Feed restriction delays developmental fast skeletal muscle myosin heavy chain isoforms in turkey poult selected for differential growth

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ABSTRACT Genetic selection has been very successful at significantly increasing BW and breast muscle proportion in commercial broiler and turkey strains. The mechanisms of breast muscle growth in poultry and the interactive effects of nutritional status and selection are not fully understood. The hypothesis underlying the current study is that feed restriction, simply as a vehicle for controlling early growth, would delay the temporal expression pattern of neonatal (nMyHC) and adult (aMyHC) fast skeletal muscle myosin heavy chain (MyHC) isoforms in the pectoralis major muscle of turkey poult. The poultry growth model used to evaluate this hypothesis consisted of a randombred control turkey line (RBC2) that represents commercial turkeys of the 1960s and a line developed from the RBC2 by selection for BW at 16 wk of age (F line). The F line has significantly heavier breast muscles than the RBC2 concomitant with increased BW, but the proportion of breast muscle relative to BW is similar. A quantitative indirect ELISA using fast skeletal MyHC isoform specific monoclonal antibodies revealed no significant line differences in the temporal expression of posthatch fast skeletal muscle MyHC in ad libitum fed poult. Feed restriction, however, altered the temporal expression patterns of nMyHC and aMyHC in both F line and RBC2 poult compared with the poult fed ad libitum.

Key words: turkey, breast muscle, feed restriction, ELISA, myosin isoform

INTRODUCTION

Within commercial broiler and turkey strains, geneticists have been very successful at increasing BW gain over the course of a commercial production cycle as well as the proportion of breast muscles. The latter is in response to market demand for more white meat. The associated changes or improvements in market traits are not without biological tradeoffs, and there are many studies on the negative or tradeoff effects of selection that may also result in negative collateral effects on protein functionality, which is important to the quality of further processed breast meat products (Liu et al., 2002; Woelfel et al., 2002; Updike et al., 2005; Owens et al., 2009; Smith and Northcutt, 2009; Strasburg and Chiang, 2009).

White meat in poultry is largely derived from the breast muscle (pectoralis major) and is primarily composed of fast-twitch white muscle fibers. The growth of the pectoralis major muscle is accompanied by the temporal expression of muscle specific protein isoforms (Schiaffino and Reggiani, 1996), including the development of fast skeletal muscle myosin heavy chain isoforms (Bandman, 1985; Tidyman et al., 1997). The contribution of these proteins to the growth, development, and function of muscle remains an active but, as yet, unresolved area of investigation (Bottinelli, 2001). Fast skeletal myosin heavy chain (MyHC) isoforms have been characterized in chickens by immunochemical analysis using fast skeletal MyHC isoform specific monoclonal antibodies (Cerny and Bandman, 1987; Bandman and Bennett, 1988; Bourke et al., 1991; Moore et al., 1992). Chicken fast skeletal MyHC specific monoclonal antibodies cross react with the turkey MyHC isoforms, and the temporal and tissue specific expression of the MyHC isoforms in turkeys is similar to that reported in chickens (Maruyama and Kanemaki, 1991; Maruyama et al., 1993).

Two turkey lines that have been commonly used to study the effects of genetic selection for growth are a randombred control line representative of the commercial turkey of the late 1960s (RBC2) and the F line, which was developed from the RBC2 by selection for BW alone at 16 wk of age (Nestor, 1977). It was reported that feed restriction and controlled early growth equalized mortality following a disease challenge in F line and ad libitum fed RBC2 poult, and both were significantly lower than F line control poult fed ad libitum (Nestor et al., 1999). A recent study associated
feed restriction on the temporal expression of skeletal muscle specific proteins and the concomitant reduction in growth (Zapata et al., 2011). Supported by these evidence, the objective of the current study was to quantify the temporal expression of fast skeletal neonatal MyHC (nMyHC) and fast skeletal adult MyHC (aMyHC) isoforms in the pectoralis major from restricted fed and ad libitum fed poults from RBC2 and F line turkey poults. A better understanding of maturation mechanisms and how feed restriction affects the maturation onset can help improve poultry breeding practices.

MATERIALS AND METHODS

Bird and Dietary Protocol

Turkey poults from the RCB2 and F line were used in this experiment. The poults from each line were reared in heated Petersime battery brooders. All birds were fed the Ohio State University turkey starter diet, which met or exceeded the NRC nutrient recommendations for turkeys (NRC, 1994). Poults were allowed either ad libitum access to feed or given access to feed for 30 min per day beginning at 4 d of age (restricted fed). At the beginning of the experiment, 50 RBC2 and 50 F line poults were randomly assigned to 16 pens (8 pens for RBC2 and 8 pens for F line). Of the 8 pens assigned to each turkey line, the poults in 4 pens had ad libitum access to feed, whereas the other 4 pens were restricted fed. To accommodate for replication and avoid having each pen represent a single experimental unit, one bird per pen was randomly selected from each of the pens at each sample age for the respective line and treatment combination. During the course of the experiment, some animals died, so the samples collected taken at later ages represented a reduced number of poults.

Poults were individually weighed and euthanized following standard procedures (FASS, 1998). Muscle samples were removed from the pectoralis major, flash frozen in liquid nitrogen, and subsequently stored at −80°C until further analysis. In summary, the experimental design was a 2 (genotype) × 2 (diet) × 5 (age) factorial arrangement of treatments with 4 to 5 biological replicates within each treatment combination. In total, 48 RBC2 poults and 50 F line poults were analyzed. All animals were handled in compliance with Institutional Animal Care and Use Committee policies and guidelines at The Ohio State University.

Myofibrillar Protein Preparation

A modified myofibrillar protein enriched muscle extract from 7, 11, 14, 17, and 21 d posthatch poults was prepared and analyzed as previously described, with modifications (Reddish et al., 2005). Briefly, 1 part muscle was mined in 2 parts ice-cold low-salt buffer (0.02 M KCl, 0.002 M KH₂PO₄, and 0.001 M EGTA, pH 6.8) and centrifuged at 10,000 × g, 10 min, 4°C. The supernatant, containing sarcoplasmic proteins was removed and the pellet, containing myosin for analysis, was solubilized in a high-salt buffer solution (0.04 M Na pyrophosphate, 0.001 M MgCl₂, and 0.002 M EGTA, pH 9.5; Figure 1). Solubilized myosin was precipitated by dialysis in low-salt buffer and solubilized in storage buffer (0.04 M sodium pyrophosphate, 0.002 M MgCl₂, and 0.002 M EGTA, pH 9.5, and 50% glycerol) and stored at −20°C. The protein concentration of individual muscle extracts was determined by bicinchoninic acid assay according to the manufacturer’s protocol using column purified myosin as a standard (Pierce Endogen, Rockford, IL).

Column Purified Myosin Standards

Chicken fast skeletal muscle myosins were prepared from both 5-d posthatch (nMyHC) and 42-d posthatch (aMyHC) chicken pectoralis major muscle and purified by diethylethylaminoethyl chromatography (Margosian and Lowey, 1982) and precipitated by dialysis in 0.1 M KCl, 0.01 M imidazole, 0.005 M MgCl₂, 0.005 M adenosine triphosphate, 0.001 M dithiothreitol, 0.001 M phenylmethanesulfonyl fluoride at 4°C for 18 h. The pellet was solubilized in 0.5 M KCl, 0.01 M imidazole, 0.005 M MgCl₂, 0.005 M adenosine triphosphate, 0.001 M dithiothreitol, 50% glycerol and stored at −20°C.

Protein Purity

The purity of the column purified myosins and muscle extracts was evaluated by 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue G250 and subsequently destained with 10% acetic acid.
**Immunohistochemistry**

Standard curves were generated independently for nMyHC and aMyHC from column-purified myosin standards. The quantity of nMyHC and aMyHC was determined by a modification of the semiquantitative ELISA reported previously (Wick et al., 2003; Reddish et al., 2005). Briefly, each sample and standard (200 ng of protein) was plated in triplicate onto a 96-well EIA/RIA plate (Costar Corp., Cambridge, MA), incubated for 30 min at 37°C in a humidified chamber, then washed with PBS, 0.15% Tween-20 (Fisher Biotechnology, Fair Lawn, NJ). Plates were subsequently blocked in 3% Tween-20 in PBS (PBST) for 30 min at 37°C in a humidified chamber and then incubated with the monoclonal antibodies; 2E9, specific for chicken/turkey fast skeletal muscle nMyHC, and AB8, specific for chicken/turkey fast skeletal muscle aMyHC (Maruyama and Kanemaki, 1991; Moore et al., 1992; Maruyama et al., 1993). The antibodies were used at: 1:5 for 2E9 and 1:1,000 for AB8 and incubated for 30 min at 37°C. Plates were subsequently washed with PBST. Bound mAb was detected with horseradish peroxidase conjugated goat anti-mouse IgG (H + L; Pierce-Endogen, Rockford, IL) at a dilution of 1:1,000 in PBST and incubated for 30 min at 37°C. Plates were washed with PBST, and 100 μL of ABTS peroxidase substrate solution (KPL Inc., Gaithersburg, MD) was added to each well for 10 min. The absorbance of each well was scanned using Laboratory systems Multiskan EX at 405 nm (version 1.0, Labsystems, Vantaa, Finland). Each ELISA plate contained standard curves based on known amounts of column-purified chicken neonatal and adult myosin previously determined to be within the linear dynamic range of the antibody titers used. Data were reported as a ratio of the individual myosin isoform divided by the sum of the neonatal and adult MyHC isoform concentrations.

**Statistical Analysis**

**BW**. Data were analyzed using the MIXED model of SAS V.9.2 (SAS Institute Inc., Cary, NC). Body weight estimation was previously described in Zapata et al. (2011).

**Myosin Isoform Ratios**. Data were analyzed using the mixed model of SAS V.9.2 (SAS Institute Inc., Cary, NC). Myosin ratio differences across treatments and days were estimated by including the fixed effects of turkey line, dietary treatment, age, and all their interactions. Because the ratios of each isoform are not independent from each other, only the aMyHC isoform was analyzed. From the percentage of adult MyHC isoform, the percentage of nMyHC isoform can be derived. In addition, the experiment included a repeated measurement term to identify the birds that were raised together in the same battery pen. Only the 3-way interaction terms were estimated. The model is described as follows:

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\text{adult myosin ratio}_{ijkl} = \frac{\text{line}_i \times \text{treatment}_j \times \text{day}_k + \epsilon_{ijkl}}{\epsilon_{ijkl}}
\]

where adult myosin ratio\(_{ijkl}\) is the dependent variable measured on a poult of line \(_i\) (i.e., RBC2 or F) from treatment\(_j\) (full ad libitum) fed or restricted fed) on day\(_k\) (time point \(k = 7, 11, 14, 17,\) and \(21\) d) from bird \(l\). Line\(_i\) is the effect of the turkey line; treatment\(_j\) is the effect of the treatment; day\(_k\) is the effect of the day; and \(\epsilon_{ijkl}\) is the random error inherent to each measurement, which is assumed to be independent of other observations and normally distributed with mean zero and SD of \(\sigma^2\).

**RESULTS AND DISCUSSION**

**Weight Gain**

In the present study, the expected genotype differences in BW in the ad libitum fed poult was evident throughout the study as has been reported previously (Nestor, 1984; Nestor et al., 2008). Body weight data used in this study are a subset of a data set previously published (Zapata et al., 2011) with exceptions noted in the Materials and Methods section.

**Myofibrillar Protein Preparation**

A modified myofibrillar protein enriched muscle extract from 7, 11, 14, 17, and 21 d posthatch pouls was prepared and analyzed as previously described (Reddish et al., 2005), with modifications described in the Materials and Methods section. A representative gel image is presented in Figure 1 indicating the removal of sarcoplasmic proteins (Figure 1S) with concomitant enrichment of myosin in the myofibrillar fraction (Figure 1M).

**Fast Skeletal Muscle MyHC Isoform Expression**

Myosin is the major contractile protein of muscle and is a member of a multigene family of contractile proteins. Fast skeletal muscle myosin is expressed in a spatially and temporally specific manner in turkeys and chickens with the nMyHC isoform preceding the aMyHC during skeletal muscle development (Bandman and Bennett, 1988; Maruyama et al., 1993; Tidyman et al., 1997; Rushbrook et al., 1998). The percentage of the aMyHC isoform (Figures 2 and 3) is reported as the ratio of the aMyHC isoform concentration divided by the sum of both the aMyHC + nMyHC concentrations for each turkey line, treatment, and sample age. In the F line and RBC2 ad libitum fed pouls, there was a similar increase in the ratio of aMyHC between 7 d (0.014 ± 0.006 and 0.025 ± 0.011, respectively; Figures 2 and 3, dark gray bars) and 21 d (0.583 ± 0.058 to 0.507 ± 0.042 respectively; Figures 2 and 3, dark gray bars). These results are consistent with turkey muscle data reported by Merly et
al. (1998), who reported no differences in the posthatch temporal expression of the MyHC isoforms in light-weight and heavyweight turkey lines. These investigators also noted that selection based on growth rate did not modify muscle fiber maturation. Our data are also consistent with Maruyama et al. (1993), who showed that nMyHC expression peaked at 7 d posthatch and then declined through 21 d. Our data, however, are inconsistent with our previous studies in selected broiler genotypes that transitioned to the adult MyHC isoform at an earlier age than slow-growing leghorn chicks (Wick et al., 2003; Reddish et al., 2005). However, studies comparing leghorns with broilers and the isoform transitions in animals under nutritional challenge in the current turkey lines may not be completely appropriate.

The graphs in Figures 2 and 3 (light gray bars) also show that the effect of feed restriction on the temporal expression of the developmental fast skeletal muscle MyHC isoforms in the breast muscle in the F line and RBC2 pouls is the same \( (P > 0.05) \). Both lines exhibit a significant delay in the transition to the aMyHC isoform. The ratio of aMyHC increased from 0.004 ± 0.003 at 7 d posthatch to 0.368 ± 0.020 at 21 d posthatch in the F line restricted fed pouls (Figure 2, light gray bars) and 0.010 ± 0.006 at 7 d posthatch to 0.186 ± 0.046 at 21 d posthatch in the restricted-fed RBC2 pouls (Figure 3, light gray bars). These ratios are consistent with the calculated BW of the restricted-fed pouls in both lines. Thus, when pouls were restricted fed to a constant BW, the neonatal isoform remained the predominant isoform, suggesting that the muscle tissue had not yet transitioned to the adult stage within the myofibrillar organelle of skeletal muscle.

The growth and development of the pectoralis major is accomplished by the temporal expression of fast skeletal muscle specific proteins. The predominant fast skeletal muscle specific protein is myosin. During muscle development, myosin is temporally expressed as a series of 6 isoforms (Tidyman et al., 1997). Therefore, it is likely that the growth of fast skeletal muscle is associated, in an as yet an unknown way, with the expression of the developmental fast skeletal muscle myosin isoforms. The problems associated with comparative developmental studies in animals using chronological age have been known and addressed for over 60 yr (Hamburger and Hamilton, 1992). Hamburger and Hamilton (1992) developed a staging system during embryonic development of chickens to compare animals at similar developmental stages rather than the same chronological age. There is a need for a comparable staging system for studying posthatch animals at similar developmental stages independent of chronological age. Thus, the relative concentrations of the developmental fast skeletal muscle MyHC isoforms were used as molecular markers to document the effect of feed restriction on the early developmental stages of a fast growing commercial turkey line and an unselected turkey line developed and maintained at The Ohio State University that led to a comparison of the growth of the pectoralis major, between the 2 lines of turkeys undergoing different nutritional planes.

Although these results are preliminary they are consistent with those reported by Maruyama and Kameda (1991) who, using the same myosin isoform specific monoclonal antibodies used in the current study, showed that in skeletal muscles of turkeys, the MyHC

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**Figure 2.** Temporal expression ratios of the adult myosin heavy chain (MyHC) isoform in the pectoralis major of F line turkeys in full-fed (dark gray bars) and restricted-fed (light gray bars) diets. Different letters indicate significant differences at \( P > 0.05 \). Asterisks indicate significant differences within day across fed regimens; \( a, b, c \) and \( d \) indicate significant differences among different days within the full-fed treatment group; \( w, x, y, \) and \( z \) indicate significant differences among different days within the restricted-fed treatment group; \( a, b, d \) were not compared against \( w, x, y, \) and \( z \) because the comparisons are biologically irrelevant.

**Figure 3.** Temporal expression ratios of the adult MyHC isoform in the pectoralis major of RBC2 turkeys in full-fed (dark gray bars) and restricted-fed (light gray bars) diets. Different letters indicate significant differences at \( P > 0.05 \). Asterisks indicate significant differences within day across fed regimens; \( a, b, c, \) and \( d \) indicate significant differences among different days within the full-fed treatment group; \( x, y, \) and \( z \) indicate significant differences among different days within the restricted-fed treatment group; \( a, b, c, \) and \( d \) were not compared against \( x, y, \) and \( z \) because the comparisons are biologically irrelevant.
exists as isoforms and the expression of isoforms is dependent on the developmental stage, as well as the muscle fiber type. Maruyama et al. (1993) showed that that MyHC isoforms, of which amino acid sequences vary by a small number of substitutions, were expressed in an orderly sequence and their appearance coincides with changes in the circulating thyroid hormone concentration, which is postulated to influence muscle-specific gene expression. In addition, previous studies by Reddish et al. (2005), using fast skeletal muscle MyHC specific monoclonal antibodies, showed that genetic selection for increased breast yield in broilers resulted in accelerated temporal expression of developmental fast skeletal MyHC isoforms and that this acceleration of developmental is likely necessary for muscle specific growth, regardless of whole muscle growth. In addition, Agbulut et al. (2003) analyzed myosin isoform transitions in several muscles in mice and hypothesized that muscle activity, innervation, and hormonal status work in concert to produce the highly specialized shifts in MyHC isoform expression during postnatal development characteristic for each muscle.

Finally, the results of this study and previous research supports the hypothesis that genetic and intrinsic/extrinsic environmental factors influence fast skeletal muscle development and that this growth is related to the temporal expression of not only the developmental MyHC but also potentially other muscle specific protein isoform transitions. This methodology combined with functional genomic methodologies could potentially lead to knowledge of the genes and protein expression patterns associated with the sequential events during muscle development. This knowledge is crucial to understanding the cellular and molecular mechanisms underlying fast skeletal muscle development, which ultimately is responsible for turkey meat quality.

**Conclusion**

The results of this study support the hypothesis that the temporal expression of the developmental fast skeletal MyHC isoforms may be associated with other molecular mechanisms controlling muscle growth in poultry. Furthermore, using the developmental MyHC isoforms as signal markers may be useful in identifying the transcriptional regulators that control the growth and development of fast muscle tissue.

Posthatch staging during posthatch poultry muscle development combined with “omic” analysis should ultimately lead to knowledge of the proteins associated with the sequential events during muscle development. This knowledge is crucial to understanding the cellular and molecular mechanisms underlying fast skeletal muscle development and the concomitant impact on pale poultry muscle syndrome in poultry.

**REFERENCES**


