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Relating Maximal Oxygen Consumption to Skeletal Muscle Mitochondria in Horses

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Summary

Generally, large animals have smaller capacities for weight-specific resting and maximal oxidative metabolism than small animals, though horses represent a remarkable exception to that rule. Despite a body mass of several hundred kilograms, their weight-specific maximal oxygen consumption ($\dot{V}O_2max/Mb$) is similar to that predicted for a 100 g animal. To address the question as to how horses achieve these extremely high rates of oxygen consumption, $\dot{V}O_2max$ was determined in 4 Standardbred horses using an open flow system. The horses were trained to run on an inclined treadmill and reached $\dot{V}O_2max$ (130 ± 5.8 ml O_2/kg/min) while trotting. After the physiological experiments the horses were sacrificed and skeletal muscle tissue samples were obtained for ultrastructural morphometry. The entire musculature of the horses was sampled according to a statistical procedure which yields representative results for the volume density of mitochondria, $V_V(mt,f)$, of the whole body skeletal muscle tissue. $V_V(mt,f)$ for the whole musculature of the four horses analyzed was 7.10 ± 0.36%. Assuming that at $\dot{V}O_2max$ all the oxygen is consumed by skeletal muscle tissue, a cm^3 of skeletal muscle mitochondria consumed on average 4.75 ± 0.36 ml O_2/min. This is not significantly different from the value of average whole body mitochondrial oxygen consumption of the European woodmouse exercising at $\dot{V}O_2max$ (4.9 ml O_2/min/cm^3; body mass 20 g). These data suggest that per unit volume, mitochondria consume oxygen at fixed and constant rates and that the high aerobic capacity of horses is thus commensurate with the high mitochondrial content of their musculature.

Index terms: Morphometry, exercise.

Introduction

Horses are considered to be among the elite athletes of the animal kingdom (Snow, 1985), capable of sustaining speeds of 47 km/h for over 9 minutes during a steeplechase (RED-RUM, Grand National, Aintree, 1973). Under these extreme exercise conditions the contracting muscles are fueled by aerobic and anaerobic metabolic processes imposing extraordinary demands on skeletal muscle tissue as well as on the cardiovascular system. The horse's ability to deliver high mechanical power outputs over prolonged periods of time is related to its high weight-specific maximal oxygen uptake capacity ($\dot{V}O_2max/Mb$), which is reported to be in excess of 120 ml O_2/min/kg in ponies (Parks and Manohar, 1983; Manohar, 1986) and possibly higher in Thoroughbred horses. $\dot{V}O_2max$ is thus much higher in equines than in the very best of human endurance athletes (≈80 ml O_2/min/kg; c.f. Saltin and Gollnick, 1983).

The exceptional aerobic capacity of horses is even more surprising in light of the general rule that $\dot{V}O_2max/Mb$ decreases with increasing body size (Taylor *et al.*, 1981). Solving equations given by Taylor *et al.* (1981) an animal with a $\dot{V}O_2max/Mb$ of 120 ml O_2/kg/min should have a body mass of 100 g. Thus, for their size, horses are unusually effective at moving oxygen from environmental air to skeletal muscle mitochondria, that generate all aerobically derived ATP for the contractile machinery.

To study the interdependence of whole body oxygen consumption and the respiration of skeletal muscle mitochondria in horses under these conditions we have related $\dot{V}O_2max$ in 4 Standardbred horses to the mitochondrial volume determined morphometrically in their entire musculature. The latter determination was achieved by using a statistical sampling scheme previously established (Hoppeler *et al.*, 1984a). This approach allowed us to calculate unbiased estimates of mitochondrial volumes for a number of body compartments such as hind- and forelimbs, trunk, head and neck.

Materials and Methods

Four Standardbred horses were used in this study (one female, two stallions, one gelding). The age of the animals was 7.5 ± 2.3 (SEM) years. Three of the horses had been trained systematically for several years (by their previous owners) for trotting, while one of the stallions had never been formally trained. The three trotters could be purchased for the current experiment because of their limited success in racing, although one of them had occasionally won races on a local level. The body mass of the animals ranged from 397–516 kg; mean 461 ± 29 (SEM) kg.

Oxygen consumption. Oxygen consumption was measured with an open flow mask system as described by Fedak *et al.* (1981). One month prior to the beginning of experiments the animals were familiarized with the treadmill on which they would run and with the masks and probes they would wear during an experiment. Once familiarized with the procedures the animals began a training regime during which oxygen consumption ($\dot{V}O_2$) was measured. Each $\dot{V}O_2$ run lasted 6 minutes and running speed was adjusted so that the animals would reach, but not exceed, $\dot{V}O_2max$. Animals were rested at least 1 day after each run at or near $\dot{V}O_2max$. Animals were considered trained, when plateauing of oxygen consumption occurred reproducibly at a speed characteristic for each animal. The treadmill was inclined by 6%; all horses reached $\dot{V}O_2max$ while trotting at a speed of 8.1 ± 0.4 m/sec. Two blood samples for lactate determination were taken during each run; the first sample 3 minutes into the run when the animal

had reached a quasi steady-state oxygen consumption, the second sample at the end of each run. Calculations of lactate accumulation rates were based on these two samples. $\dot{V}O_2max$ was defined according to the following criteria:

1. Further increase in treadmill speed produced no increase in $\dot{V}O_2$.
2. Any additional energy required by the animal to run at a higher treadmill speed could be accounted for by anaerobic metabolism, i.e. rate of lactate accumulation in the blood.

For the final experiment each animal was deeply anaesthetized with Myolaxin® (150 mg/kg) and Na-Pentothal (10–20 mg/kg). The animal was then hoisted onto a dissection table and secured in a supine position. A tracheostomy was performed and the animal was intubated. A laparotomy was performed, the animal was curarized with tubocurarine (50–100 mg/kg) to prevent muscle seizure during lung instillation and a pneumothorax was induced. The lungs were allowed to totally collapse and the animal was killed by instillation of 2.5% glutaraldehyde into the lungs (results of lung analysis not reported here). All muscle samples for morphometry were taken within one hour after the death of the animal. Animals were totally dissected to determine the muscle mass of individual muscles as well as the masses of the individual strata as described below. The mass of the heart was not added to the total muscle weight. The dissection took 3–5 hours per animal.

Tissue fixation and sampling. Individual muscles were completely removed and weighed. Six tissue specimens were taken from each muscle in such a way that the subsequent morphometric analysis would yield results representative for the whole muscle. Transmural samples of the left ventricle and samples of the muscular flat part of the diaphragm were also removed and immersion fixed. Thin longitudinal muscle strips (1×1×5 mm) were fixed in a 6.25% solution of glutaraldehyde in 0.1 M Na-cacodylate buffer adjusted to 430 mOsm with NaCl (total osmolarity of the fixative: 1100 mOsm, pH 7.4). The muscle specimens were further processed for electron microscopy as previously described in detail (Hoppeler *et al.*, 1981). All tissue specimens were stored in a refrigerator at ≈5°C in their respective fixative solutions for 1 to 90 days before further processing (Hoppeler *et al.*, 1981).

Random sampling. Random muscle samples from 15 locations on the carcass of each animal were taken according to a systematic and volume-weighted random sampling strategy previously described (Hoppeler *et al.*, 1984a; Zeltner *et al.*, 1986) and illustrated in Fig. 1. The animal's carcass was divided into 7 strata which could be delineated reproducibly on each animal (Fig. 1A). In a first step the number of samples to be selected from each stratum was determined as a function of the proportional mass of each individual stratum. As shown in Fig. 1A, the mass of the entire skeletal musculature was represented by a column subdivided into segments proportional in length to the mass of each single stratum. An additional scale was partitioned into 15 segments of equal length. The sampling position within each of these segments was determined by a random number, as shown in Fig. 1A. The overlap of the sampling position in the segment with the column representing the mass scale, indicated from which "STRATUM" a particular sample was to be taken. In a second step, a one-digit random number determined the "DISTANCE" (proximal to distal) at which a sample had to be taken in a particular stratum. As in step one, random numbers were assigned in proportion to muscle mass. As illustrated in Fig. 1B, in both fore- and hindlimbs, approximately 90% of the muscle mass was located on the proximal limb, i.e. above elbow and stifle.

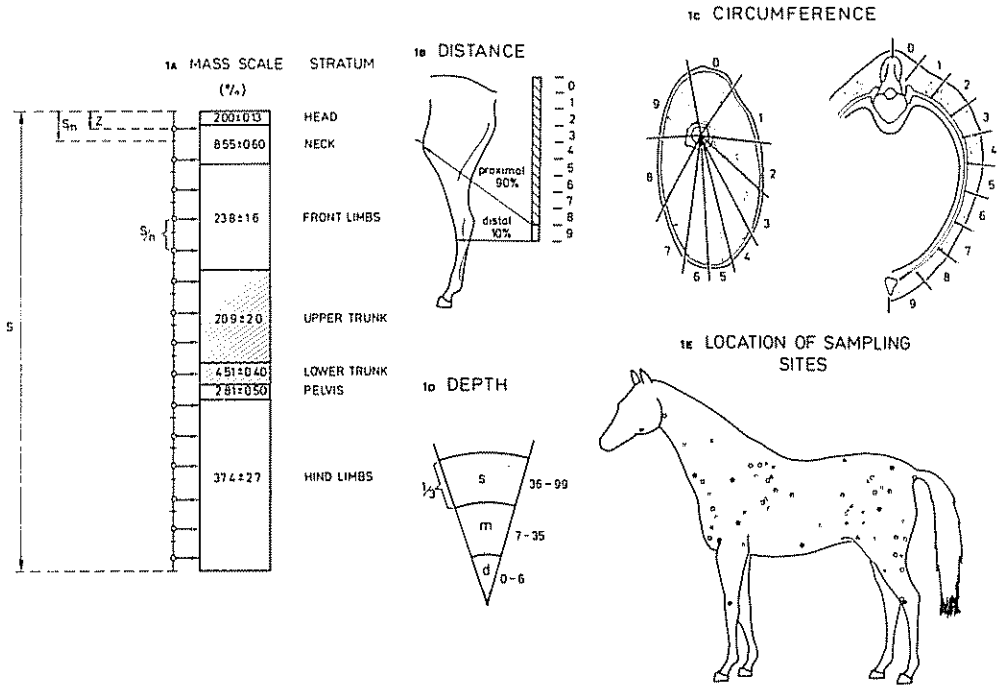

FIGURE 1. Schematic drawing illustrating the systematic volume weighted random sampling of the skeletal musculature as described in the methods section. (1A) The column represents the musculature subdivided into segments proportional to single stratum volume. A parallel scale of length (s) is partitioned into as many segments of length [s/n] as tissue specimens (n) should be selected (for the present experiment n = 15). The precise position within each segment is determined by a random number (z) chosen between zero and [s/n]. The overlap between these positions and the mass-scale determined from which "STRATUM" a sample had to be taken. (1 B,C,D) Illustrates how individual strata were subsampled for "DISTANCE," "CIRCUMFERENCE" and "DEPTH." (1E) Approximate location of sampling sites (symbols represent sampling sites in individual horses)

In a third step, a one-digit random number determined the location on the "CIRCUMFERENCE" of a stratum at which a sample was located (Fig. 1C). Finally, in a fourth step, a two-digit random number determined the "DEPTH" (superficial, middle or deep) within the stratum from which a sample had to be taken (Fig. 1D). The set of four random numbers for each sample was drawn before the final experiment started. In Fig. 1E the approximate locations of the 60 sampling sites in the four horses are shown.

Morphometry. For the stereological analysis we cut 4 randomly chosen blocks for the individual muscle analysis except for the diaphragm and the heart, where only 2 blocks were cut and analyzed. Morphometry of muscle fiber ultrastructure was carried out at a final magnification of ×24000 (Fig. 2), by analyzing 40 micrographs obtained by systematically sampling the tissue sections. The basic morphometric data were obtained by standard stereological procedures (Weibel 1979) previously described in detail

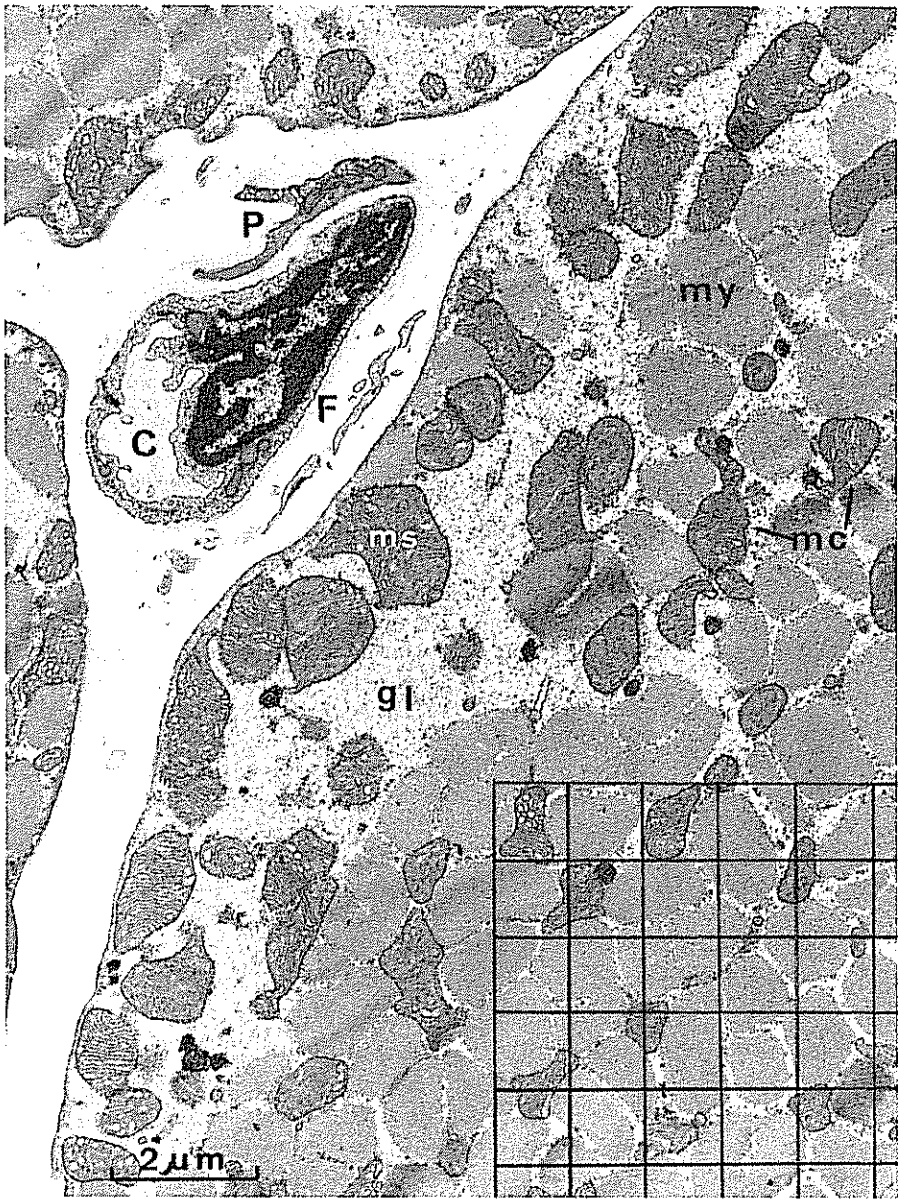

FIGURE 2. Cross-section of portions of adjacent muscle fibers of the semitendinosus muscle of a horse. A part of a morphometric test grid is superimposed (C = capillary; F = fibrocyte; P = pericyte; my = myofibrils; ms = subsarcolemmal mitochondria; mc = intermyofibrillar mitochondria; gl = glycogen)

(Hoppeler *et al.*, 1981). The absolute volume of mitochondria for each muscle, V(mt), was obtained as follows (Mathieu *et al.*, 1981):

$$V(mt) = V_V(mt,f)/Mm/\delta; \quad \text{units, ml;} \qquad (1)$$

where $V_V(mt,f)$ is the volume density of total mitochondria (= volume of mitochondria per volume of muscle fiber; unitless), Mm is the muscle mass (units, g) and δ represents the density of muscle tissue (constant, 1.06 g/ml; Mendéz and Keys 1960).

Results

Muscle volume. Skeletal muscle tissue represented between 37.4 and 43.2% of the body mass in the 4 horses analyzed. The three horses that had been trained prior to this experiment had muscle masses in excess of 42% of their body mass. More than 60% of the total muscle mass was found on the animals' limbs, i.e. on muscles which either completely or partially attached to parts of the appendicular skeleton. The trunk carried some 25% of the total skeletal muscle mass. The contribution to total muscle mass of individual muscles such as the semitendinosus or the gastrocnemius was rather small (3.0 and 1.5%, respectively). The heart and the diaphragm represented 1.8 and 1.6% of the skeletal muscle mass, respectively (heart weight was not added in determining total muscle weight). The weight of the cutaneus thoracicus muscle (responsible for twitching the skin) could not be determined because of technical difficulties in separating the muscle from the subcutaneous fat tissue.

Mitochondrial volume. The average volume density of mitochondria for the skeletal musculature of the horses was found to be 7.10 ± 0.36% (range, 6.03–8.50), individual strata showing values close to the mean of the whole animal (Table 1, Fig. 3). The volume density of mitochondria in individual samples ranged from 1.07 to 14.1%. The relative distribution of total mitochondrial volumes among the various strata (Table 1, Fig. 4) was close to that observed for the distribution of skeletal muscle mass among

TABLE 1. Quantitative structural data for muscle strata (4 animals) and individual muscles (1 animal; means ± SEM; n = number of samples analyzed)

	Muscle volume V_m (cm^3)	Volume density of mitochondria $V_V(mt,f)$ (%)	Volume of mitochondria $V(mt) = V_m \times V_V(mt,f)$ (cm^3)	Relative muscle volume (%)	Relative mitochondrial volume (%)
Whole body (n = 60)	180.000 ± 11.000	7.10 ± 0.36	12.800 ± 1.000	100.0	100.0
Limbs (n = 32)	110.000 ± 7.600	7.34 ± 0.51	8.070 ± 800	61.2	62.4
Trunk (n = 16)	45.700 ± 3.000	7.22 ± 0.64	3.300 ± 360	25.5	25.5
Pelvis (n = 4)	5.100 ± 1.800	6.38 ± 2.1	325 ± 150	2.8	2.5
Neck (n = 7)	15.400 ± 1.100	5.73 ± 0.56	882 ± 110	8.5	6.8
Head (n = 1)	3.600 ± 240	10.1	364	2.0	2.8
Heart	3.300 ± 130	26.5 ± 1.3	874 ± 51	1.83	6.84
Diaphragm	2.940 ± 140	10.5 ± 1.4	304 ± 42	1.61	2.38
M. gastrocnemius	2.700 ± 230	8.11 ± 0.97	219 ± 32	1.50	1.72
M. semitendinosus	5.490 ± 430	6.32 ± 0.57	347 ± 41	3.05	2.71
M. cutaneus thoracicus	—	4.77 ± 1.2	—	—	—

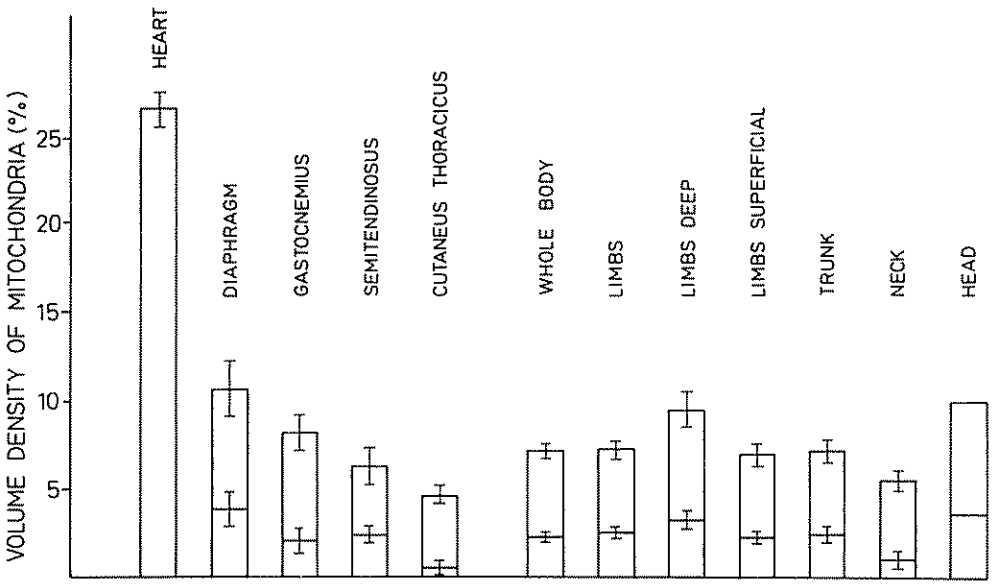

FIGURE 3 Histogram of the volume density of mitochondria in individual muscles as well as in muscle strata in the musculature of horses (the lower subdivisions of the bars represent the fraction of subsarcolemmal mitochondria; mean ± SEM).

the strata (Fig. 1A). When values for individual muscles are compared to total mitochondrial volume, it is noteworthy that due to its high volume density of mitochondria the heart contains a relatively large amount of mitochondria, while the absolute mitochondrial volume in individual skeletal muscles is quite small.

Maximal oxygen consumption. $\dot{V}O_2max/Mb$ ranged from 115 to 141 ml O_2/min/kg (mean, 130 ± 5.8). The lowest value was recorded for the horse which had not been trained before our study. At the end of the 6-minute $\dot{V}O_2max$ run, arterial lactate concentration reached 9.48 ± 0.89 mmol/l and the lactate accumulation rate was 1.1 ± 0.1 mmol/l/min.

Assuming that (1) at $\dot{V}O_2max$ all the oxygen taken up in the lungs is used by the working skeletal muscle tissue and (2) skeletal muscles consist entirely of muscle fibers, then the maximal in vivo oxygen consumption of horse skeletal muscle mitochondria can be calculated to be 4.75 ± 0.36 ml O_2/min/cm^3 (range 4.0–5.4).

Discussion

Maximal oxygen consumption. The present experiments confirm the exceptional oxygen uptake capacity of horses (Parks and Manohar, 1983; Manohar, 1986). One horse that had been trained for trotting for several years and had moderate success in local competition reached a $\dot{V}O_2max/Mb$ of 141 ml O_2/min/kg. In contrast, the one horse which had not previously been trained consumed only 115 ml O_2/min/kg. Our study offers no clues as to whether this rather large difference between these two horses was due to a difference in training, genetic background or both (c.f., Essen-Gustavsson *et al.*, 1983).

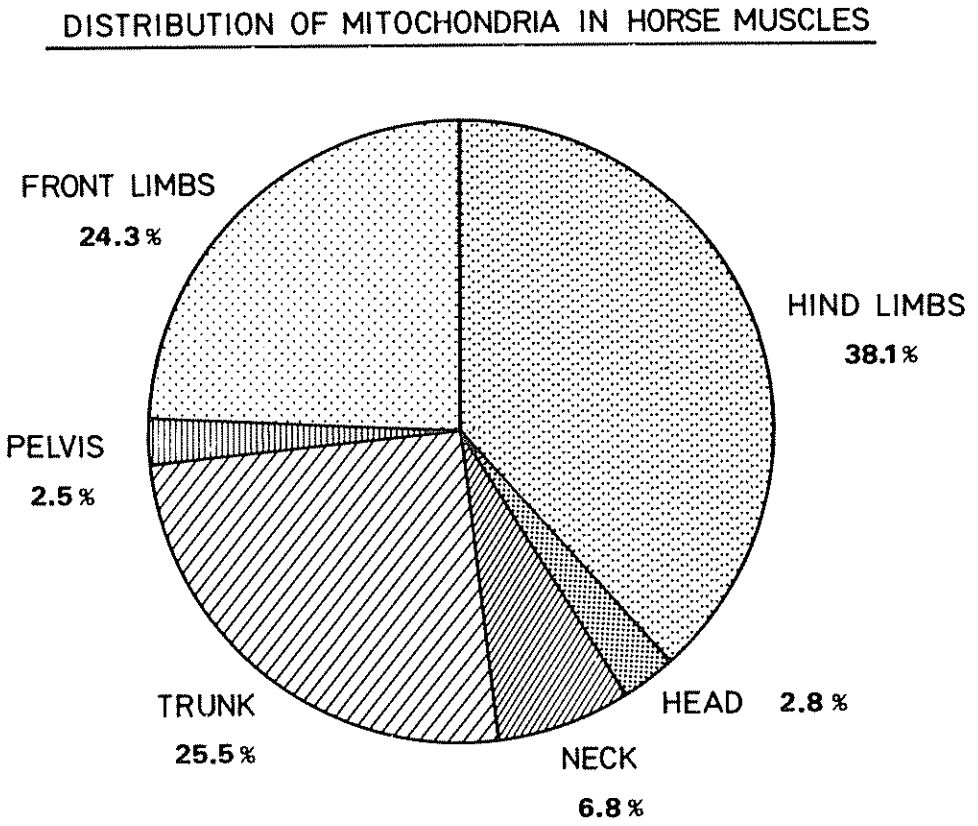

FIGURE 4. Contribution of individual strata to the total volume of mitochondria in the equine musculature.

All horses reached VO_2max with high plasma lactate concentrations at the end of VO_2max runs. The lactate accumulation rate was stoichiometrically equivalent to 5% of their power output, indicating that the animals could not likely have run aerobically at higher treadmill speeds.

Compartmental distribution of muscle mass and mitochondria. As indicated in Fig. 1A, more than 60% of a horse's muscle mass is found on the hind- and forelimbs. More than half of the remaining 40% is trunk muscles, the rest being distributed among the smaller compartments of head, neck and pelvis. Mitochondrial volume densities were not significantly different among the three compartments of limb, trunk and whole body musculature (Table 1). A significantly smaller volume density of mitochondria was found in the neck muscles. From the head only one sample was obtained. That particular sample came from the masseter muscle.

The volume density of mitochondria is the morphologic descriptor of skeletal muscle tissue's capacity to generate ATP aerobically (Pennicuick and Rezende, 1984; Hoppeler and Lindstedt, 1985) and is thus intimately linked to respiration (Kadenbach, 1986). The observation that mitochondrial volume density is very similar in equine limb and trunk muscles might indicate that these compartments work during locomotion at similar

rates. This is compatible with the contention that running quadrupeds (rats) have to engage some 70–80% of their muscle mass to reach $\dot{V}O_2$max (Mackie and Terjung, 1983), as compared to only 30% in humans (Saltin, 1986).

Our data suggest that more mitochondria are located in the deep than in the middle and superficial portions of the limb musculature (Fig. 3). This trend was anticipated from known blood flow and fiber type distributions within and among limb muscles (Laughlin and Armstrong, 1982). As previously noted for the European woodmouse (Hoppeler *et al.*, 1984a), the average volume density of mitochondria in the limbs is representative for this variable for the whole body musculature in horses as well (Table 1). However, while in woodmice the combined mass of all limb muscles represented some 40% of the total muscle mass, in horses it was over 60%. Moreover, in horses the major fraction of the muscle mass is concentrated on the proximal limbs, thus reducing considerably the problem of controlling inertial forces during high-speed locomotion.

Individual muscles. In addition to the whole body sampling, in one of the three trotters we examined a number of individual muscles (Table 1). As expected, the two locomotor limb muscles analyzed (M. gastrocnemius and M. semitendinosus) showed mitochondrial volume densities close to that observed for the whole limb. In a previous study we reported the volume density of mitochondria in equine semitendinosus to be 4.8 ± 0.45% (Straub *et al.*, 1983). This was for a group of 6 halfbred stallions after 4 weeks of a moderate exercise protocol. The higher mitochondrial density found in the present study seems to be related to the specific training history of the animals analyzed. This higher value seems to be closer to values found in populations of well trained horses.

M. cutaneous trunci, which serves no locomotory function, was found to contain less than 5% mitochondria. This is similar to the volume density of mitochondria in neck muscles which likewise do not produce power for propulsion.

Expectedly high mitochondrial densities were found in those two muscles which are continuously active throughout the lifetime of an animal, the heart (26.5%) and the diaphragm (10.5%). We have previously reported the volume density of mitochondria in an equine heart to be 25.9% (Hoppeler *et al.*, 1984b). Despite the fact that the heart mass represented less than 2% of the skeletal muscle mass (0.76 ± 0.03% of the total body mass) it contained more than 6% of the striated muscles' mitochondria; 2.5% of all skeletal muscle mitochondria were contained in the diaphragm.

Muscle mitochondria related to $\dot{V}O_2$max. The sampling design used in this study allowed us to obtain a morphometric estimate of the whole body skeletal muscle oxidative capacity. This enabled us to relate the absolute quantity of skeletal muscle mitochondria to the maximal global oxygen consumption capacity. Dividing $\dot{V}O_2$max by whole body mitochondrial volume, we found that 1 cm^3 of muscle mitochondria at $\dot{V}O_2$max consumed 4.75 ± 0.36 ml O_2/min. This is well within the range of values reported for mitochondrial oxygen consumption during maximal voluntary exercise in humans, woodmice, birds and a number of African mammals (c.f. Hoppeler and Lindstedt, 1985). What are the assumptions and sources of error in those assumptions that are necessary to make these calculations?

1. At $\dot{V}O_2$max the skeletal musculature consumes all the oxygen uptake. This is obviously not correct. However, more than 90% of the total oxygen flux at $\dot{V}O_2$max is

directed toward the active skeletal muscle mass (Shephard, 1969; Weibel 1984). If heart mitochondria consume oxygen at the same rate as skeletal muscle mitochondria, then, based on their volume, they should be responsible for about 6% of the total oxygen consumption at $\dot{V}O_2max$, the remaining 4% being consumed by all other organs.

2. We have assumed the muscle volume to consist entirely of muscle fibers, neglecting the interstitial space. The interstitial space cannot be correctly assessed on immersion fixed muscle tissue as muscle fibers tend to segregate during tissue processing (Fig. 2). Olivetti *et al.*, (1980) reported the interstitial space of optimally perfusion-fixed rat hearts to be 15% of the total muscle volume, half the space being taken up by capillaries the remainder by connective tissue. Since rat heart muscle has much smaller muscle fibers and a much higher capillary density than horse skeletal muscles, it can safely be assumed that in the latter the interstitial space accounts for less than 10% of the total muscle volume. Further, this error, like that resulting from assumption 1 is not size-dependent, thus it could only add a constant error not affecting the constancy between mice and horses. These two assumptions introduce an error on the order of 10%, but in opposite directions. Thus, in all likelihood, these errors may cancel each other.

3. It must be considered that even under conditions of $\dot{V}O_2max$ the entire skeletal musculature is not actively contracting. It has been estimated that only 30–40% of the limb musculature of rats is needed for force generation at exercise intensities eliciting $\dot{V}O_2max$ (Armstrong and Taylor, 1982). These running intensities are much below those that can be reached during maximal, short-term, anaerobic work. However, mitochondria are preferentially located in the early recruited, oxidative type I and type IIa muscle fibers (Hoppeler *et al.*, 1983). It is thus conceivable that a large fraction of the total mitochondrial pool is recruited during locomotion at speeds eliciting $\dot{V}O_2max$. Furthermore, it must be considered that mitochondria in mechanically non-active muscle fibers might process significant amounts of lactate, produced in mechanically active fibers, thereby consuming oxygen (Brooks, 1985).

In conclusion, the skeletal musculature of horses is built according to the same principles and with the same structural and functional building blocks as the musculature of other mammals. The high $\dot{V}O_2max/Mb$ of horses is commensurate with the high volume density of mitochondria in their locomotory muscles. The large oxygen flow rates are supported by a heart which weighs close to 1% of the total body mass, in contrast to less athletic "average" mammals having smaller hearts (Saltin, 1986). The present analysis indicates there is nothing "exceptional" about the horse's aerobic performance capacity other than that this animal may be built to use all its systems involved in aerobic energy supply with minimal redundancy.

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Metabolic Response to Racing Determined in Pools of Type I, II A and II B Fibers

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Summary

Histochemical and biochemical analysis of fiber pools of types I, II A and II B were performed on gluteus medius biopsies obtained, upon standstill after racing, from four Standardbred and four Thoroughbred horses. Within individuals, type I fibers possessed a lower lactate dehydrogenase (LDH) activity and a higher phosphagen level than type II fibers. The citrate synthase (CS) activity of type II B fibers was lower than that of type I and II A fibers but varied in individuals from being much lower than that in type II A fibers to almost similar levels. The lactate concentrations were 2- to 5-fold higher than reported resting concentrations and lactate levels in the three fiber pools of the same horse did not differ markedly. However, lactate, phosphagen and enzyme activities in fiber pools differed markedly between horses. The differences in lactate concentrations between horses for all fiber pools could be explained by the negative correlations between pool lactate levels and both the fiber pool oxidative capacity and the fiber type's capillarization. High lactate levels in fiber pools post race were associated with low ATP levels, particularly in type II B. It is concluded that in both Standardbred and Thoroughbred horses the oxidative capacity and capillarization of fiber types strongly influences the metabolic response to racing. Furthermore, the total number of the various fiber types that are available and subsequently recruited in muscle will influence the metabolic response to exercise. Therefore, with respect to the potential for high lactate and low adenosine triphosphate levels in type II B fibers, the metabolic profile of type II B fibers is especially important during racing when these fibers become recruited.

Index terms: Muscle; horse; fiber types; enzymes; lactate; adenosine triphosphate; creatine phosphate.

Introduction

Skeletal muscle is composed of fibers which possess different contractile and metabolic properties (Saltin and Gollnick, 1983). Functionally, these different fiber types, slow-twitch type I and fast-twitch type II A and II B, are selectively recruited in a specific pattern which varies according to the gait, speed and duration of exercise (Sullivan and Armstrong, 1978). In the horse, as the speed of exercise increases, fibers are recruited in the order, type I through II A to II B (Hodgson *et al.*, 1983; Valberg, 1986).