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# Fiber Phenotype and Coenzyme $Q_{10}$ Content in Turkey Skeletal Muscles

L.S. Nierobisz N.G. Hentz J.V. Felts P.E. Mozdziak

North Carolina State University, Raleigh, N.C., USA



**Key Words**

Coenzyme $Q_{10}$ · Mitochondria · Muscle metabolism · Muscle phenotype · Turkey

**Abstract**

Phenotypical differences between muscle fibers are associated with a source of cellular energy. Coenzyme $Q_{10}$ ($CoQ_{10}$) is a major component of the mitochondrial oxidative phosphorylation process, and it significantly contributes to the production of cellular energy in the form of ATP. The objective of this study was to determine the relationship between whole-tissue $CoQ_{10}$ content, mitochondrial $CoQ_{10}$ content, mitochondrial protein, and muscle phenotype in turkeys. Four specialized muscles (anterior latissimus dorsi, ALD; posterior latissimus dorsi, PLD; pectoralis major, PM, and biceps femoris, BF) were evaluated in 9- and 20-week-old turkey toms. The amount of muscle mitochondrial protein was determined using the Bradford assay and $CoQ_{10}$ content was measured using HPLC-UV. The amount of mitochondrial protein relative to total protein was significantly lower ($p < 0.05$) at 9 compared to 20 weeks of age. All ALD fibers stained positive for anti-slow (S35) MyHC antibody. The PLD and PM muscle fibers revealed no staining for slow myosin heavy chain (S35 MyHC), whereas half of BF muscle fibers exhibited staining for S35 MyHC at 9 weeks and 70% at 20 weeks of age. The succinate dehydrogenase (SDH) staining data revealed that SDH significantly increases ($p < 0.05$) in ALD and BF muscles and significantly decreases ($p < 0.05$) in PLD and PM muscles with age. The study reveals age-related decreases in mitochondrial $CoQ_{10}$ content in muscles with fast/glycolytic profile, and demonstrates that muscles with a slow/oxidative phenotypic profile contain a higher proportion of $CoQ_{10}$ than muscles with a fast/glycolytic phenotypic profile.



**Abbreviations used in this paper**

| | |
|---|---|
| αR | fast-oxidative muscle |
| αW | fast-glycolytic muscle |
| ALD | anterior latissimus dorsi |
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| ATPase | adenosinetriphosphatase |
| βR | slow-oxidative muscle |
| BF | biceps femoris |
| BSA | bovine serum albumin |
| $CoQ_{10}$ | coenzyme $Q_{10}$ |
| EDTA | ethylenediaminetetraacetic acid |
| F59 | fast variant of myosin heavy chain |
| GAPDH | glyceraldehydes 3-phosphate dehydrogenase |
| HPLC-UV | high-performance liquid chromatography-ultraviolet |
| MyHC | myosin heavy chain |
| NADH | nicotinamide adenine dinucleotide |
| PLD | posterior latissimus dorsi |
| PM | pectoralis major |
| SDH | succinate dehydrogenase |
| S35 | slow variant of myosin heavy chain |
| TCA | tricarboxylic acid |

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Lidia S. Nierobisz
Department of Poultry Science
North Carolina State University, Scott Hall 124
2711 Founders Drive, Raleigh, NC 27695 (USA)
Tel. +1 919 515 5530, Fax +1 919 515 2625, E-Mail lsnierob@ncsu.edu

## Introduction

Vertebrate skeletal muscle is composed of heterogeneous and highly dynamic groups of muscle fibers that vary based on their biophysical and metabolic profiles. The heterogeneity of muscle fibers is determined based on muscle fiber size, differential expression of contractile proteins, and on factors involved in regulation of cellular metabolism [Williams and Neufer, 1996; Pette and Staron, 1997]. The number, size, and type of a muscle fiber is primarily determined by the genetic and environmental factors that influence both prenatal/prehatch and postnatal/posthatch muscle development.

Although different subtypes of muscle fibers can be distinguished during embryonic development, the genetically preestablished pattern for muscle fiber types becomes determined during the early postnatal or posthatch period [Moss and Leblond, 1971; Swatland and Cassens, 1973; Miller et al., 1993; Garry et al., 1996]. During the early postnatal or posthatch period, muscle fibers undergo a maturation process that results in muscle fiber distribution similar to that found in adult animals. It has been shown that myoblasts develop into myofibers with diverse metabolic, biophysical, and contractile profiles [Gibson and Schultz, 1982].

Common histological techniques used to evaluate muscle fiber phenotype include enzymatic staining for mitochondrial succinate dehydrogenase (SDH) and for myosin adenosinetriphosphatase (ATPase). The contractile profiles of developing and adult fiber types are determined by muscle myosin heavy chain (MyHC) isoform composition [Reiser et al., 1985; Page et al., 1992]. Each myosin isoform is a product of a different MyHC gene [Schiaffino and Reggiani, 1996]. In classical fiber typing experiments [Wiskus et al., 1976; Aberle et al., 1979; Smith and Fletcher, 1988], muscle fibers were classified as α (fast-twitch, type II) or β (slow-twitch, type I), and as red (R) or white (W). The fast and slow classifications were based on histochemical staining for ATPase. However, glycolytic and oxidative fiber determination was based on succinic dehydrogenase (SDH) staining for mitochondria [Aberle et al., 1979]. Because SDH is an important component of a TCA cycle, dark SDH staining identifies muscle fibers with high oxidative potential. Wiskus et al. [1976] suggested that there are three main muscle fiber types in the turkey. Based on their differential response to ATPase and SDH staining, the three fiber types were defined as αR (fast-oxidative), αW (fast-glycolytic), and βR (slow-oxidative). Wiskus et al. [1976] demonstrated that the pectoralis major (PM) muscle was scarce in βR fibers. However, the biceps femoris (BF) muscle was composed of both oxidative (βR, αR) and glycolytic (αW), fast-twitch fibers.

The environmental cues, physical stresses, and genetic factors affect the activity of enzymes governing specific muscle fiber metabolism and control muscle fiber phenotype. One of the major determinants of skeletal muscle fiber phenotype in turkeys of the same genetic line is the location of the muscle and its corresponding functional load. Previous studies demonstrated that increased load on a muscle induces slower, more oxidative phenotype, and decreased load induces more glycolytic phenotype [Kasper et al., 1993; Mozdziak et al., 1998].

Factors that participate in mitochondrial respiration may also play a significant role in determining muscle fiber phenotype. One of the factors that plays an important role in mitochondrial respiration in skeletal muscle is coenzyme $Q_{10}$ ($CoQ_{10}$) [Pastore et al., 2005], which is a bioactive, vitamin-like molecule present in all eukaryotic cells containing mitochondria. $CoQ_{10}$ is located in the hydrophobic middle region of the phospholipid bilayer of the mitochondrial membrane and participates in the electron transport chain process, where it accepts electrons from reducing equivalents produced from fatty acid and glucose breakdown, and delivers them to electron acceptors [Bliznakov and Bhagavan, 2003]. The specific role of $CoQ_{10}$ in the electron transport chain is to transfer electrons from NADH-Q oxidoreductase (complex I) and from succinate-Q oxidoreductase (complex II) to cytochrome c oxidoreductase (complex III) [Kamzalov et al., 2003]. The movement of electrons from one complex to another results in generation of a proton gradient. The energy released when protons move back to mitochondria is used to generate ATP. Elevated concentrations of $CoQ_{10}$ have been found in organs with high energy requirements such as heart, brain and skeletal muscle [Bliznakov and Bhagavan, 2003]. $CoQ_{10}$ in its reduced form (ubiquinol) acts as a principal fat-soluble cellular antioxidant that plays an important role in neutralizing free radicals, inhibiting lipid peroxidation of membranes, and in protecting mitochondrial membrane proteins and DNA [Frei et al., 1990]. Furthermore, dietary supplementation of ubiquinol has been shown to result in elevated tissue and mitochondrial levels of α-tocopherol, which is also a powerful antioxidant [Kamzalov et al., 2003].

In this study, four functionally diverse muscles (anterior latissimus dorsi, ALD; posterior latissimus dorsi, PLD; PM and BF) were examined with consideration of their metabolic profiles. PM and PLD represent muscle groups composed of fast-twitch fibers that rely primarily

on glycolytic metabolism for energy generation. The BF muscle contains a combination of metabolically diverse muscle fibers. However, the ALD muscle is characterized by extremely low anaerobic capacity, and is composed of fibers that are entirely slow twitch [Kiessling, 1976; Wiskus et al., 1976].

Turkey performance data indicates that turkeys grow rapidly before 10 weeks of age and the difference in weight gain plateaus shortly thereafter [Anthony et al., 1990]. To account for growth-related differences in selected muscles, turkeys were evaluated at 9 weeks of age (rapid growth rate) and at 20 weeks of age (slow growth rate). Commercially raised turkeys are considered adult at 16 weeks of age and are brought to market at 20 weeks of age [Dr. Peter Ferket, pers. commun.]. Consequently, 9-week-old birds are considered to be at prepuberty stage and 20-week-old birds are considered to be adult.

Additionally, previous studies demonstrated that beyond 9 weeks of age, muscle satellite cell mitotic activity begins to diminish and muscle fiber growth occurs primarily through an increase of cytoplasmic to nuclear ratio [Mozdziak et al., 1994]. Consequently, from the cellular perspective, 9 weeks of age represents an important transition point in skeletal muscle development.

The aim of this study was to determine the interconnection between muscle phenotype, metabolic profile, mitochondrial content, and $CoQ_{10}$ content in phenotypically distinct skeletal muscles in domestic turkeys selected at two different stages of maturity.

## Materials and Methods

*Sample Collection*

All experiments involving animals were in compliance with the North Carolina State University Institutional Animal Care and Use Committee. The two groups of turkeys selected for the study consisted of young/prepuberty (9-week-old) and adult (20-week-old) toms *(Meleagris gallopavo)*. Five 9-week-old and five 20-week-old turkeys were randomly selected from a single flock and immediately sacrificed by intravenous injection of Euthasol® (Delmarva Laboratories, Midlothian, Va., USA) at a dose of 0.25 ml/kg body weight. Right ALD, PLD, PM, and BF muscles were excised and divided in half, in a transverse manner, for both high-performance liquid chromatography (HPLC) and histochemical analysis. Half of the samples were snap-frozen using isopentane cooled on dry ice, and half was fixed in 2% paraformaldehyde for further histochemical analysis.

*Mitochondrial Isolation*

The mitochondrial isolation was performed based on a procedure described by Bhattacharya et al. [1991]. Frozen muscle samples were minced with scissors until homogeneous and placed in protease solution (Subtilisin A, type VIII bacterial protease from *Bacillus licheniformis*; 12 units/mg; Sigma Cat. No. 9014-01-1). Following protease treatment, samples were transferred to ionic medium (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, 46 mM KCl) combined with 0.5% bovine serum albumin (BSA). Samples were then homogenized in a tissue grinder, placed in conical centrifuge tubes and centrifuged for 10 min at 500 *g* at 4°C. The supernatant was removed and centrifuged at 12,000 *g* for 10 min at 4°C. The cytoplasmic fraction was removed and the pellet was resuspended in fresh ionic medium/BSA buffer and centrifuged again at 12,000 *g* for 10 min at 4°C. The remaining pellet containing the mitochondrial fraction was resuspended and stored in suspension medium (230 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris-HCl, 5 mM $K_2HPO_4$).

Western blot analysis was performed on representative cytosolic and mitochondrial fractions to account for purity of isolated mitochondria [Carson and Robertson, 2006]. Protein extracts of 20 μg were loaded on 10% sodium dodecyl sulfate polyacrylamide gel. The following polyclonal primary antibodies raised in rabbit were utilized in the Western blot procedure: anti-GAPDH (Sigma-Aldrich, St. Louis, Mo., USA) at 1:1,000 dilution, and anti-Cox4 (Fisher Scientific, Pittsburgh, Pa., USA) at 1:1,000 dilution. The proteins were detected using anti-rabbit secondary antibody conjugated to horseradish peroxidase at 1:2,000 dilution. The anti-GAPDH antibody was utilized to detect any cytosolic contamination in mitochondrial fraction. The anti-Cox4 antibody was utilized to determine if mitochondrial fraction was truly mitochondrial and to confirm that mitochondrial proteins were not present in the cytosolic fraction (fig. 1).

*Mitochondrial Protein Quantification*

Bradford assay was performed on the isolated mitochondrial fractions to quantify total mitochondrial protein per gram of muscle sample. The amount of mitochondrial protein was quantified based on absorbance at 595 nm [Bradford, 1976; Zor and Selinger, 1996].

*High-Performance Liquid Chromatography-UV*

Mitochondrial and whole-tissue $CoQ_{10}$ content were quantified using HPLC (Shimadzu USA Manufacturing, Inc., Canby, Oreg., USA), which was equipped with a model LC-20AD solvent delivery pump, a model SPD-20A UV/Vis detector, a model CTO-20A column oven, and a model SIL-20C HT autosampler. The system was controlled and data were analyzed by LC solution version 1.23 (Shimadzu Corp.).

The HPLC method used in this study was modified from that described previously by Pastore et al. [2005]. Approximately 0.1 g of frozen muscle fragments (–80°C) was excised in triplicates from the most central area of the tissue and homogenized with 1,000 μl hexane and 500 μl methanol in a tissue homogenizer. The mitochondrial fraction, separated from approximately 1 g of muscle, was also mixed with 1,000 μl hexane and 500 μl methanol. The whole tissue and mitochondrial fractions were centrifuged at 10,000 *g* for 5 min at 4°C. Starting with a stock solution of 1.6 mM, the $CoQ_{10}$ standards (Sigma-Aldrich) were prepared by serially diluting 6 times in a mixture of 2/3 hexane and 1/3 methanol. The concentrations of the working standards were: 16, 8, 4, 2, 1, and 0.5 μM. The standards were then centrifuged at 10,000 *g* for 5 min at 4°C.

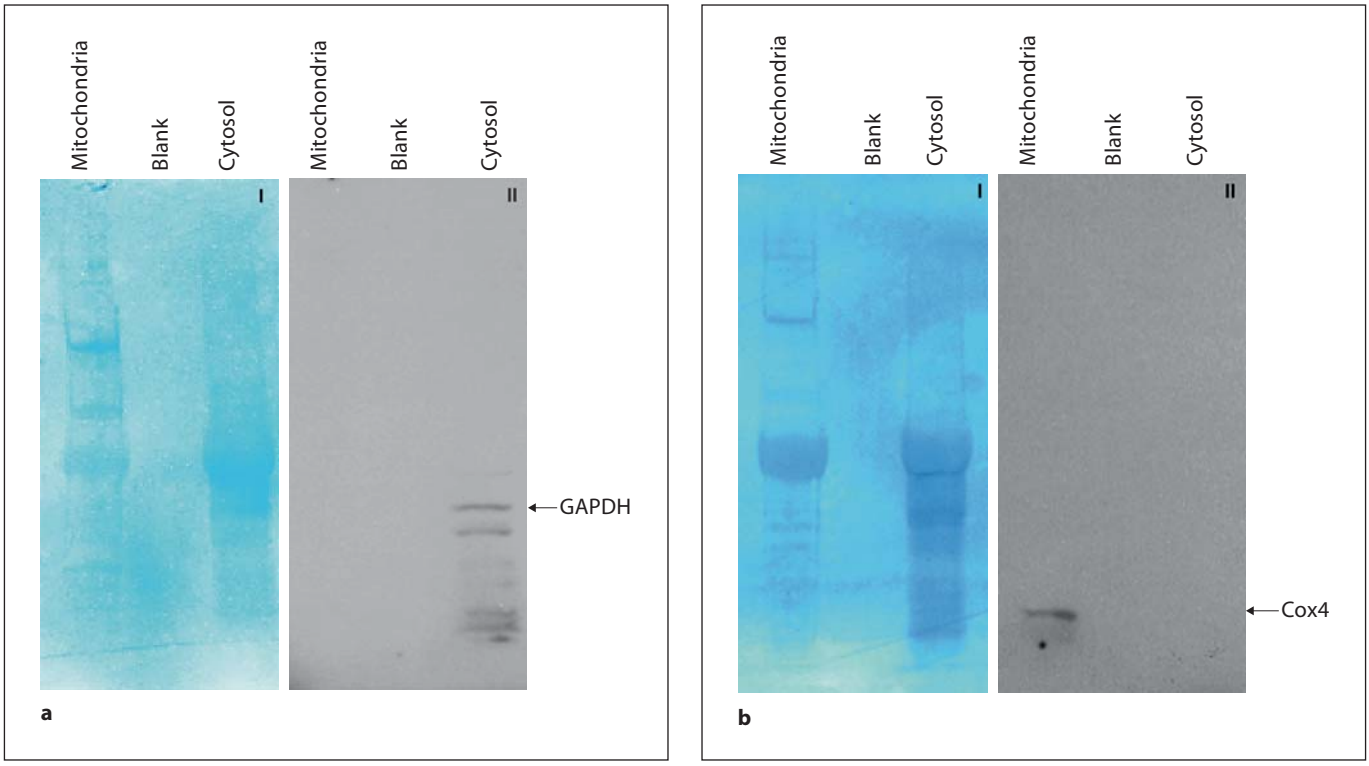


**Fig. 1.** Representative Western blot analysis of anti-GAPDH (**a**) and anti-Cox4 (**b**) in mitochondrial and cytosolic fractions of 9-week-old ALD muscle. The figure represents Coomassie blue-stained PVDF membrane (**I**) and Western blot scan (**II**). 20 μg of protein was equally loaded to each lane.

The hexane phase containing $CoQ_{10}$ standard, $CoQ_{10}$ extracted from tissue fragments, and $CoQ_{10}$ extracted from the mitochondrial fraction were transferred to glass HPLC vials and capped with Teflon-lined septa and injected in 10-μl aliquots onto the column. A 125 × 3 mm ODS Hypersil column (Thermo Scientific, Waltham, Mass., USA) was equilibrated with mobile phase containing 15 ml glacial acetic acid, 275 ml hexane, 15 ml sodium acetate, 695 ml methanol (Fisher Scientific), and 15 ml 2-propanol (Sigma-Aldrich).

The column was equilibrated with mobile phase for at least 20 min before analysis. The $CoQ_{10}$ standards and muscle extract samples were eluted isocratically at a flow rate of 1.0 ml/min and detected at 275 nm. The column temperature was held constant at 30°C. Peak area was directly proportional to concentration of standard, thus linear regression analysis was used to calculate concentration of $CoQ_{10}$ in whole muscle and mitochondrial extract.

*SDH and ATPase Histochemistry*

A modified version of the Wiskus et al. [1976] fiber staining protocol was utilized to determine SDH and slow myosin ATPase activity. Serial transverse sections (15 μm) from ALD, PLD, PM, and BF muscle samples were cut in a cryostat at –20°C, air-dried, and subjected to histochemical staining for SDH and myofibrillar ATPase at pH 3.9.

During ATPase staining procedure, tissue sections were preincubated in a solution of 0.2 M barbital acetate buffer (1.94 g sodium acetate, 2.94 g sodium barbital, Sigma-Aldrich, and 100 ml distilled water) at room temperature (19–21°C) for 5 min at pH 3.9. The acidic solution was washed in the alkali-preincubating solution (pH 9.4; 20 ml of 0.2% w/v sodium barbital, 25 ml $CaCl_2$, 55 ml distilled water) for 5 s followed by incubation in alkali solution (pH 9.4; 90 mg ATP, 12 ml barbital, 6 ml $CaCl_2$, 40 ml distilled water) for 45 min at room temperature (19–21°C). The sections were then washed in 1% $CaCl_2$, transferred to 2% $CoCl_2$, washed in 0.2% sodium barbital, and rinsed with distilled water. The reaction was developed with 1% yellow ammonium sulfide for 15 s. Sections were dehydrated, mounted in Permount®, and the cover glass was sealed with clear enamel.

The SDH staining was performed as follows: freshly dried cryostat sections were incubated for 5 min at room temperature (19–21°C) in a medium composed of 2 ml of nitroblue tetrazolium stock solution (100 ml phosphate-buffered saline at pH 7.6, 6.5 mg KCN, 185 mg EDTA, 100 mg NB), 0.2 ml succinate stock solution (2.7 g sodium succinate, 20 ml distilled water), and 0.7 mg phenazine methosulfate. The sections were rinsed in acetone gradient, cleared, and mounted in glycerin jelly.

*MyHC Immunohistochemistry*

Tissues were fixed in 2% paraformaldehyde, embedded in paraffin, dewaxed and dehydrated. Serial sections, 15 μm thick, were

**Table 1.** Correlation analysis of mitochondrial $CoQ_{10}$ content, SDH, and myosin ATPase expression levels in 9-week-old muscles and 20-week-old muscles

| | Mitochondrial $CoQ_{10}$ | SDH-L | SDH-M | SDH-H | ATPase-L | ATPase-M | ATPase-H |
|---|---|---|---|---|---|---|---|
| *9-week data* | | | | | | | |
| Mitochondrial $CoQ_{10}$ | 1.0000 | | | | | | |
| SDH-L | –0.9752 | 1.0000 | | | | | |
| SDH-M | –0.9856 | 0.9953 | 1.0000 | | | | |
| SDH-H | 0.9826 | –0.9983 | –0.9993 | 1.0000 | | | |
| ATPase-L | –0.9089 | 0.9767 | 0.9529 | –0.9635 | 1.0000 | | |
| ATPase-M | 0.5147 | –0.3158 | –0.3891 | 0.3603 | –0.1103 | 1.0000 | |
| ATPase-H | 0.6643 | –0.8099 | –0.7574 | 0.7792 | –0.9155 | –0.2989 | 1.0000 |
| *20-week data* | | | | | | | |
| Mitochondrial $CoQ_{10}$ | 1.0000 | | | | | | |
| SDH-L | –0.8459 | 1.0000 | | | | | |
| SDH-M | –0.1726 | 0.6047 | 1.0000 | | | | |
| SDH-H | 0.7559 | –0.9838 | –0.7376 | 1.0000 | | | |
| ATPase-L | –0.7205 | 0.9717 | 0.7758 | –0.9983 | 1.0000 | | |
| ATPase-M | 0.8738 | –0.4829 | 0.2983 | 0.3422 | –0.2927 | 1.0000 | |
| ATPase-H | 0.1960 | –0.6795 | –0.9506 | 0.7899 | –0.8212 | –0.3053 | 1.0000 |

Correlation coefficient of +1 indicates that two values are perfectly related in a positive manner and coefficient of –1 indicates that two values are perfectly related in a negative linear manner.

cut on a microtome and subjected to slow (S35) and fast (F59) MyHC immunohistochemical analysis. A supernatant generated from S35 and F59 MyHC hybridomas (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) was utilized to detect presence of slow and fast myosin isoforms in skeletal muscle sections. Dehydrated muscle sections were blocked for 15 min in a solution containing 0.1% BSA, 5 mM EDTA, 3% goat serum and 1 mM sodium azide to prevent nonspecific antibody binding. Subsequently, the sections were incubated in either S35 or F59 MyHC supernatant diluted 1:10 in blocking solution at 4°C in a humidified chamber for 12 h. The sections were rinsed 2 times for 5 min with IX phosphate-buffered saline buffer and were then blocked in a solution containing 0.1% BSA, 5 mM EDTA, 3% goat serum and 1 mM sodium azide and incubated for 2 h at room temperature (19–21°C) in goat anti-mouse secondary antibody conjugated to horseradish peroxidase diluted 1:50 with blocking solution. The slides were rinsed in phosphate-buffered saline and incubated in diaminobenzane and urea for 5 min. Subsequently, the slides were rinsed with distilled water, dehydrated, mounted with Permount®, and sealed with clear enamel.

*Image Analysis*

A Leica DMR light microscope (Leica Microsystems, Bannockburn, Ill., USA) was utilized to observe ALD, PLD, PM, and BF muscle sections. All images were captured at either ×20 or ×40 magnification using a Retiga 4000R fast camera (Q Imaging, Surrey, B.C., Canada). Image-Pro Plus software (Media Cybernetics, version 6, 2006) was utilized to identify muscle fiber phenotype. The criterion for accurate determination of muscle fiber phenotype was to evaluate at least 500 fibers per muscle per bird (5 birds per treatment).

The pattern of staining for myosin ATPase and for oxidative enzyme, SDH, was determined as high, middle, or low (fig. 6–8). In ATPase-stained slides, light staining was attributed to muscle fibers with low activity of slow myosin ATPase, medium staining was attributed to fibers with medium activity of slow myosin ATPase, and dark staining was attributed to muscle fibers with high activity of slow myosin ATPase (fig. 6, 7). In the SDH-stained slides, fibers stained with high intensity appeared dense blue with a band of staining inside the sarcolemma, the fibers stained with middle intensity had a band of staining inside the sarcolemma but lighter staining in the middle of the fiber, and fibers stained with low intensity for SDH lacked the sarcolemmal band and had few mitochondria dispersed throughout the section (fig. 7, 8) [Anapol and Herring, 2000]. The MyHC isoforms were evaluated based on presence or absence of staining (fig. 9).

*Statistical Analysis*

The General Linear Models procedure of SAS [SAS Institute, 1985] was performed to analyze the effect of muscle and age on the following parameters: $CoQ_{10}$ content in the whole muscle and mitochondrial fraction, muscle mitochondrial content, and on histochemically defined muscle fiber phenotype. A two-way analysis of variance (ANOVA) was performed to evaluate the effect of muscle and age (9 and 20 weeks) on each parameter (fig. 2). Means of each treatment were separated using Fisher's least significant differences. Values were considered statistically significant when $p < 0.05$. Correlation analysis was performed to identify the relationship between mitochondrial $CoQ_{10}$ content, SDH staining, and slow myosin ATPase staining (table 1).

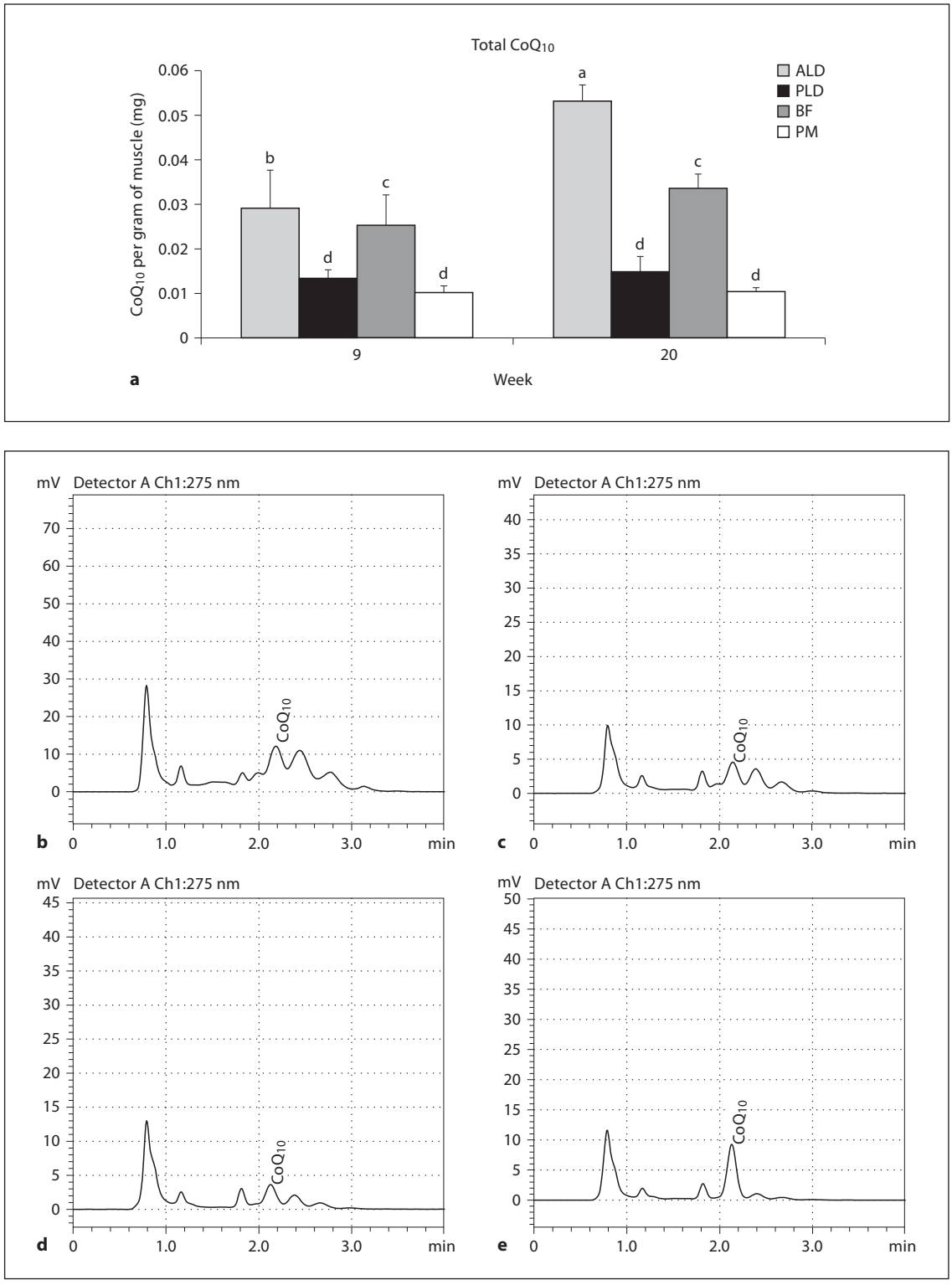


**Fig. 2. a** Total $CoQ_{10}$ (mg) per gram of 9- and 20-week-old turkey muscles determined by HPLC. Values with different superscripts represent means that are statistically different ($p < 0.05$). Values with the same superscripts represent means that do not differ statistically ($p > 0.05$). The superscripts represent values in ascending order. Error bars represent standard errors of individual means. **b–e** Examples of HPLC-UV chromatograms depicting total $CoQ_{10}$ content in ALD (**b**), PLD (**c**), PM (**d**), and BF (**e**) muscles at 20 weeks.

## Results

*Western Blot*

GAPDH and COX4 antibodies have been widely used to verify mitochondrial purity [Morrish et al., 2006]. In the cytosolic fraction, GAPDH tetramer was detected between 30 and 40 kDa with anti-GAPDH polyclonal antibody. However, there was no anti-GAPDH reactivity detected in the mitochondrial fraction, suggesting that the mitochondrial fraction is free of contamination with cytosolic components. A 17-kDa band was detected when mitochondrial fraction was reacted with anti-Cox4 antibody suggesting that mitochondria were indeed present in the mitochondrial fraction. No anti-Cox4 reactivity was detected in the cytosolic fraction, confirming that this fraction did not contain any mitochondria (fig. 1).

*HPLC Analysis*

ALD and BF muscles exhibited significantly higher ($p < 0.0001$) total $CoQ_{10}$ content than PLD and PM muscles at week 9 and 20 (fig. 2). Total $CoQ_{10}$ per gram of muscle was higher ($p < 0.0126$) in ALD muscles at 20 weeks of age as compared to 9 weeks of age. However, total $CoQ_{10}$ content remained the same in PLD, BF and PM muscles at each age.

ALD and BF mitochondrial $CoQ_{10}$ content was significantly higher ($p < 0.0157$) than PLD and PM $CoQ_{10}$ content at weeks 9 and 20 (fig. 3). The mitochondrial $CoQ_{10}$ content in ALD and BF muscles was significantly higher ($p < 0.0034$) at 20 compared to 9 weeks of age. However, the mitochondrial $CoQ_{10}$ content in PM and PLD did not change in 20-week-old birds compared to 9-week-old birds (fig. 3).

Another parameter was calculated whereby a percent ratio of $CoQ_{10}$ in mitochondria to $CoQ_{10}$ in total muscle (mitochondrial to total $CoQ_{10}$) was determined. No significant differences were revealed between the different muscles at week 9 (fig. 4). However, at week 20, the mitochondrial to total $CoQ_{10}$ was significantly higher ($p < 0.0066$) in ALD and BF muscles than in PM and PLD muscles. Mitochondrial to total $CoQ_{10}$ significantly decreased in PM and PLD muscles ($p < 0.0160$ in PLD, $p < 0.0018$ in PM), remains the same in ALD muscle, and significantly increased ($p < 0.001$) in BF muscle between weeks 9 and 20 (fig. 4).

*Mitochondrial Protein Quantification*

Data generated from Bradford assay reveals that the amount of mitochondrial protein relative to total protein significantly decreases ($p < 0.0001$) with age in all four muscles. However, there were no significant differences in mitochondrial protein content between muscles at any given time (fig. 5).

*Fiber Typing*

In the BF muscle, low, middle, and high intensity of staining for ATPase was observed, where the number of fibers stained at low intensity was significantly higher ($p < 0.0001$) than the number of fibers stained at middle and high intensity (fig. 6, 7). The staining intensity representing activity of ATPase molecule remained statistically unchanged in ALD, PLD, BF, and PM muscles over time (fig. 6, 7).

At 9 weeks of age, ALD muscle contained fibers stained at high and middle intensity for SDH. However, at 20 weeks of age, all ALD fibers were darkly stained for SDH. The percentage of PLD fibers stained at low intensity for SDH significantly increased ($p < 0.006$) over time and the percentage of PLD fibers stained at middle and high intensities for SDH were significantly lower at 20 weeks compared to 9 weeks of age (fig. 8). In the BF muscle the percentage of fibers stained at low intensity for SDH remained the same, the percentage of fibers stained at middle intensity for SDH significantly decreased ($p < 0.01$), and the percentage of fibers stained at high intensity for SDH significantly ($p < 0.04$) increased with age. The percentage of PM fibers stained at low intensity for SDH significantly increased ($p < 0.0001$), and the percentage of fibers stained at middle and high intensity for SDH significantly decreased ($p < 0.0001$) with age (fig. 7, 8).

All of the ALD fibers at 9 and 20 weeks of age exhibited positive staining for S35 MyHC. PLD muscle fibers showed a complete absence of S35 MyHC staining at 9 and 20 weeks of age. Half of BF muscle fibers exhibited positive staining for S35 MyHC at 9 weeks and 70% of BF muscle fibers exhibited positive staining for S35 MyHC at 20 weeks of age. At 9 and 20 weeks of age, over 90% of PM muscle fibers did not show any staining for S35 MyHC (fig. 9). Opposite results were observed when sequential sections from ALD, PLD, PM, and BF muscle fibers were stained for F59 MyHC (fig. 9).

*Correlation Analysis*

Correlation analysis of mitochondrial CoQ10 content, SDH, and myosin ATPase activities revealed very strong relationship between these cellular components in 9-week-old muscles. Furthermore, strong but imperfect correlations among mitochondrial $CoQ_{10}$ content, SDH, and myosin ATPase activities were observed in 20-week-old muscles (table 1).

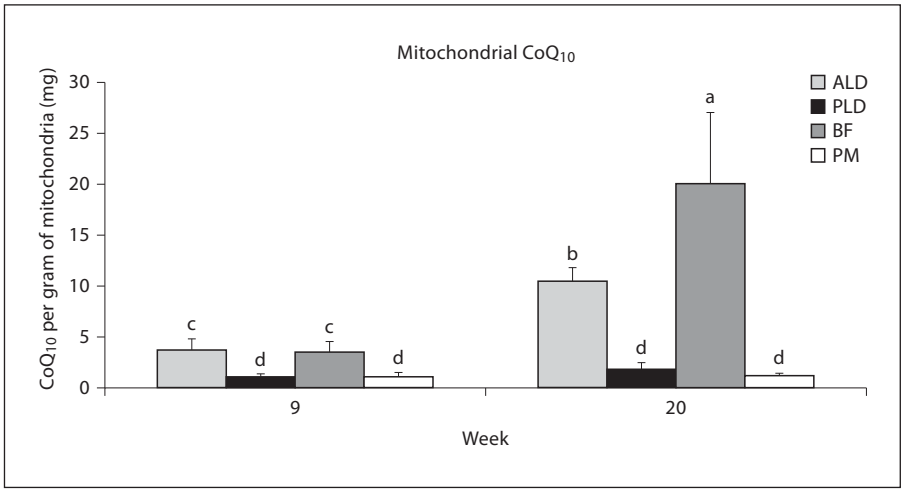


**Fig. 3.** $CoQ_{10}$ (mg) per gram of mitochondria in 9- and 20-week-old turkey muscle determined by HPLC ($p < 0.0002$). The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

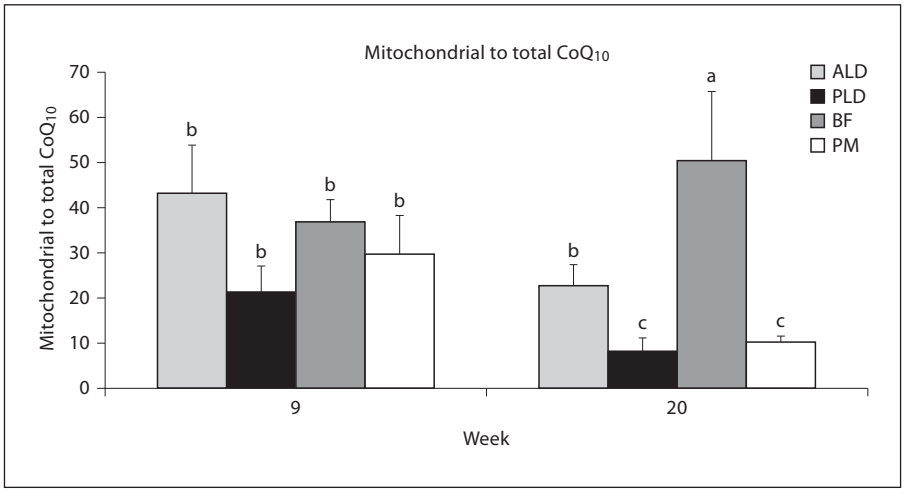


**Fig. 4.** Ratio of mitochondrial $CoQ_{10}$ to total amount of $CoQ_{10}$ in 9- and 20-week-old turkey muscle represented in percentages and determined by HPLC ($p < 0.0098$). The ratio is expressed in percentages. The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

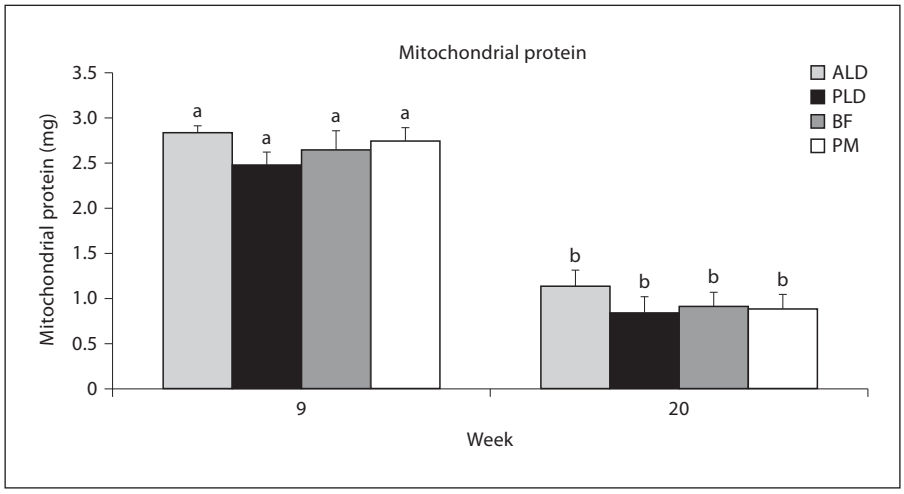


**Fig. 5.** Amount of mitochondrial protein (mg) relative to total protein in 1 g of wet muscle mass of 9- and 20-week-old turkey muscle. Protein amounts were determined by the Bradford assay ($p < 0.001$). The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

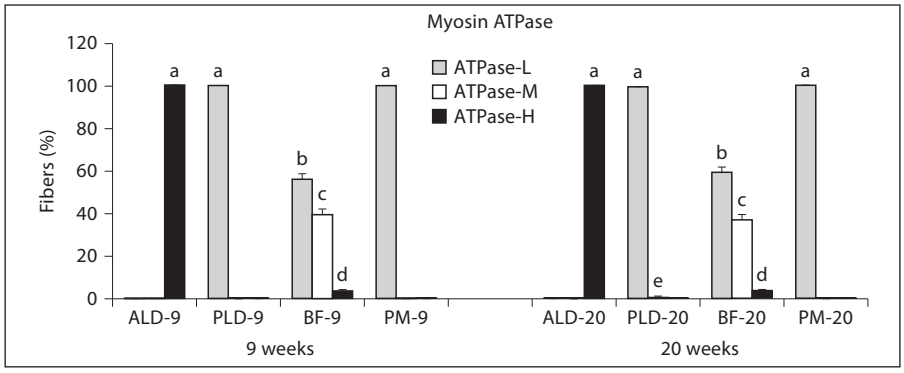


**Fig. 6.** Percentage of ALD, PLD, BF, and PM muscle fibers in 9- and 20-week-old turkey toms with low (ATPase-L), medium (ATPase-M), and high (ATPase-H) intensity staining for myofibrillar ATPase at pH 3.9 ($p < 0.0001$). The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

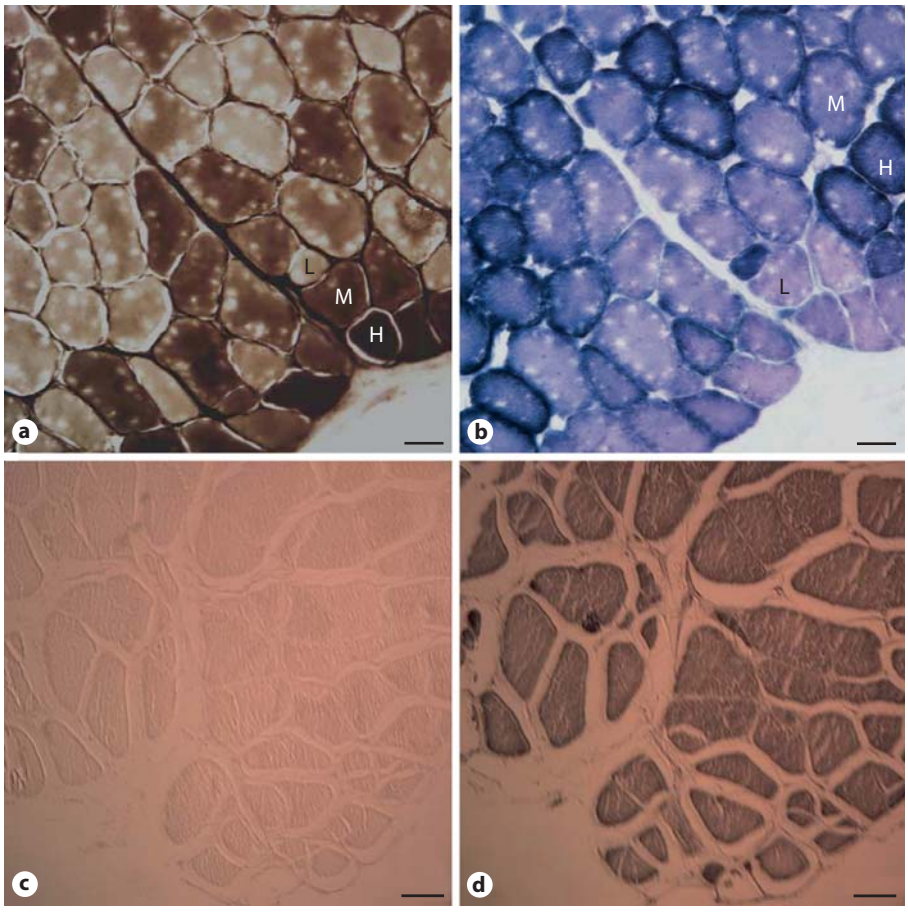


**Fig. 7.** An example of serial sections of 20-week-old BF muscle stained for slow myosin ATPase at pH 3.9 (**a**), and SDH (**b**); and 20-week-old ALD muscle reacted with F59 MyHC antibody (**c**), and S35 MyHC antibody (**d**). The figures (**a**) and (**b**) show different intensities of staining for slow myosin ATPase and SDH (L = low-intensity, M = middle-intensity, and H = high-intensity staining). Images were captured at ×20 magnification and the scale bars represent 50 μm.

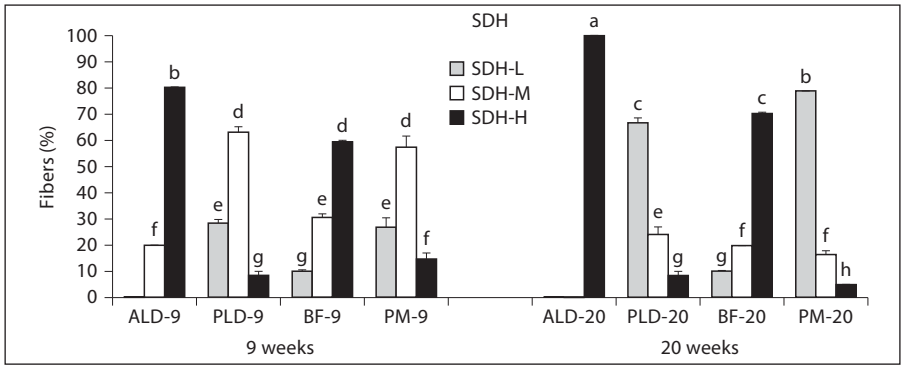


**Fig. 8.** Percentage of ALD, PLD, BF, and PM muscle fibers in 9- and 20-week-old turkey toms with low (SDH-L)-, medium (SDH-M)-, and high (SDH-H)-intensity staining for SDH ($p < 0.0001$). The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

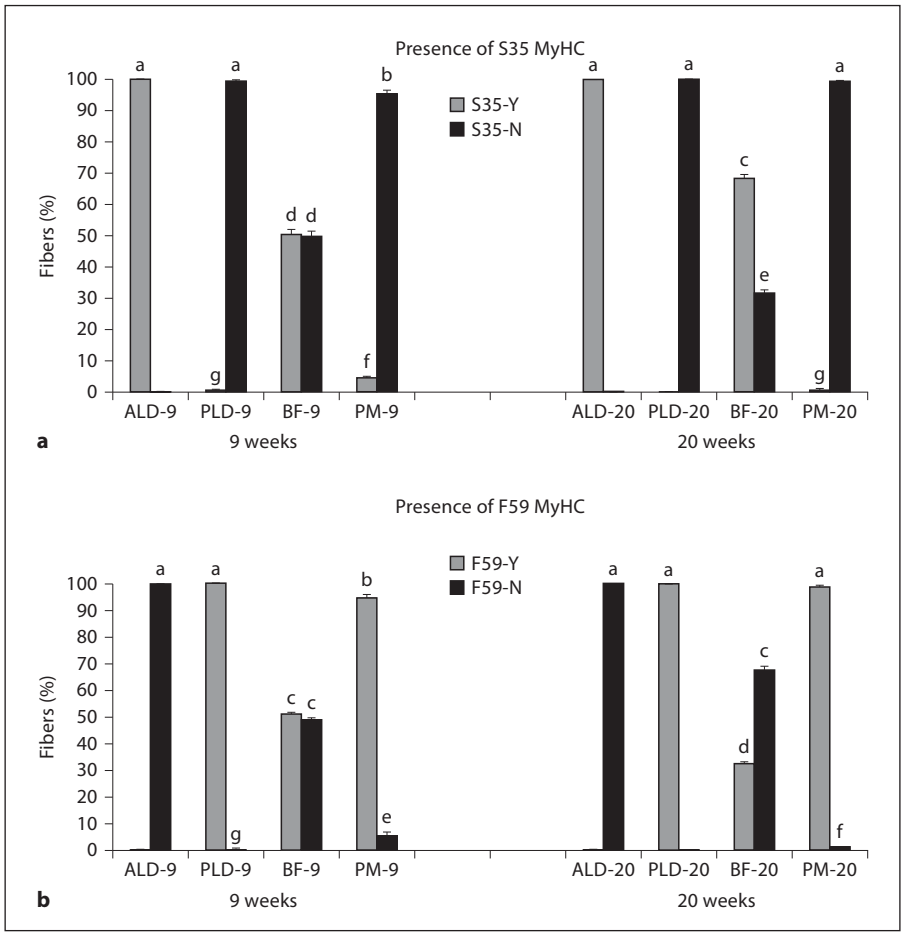


**Fig. 9.** Percentage of ALD, PLD, BF, and PM muscle fibers in 9- and 20-week-old turkey toms with (S35-Y) or without (S35-N) pronounced staining for S35 MyHC (**a**), F59 (**b**). The superscripts represent means that are significantly different. Error bars represent standard errors of individual means.

## Discussion

*Fiber Phenotype and Contractile Characteristics*

Muscle fiber location, its function, and load greatly influence the metabolic and phenotypic profile of the muscle. The total number of muscle fibers, their spatial distribution, and biochemical function vary in different muscles. Understanding metabolic differences within specialized muscles presents a useful tool in deciphering mechanisms governing skeletal muscle development. The present study focused on simultaneously selected aspects of the phenotypic, and metabolic, characteristics of ALD, PLD, BF, and PM muscles. The main objective of the current work was to examine the potential relationship between muscle $CoQ_{10}$ content, mitochondrial protein, and muscle fiber phenotype.

One of the aims of the study was to re-evaluate previously described fiber type composition in distinct muscles in adult turkey toms to further access the relationship between fiber phenotype and its mitochondrial metabolism [Wiskus et al., 1976; Beermann et al., 1978; Green et al., 1982]. Classic experiments assessing muscle fiber phenotype demonstrated that there are three main types of muscle fibers in turkey muscles, which can be defined as αR (fast-oxidative), αW (fast-glycolytic), and βR (slow-oxidative). The muscle fiber characterization described in former studies was based on muscle differential response to ATPase and SDH staining [Wiskus et al., 1976]. Previous data presented in Wiskus et al. [1976] has demonstrated that while PM is extremely scarce in βR fibers, previously considered as oxidative, BF muscle was composed of both oxidative and glycolytic fast-twitch fibers. Furthermore, Wiskus et al. [1976] demonstrated that fast-white and slow-red classification of muscle fibers may not be accurate and that certain muscles (such as BF) appear red due to high myoglobin content, but have enzymatic characteristics of fast fibers.

The fiber type of four muscle groups was re-evaluated and determined based on enzymatic staining for SDH and slow (acid-stable) myosin ATPase, followed by im-

munohistochemical labeling for slow and fast MyHC (fig. 6–9). The data confirmed that ALD, PLD, PM, and BF muscles have different enzymatic and contractile characteristics. Concurring with Wiskus et al. [1976], the present study confirmed that despite its red appearance, BF muscle contains fibers of various metabolic profiles (fig. 6–9). Additionally, ATPase and SDH staining profile in PM muscle was very similar to the data of Wiskus et al. [1976] (table 1, fig. 6, 8).

Staining for SDH revealed that at 9 weeks of age, approximately 60% of PM fibers contained medium activity of SDH, while at 20 weeks of age about 80% of PM fibers reveal low-intensity SDH staining (fig. 8). A similar staining pattern was observed in PLD muscle, suggesting that there is an age-related decrease in oxidative capacity in both PM and PLD muscle groups.

Immunohistochemical analysis of S35 and F59 MyHC isoforms was utilized to further evaluate the contractile profile of distinct muscles (fig. 9). The MyHC isoforms are the main structural proteins involved in conversion of ATP to mechanical energy required for muscle contraction [Sun et al., 2003]. In ALD muscles, all S35-positive fibers were negative for F59 antibody. The opposite staining pattern was observed in PLD and PM muscles, where all F59-positive fibers were negative for S35 antibody. The results demonstrated that slow myosin ATPase staining and S35 MyHC staining pattern is very similar in ALD, PLD, and PM muscles. In both age groups ALD muscles were predominantly slow, PLD muscles were predominantly fast, BF muscles contained both slow and fast fibers, and PM was primarily fast (fig. 9). Although the BF muscle fibers were characterized by low-, medium-, and high-intensity staining for slow myosin ATPase, the MyHC staining revealed equal amounts of slow and fast MyHC in this muscle. The medium-intensity staining for myosin ATPase in BF muscle could be related to the presence of S35 and F59 MyHC isoforms within individual muscle fibers [Rosser et al., 1996].

*Fiber Phenotype and Energy Utilization*

The capability of the muscle fibers to resist fatigue is determined by the presence and performance of mitochondria, which contain factors involved in oxidative phosporylation process [Holloszy and Coyle, 1984; Fitts, 1994]. SDH is an important component of the mitochondrial TCA cycle, and strong staining for this enzyme identifies muscle fibers with high oxidative potential. Total mitochondrial protein relative to myofibrillar protein significantly decreases between 9 and 20 weeks of age and does not differ between muscles at a given time (fig. 5). Oxidative ALD muscles contain twice as many mitochondria as the fast-twitch PLD muscles, but the ALD mitochondria are much smaller than the PLD mitochondria [Kiessling, 1976]. Therefore, it is likely that total weight of mitochondrial protein in ALD and PLD muscles is not significantly different even though there is a difference in mitochondrial number. Nonetheless, data generated based on enzymatic staining for mitochondrial enzyme, SDH, shows significant difference in the activity of this enzyme between distinct muscles and between different age groups. Consequently, other mitochondrial factors in combination with SDH should be considered when quantifying tissue mitochondria. The activity of SDH increases with age in ALD and BF muscles and decreases with age in PM and PLD muscles. Therefore, it is possible that with increased size and higher functional demands, ALD and BF muscles have an increased requirement for oxidative phosphorylation [Handel and Stickland, 1986; Hoppeler and Fluck, 2003]. Since PM and PLD muscles were already mostly relying on glycolytic metabolism for energy utilization, increased muscle size may have contributed to an increased demand for anaerobic respiration and decrease in mitochondrial function.

*Fiber Phenotype and $CoQ_{10}$ Content*

$CoQ_{10}$ represents one of the key components of mitochondrial oxidative metabolism, plays an indispensable role in ATP production in tissues with high energy requirements, and protects tissues from free radical damage [Crane, 2001; Dhanasekaran et al., 2008]. Because of $CoQ_{10}$'s role in oxidative phosphorylation and its cytoprotective properties, quantification of $CoQ_{10}$ levels has important implications in identifying a metabolic state of the skeletal muscle. HPLC-UV was utilized to determine levels of $CoQ_{10}$ in whole muscle and in the mitochondrial fractions of four different muscle groups in 9- and 20-week-old male turkeys. The HPLC-UV analysis has revealed age-related differences in $CoQ_{10}$ content where whole muscle homogenate $CoQ_{10}$ content increased with age in ALD muscles and remained unchanged in PLD, BF and PM muscles (fig. 2). Both ALD and BF muscles had significantly higher total levels of $CoQ_{10}$ than PLD and PM muscles at weeks 9 and 20. Mitochondrial $CoQ_{10}$ content in BF and ALD muscles increased with age and was significantly higher than in PLD and PM muscles. Additionally, mitochondrial $CoQ_{10}$ content in PLD and PM muscle did not change with age (fig. 3). The differences between whole tissue homogenate and mitochondrial fraction $CoQ_{10}$ content in distinct muscles may be related

to metabolic requirements of each muscle. It appears that with increasing age mitochondrial oxidative phosphorylation process requires more $CoQ_{10}$ in muscles that mostly rely on oxidative metabolism. Consequently, it can be concluded that older, oxidative muscles have higher energy requirements to support larger fiber size. Furthermore, higher energy requirements can be associated with increased free radical damage that has been observed in aging muscle [Lass and Sohal, 1998]. Additionally, with age there is an increase in reactive oxygen species generation that corresponds to oxidative damage in mitochondria [Sohal et al., 1999]. It is likely that muscle groups that rely on glycolytic metabolism possibly become more glycolytic with age; hence the lower amount of $CoQ_{10}$ in mitochondrial fraction. Since $CoQ_{10}$ has been shown to be a rate-limiting component of the oxidative phosphorylation process, further depletion of $CoQ_{10}$ may lead to reduced respiratory function of the muscle [Estronell et al., 1992; Kamzalov and Sohal, 2004].

Taken together, these results indicate the importance of $CoQ_{10}$ in oxidative metabolism. Additionally, the present study revealed a strong correlation between mitochondrial $CoQ_{10}$ content, SDH labeling, and slow ATPase labeling (table 1). Since $CoQ_{10}$ levels vary in different muscles and its levels are proportional to slow ATPase activity and mitochondrial SDH levels, it is reasonable to hypothesize that cellular levels of $CoQ_{10}$ may present a potential marker of slow-oxidative skeletal muscle phenotype.

It appears that mitochondrial to total muscle $CoQ_{10}$ ratio significantly decreases in PM and PLD muscles, remains the same in ALD muscle, and significantly increases in BF muscle between weeks 9 and 20 of age. Studies have demonstrated that aside from mitochondria, $CoQ_{10}$ is located in Golgi apparatus and in lysosomes [Nyquist et al., 1970; Sun et al., 1992; Gille and Nohl, 2000]. Although mitochondria are the major sites of $CoQ_{10}$ action, it has recently been shown that $CoQ_{10}$ also participates in lysosomal redox chain [Gille and Nohl, 2000]. Therefore, it is likely that this coenzyme exists in higher concentrations in other cellular membranes in older PLD and PM muscles [Nyquist et al., 1970; Sun et al., 1992; Gille and Nohl, 2000]. Additional research needs to be performed to identify cellular pathways involving $CoQ_{10}$ and its age-related role in the muscle.

In conclusion, the present study revealed that there is a relationship between mitochondrial and whole muscle $CoQ_{10}$ content and muscle phenotype. Furthermore, strong correlations between mitochondrial $CoQ_{10}$ content, SDH activity, and slow ATPase labeling in 9-week-old turkeys, and slightly weaker correlations between these metabolic components in 20-week-old turkeys indicate increased phenotypic variation in maturing muscle fibers. These results suggest that age-related change in functional demands may have an effect on muscle phenotype.

Additionally, current experiments indicated a strong relationship between the amount of mitochondrial protein and age of the muscle. The results of this study suggest that distinct skeletal muscles are metabolically different and that there are age-related differences in internal metabolism within the same muscles.

Enzymatic staining and immunohistochemical analysis for MyHC isoforms are useful tools in fiber type characterization. However, there are many other molecular pathways involved in regulating skeletal muscle fiber phenotype. More detailed evaluation of factors participating in muscle metabolism is required for adequate identification of specific fiber phenotype. Based on the results of the present study, it can be hypothesized that $CoQ_{10}$ may play a significant role in fiber type determination. More extensive research focusing on the impact of $CoQ_{10}$ on signaling molecules and on genes responsible for contractile and metabolic properties of the muscle will be necessary to further define the effect of $CoQ_{10}$ on muscle fiber phenotype.


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