RAPID COMMUNICATION: Differential skeletal muscle mitochondrial characteristics of weanling racing-bred horses[1]

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ABSTRACT: Responses of equine skeletal muscle characteristics to growth and training have been shown to differ between breeds. These differential responses may arise in part because muscle fiber type and mitochondrial density differ between breeds, even in untrained racing-bred horses. However, it is not known when these breed-specific differences manifest. To test the hypothesis that weanling Standardbreds (**SB**) and Thoroughbreds (**TB**) would have higher mitochondrial measures than Quarter Horses (**QH**), gluteus medius samples were collected from SB (mean ± SD; 6.2 ± 1.0 mo; n = 10), TB (6.1 ± 0.5 mo; n = 12), and QH (7.4 ± 0.6 mo; n = 10). Citrate synthase (**CS**) and cytochrome c oxidase (**CCO**) activities were assessed as markers of mitochondrial density and function, respectively. Mitochondrial oxidative (**P**) and electron transport system (**E**) capacities were assessed by high-resolution respirometry (**HRR**). Data for CCO and HRR are expressed as integrated (per mg protein and per mg tissue wet weight, respectively) and intrinsic (per unit CS). Data were analyzed using PROC MIXED in SAS v 9.4 with breed as a fixed effect. Mitochondrial density (CS) was higher for SB and TB than QH ($P \leq 0.0007$). Mitochondrial function (integrated and intrinsic CCO) was higher in TB and QH than SB ($P \leq 0.01$). Integrated CCO was also higher in TB than QH ($P < 0.0001$). However, SB had higher integrated maximum P (P_{CI+II}) and E (E_{CI+II}) than QH ($P \leq 0.02$) and greater integrated and intrinsic complex II-supported E (E_{CII}) than both QH and TB ($P \leq 0.02$), whereas TB exhibited higher integrated P with complex I substrates (P_{CI}) than SB and QH ($P \leq 0.003$) and higher integrated P_{CI+II} and E_{CI+II} than QH ($P \leq 0.02$). In agreement, TB and QH had higher contribution of complex I (CI) to max E than SB ($P \leq 0.001$), whereas SB had higher contribution of CII than QH and TB ($P \leq 0.002$). Despite having higher mitochondrial density than QH and TB, SB showed lower CCO activity and differences in contribution of complexes to oxidative and electron transport system capacities. Breed differences in mitochondrial parameters are present early in life and should be considered when developing feeding, training, medication, and management practices.

Key words: Horse, Mitochondria, Racing, Standardbred, Thoroughbred, Quarter Horse

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INTRODUCTION

Skeletal muscle fiber type composition and energetic profile of both mature trained and untrained racing-bred horses differ between breeds (Hodgson et al., 2014) and appear to diverge early in life (Bechtel and Kline, 1987; Kline and Bechtel, 1990). Stull and Albert (1980) proposed that these differences arise due to selection and adaptation for varying lengths and intensities of training and competition. Differences in adaptations to exercise have been demonstrated, and successfully raced Quarter Horses (Wood et al., 1988) and endurance horses (Rivero et al., 1993) have markedly distinctive muscle fiber type populations. Although all muscle fiber types are necessary for optimal performance, oxidative fibers and oxidative capacity are critical for longer bouts of exercise, such as Standardbred (Essèn-Gustavsson and Lindholm, 1985) and perhaps Thoroughbred (Lindholm et al., 1983) races. Conversely, fast twitch, nonoxidative fibers are extremely important for Quarter Horse racing (Wood et al., 1988), where explosive power is required, but exercise duration is shorter.

Previous studies established disparities in metabolic enzymes and muscle fiber type in mature horses of different breeds and disciplines, but there is no research comparing mitochondrial characteristics and oxidative capacity between racing breeds from a young age. Therefore, the aim of this study was to test the hypothesis that weanling Standardbreds (SB) and Thoroughbreds (TB) would have higher mitochondrial measures than Quarter Horses (QH).

MATERIALS AND METHODS

Horses

This study was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (2016-0294). Samples were collected from weanling racing-bred Thoroughbreds (**TB**; mean ± SD; 6.1 ± 0.5 mo; range 6 to 7 mo; 9 colts, 3 fillies), Quarter Horses (**QH**; 7.4 ± 0.6 mo; range 7 to 8 mo; 9 colts, 1 filly), and Standardbreds (**SB**; 6.2 ± 1.0 mo; range 5 to 8 mo; 9 colts, 1 filly) from farms in Kentucky and Texas with owner consent. Thoroughbreds were housed in pastures and brought to stalls to receive morning and evening concentrate meals. Quarter Horses were housed and fed concentrate in groups in pastures. Four Standardbreds were being housed and fed in stalls at the time of collection, and the remainder were housed and fed concentrate in groups in the pasture. No horses had received forced exercise prior to sampling.

Sample Collection and Analysis

Muscle samples were collected from the gluteus medius muscle using a 14-gauge tissue collection needle (SuperCore; Argon Medical Devices Inc., Frisco, TX) as described previously (White et al., 2016). Samples were either flash frozen in liquid nitrogen for subsequent enzyme activity analyses or placed into ice-cold BIOPS solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine; pH 7.1) and stored on ice or at 4 °C until high-resolution respirometry (**HRR**) analysis. Samples for HRR were analyzed within 24 h of collection.

Citrate synthase (**CS**) and cytochrome *c* oxidase (**CCO**) activities were analyzed as markers of mitochondrial density and function, respectively (Larsen et al., 2012). For measurement of CS and CCO activities, frozen skeletal muscle samples were prepared, and activities were measured as described previously (Spinazzi et al., 2012; Li et al., 2016). Enzymatic activities were normalized to protein content, determined using the Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Cytochrome *c* oxidase activity is presented on an integrated (per mg protein) and intrinsic (per unit CS) basis. Intrinsic CCO activity was calculated by dividing integrated CCO activity (nmol · min^{-1} · mg $protein^{-1}$) by CS activity (nmol · min^{-1} · mg $protein^{-1}$).

Immediately prior to HRR analysis, muscle fibers were isolated and permeabilized as described previously (Li et al., 2016). Oxygen flux and respiratory states were determined by HRR with the following substrate-uncoupler-inhibitor titration protocol modified from a previously described protocol for equine skeletal muscle (Li et al., 2016): 1) pyruvate (5 mM) and malate (2 mM) to support electron flow through complex I (CI) of the electron transport system (**ETS**; **LEAK** respiration); 2) adenosine diphosphate (ADP; 2.5 mM) to stimulate respiration (OXPHOS, $\mathbf{P_{CI}}$); 3) cytochrome *c* (cyt c; 10 µM) to assess outer mitochondrial membrane integrity (samples with responses to cyt c greater than 15% were excluded); 4) glutamate (10 mM) as an additional CI substrate and succinate (10 mM) to support convergent electron flow through complex II (CII) of the ETS ($\mathbf{P_{CI+II}}$); 5) uncoupler carbonyl cyanide 3-chlorophenylhydrazone (**CCCP**; 0.5 µM steps) to assess maximum ETS capacity ($\mathbf{E_{CI+II}}$); 6) rotenone (0.5 uM), an inhibitor of complex I, to measure maximal ETS capacity of complex II ($\mathbf{E_{CII}}$); 7) antimycin A (2.5 µM), an inhibitor of complex III, to measure residual oxygen flux (ROX)

independent of the ETS. Respiratory state fluxes are presented on an integrated (per mg tissue wet weight) and intrinsic (per CS activity) basis. To convert CS activity from nmol · min^{-1} · mg protein^{-1} to pmol · s^{-1} · mg tissue^{-1} for intrinsic HRR calculations, total protein for each sample was converted from mg protein/mL to mg protein/mg tissue using a known homogenization dilution factor of 1 mg tissue to 0.08 mL buffer. Subsequently, appropriate conversion factors for nmol to pmol and min to sec were used to complete the calculation. Sample flux control ratio (**FCR**) for each complex was calculated by dividing the flux in each complex by the sample's E_{CI+II} flux.

Statistical Analysis

Differences in enzyme activities and mitochondrial respiration measurements between breeds were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). Data were tested for normality using the UNIVARIATE procedure of SAS and then log-transformed prior to analysis if not normally distributed. All log transformed data were normally distributed. Intrinsic CCO and HRR, and FCR data were not transformed as they were already normalized to CS and E_{CI+II} flux, respectively. Breed was included in the model as a fixed effect. The QH and SB groups only contained one filly each, so sex was not included in the model. Additionally, because TB contained no 8-mo-old horses, and QH contained no 5- or 6-mo-old horses, age was not included in the model. However, within breed, none of the measured variables correlated with days of age ($P > 0.05$; PROC CORR in SAS 9.4; data not shown). All data are expressed as mean ± SEM. Significance was considered at $P \le 0.05$, and trends were acknowledged at $P \le 0.10$.

Table 1. Citrate synthase (CS) and integrated (per mg protein) and intrinsic (per unit CS) cytochrome *c* oxidase (CCO) activities in the gluteus medius of weanling racing-bred Quarter Horses (QH; $n = 10$), Thoroughbreds (TB; $n = 12$), and Standardbreds (SB; $n = 10$)

Enzyme	QH	TB	SB	SEM	*P*-value
CS Activity, nmol · min^{-1} · mg protein^{-1}	9.7^{a}	16.0^{b}	16.2^{b}	1.2	0.0006
Integrated CCO Activity, nmol · min^{-1} · mg protein^{-1}	15.3^{b}	25.8^{c}	9.0^{a}	1.7	<0.0001
Intrinsic CCO Activity, nmol · min^{-1} · unit CS^{-1}	1.6^{b}	1.7^{b}	0.6^{a}	0.01	<0.0001

$^{a-c}$Within a row, breeds lacking common letters differ ($P \le 0.05$).

RESULTS

Activities of CS and CCO are presented in Table 1. Citrate synthase activity was used as a marker of mitochondrial density (Larsen et al., 2012). Standardbreds and TB had higher CS activity than QH ($P \le 0.0007$). Citrate synthase activity did not differ between SB and TB. Cytochrome *c* oxidase activity was used as a marker of mitochondrial function (Larsen et al., 2012). Integrated CCO activity was higher in TB than QH ($P < 0.0001$) and SB ($P < 0.0001$) and was also higher in QH than SB ($P = 0.01$). Intrinsic CCO was lower in SB than QH and TB ($P < 0.0001$; Table 1) but did not differ between QH and TB.

Mitochondrial respiratory capacities were determined by HRR. Thoroughbreds had higher integrated ($P = 0.002$; Figure 1A) and intrinsic ($P = 0.02$; Figure 1B) LEAK than QH. Additionally, TB tended to have higher integrated ($P = 0.07$; Figure 1A) and intrinsic LEAK ($P = 0.07$) compared with SB. Integrated and intrinsic LEAK did not differ between SB and QH.

Integrated P_{CI} was higher for TB than SB and QH ($P \le 0.003$; Figure 1A) but did not differ between SB and QH. Intrinsic P_{CI} was not different between breeds (Figure 1B). Integrated P_{CI+II} was lower for QH than TB and SB ($P \le 0.01$; Figure 1A) but did not differ between SB and TB. A trend for an effect of breed ($P = 0.1$) for intrinsic P_{CI+II} suggested that SB was higher than TB ($P = 0.03$), whereas QH did not differ from SB or TB (Figure 1B). Integrated E_{CI+II} was lower for QH than TB and SB ($P \le 0.02$; Figure 1A) but did not differ between TB and SB. However, a trend for a main effect of breed ($P = 0.07$) on intrinsic E_{CI+II} showed that SB was higher than TB ($P = 0.02$), whereas QH did not differ from SB or TB (Figure 1B). Integrated (Figure 1A) and intrinsic (Figure 1B) E_{CII} were higher for SB than QH ($P \le 0.005$) and TB ($P \le 0.02$), and integrated E_{CII} was higher for TB than QH ($P = 0.04$; Figure 1A).

Thoroughbreds had higher FCR for LEAK than QH and SB ($P \le 0.01$) but LEAK FCR did not differ between SB and QH (Figure 1C). The FCR for P_{CI} was higher for TB and QH than SB ($P \le 0.001$) but did not differ between TB and QH (Figure 1C). A trend for an overall effect of breed ($P = 0.09$) indicated that the FCR for P_{CI+II} was lower for QH than TB ($P = 0.03$) and tended to be lower for QH than SB ($P = 0.1$) but did not differ between TB and SB. The FCR for E_{CII} was higher in SB than QH and TB ($P \le 0.002$; Figure 1C) but did not differ between QH and TB.

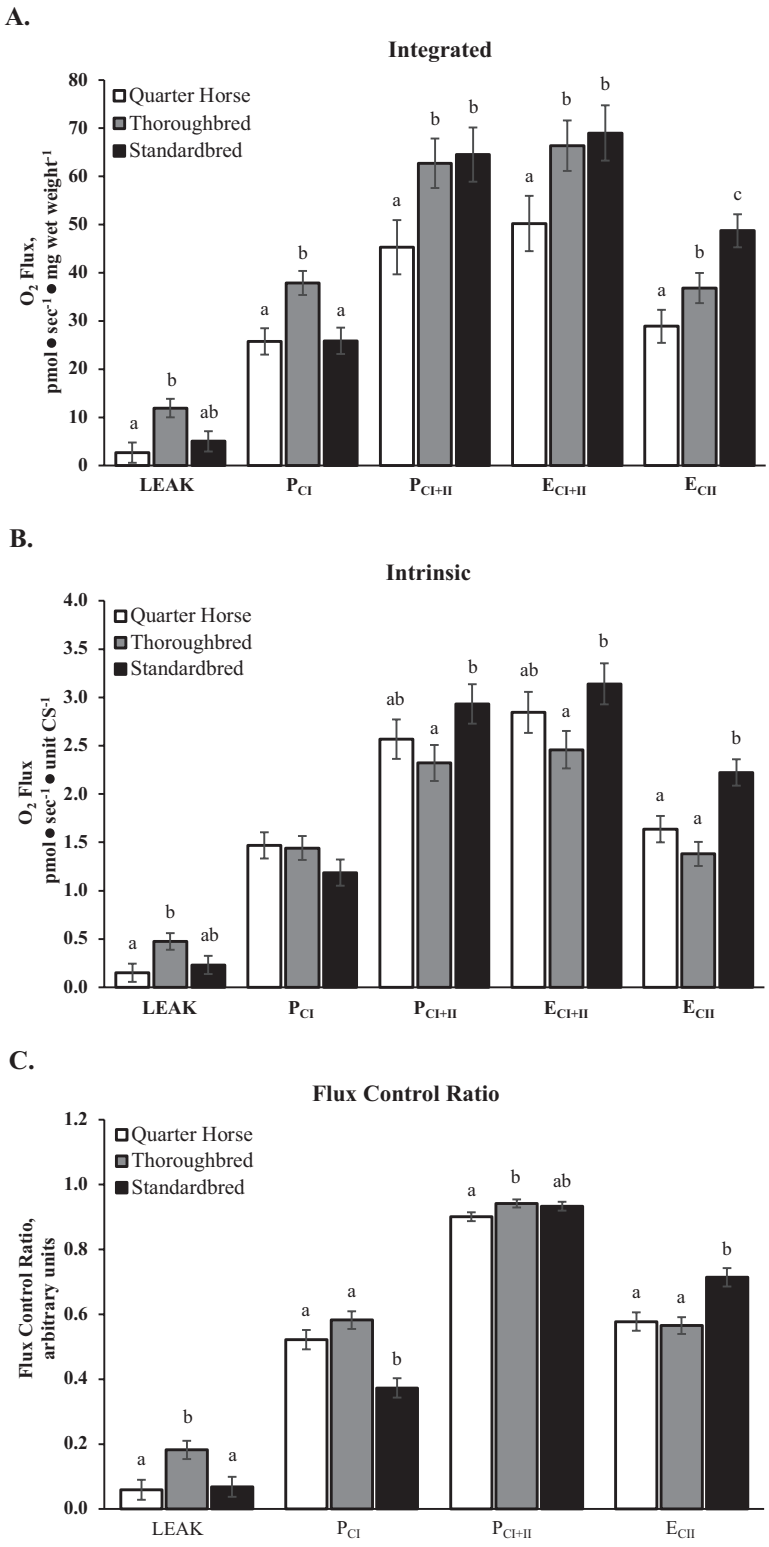

Figure 1. Mitochondrial respiration of permeabilized fibers from the gluteus medius of weanling racing-bred Quarter Horses (QH; n = 10), Thoroughbreds (TB; n = 12), and Standardbreds (SB; n = 10) via high-resolution respirometry. Mass specific O_2 flux (A; pmol · s^{-1} · mg wet $weight^{-1}$), O_2 flux normalized to CS activity (B; pmol · s^{-1} · unit CS^{-1}), and O_2 flux normalized to maximum electron transport system (ETS) capacity (C; arbitrary units) with LEAK respiration (LEAK), oxidative phosphorylation (OXPHOS) capacity of complex I (P_{CI}), OXPHOS capacity of complex I and II (P_{CI+II}), maximum ETS capacity (E_{CI+II}), and maximum ETS capacity of complex II (E_{CII}). [a,b]Within a variable, bars lacking common letters differ ($P \leq 0.05$).

DISCUSSION

Oxidative capacity of skeletal muscle depends primarily on mitochondrial density and the capacities of five main protein complexes in the mitochondria, which are responsible for electron transport and, ultimately, adenosine triphosphate (ATP) production.

The present study demonstrates that racing-bred QH, TB, and SB differ in their skeletal muscle mitochondrial density and capacities as early as 6 mo of age. This is unsurprising, as individuals have been selected within each breed based on excellence at unique race distances (1/4 mile for QH, longer distances for TB and SB) that have disparate oxidative respiration requirements. Young and mature sedentary QH have previously been shown to have a lower percentage of slow twitch, oxidative muscle fibers and lower measures of mitochondrial density when compared with SB and TB (Stull and Albert, 1980; Bechtel and Kline, 1987; Kline and Bechtel, 1990). In agreement, we found mitochondrial density and several measures of integrated oxidative capacity to be lower in QH weanlings than TB and SB. Given that successful racing QH have a higher percentage of fast twitch, nonoxidative fibers (Wood et al., 1988) and rely more heavily on anaerobic metabolism for work and competition, our findings suggest that these metabolic characteristics have been selected for and are present from a young age. The current study did not investigate muscle fiber type, but the lower mitochondrial markers suggest a lower presence of oxidative type I fibers in QH even as weanlings.

Reduction of NADH at complex I (CI) and succinate at complex II (CII), and the subsequent transfer of electrons through the ETS to oxygen as the terminal electron acceptor ultimately results in ATP synthesis. However, insufficient concentrations of ADP, damaged cellular membranes, or the presence of uncoupling proteins may lead to ETS activity uncoupling from ATP synthesis. Instead, protons "leak" across the inner mitochondrial membrane, consuming cellular oxygen and as such, potentially mitigating reactive oxygen species (ROS)-induced oxidative damage (Brand, 2000). Increased CI activity has been associated with increased mitochondrial ROS production (St-Pierre et al., 2002) that may be related to anabolic signaling and adaptation following exercise (Seifert et al., 2012). The elevated LEAK respiration exhibited by TB in the current study may be related to TB also exhibiting higher integrated capacity with CI substrates; greater proton leak could alleviate CI-linked ROS production.

Standardbreds showed the lowest FCR for CI respiration, along with the highest integrated and intrinsic ETS capacity with CII, and the highest FCR for E_{CII}. Calculation of the flux control factor (**FCF**) for oxidative phosphorylation capacity with complex II (P_{CII}) in the present study ($\frac{P_{CI+II}-P_{CI}}{P_{CI+II}}$) revealed that SB also had a higher FCF for CII (0.60 ± 0.03) than TB (0.38 ± 0.03) and QH (0.42 ± 0.03; $P \leq 0.0005$). Furthermore, SB had higher intrinsic maximum oxidative and electron transport system capacities than TB. Complex II is a source of reserve respiratory capacity that promotes cell survival during times of stress, such as when energy demands exceed supply (Pfleger et al., 2015). Therefore, it is possible that because SB typically undergo longer bouts of exercise during both training and competition, they have been selected to achieve a higher reserve respiratory capacity to maintain energy production during prolonged bouts of physical activity.

Despite having comparable integrated maximum oxidative and ETS capacity with TB, SB showed lower CCO activity. To the best of our knowledge, this is the first study to examine breed differences in CCO activity in weanling horses. Although CCO activity did not increase with growth in 18-mo-old QH (White et al., 2017), it is possible that CCO activity increases with growth of younger horses, in concert with observed increases in percentages of oxidative fibers and other enzymes for oxidative metabolism (Yamano et al., 2005). To support this idea, CCO activity before exercise training in the GM of 18-mo-old QH in the aforementioned study was considerably higher (approximately 80 nmol · min^{-1} · mg $protein^{-1}$; White et al., 2017) than the observed CCO activity in the GM of untrained 6-mo-old QH in the present study (15.3 ± 1.7 nmol · min^{-1} · mg $protein^{-1}$). Therefore, the observed lower CCO activity of weanling SB may increase with age.

The present study provides insight into mitochondrial biology of young racing bred horses. This insight may be used to help inform medication, training, and other management strategies in different breeds of racing horses. An applicable example is the use of clenbuterol in performance horses. In SB, clenbuterol treatment increased fat free mass (Kearns et al., 2001) but impaired aerobic performance (Kearns and Mckeever, 2002). Similarly, clenbuterol failed to improve aerobic performance in TB (Rose and Evans, 1987). The current study demonstrates a greater capacity for aerobic metabolism from a young age in SB and TB when compared with QH. This finding supports the idea that clenbuterol, which acts as a repartitioning agent (Kearns et al., 2001) by increasing the percentage of myosin heavy chain (MyHC) type IIx (fast twitch, nonoxidative) in skeletal muscle (Beekley et al., 2003), would be unlikely to enhance performance in breeds that rely heavily on oxidative fibers. The effects of clenbuterol treatment on performance measures in QH have not been specifically examined. However, an increase in the percentage

of MyHC IIx may benefit racing QH, as successfully raced QH rely more heavily on this anaerobic fiber type (Wood et al., 1988).

In this study, we found that SB and TB weanlings had higher mitochondrial density and integrated maximum oxidative capacity compared with QH, as well as differences in contribution of complexes to oxidative and electron transport system capacities between breeds. Breed differences in mitochondrial parameters appear to be present early in life, but their changes during growth and training programs are not well studied and remain poorly understood. Inherent breed differences likely affect responses to feeding, training, medications, and other management strategies. The impact of interventions on different breeds during growth, training, and competition must be further examined in order to construct informed regulation and management practices that maximize welfare of racing horses of all breeds.

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