Mitochondrion 24 (2015) 99–104

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Determination of muscle mitochondrial respiratory capacity in Standardbred racehorses as an aid to predicting exertional rhabdomyolysis

Rosa Houben [a], Claire Leleu [b], Audrey Fraipont [a], Didier Serteyn [a,c], Dominique-M. Votion [a,*]

[a] Equine Department, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, Bat B41 & B42, University of Liege, Sart Tilman, 4000 Liège, Belgium
[b] EQUI-TEST, Grez-en-Bouère, France
[c] Center for Oxygen Research and Development (CORD) Institute of Chemistry Bat B6a, Liège University, Sart Tilman, 4000 Liège, Belgium

ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 6 July 2015
Accepted 20 July 2015
Available online 26 July 2015

Keywords:
Horse
Muscle
Mitochondria
High-resolution respirometry
OXPHOS
Exertional rhabdomyolysis

ABSTRACT

This prospective cohort study evaluated the potential of high-resolution respirometry applied to permeabilized muscle fibers for fitness evaluation in French Standardbred racehorses.
Fitness evaluation by means of respirometric parameters did not correlate with racing performance registered over the following racing season. However, altered mitochondrial energy metabolism was associated with higher risk of developing exertional rhabdomyolysis, a common cause of exercise intolerance in racehorses. These data represent a first step towards establishing reference values for muscle OXPHOS capacity in this breed.

1. Introduction

Means to evaluate horses' performance ability, level of fitness and causes of unsatisfactory performance have been the subject of extensive scientific research (Couroucé, 1999; Couroucé et al., 1997; Davie and Evans, 2000; Davie et al., 2002; Kobayashi et al., 1999; Leleu et al., 2005; Lindner, 2010; Martin et al., 2000; McGowan, 2008; Ronéus et al., 1999).

Standardized exercise tests (SETs) as a means of determining a horse's fitness and performance ability have been developed for multiple breeds, tailored to different types of exercise (Davie and Evans, 2000; Davie et al., 2002; Fraipont et al., 2012; Munoz et al., 1997). These SETs, performed either on a treadmill or in the field, may reveal (subclinical) myopathies, as well as evaluate a horse's fitness by determining *via* a regression slope the velocity at onset of blood lactate accumulation, set at blood lactate concentration >4 mmol/l (VLa4). A field SET for French Standardbreds racehorses was previously described and validated, where it was demonstrated that VLa4 correlates with racing performance (Couroucé, 1999; Leleu et al., 2005).

When poor performance is being investigated in sport horses, evaluation of muscle function is usually included and comprises observing locomotion of the horse at work as well as pre- and post-exercise measurement of serum creatine kinase (CK) activity. A common muscle pathology associated with exercise intolerance in racehorses is exertional rhabdomyolysis (ER), which manifests clinically as muscle stiffness, muscle swelling and myoglobinuria occurring during or shortly after exercise. Signs can vary from mild to life-threatening.

A study using SETs to investigate poor performance in 348 predominantly Thoroughbred and Standardbred racehorses (Martin et al., 2000), indicated that less than 3% of all cases showed overt clinical signs consistent with ER but about 15% of horses with exercise intolerance had increased post-exercise serum CK activity without any overt signs. This subclinical myopathy may have been the cause of the poor performance of these horses but the underlying pathologic process remains unknown. A later case–control study investigating performance in 38 underperforming French Standardbred racehorses and 20 healthy controls (Richard et al., 2010), revealed that 24% of poor performers were affected by subclinical myopathy. It should be noted however that in both studies the cause of poor performance was often multi-factorial, with respiratory tract abnormalities being the most prevalent co-pathology.

Abbreviations: BCS, body condition score; CK, creatine kinase; ETS, electron transfer system; ER, exertional rhabdomyolysis; *FCR*, flux control ratio; HRR, high-resolution respirometry; ITR, performance index (for "Index de Trot"); OXPHOS, oxidative phosphorylation; Pfi, permeabilized muscle fibers; *SCR*, substrate control ratio; SET, standardized exercise test; SUIT, substrate–uncoupler–inhibitor titration; VLa4, velocity at onset of blood lactate accumulation.

* Corresponding author at: Equine Department, Faculty of Veterinary Medicine, Boulevard de Colonster 20, Bat B42, Liège University, Sart Tilman, 4000 Liège, Belgium.
E-mail address: dominique.votion@ulg.ac.be (D.-M. Votion).

http://dx.doi.org/10.1016/j.mito.2015.07.006
1567-7249/

In competing Standardbred racehorses, ER is fairly common with an estimated annual incidence of 6% and is considered a significant problem in this breed by a majority of trainers (Isgren et al., 2010). In horses thus far, very few etiogenic pathways for ER have been elucidated. Currently available diagnostic modalities are likewise limited beyond the above mentioned evaluation of muscle enzyme activities, whole-body electrolyte and anti-oxidant status, muscle histopathology and for ER affecting specific breeds such as Quarter horses and related breeds, genetic testing to diagnose type 1 polysaccharide storage myopathy (McCue et al., 2008). Our fundamental understanding of the pathophysiology of inherited and acquired equine myopathies requires improvement.

In horses, muscle response to exercise and/or training has typically been evaluated by the activity of mitochondrial enzymes such as succinate dehydrogenase, by examining the capillary supply of muscles and/or by determining mitochondrial volume density (Kim et al., 2005; Rivero et al., 1995a; Rivero et al., 2001; Rivero et al., 2007). These techniques have contributed to defining athletic potential (Rivero and Henckel, 1996; Rivero et al., 1995b). They have also highlighted the possible contribution of muscle aerobic capacity on determinants of maximal metabolic rate in mammals (Kayar et al., 1989; Weibel et al., 2004).

Recently, reference protocols to study mitochondrial function in muscle microbiopsies by high-resolution respirometry (HRR) have been described and the relationship between mitochondrial function and physical fitness has been documented (Votion et al., 2012). In healthy horses, muscle oxidative phosphorylation (OXPHOS) and electron transfer system (ETS) capacities vary according to fitness level (Votion et al., 2012). To which extent ER and/or exercise intolerance in horses are related to mitochondrial respiratory dysfunctions is poorly known. In a case report (Valberg et al., 1994), exercise intolerance in an Arabian horse has been attributed to abnormally low activity of complex I of the ETS. Since then, the study of mitochondrial function in horses has been limited to the effect of training and racing in endurance horses, where significant alterations in mitochondrial respiratory function induced by racing have been observed (Votion et al., 2010). Standardbred races, although of markedly higher intensity and shorter duration than endurance events, do also depend on aerobic energy production (Hinchcliff and Geor, 2008), and it is currently believed that energy is derived from both aerobic and anaerobic pathways in nearly all types of exercise (Gastin, 2001).

The aims of this study were to evaluate the potential of respirometry for fitness evaluation and early detection of impaired mitochondrial function possibly leading to ER. In addition, this study establishes the first HRR reference values for trained French Standardbred racehorses.

2. Materials and methods

2.1. Animals

French Standardbred racehorses in active racing training, in good general health and orthopedically sound as determined by veterinary examination and without a known history of previous episodes of ER were enrolled in this prospective cohort study. Ethical approval for the muscle tissue sampling procedure was granted by the institutional Animal Ethics Committee (agreement no 07–629) and owners' informed consent was sought for each horse before enrollment in the study.

2.2. Study protocol

Muscle tissue sampling was performed mid-May and was followed by the SETs within 10 days. The Standardbred competitive racing season started early June and was completed end of September.

Variables recorded included horse's sex, age, body condition score (BCS, on a scale of 0–5 where 0 is emaciated and 5 is obese) and *a posteriori*, the “Index de Trot” (ITR) for the following racing season. The ITR is an annual performance index based on the log of earnings over a year, expressed as population mean $= 100 \pm 20$; a score of >120 is considered good performance and <100 indicates poor performance (Leleu et al., 2005).

2.2.1. Muscle tissue sampling

Skeletal muscle microbiopsies were obtained from the *m. triceps brachii* in unsedated horses at a previously described standardized location at the intersection between a vertical line raised from the tricipital line and a line running from the point of the shoulder to the elbow, using a semi-automatic guillotine with a 14 G needle for soft-tissue biopsy and at a sampling depth of 50 mm (Votion et al., 2010; Votion et al., 2012). Samples were immediately placed in ice-cold relaxing solution (BIOPS (Letellier et al., 1992)) containing 10 mM CaK_2-EGTA, 7.23 mM K_2-EGTA, 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 adjusted to pH 7.1. The fibers were stored and transported at 4 °C until further preparation and analyzed within 72 h of sampling. Equine muscle samples can be stored this way for at least four days with good preservation of mitochondrial respiratory function (Votion et al., 2012).

2.2.2. High-resolution mitochondrial respirometry

Muscle microbiopsy samples were dissected using two pairs of forceps with sharp tips. Chemical permeabilization of the plasma membrane was then established by gentle agitation for 30 min at 4 °C in 2 ml of BIOPS solution containing 50 μg/ml saponin. The fiber bundles were washed by agitation for 10 min in ice-cold mitochondrial respiration medium (MiR05; 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose and 1 g/l BSA essentially fatty acid free adjusted to pH 7.1) (Gnaiger et al., 2000). The permeabilized muscle fibers (Pfi) were immediately used for HRR.

Mitochondrial OXPHOS and ETS capacities were determined in Pfi using two substrate-uncoupler–inhibitor titration (SUIT1 and SUIT2; Table 1) protocols extensively described elsewhere (Votion et al., 2012) using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). The protocols were performed at 37 °C with 1.5 mg of permeabilized fibers per respiration chamber. Oxygen limitation of fibers was avoided by maintaining oxygen levels over 200 μM O_2. ADP and substrate concentrations were determined prior to the protocol to ensure no limitation.

The two SUIT protocols differed by the addition or not of pyruvate in order to investigate the contribution of the pyruvate dehydrogenase complex (PDH) to mitochondrial respiration. Datlab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition and analysis and the data were presented as muscle mass-specific oxygen fluxes in pmol $O_2 \cdot S^{-1} \cdot mg^{-1}$ wet weight.

Oxygen fluxes were corrected for oxygen flux due to instrumental background and for residual oxygen consumption (ROX) after inhibition of complex III with antimycin A. The mitochondrial steady-state fluxes considered for statistical analysis are outlined in Table 1. Flux control ratio (*FCR*, with normalization for ETS) and substrate control ratio (*SCR*, flux ratios at constant coupling state) were also calculated within SUITs 1 and 2. In our study, *FCR*'s aim is to provide information about coupling and substrate control independently of mitochondrial content of muscle samples and without the need of mitochondrial markers such as citrate synthase. The calculated *SCR* are *FCR* at a constant mitochondrial coupling state. More detailed information is available elsewhere (Votion et al., 2012).

2.2.3. Standardized exercise test, performance index and follow-up

The SET used in this study has previously been described and validated (Couroucé, 1999; Couroucé et al., 2002). The SET was performed on a racetrack with horses harnessed and driven by their usual driver. In brief, it consists of a 10 minute warm-up at trot at 350 m/min,

Table 1
Substrate–uncoupler–inhibitor titration (SUIT1 and SUIT2) protocols applied on permeabilized muscle fibers (Pfi) taken by microbiopsy in the *triceps brachii*.

Substrates and inhibitors	SUIT 1	SUIT 2
Pfi	± 2 mg	± 2 mg
O_2	500 μM	500 μM
Glutamate	10 mM	---
Malate	2mM	2mM
Pyruvate	---	5 mM
ADP	2.5 mM	2.5 mM
Cytochrome *c*	10 μM	10 μM
Steady-state: CI_P	GM_P	PM_P
Glutamate	---	10 mM
Steady-state: CI_P	---	CI: PGM_P
Succinate	10 mM	10 mM
Steady-state: $CI\&II_P$	GMS_P	$PMGS_P$
FCCP	0.05 + 0.025 mM*	0.05 + 0.025 mM*
Steady-state: $CI\&II_E$	GMS_E	$PMGS_E$
Rotenone	0.5 μM	0.5 μM
Steady-state: CII_E	$S(Rot)_E$	$S(Rot)_E$
Antimycin A	2.5 μM	2.5 μM
Steady-state	ROX	ROX

The oxidative phosphorylation (OXPHOS) and electron transfer system (ETS) capacities per muscle mass were determined by sequential addition of different substrate combinations that furnish electrons upstream of the Q-junction. In SUIT1, electron flow through complex I (CI) necessitates the presence of ADP and was supported by the NADH-linked substrates glutamate&malate (GM) in SUIT1 and pyruvate&malate (PM) in SUIT2 with the subsequent addition of glutamate (G; *i.e.* PGM: pyruvate&glutamate&malate).
At steady-state, ADP stimulated respiration represents OXPHOS capacity ($_P$). By adding cytochrome *c*, it was checked that the integrity of the outer mitochondrial membrane was preserved. Injury of the outer mitochondrial membrane lead to loss of cytochrome *c* from the mitochondria, and would lead to significant stimulation of respiration following addition of exogenous cytochrome *c* to the respiration medium. At the next step, convergent electron flow through complexes I and II (CI&II) is obtained by adding succinate (in the mitochondria, succinate is formed in the TCA cycle and is a substrate of complex II (CII), reacting to fumarate and feeding electrons into the Q-junction through flavin adenine dinucleotide ($FADH_2$)). Thus, in SUIT1, CI&CII sustained respiration was obtained by the combination of glutamate&malate&succinate (GMS) in SUIT1 and by the combination of pyruvate&malate&glutamate&succinate (PMGS) in SUIT2. By stepwise addition of the uncoupler FCCP* (induces the dissipation of the electrochemical proton gradient thus, electron flow is not anymore coupled to OXPHOS), the ETS capacity ($_E$), with convergent electron flow through CI&II was obtained. Electron input into the Q-junction through CII alone was subsequently induced by inhibition of CI by rotenone (the abbreviation S(Rot) means succinate in the presence of rotenone). Finally, residual oxygen consumption (ROX) was obtained by addition of antimycin A to block electron transfer through complex III (CIII).
The mitochondrial steady-state fluxes considered for statistical analysis (after ROX subtraction) are highlighted in gray.
* Stepwise addition of the uncoupler FCCP (0.05 μM, followed by 0.025 μM steps) until maximal oxygen flux was reached.

followed by a 3-stage protocol of increasing speed, at 500, 580 and 660 m/min for 3 min each, with 1 min rest between stages. The horses then performed a fourth stage of 1 min 30 s at maximal speed. The final, fastest stage usually induces lactate accumulation (blood lactate > 4 mmol/l) (Leleu et al., 2005). Blood samples were obtained by jugular venipuncture pre-exercise, within 30 s of finishing each stage and 2 hours post-exercise. Plain tubes were used to collect serum for pre- and post-SET CK activity measurement. Speed and heart rate were continuously recorded using a combined GPS (Forerunner 305, Garmin) and heart rate monitoring device (Polar, RS 800 G3 & Precision Performance SW); heart rate and speed were averaged for each stage, and combined with measured blood lactate concentration for determination of VLa4, using previously described regression models (Leleu et al., 2005). A handheld portable analyzer (Accutrend Lactate; Roche, Mannheim, Germany) validated for horses (Tennent-Brown et al., 2007) was used for immediate determination of blood lactate concentration.

The ITR score for each horse by the end of the season was recorded from public racing records. Occurrence of any adverse events during the racing season was also recorded.

2.3. Statistical analysis

After testing for equal variance, comparisons of means by sex were performed using unpaired *t*-tests. Spearman's rank correlation test was used to test for association between HRR parameters *vs* proxies for fitness and competitive performance VLa4 and ITR respectively. Univariate logistic regression was performed for subsequent occurrence of rhabdomyolysis *vs* HRR parameters. Significance was set at $P < 0.05$. As this was a preliminary hypothesis-generating study, with few observations on a large number of variables, no correction for multiple comparisons was applied. Commercially available statistical software was used for all analyses (STATA® 13.1, StataCorp, College Station, Texas, USA).

3. Results

3.1. Animals

Ten French Standardbred racehorses aged 4–7 years were enrolled in the study: four mares, five geldings and one stallion. All horses presented a normal BCS of 2.5–3. Horses were housed and trained in France at two separate training yards A (n = 5, horses 1–5) and B (n = 5, horses 6–10).

3.2. Standardized exercise test, performance index and follow-up

For all participating horses, VLa4 (mean 635 m/min, range 581–677) and post-exercise serum CK activity (mean 131 IU/l, range 84–221) were within expected limits. There was an effect of sex (female *vs* male) on VLa4 (mean ± SD of 604 ± 23 m/min for female horses, 646 ± 28 m/min for male horses). Performance index ITR scores ranged from 110–124 with a mean score of 118. Again there was an effect of sex on ITR with male horses having a mean of 120 ± 4 *vs* 116 ± 5 for female horses. No ITR score was available for horse 4 which was sold by its owner during the racing season.

3.3. Muscle tissue sampling and mitochondrial respirometry

Usable muscle samples were obtained on each sampling occasion. None of the horses developed complications following the muscle biopsy procedure. Table 2 displays the mean (±SD) mitochondrial

Table 2
Steady-state fluxes (mean ± SD) at different substrates states and coupling states ($_P$ for OXPHOS capacity and $_E$ for electron transfer system capacity) for substrate–uncoupler–inhibitor titration (SUIT1 and SUIT2) protocols ($N = 8$; horses 2 and 6 are not included as they developed exertional rhabdomyolysis during the following racing season; *triceps brachii* muscle).

Steady-state fluxes	SUIT 1	SUIT 2
CI: GM_P	59 ± 13	–
CI: PM_P	–	90 ± 16
CI: PGM_P	–	96 ± 19
$OXPHOS_{Max}$	128 ± 19	145 ± 33
ETS	148 ± 21	195 ± 38
CII: $S(Rot)_E$	104 ± 18	104 ± 27

Mitochondrial steady-state fluxes (pmol $O_2 \cdot S^{-1} \cdot mg^{-1}$ wet weight) after subtracting residual oxygen consumption. Abbreviations: CI: complex I; CII: complex II; GM: glutamate&malate; PM: pyruvate&malate; PGM: pyruvate&glutamate&malate; $OXPHOS_{MAX}$ and ETS, maximal OXPHOS and electron transfer system capacities with glutamate&malate&succinate in SUIT1 and pyruvate&malate&glutamate&succinate in SUIT2, respectively; S(Rot): succinate in the presence of rotenone.

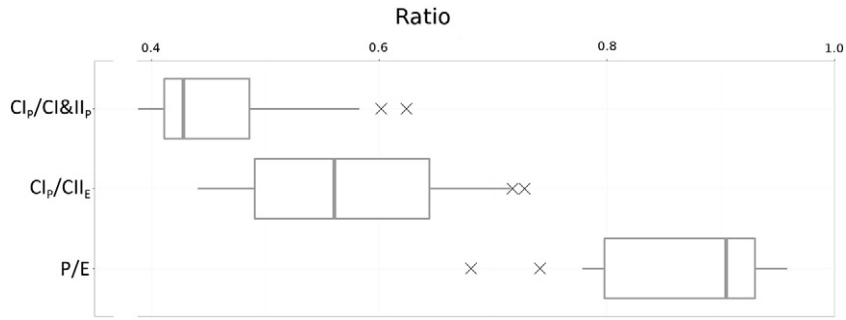

Fig. 1. Boxplot (range, median, quartiles) of flux control ratios (*FCR*) *P/E*, $CI_P/CI\&II_P$ and CI_P/CII_E for unaffected racehorses. Horses which later developed exertional rhabdomyolysis (ER) are added to the graph and indicated by an X. ER-affected animals had higher $CI_P/CI\&II_P$ and CI_P/CII_E and lower *P/E* than unaffected horses.

steady-state fluxes for each step of SUIT1 and SUIT2. This table excludes two horses (horses 2 and 6) who developed clinical signs consistent with rhabdomyolysis during the study; horse 2 had a severe episode one month into the study; horse 6 suffered several episodes and was later withdrawn from training and competition for the remainder of the season.

There was no evidence for an effect of absolute OXPHOS and ETS capacities on the probability of the occurrence of ER ($P = 0.052$ and 0.53, respectively). However, *FCR* and *SCR* in SUIT1 (Table 3a) enabled the discrimination of ER-affected *vs* unaffected horses at sampling time, *i.e.* before the start of the racing season. Univariate logistic regression showed that a cut-off of ≤0.74 for the ratio $[CI\&II_P]/[CI\&II_E]$ also named *P/E* ratio (ER if below the cut-off), a cut-off of >0.58 for $CI_P/CI\&II_P$ (ER if above this cut-off) and a cut-off of >0.72 for CI_P/CII_E (ER if above this cut-off) (Fig. 1) were predictive of ER.

No differences were noted between affected and unaffected horses for the SUIT2 respirometric parameters (Table 3b), indicating no influence by the PDH metabolic pathway on ER occurrence in these two horses.

No statistically significant correlations were found between the HRR variables and either VLa4 or ITR. No statistically significant difference was observed in HRR parameters between male and female horses.

4. Discussion

Results of our study revealed that altered muscle mitochondrial energy metabolism may predispose horses to ER. These alterations in mitochondrial metabolism were detectable in apparently healthy horses in training, before apparition of clinical signs.

This study applied two reference protocols (*i.e.* SUITs 1 and 2) previously validated for horses for the investigation of mitochondrial function in muscle microbiopsies by HRR (Votion et al., 2012) to French Standardbred racehorses. The measured OXPHOS and ETS capacities found in these racehorses were within the range of previously published respirometric parameters in horses (Votion et al., 2010; Votion et al., 2012). This study demonstrated the practical possibility and procedural safety of HRR, performed on muscle biopsies obtained under field conditions in racing horses, without interfering with training or competition (Votion et al., 2010). These data represent a first step towards establishing HRR reference values in this breed but further studies are required to obtain a pool of data enabling studies matched for sex and/or age, both variables known to affect ITR (Cheetham et al., 2010; Physick-Sheard, 1986) and potentially also HRR parameters. Based on key enzymatic activities in biopsies, the oxidative capacity of equine skeletal muscle has been found to decrease with age (Kim et al., 2005; Ronéus et al., 1991) and an age effect on HRR parameters is therefore likely. However, Ronéus at al. (1991) observed no differences in enzyme activities between stallions and mares. Nevertheless, sex has been shown to have a significant impact on ITR in Standardbred racehorses with mares consistently earning less than stallions and geldings these latter having the highest mean money won (Cheetham et al., 2010; Physick-Sheard, 1986). Regardless of species, few studies have examined the effect of sex on muscle oxidative capacity and, to the authors' knowledge, none using HRR. The correlation between HRR parameters and gender deserves additional study.

4.1. Mitochondrial respirometry vs exertional rhabdomyolysis

To qualitatively assess mitochondrial respiration in the *m. triceps brachii* of our cohort of horses, *FCR* and *SCR* ratios were determined. The coupling control ratio *P/E* which expresses the degree of limitation of OXPHOS by the capacity of the phosphorylation system appears to be invariable when measured in horses of widely ranging fitness (Votion et al., 2012). The *P/E* ratios calculated in our study were in the same range as those previously published for trained horses of various breeds (Votion et al., 2012). In contrast, a low *P/E* ratio in SUIT 1 was found in the two ER-affected horses (0.88 ± 0.08 for unaffected horses *vs* 0.68 and 0.74 for horses 2 and 6 that went on to develop ER) indicating a stronger limitation by the phosphorylation system over coupled respiration.

The *SCR* express the relative contribution of different mitochondrial complexes (Gnaiger, 2009). Previous studies performed on endurance

Table 3a
Calculated respiratory parameters from substrate–uncoupler–inhibitor titration protocol 1 (SUIT1) applied on equine permeabilized muscle fibers of *triceps brachii*; mean ± SD among healthy horses (HH); horses 2 (ER 2) and 6 (ER 6) went on to develop exertional rhabdomyolysis.

Horses	*FCR*		*SCR*			
	$CI_P/CI\&II_E$	$CI\&II_P/CI\&II_E$ (*P/E*)	$CII_E/CI\&II_E$	$CI_P/CI\&II_P$	$CII_E/CI\&II_P$	CI_P/CII_E
HH	0.40 ± 0.04	0.88 ± 0.08	0.71 ± 0.09	0.46 ± 0.07	0.81 ± 0.05	0.58 ± 0.10
ER 2	0.41	0.68	0.56	0.60	0.83	0.73
ER 6	0.46	0.74	0.64	0.62	0.87	0.72

Flux control ratios (*FCR*) are ratios of oxygen flux in different respiratory control states. In this study, *FCR* were obtained by dividing OXPHOS capacity (CI_P or $CI\&CII_P$) by electron transfer system (ETS) capacity (*i.e.* $CI\&II_E$). Thus, the oxygen fluxes are normalized for ETS, the maximum flux. *FCR* (ranging from 0 to 1) expresses the respiratory control independently of mitochondrial content of the sample.
Substrate control ratios (*SCR*) are ratios obtained from fluxes at the same coupling sate (assuming $CII_E = CII_P$). *SCR* may be larger or smaller than 1.0 and indicate the relation between the two components of the ratio.

Table 3b
Respiratory parameters from substrate–uncoupler–inhibitor titration protocol 2 (SUIT2) applied on equine permeabilized muscle fibers of *triceps brachii*:flux control ratios (*FCR*, with normalization for $CI\&II_E$) and substrate control ratio (*SCR*, assuming $CII_E = CII_P$) (mean ± SD) among healthy horses (HH); horses 2 (ER 2) and 6 (ER 6) went on to develop exertional rhabdomyolysis.

Horses	*FCR*			*SCR*					
	$PM_P/CI\&II_E$	$PGM_P/CI\&II_E$	$CI\&II_P/CI\&II_E$ (*P*/*E*)	$CII_E/CI\&II_E$	$PM_P/CI\&II_P$	$PGM_P/CI\&II_P$	$CII_E/CI\&II_P$	$PM_P/CI\&II_E$	PM_P/CII_E
HH	0.47 ± 0.05	0.49 ± 0.05	0.74 ± 0.05	0.53 ± 0.05	0.63 ± 0.07	0.67 ± 0.05	0.72 ± 0.06	0.89 ± 0.16	0.94 ± 0.13
ER 2	0.45	0.46	0.74	0.46	0.60	0.62	0.62	0.97	0.73
ER 6	0.58	0.59	0.81	0.55	0.72	0.72	0.68	1.05	0.67

See Table 3a for definition of flux control ratios (*FCR*) and substrate control ratios (*SCR*).

horses showed an increase of OXPHOS and ETS capacities with a significantly greater contribution of complex I sustained respiration with training (Votion et al., 2010) and higher level of fitness (Votion et al., 2012). In our study, an increase OXPHOS capacity with complex I substrates, when expressed over maximal capacity with complexes I & II and with complex II only, was significantly associated with ER. Given the limited number of observations as well as the close proximity of values for ER-affected and healthy horses, the cut-off values in our study should not be over-interpreted. Furthermore, the exact implications of the association between low *P*/*E* combined with increased contribution of NADH-related substrates and ER in our study is currently difficult to stipulate beyond an apparently relative decrease of muscle mitochondrial complex II capacity and/or a shift towards dependence on NADH-related substrates by the mitochondria. So, as opposed to the report found in the literature where profound exercise intolerance in an Arabian horse (*i.e.* a breed made for endurance racing) was associated to complex I deficiency (Valberg et al., 1994), complex I sustained respiration was not severely depleted in our two ER-affected horses and contributed significantly to OXPHOS capacity. These preliminary results highlight the need to study mitochondrial function in horses affected with skeletal muscles disorders. It is likely that a multitude of different underlying pathological processes that can lead to the syndrome of ER in horses exists.

Absolute OXPHOS and ETS capacities had no statistically significant effect on the probability of the occurrence of ER. Horse 2 had respirometric values lower than those of unaffected horses whereas horse 6 had values within the range of the unaffected horses. In ER-affected horses, severe alterations in OXPHOS and ETS capacities in both ER-affected horses might have been expected, however at time of sampling all horses were clinically healthy and in training (for example, horse 6 aged of 7 years old was in training since several years without any history of rhabdomyolysis). The relatively high OXPHOS capacity in these ER-affected horses is perhaps a sign of compensatory mechanism which further met its limitation when clinical signs became overt. These two horses which presented clinical signs of ER during the study were also not necessarily the lesser performers in the cohort, having a performance score ITR of 117 and 115, respectively. This is perhaps not surprising given findings by Isgren et al. (2010) where susceptibility to ER was associated with better racing performance.

4.2. Mitochondrial respirometry vs fitness and performance

As no correlation was found between the measured HRR parameters *vs* fitness and performance indicators VLa4 and ITR, no claims can currently be made as to HRR's utility in fitness assessment or prediction of racing performance in Standardbreds. One factor which may have contributed to the failure to detect any correlations is the limited range of performance ability of the horses in the study: the range of ITR from 110–124 indicates that all horses were moderate to good performers; inclusion of more moderate or bad performers might have increased chances for detection of an effect. Insufficient numbers of animals were included in our study to detect differences between HRR parameters by gender.

4.3. Conclusion

Our results suggest that Standardbreds with a predisposition to ER have altered mitochondrial energy metabolism which may be detectable before overt clinical signs are manifested. Better understanding of causal pathways leading to this syndrome may in the future lead to better, more adapted preventive strategies targeting improvement of mitochondrial function (DiMauro and Schon, 2003).

Acknowledgments

The authors wish to express their sincere gratitude to the owners of the two training yards. This work was funded by the Haras Nationaux (France). The authors also acknowledge Oroboros Instruments (Austria) for their technical support and scientific advice regarding HRR. We gratefully acknowledge the valuable help received from Prof. Helene Lemieux from Campus Saint-Jean, University of Alberta (Canada) in revising this manuscript and Rein Houben for fruitful discussions on statistical analyses.

References

Cheetham, J., Riordan, A.S., Mohammed, H.O., McIlwraith, C.W., Fortier, L.A., 2010. Relationships between race earnings and horse age, sex, gait, track surface and number of race starts for Thoroughbred and Standardbred racehorses in North America. Equine Vet. J. 42, 346–350. http://dx.doi.org/10.1111/j.2042-3306.2010.00032.x.

Couroucé, A., 1999. Field exercise testing for assessing fitness in French Standardbred trotters. Vet. J. 157, 112–122. http://dx.doi.org/10.1053/tvjl.1998.0302.

Couroucé, A., Chatard, J.C., Auvinet, B., 1997. Estimation of performance potential of Standardbred trotters from blood lactate concentrations measured in field conditions. Equine Vet. J. 29, 365–369. http://dx.doi.org/10.1111/j.2042-3306.1997.tb03140.x.

Couroucé, A., Chrétien, M., Valette, J.P., 2002. Physiological variables measured under field conditions according to age and state of training in French trotters. Equine Vet. J. 34, 91–97. http://dx.doi.org/10.2746/042516402776181141.

Davie, A.L., Evans, D.J., 2000. Blood lactate responses to submaximal field exercise tests in Thoroughbred horses. Vet. J. 159, 252–258. http://dx.doi.org/10.1053/tvjl.1999.0420.

Davie, A.J., Priddle, T.L., Evans, D.L., 2002. Metabolic responses to submaximal field exercise tests and relationships with racing performance in pacing Standardbreds. Equine Vet. J. 34, 112–115. http://dx.doi.org/10.1111/j.2042-3306.2002.tb05401.x.

DiMauro, S., Schon, E.A., 2003. Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348, 2656–2668. http://dx.doi.org/10.1056/NEJMra022567.

Fraipont, A., Van Erck, E., Ramery, E., Fortier, G., Lekeux, P., Art, T., 2012. Assessing fitness in endurance horses. Can. Vet. J. 53, 311–314.

Gastin, D.P.B., 2001. Energy system interaction and relative contribution during maximal exercise. Sports Med. 31, 725–741. http://dx.doi.org/10.2165/00007256-200131100-00003.

Gnaiger, E., 2009. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int. J. Biochem. Cell Biol., Mitochondrial Dyn. Funct. Biol. Med. 41, 1837–1845. http://dx.doi.org/10.1016/j.biocel.2009.03.013.

Gnaiger, E., Méndez, G., Hand, S.C., 2000. High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc. Natl. Acad. Sci. U. S. A. 97, 11080–11085.

Hinchcliff, K.W., Geor, R.J., 2008. Chapter 1.1 — the horse as an athlete: a physiological overview. In: Kaneps, K.W.H.J.G.J. (Ed.), Equine Exercise Physiology. W.B. Saunders, Edinburgh, pp. 2–11.

Isgren, C.M., Upjohn, M.M., Fernandez-Fuente, M., Massey, C., Pollott, G., Verheyen, K.L.P., Piercy, R.J., 2010. Epidemiology of exertional rhabdomyolysis susceptibility in Standardbred horses reveals associated risk factors and underlying enhanced performance. PLoS One 5. http://dx.doi.org/10.1371/journal.pone.0011594.

Kayar, S.R., Hoppeler, H., Lindstedt, S.L., Claassen, H., Jones, J.H., Essen-Gustavsson, B., Taylor, C.R., 1989. Total muscle mitochondrial volume in relation to aerobic capacity of horses and steers. Pflugers Arch. — Eur. J. Physiol. 413, 343–347.

Kim, J., Hinchcliff, K.W., Yamaguchi, M., Beard, L.A., Markert, C.D., Devor, S.T., 2005. Exercise training increases oxidative capacity and attenuates exercise-induced ultrastructural damage in skeletal muscle of aged horses. J. Appl. Physiol. 98, 334–342. http://dx.doi.org/10.1152/japplphysiol.00172.2003.

Kobayashi, M., Kuribara, K., Amada, A., 1999. Application of V200 values for evaluation of training effects in the young Thoroughbred under field conditions. Equine Vet. J. 31, 159–162. http://dx.doi.org/10.1111/j.2042-3306.1999.tb05208.x.

Leleu, C., Cotrel, C., Courouce-Malblanc, A., 2005. Relationships between physiological variables and race performance in French Standardbred trotters. Vet. Rec. 156, 339–342. http://dx.doi.org/10.1136/vr.156.11.339.

Letellier, T., Malgat, M., Coquet, M., Moretto, B., Parrot-Roulaud, F., Mazat, J.P., 1992. Mitochondrial myopathy studies on permeabilized muscle fibers. Pediatr. Res. 32, 17–22. http://dx.doi.org/10.1203/00006450-199207000-00004.

Lindner, A.E., 2010. Relationships between racing times of Standardbreds and v4 and v200. J. Anim. Sci. 88, 950–954. http://dx.doi.org/10.2527/jas.2009-2241.

Martin, B.B., Reef, V.B., Parente, E.J., Sage, A.D., 2000. Causes of poor performance of horses during training, racing, or showing: 348 cases (1992–1996). J. Am. Vet. Med. Assoc. 216, 554–558. http://dx.doi.org/10.2460/javma.2000.216.554.

McCue, M.E., Valberg, S.J., Miller, M.B., Wade, C., DiMauro, S., Akman, H.O., Mickelson, J.R., 2008. Glycogen synthase (GYS1) mutation causes a novel skeletal muscle glycogenosis. Genomics 91, 458–466. http://dx.doi.org/10.1016/j.ygeno.2008.01.011.

McGowan, C., 2008. Clinical pathology in the racing horse: the role of clinical pathology in assessing fitness and performance in the racehorse. Vet. Clin. North Am. Equine Pract., Clin. Pathol. 24, 405–421. http://dx.doi.org/10.1016/j.cveq.2008.03.001.

Munoz, A., Santisteban, R., Rubio, M.D., Vivo, R., Aguera, I., Escribano, B.M., Castejon, F.M., 1997. The use of functional indexes to evaluate fitness in Andalusian horses. J. Vet. Med. Sci. 59, 747–752. http://dx.doi.org/10.1292/jvms.59.747.

Physick-Sheard, P.W., 1986. Career profile of the Canadian Standardbred II. Influence of age, gait and sex upon number of races, money won and race times. Can. J. Vet. Res. 50, 457–470.

Richard, E.A., Fortier, G.D., Pitel, P.-H., Dupuis, M.-C., Valette, J.-P., Art, T., Denoix, J.-M., Lekeux, P.M., Erck, E.V., 2010. Sub-clinical diseases affecting performance in Standardbred trotters: diagnostic methods and predictive parameters. Vet. J. 184, 282–289. http://dx.doi.org/10.1016/j.tvjl.2009.04.016.

Rivero, J.L., Henckel, P., 1996. Muscle biopsy index for discriminating between endurance horses with different performance records. Res. Vet. Sci. 61, 49–54.

Rivero, J.L., Ruz, M.C., Serrano, A.L., Diz, A.M., 1995a. Effects of a 3 month endurance training programme on skeletal muscle histochemistry in Andalusian, Arabian and Anglo-Arabian horses. Equine Vet. J. 27, 51–59.

Rivero, J.L., Serrano, A.L., Henckel, P., 1995b. Activities of selected aerobic and anaerobic enzymes in the gluteus medius muscle of endurance horses with different performance records. Vet. Rec. 137, 187–192.

Rivero, J.L., Serrano, A.L., Quiroz-Rothe, E., Aguilera-Tejero, E., 2001. Coordinated changes of kinematics and muscle fibre properties with prolonged endurance training. Equine Vet. J. Suppl. 104–108.

Rivero, J.-L.L., Ruz, A., Martí-Korff, S., Estepa, J.-C., Aguilera-Tejero, E., Werkman, J., Sobotta, M., Lindner, A., 2007. Effects of intensity and duration of exercise on muscular responses to training of thoroughbred racehorses. J. Appl. Physiol. 102, 1871–1882. http://dx.doi.org/10.1152/japplphysiol.01093.2006.

Ronéus, M., Lindholm, A., Asheim, A., 1991. Muscle characteristics in Thoroughbreds of different ages and sexes. Equine Vet. J. 23, 207–2010.

Ronéus, N., Essén-Gustavsson, B., Lindholm, A., Persson, S., 1999. Muscle characteristics and plasma lactate and ammonia response after racing in Standardbred trotters: relation to performance. Equine Vet. J. 31, 170–173. http://dx.doi.org/10.1111/j.2042-3306.1999.tb03811.x.

Tennent-Brown, B.S., Wilkins, P.A., Lindborg, S., Russell, G., Boston, R.C., 2007. Assessment of a point-of-care lactate monitor in emergency admissions of adult horses to a referral hospital. J. Vet. Intern. Med. Am. Coll. Vet. Intern. Med. 21, 1090–1098.

Valberg, S.J., Carlson, G.P., Cardinet, G.H., Birks, E.K., Jones, J.H., Chomyn, A., DiMauro, S., 1994. Skeletal muscle mitochondrial myopathy as a cause of exercise intolerance in a horse. Muscle Nerve 17, 305–312.

Votion, D.-M., Fraipont, A., Goachet, A.G., Robert, C., van Erck, E., Amory, H., Ceusters, J., de la Rebière de Pouyade, G., Franck, T., Mouithys-Mickalad, A., Niesten, A., Serteyn, D., 2010. Alterations in mitochondrial respiratory function in response to endurance training and endurance racing. Equine Vet. J. Suppl. 268–274. http://dx.doi.org/10.1111/j.2042-3306.2010.00271.x.

Votion, D.-M., Gnaiger, E., Lemieux, H., Mouithys-Mickalad, A., Serteyn, D., 2012. Physical fitness and mitochondrial respiratory capacity in horse skeletal muscle. PLoS One 7, e34890. http://dx.doi.org/10.1371/journal.pone.0034890.

Weibel, E.R., Bacigalupe, L.D., Schmitt, B., Hoppeler, H., 2004. Allometric scaling of maximal metabolic rate in mammals: muscle aerobic capacity as determinant factor. Respir. Physiol. Neurobiol. 140, 115–132. http://dx.doi.org/10.1016/j.resp.2004.01.006.