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Skeletal Muscle Fiber Type Composition and Citrate Synthase Activity in Fit and Unfit Warmbloods and Quarter Horses

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ABSTRACT

Selective breeding and discipline specific training has led to equine breeds adept at various athletic disciplines. Breed-specific skeletal muscle adaptations have been studied in many breeds but not Warmbloods (WB). We evaluated gluteal muscle contractile muscle fiber types and citrate synthase activity (CS), a marker for mitochondrial volume density, in WB trained for dressage (second level-Grand Prix) contrasted with Quarter Horses (QH). Gluteus medius muscle biopsies from 14 unfit/18 fit dressage-trained WB and 20 unfit/16 fit reining/working cow QH were analyzed fluorometrically and fiber types determined by ATPase activity. Comparisons were made by one-way ANOVA. Unfit and fit WB had significantly higher % type 1 and lower % type 2X fibers than QH. Unfit WB had significantly higher CS than unfit QH but CS did not differ between fit WB and fit QH. CS was only significantly higher in fit versus unfit QH, not fit versus unfit WB. In conclusion, WB gluteal muscle has an inherently high % type 1/low % type 2X fibers and high mitochondrial content whether unfit or trained for dressage, contrasting QH with an inherently low % type 1/high % type 2X and low mitochondrial content, that was enhanced in fit QH. Similar CS activity in fit WB versus QH despite a two-fold difference in % type 2X fibers indicates that mitochondrial volume density cannot accurately be predicted from contractile fiber type composition.

1. Introduction

Discipline-specific training and hundreds of years of selective breeding have resulted in breeds of horses that are adept at a variety of athletic disciplines. Quarter Horses (QH) for example, excel at races over sprint distances of ¼ mile (402 m) whereas Arabians excel at races of 50–100 miles (80–160 km) [1,2]. Warmblood (WB) horses, in contrast, have been purpose bred to excel at dressage and jumping which requires power and precision in tests that last 5–10 minutes [3,4]. Athletic performance is dependent upon optimal skeletal muscle function [5]. Muscle fiber type composition, the percentage of slow-twitch type 1, fast-twitch type 2A and fast-twitch type 2X fibers, affects the speed of muscle contraction and thereby performance [6,7]. Breed specific differences in muscle fiber type composition exist, with QH known to have a high percentage of the fastest contracting fibers in locomotor muscles, type 2X, and a low percentage of slow contracting type 1 fibers [1,5]. Muscle fiber type composition has been reported in Dutch WB foals but, surprisingly, has not been reported to the authors' knowledge in adult WB [8,9].

The mitochondrial content of muscle fibers also has a strong impact on performance, influencing a horse's ability to sustain a given speed or duration of exercise [5]. The activity of citrate synthase (CS), the first enzyme in the mitochondrial tricarboxylic acid cycle, has been used in many equine exercise physiology studies as a marker for muscle oxidative capacity [2,10–22]. CS is a marker

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Ethical Statement: Muscle biopsy samples had been collected by one author (SJV) and the research protocols were approved by the Institutional Animal Care And Use Programs (IACUC) at the University of Minnesota or Michigan State University in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals.

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Table 1
The fitness status-breed, number of horses, age, sex, and citrate synthase (CS) activity of horses in the study presented as mean ± SD. Lower case letters indicate a significant difference in ages or CS activity.

Training Status	N	Age (yrs)	Sex	CS (μmol/g/min)
QH-UF	20	4.9 ± 3.9ac	15M, 3G, 2S	25.2 ± 6.4a
QH-F	16	6.4 ± 4.0ac	3M, 12G, 1S	34.2 ± 10.3b
WB-UF	14	8.6 ± 6.0abc	9M, 4G, 1S	37.7 ± 7.9b
WB-F	18	10.8 ± 4.3bc	8M, 8G, 2S	38.5 ± 9.0b

QH, Quarter Horse; WB, Warmblood; UF, unfit; F, fit; M, mare; G, gelding; S, stallion.

for mitochondrial volume density and previous studies have shown a correlation between muscle CS activity and maximal oxygen uptake in horses [9,23–25]. CS activity has been measured in muscle from QH, Thoroughbreds, Standardbreds, Arabians, Andalusians, and WB foals but not unfit or fit adult WB trained for their athletic discipline [2,8,10–18,20–22,26–39]. Oxidative capacity of fiber types has been subjectively assessed in many breeds based on mitochondrial staining [11,14,22,26]. Fibers can be classified as type 1 or type 2 based on ATPase staining and type 2 fibers subdivided as high or low oxidative based on mitochondrial stains [13]. A dark mitochondrial stain or high CS activity in a type 2 fiber does not necessarily correlate with myosin ATPase staining as a type 2A fiber [40,41].

The purpose of this retrospective cross-sectional study was to compare gluteal muscle contractile fiber type composition and CS activity in adult WB bred and trained for dressage with QH bred and trained for reining/ranch work. The first objective of our study was to determine muscle fiber type composition in gluteal muscle of unfit and fit WB and QH. The second objective was to compare CS activity in unfit and fit adult WB and QH.

2. Materials and Methods

2.1. Horses

Snap frozen gluteus medius muscle biopsy samples had been obtained during previous research studies from 32 healthy WB and 36 healthy QH (Table 1). WB were a variety of European WB types including nine German Sport Ponies (Connemara/Hanoverian/Trakehner/Mustang blood lines), five Hanoverians, five Hanoverian crosses (with Thoroughbred/Trakehner/Canadian Warmblood), one Trakehner, one Trakehner x Thoroughbred, two Danish WB, two Oldenburg, two Dutch WB, one Swedish WB, one Westphalian, one Holsteiner, and two unspecified WB. WB were housed on two farms, QH were housed on four farms. Of the 14 unfit WB, six were not previously trained and eight had been out of work for more than a year (Table 1). The 18 fit WB were trained for 20–45 min/day, 3–5 d/wk for second level through Prix St George dressage (Table 1). Of the 20 unfit QH, 12 were not previously trained and eight were out of work for more than a year. Of the 16 fit QH, 14 were trained for working cow disciplines and two reining (Table 1). QH were worked 5–6 d/wk with 10–15 minutes of relaxed walk/trot/lope, 5–10 minutes of suppling work (10–15 m circles with haunches in/out, shoulders in/out and leg-yielding-type work) and stop/go/rein back/go work at a lightly/moderately collected trot/jog/walk and then performed 10–15 minutes working live cattle or following the training flag. All the work was done in the arena with 10–15 cm of watered and groomed natural sand. As a positive control for oxidative capacity, CS activity was also assessed in gluteal muscle biopsies from 17 Arabian mares trained for 50–100-mile endurance races (mean age 8.3, range 6–22 years).

2.2. Muscle Biopsy Procedure

Muscle biopsy samples had been collected by one author (SJV) during previous research trips and approved by the Institutional Animal Care and Use Programs (IACUC) at the University of Minnesota or Michigan State University. Intravenous sedation with 200 mg xylazine (AnaSed, Santa Cruz Animal Health, Santa Cruz, CA) was used when necessary. Percutaneous gluteus medius muscle biopsies were obtained at a depth of 8 cm, and a site 16 cm distal to the highest point of the tuber coxae on a line from the tuber coxae to the tail head as previously described [42]. Approximately 200 mg of skeletal muscle was divided into two aliquots, one aliquot for biochemical analyses was immediately frozen in liquid nitrogen and the other for histochemistry was rolled in talc before freezing in liquid nitrogen. Muscle was stored at -80°C until analysis.

2.3. Muscle Fiber Type Composition

Gluteal muscle samples from 10 fit WB and 10 fit QH as well as four unfit WB and five unfit QH were sectioned 7 μm thick and stained for myosin adenosine triphosphatase (ATPase) following pre-incubation at pH 4.4 then lightly counterstained with eosin [43]. Samples were selected based on the quality of the preparation for histochemistry and degree of freeze-artifacts in the samples. Muscle fiber type composition of type 1, 2A, and 2X fibers was determined by dividing the number of fibers of each type by the total number of muscle fibers using a minimum total count of 250 muscle fibers per sample [44].

2.4. Citrate Synthase Analysis

Approximately 10–20 mg of muscle was rapidly weighed on a digital microbalance and then ultrasonically homogenized in ice-chilled 0.1 M potassium buffer (pH 7.3) (10mg:500μL dilution). The fluorometric method for CS activity was adapted from Essen-Gustavsson and Henriksson [45]. CS activity was determined at 25°C in 96 well plates with reagent volumes of 250 μL per well. The reagent mixture consisted of Tris-hydrochloride (TRIS-HCI) 100 mM pH 8.0, ethylenediaminetetraacetic acid (EDTA) 2.5 mM, nicotinamide adenine dinucleotide (NAD) 0.5 mM, L-malate 1 mM, malate dehydrogenase 8 μg/mL and 3 μL of muscle homogenate. The reaction was started with 5 μL of 10 m acetyl CoA.

2.5. Statistical Analysis

Fiber type composition was compared across breeds using one-way ANOVA. CS activity was normally distributed based on Kolmogorov-Smirnov testing. Differences in CS activity were first compared between never-been-trained and retired horses within breed using a t-test to ensure that they could be combined in one unfit category per breed. Differences in CS activity between 7 unfit German Sport Ponies and 6 unfit Hanoverian/Trakehners were made to determine potential differences between WB types. Differences in CS activity among fit and unfit WB and QH as well as fit Arabians were determined by one-way ANOVA. An association between CS activity and the percentage of type 1, 2A or 2X fibers was investigated using a Pearson's correlation. Data are presented as mean ± SD.

3. Results

3.1. Muscle Fiber Types

The mean % type 1 fibers was 4-fold higher in unfit WB versus unfit QH ($P < .0001$) and 1.7-fold higher in fit WB versus fit

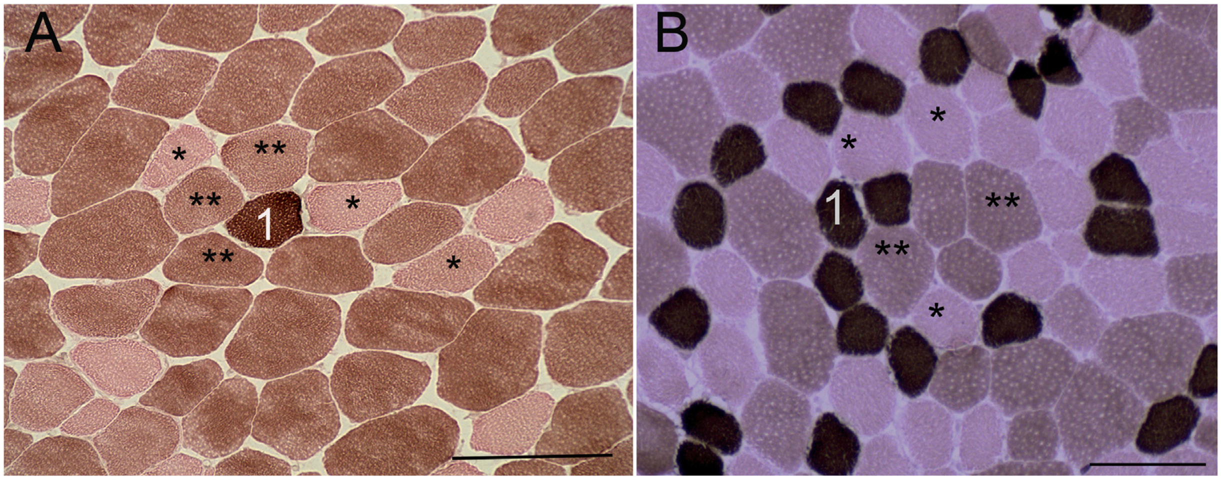

Fig. 1. Cross section of gluteal muscle from a Quarter Horse and a Thoroughbred stained with myosin ATPase pH 4.4 and counterstained with eosin. A. The Quarter Horse has a low proportion of type 1 (1) and 2A (*) fibers and a high proportion of type 2X (**) fibers. B. The Warmblood has a high proportion of type 1 (1) and 2A (*) fibers and a low proportion of type 2X (**) fibers.

Table 2
Muscle fiber type composition of gluteal muscle. Differences in lower case letters indicate significant differences within columns.

	N	Type 1 (%)	Type 2A (%)	Type 2X (%)
QH-UF	5	8.0 ± 7.9a	29.6 ± 8.2ab	62.2 ± 7.9a
QH-F	15	21.1 ± 5.3a	24.7 ± 7.8a	54.3 ± 8.0a
WB-UF	4	35.0 ± 4.2b	35.0 ± 2.2ab	29.8 ± 6.3b
WB-F	10	36.0 ± 5.4b	35.7± 7.9b	28.3 ± 5.5b

QH (P = .0003) (Fig. 1, Table 2). The % type 2A fibers was 1.4-fold higher in fit WB versus fit QH (P = .02) but not different in unfit WB and QH (P = .99) (Fig. 1, Table 2). The % type 2X fibers was twofold lower in unfit WB versus unfit QH (P =< .0001) and fit WB versus fit QH ($P < .0001$) (Fig. 1, Table 2).

3.2. Citrate Synthase Activity

3.2.1. Unfit Horses

There were no significant differences in CS activity between WB with no previous training (34.9 ± 5.9 µmol/g/min, N = 6) and retired WB (40.1 ± 9.0 µmol/g/min, N = 8, P = .3) or QH with no previous training (26.6 ± 3.7 µmol/g/min, N = 12) and retired QH (23.1 ± 9.1 µmol/g/min, N = 8, P = .3). Therefore, not previously trained and retired horses were combined to form one unfit group per breed (Table 1). The mean CS activity of unfit German Sports Ponies (34.3 ± 5.8 µmol/g/min n = 7) was not significantly different from Hanoverian/Trakehners 41.7 ± 8.5 µmol/g/min, P = .1 n = 6).

The ages of unfit versus fit WB (P = .55), unfit versus fit QH (P = .76) and unfit WB versus unfit QH (P = .11) did not differ significantly (Table 1). Fit WB were significantly older that fit QH (P = .026) (Table 1).

3.2.2. CS Activity

Unfit WB had 1.5-fold higher CS activity than unfit QH (P = .0005) (Fig. 2). CS activity did not differ between fit WB versus fit QH (P = .68) or between unfit versus fit WB ($P > .99$) (Fig. 2). Fit QH had 31% higher CS activity than unfit QH (P = .05) (Fig. 2). Endurance-trained Arabians had 1.3–1.5-fold higher CS activity than fit WB (P = .006) and QH ($P < .0001$).

3.2.3. CS activity versus age and fiber types

There was no significant correlation between age and CS activity across breeds (r = 0.12, P = .33). When including only fit

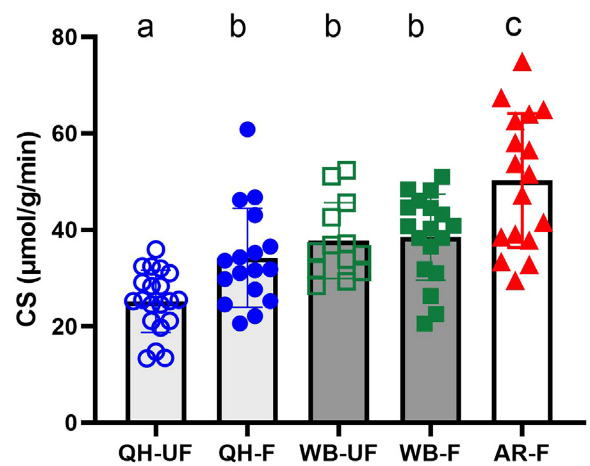

Fig. 2. Gluteal muscle citrate synthase (CS) activity in unfit (UF) and fit (F) Warmbloods (WB), Quarter Horses (QH) and endurance-trained Arabians (AR). Different letters indicate significant differences between groups. Unfit QH had the lowest CS activity and Arabians the highest. There was no significant difference in CS activity between unfit WB, fit WB and fit QH.

horses, there was a significant correlation between CS activity and type 1 fibers (r = 0.54, P = .01) but not for type 2A (r = -0.03, P = .89) or type 2X fibers (r = -0.40, P = .9). With fit and unfit horses combined, CS correlated positively with % type 1 (r = 0.53, P = .003) and negatively with % type 2X (r = -0.44, P = .017) with considerable variability in CS activity relative to fiber type (Fig. 3). For example, WB with 20% type 2X fibers could have similar CS activity to fit QH with 70% type 2X fibers.

4. Discussion

The goal of the present study was to characterize the contractile muscle fiber type composition and oxidative capacity of gluteal muscle in unfit and fit WB trained in the field for second to Grand Prix level dressage. This was contrasted with QH, previously demonstrated to have a low muscle oxidative capacity, and endurance-trained Arabians, previously demonstrated to have a high oxidative capacity [2,19,26,27]. The results of our study show that WB have inherently high CS activity and high % of type 1/low % type 2X fibers with no differences in CS activity or fiber types between unfit WB and fit WB trained for dressage.

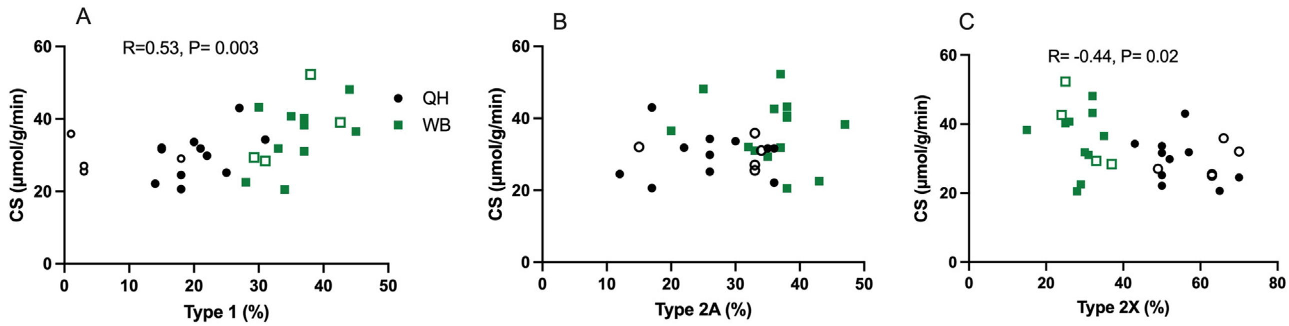

Fig. 3. The correlation between citrate synthase (CS) activity and muscle fiber type composition**.** A. CS activity was significantly correlated with the % of type 1 fibers. B. CS activity was not significantly correlated with the % of type 2A fibers. C. CS activity was significantly negatively correlated with the % of type 2X fibers. Note, however, that CS activity for a given fiber type could vary by at least twofold.

Warmblood breed organizations have open studbook policies with the goal of selecting and breeding horses highly adept at dressage, show jumping or driving [4,46]. Several breeds of horses with closed stud books have been incorporated into WB types and this was evident from the variety of WB and included in our study [47]. The heritability of muscle fiber type composition has been estimated to be 13%–25% in Anglo-Arabians and Spanish horses and 45% in humans [7,48,49]. The WB in our study had a unique muscle fiber type composition, a high percentage of type 1 (36%) and 2A (36%) fibers and a low percentage of type 2X (28%) fibers. The abundance of type 1 and 2A fibers in WB gluteus muscle is well aligned with fiber types likely recruited during dressage movements. A survey of 2,554 dressage riders in the United Kingdom found that during dressage training, horses spend 21% of time at walk, 45% at trot and 32% at canter [50]. At these speeds, type 1 and 2A fibers are primarily recruited, in line with their preponderance in WB, with 2X fibers only recruited as power, speed, or duration of exercise increases [51]. The similar fiber type compositions reported previously in Dutch WB yearlings and lack of difference in fiber types in unfit and fit WB together support the fiber type composition of WB being an inherent characteristic [8].

In contrast to WB, QH were originally selected for their ability to sprint short distances and, in line with this performance expectation, QH had a high percentage of the fastest contracting fiber type, type 2X, comprising over 50% of muscle fibers in gluteal muscle in this and other studies [1]. It is important to recognize that beyond fiber type composition, there are many other factors important for aptitude for a given athletic discipline. This is illustrated by the fact that Arabian horses have a lower % of type 1 fibers (24%) than WB, yet they are superior endurance horses to WB [2,26].

Repeated recruitment of muscle fibers stimulates an increase in CS activity [52] and controlled training trials have shown that exercise of sufficient duration and intensity induces an increase in muscle CS activity in Thoroughbreds, Standardbreds and QH [13,14,18,30,36,53,54]. A training effect likely contributed to the high CS activity in endurance trained Arabians in our study and to the higher CS activity in fit versus unfit QH. Notably, however, no difference in CS activity was found between unfit and fit WB. One explanation for this could be that retired horses were included in the unfit WB group. Statistical comparison, however, found no difference in CS activity between not previously trained and retired WB, or between trained and retired QH. Further, a decline in CS activity to pretraining levels has been shown to occur within 3 months of rest following training [18]. Thus, inclusion of previously trained horses is unlikely to have a major impact on unfit CS activity.

Another explanation for the lack of difference in CS activity between unfit and fit WB could be the intensity and duration of dressage training. Maximal heart rates below 155 bpm are reported during dressage [55]. In contrast, heart rates reach 180 bpm during QH reining competitions and 209 bpm during QH cutting competitions, indicating a higher level of exertion in QH training versus dressage training [56]. The amount of exercise each horse received in our study was not controlled but determined on a day-to-day basis by riders. In a survey of 2,554 dressage riders in the United Kingdom, dressage training usually involved on average 36 minutes of exercise 3–4 times a week with a mean warm-up time of 16 minutes and cool-down of 11 minutes at all levels [50]. Novice, Intermediare I and Grand Prix horses trotted most and cantered least during training amongst the different levels [50]. It would appear from our study that the focus of dressage training on developing suppleness, rhythm, relaxation, connection, impulsion, and collection does not result in a change in muscle oxidative capacity [55]. In contrast, the focus of training in QH on speed and motor skills appears to enhance muscle oxidative capacity [1]. To definitively determine if dressage training evokes an increase in muscle oxidative capacity, a longitudinal training study of the same horses in a dressage training program would be necessary and of great interest to pursue. What can be concluded from the present study is that the amount of effort and time average riders in the USA devote to dressage does not result in gluteal muscle CS activity that is higher than that of unfit horses on the same farms.

The lowest muscle CS activity amongst breeds was found in unfit QH suggesting that a low gluteal muscle mitochondrial volume density appears to be inherent in the QH breed in agreement with a study of QH foals [57]. CS activity was, however, substantially higher by a mean of 50% in fit versus unfit reining/working cow horses with CS activity in fit QH being similar to that of fit WB. Working QH training incorporates both aerobic and anaerobic training generally lasting for 30 minutes, 6 days per week achieving maximum heart rates as high as 180 bpm [58]. Muscle CS activity in response to training in QH varies in controlled trials [30,59]. No change in gluteal muscle CS activity was noted in 2 year old QH trained for 8–9 weeks at submaximal speeds, 4 days per week for 30 minutes in a round pen, however, coupling of mitochondrial oxidative phosphorylation was enhanced [59]. In aged QH, 10 weeks of training at speeds achieving up to 80% VO_{2max} resulted in a 40% increase in CS activity in the triceps with no change in CS in the semimembranosus muscle [30]. Our study suggests that training of reining and ranch horses in the field increases the naturally low muscle CS activity and mitochondrial volume density by approximately 50% in unfit QH.

An important finding in the present study was that muscle fiber type composition did not always reflect the mitochondrial content of gluteal muscle across breeds captured as CS activity. There was a moderate negative correlation between CS and % type 2X fibers across fit and unfit WB and QH. However, CS activity could differ almost twofold in fit QH and WB with the same % type 2X fibers. This seems puzzling because type 2X fibers are reported to have the lowest oxidative capacity of the three fiber types [5,60]. One potential explanation for this is that the CS activity of type 2X fibers or type 2AX hybrid fibers increased with training in the QH but not WB. Hybrid 2AX fiber type could not be detected by the ATPase method used for fiber typing in our study [61]. In support, an increase in mitochondrial staining of fast-twitch fibers has been shown to occur with training that includes high speed exercise, an intensity needed to recruit type 2X fibers and not achieved in WB dressage [13,51]. The high degree of variability in CS activity for a given fiber type composition in fit WB versus fit QH indicates that suppositions regarding differences in muscle oxidative capacity based on ATPase contractile fiber type composition should be made with caution.

The design of our retrospective cross-sectional study that used convenience sampling of horses in the field introduced several limitations. All WB in our study were not of the same type, and this could have introduced variability in our data. Many of the unfit WB were German Sport Ponies. This WB type had a trend ($P = .1$) toward lower CS activity than Hanoverians and Trakehners. Thus, inclusion of the German Sport Ponies should have increased the likelihood of seeing differences in CS activity between unfit and fit WB, yet no significant differences were found. This supports our conclusion that dressage training in WB has little impact on muscle mitochondrial volume. The daily intensity or duration of training was not controlled, and a training effect was inferred by comparing fit versus unfit horses. A longitudinal study following the same individuals during discipline-specific training would be necessary to conclusively determine if differences in muscle oxidative capacity between fit and unfit horses were the result of training. A longitudinal study of training in the field, however, is difficult to perform and has only been accomplished to our knowledge in Thoroughbred horses in race training [35].

Analyzing electron transfer capacity during oxidative phosphorylation is a method that could have captured mitochondrial function rather than potential oxidative capacity, however, this analysis requires fresh muscle which was not available for our retrospective study [59]. Further, a more accurate method for determining fiber types would have been immunohistochemical staining for myosin heavy chain composition [62]. This method would also have distinguished hybrid 2A/X fibers that can be associated with both fiber type transitions from type 2A to 2X fibers during training and an increase in mitochondrial staining [63]. Unfortunately, this technique is technically more difficult, labor intensive and more expensive than ATPase staining which is therefore commonly used in equine exercise physiology studies [11,12,15,22,33,39,54]. CS activity in our study was expressed per wet weight of tissue. Because connective tissue could have been incorporated within our sample, CS activity could have been underestimated in some samples. The risk of this was likely equal across fit and unfit groups and between breeds, and the inclusion of 14–20 horses per group helped to mitigate this potential complicating factor.

5. Conclusions

In conclusion, gluteal muscle in WB horses has an inherently high muscle oxidative capacity based on CS activity and has a high percentage of type 1 fibers and low % type 2X fibers. The focus on developing suppleness, rhythm, relaxation, connection, impulsion, and collection in dressage training does not result in gluteal muscle CS activity being higher in fit versus unfit horses on the same farms. This contrasts the higher CS activity found in fit versus unfit reining/ranch horses. Similar CS activity in fit WB versus fit QH despite a twofold difference in % type 2X fibers indicates that muscle oxidative capacity cannot accurately be predicted from contractile fiber type composition across breeds using ATPase staining methods.

Authors' contribution

SV: Conceptualization; Data curation; Funding acquisition; Methodology; Writing original draft, review & editing; final approval of manuscript; **HI:** Data curation; Formal analysis; Methodology; final approval of manuscript; **MH:** Formal analysis; Supervision; review & editing; final approval of manuscript; **AS:** Data curation; Writing - review & editing; final approval of manuscript; **EM:** Data acquisition; Writing - review & editing; final approval of manuscript.

Declaration of Competing Interest

None

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