dose of 15N-Phe (150 mM, 40% APE) with 10 ml/kg. After 10 min, birds were slaughtered and the breast muscle (pectoralis major) excised and frozen in liquid nitrogen for protein turnover and gene expression analysis. Excreta was also collected and frozen. The acid-soluble fraction (2% perchloric acid) containing free amino acids from muscle was separated from the protein precipitate. The ratio of 15N:14N of each fraction was determined via GCMS. Quantification of 3-methylhistidine in both muscle and excreta was also determined via GCMS. Gene expression was measured using real-time quantitative PCR. All birds were scanned for body composition before sampling. There was only an age effect regarding fractional synthesis rate (FSR). The FSR significantly increased from 6 week pullets to 10 and 12 week pullets (3.62 %, 10.65 % and 10.93 %, respectively; P. value = 0.01) and then decreased at 16, 21, 25 and 31 weeks of age (6.81 %, 8.67%, 4.69%, 5.77%, respectively), then increased significantly at week 46 and 66 (6.25%/day and 11.76%/day, respectively; P. value = 0.002). There was no significant difference in FSRs between dietary treatments. There was only
an effect regarding fractional breakdown rate (FBR). However FBR was significantly higher for the pullets underfed when contrasted to the other treatments at 10 and 12 weeks of age (P. value = 0.03). FBR significantly increased from week 21 (5.70%) to week 25 (13.81 %, first egg) and 31 (22.46%, peak egg production) (P. value < 0.001), then it significantly decreased at week 46 and 66 (5.44%/day and 6.53%/day, respectively; P. value < 0.0001). FSR in all birds decreased from 12 to 16 wk. of age but the decrease in FBR was larger for the same period thus allowing both protein and fat deposition as they prepared for sexual maturity. The expression of the genes related to protein degradation (atrogin-1, murf-1) was significantly higher at peak egg production. There is a large increase in FBR during the transition for the pullet to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in % lean mass body content during this period of time. Broiler breeders may rely on skeletal muscle tissue as a source of nutrients for egg production.

Key words: Fractional breakdown rate, fractional synthesis rate, protein turnover, 15N phenylalanine, GC-MS.
INTRODUCTION

The net result of the protein synthesis and breakdown is the accretion of the body protein. The dynamic nature of protein metabolism has been known for 60 years thanks to the pioneering work of Schoeheimer and others (Schoeheimer et. al., 1939). Using stable isotopes of amino acids, they demonstrated that proteins continually were being broken down and resynthesized. In addition they reported that different organs have different rates of protein synthesis. The dynamic process by which body proteins are continually synthesized and broken down is protein turnover.

Studies on the growth of body protein have been a major area of research. The main reason for this is that, for example, up to 20-25% of the muscle protein can be broken down per day early in the life of humans and farm animals. This rate slows with age to 1-2 % day -1 in adults. Rates of synthesis and breakdown are influenced not only by age, but by plane of nutrition, stress, disease, hormones, exercise, and inactivity.

Since the first indication by Schoenheimer (1939) for the dynamic state of body protein, it is well established that intracellular proteins are subjected to substantial synthesis and degradation processes. As a result, accretion of protein in the whole body of the animals is the reflection of small differences between synthesis and degradation rates (Muramatsu, 1990). Because protein accretion, hence overall growth at early stages or egg, wool and milk production at maturity, is one of the major concerns in livestock husbandry, therefore the interest in the regulation of these processes has increased in the recent years.

Protein turnover in broiler breeders has been studied by Ekmay et al. (2012). The researchers showed that protein degradation rate of breast meat increased at sexual maturity and then declined with additional egg production. Furthermore, Vignale et al. (2014) evaluated protein
turnover in broiler breeders pure lines and were able to conclude that fractional breakdown rate significantly increased as the hens enter sexual maturity and during peak egg production. However this phenomena is still under research, whereas in mammals it has been largely studied. Overton and coworkers (1998) found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition. In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

It is still under research how gene expression of genes related to protein synthesis and degradation (IGF-1, ampk, antrogin-1, MURF1, and Cathepsin B) changes due to changes in protein turnover in broiler breeders. These genes have already been sequenced for chickens (Dupont et al., 1998; Heck et al., 2003; Bigot et al., 2003; Tosca et al., 2006 and Tesseraud et al., 2007). Similar genes has been already studied in mammals. Clowes et al. (2005) using a biopsy technique to collect triceps muscle samples from sows in late gestation and midlactation and
within 3 h of weaning from sows were able to find that muscle RNA concentrations decreased 10-15% between late gestation and midlactation in all treatments. Muscle mRNA concentrations of several key elements of the ATP-ubiquitin proteasome-dependent proteolytic pathway in muscle increased as lactation progressed. The 1.2-kb 14-kDa E2 transcript showed the most marked increase. This transcript more than doubled between late gestation and midlactation in all treatments. Furthermore, in all treatments, 2.6-kb ubiquitin transcript expression increased by 40-60% over lactation. Also, in agreement with this findings, most muscle free amino acids concentrations increased by ~ 30% between late gestation and midlactation. The questions arising here would be: Does muscular protein degradation also increase in broiler breeders as they enter sexual maturity i.e.: production? Is this a common phenomena between birds and mammals? Does this phenomena occur in any animal in production?

The objective of the present study were: 1) To determine the effect of four different feeding programs: skip a day, normal feeding, under feeding and over feeding on protein turnover in parent stock breeders as pullets and during and after sexual maturity. 2) To determine the effect of sexual maturity on gene expression of genes related to protein synthesis and degradation in parent stock breeders.
MATERIALS AND METHODS

Stock and Management

A flock of 600 Cobb 500 breeder pullets were reared to four feeding programs (150 birds/program) based on body weight curves as follows: Everyday feeding, skip a day feeding (Cobb Standard body weigh curve), under feeding (body weigh curve 20% under) and over feeding (body weigh curve 20% over). The Cobb Breeder Management Guide (Cobb-Vantress, 2005) was used as a reference for all management conditions. The flock was placed in 2.38 m X 1.83 m floor pens and fed ad libitum for the first 2 wk. From 2 to 4 wk., all birds were fed restricted amounts of feed every day. From 4 wk. onward, all birds were fed on a skip-a-day regimen but the ones following the everyday feeding program. Feed allocation was based on breeder recommended guidelines to reach target BW. Birds were weighed weekly by pen and feed allocation adjusted to ensure target BW was met. At 21 wk., 520 birds (130 from each growth curve) were transferred to a production house and individually caged. Cages (47 cm high, 30.5 cm wide, and 47 cm deep) were each equipped with an individual feeder and nipple drinker. All breeders were put on an everyday feeding system. The energy concentration utilized was 450 kcal, and the protein intake was 22 g/d at peak intake. Experimental diets are shown in tables 1, 2, and 3.

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee (protocol # 13002).
**Experimental Procedure**

*Infusion procedure*

A total of 5 birds (4 infused, one control) per treatment were infused with 15N PHEN at the following ages: 6, 10, 12, 16, 21, 25 (first egg), 31 (peak), 37, 46, 66 weeks of age with a dose of 10 ml/kg BW of a 40% APE 15N phenylalanine solution. Each bird was culled after 10 minutes of infusion. One sample of the breast muscle (*pectoralis major*) was taken, put in a label sample bag, and the bag was saved in liquid N. A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the sample were storage at -80 C, until samples analysis.

*Body composition*

All birds were scanned for body composition using a DEXA equipment as previously described (Salas et al., 2012).

*Sample Processing*

The acid-soluble fraction containing free amino acids was removed by addition of 2% (w/v) perchloric acid. After homogenization, samples were centrifuged at 3,000 x g and the supernatant, containing free amino acids, removed. The protein precipitate was washed three times with 2% perchloric acid before being hydrolyzed in 6N HCl. The supernatant and precipitate, respectively, was then run through an ion-exchange column packed with Dowex 50WX8-200. Phenylalanine and 3-methylhistidine (3-MH) were eluted with 2ml of 4N NH4OH and 1ml of nanopure H2O into a new vial and dried under vacuum. The tert-butyldimethylsilyl
(tBDMS) derivative was formed by addition of 800 µl of C2CH3CN-MTBSTFA (1:1) and incubation at 110 ºC for 120 min. Excreta was processed without the removal of the acid-soluble fraction.

**GC/MS analysis**

Analysis of the protein precipitate of breast and legs samples and free amino acids was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature was 150°C and increased 50°C/min to 200°C, after which temperature was increased 20° C/min to 270° C and was held for 5.5 min. The mass spectrometer was operated under EI and SIM modes. The 394, and 395 m/z fragments, representing the M, and M+1 fragments of phenylalanine, was monitored. Standard solutions (in 0.1M HCl) were prepared from the same phenylalanine stock to validate APE linearity.

3-MH was determined on the same mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature will be 110°C and held for 0.65 min, then temperature was increased 30° C/min to 250°C, and held for 10 min. The mass spectrometer was operated under EI and SIM modes. The 238 m/z fragment of 3-MH was monitored.

Fractional synthesis rate was calculated as:

\[
K_s = \frac{APE_b}{APE_f} \times \frac{1}{t} \times 100
\]

Where APEb, =15N atom percent excess (relative to natural abundance) of phenylalanine in protein
APEf = 15N atom percent excess of free phenylalanine in tissues, assumed as the Precursor pool
t = time [d].

Fractional degradation rate was calculated as:

\[ kd = \frac{3-MH \text{ daily excretion}}{3-MH \text{ muscle pool}} \times 100 \]

**RNA isolation, reverse transcription, and real-time quantitative PCR**

A small sample of breast was taken at the same time the sample for protein turnover analysis was taken. The sample was put in a 1.5 ml tube and frozen in liquid nitrogen. All samples were stored overnight at -80°C until sample analysis.

Total RNA were extracted from muscle tissues by Trizol reagent (Life Technologies, grand Island, NY) according to manufacturer’s recommendations, DNase treated and reverse transcribed (Quanta Biosciences, Gaithersburg, MD). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix. Oligonucleotide primers specific for chicken Atrogin-1, forward, 5’-CCTTCCACCTGCTCACATCTC-3’ and reverse, 5’-CACAGGCAGGTCCACAAA-3’; Murf-1, forward, 5’-TGGAGAAGATTGAGCAAGGCTAT-3’ and reverse, 5’-GCGAGGTGCTCAAGACTGACT-3’; cathepsin B, forward, 5’-GCTACTCGCCTTCTCACAAGGA-3’ and reverse 5’-GCGAGGGACACCGTAGGAT-3’;
IGF-1, forward, 5’-GCTGCCGCCCCAGAA-3’ and reverse, 5’-
ACGAACTGAAGAGCATCAACCA-3’; AMPK alpha 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK beta 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK gamma 3, forward, 5’-
ACGAACTGAAGAGCATCAACCA-3’; AMPK alpha 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK beta 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK gamma 3, forward, 5’-
ACGAACTGAAGAGCATCAACCA-3’; AMPK alpha 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK beta 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK gamma 3, forward, 5’-
ACGAACTGAAGAGCATCAACCA-3’; AMPK alpha 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK beta 2, forward, 5’-
TGTAAGCATGGACGTGTGAAGA-3’ and reverse, 5’-
GCGGAGAATCTGCTGGAA-3’; AMPK gamma 3, forward, 5’-
or negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the 2−ΔΔCt method (Schmittgen and Livak, 2008).

**Experimental Design**

The experimental design for this trial was a CRD with 4 treatments and 10 time periods (four feeding programs, ten ages). Each treatment/time periods had five replications.

The model to be used was the following:

\[ Y_{ijk} = u + A + B + AB + e \]

Where:
u = general media
A = feeding program
B = age of the bird
AB = interaction feeding program x age
e = random error

Analysis off variance was performed using JMP software, and means were separated by Tukey test when p Value < 0.05.

RESULTS

Fractional synthesis and breakdown rate

Broiler breeder breast muscular fractional synthesis and breakdown rate by feeding program and age are shown in Tables 4 and 5 and Figures 1 and 2. No statistical interaction was found for the breast fractional synthesis rates (FSR) between feeding programs and ages. However a significant age effect was found. FSR significantly increased during the rearing period from week 6 to week 12 (3.62%/d and 10.93 %/d, respectively; P. value = 0.002). This rate in all birds decreased from 12 to 16 wk. of age but the decrease in FBR was larger for the same period thus allowing both protein and fat deposition as they prepared for sexual maturity. FSR significantly decreased from week 25 (first egg) to week 31 (peak egg production) (4.67 %/d and 5.77 %/day, respectively; P. value = 0.002), then it increased significantly later on at week 45 and 66 (6.25%/d and 11.76%/day, respectively; P. value = 0.002). There was a significant age effect on fractional breakdown rate (FBR). The FBR in breast skeletal tissue did not show differences during the rearing period. A contrast was conducted between the under fed birds and the ones over fed, and fed every day and skip a day. The birds that were under fed showed significantly higher FBR than the other three treatments at 10 weeks of age (11.81 %/day vs 3.38 %/d, 3.19
of age (first egg) to week 31 (peak egg production) and remained the same at week 37 (13.81 %/day, 22.46 %/day and 24.29 %/day, respectively; P. value < 0.0001), then it decreased significantly at week 46 (5.44 %/day; P. value < 0.0001) and again at week 66 (6.53 %/day; P. value <0.0001).

**Gene Expression**

The relative expression of MURF-1, atrogin-1, IGF-1, cathepsin B, AMPK alpha 2, AMPK beta 2 and AMPK gamma 3 from 16, 31 and 66 old hens breast muscle are shown in figures 3, 4, 5, 6, 7, 8 and 9. MURF-1 relative expression in skeletal muscle was higher but not significant at 31 wks. old hens when compared to the one from 16 week old hens. There was no significantly difference in this gene relative expression between 16 week old hens and 66 week old ones.

Atrogin relative expression in breast muscle was significantly higher at 31 week old hens when compared to the 16 weeks old ones ( P. value < 0.0001). No significantly difference was found for the expression of the same gene between 16 week old hens and 66 week old ones.

No significantly differences were found in the relative expression of cathepsin at any ages in skeletal muscle. IGF-1 relative expression was significantly lower for 31 week old hens when compared to the 16 week old ones ( P. value = 0.0140), however no differences were found in the relative expression of this gene in 66 week old hens when compared to the 16 week old ones. No differences were found at any ages for the AMPK beta 2 relative expression. AMPK alpha 2 was significantly less expressed in 31 and 66 week old hens when compared to the 16 week old ones (P. value < 0.0001). The relative expression of AMPK gamma 3 was significantly higher in 31 week old hens when compared to the 16 week old ones (P. value = 0.027). The relative
expression for the same gene in 66 week old hen skeletal muscle was not different from the 16 week old ones.

**Body composition**

Broiler breeder body composition by feeding program and age are shown in Figures 10 and 11; tables 6, 7 and 8. There was an interaction between age and feeding program on fat mass. Underfed birds showed significantly lower fat mass in comparison to the other feeding programs at 12 and 16 weeks old. (P. value = 0.002). Fat mass tend to decrease between 25 and 31 weeks. There was no interaction between age and feeding program on lean mass. However an age effect and a treatment effect was seen. Lean mass significantly increased from 6 week old pullet to 25 weeks old hen (P. value < 0.001). No significantly changes in lean mass were seen during 25 week (first egg) and 37 week (post peak egg production). The birds that were underfed showed significantly lower lean mass when compared to the other treatments (P. value < 0.001). There was only an age effect regarding total mass. This variable significantly increased with age.

**DISCUSSION**

Results of the present study show that muscular protein fractional synthesis rate (FSR) during the rearing period significantly increased 6 weeks old pullets to 12 week old ones, as they gained weight. Interestingly there is a drop in this variable between week 12 and week 16, which is in agreement with the fact that the lean mass did not change much during that period of time and that there was an increased in fat mass, which might suggest that these pullets were getting building their reserves for the sexual maturity period. The birds that were underfed during the rearing period showed significantly higher muscular fractional degradation rate when contrasted
with the other treatments. This results are in agreement with the study conducted by De Beer et al. (2008) to determine the effects of different feeding regimens on plasma hormone and metabolite concentrations in 16-wk-old broiler breeder pullets. In that study the birds were divided in 2 at 28 d of age and fed either every day or skip-a-day from 28 to 112 d of age. The authors found that IGF-1 (insulin like growth factor 1) concentrations were higher in birds fed every day. In our study, the birds that were underfed, were also fed skip a day, and the fact that they showed more FBR, is in agreement with De Beer et al. (2008) findings, since low concentrations of IGF-1 allows muscular degradation to happen (Sacheck et al., 2004). In addition, birds fed according to the standard growth curve but skip a day, also showed slightly higher FBR, again in agreement with De Beer et al. (2008) results. FSR in broiler breeders significantly decreased as they approached peak egg production and the degradation rate significantly increased during the same period of time. After egg production slowed down, muscular fractional synthesis rate tended to increase whereas the breakdown rate decreased significantly. These results are in agreement with what was reported by Manangi and Coon (2006). These authors reported an increase in fractional degradation rates from 22 to 26 wk. The authors suggested that there is increased mobilization of skeletal muscle for egg formation. The rise in FBR in the present study as hens enter sexual maturity suggests that this may be the case. Furthermore, Vignale et al. (2014a) were able to find a similar trend in muscular FSR and FBR when these variables were evaluated in broiler breeder pure lines. Salas et al. (2013) conducted a trial where a group of pullets were fed a daily 15 mg dose of U-13C-g glucose 10 d prior to sexual maturity continuing through 10 d following first egg. The authors were able to conclude that the U-13C-g glucose is being utilized for yolk fatty acid synthesis in a higher concentration at the beginning of the production period. This indicates that glucose is needed for yolk fatty acid
synthesis. The fact that more FBR is seen in the present study might suggest that birds are degrading protein for gluconeogenesis. The dramatic increased in FBR might be due to the feed restriction these birds are facing. Boonsinchai et al. (2014) conducted a study in broiler breeders where they were testing six different feeding programs. The programs consisted in regular breeder diets with or without feed withdrawal after peak egg production and increasing feed intake at 32, 35 or 40 weeks of age. The authors found that maintaining and increasing feed after peak enhanced persistency of lay. They were able to conclude that either maintaining or increasing feed after peak resulted in higher body weigh which is corresponding to bigger eggs and hatched chicks. Also, feed withdrawal after peak (as commercial practice) does not provide enough energy for breeder hens to produce eggs. These results support our findings that there was more FBR. Since the hens in the present trial were subjected to feed withdrawal and no extra feed was added at any point of the laying period, the data clearly show how much muscular protein the hens were degrading. The results from Boonsinchai et al. (2014) study indirectly imply that the birds facing no feed withdrawal, but feed addition while laying eggs might have not been as restricted as the hens in the present study and that is why the persistency was enhanced. The birds in that study may not have been forced to use their body muscular reserves to produce eggs. Unfortunately, no protein turnover has been measured in hens that are not subjected to feed withdrawal or feed addition; it may be that these hens may not degrade as much as the ones in the present study. The results in the present study are also in agreement with Overton and coworkers (1998) who found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance
decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition, which is in agreement with the present results.

In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

Lactation imposes a unique challenge to mammalian protein metabolism, especially for species such as the pig, which support high growth rate in their 10-12 offspring. This state represents a physiological maximum in protein anabolism, with high rates of net protein export to the mammary gland (Clowes, et al., 2005). Because of the high rate of net protein export from the dam in the form of milk, lactation can represent a physiological maximum in protein mobilization in the dam, as dietary intakes are often inadequate. This statement can be also applied for a broiler breeder hen when is facing peak egg production as they are feed restricted being fed only every 24 hours. Clowes et al. (2005) using a biopsy technique to collect triceps muscle samples in late gestation and midlactation and within 3 h of weaning from sows were able to find that muscle RNA concentrations decreased 10-15% between late gestation and midlactation in all treatments. Muscle mRNA concentrations of several key elements of the ATP-ubiquitin proteasome-dependent proteolytic pathway in muscle increased as lactation
progressed indicating muscular protein degradation was taking place. The 1.2-kb 14-kDa E2 transcript showed the most marked increase. This transcript more than doubled between late gestation and midlactation in all treatments. Furthermore, in all treatments, 2.6-kb ubiquitin transcript expression increased by 40-60% over lactation. Also, in agreement with these findings, most muscle free amino acids concentrations increased by ~ 30% between late gestation and midlactation. This later study is in agreement with our findings regarding relative expression of genes related to protein degradation (murf-1 and atrogin-1) which were high at peak egg production. Li et al. (2011) evaluated expression of atrogin-1 and murf-1 during fasting and refeeding in chick skeletal muscle. The authors found that the expression of atrogin-1 and murf-1 significantly increased after 24 h fasting. These results are in agreement with the ones in the present study since the hens were fed just once a day every 24 h. It is know that birds are able to retain feed in the crop. De Beer and coworkers (2008) measure feed retention in the crop at different intervals and concluded that in every day fed birds, the crop was empty by 12 h. being the amount of feed in the crop significantly reduced after 6 h post feeding. In the present study we found that muscular relative expression of IGF-1 is significantly low in 31 week old breeders when compared to the 16 week old ones. These results are in agreement with Sacheck et al. (2004) who concluded that IGF-1 suppresses protein breakdown and the expression of murf-1 and atrogin-1. The fact that IGF-1 relative expression in our study is low at 31 weeks, suggest that this later component is not suppressing neither atrogin-1 nor murf-1, allowing these genes to be highly expressed in these birds. These results support our FBR findings. Furthermore, Bell et al. (2000) found that the IGF-1 concentrations in cows decreases as lactation starts taking place. The later findings are also in agreement with our IGF-1 gene expression results. It is well known that AMPK activation stimulates myofibrillar protein degradation and expression of ubiquitin
ligases (i.e. murf-1 and atrogin-1) (Nakashima et al., 2007). The gamma-subunits appear to play a role in determining sensitivity to AMP (Winder, 2001). In the present study we found that the relative expression of the AMPK gamma 3 subunit is significantly higher at peak egg production suggesting that AMP/ATP ratio is high and that glucose is needed, thus muscular protein is being degraded for gluconeogenesis which is in agreement with Kazuki et al. (2007) findings and explains how the ubiquitin ligases genes in our study are being up regulated. Chau and Zierath (2006) reported that total AMPK gamma 3 subunit knockout mice impaired fasting induced skeletal muscle metabolic gene expression which supports our current findings. The fact that in the present study no changes were seen in the AMPK beta 2 subunits and that the AMPK alpha subunit is lower expresses at peak egg production and at 66 weeks, may suggest that the AMPK gamma 3 subunit is the one responsible for enhancing the ubiquitin ligases gene expression. Further studies need to be conducted to better understand how AMPK subunits work in the chicken muscle.

In the present study we found that lean mass increased during the rearing period up to around 37 weeks. However between 25 and 37 weeks no much increase was seen. All birds declined in lean deposition from 37 weeks to 45 weeks and increased it until 66 weeks. Fat mass presented a different tendency; all birds gained fat mass until 46 weeks; afterwards all groups decreased fat deposition until the end of the production period at wk. 65. These results are in agreement with Salas et al. (2013) who determined body composition in breeder hens reared on 3 different growth curves and fed different energy concentrations and were able to see the same trend. Figure 12 shows the pooled breast muscular FSR, FBR, lean mass and egg production. This graph shows us that as egg production increases, FSR decreases, FBR increases and lean mass
(grams) does not either increase or decrease, whereas when egg production decreases (week 37), FSR increases, FBR decreases and lean mass increases.

Together our results and the one found in other species such as mammals that are also feed restricted (during pregnancy) suggest that the dramatic increased in muscular fractional degradation rate is a phenomena related more to a production state and a feed restriction issue than as to the specie itself and it might be triggered by the fact that the animals are not getting the nutrients they need to produce either eggs or milk.

There is a large increase in FBR during the transition for the pullet to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in lean mass body content during this period of time. Broiler breeders may rely on skeletal muscle tissue as a source of nutrients for egg production. Further studies need to be conducted in broiler breeders using different feeding programs involving frequency of feeding and evaluating muscular protein degradation to better understand why these birds have such a dramatic degradation rate during production. The answer might be a balance between feed frequency and quantity to optimize nutrient utilization for production without increasing maintenance requirements.
Table 1: Pullet Broiler Breeder Experimental diet: Starter

<table>
<thead>
<tr>
<th>INGRIDIENT, %</th>
<th>Nutrient, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>61.49</td>
</tr>
<tr>
<td>soybean meal</td>
<td>29.27</td>
</tr>
<tr>
<td>Wheat midds</td>
<td>5.91</td>
</tr>
<tr>
<td>Fat</td>
<td>0.25</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.57</td>
</tr>
<tr>
<td>dicalcium phosphate</td>
<td>1.86</td>
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<tr>
<td>Salt</td>
<td>0.26</td>
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<tr>
<td>Lysine</td>
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<tr>
<td>Alimet</td>
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<tr>
<td>Choline</td>
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<tr>
<td>vitamin premix</td>
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<tr>
<td>Threonine</td>
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</tr>
<tr>
<td>mineral premix</td>
<td>0.075</td>
</tr>
<tr>
<td>TOTAL %</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Pullet Broiler Breeder Experimental diet: Grower

<table>
<thead>
<tr>
<th>INGRIDIENT,%</th>
<th>Nutrient,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
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</tr>
<tr>
<td>soybean meal</td>
<td>17.3</td>
</tr>
<tr>
<td>Wheat midds</td>
<td>17.09</td>
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<tr>
<td>Fat</td>
<td>0.5</td>
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<tr>
<td>Limestone</td>
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<tr>
<td>dicalcium phosphate</td>
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<tr>
<td>Salt</td>
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<td>Alimet</td>
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<tr>
<td>Choline</td>
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<tr>
<td>vitamin premix</td>
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</tr>
<tr>
<td>Sodium Carbonate</td>
<td>0.05</td>
</tr>
<tr>
<td>mineral premix</td>
<td>0.075</td>
</tr>
<tr>
<td>TOTAL %</td>
<td>100</td>
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</tbody>
</table>

Analyzed:
Crude Protein 16.1
Crude Fat 3.59
Ash 5.35
Calcium, ppm 9046
Table 3: Broiler breeder hen experimental diet

<table>
<thead>
<tr>
<th>INGRIDIENT, %</th>
<th>Nutrient, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>66.28</td>
</tr>
<tr>
<td>soybean meal</td>
<td>23.11</td>
</tr>
<tr>
<td>Fat</td>
<td>1.31</td>
</tr>
<tr>
<td>Limestone</td>
<td>6.78</td>
</tr>
<tr>
<td>dicalcium phosphate</td>
<td>1.81</td>
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<tr>
<td>Salt</td>
<td>0.17</td>
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<tr>
<td>Alimet</td>
<td>0.19</td>
</tr>
<tr>
<td>Choline</td>
<td>0.1</td>
</tr>
<tr>
<td>vitamin premix</td>
<td>0.07</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>0.1</td>
</tr>
<tr>
<td>mineral premix</td>
<td>0.075</td>
</tr>
<tr>
<td>TOTAL %</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

  Analyzed:                      |
  Crude Protein                 | 15.1            |
  Crude Fat                     | 4.83            |
  Ash                           | 10.27           |
  Calcium, ppm                  | 33664           |
Figure 1: Muscular fractional synthesis rate by age and feeding program

Table 4: Muscular fractional synthesis rate (FSR) by age

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>FSR %/day</th>
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</thead>
<tbody>
<tr>
<td>6</td>
<td>3.62(^b)</td>
</tr>
<tr>
<td>10</td>
<td>10.65(^a)</td>
</tr>
<tr>
<td>12</td>
<td>10.93(^a)</td>
</tr>
<tr>
<td>16</td>
<td>6.81(^a)</td>
</tr>
<tr>
<td>21</td>
<td>8.68(^a)</td>
</tr>
<tr>
<td>25</td>
<td>4.68(^b)</td>
</tr>
<tr>
<td>31</td>
<td>5.78(^b)</td>
</tr>
<tr>
<td>37</td>
<td>9.18(^a)</td>
</tr>
<tr>
<td>46</td>
<td>6.269(^a)</td>
</tr>
<tr>
<td>60</td>
<td>11.76(^a)</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.

P. Value < 0.0001, SEM: 1.55
Figure 2: Muscular fractional degradation rate by age and feeding program

![Graph showing muscular fractional degradation rate by age and feeding program.](image)

Table 5: Muscular fractional breakdown rate (FBR) by age

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>FBR, %/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>4.90&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>5.95&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>12</td>
<td>6.28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>4.22&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>21</td>
<td>5.70&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>25</td>
<td>13.81&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>31</td>
<td>22.46&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>37</td>
<td>24.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>46</td>
<td>5.44&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>6.53&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different. P. Value < 0.0001, SEM: 1.01
Figure 3: MURF-1 relative expression

![Figure 3: MURF-1 relative expression](image)

Figure 4: Atrogin-1 relative expression

![Figure 4: Atrogin-1 relative expression](image)
Figure 5: IGF-1 relative expression

![Bar chart showing IGF-1 relative expression across different weeks (16 W, 31 W, 65 W).](chart)

- 16 W (Control) shows the highest expression.
- 31 W and 65 W show lower expression, with 31 W showing a statistical difference compared to control (indicated by an asterisk).

Figure 6: Cathepsin relative expression

![Bar chart showing Cathepsin relative expression across different weeks (16 W, 31 W, 65 W).](chart)

- 16 W (Control) shows the highest expression.
- 31 W and 65 W show lower expression, with 65 W showing a statistical difference compared to control.
Figure 7: AMPK alpha 2 relative expression

![Bar chart showing AMPK alpha 2 expression levels](image)

- 16 W (Control)
- 31 W
- 65 W

Figure 8: AMPK beta 2 relative expression

![Bar chart showing AMPK beta 2 expression levels](image)

- 16 W (Control)
- 31 W
- 65 W
Figure 9: AMPK gamma 3 relative expression

![AMPK gamma 3 relative expression graph](image)

Figure 10: Lean mass (g) by feeding program and age

![Lean mass by feeding program and age graph](image)
Table 6: Lean mass (g) by age

<table>
<thead>
<tr>
<th>age, weeks</th>
<th>Lean mass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>589.76\textsuperscript{g}</td>
</tr>
<tr>
<td>10</td>
<td>866.03\textsuperscript{f}</td>
</tr>
<tr>
<td>12</td>
<td>1237.66\textsuperscript{e}</td>
</tr>
<tr>
<td>16</td>
<td>1337.03\textsuperscript{e}</td>
</tr>
<tr>
<td>21</td>
<td>1977.38\textsuperscript{d}</td>
</tr>
<tr>
<td>25</td>
<td>2550.53\textsuperscript{bc}</td>
</tr>
<tr>
<td>31</td>
<td>2664.17\textsuperscript{ab}</td>
</tr>
<tr>
<td>37</td>
<td>2743.12\textsuperscript{a}</td>
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<td>46</td>
<td>2452.31\textsuperscript{c}</td>
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<tr>
<td>66</td>
<td>2671.41\textsuperscript{ab}</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different. P. Value < 0.001, SEM: 60.22
### Table 7: Lean mass (g) by feeding program

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lean Mass, g</th>
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<tbody>
<tr>
<td>Normal ED</td>
<td>2014 b</td>
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<tr>
<td>Normal SD</td>
<td>1876.22 b</td>
</tr>
<tr>
<td>Over feeding</td>
<td>1992.69 b</td>
</tr>
<tr>
<td>Under feeding</td>
<td>1752.86 a</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different. P. Value < 0.001, SEM: 38.08

### Table 8: Fat mass (g) by feeding program and by age

<table>
<thead>
<tr>
<th>age, wks</th>
<th>Normal ED</th>
<th>Normal SD</th>
<th>Under</th>
<th>Over</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>74.92 j</td>
<td>75.39 j</td>
<td>73.22 j</td>
<td>81.51 j</td>
</tr>
<tr>
<td>10</td>
<td>109.58 ij</td>
<td>122.15 ij</td>
<td>98.64 j</td>
<td>124.94 i</td>
</tr>
<tr>
<td>12</td>
<td>132.61 j</td>
<td>325.1 fghi</td>
<td>43.24 j</td>
<td>128.42 i</td>
</tr>
<tr>
<td>16</td>
<td>313.69 ghi</td>
<td>268.77 ghi</td>
<td>88.62 j</td>
<td>524.8 efghi</td>
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<tr>
<td>21</td>
<td>411.3 efg h</td>
<td>296.84 ghi</td>
<td>181.65 hi</td>
<td>411.96 efg h</td>
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<tr>
<td>25</td>
<td>620.75 efg</td>
<td>574.75 efg</td>
<td>527.04 efg h</td>
<td>705.73 def</td>
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<tr>
<td>31</td>
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<td>607.15 efg</td>
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<tr>
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<td>593.1 efg</td>
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<tr>
<td>46</td>
<td>1115.97 ab</td>
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<td>66</td>
<td>1021.9 abcd</td>
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<td>1109.33 ab</td>
</tr>
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</table>

Concentrations not connected by same letter are significantly different. P. Value < 0.002, SEM: 62.22
Figure 11: Fat mass (g) by feeding program and by age
Figure 12: Pooled lean mass, FSR, FBR and egg production
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IV. The effect of sexual maturity on protein turnover in laying hens
ABSTRACT Protein turnover in skeletal tissue for broiler breeders has been shown to increase at sexual maturity and then decline with increased egg production (Ekmay et al., 2012). The authors believe the increased protein degradation rate at sexual maturity is to provide amino acids for egg production. The protein turnover of skeletal tissue for commercial layers at sexual maturity and during the production period has not been determined. Two studies were conducted in order to evaluate the effect of sexual maturity on protein turnover in laying hens. Protein turnover was determined in commercial layers (Bovans Brown) at 16, 21, 31, and 60 weeks of age (Experiment 1) and at 16, 25, 31, 43, and 60 weeks of age (Experiment 2). Ten laying hens at each age were given an intravenous flooding-dose of 15N-Phe (125 mM, 25% APE (exp 1) and 150 mM, 40 APE (Exp. 2)) at 10 ml/kg. After 10 min, birds were slaughtered and the breast muscle (pectoralis major) excised and frozen in liquid nitrogen. Excreta was also collected and frozen. The acid-soluble fraction (2% perchloric acid) containing free amino acids from muscle was separated from the protein precipitate. The ratio of 15N:14N of each fraction was determined via GCMS. Quantification of 3-methylhistidine in both muscle and excreta was also determined via GCMS. Fractional synthesis rate (FSR) in experiment 1 significantly increased from 16 week pullets to 21 week layers (first egg) (4.46 %/day and 7.11 %/day, respectively; P. value = 0.005), then it significantly decreased at week 31 (post peak egg production) and increased again at week 60 (2.61 %/day and 6.5 %/day, respectively; P. value = 0.005). There was a slight increase in fractional breakdown rate (FBR) from 16 week pullets to 21 week layers (first egg) (11.66 %/day and 12.15 %/day, respectively). FBR decreased, but not significantly, from 21 week (first egg) layers to 31 week (post peak) ones (12.15 %/day and 8.51 %/day, respectively), then it significantly decreased to week 60 (5.36 %/day, P. value = 0.002). In experiment 2, FSR significantly increased for 25 week layers (peak egg production) compared to
16 week pullets (7.81 %/day and 2.95 %/day, respectively; P value < 0.0001). There was no significant change for this variable between 25 week (peak egg production) layers and 31 week (post peak egg production) ones (7.81 %/day and 6.35 %/day). Fractional synthesis rate significantly increased from 31 to 43 and 63 week old layers (6.35 %/day, 10.30 %/day and 13.52 %/day, respectively; P.value < 0.0001). FBR significantly increased for 25 week (peak egg production) layers compared to 16 week pullets (4.50 %/day and 15.32 %/day, respectively; P. value < 0.0001), then it significantly decreased at 31, 43 and 63 week (7.03 %/day, 6.29 %/day and 6.11 %/day, respectively). Further studies are needed to determine regulatory control of the fractional synthesis rate and fractional degradation rate for commercial layers to better understand how they are using the tissue amino acids. Laying hens may rely on skeletal muscle tissue as a source of protein for egg production as they reach peak egg production.

Key words: Fractional breakdown rate, fractional synthesis rate, protein turnover, 15N phenylalanine, GC-MS.
INTRODUCTION

The net result of the protein synthesis and breakdown is the accretion of the body protein. The dynamic nature of protein metabolism has been known for 60 years thanks to the pioneering work of Schoeheimer and others (Schoeheimer et. al., 1939). Using stable isotopes of amino acids, they demonstrated that proteins continually were being broken down and resynthesized. In addition they reported that different organs have different rates of protein synthesis. The dynamic process by which body proteins are continually synthesized and broken down is protein turnover.

Studies on the growth of body protein have been a major area of research. The main reason for this is that, for example, up to 20-25% of the muscle protein can be broken down per day early in the life of humans and farm animals. This rate slows with age to 1-2 % day -1 in adults. Rates of synthesis and breakdown are influenced not only by age, but by plane of nutrition, stress, disease, hormones, exercise, and inactivity.

Since the first indication by Schoenheimer (1939) for the dynamic state of body protein, it is well established that intracellular proteins are subjected to substantial synthesis and degradation processes. As a result, accretion of protein in the whole body of the animals is the reflection of small differences between synthesis and degradation rates (Muramatsu, 1990). Because protein accretion, hence overall growth at early stages or egg, wool and milk production at maturity, is one of the major concerns in livestock husbandry, therefore the interest in the regulation of these processes has increased in the recent years.

Protein turnover in broiler breeders has been studied by Ekmay et al. (2012). The researchers showed that protein degradation rate of breast meat increased at sexual maturity and then declined with additional egg production. Furthermore, Vignale et al. (2014a, b) evaluated protein
turnover in broiler breeder pure lines and parent stock. The authors were able to conclude that fractional breakdown rate significantly increased as the hens enter sexual maturity and during peak egg production. However this phenomena is still under research, whereas in mammals it has been largely studied. Overton and coerces (1998) found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition. In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

The questions arising here would be: Does muscular protein degradation also increase in broiler breeders as they enter sexual maturity i.e.: production? Is this a common phenomenon between birds and mammals? Does this phenomena occur in any animal in production?

The protein turnover of skeletal tissue for commercial layers at sexual maturity and during the production period has not been determined. The objective of the present study was to evaluate the effect of sexual maturity on protein turnover in laying hens.
MATERIALS AND METHODS

Stock and Management

Experiment 1 and 2

A total of 40 laying hens for experiment 1 and 50 for experiment 2 (Bovans Brown) from the company Benton County foods were used for this trial. Birds were raised at the company’s facilities under the bovans brown management guide (Bovans Brown Management Guide, 2012). The proximal analysis of the diet that was provides to the hens is shown in table 1.

Experimental Procedure

Infusion procedure: Experiment 1

A total of 10 birds (9 infused, one control) per treatment were infused with a 25% APE (atom percent excess) 15N PHEN solution at the following ages: 16, 21, 31 and 60 weeks of age with a dose of 10 ml/kg BW of 15N phe. Each bird was culled after 10 minutes of infusion. One sample of the breast muscle (pectoralis major) was taken, put in a label sample bag, and the bag will be save in liquid N. A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the samples were stored at -20 C, until samples analysis.
Infusion procedure: Experiment 2

A total of 10 birds (9 infused, one control) per treatment were infused with a 40% APE (atom percent excess) 15N PHEN solution at the following ages: 16, 25, 31, 43, and 63 weeks of age with a dose of 10 ml/kg BW of 15N phe. Each bird was culled after 10 minutes of infusion. One sample of the breast muscle (*pectoralis major*) was taken, put in a label sample bag, and the bag will be save in liquid N. A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the samples were stored at -20 C, until samples analysis.

Sample Processing: Experiment 1 and 2

The acid-soluble fraction containing free amino acids was removed by addition of 2% (w/v) perchloric acid. After homogenization, samples were centrifuged at 3,000 x g and the supernatant, containing free amino acids, removed. The protein precipitate was washed three times with 2% perchloric acid before being hydrolyzed in 6N HCl. The supernatant and precipitate, respectively, were then run through an ion-exchange column packed with Dowex 50WX8-200. Phenylalanine and 3-methylhistidine (3-MH) were eluted with 2ml of 4N NH₄OH and 1ml of nanopure H₂O into a new vial and dried under vacuum. The tert-butyldimethylsilyl (tBDMS) derivative was formed by addition of 800 µl of C₂CH₃CN-MTBSTFA (1:1) and incubation at 110° C for 60 min. Excreta was processed without the removal of the acid-soluble fraction.

GC/MS analysis: Experiment 1 and 2

Analysis of the protein precipitate of breast samples and free amino acids was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer. Helium was used as
the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature was 150°C and increased 50°C/min to 200°C, after which temperature was increased 20° C/min to 270° C and was held for 5.5 min. The mass spectrometer was operated under EI and SIM modes. The 394, and 395 m/z fragments, representing the M, and M+1 fragments of phenylalanine, were monitored. Standard solutions (in 0.1M HCl) were prepared from the same phenylalanine stock to validate APE linearity.

3-MH was determined on the same mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature was 110°C and held for 0.65 min, then temperature was increased 30° C/min to 250°C, and held for 10 min. The mass spectrometer was operated under EI and SIM modes. The 238 m/z fragment of 3-MH was monitored.

Fractional synthesis rate was calculated as:

\[ KS = \frac{\text{APE}_b}{\text{APE}_f} \times \frac{1}{t} \times 100 \]

Where \( \text{APE}_b \) = \(^{15}\)N atom percent excess (relative to natural abundance) of phenylalanine in protein

\( \text{APE}_f \) = \(^{15}\)N atom percent excess of free phenylalanine in tissues, assumed as the precursor pool

t = time [d].

Fractional degradation rate was calculated as:

\[ KD = \frac{3-\text{MH daily excretion}}{3-\text{MH muscle pool}} \times 100 \]
Body composition: Experiment 2

Five birds per treatment were analyzed for body composition by chemical analysis according to Salas et al. (2012). Prior to chemical analysis, the carcasses were thawed and transferred to individual aluminum tubs, weighed and a small amount of water was added. The tubs were covered with aluminum foil and autoclaved at 110°C with 1 atmosphere of pressure from 1 to 5 hours depending on the size of the bird (adapted from Sibbald and Fortin, 1982). After autoclaving, the tubs containing the autoclaved hens were cooled to room temperature and reweighed. Any weight loss during this process was assumed to be water. The autoclaved carcasses (including feathers) were transferred into a heavy duty blender (Waring Laboratory, Blender LBC15, Model CB15) for homogenization. Once homogenized, a sample of approximately 150 g was obtained and freeze dried. After freeze drying, samples were reweighed and ground for further analysis. The total ash, ether extract, and crude protein of the carcasses were determined according to AOAC approved methods (AOAC, 1990, 1995). As suggested by Barker and Sell (1994), the carcass dry matter was calculated from the weight of the hen and recorded amounts of additions and losses of water during autoclaving and after freeze drying. The quantitative amount of total ash, ether extract and crude protein of the hens carcasses were converted into as is body composition (with water) data.

EXPERIMENTAL DESIGN: EXPERIMENT 1 AND 2

The experimental design for this trial was a CRD with 4 treatments (experiment 1) and 5 treatments (experiment 2), being each treatment represented by the age of the hen. Each treatment had ten replications, where each hen per treatment represented one replicate.
The model used was the following:

\[ Y_{ijk} = u + A + e \]

Where:

- \( u \) = general media
- \( A \) = bird age
- \( e \) = random error

Analysis of variance was performed using JMP software, and means were separated by Tukey test when \( p \) Value < 0.05.

RESULTS

Experiment 1

**Muscular Fractional synthesis and breakdown rate**

Laying hens breast muscular fractional synthesis and breakdown rate by age are shown in Table 2 and Figure 1. Fractional synthesis rate (FSR) significantly increased from 16 week pullets to 21 week layers (first egg) (4.46 %/day and 7.11 %/day, respectively; \( P \) value = 0.005), then it significantly decreased at week 31 (post peak egg production) and increased again at week 60 (2.61 %/day and 6.5 %/day, respectively; \( P \) value = 0.005). There was a slight increase in fractional breakdown rate (FBR) from 16 week pullets to 21 week layers (first egg) (11.66 %/day and 12.15 %/day, respectively). FBR decreased, but not significantly, from 21 week (first egg)
layers to 31 week (post peak) ones (12.15 %/day and 8.51 %/day, respectively), then it significantly decreased to week 60 (5.36 %/day, P. value = 0.002).

**Experiment 2**

**Muscular Fractional synthesis and breakdown rate**

Laying hens breast muscular fractional synthesis and breakdown rate by age are shown in Table 3 and Figure 2. FSR significantly increased for 25 week layers (peak egg production) compared to 16 week pullets (7.81 %/day and 2.95 %/day, respectively; P value < 0.0001). There was no significant change for this variable between 25 week (peak egg production) layers and 31 week (post peak egg production) ones (7.81 %/day and 6.35 %/day). Fractional synthesis rate significantly increased from 31 to 43 and 63 week old layers (6.35 %/day, 10.30 %/day and 13.52 %/day, respectively; P.value < 0.0001). FBR significantly increased for 25 week (peak egg production) layers compared to 16 week pullets (4.50 %/day and 15.32 %/day, respectively; P. value < 0.0001), then it significantly decreased at 31, 43 and 63 week (7.03 %/day, 6.29 %/day and 6.11 %/day, respectively).

**Body composition: Experiment 2**

The body composition of the birds is shown in table 4, and figure 3. No significantly differences were found in fat mass, however it tended to increase through 64 week layers. Protein mass significantly increased for 25 weeks old layers when compared to 31 week old ones (315.48 g and 379.82 g, respectively. P. value = 0.02), then it decreased at week 43 (286.41 g) to then increased through week 63 (316.63 g, not significant).
DISCUSSION

Results of the present study show that muscular protein fractional synthesis rate (FSR) in laying hens in experiment 1 significantly decreased as they approached peak egg production and the degradation rate slightly increased as they start laying eggs (21 wks., first egg). After egg production slowed down, muscular fractional synthesis rate increased significantly towards 60 week of age whereas the breakdown rate decreased significantly for the same period of time. The response for laying hens in experiment 2 was similar to experiment 1 with slight variations. FSR in these hens also increased significantly from week 16 to week 25, no significant changes were seen for this variable between 25 and 31 week old hens. Suggesting that no muscular accretion was taking place. However, FBR significantly increased as the hens approach peak egg production. In experiment 1 just a slight increase in FBR was seen, but this variable was measured at first egg, whereas in experiment 2 we were able to measure FBR at peak egg production. This may be the reason why we are seeing significant changes in this variable in comparison to experiment 1. As hens passed peak egg production and more feed was available FSR significantly increased to week 43 and 60, which follows a similar trend tan experiment 2. These results are in agreement with what was reported by Manangi and Coon (2006). These authors reported an increase in fractional degradation rates from 22 to 26 wk. The authors suggested that there is increased mobilization of skeletal muscle for egg formation. The rise in FBR in the present study as hens enter sexual maturity suggests that this may be the case. Furthermore, Vignale et al. (2014a, b) were able to find a similar trend in muscular FSR and FBR when these variables were evaluated in broiler breeder pure lines and in broiler breeder’s parent stock. However in the two studies conducted by Vignale et al. (2014a,b) the authors were able to see that the birds hold their FBR for almost all the peak egg production (31 wk. to 37
wk.), which is not in agreement with the present findings. FBR for laying hens significantly decreased after they hit peak egg production (25 weeks). The reason why we are seeing this difference response, might be due to an increase in feed as they approach peak egg production, whereas in broiler breeders feed withdrawal takes place during this period of time. Salas et al. (2013) conducted a trial where a group of pullets were fed a daily 15 mg dose of U-13C-glucose 10 d prior to sexual maturity continuing through 10 d following first egg. The authors were able to conclude that the U-13C-glucose is being utilized for yolk fatty acid synthesis in a higher concentration at the beginning of the production period. This indicates that glucose is needed for yolk fatty acid synthesis. The fact that more FBR is seen in the present study as birds approach peak egg production might suggest that birds are degrading protein for gluconeogenesis. The results in the present study are also in agreement with Overton and coworkers (1998) who found in a study conducted in lactating cows that the ratio of 3-methyl histidine to creatine in urine increased markedly shortly postpartum and then declined until d + 21, indicating substantial degradation of skeletal muscle protein during the early postpartum period. Bell et al. (2000) found in lactation cows that the calculated protein balance decrease to a nadir of about -600 g/d at 7 d postpartum, then steadily increased for the next 3 weeks, with zero balance apparently achieved at about 23 d postpartum. Furthermore, Blum et al. (1985) found in lactation cows that plasma 3-methyl histidine increased rapidly from relatively high before parturition to maximal concentrations at 1 wk. after calving, then drastically decreased to a relative low attained 5 wk. after parturition, which is in agreement with the present results. In a study conducted by Baracos et al. (1991) in lactating and dry goats where they measured muscular protein synthesis in both group of animals using the phenylalanine approach, they found that absolutes rates of synthesis of skin protein was 48% lower, of muscle protein 34% lower and bone marrow 29% lower (not
significant) in the lactating group which is in agreement with our muscular fractional synthesis rate results.

Lactation imposes a unique challenge to mammalian protein metabolism, especially for species such as the pig, which support high growth rate in their 10-12 offspring. This state represents a physiological maximum in protein anabolism, with high rates of net protein export to the mammary gland (Clowes, et al., 2005). Because of the high rate of net protein export from the dam in the form of milk, lactation can represent a physiological maximum in protein mobilization in the dam, as dietary intakes are often inadequate. Clowes et al. (2005) using a biopsy technique to collect triceps muscle samples in late gestation and midlactation and within 3 h of weaning from sows were able to find that muscle RNA concentrations decreased 10-15% between late gestation and midlactation in all treatments. Muscle mRNA concentrations of several key elements of the ATP-ubiquitin proteasome-dependent proteolytic pathway in muscle increased as lactation progressed indicating muscular protein degradation was taking place. The 1.2-kb 14-kDa E2 transcript showed the most marked increase. This transcript more than doubled between late gestation and midlactation in all treatments. Furthermore, in all treatments, 2.6-kb ubiquitin transcript expression increased by 40-60% over lactation. Also, in agreement with these findings, most muscle free amino acids concentrations increased by ~ 30% between late gestation and midlactation. The later findings are also in agreement with our current results.

In the present study we found that lean mass decreased from 16 week old pullets through peak egg production (25 weeks old layers). All birds declined in lean deposition from 31 weeks to 43 weeks and increased it until 63 weeks. Fat mass presented a different tendency; all birds gained fat mass until 31 weeks; afterwards all groups decreased fat deposition at week 43 and then increased until the end of the production period at wk. 63 (Figures 3). These results are in
agreement with Salas et al. (2010) who determined body composition in breeder hens reared on 3 different growth curves and fed different energy concentrations and were able to see the same trend. However, they saw a decline in fat mass through the end of production, which is not in agreement with our current results. The difference might be because here we are dealing with laying hens, which might behave slightly different than broiler breeder hens. Figure 4 shows the breast muscular FSR, FBR, lean mass, egg production and feed intake. This graph shows us that as egg production increases, FSR does not increase too much, FBR increases and lean mass (grams) decreases slightly but not significantly. Feed intake increases as peak egg production is reached and FBR decreases. Whereas when egg production decreases, FSR increases, FBR decreases and lean mass increases.

Together our results and the one found in other species such as mammals that are also feed restricted (during pregnancy) suggest that the dramatic increased in muscular fractional degradation rate is a phenomena related more to a production state and a feed restriction issue than as to the specie itself and it might be triggered by the fact that the animals are not getting the nutrients they need to produce either eggs or milk.

There is a large increase in FBR during the transition for the pullet to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in lean mass body content during this period of time. Laying hens may rely on skeletal muscle tissue as a source of nutrients as they reach egg production. These hens showed a similar response as broiler breeders regarding FBR, however a less dramatic change in FBR was seen, maybe due to the fact that after peak egg production is reached, more feed is available.
Table 1: Laying hen commercial diet chemical analysis

<table>
<thead>
<tr>
<th>Nutrient, %</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>18.3</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>3.86</td>
</tr>
<tr>
<td>Ash</td>
<td>15.8</td>
</tr>
</tbody>
</table>

Table 2: Fractional synthesis and degradation rate by age: Experiment 1

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>FSR%/day</th>
<th>FBR%/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>4.46 b c</td>
<td>11.66 a</td>
</tr>
<tr>
<td>21</td>
<td>7.11 a</td>
<td>12.15 a</td>
</tr>
<tr>
<td>31</td>
<td>2.61 c</td>
<td>8.51 a b</td>
</tr>
<tr>
<td>60</td>
<td>6.5 a b</td>
<td>5.36 b</td>
</tr>
<tr>
<td>P Value</td>
<td>0.0055</td>
<td>0.0202</td>
</tr>
<tr>
<td>SEM</td>
<td>0.9</td>
<td>1.61</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.
Figure 1: Fractional synthesis and degradation rate by age: Experiment 1

Table 3: Fractional synthesis and degradation rate by age: Experiment 2

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>FSR%/day</th>
<th>FBR%/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>2.95d</td>
<td>4.5b</td>
</tr>
<tr>
<td>25</td>
<td>7.81bc</td>
<td>15.32a</td>
</tr>
<tr>
<td>31</td>
<td>6.35c</td>
<td>7.03b</td>
</tr>
<tr>
<td>43</td>
<td>10.31b</td>
<td>6.29b</td>
</tr>
<tr>
<td>60</td>
<td>13.53a</td>
<td>6.11b</td>
</tr>
<tr>
<td>P Value</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SEM</td>
<td>0.66</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.
Figure 2: Fractional synthesis and degradation rate by age: Experiment 2

![Graph showing fractional synthesis and degradation rate by age for Experiment 2.](image)

Table 4: Laying hens body composition by age: Protein and Fat content

<table>
<thead>
<tr>
<th>Age</th>
<th>Lean mass, g</th>
<th>Fat mass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>347.43&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>131.945</td>
</tr>
<tr>
<td>25</td>
<td>315.48&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>160.723</td>
</tr>
<tr>
<td>31</td>
<td>379.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>174.068</td>
</tr>
<tr>
<td>43</td>
<td>286.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108.978</td>
</tr>
<tr>
<td>63</td>
<td>316.63&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>170.925</td>
</tr>
</tbody>
</table>

p. Value 0.02 0.3
SEM 17.6 24.1
Figure 3: Laying hens body composition by age: Protein and Fat content

![Graph showing protein and fat content by age](image)

Figure 4: Laying hen FSR, FBR, egg production, feed intake and body protein content

![Graph showing FSR, FBR, egg production, feed intake, and body protein content](image)
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V. Tracking What Goes From Muscle To The Egg: 13c Leucine Approach
ABSTRACT A study was conducted to evaluate the amino acid mobilization from the breast muscle to the egg in broiler breeders. At week 34, twelve birds were dosed with 20 mg of 13C Leucine for a period of 14 days. After this period of time, a two day rest period was ensued. First egg laid after this rest period was saved, but not analyzed. When the second egg was laid, a group of 4 hens plus 1 control (4 dosed with 13C leu, and 1 control) were selected and slaughtered after this and the left pectoralis muscle was excised and snap frozen in liquid nitrogen. Eggs were broken out and the yolk separated from the albumen. Muscle and egg contents were stored at -20 C. After the third egg (3rd egg) was laid, another 4 hens and eggs were sampled as described. Finally, after the fourth egg (4th egg) was laid, the remaining 4 hens and eggs were sampled as described. All samples were analyzed using GCMS. A consistent decrease in the 13C leucine in breast muscle from the 2nd to the 3rd egg was observed, while also seeing an increase in the 13C leucine in the egg from the 3rd to the 4th egg. These findings suggest that broiler breeders are utilizing their skeletal muscle reserves for egg formation.

Key words: 13C leucine, GCMS, amino acid, egg, broiler breeder hen
INTRODUCTION

Since the first indication by Schoenheimer (1939) for the dynamic state of body protein, it is well established that intracellular proteins are subjected to substantial synthesis and degradation processes. As a result, accretion of protein in the whole body of the animals is the reflection of small differences between synthesis and degradation rates (Muramatsu, 1990). Because protein accretion, hence overall growth at early stages or egg, wool and milk production at maturity, is one of the major concerns in livestock husbandry, therefore the interest in the regulation of these processes has increased in the recent years.

Protein turnover in broiler breeders has been studied by Ekmay et al. (2012). The researchers showed that protein degradation rate of breast meat increased at sexual maturity and then declined with additional egg production. Furthermore, Vignale et al. (2014a, b) evaluated protein turnover in broiler breeders purelines and parentstock. The authors were able to conclude that fractional breakdown rate significantly increased as the hens enter sexual maturity and during peak egg production. Ekmay et al. (2014) looked at lysine partitioning using 15N lysine in broiler breeders. The authors were able to conclude that endogenous muscle was the main source of lysine for yolk and albumen formation at week 25 and week 45. Furthermore, the later authors were also able to observe a consistent decrease in the 15N lysine in breast muscle from second to third egg, while also seeing an increase in 15N lysine in the egg from third to fourth egg. Hiramoto et al. (1990) looked into changes in protein synthesis in the liver and oviduct as an ovum passes through the magnum in laying hens. The group found no changes in liver protein synthesis but noted differences in protein synthesis in the oviduct when the ovum was in the magnum.
The question arising here would be: Are broiler breeders relaying on their muscular reserves for egg formation?

The objective of the present study was to determine the amino acid movement from the muscle to the egg.

MATERIALS AND METHODS

Stock and Management

A flock of 50 Cobb 500 breeder pullets were reared to an everyday feeding program (Cobb Standard body weigh curve). The Cobb Breeder Management Guide (Cobb-Vantress, 2005) was used as a reference for all management conditions. The flock was placed in 2.38 m X 1.83 m floor pens and fed ad libitum for the first 2 wk. From 2 wk onward, all birds were fed the everyday feeding program. Feed allocation was based on breeder recommended guidelines to reach target BW. Birds were weighed weekly by pen and feed allocation adjusted to ensure target BW was met. At 21 wk, all birds were transferred to a production house and individually caged. Cages (47 cm high, 30.5 cm wide, 47 cm deep) were each equipped with an individual feeder and nipple drinker. All breeders were put on an everyday feeding system. The energy concentration utilized was 450 kcal, and the protein intake was 22 g/d at peak intake. Experimental diet is shown in table 1.

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee (protocol # 13002).
EXPERIMENTAL PROCEDURE

At week 34, twelve birds were dosed with 20 mg of 13C Leucine for a period of 14 days. After this period of time, a two day rest period was ensued. First egg laid after this rest period was saved, but not analyzed. When the second egg was laid, a group of 4 hens plus 1 control were selected and slaughtered after this and the left Pectoralis muscle was excised and snap frozen in liquid nitrogen. Eggs were broken out and the yolk separated from the albumen. Muscle and egg contents were stored at -20 C. After the third egg (3rd egg) was laid, another 4 hens and eggs were sampled as described. Finally, after the fourth egg (4th egg) was laid, the remaining 4 hens and eggs were sampled as described.

Sample Processing and Derivitization

A 0.5g sub-sample of yolk and albumen and 1g (ml) sub-sample of breast muscle were, respectively, hydrolyzed in 6N HCl. Samples were centrifuged at 3,000 x g and the supernatant separated into a new vial. The supernatant was then run through an ion-exchange column packed with Dowex 50WX8-200. Leucine was eluted with 2ml of 4N NH4OH and 1ml of nanopure H2O into a new vial and dried under vacuum. The tert-butyldimethylsilyl (tBDMS) derivative was formed by addition of 800 µl of C2CH3CN-MTBSTFA (1:1) and incubation at 110 °C for 120 min.
**GC/MS Analysis**

Analysis was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 µl volume was injected in splitless mode. Starting oven temperature was 150 ºC and increased 50º C/min to 200ºC, after which temperature was increased 20 ºC/min to 270 ºC and held for 5.5 min. The mass spectrometer was operated under EI and SIM modes. The 359 and 365 m/z fragments, representing the M and M + 6 fragments, were monitored. The retention time for leucine and norleucine was 5.01 min and 4.8 min respectively. The amount of label leucine from each tissue was calculated as follows:

\[ \mu g \text{ 6C13 leucine} = \frac{[L]}{\text{sample mass}} \times R \]

Where:

\[ [L] = \mu g \text{ of leucine}, \text{ was calculated as:} \]

\[ \text{Rf} \times \text{leucine peak area} \]

\[ \text{Rf: response factor, was calculated as:} \]

\[ \text{Rf} = \frac{[NL]}{\text{nor leucine peak area}} \]

\[ [NL] = \mu g \text{ nor leucine} \]

\[ R \text{ was calculated as:} \]

\[ \frac{(M+n)}{((M+n) + \text{Msample})} - \frac{(M+n)}{((M+n) + \text{Mnatural})} \]
EXPERIMENTAL DESIGN

A CRD was conducted with 3 treatments and 4 replications per treatment. Each treatment was represented by each egg collection (2nd, 3rd and 4th egg).

The model used was the following:

\[ Y_{ijk} = u + A + e \]

Where:

- \( u \) = general media
- \( A \) = egg
- \( e \) = random error

Analysis of variance was performed using JMP software, and means were separated by Tukey test when \( p \) Value < 0.05.

RESULTS

The amount of 6C13 leucine in breast muscle, yolk, albumen and whole egg (yolk plus albumen) and the percentage of 6C13 leucine from breast in yolk, albumen and whole egg are shown in tables 2,3 and figures 1, 2 and 3. No differences were found regarding 6C13 leucine content in the breast muscle sample between birds who laid the second, third and fourth egg. The 6C13 leucine content in the yolk significantly increased from the second to the fourth egg \((1.35 \mu g \text{ and } 3.16 \mu g, \text{ respectively; } P. \text{ value } = 0.008)\), a similar trend was seen in the albumen, however is was not significant \((0.80 \mu g \text{ and } 1.33 \mu g, \text{ respectively})\). When the 6C13 leucine content from yolk and albumen was added, this content significantly increased from the second to the fourth egg.
(2.45 µg and 4.50 µg, respectively; P. value = 0.001). The percentage of the 6C13 leucine from the breast in the yolk significantly increased from the second to the fourth egg (25.18% and 61.2%, respectively; P. value = 0.00005), a similar trend was seen in the albumen, however it was not significant (16.60% and 26.07%, respectively). Furthermore, when the percentage of 6C13 leucine from the breast in the yolk and albumen was added, this percentage significantly increased from the second to the fourth egg (41.97 % and 88.2%, respectively; P. value = 0.0038).

**DISCUSSION**

Results in the present study show that there was a decrease in the 13C leucine content in breast muscle from second to third egg and that the amount of 13C leucine in the egg significantly increased from second to fourth egg suggesting that birds are using their breast muscle protein reserves for egg protein formation. Furthermore, the percentage of 13C leucine from the breast muscle significantly increased up to approximately 67% from second egg to fourth egg, suggesting that the birds are actively degrading their muscle protein and mobilizing the amino acids for egg production. Vignale et al. (2014 a, 2014b) conducted two studies looking into the effect of sexual maturity on protein turnover in broiler breeder’s pure lines and parent stock. The authors were able to conclude in both studies that muscular protein degradation rate significantly increased for broiler breeders through peak egg production. Our results in the present study are in agreement with the later authors since the 13C leucine mobilization that we were able to see is a reflection of protein degradation, moreover the present study was conducted during peak egg production. Our results are also in agreement with Ekmay et al. (2014) who looked at lysine partitioning using 15N lysine in broiler breeders. The authors were able to conclude that endogenous muscle was the main source of lysine for yolk and albumen formation at week 25.
and week 45. Furthermore, the later authors were also able to observe a consisted decrease in the 15N lysine in breast muscle from second to third egg, while also seen an increase in 15N lysine in the egg from third to fourth egg. Hiramoto et al. (1990) looked into changes in protein synthesis in the liver and oviduct as an ovum passes through the magnum in laying hens. The group found no changes in liver protein synthesis but noted differences in protein synthesis in the oviduct when the ovum was in the magnum. The broiler breeder data reported herein suggests a large role of skeletal tissue in protein utilization. Further studies are needed to evaluate the role of skeletal tissue in protein utilization in layers, and to better understand why broiler breeders are relying in such extend to skeletal muscle tissue for egg production. A feed restriction issue might be forcing the hens to utilize that much skeletal muscle protein for egg formation. More research need to be conducted in broiler breeders using different feeding programs involving frequency of feeding and evaluating muscular protein degradation to better understand why these birds have such a dramatic degradation rate during production. The answer might be a balance between feed frequency and quantity to optimize nutrient utilization for production without increasing maintenance requirements.
### Table 1: Experimental diet

<table>
<thead>
<tr>
<th>INGRIDIENT, %</th>
<th>Nutrient, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>66.28</td>
</tr>
<tr>
<td>soybean meal</td>
<td>23.11</td>
</tr>
<tr>
<td>Fat</td>
<td>1.31</td>
</tr>
<tr>
<td>Limestone</td>
<td>6.78</td>
</tr>
<tr>
<td>dicalcium phosphate</td>
<td>1.81</td>
</tr>
<tr>
<td>Salt</td>
<td>0.17</td>
</tr>
<tr>
<td>Alimet</td>
<td>0.19</td>
</tr>
<tr>
<td>Choline</td>
<td>0.1</td>
</tr>
<tr>
<td>vitamin premix</td>
<td>0.07</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>0.1</td>
</tr>
<tr>
<td>mineral premix</td>
<td>0.075</td>
</tr>
<tr>
<td>TOTAL %</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Calculated :</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME, Kcal/kg</td>
<td>2860</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>15.5</td>
</tr>
<tr>
<td>Dig. Lysine</td>
<td>0.76</td>
</tr>
<tr>
<td>Dig. Meth &amp; Cyst</td>
<td>0.67</td>
</tr>
<tr>
<td>Dig. Methionine</td>
<td>0.42</td>
</tr>
<tr>
<td>Dig. Threonine</td>
<td>0.52</td>
</tr>
<tr>
<td>Dig. Tryptophan</td>
<td>0.16</td>
</tr>
<tr>
<td>Dig. Arginine</td>
<td>0.95</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>3.38</td>
</tr>
<tr>
<td>Calcium</td>
<td>3.25</td>
</tr>
<tr>
<td>Phosphorus-Non</td>
<td>0.41</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Analyzed:**
- Crude Protein: 15.1
- Crude Fat: 4.83
- Ash: 10.27
- Calcium, ppm: 33664

### Table 2: 6C13 Leucine content in breast muscle, yolk, albumen, and whole egg

<table>
<thead>
<tr>
<th></th>
<th>µg 6C13 Leu</th>
<th>µg 6C13 Leu yolk</th>
<th>µg 6C13 Leu alb</th>
<th>total µg 6C13 Leu in egg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>breast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>second egg</td>
<td>5.36</td>
<td>1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89</td>
<td>2.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>third egg</td>
<td>4.94</td>
<td>1.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86</td>
<td>2.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>fourth egg</td>
<td>5.1</td>
<td>3.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.33</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. value</td>
<td>0.93</td>
<td>0.008</td>
<td>0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>SEM</td>
<td>0.81</td>
<td>0.34</td>
<td>0.16</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.
Table 3: 6C13 Leucine percentage from breast muscle in yolk, albumen, and whole egg

<table>
<thead>
<tr>
<th></th>
<th>% breast 6C13 Leu in yolk</th>
<th>% breast 6C13 Leu in alb</th>
<th>% breast 6C13 Leu in egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>second egg</td>
<td>25.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.6</td>
<td>41.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>third egg</td>
<td>33.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.4</td>
<td>50.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>fourth egg</td>
<td>61.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.07</td>
<td>88.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. value</td>
<td>0.0005</td>
<td>0.26</td>
<td>0.02</td>
</tr>
<tr>
<td>SEM</td>
<td>4.47</td>
<td>4.4</td>
<td>8.81</td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.

Figure 1: 6C13 Leucine content in breast muscle, yolk, albumen, and whole egg
Figure 2: 6C13 Leucine percentage from breast muscle in yolk and albumen

Figure 3: 6C13 Leucine percentage from breast in whole egg
REFERENCES


Scherwin, R.S. (1978) J. Clin. Invest. 61, 1471-1481


Waterlow, J.C., P.J. Garlick, and D.J. Millward (1978) Protein turnover in Mammalian Tissues and in the Whole body. Elsevier North-Holland, Amsterdam.
VI. The effect of White striping on protein turnover
ABSTRACT A study was conducted to evaluate the effect of white striping in protein turnover and gene expression of genes related to protein degradation and fatty acid synthesis. The objective of this study was to elucidate why white stripes appear in older (60 d old) broilers. A total of 560 1 day old male broiler chickens Cobb 500 were allocated in a total of 16 pens, 35 chickens per pen. Birds were fed under Cobb nutritional recommendations. Chickens were raised until 60 days of age. All chickens were weighted at 1, 21, 42, and 60 days of age. Feed intake and FCR was also recorded. A CRD was conducted with a 2X3 factorial arrangement (two scores: severe and normal, and 3 samples sites). At day 60, 20 birds were randomly selected, culled and scored for white striping. Scoring was either normal (no white striping) or severe (severe white striping). After scoring, birds were scanned for body composition determination. Also, the same day, 17 birds (16 infused one control) were randomly selected and infused with a solution of 15N Phen 40% APE (atom percent excess). As small piece of breast muscle tissue was taken for gene expression analysis of the following genes: murf-1, atrogin-1, IGF-1, insulin receptor (IR), fatty acid synthetase, and acetyl CoA carboxylase (ACC). Blood samples were also taken from the same birds for insulin and VLDL plasma concentrations analysis. Each bird was culled after 10 minutes of infusion and scored for white striping (normal or severe). Samples of the breast muscle (pectoralis major) were taken at different layers (3 samples per bird, top, mid, bottom), put in a label sample bag, and the bag will be save in liquid N. A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the samples were stored at -20 C, until samples analysis. Out of the 16 samples taken, only ten were selected for analysis based on the white striping score (5 normal and 5 severe). Fractional synthesis rate (FSR) and Fractional breakdown rate (FBR) were determined using GCMS. No significant differences were found in fractional synthesis rate (FSR) between with severe white
striping score and normal ones and samples sites. There was only a score effect on FBR. This variable was significantly higher in birds with severe white striping when compared to the ones with normal score (8.2 and 4.28, respectively, P. value < 0.0001). Birds with severe white striping showed significantly higher relative expression of murf-1 and higher but not significant relative expression of atrogin-1 than the birds with normal score. These birds also showed lower relative expression of IGF-1 than birds with normal score. Insulin receptor (IR) relative expression was higher for birds with severe white striping when compared to the ones with normal score. Furthermore, birds with severe white stripes showed significantly lower relative expression of fatty acid synthetase and ACC than the control (normal score). No significantly differences were found between treatments regarding plasma VLDL concentrations. Birds with severe white striping showed higher, but not significant plasma insulin concentrations than the control (13.10 µl/ml and 11.50 µl/ml, respectively; P. value = 0.17). No significantly differences were found between groups regarding body composition. Further studies are needed to better understand why birds with severe white striping are degrading more muscular protein and mobilizing more fat.

Key words: white striping, protein turnover, gene expression, VLDL, insulin
INTRODUCTION

Meeting the substantial increase in food demand, that is being driven by increased human population growth, severe drought conditions and diversion of grain to ethanol-biofuel production (Steinfel et al., 2006; Chen et al., 2011; Mora et al., 2013) is very challenging. There is, thus, a need to identify strategies (nutritional, genetic and/or management) to improve livestock (meat, egg, milk, etc) production at both quantitative and qualitative concentrations. White striping is the white striation occasionally observed parallel to the direction of muscle fibers in broiler breast fillets and thighs at the processing plant. Broiler breast fillets can be categorized as normal (NORM), moderate (MOD), or severe (SEV) based on the degree of white striping. Histologically, SEV fillets are characterized by the highest degree of degeneration of muscle fibers along with fibrosis and lipidosis when compared with NORM (Kuttappan et al., 2013 a, b). It has been found in breast muscle proximal analysis in birds with white striping that, as the degree of white striping increases, the fat and protein contents of muscle increased (P < 0.05) and decreased (P < 0.05), respectively (Kuttappan et al., 2013 a, b).

It is known that white striping could be a potential reason for the rejection of raw breast fillets in the market (Kuttappan et al., 2012). However, it is still not clear what the origin of the problem is, or what is happening in the breast meat that make this white stripes to appear.

The objective of this study is to elucidate why white stripes appear in older (60 d old) broilers.
MATERIALS AND METHODS

Stock and Management

A total of 560 1 day old male broiler chickens Cobb 500 were allocated in a total of 16 pens, 35 chickens per pen (3.0 m x 1.5 m). Birds were fed under Cobb nutritional recommendations. Diet composition and nutritional content are shown in table 1.

Chickens were raised until 60 days of age. All chickens were weighted at 1, 21, 42, and 60 days of age. Feed intake and FCR was also recorded.

The present study was conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee (protocol # 12041).

Experimental Procedure

Body composition determination

At day 60, 20 birds were randomly selected, culled, and scored for white striping. Scoring was either normal (no white striping) or severe (severe white striping). After scoring, birds were scanned for body composition determination as previously described (Salas et al., 2012).
**Infusion procedure**

At day 60, 17 birds (16 infused, one control) were randomly selected and infused with a 150mM solution of 15N Phen 40% APE (atom percent excess). Each bird was culled after 10 minutes of infusion and scored for white striping (normal or severe). Samples of the breast muscle (*pectoralis major*) were taken at different layers (3 samples per bird, top, mid, bottom), put in a label sample bag, and the bag will be save in liquid N. A sample of excreta of each bird was taken, put in a labeled sample bag, and the bag was saved in liquid N. All the samples were stored at -20 C, until samples analysis. Out of the 16 samples taken, only ten were selected for analysis based on the white striping score (5 normal and 5 severe).

**Sample Processing**

The acid-soluble fraction containing free amino acids was removed by addition of 2% (w/v) perchloric acid. After homogenization, samples were centrifuged at 3,000 x g and the supernatant, containing free amino acids, removed. The protein precipitate was washed three times with 2% perchloric acid before being hydrolyzed in 6N HCl. The supernatant and precipitate, respectively, were then run through an ion-exchange column packed with Dowex 50WX8-200. Phenylalanine and 3-methylhistidine (3-MH) were eluted with 2ml of 4N NH4OH and 1ml of nanopure H2O into a new vial and dried under vacuum. The tert-butyldimethylsilyl (tBDMS) derivative was formed by addition of 800 µl of C2CH3CN-MTBSTFA (1:1) and incubation at 110º C for 60 min. Excreta was processed without the removal of the acid-soluble fraction.
**GC/MS analysis**

Analysis of the protein precipitate of breast samples and free amino acids was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 μl volume was injected in splitless mode. Starting oven temperature was 150°C and increased 50°C/min to 200°C, after which temperature was increased 20° C/min to 270° C and was held for 5.5 min. The mass spectrometer was operated under EI and SIM modes. The 394, and 395 m/z fragments, representing the M, and M+1 fragments of phenylalanine, were monitored. Standard solutions (in 0.1M HCl) were prepared from the same phenylalanine stock to validate APE linearity.

3-MH was determined on the same mass spectrometer. Helium was used as the carrier gas at 1 ml/min; a 1 μl volume was injected in splitless mode. Starting oven temperature was 110°C and held for 0.65 min, then temperature was increased 30° C/min to 250°C, and held for 10 min. The mass spectrometer was operated under EI and SIM modes. The 238 m/z fragment of 3-MH was monitored.

Fractional synthesis rate was calculated as:

\[ K_s = \frac{APE_b}{APE_f} \times \frac{1}{t} \times 100 \]

Where \( APE_b \) = 15N atom percent excess (relative to natural abundance) of phenylalanine in protein

\( APE_f \) = 15N atom percent excess of free phenylalanine in tissues, assumed as the precursor pool

\( t \) = time [d].
Fractional degradation rate was calculated as:

$$kd = \frac{3-\text{MH daily excretion}}{3-\text{MH muscle pool}} \times 100$$

**RNA isolation, reverse transcription, and real-time quantitative PCR**

A small sample of breast was taken at the same time the sample for protein turnover analysis was taken. The sample was put in a 1.5 ml tube and frozen in liquid nitrogen. All samples were stored overnight at -80°C until sample analysis.

Total RNA were extracted from muscle tissues by Trizol reagent (Life Technologies, Grand Island, NY) according to manufacturer’s recommendations, DNAse treated and reverse transcribed (Quanta Biosciences, Gaithersburg, MD). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix. Oligonucleotide primers specific for chicken: FAS, forward, 5’-

ACTGTGGGCTCCAAATCTTCA-3’ and reverse, 5’-CAAGGAGCCATCGTGAAGC-3’,

ACC alpha , forward, 5’-CAGGTATCGCATCAGTATAGGTAACAA-3’ and reverse, 5’-

GTGAGCGCAGAATAGAAGGATCA-3’, Atrogin-1, forward, 5’-

CCTTCCACCTGTCACATCTC-3’ and reverse, 5’-CACAGGCAGGTCCACAA-3’; Murf-1, forward, 5’-TGGAGAAGATTGAGCAAGGCTAT-3’ and reverse, 5’-

GCGAGGTGCTCAAGACTGACT-3’; IGF-1, forward, 5’-GCTGCGGAGGCAGAA-3’ and reverse, 5’-ACGAACTGAAGAGCATCAACCA-3’; Insulin receptor (IR), forward, 5’-
CGGA\textit{ACTGCATGGTGCA-3'} and reverse, 5'-\textit{TCTCTGGTCACTGCGAATCT-3'}; ribosomal 18S, forward, 5'-\textit{TCCCCTCCGGTTACTGAT-3'} and reverse, 5'-\textit{GCGCTCGTCGGCATGTA-3'}, as a housekeeping gene were used. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the 2–\textit{ΔΔCt} method (Schmittgen and Livak, 2008).

Out of the 16 samples taken, only ten were selected for analysis based on the white striping score (5 normal and 5 severe).

\textit{VLDL Analysis}

Blood samples were taken from the birds to be infused at day 60. Out of the 16 samples taken, only ten were selected for analysis based on the white striping score (5 normal and 5 severe). Plasma VLDL was analyzed using and Elisa kit specific for chicken from NeoBioLab.

Briefly, 50 \textmu L of sample or standard was added to the appropriate wells in the supplied microtiter plate. Plate was incubated for 1 hour at room temperature. Wells were emptied and washed 3-5 times with 300-400 \textmu L 1x wash solution per well. After last wash 100 \textmu L of conjugate per well was added and mixed. Plate was covered and incubated 1 hour at 37°C in a humid chamber. Each well was washed 5 times with 1x wash solution. After the last wash the
plate was inverted and blot dry by tapping on absorbent paper. 50 µL substrate A was added to each well followed by addition of 50 µL substrate b. Plate was covered and incubated for 10-15 minutes at room temperature. After that 50 µL of stop solution was added to each well. Finally the optical density (O.D.) at 450 nm on a plate reader. The mean blank value was subtracted from each sample or standard value and the mean was calculated for duplicate wells. A standard curve was calculated using excel, and the VLDL plasma were calculated by using the standard curve equation.

*Insulin plasma concentrations analysis*

Blood samples were taken from the birds to be infused. Out of the 16 samples taken, only ten were selected for analysis based on the white striping score (5 normal and 5 severe). Plasma insulin was analyzed using and Elisa kit specific for chicken from Cusabio Lab. Insulin was evaluated using an ELISA specific kit for chicken. This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with and antibody specific to insulin. Standards or samples were added to the appropriate microtiter plate wells with Biotin-conjugated Insulin or IGF-1. A competitive inhibition reaction was launched. The more hormones in the sample, the fewer antibodies bound to by Biotin-conjugated hormone. After washing avidin conjugated Horseradish peroxidase (HRP) was added to the wells. Substrate solution was added to the wells and the color develops in opposite to the amount of hormone in the sample. The color development was stopped and the intensity of the color was measured in a plate reader at 450 nm. A standard curve was built and insulin concentrations were calculated by used of the standard curve equation.
Experimental Design

The experimental design for this trial was a CRD with a factorial arrangement 2x3, 2 treatments and three sample sites being each treatment represented by the white striping score (normal or severe) and each sample site represented by top, middle and bottom. Each treatment had 5 replications, where each bird per treatment represented one replicate.

The model to be used was the following:

\[ Y_{ijk} = u + A + B + AB + e \]

Where:
- \( u \) = general media
- \( A \) = score
- \( B \) = sample site
- \( AB \) = interaction score x sample site
- \( e \) = random error

Analysis of variance was performed using JMP software, and means were separated by Tukey test when \( p \) Value < 0.05.

Analysis of variance was performed using JMP software, and means were separated by Student test when \( p \) Value < 0.05.

RESULTS

Fractional synthesis and breakdown rate: breast muscle

Broiler breast muscular fractional synthesis and breakdown rate by white striping score are shown in Table 2. There was no significant differences in fractional synthesis rate (FSR)
between with severe white striping score and normal ones and samples sites. There was only a score effect regarding fractional breakdown rate (FBR). This variable in birds with severe white striping was significantly higher when compared to the ones with normal score (8.2%/d and 4.28%/d, respectively, P. value < 0.0001).

**Plasma Insulin and VLDL concentrations**

Insulin and VLDL plasma concentrations are shown in table 5 and figures 1 and 2. No significantly differences were found between treatments regarding plasma VLDL concentrations. However, birds with severe white striping showed higher but not significant plasma insulin concentrations than the control (13.10 µl/ml and 11.50 µl/ml, respectively).

**Gene expression**

Relative gene expression of Murf-1, atrogin-1, IGF-1, insulin receptor (IR), fatty acid synthethase and Acetyl CoA carboxilase (ACC) are shown in table 6 and figures 3, 4, 5, 6, 7 and 8. Birds with severe White striping showed significantly higher relative expression of murf-1 and higher but not significant relative expression of atrogin-1 than the birds with normal score. These birds also showed lower relative expression of IGF-1 than birds with normal score. IR relative expression was higher for birds with severe white striping when compared to the ones with normal score. Furthermore, birds with severe white stripes showed significantly lower relative expression of fatty acid synthetase and ACC than the control (normal score).
**Body composition**

Body composition from birds with severe and normal white striping scores are shown in table 4. No significantly differences were found between groups regarding fat mass, lean mass and total mass.

**DISCUSSION**

Based on visual evaluation of the intensity of white striping, breast fillets can be categorized into normal (NORM), moderate (MOD), and severe (SEV) categories (Kuttappan et al., 2012a, b, 2013a, b). Results of the present study shows that birds with severe white striping showed significantly higher FBR than that those with normal score. Kuttappan et al., (2012a and 2013b) conducted a study to evaluate the details of changes in histology as well as proximate composition occurring in the fillets with respect to the 3 degrees of white striping. The authors were able to find that there was an increase \((P < 0.05)\) in mean scores for degenerative or necrotic lesions, fibrosis, and lipidosis as the degree of white striping increased from NORM to SEV. The results from the histopathological study were supported by the findings from proximate analysis confirming that the fat and protein contents of muscle increased \((P < 0.05)\) and decreased \((P < 0.05)\), respectively, as the degree of white striping increased. Our results showing higher muscular degradation rate are in agreement with the later study since less muscular protein content was seen in birds with severe white striping which can be related to more FBR. In the present study birds with severe white striping also showed significantly higher relative expression of murf-1 and higher but not significant atrogin-1 relative expression (ubiquitin-proteasome pathway related genes) than birds with normal score. These results are in support of our FBR findings. Furthermore, Vignale et al., (2014a, 2014b) evaluated protein
turnover in broiler breeders and were able to conclude that the relative expression of murf-1 and atrogin-1 was significantly higher in birds with higher FBR supporting our gene expression and FBR findings. We were able to find that birds with severe white striping have lower relative expression of IGF-1 than the control (normal score). Sacheck et al. (2004) concluded that IGF-1 down regulate murf-1 and atrogin-1. Whereas Vignale et al. (2014b) found that the relative expression of IGF-1 was significantly lower in birds with higher FBR supporting our gene expression and FBR findings. We were able to find in the present study that the plasma insulin concentrations (not significant) as well as the relative expression of the insulin receptor were higher for the birds with severe white striping in comparison to the control. This results are in agreement with the fact that we saw in the same group of birds a lower relative expression of fatty acid synthetase and ACC, suggesting that fat is being mobilized to instead of synthesized in the muscle. In general the birds with severe white striping and normal score did not show differences in body composition (fat mass, lean mass and total mass) which is in agreement with Kuttappan et al. (2013a and 2013b) who did not find differences in body weight between birds with different score. Unfortunately we did not do any proximal analysis of the fillets, but the fact that we are seeing more muscular FBR in the birds with severe white striping in comparison to the ones with normal score, suggests that the composition of those fillets might also be different. Together our results suggest that one of the possible reasons why white striping is occurring might be a higher muscular degradation rate that leave space for fat deposition as well as more fat mobilization. Further studies are needed to better understand why birds with severe white striping are degrading more muscular protein and mobilizing more fat.
Table 1: Experimental Diet composition and Nutrient content

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>FEEDING PHASE</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STARTER</td>
<td>GROWER</td>
<td>FINISHER 1</td>
<td>FINISHER 2</td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>56.3</td>
<td>61.5</td>
<td>65.45</td>
<td>67.9</td>
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<tr>
<td>soybean meal</td>
<td>35.91</td>
<td>30.54</td>
<td>26.41</td>
<td>23.94</td>
<td></td>
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<tr>
<td>Fat</td>
<td>3.75</td>
<td>4.16</td>
<td>4.64</td>
<td>4.67</td>
<td></td>
</tr>
<tr>
<td>Limestone</td>
<td>1.19</td>
<td>1.13</td>
<td>1.04</td>
<td>1.04</td>
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<tr>
<td>dicalcium phosphate</td>
<td>1.17</td>
<td>1.06</td>
<td>0.88</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>0.38</td>
<td>0.38</td>
<td>0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.36</td>
<td>0.32</td>
<td>0.29</td>
<td>0.27</td>
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</tr>
<tr>
<td>Lysine</td>
<td>0.26</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
<td>Choline</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>vitamin premix</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
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</tr>
<tr>
<td>Threonine</td>
<td>0.12</td>
<td>0.11</td>
<td>0.13</td>
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<td>mineral premix</td>
<td>0.1</td>
<td>0.1</td>
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<tr>
<td>selenium premix .06%</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td>Monsanto Sanoquin 6 ethox</td>
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<td>0.02</td>
<td>0.02</td>
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<tr>
<td>hiphos phytase</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
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<tr>
<td>TOTAL %</td>
<td>100</td>
<td>100.01</td>
<td>100</td>
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Nutrient,%
Calcuated:

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<th>FINISHER 2</th>
</tr>
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<tr>
<td>ME, Kcal/kg</td>
<td>3035</td>
<td>3108</td>
<td>3180</td>
<td>3203</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>22.37</td>
<td>20.17</td>
<td>18.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Dig. Lysine</td>
<td>1.18</td>
<td>1.05</td>
<td>0.95</td>
<td>0.9</td>
</tr>
<tr>
<td>Dig. Meth &amp; Cyst</td>
<td>0.88</td>
<td>0.8</td>
<td>0.74</td>
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<tr>
<td>Dig. Methionine</td>
<td>0.62</td>
<td>0.56</td>
<td>0.52</td>
<td>0.49</td>
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<tr>
<td>Dig. Threonine</td>
<td>0.77</td>
<td>0.69</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>Dig. Isoleucine</td>
<td>0.8</td>
<td>0.71</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>Dig. Valine</td>
<td>0.83</td>
<td>0.75</td>
<td>0.69</td>
<td>0.65</td>
</tr>
<tr>
<td>Dig. Tryptophan</td>
<td>0.24</td>
<td>0.21</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td>Dig. Arginine</td>
<td>1.24</td>
<td>1.1</td>
<td>0.99</td>
<td>0.93</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>6.07</td>
<td>6.62</td>
<td>7.19</td>
<td>7.28</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.9</td>
<td>0.84</td>
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<td>0.76</td>
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<tr>
<td>Phosphorus-Non</td>
<td>0.45</td>
<td>0.42</td>
<td>0.54</td>
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<tr>
<td>Sodium</td>
<td>0.16</td>
<td>0.16</td>
<td>0.5</td>
<td>0.15</td>
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Analyzed:

<table>
<thead>
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<th>FINISHER 1</th>
<th>FINISHER 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>20.4</td>
<td>19.9</td>
<td>17.6</td>
<td>16.5</td>
</tr>
<tr>
<td>Crude Fat</td>
<td>5.87</td>
<td>5.89</td>
<td>7.04</td>
<td>5.1</td>
</tr>
<tr>
<td>Ash</td>
<td>5.11</td>
<td>4.81</td>
<td>4.51</td>
<td>3.95</td>
</tr>
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</table>
Table 2: Fractional Synthesis rate (FSR) and fractional breakdown rate (FBR) by score and simple site

<table>
<thead>
<tr>
<th>Score</th>
<th>Sample site</th>
<th>FBR %</th>
<th>FSR %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>TOP</td>
<td>4.07a</td>
<td>14.01</td>
</tr>
<tr>
<td>Normal</td>
<td>MID</td>
<td>4.7a</td>
<td>7.20</td>
</tr>
<tr>
<td>Normal</td>
<td>BOTTOM</td>
<td>4.03a</td>
<td>10.03</td>
</tr>
<tr>
<td>Severe</td>
<td>TOP</td>
<td>9.24b</td>
<td>8.45</td>
</tr>
<tr>
<td>Severe</td>
<td>MID</td>
<td>7.54b</td>
<td>8.08</td>
</tr>
<tr>
<td>Severe</td>
<td>BOTTOM</td>
<td>7.82b</td>
<td>12.04</td>
</tr>
</tbody>
</table>

P. Value < 0.001
SEM 0.38

Concentrations not connected by same letter are significantly different.

Table 3: Body weight and Feed Conversion Ratio (FCR)

<table>
<thead>
<tr>
<th>Body weight gain, g</th>
<th>FCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3973</td>
<td>1.88</td>
</tr>
</tbody>
</table>

Table 4: Body composition by score

<table>
<thead>
<tr>
<th>SCORE</th>
<th>Fat mass, g</th>
<th>lean mass, g</th>
<th>total mass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>438.61</td>
<td>2925.63</td>
<td>4254.65</td>
</tr>
<tr>
<td>severe</td>
<td>377.65</td>
<td>2787.70</td>
<td>4091.66</td>
</tr>
<tr>
<td>p Value</td>
<td>0.25</td>
<td>0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>SEM</td>
<td>35.74</td>
<td>75.05</td>
<td>109.74</td>
</tr>
</tbody>
</table>

Table 5: Insulin and VLDL plasma concentrations by score

<table>
<thead>
<tr>
<th>VLDL ng/ml</th>
<th>Normal (C)</th>
<th>Severe</th>
<th>p Value</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin ug/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

167
Figure 1: Plasma insulin concentrations by score (µg/ml)
Figure 2: Plasma VLDL concentrations by score (ng/ml)

Table 6: Relative expression of Murf-1, atrogin-1, IGF-1, Insulin Receptor, Fatty acid synthetase and acetyl CoA carboxilase by score

<table>
<thead>
<tr>
<th>GENE</th>
<th>Relative gene expression</th>
<th>Normal (C)</th>
<th>Severe</th>
<th>p Value</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MURF-1</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>1</td>
<td>1.4</td>
<td>0.076</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Fatty acid synthetase</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.032</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Acetyl CoA carboxylase</td>
<td>1</td>
<td>0.67</td>
<td>0.23</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004</td>
<td>0.145</td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

Concentrations not connected by same letter are significantly different.
Figure 3: Murf-1 relative expression by score

![Murf-1 relative expression by score](image)

Figure 4: Atrogin-1 relative expression by score

![Atrogin-1 relative expression by score](image)
Figure 5: IGF-1 relative expression by score

Figure 6: Insulin Receptor relative expression by score
Figure 7: Fatty acid synthetase relative expression by score

Figure 8: Acetyl CoA carboxilase relative expression by score
REFERENCES


Waterlow, J.C., P.J. Garlick, and D.J. Millward (1978) Protein turnover in Mammalian Tissues and in the Whole body. Elsevier North-Holland, Amsterdam.

VII. 25-hydroxycholecalciferol enhances broiler breast meat yield through mechanistic target of rapamycin (mTOR) pathway
ABSTRACT In recent years, there has been a growing body of evidence indicating that replacing vitamin D3 with 25-hydroxycholecalciferol (25(OH) D3) through dietary supplementation enhances muscle breast yield in broiler chickens. However, the underlying molecular mechanisms are still unknown. We investigated here the effect of 25(OH) D3 on broiler growth performance (body weight, feed intake, feed conversion ratio and breast meat yield), muscle protein synthesis and the potential underlying molecular mechanisms. Male broiler chickens Cobb 500 were randomly divided into four treatment groups (n = 360 birds/treatment): Control diet (normal D3, 2760 IU/kg feed), Diet with high concentrations of vitamin D (VD3, 5520 IU/kg feed), Diet with 25(OH) D3 during 42 days (HYD-42, 5520 IU/kg feed), and Diet with 25(OH) D3 during 21 days (HyD-21). Functional studies were performed in vitro using quail myoblast cell line. 25(OH) D3 enhanced breast muscle yield (P<0.05) and increased the fractional rate of protein synthesis (FSR) by 10% (P < 0.05) compared to the control and vitamin D-supplemented groups. Molecular analyses revealed that 25(OH) D3 phosphorylated mTOR, the master growth promoter, at Ser2481 and ribosomal P70 S6K at Thr421/Ser424 and increased the protein expression of the proliferation marker Ki67. In line with the in vivo data, in vitro functional studies showed that blocking mTOR pathway by rapamycin reversed the beneficial effects of 25(OH) D3 on muscle cell proliferation and Ki67 protein expression. Taken together, our findings provide evidence that the beneficial effects of 25(OH) D3 on broiler breast muscle is mediated through mTOR-S6K pathway.

Key words: 25(OH) D3, broiler breast muscle, mTOR pathway, gene expression
INTRODUCTION

Meeting the substantial increase in food demand, that is being driven by increased human population growth, severe drought conditions and diversion of grain to ethanol-biofuel production (Steinfel et al., 2006; Chen et al., 2011; Mora et al., 2013) is very challenging. There is, thus, a need to identify strategies (nutritional, genetic and/or management) to improve livestock (meat, egg, milk, etc) production at both quantitative and qualitative concentrations.

Cholecalciferol is a common commercial source of vitamin D3 in poultry nutrition. The metabolism of vitamin D3 has been extensively studied and it is well understood, at least in mammals (DeLuca et al., 1988). Vitamin D3 is metabolized by sequential hydroxylations: first in the liver at the 25 position to form 25-hydroxyvitamin D3 (25(OH) D3) and then in the kidney mainly at the 24R and 1α-positions to form 24R, 25-dihydroxyvitamin D3 (24R, 25(OH) 2D3) and 1α, 25-dihydroxyvitamin D3 (1α, 25(OH) 2D3) respectively. Since its commercialization, the use of supplemental 25(OH) D3 or HyD, the recommended metabolite for commercial poultry use, has proven effective in improving growth performances. It has been shown, that compared to vitamin D3, dietary supplementation with 25(OH) D3 improves body weight gain and feed efficiency in broiler chickens (Yarger et al., 1995a, b). 25(OH) D3 has also been reported to improve breast muscle yield in broiler chickens (Yarger et al., 1995a) however the underlying molecular mechanisms for this improvement are still unknown.

The mechanistic target of rapamycin (mTOR) signaling pathway senses and integrates a variety of nutritional and environmental cues to regulate organismal growth and homeostasis (for review (Laplante et al., 2012). It is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family (Sabatini et al., 1994) and interacts with
several proteins to form two complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2).

Both complexes share the catalytic mTOR subunit, the mammalian lethal with sec-13 protein 8 (mLST8), the DEP domain containing mTOR-interacting protein (DEPTOR), and the Tti/Tel2 complex (Kim et al., 2003; Peterson et al., 2009). While the regulatory-associated protein of mTOR (raptor) and the proline-rich Akt substrate 40 KDa (PRAS40) are only part of mTORC1 (Kaizuka et al., 2010; Sancak et al., 2007), the rapamycin-insensitive companion of mTOR (rictor), the mammalian stress-activated map kinase-interacting protein 1 (mSin1) and the protein observed with rictor 1 and 2 (protor 1/2) are specific for mTORC2 (Sarbassov et al., 2005; Frias et al., 2006; Pearce et al., 2007).

Although the two mTOR complexes have different upstream inputs and downstream outputs, protein synthesis is the best-characterized process controlled by mTOR. mTOR phosphorylates the translational regulators eukaryotic translation initiation factor 4E (elF4E)-binding proteins (4E-BP1 and 4E-BP2) and S6 kinase 1 (S6K1), which in turn enhances mRNA biogenesis as well as translational initiation and elongation leading thereby to an increase in protein synthesis (Ma et al., 2009). mTOR also promotes protein synthesis through other pathways including 1) activation of the regulatory element tripartite motif-containing protein-24 (TIF-1A) which induces its interaction with RNA Polymerase I (Pol I) and the expression of ribosomal RNA (Mayer et al., 2004) and 2) inhibition of the Pol III repressor, Maf1, and induction of the 5S rRNA and tRNA transcription (Kantidakis et al., 2010; Shor et al. 2010).

Therefore, we sought to determine, in the present study, the effect of 25(OH) D3 on broiler growth performances, breast muscle yield and protein synthesis and whether this effect is mediated through the mTOR pathway or not.
MATERIALS and METHODS

Animals and experimental design

A total of 1440 one-day old male broiler chickens Cobb 500 were allocated in a total of 48 pens, 30 chickens per pen (3.0 m x 1.5 m). Chickens were raised, fed, and handled in accordance with Cobb management guide recommendations. Chickens were randomly divided into four treatment groups with 12 replicates per treatment (360 birds/treatment, 30 birds/replicate). The treatments tested were: Control diet (normal D3, 2760 IU/kg feed), Diet with high concentrations of D3 (VD3, 5520 IU/kg feed), Diet with 25(OH) D3 during 42 days (HyD-42, 5520 IU/kg feed), and Diet with 25(OH) D3 during 21 days (HyD-21, 5520 IU/kg feed). The diet composition is summarized in Table 1. Chickens had ad libitum access to feed and clean water. All chickens were weighted at 1, 21, and 42 days of age. Feed intake was recorded and FCR was calculated. Blood samples were collected from wing vein to measure the circulating concentrations of 25(OH) D3. At days 21 and 42, 10 birds per treatment were infused with a solution of 15N Phen 40% atom per excess (APE), killed after 10 min, and breast samples were taken and snap frozen in liquid nitrogen for protein turnover determination and gene and protein expression. Excreta sample were also collected and frozen in liquid nitrogen for 3-methylhistidine determination. At day 42, 50 chickens per treatment were processed and the breast meat yield was determined.

The present study was conducted in accordance with the recommendations in the guide for the Care and use of laboratory animals of the National Institutes of Health and the protocol was approved by the University of Arkansas Institutional Animal Care and Use Committee (protocol # 12041).
Measurement of Protein Turnover

Muscle protein synthesis was measured according to the flooding-dose technique previously described (Ekmay et al., 2013). Briefly, the birds (n=10) were weighed and then intravenously injected with 1 mL/100 g BW of a phenylalanine solution (L-Phenylalanine-15N, 98% atom %15N, Sigma-Aldrich, St. Louis, MO). Ten minutes after injection, birds were humanly euthanized and the breast muscle (pectoralis major) was quickly removed, immediately snap frozen in liquid nitrogen, and stored at -80°C until analysis. Total excreta was collected for 3-methylhistidine analysis. To determine the free and protein-bound phenylalanine specific activities, frozen muscle sample (1g) was homogenized in perchloric acid (2% w/v) and centrifuged (3,000g, 20 min, 4°C). The supernatant (containing the free pool) was applied to an ion-exchange column packed with Dowex 50WX8-200 (Alfa Aesar, Ward Hill, MA) and free phenylalanine was eluted with 4N of NH4OH. The pellet which containing the bound pool was washed 3 times in 2% (w/v) perchloric acid, hydrolyzed in 6N HCl and processed as described for the free pool. A 0.5g sample of freeze-dried excreta was hydrolyzed in 6N HCl. One mL of the hydrolyzed product was run through an ion-exchange column packed with Dowex 50WX8-200. 3-methylhistidine (3-MH) was eluted with 2mL of 4N NH4OH and 1mL of nanopure H2O into a new vial and dried under vacuum. The tert-butyldimethylsilyl (tBDMS) derivative was formed by addition of 800 µl of C2CH3CN-MTBSTFA (1:1) and incubation at 110° C for 60 min. Analysis of free and bound phenylalanine in breast muscle samples was carried out on an Agilent 7890A GC system attached to an Agilent 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA). Helium was used as the carrier gas at 1 mL/min; a 1 µL volume was injected in splitless mode. Starting oven temperature was 150°C and increased 50°C/min to 200°C, after which temperature was increased 20° C/min to 270° C and held for 5.5 min. The
mass spectrometer was operated under EI and SIM modes. The 394 and 395 m/z fragments, representing the M and M+1 fragments of phenylalanine, were monitored. Standard solutions (in 0.1M HCl) were prepared from the same phenylalanine stock to validate APE linearity.

3-MH was determined on the same GC-MS system. Helium was used as the carrier gas at 1 ml/min; a 1 µL volume was injected in splitless mode. Starting oven temperature was 110°C and held for 0.65 min, then temperature was increased 30°C/min to 250°C, and held for 10 min. The mass spectrometer was operated under EI and SIM modes. The 238 m/z fragment of 3-MH was monitored for identification. Standard curves were generated using commercially acquired stock (Sigma-Aldrich, St. Louis, MO). The fractional synthesis rate was calculated as: 

$$\text{FSR} = \frac{\text{APEb}}{\text{APEf}} \times \frac{1}{t} \times 100$$

Where APEb is 15N atom percent excess (relative to natural abundance) of phenylalanine in protein, APEf is 15N atom percent excess of free phenylalanine in tissues, assumed as the precursor pool, and t is the time [d]. The fractional breakdown rate was calculated as: 

$$\text{FBR} = \frac{(3-\text{MH daily excretion})}{(3-\text{MH skeletal muscle} \times \text{estimated lean mass in g})} \times 100$$

**Measurement of circulating 25(OH) D3**

Circulating concentrations of 25(OH) D3 were determined by radioimmunoassay (RIA) at Heartland assays LLC (Ames, IA).

**Cell culture**

Quail myoblast (QM7) cells were grown in M199 medium (Life technologies, grand Island, NY) complemented with 10% fetal bovine serum (Life technologies, grand Island, NY), 10% tryptose phosphate (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. At 80-90% confluence,
cells were synchronized overnight in serum free medium and treated with two doses (1 and 10 nM) of vitamin D3 or 25(OH) D3 (DSM Nutritional products) for 24h. Untreated cells were used as control. The dose and duration of treatments were chosen based on pilot and previous published experiments (22). For the functional studies, cells were pretreated with rapamycin (100 nM, Sigma-Aldrich, St Louis, MO).

**RNA isolation, reverse transcription, and real-time quantitative PCR**

Total RNA were extracted from tissues and cells by Trizol reagent (Life Technologies, grand Island, NY) according to manufacturer’s recommendations, DNAse treated and reverse transcribed (Quanta Biosciences, Gaithersburg, MD). RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 micro volume plate using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix. Oligonucleotide primers specific for chicken mTOR : forward, 5’-CATGTCAGGCACTGTGTCTATTCTC -3’ and reverse, 5’-CTTTCCGCCCTTGTTTCTTCACT -3’; S6K, forward, 5’-GTCAGACATCATTGGGTTAGAGAAAG -3’ and reverse, 5’-ACGCCCTCGCCCTTGT -3’; And ribosomal 18S, forward, 5’-TCCCCCTCCCGTTACTTGGAT-3’ and reverse, 5’-GCGCTCGTGGCATGTA-3’, as a housekeeping gene were used. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection
system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the 2−ΔΔCt method (Schmittgen and Livak, 2008).

**Western blot analysis**

Muscle tissues and QM7 cells were homogenized in lysis buffer (10 mM Tris base, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, protease and phosphatase inhibitor cocktail). Protein concentrations were determined using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Proteins (70 µg) were run on 4–12% Novex bis–Tris gels (Life Technologies, grand Island, NY). The transferred membranes were blocked for 1 h at room temperature, and incubated with primary antibodies (diluted 1:500-1:1000) at 4°C overnight. The rabbit polyclonal anti-VDR, anti-phospho mTORSer2481, anti-mTOR, anti-phospho S6KThr421/Ser424, anti-S6K, anti-Ki67 antibodies were used. Protein loading was assessed by immunoblotting using rabbit anti-β actin or rabbit anti-vinculin. Prestained molecular weight marker (precision plus protein Dual color) was used as standard (BioRad, Hercules, CA). All the primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX), except for anti-phospho mTOR, mTOR and β actin from Cell Signaling Technology (Danvers, MA), anti-vinculin from Sigma-Aldrich (St. Louis, MO) and anti-ki67 from abcam (Cambridge, MA). The secondary antibodies were used (1:5000) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FluorChem M MultiFluor
System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993-2011, Proteinsimple, Santa Clara, CA).

**Immunofluorescence**

Immunofluorescence was performed as previously described (Dridi et al., 2012). Briefly, cells were grown to 50-60% confluence and treated with 25(OH) D3 as described above in chamber slides (Lab-Tek, Hatfield, PA) and fixed in methanol for 10 min at –20°C. Cells were blocked with protein block serum free blocking buffer (Dako, Carpinteria, CA) and incubated with rabbit anti-VDR, anti-phospho-mTOR, anti-phospho S6K or anti-Ki67 antibody overnight at 4°C and visualized with Alexa Fluor 488- or 594 conjugated secondary antibody (Molecular probes, Life Technologies, grand Island, NY). After DAPI counterstaining, slides were cover slipped in Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using the Zeiss Imager M2 with a 20X Plan-APOCHROMAT 20x/0.8 objective and a 100X EC PLAN-NEOFLUOR 100x/1.3 oil objective. Differential interference contrast images were collected using DIC M27 condensers. All analysis was performed using AxioVision SE64 4.9.1 SP1 software (Carl Zeiss Microscopy GmbH 2006-2013).

**Cell proliferation**

MTS assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instruction and as previously described (Dridi et al., 2012).
Statistical analyses

Data were analyzed by one-factor ANOVA. Significant differences among individual group means were determined with Student-Newman-Keuls (SNK) or Tukey’s multiple range test using the Graph Pad Prism version 6.00 for Windows, Graph Pad Software (La Jolla, CA) or JMP software (Cary, NC). Significance was set at P<0.05. Data are expressed as the mean ± SEM.

RESULTS

25(OH) D3 enhances breast meat yield in broiler chickens

Broiler growth performances in relation to dietary supplementation with vitamin D3 or 25(OH) D3 are shown in Figure 1. Body weight gain, cumulative feed intake, and FCR were not affected by all treatments (Fig. 1a-c). Interestingly, dietary supplementation with 25(OH) D3 for 42 days significantly increased breast meat yield compared to the control and vitamin D3 supplemented groups (Fig. 1d). Supplementation with 25(OH) D3 for 21d only, however, moderately increased the breast meat yield at 42d, but the effect was not statistically discernable (Fig. 1d).

25(OH) D3 enhances fraction synthesis rate in broiler breast muscle

Dietary supplementation with 25(OH) D3 for 42d significantly increased the circulating concentrations of 25(OH) D3 (P < 0.05, Fig. 2a) and the fractional rate of protein synthesis (FSR) by about 10% in broiler breast muscle (P < 0.05, Fig. 2b). No differences in fractional breakdown rate (FBR) were observed between all treatments (Fig. 2c).
25(OH) D3 activates mTOR pathway in broiler breast muscle

Dietary supplementation with 25(OH) D3 for 42d induced the expression of VDR protein and the phosphorylated concentration of mTOR and S6K at Ser2481 and Thr421/Ser424, respectively (Fig. 3a). Concomitant with these changes 25(OH) D3 significantly up-regulated the expression of mTOR and S6K genes in broiler breast muscle (P<0.05, Fig. 3b and c).

25(OH) D3 activates mTOR pathway in avian QM7 cell line

To better understand the mechanism by which 25(OH) D3 enhanced breast muscle yield in broilers and whether mTOR signaling is involved or not, we conducted functional studies using quail myoblast (QM7) cell line. Treatment of cells with 25(OH) D3 at 1 and 10 nM induced VDR protein expression compared to the control and vitamin D-treated groups (Fig. 4a). Immunofluorescence staining confirms the Western blot data and showed that 25(OH) D3 administration induced nuclear translocation of VDR (Fig. 4b). Similar to the in vivo study, 25(OH) D3 treatment induced the phosphorylation of mTOR at Ser2481 and S6K at Thr421/Ser424 in QM7 cells (Fig. 5a). Importantly, 25(OH) D3 treatment increased the protein abundance of the proliferation marker Ki67 (Fig. 5a). In line with these data, immunofluorescence staining showed that 25(OH) D3 treatment increased the concentrations of phospho-mTOR, pospho-S6K, and Ki67 compared to untreated QM7 cells (control) (Fig. 5b). In contrast to the in vivo data, 25(OH) D3 administration did not affect mTOR or S6K mRNA abundance (Fig. 5c).

25(OH) D3 significantly induced QM7 cell proliferation in a dose-dependent manner (Fig. 6a) and this effect was abolished by rapamycin, the mTOR inhibitor, pretreatment (Fig. 6b). Consistent with these observations, rapamycin inhibited 25(OH) D3-induced mTOR and S6K
phosphorylation and Ki67 expression (Fig. 6c). Together, our data showed evidence that 25(OH)D3 enhanced breast muscle yield in broiler chickens through mTOR activation-induced protein synthesis.

**DISCUSSION**

Maximizing feed efficiency and meat production is a major objective of modern poultry and animal agriculture. Compelling evidence indicates that replacing vitamin D3 with 25(OH)D3 in dietary supplementation is beneficial to both mammalian and avian species in enhancing muscle tissue development and growth. The increase in breast meat yield by 25(OH)D3 supplementation observed here corroborates previous studies in avian (Yarger et al., 1995a) and in mammalian species (Buitrago et al., 2003; Buitrago et al., 2001; Buitrago et al., 2002). However, it disagrees with previous reports (Stevens et al., 1984; Atencio et al., 2005a; Atencio et al., 2005b) in terms of body weight gain. The mechanism behind this discrepancy is not clear and could be due to subtle line/species-differences (broiler vs layer and turkey vs chickens) and/or experimental conditions (dose of 25(OH)D3, diet composition, environmental conditions, and management).

Since body weight gain is the net result of the difference between energy intake and energy expenditure, the increase in breast muscle yield without alteration in feed intake and body weight in the present study indicates that 25(OH)D3 may affect the energy expenditure and/or the intermediary metabolism as previously reported (Wong et al., 2009). For instance, a bidirectional genetic and prospective studies have suggested that lower blood concentrations of 25(OH)D3 were associated with high body mass index (BMI) and greater adiposity (Gonzales-Molero et al., 2013; Vimaleswaran et al., 2013; Liel et al., 1988), indicating that 25(OH)D3 may
regulate fat and energy metabolism (Bhat et al., 2014). Using an adipocyte-specific VDR transgenic model, Wong and co-workers (Wong et al., 2011) showed that adipocyte VDR suppresses energy expenditure and promotes obesity. As 25(OH)D3 has been shown to up-regulate leptin expression in rodent adipose tissue (Kong et al., 2013) and leptin has been reported to exert a direct lipolytic effect on mouse adipocytes (Fruhbeck et al., 1997; Siegrist-Kaiser et al., 1997). We hypothesize that 25(OH)D3 mobilizes fat and enhances protein synthesis in our experimental conditions. Although it is beyond the aim of the present study, the lack of body composition (fat mass) data represents a limitation and further investigations are warranted.

Chickens supplemented with 25(OH)D3 for 42d had significant higher circulating 25(OH)D3 concentrations compared to those provided diets containing vitamin D3 for 42d or 25(OH)D3 for 21d. These data are in agreement with previous studies (Yarger et al., 1995b; Hutton et al., 2014), and indicate that the treatment for 42d was sufficient to improve vitamin D status in broiler chickens. The significant high blood concentrations of 25(OH)D3 in 25(OH)D3-compared to vitamin D3-supplemented broilers might be due to more efficient absorption of this metabolite in the upper small intestine as a direct result of the increased polarity of the hydroxylated metabolite (Hutton et al., 2014; Bar et al., 1980). Because dietary vitamin D3 is fat soluble once it is ingested, it is incorporated into the chylomicron fraction, absorbed via the lymphatic system (Holick et al., 1995), enters the circulation and accumulates in the liver for hydroxylation by vitamin D-25-hydroxylase and thereafter it appears in the circulation as 25(OH)D3 (Tanaka et al., 1984). It is conceivable that the increase of circulating 25(OH)D3 after vitamin D3 supplementation is relatively modest compared with cumulative intake of vitamin D3 because 25(OH)D3 regulates vitamin D-25-hydroxylase by negative feedback
(Holick et al., 1996). The half-life of 25(OH) D3 in the circulation is approximately 10 days to 3 weeks (Mawer et al., 1971; Vicchio et al., 1993) which might explain the similar observed concentration of 25(OH) D3 in the blood of 25(OH) D3 supplemented group for 21d compared with the control and the vitamin D3 groups.

Although the biological actions of 25(OH)D3 on avian muscle development and growth are being documented and recognized (Yarger et al., 1995a, Hutton et al., 2014), the underlying molecular mechanisms are still unclear. Here we showed firstly that VDR, the central mediator of vitamin D action in all tissues (Bouillon et al., 2008), is expressed in avian muscle both in vivo (broiler muscle tissue) and in vitro (QM7 cell line), corroborating previous studies in mammals (Srikue et al., 2012; Girgis et al., 2014) and in chickens (Zanello et al., 1997). The up-regulation of VDR expression in vivo and in vitro following 25(OH) D3 treatment and its apparent translocation in QM7 cell nuclei indicate that VDR is capable of mediating a direct effect of 25(OH) D3. These observations are in accordance with previous studies in humans and rats where 25(OH) D3 has been shown to regulate the VDR gene expression through intronic and upstream enhancers (Pike et al., 2010).

VDR is a DNA-binding transcription factor which contains two overlapping ligand binding sites, a genomic (VDR-GP) and an alternative pocket (VDR-AP) that respectively bind a bowl-like ligand configuration for genomic response or a planar-like ligand for rapid non genomic response (Haussler et al., 2011). The increased concentrations of muscle protein fractional synthesis rate (FSR) by 25(OH) D3 supplementation indicated that protein synthesis machinery is switched on. As a step towards defining the downstream mechanism through which 25(OH) D3 exerts its beneficial action on muscle growth in broiler chickens, we examined whether mTOR pathway is activated or not. mTOR, the nutrient sensor and the master growth promoter,
integrates a variety of environmental cues to regulate organismal growth and homeostasis [reviewed in (Laplante et al., 2012)]. It phosphorylates the translational regulators S6K and the eukaryotic translation initiation factor binding proteins (4E-BP1 and 4E-BP2) to regulate processes that control cell growth and proliferation including protein synthesis (Laplante et al., 2012). Alongside with previous reports (Birge et al., 1975; Salles et al., 2013), our in vivo data pointed to the conclusion that 25(OH) D3 supplementation enhanced chicken muscle growth and proliferation through activation of mTOR-S6K pathway. This conclusion has been further confirmed by in vitro functional studies where inhibition of mTOR pathway by rapamycin (sirolimus) blocked 25(OH) D3-induced cell proliferation and -Ki67 protein expression in QM7 cell line.

The signaling of 25(OH) D3 via VDR in our experimental conditions seemed to be both nongenomic and genomic, because it rapidly activated (1 to 2h) mTOR and S6K (data not shown), and increased their respective mRNA concentrations after 24h. In line with our data, several previous studies showed that 25(OH)D3 can initiate rapid response pathways including rapid stimulation of calcium absorption (Norman et al., 1997;), insulin secretion (Kajikawa et al., 1999), and exocytosis and voltage-gated Cl-channel opening (Zanello et al., 2004). Together, these data could be supported by the association of VDR with the plasma membrane caveolae (Huhtakangas et al., 2004).

Regarding the genomic mechanism, VDR is a transcription factor which, upon binding to its ligand, is translocated to the nucleus where it is heterodimerized with 9-cis-retinoic acid receptor (RXR) and this heterodimer DVR-RXR modulates gene expression via binding to specific target gene promoter regions, known as vitamin D response elements (VDREs), to activate or suppress their expression (Deeb et al., 2007). VDREs possess either a direct repeat of two hexanucleotide
half-elements with a spacer of three nucleotides (DR3) or an everted repeat of two half-elements with a spacer of six nucleotides (ER6) motif, with the DR3 being the most common (Haussler et al., 2011). Intriguingly, using a computational analysis (GeneQuest, DNASTAR software) we could not find a known consensus VDRE sequences in the mTOR promoter region. This suggest that either VDRE might exist in other regions of the mTOR gene such as intronic regions (Kang et al., 2012) or activated VDR recruits other transcription factors and co-activators (Haussler et al., 2012) to cooperatively regulate mTOR gene expression.

To conclude, our findings reveal an important role for mTOR pathway in mediating the genomic and nongenomic effects of 25(OH) D3 on chicken muscle proliferation and development. Further studies are definitely needed to 1) define the upstream cascade through which 25(OH) D3 phosphorylate mTOR; 2) determine whether satellite cell-mediated muscle hypertrophy (Hutton et al., 2014) are controlled by mTOR pathway in response to 25(OH) D3 supplementation and; 3) identify potential cross-talk between mTOR and other pathways in regulating chicken muscle growth following vitamin D status improvement.
**Table 1: Experimental Diets and Nutritional Content**

<table>
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<tr>
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<td>STARTER</td>
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<td>FINISHER</td>
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<tr>
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<tr>
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**NUTRIENT, %**

Calculated:

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<tr>
<td>ME, Kcal/kg</td>
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<td>3180</td>
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<tr>
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Analyzed:

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<tr>
<td>Ash</td>
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Figure 1: Effects of 25(OH) D3 diet supplementation on broiler performances.
Figure 2: Effects of 25(OH) D3 diet supplementation on muscle protein synthesis and circulating 25(OH) D3 concentrations.
Figure 3: 25(OH) D3 diet supplementation increases VDR expression and activates mTOR pathway in broiler breast muscle.
Figure 4: 25(OH) D3 treatment increases VDR expression in quail myoblast (QM7) cells.
Figure 5: 25(OH) D3 treatment activates mTOR pathway and increases the protein expression of the proliferation marker Ki67 in quail myoblast (QM7) cells.
Figure 6: Blocking mTOR pathway inhibits QM7 cell proliferation-induced by 25(OH) D3.
REFERENCES


CONCLUSIONS

Together our results and the one found in other species such as mammals that are also feed restricted (during pregnancy) suggest that the dramatic increased in muscular fractional degradation rate in broiler breeder and laying hens is a phenomena related more to a production state and a feed restriction issue than as to the specie itself and it might be triggered by the fact that the animals are not getting the nutrients they need to produce either eggs or milk.

There is a large increase in FBR during the transition for the pullet layer and breeder hen to sexual maturity with increases in FBR through peak egg production, which is related to a decreased in lean mass body content during this period of time. Laying and broiler breeder hens may rely on skeletal muscle tissue as a source of nutrients as they reach egg production. Laying hens showed a similar response as broiler breeders regarding FBR, however a less dramatic change in FBR was seen, maybe due to the fact that after peak egg production is reached, more feed is available. Further studies are needed to evaluate the role of skeletal tissue in protein utilization in layers, and to better understand why broiler breeders are relying in such extend to skeletal muscle tissue for egg production.

Our broiler results suggest that one of the possible reasons why white striping is occurring might be a higher muscular degradation rate that leave space for fat deposition as well as more fat mobilization. Our findings reveal an important role for mTOR pathway in mediating the genomic and nongenomic effects of 25(OH) D3 on chicken muscle proliferation and development.
MEMORANDUM

TO: Craig N. Coon
FROM: Carol Rodlin, Program Manager
Institutional Animal Care
And Use Committee
DATE: February 15, 2013

SUBJECT: IACUC Modification Request APPROVAL
Expiration date: July 31, 2015

The Institutional Animal Care and Use Committee (IACUC) has APPROVED the modification request (to add the use of metabolism chambers) to Protocol #13002- "Evaluation of broiler breeder feeding regimes for pure-line and commercial type stock during rearing and production phases and calcium requirement during the production period." You may implement this modification immediately.

In granting its approval, the IACUC has approved only the modification request provided. Should there be any additional changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request Form] prior to initiating the changes.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cc: Animal Welfare Veterinarian
MEMORANDUM

TO: Craig N. Coon
FROM: Carol Rodlun, Program Manager
       Institutional Animal Care
       And Use Committee
DATE: February 15, 2013

SUBJECT: IACUC Modification Request APPROVAL;
         Expiration date: May 14, 2015

The Institutional Animal Care and Use Committee (IACUC) has APPROVED the modification request (to add the use of metabolism chambers) to Protocol #12041- “THE EFFECT OF FEEDING COMMERCIAL EXOGENOUS ENZYMES TO BROILERS TO IMPROVE PERFORMANCE AND DIGESTION OF NUTRIENTS.” You may implement this modification immediately.

In granting its approval, the IACUC has approved only the modification request provided. Should there be any additional changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request Form] prior to initiating the changes.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cc: Animal Welfare Veterinarian