MOORE, DANIEL TODD. The Influence of Early Nutrition on Muscle Development in the Poult. (Under the direction of Peter R. Ferket and Paul E. Mozdziak)

ABSTRACT

The focus of the dissertation is on myonuclear accretion of the early post-hatch poult because an increase in myofiber size is limited to the number of myonuclei present and the ability of the myofiber to acquire new nuclei is not consistent from hatch to market.

The objective of the first experiment was to develop a technique to manipulate the turkey fetus three days prior to hatch. In order to understand satellite cell mitotic activity, an injection of 5-Bromo-2'-deoxyuridine (BrdU), a thymidine analog, can be given and detected via immunohistochemistry. The post-hatch bird can be given an intra-peritoneal injection of BrdU; however, the hard shell surrounding the avian fetus makes it difficult to administer BrdU to a precise location in the fetus during late development in the turkey. A successful method was accomplished and employed in experiment two.

The objectives of the second experiment were to study the injection of nutrients before hatch to the turkey fetus and to determine the influence of myogenic satellite cell mitotic activity and muscle development before hatch and immediately post-hatch. The nutrient injection did not improve muscle development **($P \geq 0.05$)** following hatch when compared to a saline injected control. However, satellite cell mitotic activity was highest at day of hatch and one day of age **($P \leq 0.05$)** when compared to one week of age indicating the importance of the first week post-hatch in muscle development.

The objective of the third experiment was to determine the influence of β-hydroxy β-methylbutyrate (HMB) and fasting during the first week period on satellite cell mitotic activity, and muscle development. This experiment also employed BrdU injection for satellite cell mitotic activity determination. The detection of the protein Pax7, found in quiescent myogenic satellite cells and recently activated satellite cells, was employed in this experiment. Immediately fed poults given a diet containing HMB had higher body weights (***P*** ≤ **0.01)** at 48 hours and one week of age, and had higher satellite cell mitotic activity at 48 hours of age (***P*** ≤ **0.01)** compared to the immediately fed poults on a standard industry based starter diet and fasted poults. However, the fasted poults had the lowest amount of satellite cell mitotic activity (***P*** ≤ **0.01)** at 48 hours post-hatch than the other two groups. Therefore, HMB may play an anabolic role in early post-hatch muscle development.

The objectives of the fourth experiment were to examine the influence of fasting on skeletal muscle dynamics of the immediately post-hatch poult and to understand the skeletal muscle dynamics involving the satellite cell population. The experiment used BrdU, Pax7, Bcl-2, a cell marker found underneath the basal lamina of the myofiber. Fed poults had higher body weights throughout the experiment **(P ≤ 0.01)** and had higher muscle weights **(P ≤ 0.01)** at ten days of age than the fasted poults. Fed poults had higher satellite cell mitotic activity at 72 hours and four days of age **(P ≤ 0.01)** compared to the fasted poults. However, Pax7 labeling index was higher in the fasted poults **(P ≤ 0.01)** at 72 hours and four days post-hatch than the fed group. The results indicate that a level of fasting and nutrition during the first week post-hatch may influence the overall dynamics

of muscle development involving satellite cell mitotic activity and the overall satellite cell population.

THE INFLUENCE OF EARLY NUTRITION ON MUSCLE DEVELOPMENT IN THE POULT

By

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BIOGRAPHY

Daniel Todd Moore was born November 17, 1974, in Rockford, Illinois. He graduated from Kirksville Senior High School in Kirksville, Missouri in 1993. He received a Bachelor of Science degree in Animal Science in 1997 from the University of Missouri-Columbia in Columbia, Missouri. In 2000, he completed a Master of Science degree in Animal Science emphasizing poultry nutrition at the University of Missouri-Columbia. From 2000 until the present he has been compiling the requirements for a Ph.D. degree in Nutrition with a minor in Biotechnology at North Carolina State University in Raleigh, North Carolina.

The author is married to Amanda Fowler Moore. I appreciate the support and encouragement of my wife during the completion of my experiments and preparation of this thesis.

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1. LITERATURE REVIEW

1.1 BREAST MUSCLE YIELD IN TURKEYS

Breast meat yield is becoming an important aspect of the turkey industry because the goal of many operations is to maximize the amount of breast meat yield at market age by selecting for fast growing turkey lines and feeding high levels of proteins. The use of varying levels of protein and amino acids for breast meat production has been extensively studied (Noll, 2002), and maximizing production through conventional dietary manipulations will reach a limit. Other avenues of improving breast meat yield will need to be explored to go beyond the conventional nutritional and genetic programs.

1.11 BREAST MUSCLE PRODUCTION

A simplistic way to view breast muscle production is calculating the rate of protein deposition as the difference between protein synthesis and protein degradation. Kang et al. (1985) studied that relationship in turkey poults from one to eight weeks of age using regression equations of protein content and muscle weights on body size. Protein synthesis decreased from 56% to 10%/day and protein degradation decreased from 22% to 7%/day in the *Pectoralis thoracicus* over the seven week period (Kang et al., 1985). Consequently, the rate of protein deposition also fell from 34% to 4%/day over the same period, which was higher than protein deposition in leg muscle (Kang et al., 1985), indicating an age related decrease in protein deposition. It is reasonable to

assume the deposition rate will remain lowered throughout the growth period beyond eight weeks of age.

Another method of evaluating breast muscle production is to observe breast muscle yield as a percentage of carcass weight or live weight. Adams and Stadelman (1991) measured breast meat yield as a percentage of preslaughter and chilled carcass weight in turkeys of three different size categories at market age. It was found that breast meat yield increases as a percentage of both weights in larger turkeys of the same age (Adams and Stadelman, 1991). Essentially, the amount of breast meat yield is directly related to the size of the bird. Interestingly, the percentage of breast meat yield continues to increase as the birds become larger, which is not true of other muscle groups in the turkey (Adams and Stadelman, 1991). This may be due to the increased levels of protein deposition in the breast muscle compared to other muscles at market age (Kang et al., 1985). However, Kang et al. (1985) used mixed sex samples for their determination, in contrast to Adams and Stadelman (1991) who employed only tom turkeys, which may influence breast muscle yield and protein deposition. Since an increase in breast meat yield is related to bird size, turkeys can be marketed at a younger age if a target size is reached without negatively influencing breast meat yield.

1.12 INFLUENCE OF FAST GROWING LINES ON BREAST MUSCLE

Breast muscle yield is directly related to the size of the turkey, and it is reasonable to assume that turkeys selected for a faster growth rate would also have a higher level of breast muscle yield than slower growing lines of turkeys at the same age. Broiler chicks

that were heavier at hatch had higher pectoralis muscle weights at market age (Sklan et al., 2003), indicating a similar trend in chickens. To further the understanding of selecting rapid growth lines of chickens, Remignon et al. (1995) studied the difference in characteristics of the *Pectoralis thoracicus* between fast growing and slow growing lines of chicken. The authors found no differences in the distribution of fiber types between the lines (Remignon et al., 1995). However, birds from the faster growing lines exhibit larger myofiber areas (Remignon et al., 1995). In turkeys, differences in muscle organization between sexes and between lines selected for different growth rates exist at 25 days of incubation (Velleman et al., 2002), suggesting an embryonic frame for programming muscle.

1.13 CHARACTERISTICS OF BREAST MUSCLE

Turkey breast muscle is composed of 90-100% white muscle fibers (Wiskus et al., 1976). Interestingly, fibers in other muscles in the turkey appear red from a high myoglobin content but have enzymatic characteristics of white fibers (Wiskus et al., 1976), indicating a phenotypic difference in turkey fiber types that has not been studied. White fibers are type IIB fast twitch, glycolytic fibers that are quicker to fatigue than the oxidative fiber type, but have a higher capability of performing powerful bursts. White fibers have less capillary density surrounding the fiber than red fibers (Sosnicki et al., 1991), which may be due to the glyoclytic pathway used by a white fiber pathway. Glycolytic fibers also have a higher rate of rigor mortis following slaughter (Dransfield and Sosnicki, 1999). An unusually high rate of postmortem glycolysis results in pale,

soft, and exudative (PSE) meat in pork (Greaser, 1986). The PSE condition is also found in turkeys, and may be more prominent in fast growing lines of turkeys (Sosnicki and Wilson, 1991). Elevated levels of postmortem glycolysis in pigs and turkeys results in a rapid decrease in postmortem pH (Pietrzak et al., 1997). In turkeys PSE, may be counteracted by quickly chilling the carcass (Dransfield and Sosnicki, 1999).

1.2 IMPACT OF EARLY NUTRITION ON POULTRY

In normal commercial production practices, poults often do not have access to feed and water for 48 to 72 hours. The energy required to emerge from the shell leaves the newly hatched poult in a nutrient deficient state (Uni and Ferket, 2004). Glycogen is the primary energy source available to the fetus when hatching; however, upon completion of the hatching process, the poult has greatly decreased its glycogen stores (John et al., 1987; John et al., 1988) increasing the need for nutrients. It is often thought that the residual yolk found in the poult or chick is sufficient to maintain the bird until feed is offered. However, it has been suggested that the initiation of growth may be more dependent on feed consumption than the nutrients found in the yolk post-hatch (Nir and Levanon, 1993), and when feed consumption occurs early post-hatch the nutrients provided by the feed are complementary to the yolk nutrients (Murakami et al., 1992). Therefore, it is extremely important for the poult to consume nutrients as close to hatch as possible in order to improve muscle development.

1.21 ASPECTS OF EARLY NUTRITION

Providing poults and chicks with feed, aids in the development of the gastrointestinal tract, and can up-regulate brush border enzymes (Uni, 1998). Organ systems also have high levels of growth during the first week post-hatch in poults relative to body weight, indicating a need for nutrients (Lilburn, 1998). Different sources of protein and energy have varying levels of impact on poults (Lilburn, 1998), showing a need for more digestible nutrients. Noy and Sklan (1999a) found that offering nutrients to poults in solid, semi-solid, or liquid form immediately post-hatch improved body weight and breast meat percentage of body weight at market age. Finally, without access to feed and water, the development of the early poult is dependent on residual nutrients found in the yolk sac that have been depleted during the hatching process (Uni and Ferket, 2004), which can result in a mortality rate of about 5%, poor growth, decreased disease resistance, and impaired levels of muscle development (Uni and Ferket, 2004).

1.22 IN OVO FEEDING

The term in ovo feeding refers to the administration of nutrients to the embryo before hatch. It is proposed that in ovo feeding will help bridge the nutrient gap between hatch and placement at the farm (Uni and Ferket, 2004). Al-Murrani (1982) injected amino acids *in ovo* to chicken embryos and found an increase in body weight from hatch to market when compared to non-supplemented chicks. Amino acid injection into broiler chicks via the yolk or air cell also has been shown to increase chick weight at hatch (Ohta et al., 1999). The in ovo administration of vitamin E resulted in enhanced antibody and

macrophage response in poults and chicks (Gore and Qureshi, 1997). Recombinant human insulin-like growth factor-I (rhIGF-1) was injected into incubating chicken eggs to determine the influence on skeletal muscle development (Kocamis et al., 2002). The rhIGF-I injection altered the expression of skeletal muscle development factors, myostatin and transforming growth factor-β2 (TGF-β2) in ovo (Kocamis et al., 2002). However, little other research has been performed providing nutrients in ovo or via in ovo feeding.

1.23 β-HYDROXY-β-METHYLBUTYRATE (HMB) IN FEED

One possible nutrient that may be used in early post-hatch diets to improve muscle development is HMB, which is a leucine metabolite that is formed endogenously by the oxidation of α-ketoisocaproate (KIC) and is finally converted to HMG-CoA used in cholesterol synthesis needed for membrane production and repair (Nissen and Abumrad, 1997). An alternative pathway for HMB utilization that has not been shown or tested is through its action as a butyrate. Butyrates have been shown to inhibit the activity of the enzyme histone deacetylase (HDAC; Davie, 2003). The decrease in HDAC results in an increase in follistatin and ultimately an increase in muscle growth following a period of stress (Iezzi et al., 2004).

Following an injection of HMB to lambs and pigs, a recovery rate of only 34% in the urine was observed, indicating the use of the metabolite by animals (Van Koevering and Nissen, 1992). Leucine may have more important physiological roles than the formation of HMB; therefore it may be possible that an exogenous source of HMB may be beneficial. HMB has been shown to decrease the amount of proteolysis in muscle, as

well as, decrease muscle damage during a period of stress to the muscle (Gallagher et al., 2000; Panton et al., 2000; Vukovich et al., 2001), which may be beneficial to animal agriculture.

In vitro, cultures of whole chicken muscles showed a decrease in proteolysis after the administration of HMB (Ostaszewski, 2000). It has also been shown that HMB decreases proteolysis in young rat muscle following a period of stress by altering the activity of proteolytic enzymes (Jank et al., 2000). However, HMB does not seem to be as effective in more mature animal muscle because broiler chickens showed no increase in body weight and carcass yield at 42 days following HMB supplementation, and weanling pigs did not show an increase in average daily gain following HMB supplementation (Nissen et al., 1994; Gatnau et al., 1995).

1.24 IMPACT OF FASTING ON AVIAN MUSCLE DEVELOPMENT

Glycogen is considered a primary energy source available to the embryo while hatching; therefore, glycogen stores are greatly reduced during the emergence from the shell (John et al., 1987; John et al., 1988), resulting in a newly hatched poult with low glycogen reserves. However, phosphorylase a, a glycogen degradation enzyme involved in the mobilization of glycogen, is at a high level in the one-day-old poult when compared to the embryo (Rosebrough et al., 1978), indicating a need for glycogen immediately post-hatch. Donaldson and Christensen (1991) showed a dose response decrease in glucose-6-phophatase activity in poults fed increasing levels of dietary carbohydrates compared to poults fasted for the first 24 hours post-hatch. The decrease in glucose-6-phosphatase indicates a decreased dependence on gluconeogenesis and a

possible increase in glycogen reserves needed for a period of stress. If glycogen reserves are depleted at hatch and mild stressors can cause an increase in plasma glucose levels (Donaldson and Christensen, 1991), few energy sources remain for secondary physiological functions such as muscle development. It also has been shown that both chicks and poults with delayed access to feed and water for 48 hours resulted in a severe shortage of energy that altered body composition (Pinchasov and Noy, 1993).

Body weights are also lower because of the shortage of energy as a result of fasting. Considerable research has shown that chicks with delayed access to feed and water for the first 48 hours post-hatch had lower body weights that were present at market age (Nir and Levanon, 1993; Noy and Sklan, 1999b; Vieira and Moran 1999a). This change in body weight following fasting immediately post-hatch also has resulted in a reduction in meat yield at market age (Vieira and Moran, 1999b). A decrease in all aspects of intestinal development also has been shown to exist following immediate post-hatch fasting (Geyra et al., 2001), which along with decreased energy status results in a lower performance to market age.

Withholding feed during the immediate post-hatch (2-3 days post-hatch) period also negatively impacts muscle development of poultry. In chickens not provided feed for 48 hours following hatch, protein synthesis in the *Pectoralis thoracicus* was significantly reduced when compared to fed chicks (Yaman et al., 2000). Also, *Pectoralis thoracicus* IGF-I and myostatin mRNA levels increased from hatch to two days of age in chicks that had immediate access to feed and water; however, fasted chicks showed no change in IGF-1 and myostatin mRNA levels over the same time period (Guernec et al., 2004), indicating reduced muscle development as a result of fasting.

Apoptosis of chicks fasted for three days was 88.8% of all nuclei with the amount of apoptosis occurring in fed chicks at the same age was only 1.1% (Mozdziak et al., 2002), giving further indication to the detrimental effect of immediate post-hatch fasting on muscle development.

1.3 MYOGENIC SATELLITE CELLS

Postnatal or post-hatch myofibers contain myonuclei that are incapable of mitotic division (Stockdale and Holtzer, 1961). However, the acquisition of new DNA units, considered the myonucleus and the surrounding cytoplasm, or the increase of existing DNA unit size are the only two mechanisms for post-hatch myofiber hypertrophy. Since there is a limit to DNA unit size and myonuclei are post-mitotic, satellite cell fusion is the mechanism driving muscle growth (Horsley et al., 2001; McCall et al., 1998; Roy et al., 1999). Myonuclear accretion is regulated by the surrounding myofibers (Bischoff, 1990; Mozdziak et al., 1998a). Satellite cells are located between the basal lamina and the sarcolemma of the myofiber and run the entire length of the fiber (Mauro, 1961; Campion, 1984). There has been increasing evidence satellite cells are a heterogeneous population (Zammit et al., 2004).

1.31 MYOGENIC SATELLITE CELLS IN MUSCLE DEVELOPMENT

Embryological muscle development begins with the fusion of mononucleated myoblasts to form myotubes, which mature into myofibers (Schultz and McCormick, 1994). During avian development, a distinct population of satellite cells is present at the midfetal stages of development (Feldman and Stockman, 1992), indicating the importance of satellite cells from an early developmental time. The developmental origin of satellite cells remains unclear (Buckingham et al., 2003; Chen and Goldhamer, 2003). However, a majority skeletal muscle progenitors arise in the somites (Chen and Goldhamer, 2003), therefore, it has been suggested they arise from the mesenchymal cells in the somites. Myoblasts found in the chicken embryo during late embryogenesis are predominantly adult myoblasts (Hartley et al., 1992). Post-hatch, normal skeletal muscle growth does not occur through an increase in myofiber number, but through an increase in myofiber size (Remignon et al., 1995). Myofiber number does not increase during normal postnatal growth because myonuclei are postmitotic, and they can not synthesize DNA (Stockdale and Holtzer, 1961). An increase in fiber size in postnatal vertebrates is directly related to an increase in myonuclei (Allen et al., 1979). Therefore, the cell population available to donate myonuclei is the satellite cell population.

1.32 AGE RELATED CHANGES IN MYOGENIC SATELLITE CELLS

Satellite cells are first associated with myofibers at 19 days of gestation in the mouse with an estimated decrease in satellite cell mitotic activity from 32% to 6% from birth to adulthood (Cardasis and Cooper, 1975), showing a decrease in age-related satellite cell mitotic activity. Mozdziak et al. (1994) show a similar age-related *in vivo* decrease in satellite cell mitotic activity from 3 to 9 weeks of age in the turkey. As a result of high levels of postnatal satellite cell mitotic activity, a reduction in muscle size at maturity can result from a short-term reduction in satellite cell mitotic activity early in life (Mozdziak et al., 1997; Mozdziak et al., 2000). *In vitro*, satellite cells from mature turkeys retain their proliferative capacity (Doumit et al., 1990), indicating that satellite cells have the ability to proliferate in the older animal but the *in vivo* environment may not favor a proliferative state. The classical model for myofiber growth was that satellite cells were orderly added to the fibers to maintain a constant DNA unit size (Moss, 1968). However, from 9 to 26 weeks of age, myofiber growth in turkeys occurred mainly through an increase in the cytoplasmic volume to myonucleus ratio (Mozdziak et al., 1994), indicating that muscle growth also can occur through an increase in DNA unit size.

1.33 MYOGENIC SATELLITE CELL ACTIVITY IN VARIOUS STRAINS OF POULTRY

If satellite cell mitotic activity plays a major role in governing mature muscle size, then it is possible that genetic lines of poultry selected for different levels of production may have varying levels of satellite cell mitotic activity. Chicks that had the same body weight at hatch, showed no differences in satellite cell mitotic activity in the *Pectoralis thoracicus* (Sklan et al., 2003). However, chicks that were heavier in body weight at hatch had higher satellite cell mitotic activity in the pectoralis muscles that and had heavier pectoralis muscle weights at five days post-hatch and at market age (Sklan et al., 2003). In turkeys, birds selected for fast growth showed an increase in satellite cell proliferation *in vitro* when compared to a randomly bred line of turkeys (Velleman et al., 2000), suggesting a difference in satellite cell dynamics between two different lines of turkeys. The dynamics of satellite cells in the *Pectoralis thoracicus* was also studied *in vitro* between Nicholas poults (a fast growing line) and Merriam's poults (a slow growing line) (McFarland et al., 1993a). A greater growth rate of the Nicholas poults was observed when compared to the Merriam's poults that was associated with an increase in satellite cell proliferation and an increased responsiveness of satellite cells to mitogenic stimuli (McFarland et al., 1993a), suggesting a relationship to satellite cell dynamics at muscle development. Interestingly, the Merriam's poults had a higher responsiveness to signals associated with differentiation *in vitro* (McFarland et al., 1993a), which may indicate the difference in performance between the two strains could be related to a decreased ability of satellite cells to proliferate prior to differentiation.

1.34 GROWTH FACTORS INVOLVED IN MYOGENIC SATELLITE CELL DYNAMICS

Growth factors are small polypeptides that are usually secreted by cells to influence themselves or adjacent cells via autocrine or paracrine regulation (Dodson et al., 1996). A family of growth factors is the insulin-like growth factors (IGF), which is found in two forms, IGF-I and IGF-II (McFarland, 1999). In rat satellite cells *in vitro*, IGF-I alone stimulated proliferation and differentiation (Allen and Boxhorn, 1989). Other *in* vitro research involving rat satellite cells supports these findings showing IGF-I activates proliferation then stimulates events leading to differentiation (Engert et al., 1996). IGF-I and IGF-II both stimulate proliferation of turkey satellite cells (McFarland et al., 1993b), which was further supported by the fact that the turkey satellite cell cultures showed higher levels of mRNA expression for IGF-II and insulin-like growth factor binding protein-2 (IGFBP-2) during proliferation with decreased levels at the onset of differentiation (Ernst et al., 1996). The influence of growth factors on satellite cell development and muscle growth is evident because satellite cells from Nicholas poults are more responsive to IGFs when compared to Merriam's poults (McFarland et al., 1995).

Another growth factor family is fibroblast growth factor (FGF). In rat satellite cell cultures FGF stimulates proliferation and depresses differentiation (Allen and Boxhorn, 1989). Mitogenic responses from FGF have also been observed in turkey satellite cells (Dodson et al., 1996). The slow growing Merriam's turkey derived satellite cells had a greater response to FGF than satellite cells derived from the faster growing

Nicholas poults (McFarland et al., 1995). In contrast to the evidence presented by Allen and Boxhorn (1989), Marcelle et al. (1995) found that the avian FGF receptor, FREK, was prominently expressed during differentiation, suggesting a possible role of FGF in differentiation in the bird.

A more recently identified growth factor that influences satellite cell dynamics is hepatocyte growth factor (HGF). HGF stimulates satellite cell proliferation of rat muscle *in vitro* (Allen et al., 1995). The HGF receptor c-met is present in quiescent satellite cells (Allen et al., 1995). Gal-Levi et al. (1998) discovered that HGF increases cell proliferation and inhibits cell differentiation of cultured chicken myoblasts. HGF *in vitro* has also been shown to stimulate turkey satellite cell proliferation and inhibit satellite cell differentiation (Zeng et al., 2002).

Another mitogenic growth factor is platlet-derived growth factor (PDGF). Turkey *Pectoralis thoracicus* satellite cells show a greater responsiveness to PDGF, and a greater binding affinity to PDGF than satellite cells derived from the *Biceps femoris* (McFarland et al., 1997), suggesting a greater proliferation rate of *Pectoralis thoracicus* satellite cells.

Finally, the growth factor transforming growth factor beta (TGF-β) has been shown to decrease proliferation and inhibit differentiation of rat satellite cells *in vitro* (Allen and Boxhorn, 1989). Similarly, TGF-β inhibited proliferation and differentiation in turkey satellite cells (Yun et al., 1997).

Growth factors rarely are present in the absence of other growth factors, and the presence of multiple factors may alter the mode of action of some growth factors. Allen and Boxhorn (1989) studied the interaction between IGF-I, FGF and TGF-β in satellite

cells. TGF-β continued to inhibit differentiation in the presence of IGF-I and FGF; however, TGF-β could not suppress the proliferation stimulated by FGF. The combination of IGF-I and FGF resulted in the highest level of proliferation and the highest level of fusion. HGF when combined with IGF-I and FGF did not have any additive of synergistic effects on the proliferation of satellite cells (Zeng et al., 2002). It is important to understand the varying levels of growth factor combination when determining satellite cell dynamics.

1.35 OTHER FACTORS IMPACTING MYOGENIC SATELLITE CELL DYNAMICS

Satellite cells in vivo respond to a variety of stimuli that can increase or decrease satellite cell mitotic activity. The irradiation of turkey *Pectoralis thoracicus* at two weeks of age decreased satellite cell mitotic activity one and four weeks after irradiation when compared to nonirradiated muscle (Mozdziak et al., 1997). The loss in satellite cell mitotic activity was associated with the lower muscle weight of the irradiated group when compared to the nonirradiated group at fifteen weeks post-irradiation (Mozdziak et al., 1997), suggesting satellite cell mitotic activity early in life impacts adult muscle weight. Hindlimb suspension performed on rats results in lower satellite cell mitotic activity when compared to weight-bearing control rats (Darr and Schultz, 1989; Mozdziak et al., 1998a; Mozdziak et al., 2000). Similar to the turkey, rats with a temporarily lower level of satellite cell mitotic activity early in life exhibit a lower muscle weight in the adult muscle (Mozdziak et al., 2000), once again suggesting the importance of early satellite

cell mitotic activity on mature muscle size. Feed deprivation of chicks early post-hatch resulted in a decreased level of satellite cell proliferation and decreased muscle weight at market age (Halevy et al., 2000), which is important to the poultry industry that has a practice of delaying the placement of poults and chicks.

Interestingly, new evidence is emerging that suggests a mild stress to the bird may be beneficial to satellite cell mitotic activity and muscle development. Chicks that were exposed to a mild heat stress at three days of age resulted in an increase in satellite cell mitotic activity that resulted in increased body weight and percent breast muscle yield at market age (Halevy et al., 2001). Pophal et al., (2004) fed chicks varying levels of digestible lysine ranging from a slightly deficient level to a level well over the requirement. The results showed the highest level of satellite cell mitotic activity was in the chicks fed a diet slightly deficient in lysine. The idea of mildly stressing young animals to increase satellite cell mitotic activity is a relatively novel idea that may have a major impact in animal agriculture.

Other factors improve satellite cell mitotic activity. Muscles from overloaded rats that had been supplemented with creatine had higher satellite cell mitotic activity than overloaded rats on a control diet (Dangott et al., 2000). It has also been shown that chicks that had immediate access to feed and water had higher satellite cell mitotic activity during the feed deprived period, and had increased body and muscle weights at market age when compared to chicks with delayed access to feed and water (Halevy et al., 2003). It is evident that influencing satellite cell mitotic activity early in life is important in determining mature muscle size.

1.36 SUB-POPULATIONS OF MYOGENIC SATELLITE CELLS

Original studies of satellite cells described them as a population of cells residing between the basal lamina and sarcolemma of the myofiber, but more recent studies suggest there are sub-populations of satellite cells. Schultz and Lipton (1982) were the first researchers to suggest satellite cell heterogeneity. Morgan and Partridge (2003) suggest that a population of satellite cells exist that may be derived from a more primitive stem cell that can adopt a more divergent phenotypic fate than a majority of the population of satellite cells committed to muscle repair. However, even though satellite cell heterogeneity may exist, a majority of cells contributing to muscle do not appear to arise from hematopoietic or other bone marrow precursors (Sherwood et al., 2004). A recent study has confirmed the idea of a sub-population by using a clonal culture system of mouse muscle satellite cells and identifying two types of cells, “round cells” and “thick cells”, with the round cells forming clusters and producing the thick cells that committed to myogenic and osteogenic pathways (Hashimoto, 2004). Schultz (1996) found that 80% of the myogenic satellite cell population provided myonuclei to myofibers and underwent a more rapid level of proliferation. The remaining 20% of the satellite cells underwent a slower level of proliferation, suggesting that this group of cells is a proliferative reserve maintained to create new satellite cells. Other research also found a satellite cell sub-population consisting of 28% of all satellite cells that was composed primarily of large type cells (Molnar et al., 1996). Through the use of satellite cell expression factors, it has been determined that a majority of satellite cells are activated, proliferate, and eventually differentiate (Zammit et al., 2004). However, a small population of cells withdraws from the cell cycle during proliferation, and once

again become quiescent satellite cells (Zammit et al., 2004) displaying a proliferative reserve of satellite cells that maintain the ability to replenish the satellite cell population. Finally, it has been determined that a proliferative reserve of satellite cells exist in turkey muscle that can be identified by their proliferation rate and fusion capabilities (Rouger et al., 2004).

1.37 REGULATORY FACTORS INVOLVED WITH MUSCLE DEVELOPMENT AND MYOGENIC SATELLITE CELLS

Myogenic regulatory factors (MRFs) are DNA binding proteins that induce a myogenic phenotype. The main MRFs include MyoD, Myf-5, myogenin, and MRF4. It has been shown in mice lacking MyoD and Myf-5 that myoblasts are not present, and myoblast found in mice lacking myogenin and MRF4 cannot differentiate (Rudnicki and Jaenisch, 1995). In tissue culture, most myoblasts express MyoD and Myf-5, but up-regulate myogenin during differentiation (Weintraub, 1993), further indicating myogenin's role downstream of MyoD and Myf-5. Cornelison and Wold (1997) showed that once satellite cells were activated MyoD or Myf-5 were expressed, followed by the expression of myogenin. At some time points, all four MRFs were expressed (Cornelison and Wold, 1997), suggesting a cascade of regulatory factors during muscle development.

A better understanding of the myogenic regulatory factors can be accomplished by determining expression during muscle growth or regeneration. Pharmacologically induced hypertrophy resulted in the induced expression of MyoD and the depressed expression of myogenin (Mozdziak et al., 1998b), indicating an induced state of

proliferation. During proliferation, satellite cells express MyoD and a portion of the population expresses Myf-5 (Cooper et al., 1999). In regenerating mouse muscle following a muscle graft, MyoD and myogenin were not detected until after fusion into the myotubes, and they were not express in nonregenerating muscle (Fuchtbauer and Westphal, 1992), suggesting the presence of MyoD and myogenin only in activated satellite cells.

Quiescent satellite cells have been suggested to express c-met, m-cadherin, Pax7, and CD34, (Irintchev et al., 1994; Allen, 1995; Beauchamp et al., 2000; Zammit and Beauchamp, 2001; Asakura et al., 2002). However, c-met and Myf5 have been found in satellite cells further down the cascade of fusion with myofibers (Cornelison and Wold, 1997). The most important function of m-cadherin is to regulate the fusion of the satellite cell with the myofiber (Irintchev et al., 1994). CD34 expression has also been suggested to indicate muscle-derived stem cells (Hwang et al., 2004). Pax7 has been shown to be expressed in quiescent satellite cells and in a small population of satellite cells that quickly return to a quiescent state (Zammit, 2004), making Pax7 an ideal marker for studying the proliferative reserve of satellite cells.

Finally, a paradigm that represents postnatal muscle development, via satellite cells, has been developed by Seale et al. (2000). Pax7 is responsible for the specification of satellite cells in postnatal muscle, and shows the commitment to the myogenic lineage. Pax7 also is expressed upstream of all of the MRFs, suggesting its importance in the beginning of satellite cell myonuclear donation. Once the satellite cell is activated by stimuli, the generation of daughter myogenic precursor cells (proliferation) is marked by the expression of MyoD and Myf-5. Then the expression of myogenin and MRF4

indicates terminally differentiation (differentiation) of the muscle precursor cells into myotubes.

1.4 PAX7 INVOLVEMENT IN MYOGENIC SATELLITE CELLS

Pax7 is a protein expressed in quiescent satellite cells (Zammit and Beauchamp, 2001; Asakura et al., 2002). However, more recently it has been discovered to be present in satellite cells that are active for a brief period before reentering the quiescent state (Zammit, 2004), making Pax7 a reliable marker for studying and understanding a proliferative reserve of satellite cells (Schultz, 1996). Understanding the role of Pax7 is an integral part of understanding satellite cell dynamics in postnatal muscle and is made easier by the development of a Pax7 antibody (Kawakami et al., 1997).

1.41 STRUCTURE OF PAX7

Pax transcription factors have a conserved octapeptide and a transactivation domain that is encoded by eight or nine exons (Balczarek et al., 1997). The transactivation domain is located at the C-terminus of the Pax protein and is a Proline, Threonine, and Serine rich area (Jostes et al., 1991). Pax7 also contains a paired-box and a homeobox that encode for the paired domain and homeodomain (Balczarek et al., 1997). These N-terminal domains bind to the target DNA (Chi and Epstein, 2002), once binding takes place, activation of the target gene occurs via the formation of a protein-

DNA pre-initiation complex (Lamey et al., 2004). In binding, the paired box domain utilizes its two helix-turn-helix motif, and the homeodomain utilizes its helix-turn-helix motif (Lamey et al., 2004). Binding specificity of the homeodomain is influenced by the binding specificity of the paired domain (Mishra et al., 2002).

1.42 PAX7 TRANSCRIPTS

It has been known that Pax7 is involved in muscle development, as well as, development of the nervous system (Jostes et al., 1991). However, recently it appears that there are varying forms of Pax7 that may perform different functions. First, four alternative forms of Pax7 were discovered, and different forms were found in different tissues (Ziman et al., 1997). The four alternative forms were found to differ in their paired-box sequence (Ziman et al., 2001). All forms were expressed in the myogenic lineage, but only two forms were expressed in the neurogenic lineage (Ziman et al., 2001), suggesting the importance for Pax7 in skeletal muscle. Alternative forms of Pax7 were found to be expressed in myogenic cell lines versus tumor cell lines, which consisted of eight exons or nine exons, respectively (Barr et al., 1999). Recently, these two alternative forms were shown to differ because of differential RNA cleavage-polyadenylation and splicing, resulting in inclusion or exclusion of the conserved carboxy-terminal domain (Vorobyov and Horst, 2004).

1.43 COMMITMENT TO THE MYOGENIC LINEAGE OF CELLS EXPRESSING PAX7

Once a cell expresses Pax7, it becomes committed to the myogenic lineage (Seale et al., 2000). Pax7 has been shown to be expressed in quiescent satellite cells (Zammit and Beauchamp, 2001; Asakura et al., 2002). Mice that are Pax7-/- do not contain satellite cells (Seale et al., 2000), which suggests that Pax7 is necessary for the formation or maintenance of satellite cells. Recently, stem cells resident in adult muscle underwent myogenic specification following the expression of Pax7 (Seale et al., 2004). These studies demonstrate the importance of Pax7 to satellite cell dynamics.

1.44 PAX7 EXPRESSION MAINTAINS A PROLIFERATIVE RESERVE OF SATELLITE CELLS

Recently, Pax7 expression has been shown not only in quiescent satellite cells, but also briefly during activation and is coexpressed for a short time with MyoD (Zammit et al., 2004). Pax7 expression was up-regulated at the onset of proliferation following a period of stress to muscle (Zhao and Hoffman, 2004), indicating an increase in the activation of a proliferative reserve needed to replenish the satellite cell population. The absence of Pax7 can result in a decrease in the number of satellite cells because the mechanism for satellite cell self-renewal is not present. Oustanina et al. (2004) showed that mice lacking Pax7 were populated by satellite cells, but they were incapable of muscle regeneration. Pax7 and myogenin expression are mutually exclusive (Olguin and Olwin, 2004), suggesting that myogenin is a marker differentiation and donation of the

nuclei to the existing myofiber and Pax7 may be a marker for the maintenance of a renewal population of satellite cells. The overexpression of Pax7 also down-regulates the expression of MyoD and prevents myogenin expression (Olguin and Olwin, 2004), once again demonstrating the role of Pax7 to maintain a proliferative reserve population. Further showing the need for Pax7 in maintaining a self-renewal population, cells that escape differentiation and exit the cell cycle up-regulate the expression of Pax7 (Olguin and Olwin, 2004). During the activation process of quiescent satellite cells, there is a brief period of co-expression of Pax7 and MyoD, but Pax7 is quickly down-regulated during proliferation (Zammitt et al., 2004). However, some cells during proliferation continue to express Pax7, but not MyoD, and then leave the cell cycle prior to differentiation (Zammitt et al., 2004). Once they leave the cell cycle, they regain their quiescent satellite cell status (Zammitt et al., 2004). It may be possible that this population of cells produces satellite cells during proliferation to further differentiate, but retain the ability to divide again at a later time. Similar findings that indicate Pax7 is an early marker of proliferation have been observed in chickens (Halevy et al., 2004). *In vitro*, Halevy et al. (2004) found that at all time frames tested there was a population of Pax7+/MyoD- cells indicating the proliferative reserve is maintained throughout the early post-hatch period. A similar relationship of Pax7 to myogenin found in the mouse has also been observed in the chicken (Halevy et al., 2004). All of these recent data indicates the most important role of Pax7 in postnatal muscle is to maintain a proliferative population of satellite cells that can be up-regulated following a period of stress to form daughter satellite cells.

1.5 STEM CELLS IN SKELETAL MUSCLE

Stem cells are cells that have a high level of plasticity and can differentiate into a variety of cell types. Stem cells in skeletal muscle can either be referred to as stem cells that give rise to skeletal muscle, presumably through a satellite cell lineage, or cells that originate in muscle and differentiate into other tissues. Both situations have been suggested by previous work (Asakura et al., 2001, Ferrari et al., 1998; Grounds et al., 2002). However, the location of these cells and their status as satellite cells remains unclear because many of the studies have not identified cellular location before isolating cells and determining stem cell capabilities.

1.51 STEM CELLS WITHIN THE SATELLITE CELL POPULATION

As mentioned previously, satellite cells are sub-laminal cells that are mitotically active and can donate myonuclei to the existing myofiber. However, the ability to be mitotically active and the presence of a self-renewing population of satellite cells has indicated to some researchers that certain populations of satellite cells may have stem cell/multipotential cell capabilities. Cells isolated from skeletal muscle can reconstitute the hematopoietic compartment that participates in muscle regeneration, has led some researchers to conclude that pluripotential stem cells are present in adult skeletal muscle within the satellite cell population. (Seale and Rudnicki, 2000) Satellite cells have been considered to have the ability to exhibit multipotential mesenchymal stem cell activity because under optimal conditions *in vitro* myogenic satellite cells differentiated into adipogenic and osteogenic lineages (Asakura et al., 2001). Shefer et al. (2004) concluded

that myogenic satellite cells possess mesenchymal stem cell capabilities because cells expressing Pax7 committed to myogenic and non-myogenic pathways *in vitro*. However, it also has been observed in isolated muscle fibers that markers present in multipotent adult skeletal muscle-derived stem cells were not coexpressed in cells expressing Pax7 (Zammit and Beauchamp, 2001), suggesting a population of stem cells that is separate from satellite cells lying outside the lamina. Grounds et al. (2002) through an investigation of skeletal and cardiac muscle, and the literature concluded that satellite cells did not represent a population of multipotential stem cells. At this time, it is difficult to dissect the literature to identify satellite cells with stem cell capabilities.

Stem cells also have been proposed to originate from connective tissue found in skeletal muscle. In skeletal muscle isolated from chicken embryos, it was concluded that a population of mesenchymal stem cells was present in connective tissue (Young et al., 1993). Postnatal mammals also contain pluripotent mesenchymal stem cells derived from skeletal muscle connective tissue (Young et al., 2001a). Finally, mesenchymal stem cells present in skeletal muscle connective tissue derived from fetal, adult, and geriatric donors differentiated into muscle, adipocytes, carilage, bone, fibroblasts, and endothelial cells (Young et al., 2001b), indicating the presence of stem cells within the skeletal muscle tissue.

Finally, bone marrow-derived stem cells have been implicated in muscle regeneration. Corbel et al. (2003) found that hematopoietic stem cells gave rise to both blood and muscle tissue. A low incidence of bone marrow-derived stem cells incorporated into muscle tissue has also been reported (Ferrari et al., 1998). Other studies have shown a similar low rate of differentiation of bone marrow-derived stem

cells into muscle tissue (Bittner et al., 1999; Gussoni et al., 1999). LaBarge and Blau (2002) suggested that bone marrow-derived stem cells not only differentiate into muscle but also contribute to the satellite cell population.

It is difficult to determining if cells with stem cell capabilities found in muscle arise from the heterogeneous satellite cell population or are associated with connective tissue because they have not clearly been identified. A weakness of these intriguing studies was that many employed whole muscle homogenates and did not identify cellular location before identifying multipotential cells in muscle.

1.52 PROTEINS EXPRESSED IN STEM CELLS FOUND IN SKELETAL MUSCLE

Some proteins expressed in myogenic cells with stem cell potential are CD45, stem cell antigen-1 (SCA-1), CD34, and Bcl-2. Most of the stem cell properties exhibited by skeletal muscle are due to its hematopoietic potential of cells expressing the hematopoietic markers Sca-1 and CD45 (Jackson et al., 1999). A side population of muscle cells considered to have stem cell properties that consists of 92% Sca-1+ cells, 16 % CD45+ cells, 9.2% Sca-1+/CD45+, and 6.8% Sca-1-/CD45+ (Asakura et al., 2002). CD45+ cells are more responsible for the hematopoietic activity exhibited in muscle than CD45- cells (Charge and Rudnicki, 2004). CD45+ stem cells from uninjured muscle are nonmyogenic, but CD45+ cells from injured muscle are readily myogenic (Polesskaya et al., 2003), and are activated by Pax7 (Seale et al., 2004). Sca-1 positive cells have been

found outside the basal lamina, and CD45 and Sca-1 are not specifically myogenic (Asakura et al., 2002).

CD34+/CD45- cells isolated from skeletal muscle possessed a small potential to differentiate into adipocytes and endothelial cells (Tamaki et al., 2003), suggesting the stem cell capabilities of CD34+/CD45- cells. In the same experiment, colony forming units that were CD34-/CD45- expressed CD34 prior to the expression of MyoD when forming myogenic cells (Tamaki et al., 2003), indicating its upstream location in the myogenic cascade. Jankowski et al. (2002) found that CD34+ cells, that were considered to be in the stem cell compartment by plating techniques, led to myogenic progenitors and differentiation in regenerating muscle. CD34+/Sca-1+ cells exhibited stem cell properties when injected into *mdx* mice resulting in the restoration of dystrophin following an injection of the cells (Torrente et al., 2001). Hwang et al. (2004) showed the stem cell capabilities of skeletal muscle CD34+ or Sca-1+ and CD45- cells by co-culture cells with smooth muscle resulting in the differentiation into smooth muscle.

Bcl-2 is expressed in about 1-4% of cells in young muscle, and is found in mononucleated cells (Dominov et al., 1998). Dominov et al. (1998) also showed that cells expressing Bcl-2 were found in the early stages of myogenesis. It is evident that Bcl-2 expressing cells express CD34, but do not express satellite cell markers, such as m-cadherin and c-met (Deasy et al., 2001; Qu-Petersen et al., 2002). Bcl-2+ cells reside within in the basal lamina meeting the definition of satellite cells (Lee et al., 2000), suggesting they are satellite cells. Therefore, it seems reasonable to use Bcl-2 or CD34 as a marker for identifying the heterogeneous satellite cell population involved in muscle dynamics because of its location and possible commitment to the myogenic lineage.

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2. OVERALL OBJECTIVE

The goal of many turkey operations is to maximize the amount of breast meat yield at market age by management and feeding high levels of protein, which have been extensively studied (Noll, 2002). However, maximizing production through conventional dietary manipulations and management will reach a limit, and other avenues of increasing meat production must be explored.

An area scientifically important for the turkey industry is the muscle development of the turkey. Post-hatch myofibers contain myonuclei that are incapable of mitotic division (Stockdale and Holtzer, 1961), which means an external source of myonuclei must be available for the myofiber to acquire myonuclei. Satellite cells are all cells found between the basal lamina and the sarcolemma of the myofiber (Mauro, 1961), and are mitotically active post-hatch (Feldman and Stockdale, 1992). In the turkey, satellite cell mitotic activity decreased from 3 to 9 weeks of age, and from 9 to 26 weeks of age, myofiber growth mainly occurs through an increase in DNA unit size (Mozdziak et al., 1994). Therefore, the number of myonuclei present in the muscle early in life can influence mature muscle size.

Satellite cells are heterogeneous (Schultz, 1996). These sub-populations are responsible for muscle regeneration, and possibly satellite cell self-renewal (Rouger et al., 2004; Zammitt et al., 2004). It has also been suggested that a small portion of satellite cells exhibit stem cell properties (Asakura et al., 2001; Morgan and Partridge, 2003). An understanding of satellite cell and stem cell dynamics is the next step towards maximizing muscle development.

Finally, the influence of nutrition on satellite cell dynamics is important in improving muscle development. Feed deprivation in chicks early post-hatch has a negative impact satellite cell proliferation and a decrease in muscle weight at market age (Halevy, et al., 2000). Numerous studies have shown similar results (Nir and Levanon, 1993; Vieira and Moran 1999a; Vieira and Moran 1999b). Conversely, offering nutrients to poults in solid, semi-solid, or liquid form immediately post-hatch improves body weight and breast meat percentage of body weight at market age Noy and Sklan (1999a).

The specific objectives of this thesis were:

1. To develop a new method for BrdU delivery into the avian fetus that allows for the precise location of the delivery to each fetus.
2. To test the *in ovo* injection of nutrients to the turkey embryo and the influence *in ovo* feeding has on satellite cell mitotic activity immediately post-hatch.
3. To examine the influence of HMB on satellite cell mitotic activity in the newly hatched poult.
4. To examine the influence of fasting on skeletal muscle dynamics of the immediately post-hatch poult.
5. To understand the skeletal muscle dynamics involving the satellite cell population.

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3. BROMODEOXYURIDINE ADMINISTRATION TO AVIAN FETUSES

3.1 ABSTRACT

The egg shell is a barrier to administering compounds to late-incubation avian fetuses. 5-bromo-2'-deoxyuridine (BrdU) is a thymidine analog that can label mitotically active cells during the S-phase of cell division. A method was developed to administer compounds to late incubation turkey fetuses. BrdU was precisely administered into the peritoneal cavity of turkey fetuses on day 25 of incubation. Subsequently, muscle was harvested from all injected birds, and BrdU was detected using immunohistochemical techniques. BrdU was successfully detected in all fetuses.

Key words: 5'-Bromo-2'-deoxyuridine (BrdU), chicken, turkey, mitotic activity, myoblast, satellite cells

3.2 INTRODUCTION

The embryological development of skeletal muscle begins with the fusion of mononucleated myoblasts to form myotubes, which mature into myofibers (Schultz and McCormick, 1994). By late embryogenesis, myoblasts found in the chicken fetus may be referred to as satellite cells, and they predominantly have adult characteristics (Hartley et al., 1992). Satellite cells are located underneath the myofiber basal lamina along the entire length of the myofiber (Campion, 1984). Satellite cells are a mitotically active cell population (Asakura et al., 2001) making it possible to label the cells with ^{3}H-thymidine, whereas pre-existing myonuclei will not be ^{3}H labeled because they do not synthesize DNA (Stockdale and Holtzer, 1961; McCormick and Schultz, 1992).

BrdU (5-bromo-2'-deoxyuridine) is a thymidine analog that labels cells entering the S-phase of the cell cycle. A viable method of studying satellite cell mitotic activity aimed at understanding early avian muscle development is to use BrdU immunohistochemistry (Mozdziak et al., 1994a; Mozdziak et al., 1994b). When administering BrdU to a post-hatch bird, delivery is accomplished by an intra-peritoneal or intravenous injection, but the hard shell surrounding the avian fetus makes it technically difficult to deliver BrdU effectively to the fetus. Applegate et al., (1999) injected BrdU at 26 days of incubation (26E) through the air cell directly into the turkey fetus; however, the exact point of delivery was unknown making it a rather imprecise method. Furthermore, BrdU would be transported throughout the fetus at a much slower rate after an intramuscular injection compared to an intra-peritoneal or intravenous injection.

The objective of this study was to develop a new method for BrdU delivery into the avian fetus that allows for the precise location of the delivery to each fetus. The ability to inject BrdU consistently to the correct fetal compartment is important because it decreases the variability associated with being unable to determine the precise orientation of the avian fetus. This study used turkey fetuses at 25 days (25E) of incubation to test a new technique for delivering BrdU *in ovo*. The technique was deemed successful when muscle from the fetuses was successfully stained for BrdU using immunohistochemistry.

3.3 MATERIALS AND METHODS

BrdU Delivery

Thirteen Hybrid turkey eggs were obtained from a commercial hatchery, and they were incubated at 37.5 °C at North Carolina State University. All fetuses were injected with a 10 mg/ml solution of BrdU (1mL/100grams of egg weight; 0.1mg BrdU/g egg weight; modified based on Mozdziak et al., 1994a; Mozdziak et al., 1994b) intra-peritoneally at 25E. The eggs were candled to determine the location of the abdomen and a mark was placed on the egg to indicate the site of entrance. Once the site of entrance was determined, the egg was washed with 70% ethanol. A small hole of approximately 5-10 mm in diameter was incised in the eggshell directly above the site of the intended BrdU injection. Fetuses were observed using incident light illumination. Aspiration of any fecal matter or blood indicated improper needle placement. If fecal matter or blood was withdrawn from an embryo before BrdU administration, then the needle was withdrawn and the injection was performed with another needle and syringe (Figure 1). Once injected, the holes in the eggshell were sealed with scotch tape, and the eggs were returned to the incubator until sampling (Figure 2). The BrdU was administered using a 22-gauge hypodermic needle fitted to a syringe for all fetuses. After the BrdU injection, two hours were allowed for the BrdU to be incorporated into the nuclei entering the S-phase of the cell cycle. The short interval between BrdU injection and euthanasia ensures that myonuclei are not labeled (Moss and Leblond, 1971; Schultz, 1996).

Tissue Sampling

The *Pectoralis thoracicus* was harvested and fixed with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) overnight then dehydrated, cleared, and embedded in paraffin. Eight-micron thick sections were cut on a microtome, and adhered to glass slides. Tissues were de-waxed, hydrated, and treated with 0.07N NaOH for three minutes. Phosphate Buffered Saline (PBS-pH 7.4) was used to neutralize the NaOH. The primary monoclonal antibody [Anti-BrdU[1] -diluted 1:20 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin) was added to the sections and incubated for two hours in a humidified chamber at room temperature to detect BrdU labeled nuclei. Negative control slides were prepared by omitting the primary antibody from some incubations. After the tissue sections were blocked with PBS containing 10% goat serum and 0.5% tween-20 for fifteen minutes to minimize background staining, the secondary antibody was added to the sections for two hours in a humidified chamber at room temperature to allow for visual observation of the BrdU labeled nuclei under a microscope. The secondary antibody was goat anti-mouse IgG conjugated to fluorescein-isothiocyanate (FITC[2]), which was diluted 1:50 with PBS containing 10% goat serum and 0.5%Tween-20. Propidium iodide[3] (50 mg/ml in PBS) was added to the sections for 20 minutes to label all nuclei. Finally, the sections were placed in mounting media [75% (vol/vol), 75mM KCL, 10mM tris(hydroxymethyl)aminomethane, 2mM $MgCl_2$, 2mM ethylene glycol-bis(*b*-amino-ethyl ether)-*N*,*N*,*N*',*N*'- tetraacetic acid, 1mM NaN_3, pH

[1] Becton Dickinson, Mountain View, CA 94039
[2] Sigma, St. Louis, MO 63103
[3] Molecular Probes Inc., Eugene OR 97402

8.5, 1 mg/ml p-phenylenediamine] (Swartz et al., 1990) and a cover slip was sealed over the section using nail polish.

Image Analysis

A Leica DMR[4] microscope with epiflorescence illumination was used to observe the tissue sections. The BrdU labeled nuclei were observed with a FITC filter set and all nuclei in the muscle were observed with a propidium iodide (PI) filter set[5]. A Spot-RT CCD[6] camera was used to capture the images of nuclei visualized by the FITC and PI filter sets. The FITC and PI labeled nuclei were counted using the Image-Pro Plus software[7]. Myofiber cross-sectional area was also determined using Image-Pro Plus software exclusively using muscle sections at a cross-sectional orientation. The number of BrdU labeled nuclei per number of PI labeled nuclei was used as an index for determining satellite cell mitotic activity (Mozdziak et al., 1994a; Mozdziak et al., 1994b). The criteria used for completing the nuclear analysis were counting at least 1000 PI labeled nuclei. Labeled nuclei clearly lying within the endomysial or the perimysial tissue compartments were excluded from analysis. Satellite cell mitotic activity for the thirteen fetuses tested was determined to be 1.4% of all nuclei (Table 1).

[4]Leica Microsystems, Bannockburn, IL 60015
[5] Omega Optical, Brattleboro, VT 05301
[6] Diagnostic Instruments Inc., Sterling Heights, MI 48314
[7] Media Cybernetics, Silver Spring MD 20910

3.4 RESULTS

Thirteen out of the thirteen 25 day-old turkey fetuses tested successfully stained for BrdU (Figure 3) and PI (Figure 4), and survived the entire two-hour post-injection period in the incubator. All fetuses were deemed viable after the two-hour incubation period because they were all alive and active when the shell was removed.

3.5 DISCUSSION

Myoblasts found in the late stage chicken fetus may be referred to as satellite cells, and predominantly have adult characteristics (Hartley et al., 1992). Satellite cells are located between the myofiber basal lamina and sarcolemma (Campion, 1984). Post-hatch myonuclei cannot be ^{3}H labeled because they do not synthesize DNA (Stockdale and Holtzer, 1961; McCormick and Schultz, 1992). However, satellite cells are a mitotically active cell population (Asakura et al., 2001) making it possible to label the cells with ^{3}H-thymidine,

The thymidine analog, BrdU, labels cells entering the S-phase of the cell cycle. BrdU immunohistochemistry is a viable method of studying satellite cell mitotic activity during muscle development (Mozdziak et al., 1994a; Mozdziak et al., 1994b). An intra-peritoneal or intravenous injection is used to deliver BrdU to a post-hatch bird. However, the hard shell surrounding the avian fetus makes it technically difficult to deliver BrdU effectively to the fetus. BrdU had been injected at 26 days of incubation (26E) through the air cell directly into the turkey fetus Applegate et al., (1999). However, it is an imprecise method because the exact point of delivery was unknown. Furthermore, BrdU would be transported throughout the fetus at a much slower rate after an intramuscular injection compared to an intra-peritoneal or intravenous injection.

The new technique for the *in ovo* administration of BrdU provides a direct and reliable method of BrdU delivery to the avian fetus. The delivery of BrdU directly into the fetus assures that the amount and volume of BrdU delivered is accurate and consistent for every fetus involved in a study. The present technique allows for the survival of the fetus for at least two hours, and it may be possible to apply fetal BrdU injections to study

BrdU labeled cell fate in post-hatch animals because viable chicks have resulted following manipulations of stage 10 to 15 chicken embryos (Hamburger and Hamilton, 1951; Giamario et al., 2003). The technique of in ovo intra-peritoneal administration of BrdU was deemed a successful means for studying satellite cell mitotic activity in the avian fetus. Methodology used in this experiment provides an accurate and easy to follow procedure for BrdU delivery.

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Table 1. Myofiber cross-sectional area (CSA), satellite cell mitotic activity, and nuclei per unit area of 25-day fetuses determined using the in ovo administration of BrdU.

Fetus Number	Myofiber CSA[A] (μm^2)	Index of Satellite Cell Mitotic Activty [B]
F1	16	0.020
F2	15	0.006
F3	16	0.022
F4	12	0.009
F5	17	0.019
F6	16	0.015
F7	12	0.017
F8	19	0.015
F9	15	0.021
F10	28	0.006
F11	37	0.013
F12	15	0.004
F13	15	0.016
Average	18	0.014
Standard Deviation	7	0.0061

[A]CSA= Cross sectional area

[B] Index of satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocynate labeled nuclei per 100 propidium iodide labeled nuclei.

Figure 1. Injection of BrdU into 25E turkey embryo in ovo.

Figure 2. Egg sealed and ready to return to incubator.

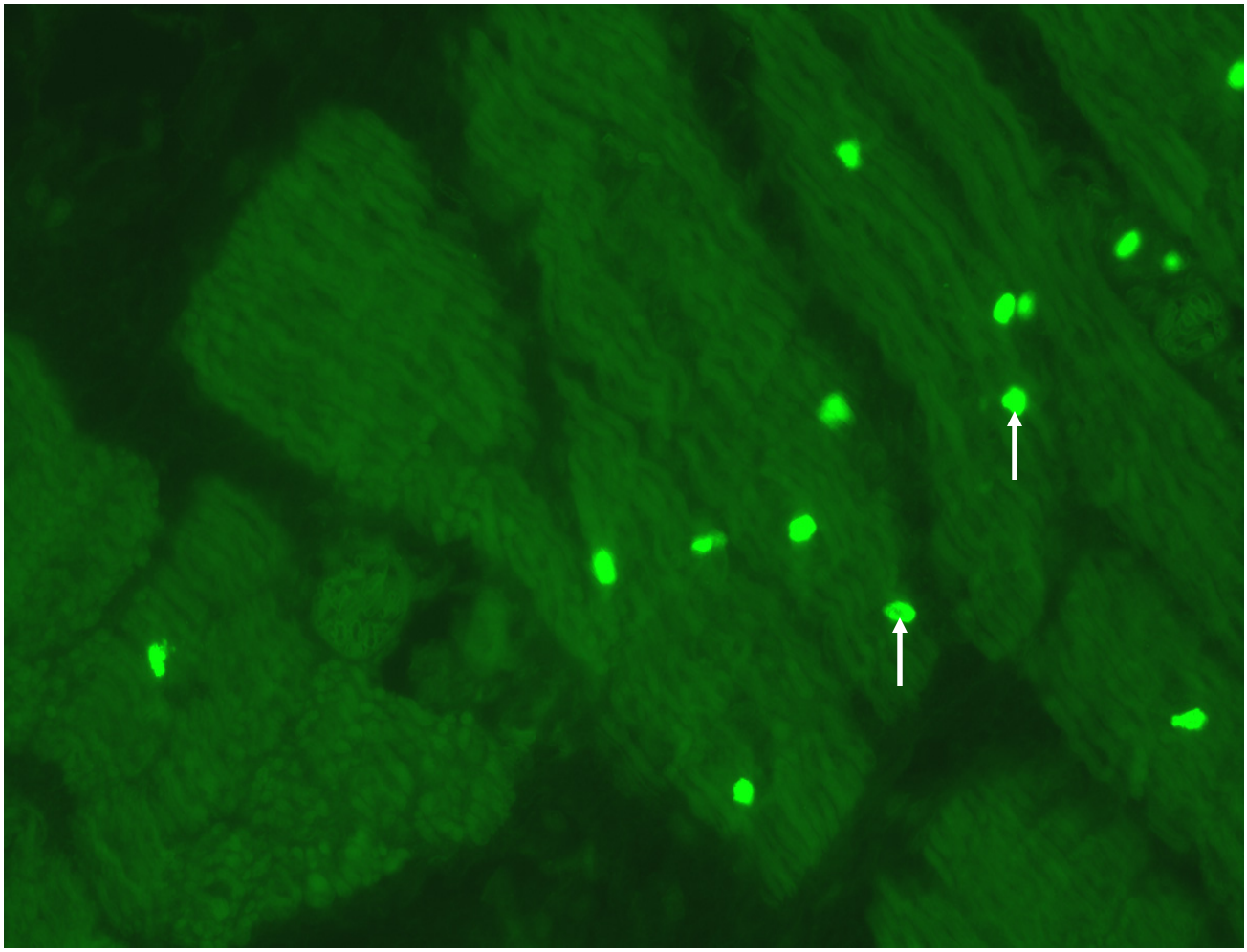

Figure 3. BrdU labeled nuclei of 25E and arrow marked nuclei correspond to arrow marked nuclei in Figure 4. Figure 3 is the same cross-section as figure 4 viewed through a FITC filter.

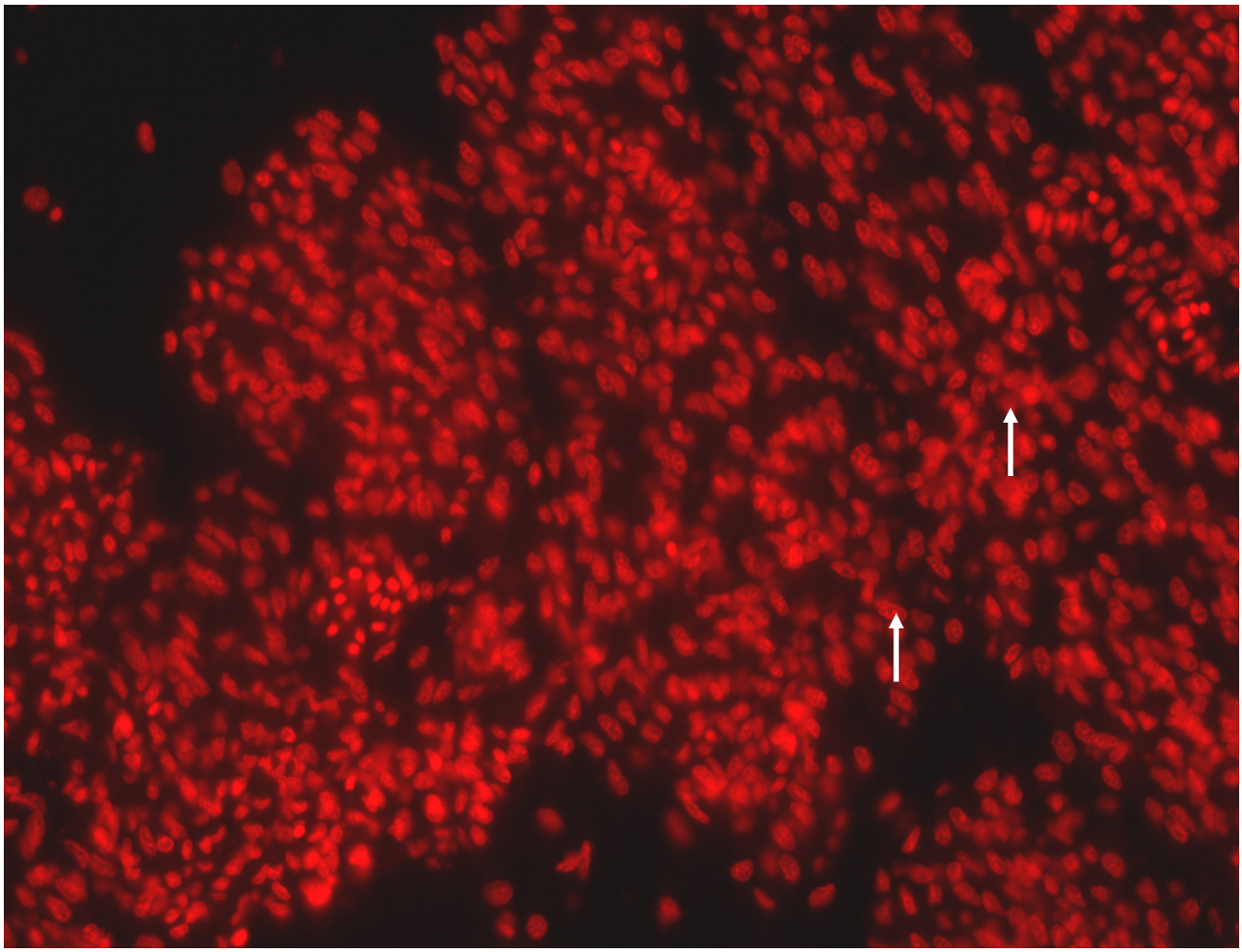

Figure 4. Propidium Iodide labeled nuclei viewed through a PI filter.

4. THE EFFECT OF IN OVO NUTRIENT INJECTION ON SATELLITE CELL MITOTIC ACTIVITY IN THE TUREKY

4.1 ABSTRACT

The objective of this experiment was to test the in ovo injection of nutrients to the turkey embryo and the influence in ovo feeding on satellite cell mitotic activity in the turkey embryo, early post-hatch period, and 1 week of age. The embryos were in ovo fed at 22 days of incubation (22E). The treatments were injected with 0.4% saline (control) or 0.4 % saline, 18% egg white protein, and 0.1% β-hydroxy-β-methylbutyrate (HMB). All poults were fed a standard corn and soybean meal based starter diet throughout the experiment. 5-bromo-2'deoxyuridine (BrdU) was injected intra-abdominally into all poults and embryos. *Pectoralis thoracicus* were taken two hours post-injection in order to determine mitotically active satellite cells. Samples were taken at 25 days of incubation (25E), day of hatch, 1 day of age, and 1 wk of age (n = 5 for all groups). BrdU immunohistochemistry along with computer-based image analysis was used to identify the mitotically active satellite cells. There were no differences (***P* ≥ 0.05**) in satellite cell mitotic activity between treatments of birds the same age. However, there were differences (***P* ≤ 0.05**) in satellite cell mitotic activity between ages of poults, suggesting a developmental window of satellite cell mitotic activity that may be manipulated.

Key Words: In Ovo Feeding, BrdU, Myoblasts

4.2 INTRODUCTION

Embryological development of the muscle begins with the fusion of mononucleated myoblasts to form a myotube, which matures into a myofiber (Schultz, 1994). Feldman and Stockman (1992) showed that during avian development a distinct population of satellite cells is present at the midfetal stages of development, and by late embryogenesis myoblasts found in the chicken embryo are predominantly adult myoblasts (Hartley et al., 1992). Once the bird is hatched and myofibers are formed, normal skeletal muscle growth occurs through an increase in myofiber size, and not an increase in myofiber number (Remignon, 1995). Myofiber number does not increase during normal postnatal growth because myonuclei are postmitotic and can not synthesize DNA (Stockdale and Holtzer, 1961). An increase in fiber size in postnatal vertebrates is directly related to an increase in myonuclei, which increases muscle fiber DNA content (Allen et al., 1979). An external cell source must donate nuclei due to the mature myofibers inability to divide. The source of myofiber DNA is the satellite cell.

Satellite cells are located beneath the basal lamina of the myofiber, and they are found throughout the length of the muscle (Mauro, 1961; Campion, 1984). Recently, it has been suggested that muscle satellite cells exhibit multipotential mesenchymal stem cell activity (Asakura et al., 2001). Moss and Leblond (1971) showed that the majority of the mitotically active satellite cells were incorporated into the myofiber immediately following mitosis in postnatal rats. Myonuclear accretion is regulated by the surrounding myofibers (Bishcoff, 1990; Mozdziak et al., 1998) suggesting stress to the myofiber may increase the mitotic activity of the satellite cell. Animal agriculture may be able to increase muscle size in turkeys by the manipulation of satellite cell nuclear donation;

however, a target period of the animal must be identified due to possible age related changes in satellite cell mitotic activity.

Moss and Leblond (1971) showed satellite cell incorporation into the myofiber in the young postnatal rat. In 1975 Cardasis and Cooper conducted a thorough study on the animal development and satellite cell activity. The findings indicated that satellite cells were first associated with myofibers at 19 days of gestation in the mouse. The same study estimated a decrease in satellite cell activity from 32% to 6% from birth to adulthood. The decreases in satellite cells in mice came between 2 weeks of age and 4 weeks of age (Cardasis and Cooper, 1975). Similar results have been reported in turkeys. Mozdziak et al., (1994) showed an age-related decrease in satellite cell mitotic activity from 3 to 9 weeks of age in the turkey. However, from 9 to 26 weeks of age myofiber growth in turkeys occurred mainly through an increase in the cytoplasmic to myonucleus ratio (Mozdziak et al., 1994). Short-term reductions in satellite cell mitotic acitvity early in life lead to a reduction in muscle size at maturity (Mozdziak et al., 1997; Mozdziak et al., 2000). Overall, it appears that early postnatal muscle development appears to be the most crucial time period for satellite cell mitotic activity.

In the poultry industry, a delay between hatching and placement of the birds in the brooder house is a common management practice. As described previously, the early post-hatch period may be a very important developmental window for myonuclear accretion and it has been shown that delayed placement results in decreased body weights (Halevy et al., 2000). Hager and Beane (1983) held broiler chicks for varying lengths of time in the incubator to simulate holding times in the industry. Chicks that were held in the incubator for 36 hours post-hatch were 10.3% lighter than chicks that were removed

immediately. The difference in body weights was still significant at 4 weeks of age (Hager and Beane, 1983; Halevy et al., 2000). It also has been shown that turkey poults held in the incubator for 48 hours post-hatch face an energy shortage resulting in differing body composition than control birds immediately removed from the incubator (Pinchasov and Noy, 1993). Further complicating the lives of poults held in the incubator, additional heat stress increased the mortality rate during the holding period (Pinchasov and Noy, 1993). Nir and Levanon (1993) found that male broiler chicks held in the incubator for 24 and 48 hours had lower body weights compared to the controls, and compensatory growth did not regain the weight lost by holding time. The difference in the weight lost was found to be 1 or 2 days body weight at market age for the 24 and 48 hours holding periods, respectively (Nir and Levanon, 1993). Male broiler chicks with a 24 hour holding time exhibit reduced yolk sac content, reduced carcass weight at 7 weeks of age, and had increased mortality 21 to 42 days after placement (Vieira and Moran, 1999a). These results suggest a prolonged holding time, as is practiced for practical reasons in the industry, has a definite negative impact on young birds. Studies involving the delayed access to feed and water have also shown decreased body weights and reduced meat yield at market age (Noy and Sklan, 1999b; Vieira and Moran 1999b). Finally, Mozdziak et al., 2002a observed a larger ratio of apoptotic myonuclei compared to total myonuclei of immediately starved post-hatched chickens when compared to fed controls. The results of delayed placement time of poults may negatively impact the satellite cell mitotic activity due to the small window of time the satellite cell is mitotically active in the young bird. As stated previously, a negative impact on satellite cell mitotic activity (i.e. starvation) at a young age decreases adult muscle weight and the

animal will never achieve its genetic potential (Halevy, 2000). Re-feeding after a starvation period of 48 hours does not compensate for the missed nutrients, and negatively impacts satellite cell mitotic activity (Mozdziak et al., 2002b). Breast muscle RNA and protein content were also reduced in an immediately starved post-hatch bird following re-feeding (Yaman et al., 2000). Evidence from this research suggests that re-feeding is not the answer to the starvation that is a result of holding time in the incubator. Immediate feeding post-hatch has been shown to increase body weights of birds when compared to poults with delayed access to feed (Noy and Sklan, 1999a). The future of turkey nutrition should focus on this early post-hatch period and the delivery of a nutritional package to young birds to allow them to reach their full genetic potential for breast muscle size.

A new technology that may reduce the problems caused by delayed placement of poults in the industry is in ovo feeding. In ovo technology may alleviate the need to feed poults immediately post-hatch and, therefore, has potential in reducing body weight deficiencies exhibited by delayed placed poults. Al-Murrani (1982) injected amino acids in ovo to chicken embryos and found an increase in body weight from hatch to market when compared to non-supplemented chicks. Amino acid injection into broiler chicks via the yolk or air cell has also been shown increase chick weight at hatch (Ohta et al., 1999). The in ovo administration of vitamin E resulted in enhanced antibody and macrophage response in poults and chicks (Gore and Qureshi, 1997). However, little to no research has been done on in ovo injection of a nutrient package to the turkey embryo.

The objective of this experiment is to test the in ovo injection of nutrients to the turkey embryo and the influence in ovo feeding has on satellite cell mitotic activity in the turkey embryo, the early post-hatch turkey poult, and the 1 week old poult.

4.3 MATERIALS AND METHODS

Turkeys

Hybrid turkey eggs were obtained from a commercial hatchery and placed in an incubator at North Carolina State University hatching facility. When the embryos were 22 days (22E) they were removed from the incubator and weighed. After weighing, two groups of seventy eggs each were formed with each group averaging between 77 and 78 grams per egg. Once the groups were formed, the eggs were labeled and placed back in the incubator. One group was designated the control group (in ovo injection of 0.4% saline) and the other group was designated the supplemented treatment [in ovo injection containing 0.4% saline, 18% egg white protein, and 0.1% β-hydroxy-β-methylbutyrate (HMB)]. Egg white protein was used to provide a digestible protein source to the embryo that would have a similar amino acid profile needed by the embryo. The nutrient mix also contained HMB because HMB has been shown to decrease mortality, increase breast meat yield, and allows for maximal cell growth and function (Nissan et al., 1994;Nissan and Abumrad, 1997).

The embryos also were fed in ovo at 22E. Eggs were candled to identify the site of injection into the amnion. The injection was performed using a 22-gauge hypodermic needle fitted to a syringe. Each embryo received 1.5ml of in ovo feed solution or 0.4% saline depending on the assigned treatment. Once the embryos were injected, the site was sealed with tape to prevent the entrance of undesirable bacteria and the eggs were placed into the incubator until further sampling or hatching. Immediately following hatching, poults were placed in floor pens and fed a standard corn and soybean meal starter diet. All birds were weighed at hatch and at seven days of age. At seven days of age, five

birds from each treatment were killed and the *Pectoralis thoracicus* and *supracoracodeus* were removed and weighed. Five birds per treatment at ages 25E, day of hatch, 1 day old, and 1 week old were randomly selected from the original population of poults and administered with 5-Bromo-2'-deoxyuridine (BrdU; 10mg/ml; 1ml/100g of bird weight), a thymidine analog. All embryos and poults were injected with BrdU intra-peritoneally. The eggs were candled to determine the location of the abdomen in the 25E embryos. Once the abdomen was located, a small hole was made in the shell directly above the site of the intended BrdU injection. Once injected, the holes were sealed with tape and the eggs were returned to the incubator until sampling. The BrdU was administered using a 22-gauge hypodermic needle fitted to a syringe for all embryos and poults. After the BrdU injection, two hours were allowed for the BrdU to be incorporated into the nuclei entering the S-phase of the cell cycle. The poults and embryos were killed at the end of two the hours by cervical dislocation.

Immunohistochemistry

The *Pectoralis thoracicus* was harvested and fixed with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) overnight in 20ml scintillation vials. The next morning the tissues were dehydrated, washed, and embedded in paraffin. Eight micron thick sections were cut out of the paraffin block on a microtome and adhered to glass slides. Tissues were then de-waxed and hydrated. Following the hydration of the tissues, 0.07N NaOH was added to the slides for three minutes. Phosphate Buffered Saline (PBS-pH 7.4) was then used to neutralize the NaOH. The primary monoclonal antibodyAnti-BrdU[1]-diluted 1:20 in PBS containing 0.5% tween-20

[1] Becton Dickinson, Mountain View, CA 94039

and 0.5% bovine serum albumin) was added to the sections and incubated for two hours in a humidified chamber. After the tissue section was blocked with PBS containing 10% goat serum and 0.5%tween-20 for fifteen minutes to minimize background staining, the secondary antibody was added to the sections for two hours in a humidified chamber to detect the BrdU labeled nuclei. The secondary antibody was goat anti-mouse IgG conjugated to fluorescein-isothiocyanate (FITC), which was diluted 1:50 with PBS containing 10% goat serum and 0.5%tween-20. Propidium iodide (50 μg/ml in PBS) was added to the sections for 20 minutes to label all nuclei. Finally, the sections were placed in mounting media [75% (vol/vol), 75mM KCL, 10mM tris(hydroxymethyl)aminomethane, 2mM $MgCl_2$, 2mM ethylene glycol-bis(*β*-amino-ethyl ether)-*N*,*N*,*N*',*N*'- tetraacetic acid, 1mM NaN_3, pH 8.5, 1mg/mlp-phenylenediamine] and a cover slip was sealed using nail polish. The slides were then placed in opaque containers for storage.

Image Analysis

A Leica DMR[2] microscope equipped with epiflorescence illumination was used to observe the tissue sections. All nuclei were observed with a propidium iodide (PI) filter set,[3] and the BrdU labeled nuclei were observed with a FITC filter set. Spot-RT CCD[4] camera was used to capture the images of nuclei visualized by the FITC and PI filter sets. The FITC and PI labeled nuclei were counted using the Image-Pro Plus software[5]. The number of BrdU labeled nuclei per 1000 PI labeled nuclei was used as an

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[2] Leica Microsystems, Bannockburn, IL 60015
[3] Omega Optical, Brattleboro, VT 05301
[4] Diagnostic Instruments Inc., Sterling Heights, MI 48314
[5] Media Cybernetics, Silver Spring MD 20910

index for determining satellite cell mitotic activity. The criteria for completing nuclear analysis were counting at least 1000 PI labeled nuclei. The area of muscle section containing the count of propidium iodide labeled nuclei was also determined. The purpose of collecting the muscle section area was to express the number of PI labeled nuclei in relation to the cross-sectional area of the muscle. Image-Pro Plus software was also used to determine myofiber cross-sectional area for at least 100 myofibers per muscle sample.

Statistics

General Linear Models procedure of SAS (SAS Institute, 1985) was used to perform a one-way analysis of variance to determine the treatment effect on day of hatch weight, 7 day weight, body weight gain, muscle weight to body weight ratio, the number of PI labeled nuclei per muscle area, myofiber cross-sectional area, and satellite cell mitotic activity. The same statistical procedure was used to determine an age effect on satellite cell mitotic activity. Means were separated using least significant differences (Zar, 1999). A contrast was also performed to determine mean separation of age related effects on satellite cell mitotic activity.

4.4 RESULTS

Growth

Body weights were not significantly different between treatments at all ages examined (Table 1). Consequently, weight gain from day of hatch to 7 days of age was not significantly different between the two groups of poults (Table 1). *Pectoralis thoracicus* and *supracoracodeus* weights per body weight of the bird were not different between treatments at seven days of age (Table 1).

Satellite Cell Mitotic Activity and Number of Total Nuclei

Myofiber area (Table 2) was the same between each treatment for each respective day sampled. Number of PI labeled nuclei per area counted was also the same between treatments at all ages tested (Table 2). The index for satellite cell mitotic activity was the number of BrdU labeled nuclei per number of PI labeled nuclei. The results suggest that there was no difference in satellite cell mitotic activity between the two treatments of poults the same age for all days sampled (Table 2). Interestingly, there appears to be a difference in mitotic activity of satellite cells between poults of different ages at the times tested in this study (Table 3).

4.5 DISCUSSION

A common practice in the poultry industry is holding poults in the hatcher for 24 to 48 hours post-hatch. This practice of holding poults was implemented out of necessity to place large numbers of turkeys in poultry houses at once. However, the result of holding poults without food or water post-hatch can be detrimental because it decreases satellite cell proliferation and overall skeletal muscle growth (Halevy et al., 2000). Growth retardation has been seen in male broiler chicks that were held in the incubator for 24 and 48 hours resulting in a decrease in body weight at market age when compared to chickens removed immediately from the incubator (Nir and Levanon, 1993). Holding time in the incubator has also been attributed to weight loss in the poult and chick that was still present at market age (Hager and Beane, 1983;Pinchasov and Noy, 1993; Halevy et al., 2000). Chicks held in the incubator also experience a decreased yolk nutrient content and increased mortality (Vieira and Moran, 1999a), which suggests a nutrient deficit in the post-hatch bird with delayed access to feed and water. Starvation trials have also been done to simulate increased holding time in the incubator and similar results to the holding trials were attained (Noy and Sklan, 1999b;Vieira and Moran, 1999b). It is likely that a developmental window in muscle development is being missed due to delayed access to feed and water because it has been shown in other models that when muscle is stressed during this time period the muscle never recovers (Mozdziak et al., 1997;Mozdziak et al., 2000).

One indicator of muscle development is satellite cell mitotic activity. Mozdziak et al. (1997) suggests that satellite cell mitotic activity and subsequent myofiber nuclear

accretion is the major factor in turkey muscle growth potential. In the turkey, there appears to be an age-related response of satellite cell mitotic activity (Mozdziak et al., 1994). Mozdziak et al. (1994) showed a decrease in satellite cell mitotic activity as the turkey grew older. Research also indicates similar responses of satellite cells in other juvenile animals (Allen et al., 1979; Moss and Leblond, 1971; Cardasis and Cooper, 1975). Then it is no surprise that a difference in satellite cell mitotic activity between ages of poults was observed in this experiment. However, the satellite cell mitotic activity is greatly increased at day of hatch and 1 day of age when compared to the 25 day embryo and satellite cell mitotic activity is significantly less at seven days of age than at one day of age. Age-related decreases in satellite cell mitotic activity suggest that the greatest influence of muscle development in the poult is the first few days post-hatch, which is the exact time frame that poults have no access to feed and water. It is therefore evident that satellite cell mitotic activity is adversely affected by extended holding time in the incubator. When young animals have a stress on the muscle satellite cell, mitotic activity decreases (Mozdziak et al., 1998; Mozdziak et al., 2000). A reduction in satellite cell mitotic activity at a young age results in a lower weight of the adult muscle (Mozdziak et al., 2000), suggesting that holding time in the incubator without food or water will not only decrease satellite cell mitotic activity, but will also result in decreased muscle weights at market age.

A solution to prevent the undo stress on the young poult is needed to assist the turkey in achieving increased satellite cell mitotic activity and ultimately achieving maximum muscle development. Re-feeding after a period of starvation results in a decrease in body weights and breast muscle weights (Halevy et al., 2000). Immediately

feeding the poults helps in increasing breast percentage (Noy and Sklan, 1999a), but may be difficult to implement in the industry. In ovo feeding may be a possible answer to the problem presented by delayed access to feed and water that is commonplace in the poultry industry. The technique of in ovo feeding involves delivering nutrients to the embryo prior to hatch. Earlier attempts of in ovo injection of amino acids to broiler embryos showed an increase in body weight of the chick (Al-Murrani, 1982; Ohta et al., 1999). Vitamin E injected in ovo in poults and chicks improved antibody and macrophage response (Gore and Qureshi, 1997). The absence of difference in the satellite cell mitotic activity between the control group and the in ovo fed group at all ages of poults tested could possibly be attributed to the fact that the poults had immediate access to water and feed upon hatching. Immediate feeding of the post-hatch poults may have resulted in a decrease in the expression of growth factors released from the myofibers. Mature myofibers control satellite cell proliferation (Bischoff, 1990) and growth factors IGF-1 and FGF stimulate satellite cell mitotic activity (Allen and Boxhorn, 1989;Hossner et al., 1997). Therefore, the positive influence in ovo feeding might have had on the poult may be negated due to immediate access to feed and water. However, there may be a protective effect of in ovo feeding in a holding situation because birds that had delayed access to feed and water experienced a 50% reduction in yolk sac content (Vieira and Moran, 1999a). The same birds had increased mortality and decreased weight at 3 weeks of age (Vieira and Moran, 1999a). In ovo feed may protect the held early post-hatch bird from yolk nutrient depletion.

The results of this study suggest that satellite cell mitotic activity in the advanced developed turkey embryo or early post-hatch poult is not significantly increased by in

ovo feeding when the poult has immediate access to food and water. However, immediate access to feed and water is not available in the poultry industry and birds may go 24 to 48 hours without feed and water. Also, this study suggests that the immediate post-hatch period may be the most critical time for satellite cell mitotic activity as shown by a significant increase in satellite cell proliferation in the day old poult when compared to a seven day old poult. The findings of this study that satellite cell mitotic activity is significantly lower ($P \leq 0.05$) at 25E than at hatch suggest a developmental window that may be manipulated by in ovo feeding. The inherent low satellite cell mitotic activity at 25E of the turkey embryo suggests the possibility of an increase in satellite cell mitotic activity by an external stimulus. It has been shown previously that muscle organizational differences between sexes in turkey embryos is shown by 25E and satellite cell proliferation and differentiation differences exist between turkeys selected for different growth rates (Velleman et al., 2000; Velleman et al., 2002). These data support the idea that approximately 25E in the turkey embryo represents an important developmental time frame in muscle development. The in ovo injection of rhIGF-I into the chicken embryo altered the expression of myostatin and TGF-β2 resulting in altered muscle development demonstrates the possibility of influencing muscle development by in ovo injection (Kocamis et al., 2002). The highly influential time for muscle development in the post-hatch bird and the common practice of delayed access to feed and water to the early post-hatch poult in the industry, suggests the importance of nutrition in the immediate post-hatch period.

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Table 1. Body weight, gain, and *Pectoralis thoracicus* weight relative to body weight at 1 wk of age, and *Pectoralis supracoracodeus* weight relative to body weight at 1 wk of age.

	Nutrient Injection (NI)	Control (C)	Sample Size
BW at Hatch (g)	55.9 ± 0.4[a]	55.6 ± 0.4[a]	NI=56 C=59
BW 1wk of age (g)	137 ± 2[a]	133 ± 2[a]	NI=50 C=54
Gain (g)	81 ± 2[a]	77 ± 2[a]	NI=50 C=54
Muscle[A] weight (g/BW g)	0.059 ± 0.0016[a]	0.058 ± 0.0010[a]	5
Muscle[B] weight (g/BW g)	0.015 ± 0.0011[a]	0.014 ± 0.0003[a]	5

[A]Muscle= *Pectoralis thoracicus*
[B]Muscle=*Pectoralis supracoracodeus*
[a] Values within rows without a common superscript are significantly different ($P \leq 0.05$). Values are means ± SE.

Table 2. Myofiber diameter, satellite cell mitotic activity, nuclei per unit area (5-Bromo-2'-deoxyuridine (BrdU) labeled for 2 hours) by treatment.

	Myofiber CSA[A] (μm^2)	Satellite Cell Mitotic Activity[B]	Nuclei per μm^{2} [C]	Sample size
Nutrient Injection				
25E	$15.0 \pm 1.44^{b,c}$	0.015 ± 0.0018^{e}	0.016 ± 0.0002^{a}	5
Day of Hatch	12.0 ± 0.96^{c}	$0.094 \pm 0.026^{b,c}$	0.013 ± 0.0003^{c}	5
1day old	9.8 ± 1.90^{c}	0.153 ± 0.018^{a}	$0.015 \pm 0.0008^{a,b}$	5
1wk old	76.6 ± 5.66^{a}	$0.042 \pm 0.038^{d,e}$	0.007 ± 0.0003^{d}	5
Control				
25E	22.4 ± 4.46^{b}	0.012 ± 0.0123^{e}	$0.015 \pm 0.0006^{a,b,c}$	5
Day of Hatch	10.5 ± 1.28^{c}	$0.062 \pm 0.019^{d,c}$	$0.014 \pm 0.001^{b,c}$	5
1 day old	7.5 ± 0.18^{c}	$0.12 \pm 0.015^{a,b}$	$0.015 \pm 0.0008^{a,b}$	5
1 wk old	82.9 ± 3.62^{a}	$0.051 \pm 0.013^{c,d,e}$	0.008 ± 0.0005^{d}	5

[A]CSA=cross-sectional area
[B]Satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocyanate labeled nuclei per 100 propidium iodide labeled nuclei.
[C]Nuclei per (m2 is the number of propidium iodide labeled nuclei per (m2 of tissue section
[a,b,c,d,e] Values within columns without a common superscript are significantly different ($P < 0.05$).
Values are means + SE.

Table 3. Satellite cell mitotic activity by age.

	25E	Day of Hatch	1 day old	1 week old
Satellite cell mitotic activity[A]	0.014 ±0.0018[d]	0.078 ±0.016[b]	0.137 ±0.013[a]	0.047 ±0.006[c]
Sample Size	10	10	10	10

[A]Satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocyanate labeled nuclei per 100 propidium iodide labeled nuclei.
[a,b,c,d] Values within rows without a common superscript are significantly different ($P < 0.05$).
Values are means ± SE.

5. THE EFFECT OF EARLY NUTRITION ON SATELLITE CELL DYNAMICS IN THE YOUNG TURKEY

5.1 ABSTRACT

Early post-hatch satellite cell mitotic activity is an important aspect of muscle development. An understanding about the interplay between nutrition and satellite cell mitotic activity will lead to more efficient meat production. The objective of this study was to test the influence of the leucine metabolite, ß-hydroxy ß-methylbutyrate (HMB), and feed deprivation on muscle development in the early post-hatch poult. Male Nicholas poults were placed into one of four treatments: Immediately fed a starter diet with 0.1% HMB (IF-HMB); Immediately fed a starter diet containing 0.1% Solka Floc for a control (IF-No HMB); Withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet (WF-HMB); Withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet (WF-No HMB). 5-bromo-2'-deoxyuridine (BrdU) was injected intra-abdominally into all poults to label mitotically active satellite cells. The *Pectoralis thoracicus* was harvested two hr following the BrdU injection. Immunohistochemistry for BrdU, Pax7, and laminin along with computer-based image analysis was used to study muscle development. IF-HMB poults had higher body weights ($P < 0.01$) at 48 hr and one wk of age, and had higher satellite cell mitotic activity at 48 hr of age ($P < 0.01$) compared to the IF-No HMB and WF poults. Therefore, dietary supplementation of HMB may have an anabolic effect on early post-hatch muscle.

Key words: ß-hydroxy ß-methylbutyrate, BrdU, feed deprivation, Pax7

5.2 INTRODUCTION

Early post-hatch nutrition (i.e. before three days of age) has an important impact on muscle development in the poult because the energy required to emerge from the shell leaves the newly hatched poult in a nutrient deficient state (Uni and Ferket, 2004). Glycogen is the primary energy source available to the fetus when hatching; however, upon completion of the hatching process, the poult has greatly decreased its glycogen stores (John et al., 1987; John et al., 1988) thereby increasing the need for nutrients. Early nutrition is also important for the development of the gastrointestinal tract and the enzymes associated with digestion (Uni, 1998). The first wk post-hatch is also a time of major organ growth and development in the poult (Lilburn, 1998). An improvement in the development of the gastrointestinal tract and organ growth will allow for a more efficient uptake of nutrients to be used for muscle development. Noy and Sklan (1999a) found that poults offered nutrients immediately following hatch in a variety of forms exhibited a higher body weight and breast meat yield at market age. Also poults fed immediately post-hatch show a higher level of satellite cell mitotic activity *in vitro* compared to feed deprived animals (Halevy et al., 2003).

Satellite cells are located between the basal lamina and the sarcolemma of the myofiber (Mauro, 1961; Campion, 1984), and they are present in the avian embryo by midfetal stages of development (Feldman and Stockdale, 1992). Once the poult has hatched, adult satellite cells are present and myonuclei are postmitotic, eliminating the possibility of myonuclear accretion via mitosis of the existing myonuclei. However, myogenic satellite cells are mitotically active, and they are the source for new myonuclei in the post-hatch muscle. A protein involved in myogenic satellite cell dynamics is Pax7,

which is important for satellite cell specification because muscle deficient in Pax7 does not contain any satellite cells (Seale et al., 2000). Quiescent satellite cells express Pax7 (Zammit and Beauchamp, 2001; Asakura et al., 2002), but do not express the hematopoieitic stem cell markers Sca-1 and CD45, suggesting that satellite cells are a distinct population of myogenic cells different from stem cells (Asakura et al., 2002). However, Pax7 expression is proposed to signal commitment of a cell to the myogenic lineage because myogenic stem cells developed into myoblasts expressing Pax7 following injury (Seale et al., 2004).

Satellite cell mitotic activity in the turkey is highest early post-hatch and decreases with age as the turkey matures (Mozdziak et al., 1994), suggesting that the immediate post-hatch period is the most important time to improve breast meat yield via myonuclear accretion because the major source of myofiber growth in the turkey greater than nine wk of age occurs via an increase in cytoplasmic volume to myonucleus ratio referred to as DNA unit size (Mozdziak et al., 1994). However, poults are commonly held for 48 to 72 hr without access to feed and water, which depresses satellite cell mitotic activity (Halevy et al., 2003).

Chicks denied access to food and water for the first 48 hr post-hatch show a smaller body weight and breast muscle weight at market age than chicks denied access to feed and water from days 2 to 4 post-hatch or days 4 to 6 post-hatch (Halevy et al., 2000) suggesting the immediate post-hatch period is the most important time for programming mature breast muscle size. Other studies have shown that delayed access to feed and water for the first 48 hr post-hatch decreases body weight and breast meat yield (Nir and Levanon, 1993; Noy and Sklan, 1999b; Vieira and Moran, 1999a; 1999b). Similar

findings in the turkey also show that early post-hatch feed deprivation decreases growth (Pinchasov and Noy, 1993), possibly programming the muscle to be smaller via decreased satellite cell mitotic activity. Poults with delayed access to feed immediately post-hatch exhibit lower satellite cell mitotic activity when compared to their fed counterparts (Mozdziak et al., 2002b; Halevy et al., 2003) suggesting the importance of early nutrition on determining muscle growth potential via early satellite cell mitotic activity. Birds with delayed access to nutrition also exhibit a smaller duodenal and jejunal crypt, as well as, the number of crypts per villus (Geyra et al., 2001), which would results in a lower digestive capacity of the small intestine, which could negatively influence muscle development.

As a result of the recently identified nutritional influence on satellite cell mitotic activity over the first few days post-hatch, early post-hatch diets should be formulated to maximize satellite cell mitotic activity. Dietary supplementation of the leucine metabolite ß-hydroxy ß-methylbutyrate (HMB) may increase satellite cell mitotic activity. HMB is endogenously derived from α-ketoisocaproate (KIC) and eventually forms HMG-CoA to form cholesterol (Nissen and Abumrad, 1997). However, it has been found that if an exogenous source of HMB is introduced to the diet, it is used by the animal beyond what is produced endogenously (Van Koevering and Nissen, 1992). Leucine may have more important physiological roles than the formation of HMB, and it has been shown to decrease the amount of proteolysis in the muscle as well as decrease muscle damage during a period of stress to the muscle (Gallagher et al., 2000; Panton et al., 2000; Vukovich et al., 2001). The lack of feed and water available to the poult immediately following hatch, as well as the energy required for the poult to emerge from

the egg, may provide a challenge to all muscles in the body. Addition of HMB to the starter diet may counteract the challenges placed on early post-hatch muscle. The first objective of this experiment was to understand satellite cell dynamics in the early post-hatch period, while the second objective was to examine the influence of HMB on satellite cell mitotic activity in the newly hatched poult.

5.3 MATERIALS AND METHODS

Turkeys

Two hundred seventy-two male Nicholas poults were obtained from a commercial hatchery immediately within four hours of hatching, and they were placed into battery cages[8] at North Carolina State University. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. Two diets were used in the study. Both diets were based upon a standard corn and soybean meal basal diet (Table 1). The basal diet either contained 0.1% HMB or 0.1% Solka Floc[9] as the control. Solka Floc is a non-nutritional cellulose fiber feed additive. The poults were divided into four treatments: immediate access to feed and water fed the HMB diet (IF-HMB), immediate access to feed and water fed the control diet (IF-No HMB), withheld from feed and water for 48 hr immediately post-hatch then placed on the HMB diet (WF-HMB), and withheld from feed and water for 48 hr immediately post-hatch then placed on the control diet (WF-No HMB). The birds in the IF group were provided feed and water within 4 hr of removal from the hatcher. Seventeen birds were housed per cage and there were four different cages for each treatment (68 birds per treatment). At least one bird from each cage was randomly chosen for each sampling interval, the remaining bird needed for sampling was randomly selected from the entire population. Conditions were closely monitored to ensure that there were similar environmental conditions (temperature) in each cage. Birds were weighed at placement, at 48 hr post-hatch, and one wk of age. *Pectoralis thoracicus* and *Pectoralis supracoracoideus* muscles were weighed at one wk of age.

[8] Alternative Design Manufacturing and Supply Inc., Silon Springs, AR
[9] Fiber Sales and Development Corp. Urbana, OH 43078

Tissue sampling

After birds were randomly chosen from each treatment group, BrdU (10mg/ml; 10mg BrdU/100g bird weight), a thymidine analogue, was administered intra-abdominally using a 27-gauge hypodermic needle. Following the injection of BrdU, the poults were maintained for two hr before sampling to allow for the incorporation of the BrdU into the nuclei entering the S-phase of the cell cycle, when the birds were euthanized with Euthasol®[10] (0.25ml/kg BW). The left *Pectoralis thoracicus* was harvested from poults at 24 hr post-hatch, 48 hr post-hatch, and one wk of age. The samples taken at 24 and 48 hr post-hatch included five poults from each immediately fed (IF) treatment and five poults from the collectively feed deprived group (WF). Five birds were sampled from all four groups at one wk post-hatch. Immediately after the tissue was harvested, it was flash frozen in cooled isopentane. The tissues were then placed in cryovials and stored at –80°C until sectioning. Eight micron cryosections were serially cut, adhered to glass slides, and allowed to warm to room temperature. Sections were stored at –20°C until staining.

Immunohistochemistry

Slides were brought to room temperature, and the sections were fixed with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for sections to be stained for BrdU. All other sections were fixed with 4% paraformaldehyde in PBS-pH 7.4. The sections were washed three times for five minutes with PBS. Sections for BrdU and Pax7 staining were treated with 0.07N NaOH for five minutes, and PBS was used to neutralize the NaOH. Three primary antibodies were added to the

[10] Delmarva Laboratories, Midlothian, VA 23113 USA

sections and incubated at 4° C in a humidified chamber overnight: monoclonal mouse anti-BrdU[11] diluted 1:20 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin; polyclonal rabbit anti-laminin[12] diluted 1:30 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin; monoclonal mouse anti-Pax7[13]-undiluted. Each primary antibody was incubated with a different serial section from each treatment group. After the tissue sections were blocked with PBS containing 10% goat serum[14] and 0.5% tween-20 for 15 minutes to minimize background staining, the secondary antibodies were added to the sections for two hr in a humidified chamber at room temperature in the dark. The secondary antibody for BrdU and Pax7 staining was goat anti-mouse IgG conjugated to FITC[15], which was diluted 1:50 with PBS containing 10% goat serum and 0.5% tween-20. Laminin was detected using goat anti-rabbit IgG conjugated to Texas Red™ [8] diluted 1:35 in PBS containing 10% goat serum[7] and 0.5% tween-20. Propidium iodide[7] (50 μg/ml in PBS) was added to all sections except for sections stained for laminin for 20 minutes to label all nuclei. Finally, the sections were placed in mounting media [75% glycerol (vol/vol), 25% dH_2O (vol/vol), 75mM KCL, 10mM tris(hydroxymethyl)aminomethane, 2mM $MgCl_2$, 2mM ethylene glycol-bis (β-amino-ethyl ether)-*N*,*N*,*N*',*N*'- tetraacetic acid, 1mM NaN_3, pH 8.5, 1mg/ml p-phenylenediamine] and a cover slip was sealed over the section using nail polish.

[11] Becton Dickinson, Mountain View, CA 94039
[12] Sigma, St. Louis, MO 63103
[13] Developmental Studies Hybridoma Bank, Iowa City, IA 52242
[14] Invitrogen, Carlsbad, CA 92008
[15] MP Biomedicals, Irvine, CA 92618

Image Analysis

A Leica DMR[16] microscope with epiflorescence illumination was used to observe the tissue sections. All nuclei and laminin were observed with PI filter set[17], and the BrdU and Pax7 labeled nuclei were observed with a FITC filter set. A Spot-RT CCD[18] camera was used to capture the images of nuclei visualized by the FITC and PI filter sets. The FITC and PI labeled nuclei were counted using Image-Pro Plus software[19]. The number of BrdU labeled nuclei per 1000 PI labeled nuclei was used as an index for satellite cell mitotic activity. The Pax7 labeling index was determined by the number of Pax7 positive nuclei per 1000 PI labeled nuclei. The criteria for completing nuclear analysis were counting at least 1000 PI labeled nuclei. The Pax7 to BrdU ratio was determined by dividing the Pax7 labeling index by the BrdU labeling index. The area of muscle section containing the count of propidium iodide labeled nuclei was also determined. The number of PI labeled nuclei was expressed relative to the section area that was analyzed. Laminin staining was used to demarcate myofiber borders. Image-Pro Plus[12] software was also used to determine myofiber cross-sectional area for at least 100 myofibers per muscle sample.

Statistics

The General Linear Models procedure of SAS (SAS Institute, 1985) was used to perform a one-way analysis of variance to determine the treatment effect on 48 hour post-hatch weight, one wk weight, body weight gain, muscle weights, muscle weight to body weight ratio, the number of PI labeled nuclei per muscle area, myofiber cross-sectional

[16] Leica Microsystems, Bannockburn, IL 60015
[17] Omega Optical, Brattleboro, VT 05301
[18] Diagnostic Instruments Inc., Sterling Heights, MI 48314
[19] Media Cybernetics, Silver Spring MD 20910

area, satellite cell mitotic activity, Pax7 labeling index, and Pax7/BrdU ratio. If population variances were unequal, then a logarithmic transformation was performed on the data before analysis. Means were separated using least significant differences (Zar, 1999). A 2X2 Factorial analysis was performed on the one week data to show diet and fasting interactions, and diet and fasting main effects. Statistical signficance was accepted at $P < 0.05$. The experimental unit for weights was pen weights, and the experimental unit for all other parameters was the bird.

5.4 RESULTS

Growth

The body weights of the IF-HMB group were significantly higher than those of the other three groups at 48 hr post-hatch (Table 2). Both of the WF groups at 48 hr post-hatch, weighed significantly less ($P < 0.01$) than either of the IF groups. At one wk of age, the pooled HMB fed birds had significantly higher body weights than the control diet fed poults (Table 3). Likewise, the pooled IF birds significantly outweighed the WF birds at one wk post-hatch. Also the IF-No HMB poults weighed significantly less than the IF-HMB birds. The WF-HMB birds were also significantly heavier than the WF-No HMB. The amount of gain at one wk of age was higher in the pooled HMB groups than the pooled poults on the control diet, and gain was also higher in the IF group than the WF group. The gain for the IF-HMB birds was higher than the IF-No HMB group; similarly, the WF-HMB group was higher than the WF-No HMB group.

At one wk of age, *Pectoralis thoracicus* weights were significantly higher in the IF-HMB compared to the IF-No HMB, IF and WF groups, and the WF-HMB and WF-No HMB groups (Table 4). However, there were no differences in the ratio of *Pectoralis thoracicus* and *Pectoralis supracoracoideus* to body weight except between the IF and WF groups. The only differences in one wk *Pectoralis supracoracoideus* weights were between the HMB pooled poults and the control fed poults, and between the IF and WF groups. Myofiber cross-sectional areas were smaller in the WF group at 24 hr post-hatch and a difference between all groups existed at 48 hr post-hatch with the IF-HMB group achieving the largest cross-sectional area (Table 5). At one wk of age, the HMB group had a larger cross-sectional area than the control fed group, as well as the IF group

having larger cross-sectional area than the WF group (Table 6). IF-HMB poults had larger cross-sectional area than the IF-No HMB birds. There was no difference in myofiber cross-sectional area between the WF-HMB poults and the WF-No HMB poults.

Satellite Cell Mitotic Activity

At 24 hr and 48 hr post-hatch, the IF-HMB poults had a higher index of satellite cell mitotic activity than the IF-No HMB and WF poults (Table 5). However, the IF-No HMB BrdU labeling index was higher compared to the WF at 48 hr. Interestingly, the WF pooled group had a higher BrdU labeling index than the IF pooled poults at one wk of age (Table 6) indicating a level of compensation following feed deprivation. However, there was no difference at one wk of age between the pooled diet treatments or the comparison of IF-HMB to IF-No HMB and WF-HMB to WF-No HMB.

At 24 hr post-hatch, there were no differences in Pax7 labeling index; however, at 48 hr post-hatch the WF group had a higher level of Pax7 labeling (Table 7). No differences were found at one wk of age (Table 8). A ratio of Pax7 to BrdU labeling index was calculated to determine the number of the satellite cells in the proliferative reserve (Halevy et al., 2004; Oustanina et al., 2004) compared to the number of cycling satellite cells by dividing the Pax7 labeling index by the BrdU labeling index. There were no differences in the Pax7/BrdU ratio at 24 hr post-hatch (Table 7). However, at 48 hr post-hatch the WF group had the highest ratio, possibly indicating a shutdown of cycling satellite cells and an increase in the number of satellite cells in the proliferative reserve awaiting activation upon feeding. There were no differences at one wk of age except the IF group had a higher ratio than the WF group (Table 8).

The number of PI labeled nuclei per area measured was not different at 24 hr post-hatch between any groups. However, the PI per area measured at 48 hr post-hatch was higher in the IF-No HMB birds than the IF-HMB (Table 5), suggesting a larger DNA unit size in the HMB fed birds. At one wk of age the pooled HMB group had a lower PI per area measured than the pooled control fed group. Both HMB fed groups had lower PI per area measured than their control fed counterparts (Table 6).

5.5 DISCUSSION

HMB is a metabolite of the amino acid leucine and may be involved in the increase in cholesterol synthesis for membrane protection after it is metabolized to HMG-CoA (Nissen and Abumrad, 1997). After the injection of HMB in lambs and pigs, only 34% was recovered in the urine indicating the use of the metabolite by animals (Van Koevering and Nissen, 1992). Other studies have concluded that the administration of HMB decreases proteolysis while it increases fat free mass (Gallagher et al., 2000; Panton et al., 2000; Vukovich et al., 2001), which may be beneficial to animal agriculture.

In vitro, chicken muscle shows a decrease in proteolysis after the administration of HMB (Ostaszewski, 2000). However, HMB does not seem to be as effective in more mature animal muscle because broiler chickens showed no increase in body weight and carcass yield at 42 days following HMB supplementation, and weanling pigs did not show an increase in average daily gain following HMB supplementation (Nissen et al., 1994; Gatnau et al., 1995). Our results indicate that HMB may be more effective in young muscle, but it is unclear if the influence will be maintained throughout production. HMB's effectiveness in young muscle may be due to the extreme growth potential and the high level of satellite cell activity of the young animal. Broiler chickens only showed a short-term improvement in growth after 0.1 % HMB administration (Nissen et al, 1994). The ability of young muscle to program ultimate size via satellite cell mitotic activity indicates the best time to feed a muscle promotant is early in life. Since satellite cell mitotic activity decreases with age (Mozdziak et al., 1994) feeding HMB in the more mature animal may be ineffective if the intention is to increase muscle size at market age

by stimulating satellite cell proliferation. Also, by programming an increase in muscle size early post-hatch in the turkey, diets may need to be reformulated to account for the increased potential to show increase in muscle performance.

HMB also has been shown to decrease proteolysis following a period of stress in the muscle by decreasing the proteolytic enzyme calpain (Jank et al., 2000). However, in chicks, calpain transcriptional activity was not influenced by feed deprivation or nutrient level (Mozdziak et al., 2002a) suggesting muscle dynamics early post-hatch influenced by HMB may be the result of another mechanism. Also, the increased level of BrdU labeling in the IF-HMB poults indicates that HMB may be acting through a mechanism other than decreased proteolysis. The increased level of satellite cell mitotic activity in the IF-HMB poults, as well as an increase in myofiber cross-sectional area, may be the result of HMB increasing the production of growth factors such as IGF-1 and FGF, which have been shown to increase satellite cell proliferation and protein synthesis in turkey satellite cells (Dodson et al., 1996; McFarland, 1999), which may result in an increase in satellite cell mitotic activity and myofiber cross-sectional area. HMB may also increase insulin production, which has been shown to increase DNA synthesis (i.e. satellite cell activity) and overall cell size (Bikopoulos et al., 2004).

It is unclear if HMB will result in an increase in meat yield at market age of poults following a period of feed deprivation. However, HMB improved gain and overall body weight in WF-HMB poults when compared to WF-No HMB fed birds. Feed deprivation decreases satellite cell mitotic activity early in life, which leads to a decrease in overall skeletal muscle growth and meat yield at market age (Halevy et al., 2000). It is detrimental to overall meat production to decrease satellite cell mitotic activity early in

life because it is the time with the highest level of DNA accretion in the poult. Further indicating the importance of satellite cell mitotic activity early in life, a decrease in activity will result in a reduction in muscle size at maturity (Mozdziak et al., 1997; Mozdziak et al., 2000). Myonuclei are not mitotically active post-hatch. Therefore, the myofibers must rely on myogenic satellite cells to fuel myonuclear accretion. Since myofibers are multinucleate, the more nuclei a muscle can acquire early in life, the larger the potential for muscle size at market age because DNA unit number governs muscle size (Mozdziak et al., 1994). Muscle growth in the turkey is a result of protein synthesis; however, the more nuclei within the muscle, the more protein synthesis that can occur. Turkeys show a decrease in satellite cell mitotic activity between 3 to 9 wk of age with virtually no growth occurring via satellite cell mitotic activity from 9 to 26 wk of age (Mozdziak et al., 1994) indicating the importance of early post-hatch satellite cell activity.

Feed deprivation caused a decrease in satellite cell mitotic activity at 48 hr post-hatch in the WF poult when compared to fed poults in this study. Similar findings have been reported in other research (Mozdziak et al., 2002b; Halevy et al., 2003), suggesting that early post-hatch feed deprivation results in a missed opportunity for muscle development. Interestingly, feed deprived poults exhibited a higher level of satellite cell mitotic activity at one wk of age supporting the work of Halevy et al. (2003) who reported a higher level of satellite cell mitotic activity after a period of feed deprivation when compared to fed poults at six days of age. However, Mozdziak et al. (2002b) did not find any compensatory satellite cell mitotic activity following the period of feed deprivation. The difference in results between Mozdziak et al. (2002b) and the current

study could be due to differences in methodology. The present experiment employed a single pulse injection of BrdU showing satellite cell mitotic activity at that particular time; whereas, Mozdziak et al. (2002b) used an infusion of BrdU via an implanted pump providing a continuous readout of satellite cell mitotic activity over one wk, which could have masked the compensatory response. The satellite cell population may be attempting to compensate for the missed development window for myonuclear accretion. However, it is unlikely that this increase in satellite cell mitotic activity would result in a complete recovery of the muscle because the critical period of satellite cell mitotic activity immediately post-hatch was missed.

Chicks that have been fed after a period of feed deprivation show a lower body weight and breast meat yield at market age when compared to chicks fed immediately (Nir and Levanon, 1993; Halevy et al., 2000). Muscle, following inactivity normally shows an increase in satellite cell mitotic activity (Mozdziak et al., 1997; Mozdziak et al., 2000). However, an increase in satellite cell mitotic activity after a period of inactivity is short-lived and does not completely compensate for the previous absence of satellite cells (Mozdziak et al., 1997; Mozdziak et al., 2000).

Another indicator of muscle development is Pax7, which is a paired box transcription factor that is essential for the development and specification of satellite cells (Seale et al., 2000; Asakura et al., 2002). Once a cell expresses Pax7, it becomes committed to the myogenic lineage (Zammit and Beauchamp, 2001). In our study, Pax7 labeling index was higher in the WF poults 48 hr after hatch compared to poults with immediate access to feed and water. The same group of poults had a low level of satellite cell mitotic activity, indicating that when there is a depressed level of satellite cell mitotic

activity, there is a relative increase in the number of quiescent cells committed to the myogenic lineage preparing for an expected time of compensatory activity. Current research has shown that uncommited cells isolated from uninjured muscle did not commit to the myogenic lineage and did not express Pax7, but in injured muscle uncommited cells were recruited to the myogenic lineage and expressed Pax7 (Seale et al., 2004). It is possible that satellite cells expressing Pax7 represent a sub-population of satellite cells that holds the proliferative reserve of the overall satellite cell population (Halevy et al., 2004). The increased level of Pax7 expression and decreased level of satellite cell mitotic activity in the WF poults compared to the IF poults at 48 hr of age shows a relative increase in the satellite cell proliferative reserve. The compensatory increase in satellite cell mitotic activity in the WF poults compared to the IF poults at one wk of age without a difference in Pax7 expression supports the notion that the proliferative reserve was being conserved at 48 hr in the WF poults.

Contradictory to past findings (Seale et al., 2000), recent research has shown that mice lacking Pax7 may contain a small number of satellite cells from embryonic formation, but these mice have fewer satellite cells at maturity than wild type mice (Oustanina et al., 2004), suggesting Pax7 is necessary for the replenishment of cycling satellite cells. Also, a recent study has shown that the expression of Pax7 in a majority of satellite cells is downregulated during proliferation (Zammit et al., 2004). However, a small population of satellite cells express Pax7 during proliferation, and subsequently do not differentiate, but leave the cell cycle and once again become quiescent satellite cells (Zammit et al., 2004) indicating that Pax7 expression marks the proliferative reserve for

satellite cell renewal, and it also supports the concept of satellite cell heterogeneity (Schultz, 1996).

HMB may improve muscle development via an increase in myogenic satellite cell mitotic activity in the immediate post-hatch turkey. Feed deprivation also detrimentally influences muscle development in the turkey. Other studies support the findings that nutrition can influence myogenic satellite mitotic activity immediately post-hatch (Mozdziak et al., 2002b). However, the exact anabolic mechanism for HMB to promote satellite cell mitotic activity is unknown. Further research may focus on understanding the relationship of myogenic stem cells and myogenic satellite cells regarding myonuclear accretion in the young animal. The area of myonuclear accretion/satellite cells/myogenic stem cells is a relatively unexplored area in animal agriculture, and may provide an effective and promising means of increasing breast meat yield in the turkey.

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Table 1. Basal diet composition

Ingredients	**Percentage of Diet**
Corn	45.5
Soybean meal (48% CP)	40.9
Limestone	1.22
Dicalcium phosphate (18.5% P)	3.38
Poultry fat	2.9
Poultry meal (60% CP)	5.0
DL-methionine	0.34
Lysine-HCL	0.36
Sodium chloride	0.27
Choline chloride (60%)	0.2
Trace mineral premix[1]	0.2
Vitamin premix[2]	0.2
Selenium[3]	0.05
Calculated Nutrient Analysis	
ME (kcal/kg)	2935
Crude protein, %	27.5
Crude fat, %	5.6
Lysine, %	1.74
Methionine, %	0.74
Met + Cys, %	1.20
Ca, %	1.4
Non-phytate P, %	0.81
Na, %	0.26

[1]Supplied the following per kilogram of feed: 120 mg Zn as $ZnSO_4$; 120 mg Mn as Mn $SO_4 \cdot H_2O$; 80 mg Fe as Fe $SO_4 \cdot H_2O$; 10 mg Cu as Cu SO_4; 2.5 mg I as $Ca(IO_3)_2$; 10 mg Co as $CoSO_4$.

[2]Supplied the following per kilogram of feed: vitamin A, 26,000 IU; cholecalciferol, 8000IU; vitamin E, 90 mg as α-tocopheryl acetate; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4 mg; pyridoxine, 16 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 4 mg; biotin, 0.500 mg; vitamin B_{12}, 0.08 mg; ethoxyquin, 200 mg.

[3]Selenium premix supplied the following per kilogram of feed: 0.2 mg Se as $NaSe_2O_3$.

Table 2. Body weights at 48 hr of age.

	Starting BW (g)[2]	Sample Size	BW at 48 hr (g)	Sample Size
IF-HMB[1]	64 ± 1 [a]	4	74 ± 1 [a]	4
IF-No HMB[1]	64 ± 1 [a]	4	67 ± 1 [b]	4
WF-HMB[1]	64 ± 1 [a]	4	53 ± 1 [c]	4
WF-No HMB[1]	64 ± 1 [a]	4	53 ± 1 [c]	4

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[2]Weights are prior to the offering of feed and water.
[a,b,c] Values within columns without a common superscript are significantly different ($P \leq 0.05$).

Table 3. Body weights at one wk of age and one wk weight gain.

	Body Weight (g)	One Wk Gain	Sample size
HMB Pooled[1]	136 ± 3 [a]	72 ± 2 [a]	8
No-HMB Pooled[1]	123± 3 [b]	58 ± 2 [b]	8
IF Pooled[2]	149 ± 3 [a]	85 ± 2 [a]	8
WF Pooled[2]	110 ± 3 [b]	46 ± 2 [b]	8
IF-HMB[3]	155 ± 3 [a]	91 ± 11 [a]	4
IF-No HMB[3]	143 ± 3 [b]	78 ± 11 [b]	4
WF-HMB[3]	118 ± 3 [a]	54 ± 7 [a]	4
WF-No HMB[3]	103 ± 3 [b]	38 ± 7 [b]	4

[1] HMB Pooled, overall mean HMB treated birds at one wk of age. No-HMB Pooled, overall mean non-HMB treated birds at one wk of age.
[2] IF Pooled, overall mean IF birds at one wk of age. WF Pooled, overall mean previously feed deprived birds at one wk of age.
[3]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[a,b] Values are means ± SE. Values within columns of the same pairing without a common superscript are significantly different ($P \leq 0.05$).

Table 4. *Pectoralis thoracicus* weight, *Pectoralis supracoracoideus* weight, *Pectoralis thoracicus*/body weight ratio and *Pectoralis supracoracoideus*/body weight ratio at one wk of age.

	Pectoralis thoracicus.	*Pectoralis thoracicus* ratio	*Pectoralis supracoracoideus*	*Pectoralis supracoracoideus* ratio
HMB Pooled[2]	3.24[a]	0.022[a]	0.75[a]	0.005[a]
No-HMB Pooled[2]	2.63[b]	0.021[a]	0.61[b]	0.005[a]
Pooled SE	0.16	0.001	0.04	0.001
IF Pooled[3]	4.01[a]	0.026[a]	0.94[a]	0.006[a]
WF Pooled[3]	1.87[b]	0.017[b]	0.43[b]	0.004[b]
Pooled SE	0.157	0.001	0.04	0.001
IF-HMB	4.42[a]	0.027[a]	1.01[a]	0.006[a]
IF-No HMB	3.61[a]	0.025[a]	0.86[a]	0.006[a]
Pooled SE	0.29	0.001	0.69	0.001
WF-HMB	2.07[a]	0.017[a]	0.49[a]	0.004[a]
WF-No HMB	1.66[b]	0.016[a]	0.37[a]	0.003[a]
Pooled SE	0.11	0.001	0.041	0.001

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[2] HMB Pooled, overall mean HMB treated birds at one wk of age. No-HMB Pooled, overall mean non-HMB treated birds at one wk of age.
[3] IF Pooled, overall mean fed birds at one wk of age. WF Pooled, overall mean previously feed deprived birds at one wk of age.
[a,b] Values within columns of the same pairing without a common superscript are significantly different ($P \leq 0.05$).
Pooled data has sample size of 10 all other data has a sample size of 5.

Table 5. Myofiber diameters, satellite cell mitotic activity, nuclei per unit area (5-Bromo-2'-deoxyuridine (BrdU) labeled for 2 hours) by treatment at 24 and 48 hours of age.

	Myofiber CSA[A] (μm^2)	Satellite Cell Mitotic Activity[B]	Nuclei per μm^2 [C]	Sample size
24 hours				
IF-HMB	19.062[a]	0.0746[a]	0.016798[a]	5
IF-control	16.796[a]	0.03391[b]	0.020728[a]	5
WF	11.144[b]	0.04167[b]	0.021740[a]	5
Pooled SE	1.567	0.00927	0.0081	
48 hours				
IF-HMB	26.865[a]	0.15891[a]	0.013299[b]	5
IF-control	19.826[b]	0.07453[b]	0.017323[a]	5
WF	12.481[c]	0.01857[c]	0.016968[a,b]	5
Pooled SE	1.675	0.0183	0.00126	

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[A]CSA=cross-sectional area.
[B]Satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei.
[C]Nuclei per μm^2 is the number of propidium iodide labeled nuclei per μm^2 of tissue section
[a,b,c] Values within columns of the same age group without a common superscript are significantly different ($\boldsymbol{P} \leq 0.05$).

Table 6. Myofiber diameters, satellite cell mitotic activity, nuclei per unit area (5-Bromo-2'-deoxyuridine (BrdU) labeled for 2 hours) by treatment at one week of age.

	Myofiber CSA[A] (μm^2)	Satellite Cell Mitotic Activity[B]	Nuclei per μm^2 [C]	Sample size
HMB Pooled[2]	114.643[a]	0.06921243[a]	0.00900618[b]	10
No-HMB Pooled[2]	63.132[b]	0.09509268[a]	0.01061951[a]	10
Pooled SE	11.743	0.0116	0.00337	
IF Pooled[3]	121.377[a]	0.06322458[b]	0.00769211[b]	10
WF Pooled[3]	56.398[b]	0.10108053[a]	0.01193357[a]	10
Pooled SE	11.743	0.0016	0.00337	
IF-HMB	155.974[a]	0.05526915[a]	0.00670423[b]	5
IF-No HMB	86.78[b]	0.0711810[a]	0.00868000[a]	5
Pooled SE	19.652	0.00805	0.00558	
WF-HMB	73.312[a]	0.08315571[a]	0.01130813[b]	5
WF-No HMB	39.484[a]	0.11900535[a]	0.01255901[a]	5
Pooled SE	12.860	0.0217	0.000379	

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[2] HMB Pooled, overall mean HMB treated birds at one wk of age. No-HMB Pooled, overall mean non-HMB treated birds at one wk of age.
[3] IF Pooled, overall mean fed birds at one wk of age. WF Pooled, overall mean previously feed deprived birds at one wk of age.
[A]CSA=cross-sectional area
[B]Satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei.
[C]Nuclei per μm^2 is the number of propidium iodide labeled nuclei per μm^2 of tissue section
[a,b] Values within columns of the same pairing without a common superscript are significantly different ($P \leq 0.05$).

Table 7. Pax7 labeling index and Pax7/Brdu labeling index ratio by treatment at 24 and 48 hours of age.

	Pax7 Labelig Index[A]	Pax7/BrdU ratio[B]	Sample size
24 hours			
IF-HMB	0.12128[a]	1.536[a]	5
IF-control	0.13275[a]	5.989[a]	5
WF	0.09607[a]	3.189[a]	5
Pooled SE	0.0135	1.63	
48 hours			
IF-HMB	0.11056[b]	0.812[b]	5
IF-control	0.09750[b]	1.878[b]	5
WF	0.15257[a]	13.064[a]	5
Pooled SE	0.0135	2.51	

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[A]Pax7 labeling index is expressed as the number of Pax7-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei.
[B]Pax7/Brdu ratio is the Pax7 labeling index/BrdU labeling index.
[a,b] Values in a column within an age group without a common superscript are significantly different ($P \leq 0.05$).

Table 8. Pax7 labeling index and Pax7/Brdu labeling index ratio at one week of age.

	Pax7 Labelig Index[A]	Pax7/BrdU ratio[B]	Sample size
HMB Pooled[2]	0.06664754[a]	1.1033[a]	10
No-HMB Pooled[2]	0.06941209[a]	0.9027[a]	10
Pooled SE	0.00375	0.1249	
IF Pooled[3]	0.07020490[a]	1.2068[a]	10
WF Pooled[3]	0.06585474[a]	0.7991[b]	10
Pooled SE	0.00375	0.1249	
IF-HMB	0.06775110[a]	1.3661[a]	5
IF-No HMB	0.07265869[a]	1.0475[a]	5
Pooled SE	0.00432	0.167	
WF-HMB	0.06554398[a]	0.8405[a]	5
WF-No HMB	0.06616550[a]	0.7578[a]	5
Pooled SE	0.00615	0.186	

[1]IF-HMB, immediately fed a starter diet with 0.1% HMB; IF-No HMB, immediately fed a starter diet; WF-HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the HMB diet; WF-No HMB, withheld feed and water for 48 hr immediately post-hatch and then fed the control starter diet.
[2] HMB Pooled, overall mean HMB treated birds at one wk of age. No-HMB Pooled, overall mean non-HMB treated birds at one wk of age.
[3] IF Pooled, overall mean fed birds at one wk of age. WF Pooled, overall mean previously feed deprived birds at one wk of age.
[A]Pax7 labeling index is expressed as the number of Pax7-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei.
[B]Pax7/Brdu ratio is the Pax7 labeling index/BrdU labeling index.
[a,b] Values within columns and the same pairing without a common superscript are significantly different ($\boldsymbol{P} \leq 0.05$)

6. THE EFFECT OF EARLY POST-HATCH FASTING ON SATELLITE CELL DYNAMICS IN THE YOUNG TURKEY

6.1 ABSTRACT

Early post-hatch satellite cell dynamics are an important aspect of muscle development, and understanding the interplay between fasting and muscle development will lead to efficient meat production. The objective of this experiment was to test the influence of immediate post-hatch fasting on satellite cells in the poult. Male Nicholas poults were placed into two treatments: a fed treatment with immediate access to feed and water upon placement and a fasted treatment with delayed access to feed and water for 72 hours. 5-bromo-2'-deoxyuridine (BrdU) was injected intra-abdominally in all poults to label mitotically active satellite cells. The *Pectoralis thoracicus* was harvested two hours following the BrdU injection. Immunohistochemistry for BrdU, Pax7, Bcl-2, Pax7 with BrdU, and determining myofiber diameters and nuclei per μm^2 along with computer-based image analysis was used to study muscle development. Fed poults had higher body weights throughout the experiment (**$P \leq 0.01$**) and had higher muscle weights (**$P \leq 0.01$**) at ten days of age than the fasted poults. Fed poults had higher satellite cell mitotic activity at 72 hours and four days of age (**$P \leq 0.01$**) compared to the fasted poults. However, Pax7 labeling index was higher in the fasted poults (**$P \leq 0.01$**) at 72 hours, four days, and five days post-hatch than the fed group. Therefore, fasting depleted proliferating satellite cells and conserved the proliferative reserve Pax7+ cells.

Key words: Fasting, Pax7, BrdU, Bcl-2

6.2 INTRODUCTION

Satellite cells are important for post-hatch muscle development because post-hatch myonuclei are postmitotic, eliminating the possibility of myonuclear accretion via mitosis of the existing myonuclei. However, myogenic satellite cells are mitotically active and are the source for new myonuclei in the post-hatch muscle. Satellite cells are located between the basal lamina and the sarcolemma of the myofiber (Mauro, 1961; Campion, 1984), and are present in the avian embryo by midfetal stages of development (Feldman and Stockman, 1992). Satellite cell dynamics is partially governed by the protein Pax7 (Seale et al., 2000). Quiescent satellite cells express Pax7 (Zammit and Beauchamp, 2001; Asakura et al., 2002), and in a sub-set of proliferating satellite cells (Zammit et al., 2004). However, satellite cells do not express the hematopoieitic stem cell markers Sca-1 and CD45, suggesting that satellite cells are a distinct population of myogenic cells different from stem cells (Asakura et al., 2002) because most of the stem cell properties exhibited by skeletal muscle are due to its hematopoietic potential of Sca-1+ and CD45+ expressing cells (Jackson et al., 1999). Pax7 expression is proposed to signal commitment of a cell to the myogenic lineage because myogenic stem cells developed into myoblasts expressing Pax7 following injury (Seale et al., 2004). A sub-population of satellite cells may also exist (Morgan and Partridge, 2003; Schultz. 1996), which may express Pax7 (Zhao and Hoffman, 2004).

The early post-hatch period in the turkey has the highest level of satellite cell mitotic activity and decreases with age as the turkey matures (Mozdziak et al., 1994), suggesting that the immediately post-hatch period is the best opportunity to improve breast meat yield via myonuclear accretion because the major source of myofiber growth

in the turkey greater than nine weeks of age occurs via an increase in cytoplasmic volume to myonucleus ratio referred to as a DNA unit size (Mozdziak et al., 1994).

However, a common practice in the turkey industry is the delayed placement of poults for 48 to 72 hours without access to feed and water that will negatively impact muscle development. Chicks denied access to food and water for the first 48 hours post-hatch showed a smaller body weight and breast muscle weight at market age than chicks denied access to feed and water from days 2 to 4 post-hatch or days 4 to 6 post-hatch (Halevy et al., 2000) suggesting the immediate post-hatch period is the most important time for programming mature breast muscle size. The negative impact of delayed placement on muscle development in chicks is manifested by a decrease in body weight and breast meat yield at market age (Nir and Levanon, 1993; Noy and Sklan, 1999; Vieira and Moran, 1999a; Vieira and Moran, 1999b). Similar findings in the turkey also show that early post-hatch feed deprivation decreases growth (Pinchasov and Noy, 1993), possibly programming the muscle to be smaller via decreased satellite cell mitotic activity. Delayed nutrition may influence muscle development by decreased crypt size in the duodenum and jejunum, as well as, the number of crypts per villus (Geyra et al., 2001), which would result in a decrease in digestive capacity of the small intestine.

To further exacerbate the problem of delayed placement, the energy required to emerge from the shell leaves the newly hatched poult in a nutrient deficient state (Uni and Ferket, 2004). Glycogen is the primary energy source available to the fetus when hatching; however, upon completion of the hatching process, the poult has greatly decreased its glycogen stores (John et al., 1987; John et al., 1988) increasing the need for nutrients.

It is likely that delayed placement negatively effects muscle development because of satellite cell dynamics. Satellite cell mitotic activity of immediately fed chicks was higher than starved animals *in vitro* (Halevy et al., 2000). Other studies have shown in poults that delayed access to feed immediately post-hatch decreases satellite cell mitotic activity when compared to their fed counterparts (Mozdziak et al., 2002a; Halevy et al., 2003) suggesting the importance of early nutrition on determining muscle growth potential via early satellite cell mitotic activity.

Another aspect of muscle dynamics that has not been studied in animal agriculture is the myogenic stem cell. It has been suggested by the use of Pax7 that a myogenic stem cell population exists within the satellite cell compartment (Zammit and Beauchamp, 2001). CD34, CD45, stem cell antigen-1 (Sca-1), and Bcl-2 are proposed myogenic stem cell markers. The presence of Sca-1 positive cells outside the basal lamina, and the noncommitment of Sca-1 and CD45 positive cells to the myogenic lineage (Asakura et al., 2002) makes it difficult to employ these markers in identifying myogenic stem cells. CD34 expression has been observed in satellite cells (Beauchamp et al., 2000). Bcl-2 is good marker for identifying multipotential cells committed to the myogenic lineage because they are also located within the basal lamina (Lee et al., 2000), and cells expressing Bcl-2 also express CD34 but do not express the quiescent satellite cell marker m-cadherin (Deasy et al., 2001; Qu-Peterson et al., 2002), suggesting a sub-population of cells expressing stem cell markers exists within the satellite cell compartment. The first objective of this experiment was to understand the skeletal muscle dynamics involving the satellite cell population, and the second objective of this experiment was to examine the influence of fasting on skeletal muscle dynamics of the immediate post-hatch poult.

6.3 MATERIALS AND METHODS

Turkeys

All experimental procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. Two hundred male Nicholas poults were obtained from a commercial hatchery within six hours of hatch. Poults were divided into two groups of one hundred. The first group (fed) was provided with a standard corn and soybean meal based basal starter diet (Table 1) and water ad libitum at placement. The second group (fasted) was withheld from feed and water for the first 72 hours of placement, and was then provided the basal diet (Table 1) and water ad libitum. Birds were weighed at placement, 72 hours post-hatch, one week of age and ten days of age.

Tissue sampling

Birds were randomly chosen from the population for each sampling interval. Conditions were closely monitored to ensure that there were similar environmental conditions (temperature) in each cage. After birds were randomly chosen, 5-Bromo-2'-deoxyuridine (BrdU; 10mg/ml; 10mg BrdU/100g bird weight), a thymidine analogue, was administered intra-abdominally using a 27-gauge hypodermic needle. Following the injection of BrdU, the poults were maintained for two hours before sampling to allow for the incorporation of the BrdU into the nuclei entering the S-phase of the cell cycle, when the birds were euthanized using Euthasol®[1] (0.25ml/kg body wt.). The left *Pectoralis thoracicus* was harvested from poults at 72 hours post-hatch, four days post-hatch, five

[1] Delmarva Laboratories, Midlothian, VA 23113 USA

days post-hatch, six days post-hatch and ten days post-hatch. Left *Pectoralis supracoracodeus* samples were also taken from poults at day ten. The samples taken for each time period included five poults from each treatment. Immediately after the *Pectoralis thoracicus* had been harvested, it was flash frozen in isopentane cooled in liquid nitrogen. The tissues were then placed in cryovials and stored at –80°C until sectioning. Eight micron thick cryosections were serially cut and adhered to glass slides, and allowed to warm to room temperature. Slides were then stored at –20°C until staining.

Immunohistochemistry

Slides were brought to room temperature, and they were fixed with either Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for sections to be stained with monoclonal BrdU, or 4% paraformaldehyde in Phosphate Buffered Saline (PBS-pH 7.4) for all other sections. The sections were washed three times for five minutes with PBS. Sections for monoclonal BrdU, Pax7, staining were treated with 0.07N NaOH for five minutes, and PBS was used to neutralize the NaOH. Polyclonal BrdU was treated with DNase I for 15 minutes.The primary antibodies [monoclonal mouse anti-BrdU[2]-diluted 1:20 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin; monoclonal mouse anti-Bcl-2[3] 1:10 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin; polyclonal rabbit anit-BrdU[4] 1:1000 in PBS containing 0.5% tween-20 and 0.5% bovine serum albumin; monoclonal mouse anti-

[2] Becton Dickinson, Mountain View, CA 94039
[3] Biovision, Mountain View, CA 94043
[4] Megabase Research Products

Pax7[5]-undiluted] were added to the sections and incubated overnight at 4°C in a humidified chamber. After the tissue sections were blocked with PBS containing 10% goat serum and 0.5% tween-20 for fifteen minutes to minimize background staining, the secondary antibodies were added to the sections for two hours in a humidified chamber at room temperature in the dark. The secondary antibody for the monoclonal BrdU and Pax7 staining was goat anti-mouse IgG conjugated to fluorescein-isothiocyanate (FITC), which was diluted 1:50 with PBS containing 10% goat serum and 0.5% tween-20. Polyclonal BrdU was detected using goat anti-rabbit IgG conjugated to Texas Red™ diluted 1:35 in PBS containing 10% goat serum and 0.5% tween-20. The secondary for Bcl-2 was goat anti-mouse biotin 1:30 in PBS containing 10% goat serum and 0.5% tween-20, then a wash period followed by 2 hours of streptavidin conjugated to FITC 1:50 in PBS for 2 hours. Propidium iodide (50 μg/ml in PBS) was added to all sections, except for sections double stained for polyclonal BrdU/Pax7 for 20 minutes to label all nuclei. Finally, the sections were placed in mounting media [75% (vol/vol), 75mM KCL, 10mM tris(hydroxymethyl)aminomethane, 2mM $MgCl_2$, 2mM ethylene glycol-bis(*β*-amino-ethyl ether)-*N*,*N*,*N*',*N*'- tetraacetic acid, 1mM NaN_3, pH 8.5, 1mg/mlp-phenylenediamine] and a cover slip was sealed over the section using nail polish.

Image Analysis

A Leica DMR[6]microscope with epiflorescence illumination was used to observe the tissue sections. All nuclei were observed with a propidium iodide (PI) filter set[7], the monoclonal BrdU Pax7, and Bcl-2 labeled nuclei were observed with a FITC filter set,

[5] Developmental Studies Hybridoma Bank, Iowa City, IA 52242
[6] Leica Microsystems, Bannockburn, IL 60015
[7] Omega Optical, Brattleboro, VT 05301

and the polyclonal BrdU labeled nuclei were observed with a Texas Red™ filter set. A Spot-RT CCD[8] camera was used to capture the images of nuclei visualized by the FITC and PI filter sets. The FITC and PI labeled nuclei were counted using Image-Pro Plus software[9]. The number of BrdU labeled nuclei per 1000 PI labeled nuclei was used as an index for satellite cell mitotic activity. The Pax7 labeling index was determined by the number of Pax7 positive nuclei per 1000 PI labeled nuclei. The Bcl-2 labeling index was determined by the number of Bcl-2 positive nuclei per 1000 PI labeled nuclei. The criteria for completing nuclear analysis were counting at least 1000 PI labeled nuclei. The Pax7 to BrdU ratio was determined by dividing the Pax7 labeling index by the BrdU labeling index. The Bcl-2 to BrdU ratio was determined by dividing the Bcl-2 labeling index by the BrdU labeling index. The area of muscle section containing the count of propidium iodide labeled nuclei also was determined as the number of PI labeled nuclei expressed relative to the area that was analyzed. Image-Pro Plus software was also used to determine myofiber cross-sectional area for at least 100 myofibers per muscle sample. Polyclonal BrdU and Pax7 double staining was performed to determine the Pax7+/BrdU+ labeling index determined by the number of Pax7+/BrdU+ nuclei per total number of Pax7 nuclei.

Statistics

General Linear Models procedure of SAS (SAS Institute, 1985) was used to perform a factorial analysis on age and treatment. The pen was the experimental unit for body weights, and the bird was the experimental unit for all other parameters. If population variances were unequal, then a logarithmic transformation was performed on

[8] Diagnostic Instruments Inc., Sterling Heights, MI 48314
[9] Media Cybernetics, Silver Spring MD 20910

the data before analysis. A one-way analysis of variance was performed on the muscle data. Means were separated using least significant differences (Zar, 1999). Statistical signficance was accepted at $P < 0.05$.

6.4 RESULTS

Growth

The body weights of the fed group were significantly higher than the fasted group at all time periods of the trial (Figure 1). All weights were significantly different among ages within each treatment (Figure 1). At ten days of age, *Pectoralis thoracicus* weights, *Pectoralis supracoracodeus* weights, and the percentage of *Pectoralis thoracicus* to body weights were significantly higher in the fed group compared to the fasted group (Table 2). There were no differences in the percentage of *Pectoralis supracoracodeus* to body weight at ten days of age (Table 2). Myofiber diameters were smaller in the fasted poults than compared to the fed poults, except at ten days of age (Figure 2). Diameters within the fed group were different between four and five days of age, and ten days of age and all other diameters (Figure 2). The diameters in the fasted group were the largest at ten days of age (Figure 2).

Satellite cell mitotic activity, Pax7, Bcl-2, and Number of nuclei per area

At 72 hours and four days post-hatch the fed poults had a higher index of satellite cell mitotic activity than the fasted poults (Figure 3). However, the fasted group had higher satellite cell mitotic activity than the fed group at six days of age (Figure 3). There were no differences in satellite cell mitotic activity at five days of age and ten days of age between the two groups. Among the fed poults satellite cell mitotic activity decreased with age, with the highest level at 72 hours of age and the lowest level at ten days of age compared to the other ages (Figure 3). However, in the fasted poults satellite cell mitotic activity increased with refeeding to six days of age and then was at a lower

level at ten days of age (Figure 3). The lowest levels of mitotic activity within the fasted poults were 72 hours, four days, and ten days of age when compared to the other ages (Figure 3). Pax7 labeling index was higher in the fasted group at 72 hours, four days, and five days of age when compared to the fed group (Figure 4). However, there were no differences in Pax7 labeling index at six days and ten days of age (Figure 4). Pax7 labeling index decreased with age in the fed poults, which is supported by Halevy et al. (2004). Similarly, the fasted poults Pax7 labeling index decreased with age with the lowest level at ten days of age (Figure 4). The multipotential cell marker, Bcl-2, had a higher labeling index in the fasted poults than the fed poults at 72 hours of age and four days of age, but there were no differences in the remaining time periods (Figure 5). In the fasted group, there were no differences among all ages in Bcl-2 labeling index (Figure 5). However, in the fed poults Bcl-2 labeling index was the lowest at 72 hours and four days of age (Figure 5) when compared to the other age groups. The number of PI labeled nuclei per area measured was different between fasted and fed poults, except at five days of age (Figure 6). The lowest level of PI labeled nuclei per area measured among all age groups for both the fasted and fed poults was ten days of age, indicating an increase in DNA unit size.

Pax7/BrdU, Pax7+/BrdU+, and Bcl-2/BrdU

The ratio of Pax7 to Brdu labeling index was calculated to determine the number of the satellite cells in the proliferative reserve (Halevy et al., 2004; Olguin and Olwin, 2004; Oustanina et al., 2004) compared to the number of cycling satellite cells. At 72 hours of age, the fasted group had the higher ratio (Figure 7), possibly indicating a shutdown of cycling satellite cells and an increase in the number of satellite cells in the

proliferative reserve awaiting activation upon feeding. At six days of age the fed group had the higher ratio, indicating a compensation period in the fasted group (Figure 7). Among the fed poults, there were no differences between age groups of the Pax7/BrdU ratio except at ten days of age the ratio was higher than any other age group (Figure 7). However, among the fasted poults, there were no differences between age groups of the Pax7/BrdU ratio except at 72 hours of age the ratio was higher than any other age group (Figure 7). At 72 hours and four days of age, the fed group had a higher Pax7+/BrdU+ labeling index than the fasted group (Figure 8). Interestingly, at six days of age the fasted group had a higher Pax7+/BrdU+ labeling index than the fed group, possibly indicating a compensation period (Figure 8). No other differences were found. The Pax+/BrdU+ labeling index was highest among all age groups of the fasted poults at six days of age (Figure 8). The Pax7+/BrdU+ labeling index was highest among all age groups of the fed poults at four and of age (Figure 8). A ratio of Bcl-2 to BrdU labeling index was also calculated. At 72 hours of age, the fasted group had a higher ratio than the fed group (Figure 9). The highest level of Bcl-2/BrdU ratio in the fed group among all ages was at ten days of age (Figure 9). However, the highest Bcl-2/BrdU ratio among all age of the fasted poults was at 72 hours of age (Figure 9).

6.5 DISCUSSION

Feeding poults immediately post-hatch is essential for the development of muscle because glycogen stores in the fetus are depleted during hatch (John et al., 1987; John et al., 1988), leaving the newly hatched poult in a nutrient deficient state. Feed offered to birds immediately post-hatch aids in the development of the gastrointestinal tract and increase brush border enzyme activity that increases efficiency of digestion (Uni, 1998), which can increase the nutrients available for muscle development. Conversely, the lack of feed and water immediately post-hatch decreases all aspects of digestive tract development, resulting in lower meat yield at market age (Geyra et al., 2001). Organ system development is also critical during the immediate post-hatch period, and it may have a greater physiological importance than the development of muscle (Lilburn, 1998), indicating the partition of nutrients to organ systems rather than to muscle development in a nutrient deficient state. Without access to feed and water the poult is reliant on the nutrient depleted yolk sac, which can result in increased mortality, poor growth and development, decreased disease resistance, and most importantly sub-optimal meat yield (Uni and Ferket, 2004).

One indicator of muscle development is overall body weight, which is lower at market age following immediate post-hatch starvation (Nir and Levanon, 1993; Noy and Sklan, 1999; Vieira and Moran, 1999a). Our results support previous studies because the poults fasted for the first 72 hours post-hatch had lower body weights compared to the fed group at all times, indicating a negative impact on muscle development following fasting. The negative impact of fasting on muscle development was evident in the muscle weights at ten days of age. The poults with immediate access to feed and water

had heavier *Pectoralis thoracicus* and *Pectoralis supracoracodeus* weights than fasted poults, as well as, increased *Pectoralis thoracicus* yield as a percentage of body weight in the fed poults, indicating a severe decrease in muscle development in the fasted poults. It is likely that the fasted poults suffered from decreased development because fasted chicks exhibit lower protein synthesis in the *Pectoralis thoracicus* (Yaman et al., 2000), as well as, increased levels of apoptosis (Mozdziak et al., 2002b). The number of PI labeled nuclei per area measured was lower in the fed poults at all time periods compared to the fasted poults, indicating an increase in DNA unit size in the fed poults compared to the fasted poults. This decrease in muscle weight and development at an early age is likely to result in decreased meat yield at market age (Vieira and Moran, 1999b).

It is probable that the lower levels of muscle development following post-hatch feed deprivation are directly related to satellite cell dynamics. The starved poults had lower satellite cell mitotic activity during starvation and one day after feeding than continuously fed poults. However, the fasted poults had a compensatory increase in satellite cell mitotic activity compared to the fed poults three days after refeeding, but the response was not sufficient to improve breast muscle weights at ten days of age. It is likely that the fasted poults will never equal their fed counterparts in breast meat yield because by ten days of age the satellite cell mitotic activity in both groups was the same. Further indication of an absence of compensatory growth that will result in similar meat yields at market age is that muscle following a period of stress or inactivity normally shows an increase in satellite cell mitotic activity, but this increase in satellite cell mitotic activity is short-lived and does not completely compensate for the previous absence of satellite cells (Mozdziak et al., 1997; Mozdziak et al., 2000). Feed deprivation of chicks

early post-hatch resulted in a lower level of satellite cell proliferation and lower muscle weight at market age (Halevy et al., 2000), indicating the compensatory period was not sufficient to overcome the lack of satellite cell mitotic activity early post-hatch.

A likely mechanism for the difference in satellite cell mitotic activity between starved and fed poults is the level of growth factors that are known to influence satellite cell dynamics. A variety of growth factors influence satellite cell dynamics, such as, insulin-like growth factors (IGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platlet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β). IGFs have been known to stimulate proliferation in turkey satellite cells (McFarland et al., 1993), but IGFs have also been shown to support events leading to differentiation following its action on proliferation (Engert et al., 1996). FGF has also been associated with satellite cell proliferation (Allen and Boxhorn, 1989; Dodson et al., 1996). McFarland (1991) demonstrated the importance of FGF to turkey satellite cells because the satellite cells do not grow in media lacking FGF. HGF has been shown to increase satellite cell proliferation and decrease satellite cell differentiation in rats, chickens, and turkeys (Allen et al., 1995; Gal-Levi et al., 1998; and Zeng et al., 2002). HGF has also been deemed important for proliferation of satellite cells because the HGF receptor, c-met, has been found in quiescent satellite cells (Allen et al., 1995). The importance of PDGF in turkey breast muscle development has been demonstrated by increased responsiveness to PDGF of cells isolated from the *Pectoralis thoracicus* when compared to cells isolated from the *Biceps femoris* (McFarland et al., 1997), suggesting a greater importance of satellite cells in the *Pectoralis thoracicus* during muscle development. TGF-β is a likely maintenance growth factor because it inhibits both

proliferation and differentiation in turkey satellite cells (Yun et al., 1997). The highest level of proliferation of satellite cells is the synergistic activity of IGF and FGF when acting together (Zeng et al., 2002). The major impact of growth factors influence on satellite cell dynamics indicates a mechanism for the decreased level of satellite cell mitotic activity found in fasted poults, which is supported by the fact that *Pectoralis thoracicus* IGF-I levels were higher from hatch to two days of age in chicks with immediate access to feed and water (Guernec et al., 2004). However, *Pectoralis thoracicus* IGF-I levels in fasted chicks from hatch to two days of age did not change, suggesting the mechanism where satellite cell mitotic activity is decreased in fasted poults and chicks immediately post-hatch is through a decrease in mitogenic growth factors.

Another important aspect of satellite cell dynamics is the idea that there is a sub-population of satellite cells that does not differentiate, but represents a proliferative reserve population of satellite cells to assist in self-renewal (Schultz, 1996). It is likely that the proliferative reserve does not commit to muscle repair (Morgan and Partridge, 2003). Hashimoto et al. (2004) found that there are indeed two types of cells derived from satellite cells, "round cells" and "thick cells", with round cells forming clusters and producing thick cells that commit to a myogenic pathway. Sherwood et al. (2004) also found a heterogeneous population of satellite cells based on morphology, phenotype, and function. Other research has also identified a heterogeneous population of satellite cells based on size (Baroffio et al., 1996; Blanton et al., 1999). It is possible that the round cells represent the proliferative reserve. Recently, it has been observed that a majority of satellite cells activate, proliferate, and differentiate; however, a small population of

satellite cells withdraw from the cell cycle become quiescent (Zammit er al., 2004). Rouger et al. (2004) has also observed a quiescent population in turkey satellite cells. Therefore, in light of all of the new evidence, a proliferative reserve population of satellite cells is present and is responsible for maintaining satellite cell populations for muscle development.

A recent marker of the proliferative reserve population is the protein Pax7, which is expressed in quiescent satellite cells and a small population of satellite cells for a brief period following activation (Zammitt et al., 2004). Also, the expression of Pax7 is increased at the onset of proliferation following a period of stress (Zhao and Hoffman, 2004). Fasting represented a stress to the muscle and results in a higher number of satellite cell nuclei expressing Pax7 at 72 hours of fasting, one day after feeding, and two days after feeding when compared to the fed poults. Suggesting that the proliferative reserve is up-regulating the potential for adding myonuclei to the myofiber during a period of compensation, which corresponded to an increase in satellite cell mitotic activity in fasted poults when compared to the fed poults at six days of age or three days after feeding the fasted poults. Pax7 and myogenin expression are mutually exculsive (Olguin and Olwin, 2004). Myogenin indicates terminal differentiation of the muscle precursor cell derived from the satellite cell (Seale et al., 2000), and is expressed downstream of Pax7. Since Pax7 and myogenin are mutually exclusive, it is possible that Pax7 is favoring the maintenance of the proliferative reserve by preventing differentiation. The overexpression of Pax7 downregulates MyoD, the myogenic regulatory factor found during proliferation, and myogenin (Olguin and Olwin, 2004), indicating that Pax7 is favoring a proliferative reserve. Halevy et al. (2004) have found a

similar relationship of Pax7 and MyoD in chickens, supporting the findings in this study that high levels of Pax7 expression in the fasted poults was accompanied by a low level of satellite cell mitotic activity. However, during the period of compensation in the fasted poult the dynamics is a little less clear.

To further investigate the relationship between the proliferative reserve population of satellite cells expressing Pax7 and the mitotically active satellite cell population, we examined the relationship between the Pax7 labeling index and the BrdU labeling index to determine the number of the satellite cells in the proliferative reserve compared to the number of cycling satellite cells. The fasted poults had a higher Pax7/BrdU ratio than fed poults at 72 hours and four days of age, exhibiting a conservation of the proliferative reserve for a later period of compensation. However, during compensation at six days of age, the fasted group had a lower Pax7/BrdU ratio than the fed poults.

Pax7+/BrdU+ labeling index was performed by double staining with Pax7 and BrdU to determine the percentage of Pax7 cells that are in the S-phase of the cell cycle. Pax7+/BrdU+ labeling index was lower in the fasted poults than the fed poults at 72 hours and four days of age. However, this is most likely due to the extremely low level of BrdU positive cells and high levels of Pax7 positive cells of the fasted poults, indicating the conservation of the proliferative reserve. Also, the Pax7+/BrdU+ ratio was higher in the fasted group than the fed group at six days, indicating the activation of the satellite cell reserve for the production of daughter cells needed for compensation. At ten days of age, it was apparent that the fasted group had reached satellite cell dynamic equilibrium because there was no difference between the fasted group and the fed group. By double

staining with Pax7 and BrdU, it was also apparent that a small population of cells expressed both during all time frames, which supports the findings that there are cells that express Pax7 during proliferation (Halevy et al., 2004; Zammitt et al., 2004), and indicates the location of the cells is within the satellite cell compartment.

In light of these findings, it is interesting to note that recent studies have found that mild stresses have increased satellite cell mitotic activity in chicks. Halevy et al., (2001) found that chicks exposed to a mild heat stress at three days of age increased levels satellite cell mitotic activity, resulting in increased body weight and percent breast meat yield at market age. Chicks fed a lysine deficient diet have also had increased levels of satellite cell mitotic activity when compared to chicks fed diets sufficient in lysine (Pophal et al., 2004). It is possible this mild stress slightly increases the activation of the proliferative reserve of satellite cells without negatively impacting the majority of satellite cells following the normal pathway of muscle regeneration.

Finally, cells expressing Bcl-2 may also play an important role in the dynamics of muscle development in the turkey. Bcl-2 was used because it is expressed by cells within the basal lamina of the myofiber and it is not co-expressed with the quiescent satellite cell expression marker, m-cadherin, possibly representing a sub-population of satellite cells (Lee et al., 2000; Deasy et al., 2001; Qu-Peterson et al., 2002). Also, Bcl-2 expressing cells are muscle specific because of the high level of expression in C2C12 cells, and high levels of co-expression with the muscle specific protein desmin (Dominov et al., 1998). It is possible that Bcl-2 is expressed upstream or in coincidence of Pax7 expression because Bcl-2 expressing cells do not express the myogenic regulatory factors myogenin, myosin, and MRF4 that are associated with mid to late stages of myogenesis (Dominov et

al., 1998), and are not found in large numbers, indicating that they are not directly involved with highly proliferative daughter cells. Bcl-2 labeling index was used in this study to determine the role of subliminal and possible multipotential cells in developing muscle dynamics. The labeling index of Bcl-2 for all time frames fell within the limit of 1-4% of all cells that was previously determined by Dominov et al. (1998). At 72 hours and four days of age, the fasted poults had a higher Bcl-2 labeling index than fed poults, suggesting a possible increase in the number of cells that can eventually donate nuclei to the myofiber following a period of stress.

In conclusion, fasting poults decreases muscle development resulting in decreased muscle weights. During a period of fasting immediately post-hatch, there is an increase in the proliferative reserve compartment of satellite cells and a decrease in satellite cell mitotic activity, which programs the muscle to stop growing. The period of compensation in poults following refeeding is marked by increased levels of satellite cell mitotic activity, which is not sufficient to improve muscle yield at ten days of age compared to poults that did not experience a stress to the muscle. Normal muscle development follows a pattern of decreasing satellite cell mitotic activity as the poult grows older. In the future, it may be necessary for the poultry industry to address satellite cell dynamics and delayed placement to increase breast meat yield at market age.

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Table 1. Basal diet composition

Ingredients	Percentage of Diet
Corn	**52.72**
Soybean meal (48% CP)	**30.0**
Limestone	**1.26**
Dicalcium Phosphate (18.5% P)	**1.83**
Gluten meal	**5.0**
Poultry meal (60% CP)	**8.0**
DL-methionine	**0.08**
Lysine-HCL	**0.42**
Sodium Chloride	**0.30**
Choline Chloride (60%)	**0.2**
Trace Mineral Premix[1]	**0.2**
Vitamin Premix[2]	**0.2**
Selenium[3]	**0.05**
Calculated Nutrient Analysis	
Kcal ME/Kg	**2920**
Crude Protein, %	**27.5**
Crude Fat, %	**3.47**
Lysine, %	**1.60**
Methionine, %	**0.55**
Met + Cys, %	**0.97**
Ca, %	**1.2**
Non-phytate P, %	**0.86**
Na, %	**0.17**

[1]Supplied the following per kilogram of feed: 120 mg Zn as $ZnSO_4$; 120 mg Mn as Mn $SO_4 \cdot H_2O$; 80 mg Fe as Fe $SO_4 \cdot H_2O$; 10 mg Cu as Cu SO_4; 2.5 mg I as $Ca(IO_3)_2$; 10 mg Co as $CoSO_4$.

[2]Supplied the following per kilogram of feed: vitamin A, 26,000 IU; cholecalciferol, 8000IU; vitamin E, 90 mg as α-tocopheryl acetate; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4 mg; pyrodoxine, 16 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 4 mg; biotin, 0.500 mg; vitamin B_{12}, 0.08 mg; ethoxyquin, 200 mg.

[3]Selenium premix supplied the following per kilogram of feed: 0.2 mg Se as $NaSe_2O_3$.

Table 2. *Pectoralis thoracicus*, *Pectoralis supracoracodeus* weights (g), *Pectoralis thoracicus*/body weight ratio and *Pectoralis supracoracodeus*/body weight ratio (g/g) at ten days of age.

	Fed Poults	**Fasted Poults**	**Sample Size**
***Pectoralis thoracicus* Wts. (g)**	$7.81^{a} \pm 0.21$	$3.88^{b} \pm 0.21$	5
***Pectoralis supracoracodeus* Wts. (g)**	$1.70^{a} \pm 0.04$	$0.94^{b} \pm 0.04$	5
***Pectoralis thoracicus* ratio**	$0.032^{a} \pm 0.0009$	$0.026^{b} \pm 0.0009$	5
***Pectoralis supracoracodeus* ratio**	$0.007^{a} \pm 0.0002$	$0.006^{a} \pm 0.0002$	5

[a,b] Values within rows of the same pairing without a common superscript are significantly different ($\boldsymbol{P} \leq 0.05$).
Values are means ± SE.

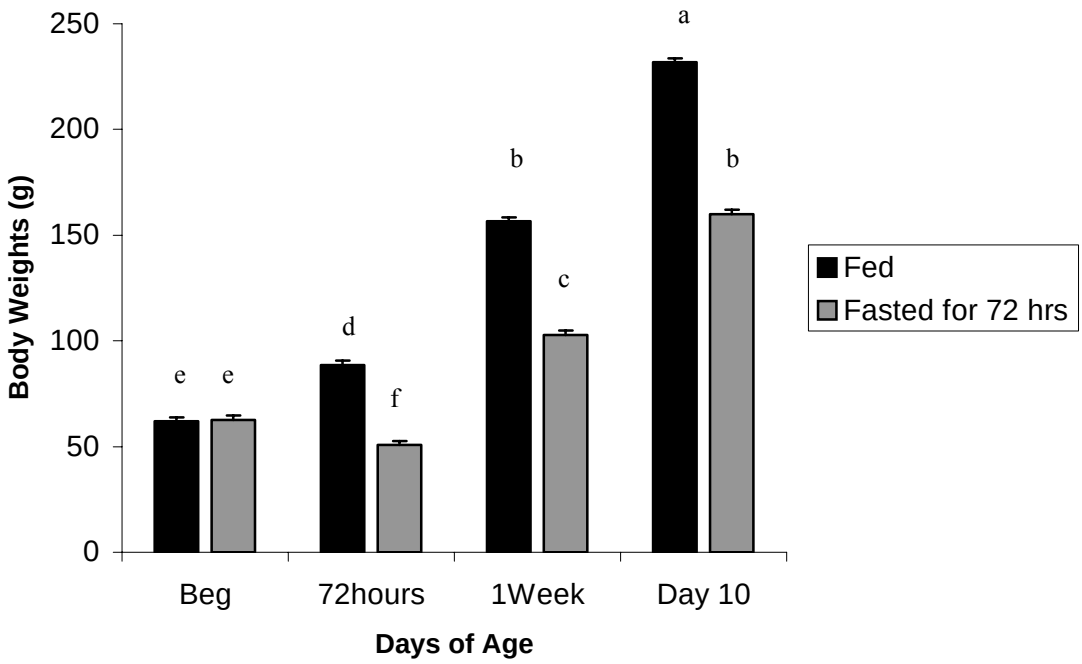

Figure 1. Body weights. Body weights are represented in grams. Values are the means ± SE. Each treatment began with 100 birds. Pens were the experimental units with 12 pens per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$.

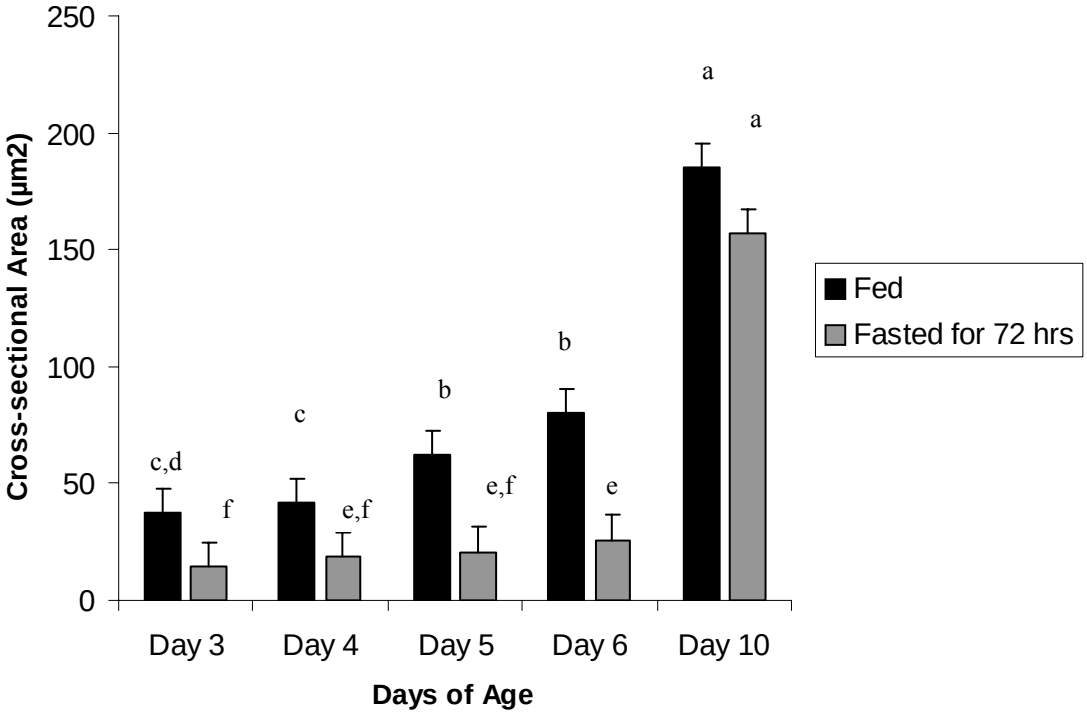

Figure 2. Myofiber diameters. Diameter is cross-sectional area (μm^2). Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0016$.

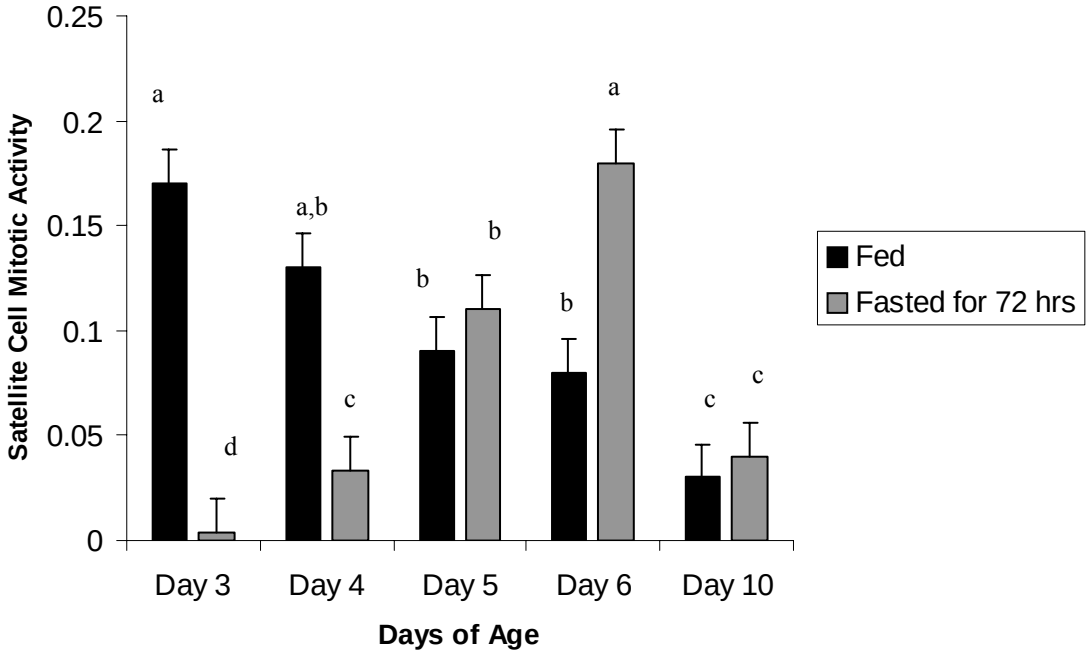

Figure 3. Satellite cell mitotic activity. Satellite cell mitotic activity is expressed as the number of BrdU-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$.

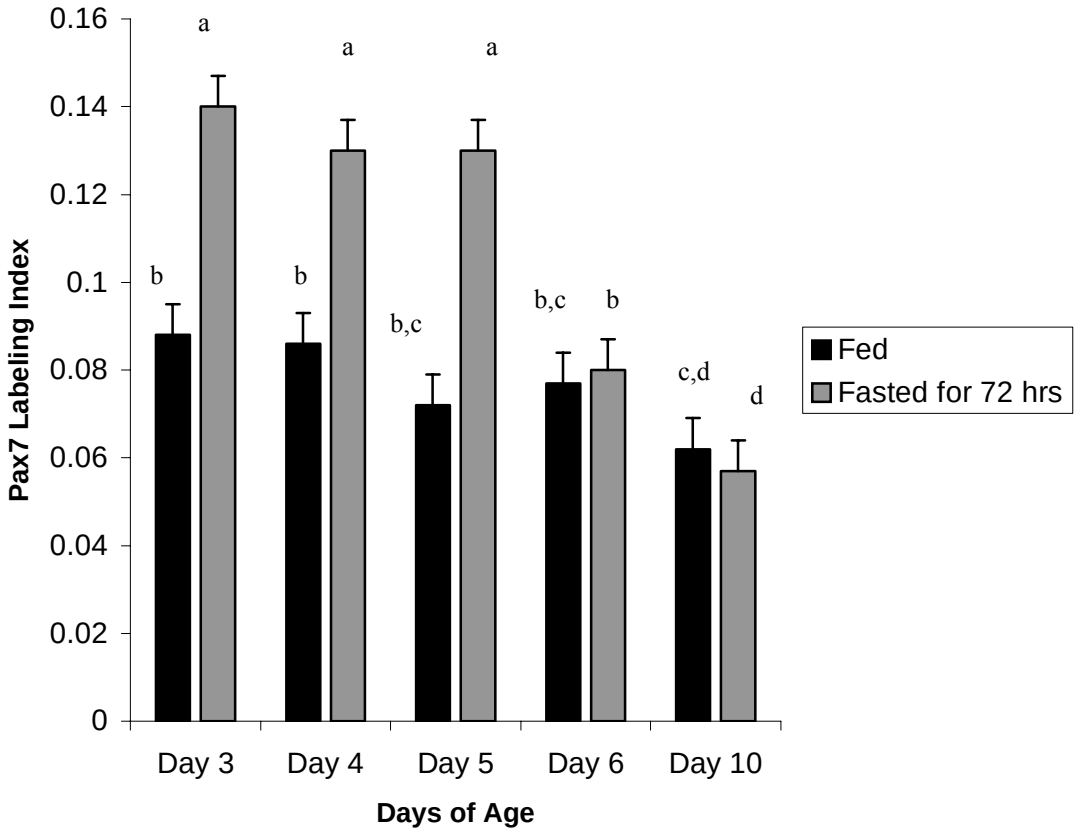

Figure 4. Pax7 labeling index. Pax7 labeling index is expressed as the number of Pax7-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0002$.

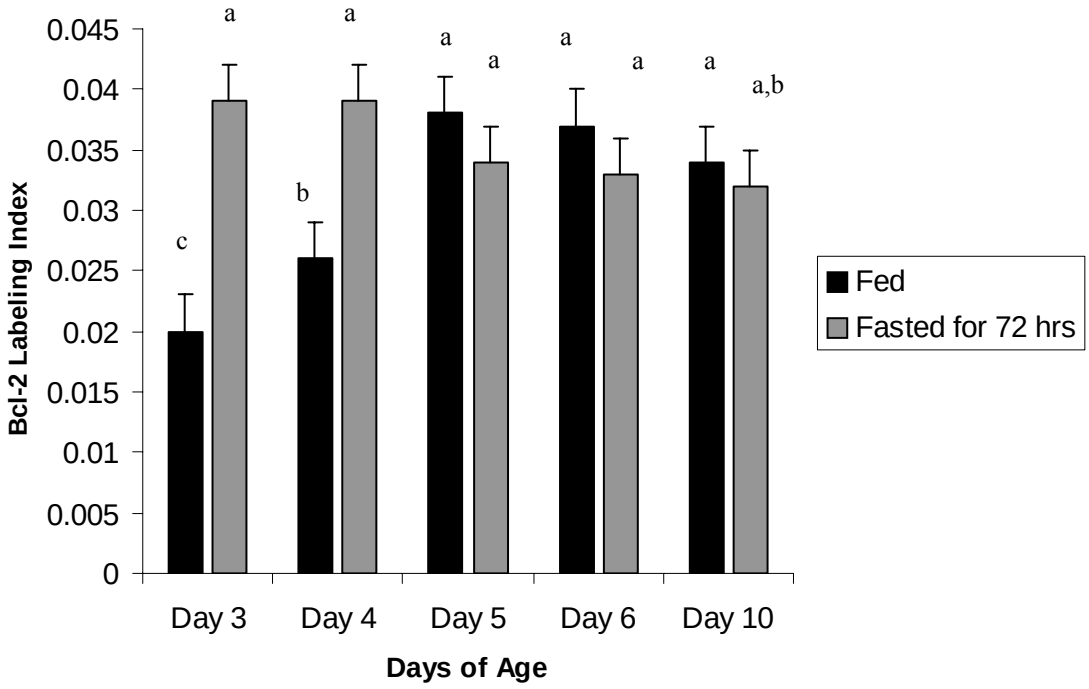

Figure 5. Bcl-2 labeling index. Bcl-2 labeling index is expressed as the number of Bcl-2-fluorescein isothiocyanate labeled nuclei per 1000 propidium iodide labeled nuclei. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$.

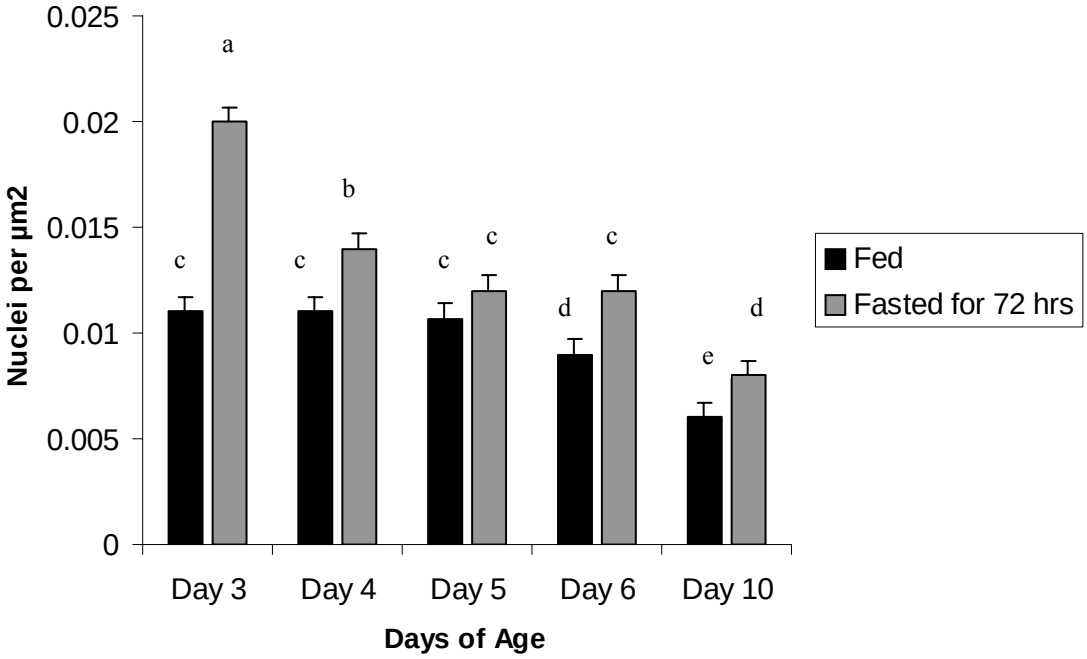

Figure 6. Nuclei per µm^2. Nuclei per µm^2 is the number of propidium iodide labeled nuclei per µm^2 of tissue section. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0038$.

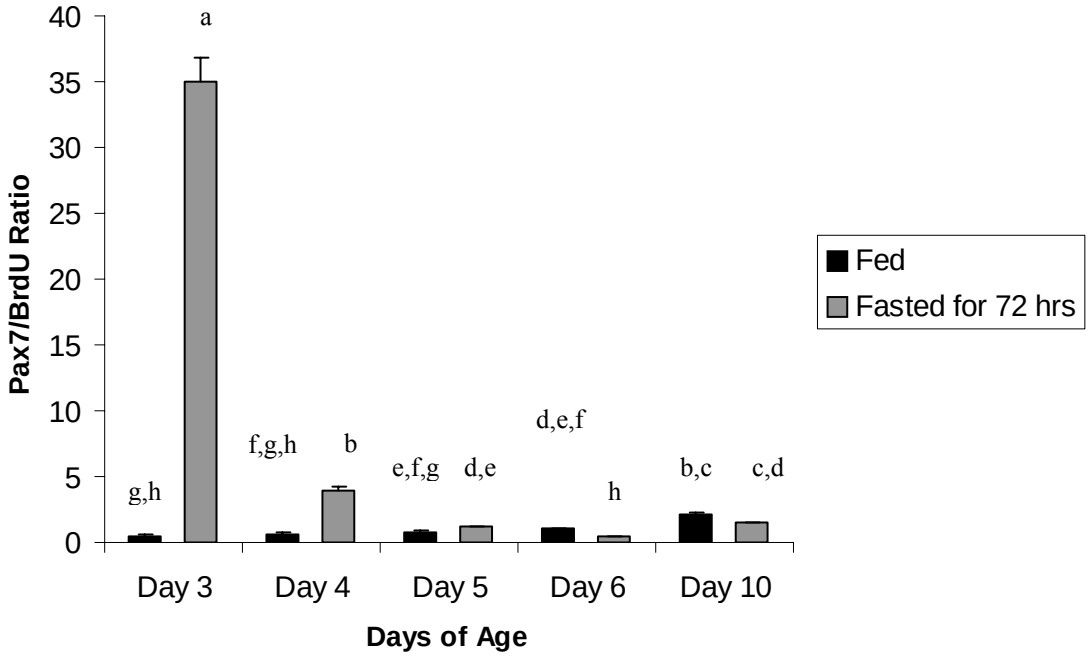

Figure 7. Pax7/BrdU labeling index ratio. Pax7/BrdU ratio is the Pax7 labeling index/BrdU labeling index. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$.

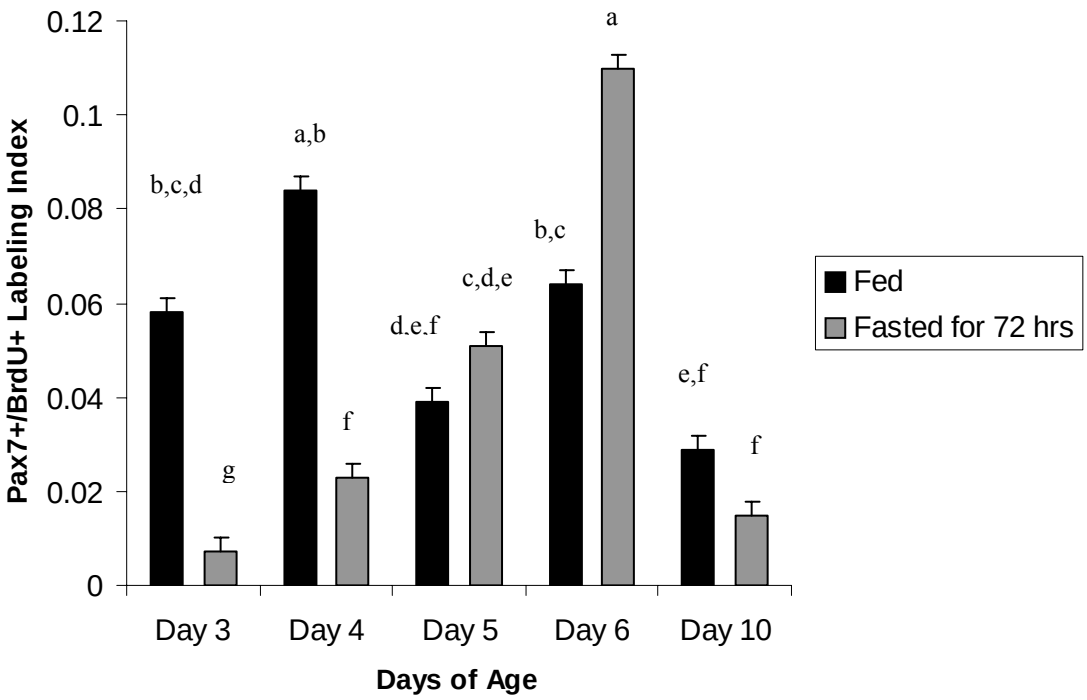

Figure 8. Pax7+/BrdU+ labeling index. Pax7+/BrdU+ index is expressed as the number of nuclei that are positive for both Pax7 and BrdU per total number of Pax7 labeled nuclei. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$

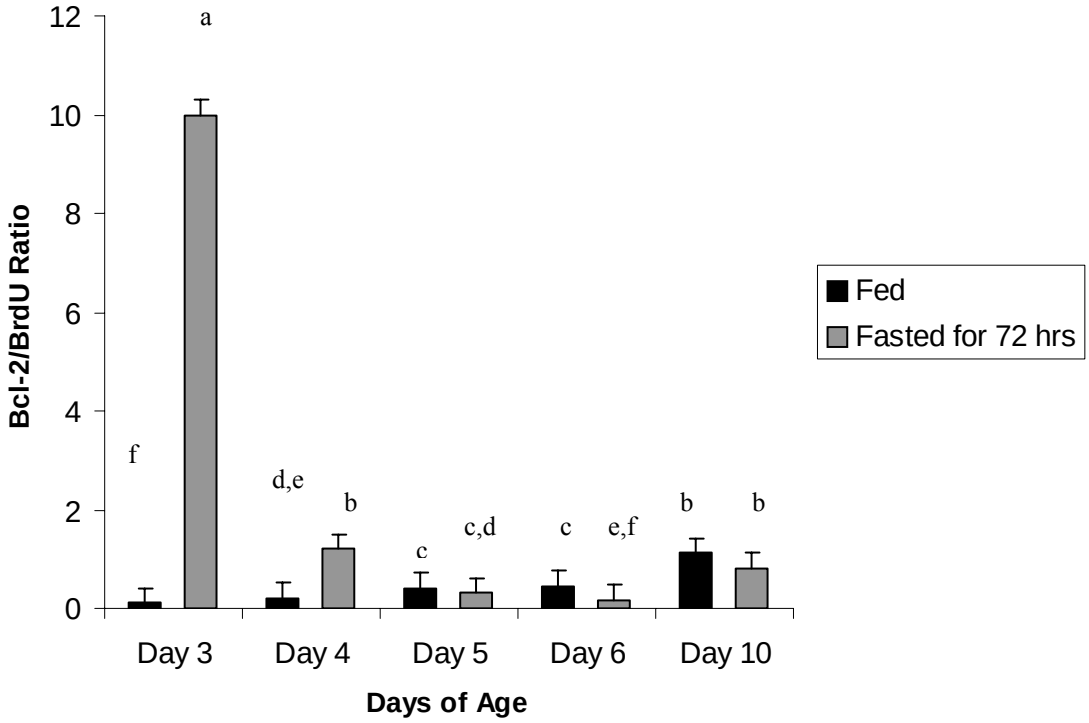

Figure 9. Bcl-2/BrdU labeling index ratio. Bcl-2/BrdU ratio is the Bcl-2 labeling index/BrdU labeling index. Values are the means ± SE of 5 birds per treatment. Values without a common letter are significantly different ($\boldsymbol{P} \leq 0.05$). P-value for interaction effects is $\boldsymbol{P} < 0.0001$.

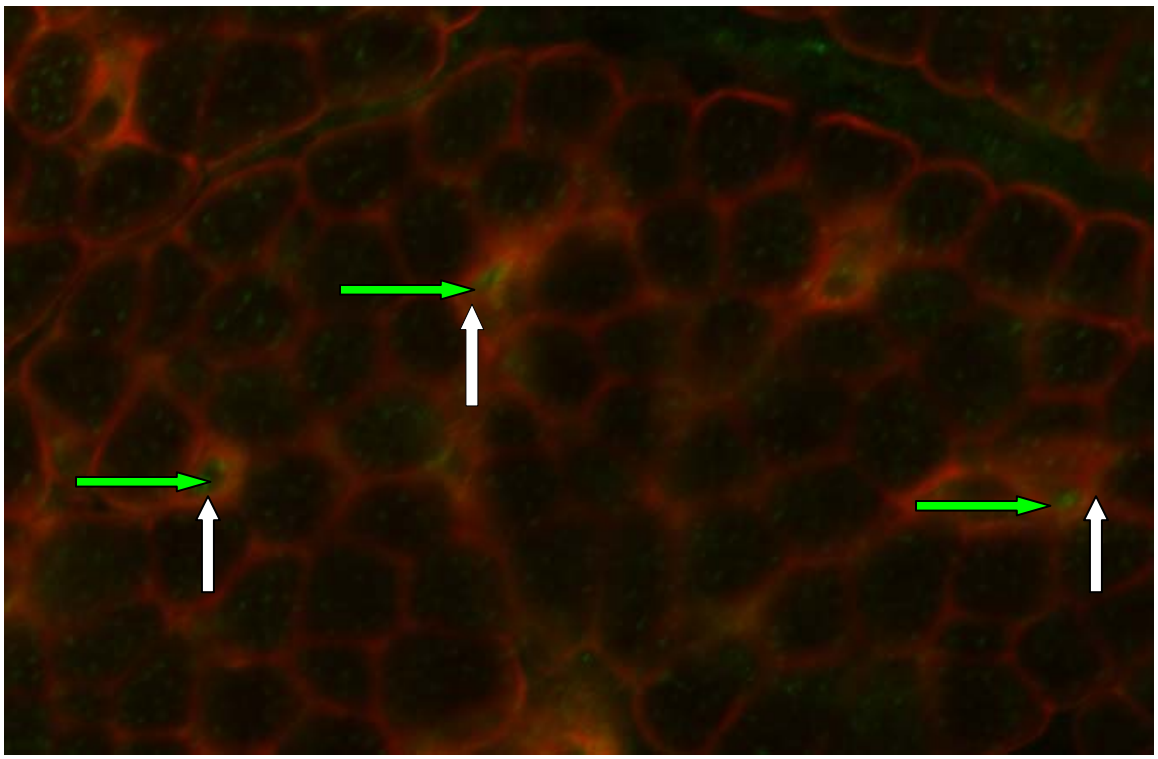

Figure 10. Bcl-2 positive cells (green arrows and green cells) residing within basal lamina (white arrows and red staining).

7. SUMMARY

A common practice in the poultry industry is the delayed placement of poults because of hatchery management and logistical limitations in transportation. It has been suggested that delayed placement reduces body weight and breast meat yield, but the mechanisms of muscle development involved are unknown. Recently, the limited understanding of muscle development has led to the investigation of the mechanisms involved with decreased performance of turkeys subjected to delayed placement and other nutritional paradigms early in life. The current mechanism commonly associated with early muscle development is satellite cell dynamics. Satellite cells are located between the basal lamina and the sarcolemma of the myofiber (Mauro, 1961; Campion, 1984), and are mitotically active post-hatch, whereas, existing myonuclei are not mitotically active post-hatch (Stockdale and Holtzer, 1961). Therefore, the only population of cells that are capable of donating nuclei during myonuclei accretion is the satellite cell population. Historically, it was suggested that the growth of adult muscle did not occur through an increase in DNA unit size (Moss, 1968), which is considered the myonucleus and the surrounding cytoplasm. However, recently in the turkey, it has been determined that an important mechanism of growth in the adult muscle is through an increase in DNA unit size (Mozdziak et al., 1994). Therefore, myonuclear accretion from satellite cell mitotic activity can program mature muscle size.

The thymidine analog, BrdU (5-bromo-2'-deoxyuridine), is used to detect mitotically active cells by labeling cells entering the S-phase of the cell cycle. However, a problem in investigating avian fetuses using BrdU arises because the surrounding

eggshell makes it difficult to inject the fetus. A method of injecting avian fetuses was accomplished and deemed a viable method for future studies.

One possible method of overcoming delayed placement would be supplying nutrients to the fetus in ovo. However, it was found that the delivery of 0.4% saline, 18% egg white protein, and 0.1% β-hydroxy-β-methylbutyrate (HMB) did not improve body weight, satellite cell mitotic activity, and muscle development when compared to saline injected controls. Interestingly, it was determined when comparing 25E, 24 hours, 48 hours and one week of age, 48 hour poults had the highest level of mitotic activity ($P \leq 0.05$) and 25E had the lowest ($P \leq 0.05$), indicating that within one week of age satellite cell mitotic activity significantly decrease from the immediate post-hatch period.

In understanding satellite cell dynamics it is also important to review a possible proliferative reserve of satellite cells expressing the protein Pax7 and the satellite cell population expressing Bcl-2. It was determined that withholding feed from poults for 48 and 72 hours resulted in a decrease in satellite cell mitotic activity ($P \leq 0.05$) and an increase in the satellite cell proliferative reserve ($P \leq 0.05$) when compared to fed counterparts at the time of starvation. After feed was offered, the poults went through a period of compensation resulting in an increase in satellite cell mitotic activity ($P \leq 0.05$). However, the compensation period was insufficient to compensate for the loss of body weight and muscle development when compared to immediately fed poults. Bcl-2 labeling index was higher ($P \leq 0.05$) in the fasted poults than the fed poults at 72 hours of age and four days of age, indicating the recruitment of cells to aid in muscle repair.

Adding the leucine metabolite, β-hydroxy-β-methylbutyrate (HMB), to immediate post-hatch diet increased satellite cell mitotic activity ($P \leq 0.05$) during the

immediate post-hatch period, as well as, body weights to one week of age ($P \leq 0.05$) when compared to poults fed a standard diet. However, the long-term impact at market age of adding HMB is unclear.

In conclusion, it is apparent that satellite cell mitotic activity and the Pax7 labeling index decrease with age through the first week post-hatch, indicating the importance of the immediate post-hatch period to manipulate mature muscle size. Delayed placement of poults results in a decrease in satellite cell mitotic activity and a preservation of the proliferative reserve satellite cell population that could result in decreased breast meat yield at market age. This evidence strongly suggests the importance of the immediate post-hatch period in muscle development. However, more in depth research involving grow-out trials is needed to fully understand the impact of satellite cell dynamics early in life on meat production at market age.

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