INTRODUCTION

Many animals (and humans) are likely to experience a certain degree of hypoxia during their lifespan. This may occur either by exposure to ambient hypoxic situations or through metabolic, respiratory or circulatory inadequacy.

Chronic ambient hypoxia (living at altitude)

At sea level, oxygen constitutes 21% atmospheric gas content whereas at altitudes of ≥ 4000 m it may comprise less than 11% total gases. High altitude thus reduces the amount of atmospheric oxygen available for delivery to cells of the body and presents an environment which, associated with reduced atmospheric pressure, is known as hypobaric hypoxia. In response to chronic hypoxia, body systems are known to adapt physiologically and biochemically. The adaptive strategies of indigenous highland populations, such as Andean and Himalayan natives, (Desplanches et al. 1996; Hochachka, 1992; Holden et al. 1995) and of highland animal species such as llamas, alpacas (Hochachka et al. 1983), pikas (Sheafor, 2003) and Andean coots (Leon-Velarde et al. 1993) have been the focus of several investigations.

Transient ambient hypoxia

While high altitude inhabitants are chronically exposed to an environment of oxygen depletion, ambient hypoxia may also be of a more transient or temporary nature. Numerous hibernating (MacDonald & Storey, 2005) and neonatal (Kanaan et al. 2005) mammals tolerate periods of oxygen depletion. Temporary hypoxia occurs also during diving activities of several species of air-breathing marine mammals. Seals, for example, successfully function for the duration of a dive under conditions of progressive asphyxia which is a combination of increasing hypoxia, hypercapnia and acidosis (Kanatous et al. 1999, 2002).

Fish such as the carp, and amphibians such as freshwater turtles and common frogs are known to survive long periods of oxygen deprivation which would prove fatal to mammals. These animals achieve this by manipulating their metabolism to one of a depressed (i.e. hypometabolic) steady state where ATP supply and ATP demand pathways are balanced (Boutilier, 2001). Such animals are regarded as oxyconformers (energy demands decrease as

oxygen availability decreases) while, on the other hand, oxyregulators maintain a high rate of energy consumption in response to hypoxia (Boutilier, 2001).

Local tissue hypoxia

Respiratory or circulatory inadequacy can produce local hypoxia within body tissues. Interrupted oxygen delivery to cells may occur due to surgery or trauma, but may also develop gradually as pathologies such as chronic pulmonary hypertension (Agusti, 2005) or ischaemic heart disease (Weir et al. 2005) progress.

The consequences of oxygen deprivation to vital organs such as the brain and heart are profound and irreversible membrane damage and loss of cellular ion homeostasis can occur (Boutilier, 2001). For heart muscle to sustain its mechanical activity there is a constant demand for ATP, which is balanced by ATP supply. Thus, cardiac metabolism is essentially oxidative. As a consequence of it being oxygen dependent, heart muscle is particularly prone to the effects of hypoxia. Owing in no small part to clinical implications, the effect of oxygen depletion on myocardial tissue has led to intensive studies (Boutilier, in review 2001; Daneshrad et al. 2000; Essop et al. 2004; Holden et al. 1995; Ngumbela et al. 2003; Novel-Chate' et al. 1998; Sharma et al. 2004).

Influence of hypoxia on metabolism and substrate selection

Glucose metabolism uses oxygen more efficiently than fat metabolism. Compared with fatty acid oxidation, carbohydrate oxidation generates more ATP per molecule of oxygen consumed and further, the glycolytic pathway can be organised for non-oxidative, anaerobic glycolysis which yields ATP and lactate or for carbohydrate oxidation (aerobic glycolysis). Pathways promoting the most efficient production of ATP per mole of oxygen should be favoured under conditions of oxygen depletion. Thus, with oxygen debt one would expect the utilisation of glucose rather than fat and the favouring of anaerobic rather than aerobic pathways. In support of this assertion, hypoxia is known to activate gene expression for glycolytic enzymes involved in anaerobic ATP supply pathways (Boutilier, 2001; Hoppeler, 2001; Ou & Leiter, 2004).

With reduction in oxygen availability, compensation in central processes leading to increased muscle blood flow and restoration of oxygen supply appear to be a primary strategy (Leon-Velarde et al. 1993; Novel-Chate' 1998; Tanaka et al. 1997). As for the effects of oxygen deprivation on muscle tissue, a review of the research offers disparate and often conflicting results. Several of these paradoxical accounts are outlined in the segments that follow.

Metabolic responses of cardiac muscle to hypoxia

Chronic hypoxia leads to alterations in myocardial metabolism that favour the use of glucose at the expense of fatty acids (Daneshrad et al. 2001). This is substantiated by the finding that gene expression of cardiac fatty acid oxidation enzymes are downregulated during hypoxia (Ngumbela et al. 2003). Moreover, it has been suggested that an elevated glucose preference in heart is a metabolic consequence of humans such as highland Quechua and Sherpa who have adapted over generations to chronic hypoxia (Holden et al. 1995).

In sharp contrast, based on gene transcriptional levels in the myocardium, Sharma et al. (2004) described a switch from fatty acid substrate to glucose only during the acute and sub-acute phases followed by a switch back to fatty acid utilisation with more prolonged hypoxia. Early work by Yoshino et al. (1990) also provided evidence for such a switch; cardiac muscle (and soleus muscle) exposed to severe, prolonged and repeated hypoxia shifted from anaerobic metabolism initially to aerobic metabolism ultimately, with acclimatisation being necessary for the shift. It was proposed that acute hypoxic exposure results in anaerobic stimulation of glycolysis while acclimation to hypoxia improves the availability of oxygen and enhances aerobic metabolism (Yoshino et al. 1990).

Metabolic responses of skeletal muscle to hypoxia and exercise

Many athletes, especially cyclists and runners, train at altitude for advantageous physiological effects such as increased red blood cell production and increased muscle myoglobin levels (Hoppeler & Vogt, 2001; Tanaka et al. 1997). Hypoxia is also known to activate gene expression for proteins involved in ventilatory and other vascular responses (Ou & Leiter, 2004; Vogt et al. 2001).

High intensity training at altitude can lead to peripheral adaptive strategies in skeletal muscle that compensate for the reduced oxygen availability during exercise. Increased reliance on metabolic pathways that favour the use of carbohydrate substrates instead of lipids is one such adaptation (Howald et al. 1990). Messenger RNA levels coding for certain glycolytic enzymes, such as phosphofructokinase, are known to increase with high intensity training in hypoxia (Hoppeler & Vogt, 2001). Along with increased glycolytic activity, strenuous exercise at high altitude (as in climbing Mt. Everest) is also known to decrease enzyme activities of terminal substrate oxidation (citric acid cycle and fatty acid oxidation) and this reduction in oxidative capacity is reportedly due to loss of mitochondrial structure which induces an aerobic to anaerobic shift of energy metabolism (Howald et al. 1990).

Conversely, in hypoxia-acclimated exercising rats, it was found that the relative contribution of carbohydrate metabolism does not increase even though increased carbohydrate utilisation would offer significant savings in oxygen. Exercise intensity, rather than hypoxia *per se*, was found to affect carbohydrate fuel selection (McClelland et al. 1998). Moreover, acclimation to high altitude was demonstrated by analysis of plasma fats, to increase the fatty acid cycle at rest as well as during exercise and is recognised as a potent stimulator of lipolysis (McClelland et al. 2001). High altitude training increased activity levels of enzymes involved in the citric acid cycle (CS) and the β oxidation of fatty acids (HAD) in fast-twitch muscles (Bigard et al. 1991).

Uniquely, under the conditions of hypoxia and ischaemia associated with diving, seals elicit a number of physiological and biochemical adaptations in their swimming muscles that allow them to sustain aerobic metabolic pathways which are adapted to maintain low levels of lipid-based metabolism (Kanatous et al 1999; 2002).

Metabolic adaptations of high altitude natives

Altitude-adapted Quechuas and Sherpas were found to have lowered maximum aerobic and anaerobic capacities as evidenced by reduced activities of both oxidative and glycolytic enzymes. Further, carbohydrate was the preferred fuel and the glycolytic pathway was adapted for carbohydrate oxidation rather than fermentation (Hochachka 1992).

Objectives of this Study: muscles and enzymes

Skeletal muscles are purported to be the most hypoxia-tolerant of all tissues (Boutilier, 2001). Skeletal muscle can be composed of two main fibre types, but also multiple subtypes (Spamer & Pette 1977, 1979; Takekura et al. 1994). Surprisingly very few studies have taken different fibre composition into account when investigating the influence of hypoxia on energy metabolism in skeletal muscle. Further, while some data exist on acute phase hypoxic exposure in cardiac muscle, few if any acute phase investigations relate to skeletal muscle. Moreover, data regarding muscular substrate utilisation in response to hypoxia is conflicting and somewhat confusing. Consequently, I decided to investigate the effect of acute hypobaric hypoxia *per se* on metabolic pathways in three clearly different types of skeletal muscles. I aimed to determine whether the immediate response is one where the anaerobic pathway is favoured over aerobic, oxidative pathways and whether carbohydrate is favoured over fat utilisation.

For this investigation, the following skeletal muscles were selected: the slow twitch *m.soleus*, the fast twitch *m.extensor digitorum longus* and the heterogenous *m.gastrocnemius* (hereafter soleus, EDL and gastrocnemius respectively). In Wistar rats, soleus comprises approximately 96% slow, oxidative, type 1 fibres while EDL comprises 95% fast, glycolytic, type 2 fibres (Soukup et al. 2002). Gastrocnemius is a mixture of slow oxidative, fast glycolytic and fast oxidative fibres (Takekura et al. 1994).

Phosphofructokinase (PFK) is a key enzyme of early glucose metabolism and PFK activity levels are indicative of the degree of commitment to the glycolytic pathway. Lactate dehydrogenase (LDH) is pivotal in the conversion of pyruvate to lactate and as such this enzyme is an indicator of anaerobic glycolysis. Citrate synthase (CS) is a key enzyme of the citric acid cycle and CS activity is an index of total mitochondrial oxidative capacity of the muscle cell. Fat utilisation is assessed by the activity of hydroxyacyl CoA dehydrogenase (HAD) an enzyme of the fatty acid β oxidation cycle. An investigation of the aforementioned enzymic activities will inform on the particular metabolic responses which occur in the different muscles in response to hypoxia.

METHODS

1. Animal Experimentation

1.1. Rats

Male Wistar rats, approximately six weeks of age, were used. Experimental animals and matched controls fell within the body weight range of 180 – 220 grams. (Burness et al. (1999) demonstrated that, in fish, enzyme activities showed dependent relationships with body-size) A total of fourteen rats were used for seven experiments with matched controls. Ethical approval for this series of experiments was obtained from the Animal Research Ethics Committee of the University of Cape Town.

1.2. Experimental conditions

A single experiment (acute hypoxic exposure vs. normoxic control) was performed per occasion. The test period lasted 48 hours and typically began at 12h00. Each rat was placed in a mouse cage, which is considerably smaller than a conventional rat cage. This was done to minimise the test animal's movement. Exposure to hypoxia tends to reduce voluntary mobile activity in the rat and in attempting to match the physical activity of experimental and control rats we deemed it appropriate to confine their environment.

Fifty grams of standard laboratory food was weighed out and placed in feeding trays at the start of experimentation. Food remaining at termination of the experiment was also weighed in order to assess consumption. Hypoxia leads to diet restriction (Daneshrad et al. 2000) and therefore, to control for the effects of hypoxia alone, we attempted to match the dietary ingestion of control rats with that of experimental animals. Water was available *ad libitum*.

The hypoxia experimental setup followed the protocol described by Sharma et al. (2004) with some modifications. The hypobaric hypoxic chamber (SciTech, Cape Town, South Africa) was custom-constructed to create an environment of 11% oxygen (45 kPa). Each experimental rat

was housed in a cage which was placed inside the chamber. As far as possible, there were no other animals in the chamber for the duration of the experiment. Control animals were maintained at ambient oxygen levels (~21%) for the same period of time (48 hours). The chamber required between three and five minutes to reach the programmed hypoxic condition when powered on, and likewise to equilibrate to ambient oxygen (normoxia) when turned off.

1.3. Dissection

Body mass was determined at the start and again at termination of each experiment.

An experienced animal handler sacrificed each rat by cervical dislocation which constituted immediate termination. This method was employed as we intended to utilise the muscle tissue for further enzymic studies. Euthanasia by gas, as well as any delay in termination is known to adversely affect enzyme integrity and would have compromised our planned investigations.

After termination, the animals were promptly dissected for EDL, soleus and gastrocnemius muscles (in this order). A clear indication of the location and characteristics of these muscles *in situ* is described in the Appendix (page 37). Both hind limbs were dissected allowing us to collect tissues in duplicate. Upon extraction from the lower leg, each muscle was rapidly immersed in liquid nitrogen for several seconds (snap frozen) and then placed in microfuge tubes for preservation at -80^{o}C. The time between euthanasia and dissection of the final piece of muscle tissue was generally less than ten minutes in total.

Body mass changes, food consumption, behavioural and other incidental observations were recorded. These data are tabulated in the Results section.

2. Preparation of Muscle Tissue

A considerable amount of time and effort was spent optimising the system and the following outline represents the optimal method of muscle preparation for enzyme activity assessment by fluorometry.

2.1. Freeze drying

Enzyme activities were assessed per gram dry weight of sample and for this reason, material was required to be freeze-dried. Several components of the freeze-dry equipment (VirTis Freezemobile 6, New York, USA) were pre-cooled in order to prevent thawing of muscle tissue samples in the initial stage. Inner core temperature was -50^{o}C and the vacuum was set at 100 milliTorr.

Each muscle, originally snap-frozen in liquid nitrogen and then stored in microfuge tubes at -80^{o}C, was transferred in liquid nitrogen to the pre-cooled freeze drier. Tube lids were perforated with small holes to facilitate maximal exposure of muscle contents to the vacuum suction. Samples were dried overnight for a period of 24 hours.

2.2. Weighing of muscle samples

All assays require that the precise weight of each muscle sample should be determined.

A slice from the middle region of the dried muscle was obtained by cutting with a clean scalpel blade while viewing through a dissection microscope. Samples were weighed to approximately 2 milligrams on a microbalance (Sartorious 4125, Germany), placed in sterile microfuge tubes and appropriately labelled. The procedure was duplicated for every muscle sample.

2.3. Homogenisation and sonication

Homogenising potassium buffer (pH 7.3) was freshly prepared on the day of use. (Refer to page 39 in Appendix for the reagent mixture.)

The required ratio of muscle to potassium buffer is 1:400 (T. Kohn pers. Comm.). The amount of buffer to add to the pre-weighed muscle samples was calculated. Since muscle samples were all weighed to approximately 2 mg, final volumes were in the region of 800 µl.

Tissue was thoroughly broken up in the microfuge tube with a clean, blunted, metal rod before the appropriate amount of buffer was added. Thereafter samples were kept on ice for at least half an hour before brief sonication, by way of two pulses of 10 seconds at medium velocity, using a Misonix (New York, USA) sonicator.

Homogenates were kept overnight at 4^{o}C for enzyme activity analysis the following day (as is required with PFK) or frozen at -20^{o}C for later analysis.

3. Enzyme Activity Determinations (LDH, PFK, CS and HAD)

Analyses were performed in two batches so as to best manage the quantity of samples and the limitations of microtitre plate capacity. Batching also helped to reduce fluorescence decay as well as the chances of enzymatic reagents heating up. Every sample was read in duplicate and each sample required 250 µl reaction buffer. For calculations, see below:

Exps 1-3	36 samples (x2) * 250 µl = 18 mls Reaction Buffer
Exps 4-7	48 samples (x2) * 250 µl = 24 mls Reaction Buffer

The reliability of muscle sampling procedures and the accuracy of fluorometric assessments was determined after subjection to statistical scrutiny. Our enzyme assay techniques and equipment proved to be technically sound. (Refer Appendix, pages 41 - 43)

3.1. Reagent preparations for enzyme analysis

Each assay required a specific reaction buffer. Refer to Appendix (pages 39 - 40) for details of reaction buffers and refer below for the respective reactions.

3.2. Biochemical basis for enzyme assays

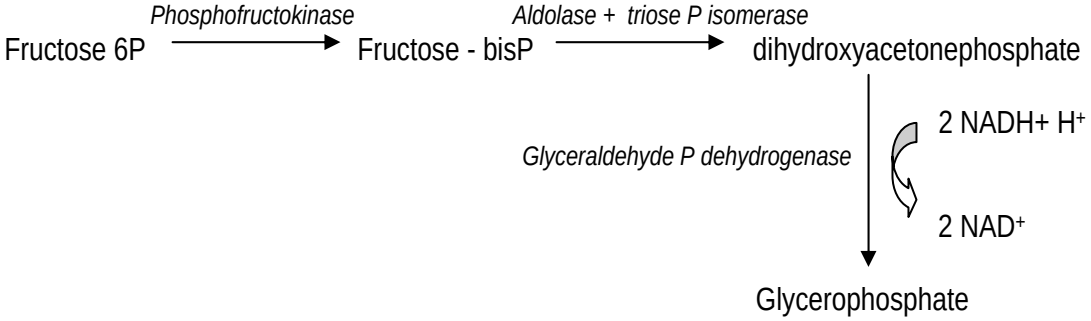
NAD^+ is a coenzyme and its reduced form is NADH. Both are electron carriers which play an essential role in enzyme catalysed oxidation–reduction reactions. These reactions involve the transfer of a hydride group to NAD^+ or from NADH. Dehydrogenases are the enzymes that facilitate these transfers. (All the analytical reactions involve NAD-dependent dehydrogenases)

Fluorescence emits from NADH, the reduced form of NAD, and this is measured in a fluorometer at 340 nm. All assays, with the exception of citrate synthase, show a decline of fluorescence over time indicating the conversion of NADH to NAD. The citrate synthase assay is based on the production of NADH from NAD and shows a positive slope of fluorescence against time.

The fluorescence signal is indicative, either directly or indirectly, of the enzyme activity as can be observed from the following reactions:

3.2.1. Phosphofructokinase (PFK)

The analysis is based on the following reaction:

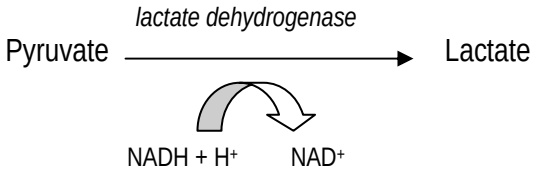


Phosphofructokinase is inherent in the muscle sample and starts the reaction, as described above. Other reagents are supplied as per the reaction buffer outlined in the Appendix.

Two molecules of NADH produce two molecules of NAD for every molecule of PFK utilised. This is taken into consideration in activity calculations. Ultimately the rate of decrease in fluorescence as NADH loses a hydride to become NAD under the influence of dehydrogenase, reflects the activity of PFK.

3.2.2. Lactate dehydrogenase (LDH)

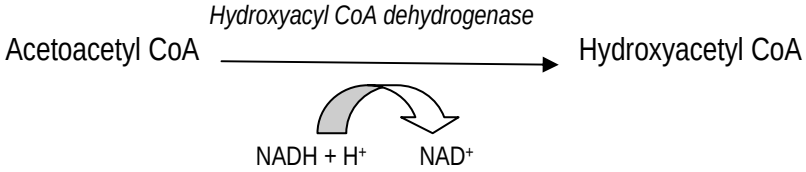
The analysis is based on the following reaction:



Pyruvate and NADH are supplied in the reaction buffer. LDH in the muscle sample, converts pyruvate to lactate in the presence of NADH. NADH has a hydride transferred by the dehydrogenase thus creating NAD and in the process there is a change (decrease) in fluorescence which reflects the activity of the enzyme.

3.2.3. Hydoxyacyl CoA dehydrogenase (HAD)

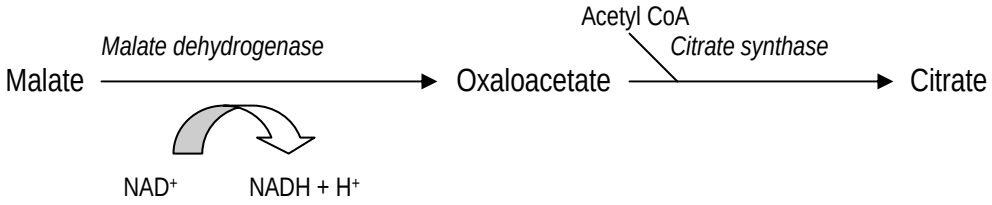
The analysis is based on the following reaction:



Acetoacetyl CoA and NADH are supplied in the reaction buffer. HAD in the muscle sample converts acetoacetyl CoA to hydroxyacteyl CoA in the presence of NADH. NADH has a hydride transferred by the dehydrogenase thus creating NAD and in the process there is a decreasing change in fluorescence which reflects the activity of the enzyme.

3.2.4. Citrate synthase (CS)

The analysis is based on the following reaction:



Malate, malate dehydrogenase, NAD and acetyl CoA are supplied in the reaction buffer. Acetyl CoA is added to start the reaction. Citrate synthase is present in the muscle sample.

Fluorescent NADH is generated from NAD and as a result fluorescence increases and the activity slope is positive. The amount of fluorescence ultimately reflects the conversion of oxaloacetate to citrate as catalysed by citrate synthase.

4. Analysis by Fluorometry

With fluorometry one measures the fluorescence emanating from reduced forms of NAD and NADH. During analysis, the fluorescence is measured at known time intervals and difference per minute is calculated. The rate of change in fluorescence is used in the calculation of maximal enzyme activity (refer to page 14).

Knowing the weight of the sample and its dilution, the enzyme activity is calculated and expressed as µmol / min / g (dry wt) substrate turnover. Statistical probabilities do not change whether enzyme activity is expressed per gram of dry tissue weight or per gram wet tissue weight (Burness et al. 1999).

4.1. Fluorometer and analytical software

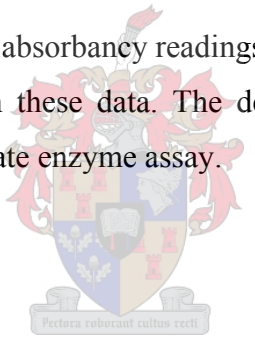
The fluorescence reader (Bioteck FLX 800, Shipley, UK) was operated by Windows-based software (KC Junior) on a linked computer. Operating instructions are included in the Appendix (page 39).

Samples were read at an excitation wavelength of 340 nm and emission wavelength of 460 nm. Light-resistant (black) multiwell microtitre plates were used in the FLX 800.

4.2. Preparation of NADH standard curve

NADH in TRIS buffer (pH 8) was prepared to approximately 5 mM. The exact concentration of NADH was confirmed by spectrophotometeric readings at 340 nm and by calculation ($A = \varepsilon cl$).

We used the fluorometer for duplicate absorbancy readings at different concentrations of NADH and generated a standard curve from these data. The derived slope value was then used in calculation of activity for the appropriate enzyme assay.



4.3. Enzyme specific set-ups

Muscle homogenate was carefully dispensed (orientation maintained throughout) into designated wells of a black microtitre plate with a P10 pipette.

Through trial and error, the number of samples that could be assayed per occasion in quick succession was optimised. Loading and reading eight samples in duplicate was standard except for the LDH assay which is based on a rapid reaction and becomes manageable only by decreasing the number of samples one reads per occasion. The table which follows on the next page, outlines the specifics of each assay.

The reaction mixture was kept on ice and in the dark until use in order to protect the integrity of the enzymes and light sensitive components. Thereafter it was poured into a mixing trough,

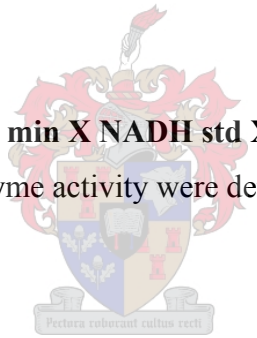
covered with foil and allowed to equilibrate to room temperature. A multichannel pipette was used to aspirate the reaction buffer from the mixing trough and to dispense into microtitre wells. Since no shaking capacity exists on the FLX800, the manner in which one dispenses reaction buffer into the sample-containing wells can facilitate mixing of the two. Air bubbles should be strictly avoided.

	sample	Buffer	Dilution factor	Recordings: frequency and total	Comments
PFK	3 µls	250 µls	84.3	every 30 secs for 2 mins (5)	
LDH	3 µls	250 µls	84.3	every 15 secs for 1 min (5)	* very quick reaction * only 2 samples/ time
HAD	5 µls	250 µls	51	every 30 secs for 2 mins (5)	
CS	5 µls	250 µls	51	every 30 secs for 2 mins (5)	* positive slope

4.4 Enzyme Activity calculations

i. Maximal Enzyme Activity: **Δ abs / min X NADH std X (sample dilution X assay dilution)**

ii. Metabolic Potentials: ratios of enzyme activity were determined by calculation



5. Statistical Analysis

Statistical comparisons between muscle types and between experimental (hypoxia) and control (normoxia) conditions were made by multifactorial analysis of variance (MANOVA). To test prior hypothesis and further analyse significant difference, we used Fisher (LSD) *post hoc* tests. In all instances confidence levels (α) were set at $p \leq 0.05$ (STATISTICA 7).

Covariance analysis was used to determine whether the amount of food consumed during the 48 hour experimental period and the weight change of the animal during this time, had any bearing on the activity of the various enzymes that were assayed.

The reliability of the assay technique was also statistically assessed by covariance analysis.

RESULTS

The different muscles were selected for their characteristic fibre type compositions: EDL comprising fast, glycolytic fibres, soleus comprising slow, oxidative fibres and gastrocnemius comprising a mixture of fibre types. Based on these different fibre profiles and muscle functions, I expected to demonstrate inherent inter-muscular differences in the capacity for utilisation of either carbohydrate metabolic pathways or fatty acid pathways.

Since glycolysis (assessed by activities of PFK and LDH) is an anaerobic process and fatty acid β oxidation (assessed by HAD activity) and the citric acid cycle (assessed by CS activity) are aerobic processes, I anticipated that oxygen depletion might affect the activity of these enzymes and would thus have a consequent effect on substrate (lipid or carbohydrate) utilisation.

Full statistical test results are presented in the Appendix section (pages 44 - 51).

1. Maximal Enzyme Activities

1. 1. Hydroxyacyl CoA dehydrogenase

HAD activity was highest in the soleus muscle (22.3 ± 2.1) (mean ± standard error) and lowest in EDL (17.2 ± 2.1) of the normoxic group (Fig. 1 and Table 1.1, Appendix, page 44). Statistically, however, this variation between muscles was not proven significant (Tables 1.2 – 1.3 in Appendix, page 44). Under hypoxic conditions, HAD activity in the soleus differed significantly only from that of the gastrocnemius.

These results do not corroborate clear inter-muscular differences in the activity of HAD, a key enzyme of fatty acid oxidation.

After acute hypoxic stress, HAD activity in the EDL was 12.2 % higher than in normoxic counterparts. There was also a small (4.1 %) increase in HAD activity in soleus of hypoxic

animals compared to normoxic controls. These alterations, however, were not statistically significant (Fig. 1 and Appendix Tables 1.1 – 1.3). In gastrocnemius, HAD activity remained more or less unchanged.

While these results hinted that acute hypoxia increased fat utilisation in some muscles, there was no statistical validation of this and therefore, based upon these data, we assert that hypoxia of short duration does not alter reliance on fatty acid oxidation in skeletal muscle.

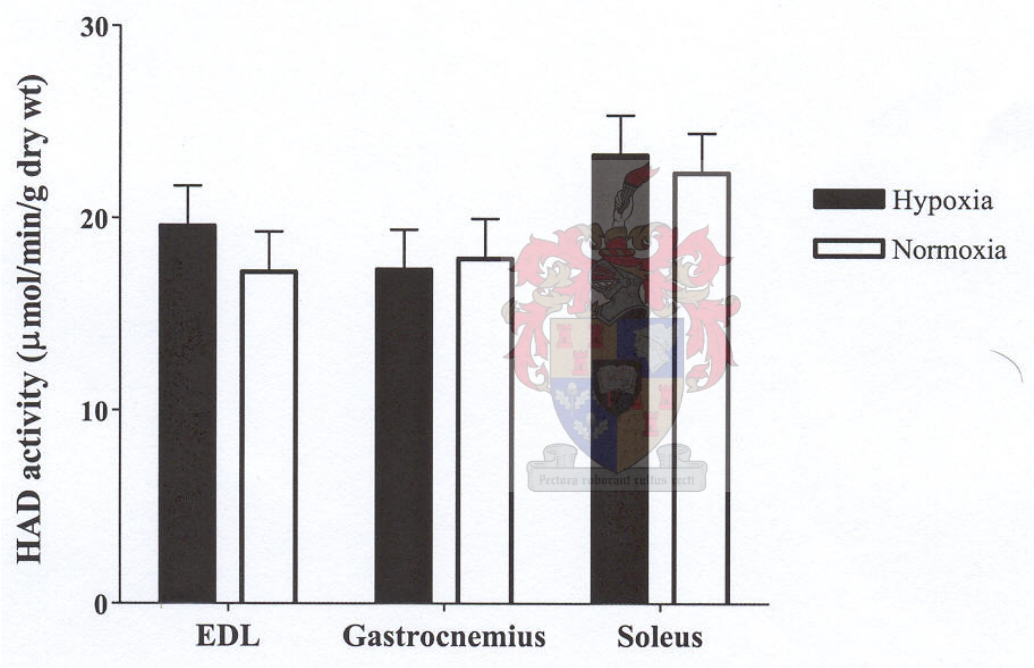


Figure 1. HAD activity (mean ± SE) for skeletal muscle exposed to hypoxia (n = 7 per group)

1.2 Citrate synthase

Of the various normoxic muscles, maximal CS activity is comparatively lowest in the fast, glycolytic EDL and highest in soleus muscle (Fig. 2) although statistics established that these marginal differences were non significant (Tables 2.1 - 2.3 in the Appendix, page 45). Thus, there appear to be no inter-muscular differences in baseline citrate synthase activity in normoxia.

Surprisingly, there was a tendency in the soleus - a muscle comprising almost entirely oxidative fibres and reliant on aerobic metabolism - for activity levels to be slightly elevated after acute hypoxic exposure (48.6 ± 3.9) compared to normoxic controls (46.7 ± 3.9) while activity levels in the other hypoxic muscles tended to be depressed. Moreover, CS activity was more elevated in soleus than all other hypoxic muscle groups. Variations were, however, not proven statistically significant leading us to conclude that CS activity was not influenced by acute hypoxia.

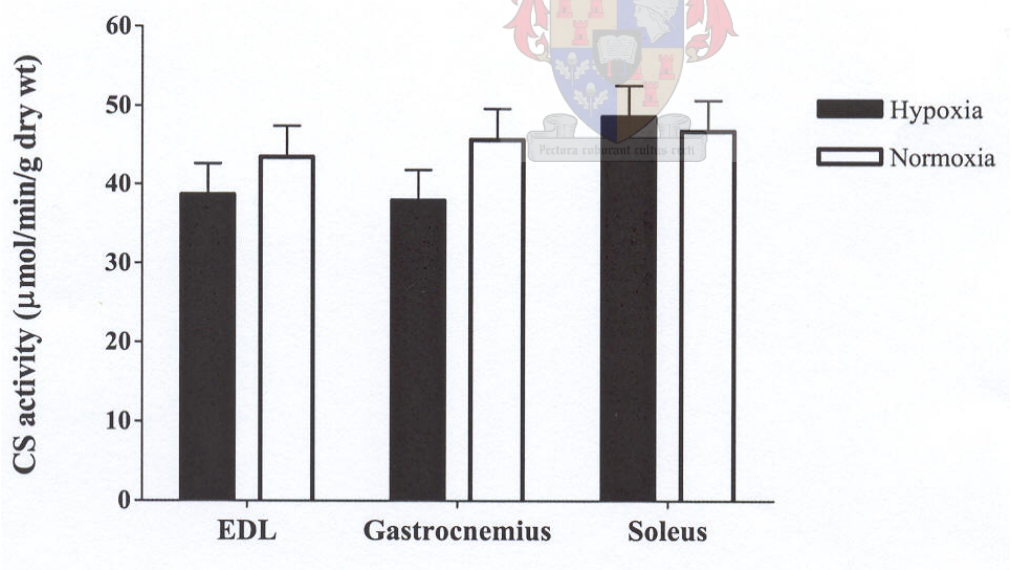


Figure 2. CS activity (mean ± SE) for skeletal muscle exposed to hypoxia (n = 7 per group)

1.3 Phosphofructokinase

There was a highly significant main effect of muscle type on PFK activity ($p < 0.0001$). PFK is most active in EDL muscle and least active in the soleus (Fig. 3 and Table 3.1 in Appendix). Fisher's *post hoc* test showed significant differences in PFK activity between all muscles under conditions of normoxia and between all, except for EDL vs. gastrocnemius, in hypoxia. (Tables 3.2 - 3.3 in Appendix). These data demonstrate inter-muscular variation in the reliance on the glycolytic pathway.

Acute hypoxia had no established effect on PFK activity although hypoxic gastrocnemius *tends* towards significantly elevated activity (intimating increased glucose metabolism) compared to normoxic counterparts ($p = 0.06$).

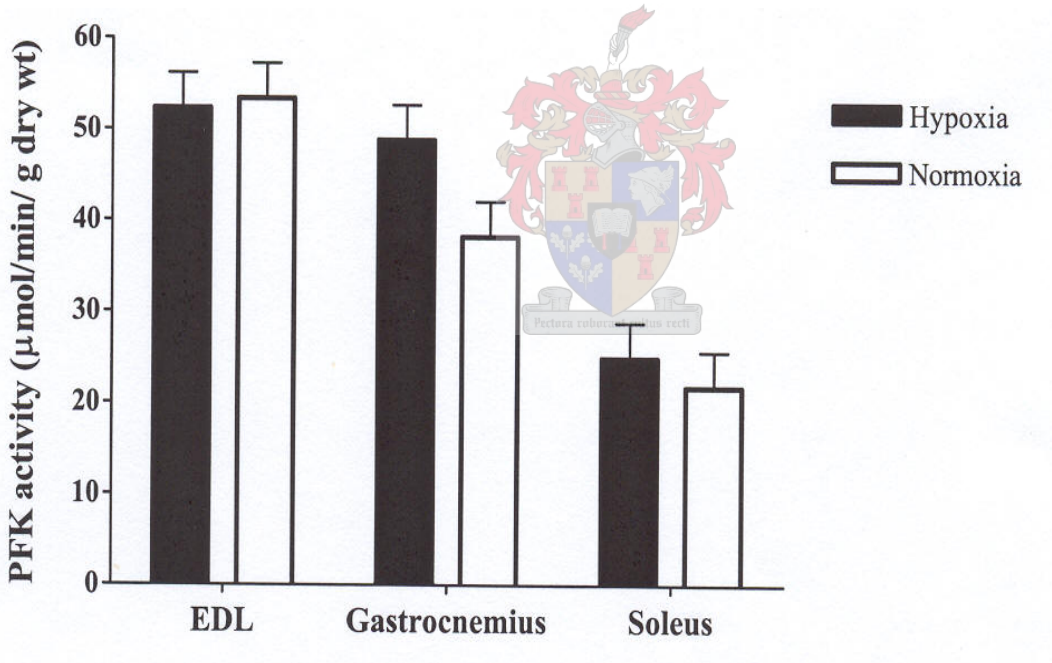


Figure 3. PFK activity (mean ± SE) for skeletal muscle exposed to hypoxia (n = 7 per group)

✶ indicates significant differences between all muscles (normoxia)

1.4 Lactate dehydrogenase

LDH is considerably more active in EDL muscle (705.2 ± 28.9) than in soleus (282.8 ± 28.9) (Figure 4, Table 4.1 in Appendix) indicating a significantly greater ability to generate lactate in anaerobic conditions. The difference in LDH activity between soleus and other muscle groups was highly significant under both of the tested environmental conditions. LDH activity in the gastrocnemius did not differ significantly from LDH activity in EDL. (MANOVA and Fisher *post hoc* tests are tabulated in Tables 4.2 - 4.3 in Appendix.). These data in conjunction with results from PFK activity analysis, indicate a considerably minimised utility of the glycolytic pathway in soleus and a significantly increased glycolytic activity in EDL.

Acute hypoxia did not effectively alter the activity of LDH enzyme although there was a slight and insignificant elevation (7%) in soleus muscle. There does not appear to be increased disposal of pyruvate to lactate in conditions of reduced oxygen although the data calculated from metabolic potentials (following section) should further elucidate this.

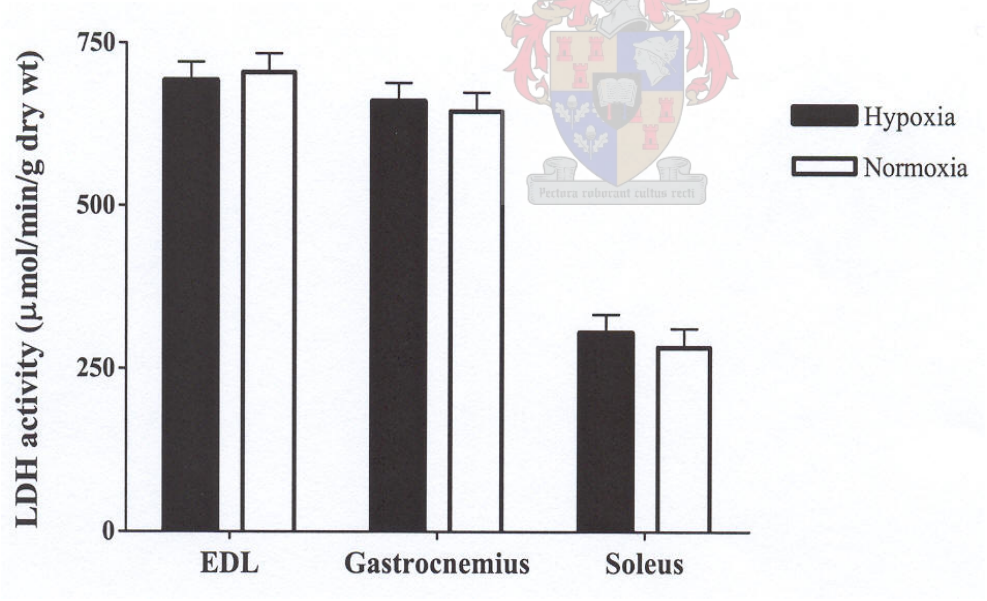


Figure 4. LDH activity (mean ± SE) for skeletal muscle exposed to hypoxia (n = 7 per group)

✶ indicates significant difference between soleus and other muscles ($p < 0.0001$)

2. Metabolic Potentials

- **anaerobic vs. aerobic metabolism**
- **carbohydrate vs. fatty acid metabolism**

2.1. Fatty acid metabolism relative to total oxidative metabolism (CS:HAD)

This ratio was calculated to assess the contribution of fatty acid metabolism (HAD) relative to total oxidative metabolism (CS). In normoxia, soleus had slightly lower CS:HAD ratio (Fig 5, Table 5.1 in Appendix). This suggests a potential in this muscle for fatty acid oxidation to contribute more towards total oxidative metabolism than is the case in the other muscle types. MANOVA, however, determined no true differences in fat oxidation potential amongst the different muscles (Tables 5.2 - 5.3 in the Appendix, page 48). All tissues exhibited CS:HAD ratios within the range 2.1 - 2.6 indicating that fatty acid oxidation does not account for the majority of aerobic metabolism in skeletal muscle of the Wistar rat.

After exposure to acute hypoxia, EDL and gastrocnemius, appeared to utilise less fat than their normoxic counterparts (13.8 % and 14.8 % respectively) (Fig. 5). This variance was not significant ($p > 0.05$) and leads to the conclusion that, in skeletal muscle, the potential for fat utilisation as part of an oxidative stategy is not affected by hypoxia of short (48 hours) duration.

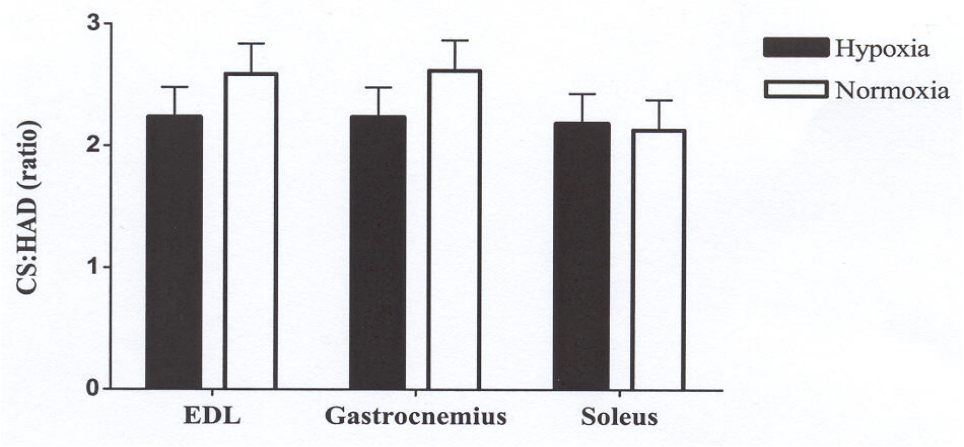


Figure 5. CS : HAD activity for skeletal muscle exposed to hypoxia (means ± SE)

2.2. Anaerobic potential versus aerobic potential. (LDH:CS)

This determination assesses the capacity of each muscle for anaerobic metabolism (LDH) with respect to its capacity for mitochondrial oxidative metabolism (CS).

Soleus exhibited the lowest anaerobic potential of all muscle groups (Fig.6). This was statistically determined ($p = 0.0001$) to be highly variant from both EDL and gastrocnemius muscle (Tables 6.1 - 6.3 in Appendix, page 49).

After exposure to hypoxia, EDL and gastrocnemius muscle seemed to slightly increase their anaerobic potential above the normoxic (13.8 % and 15 % respectively) (Fig. 6). This was however not statistically validated. The anaerobic potential of soleus remained unaltered.

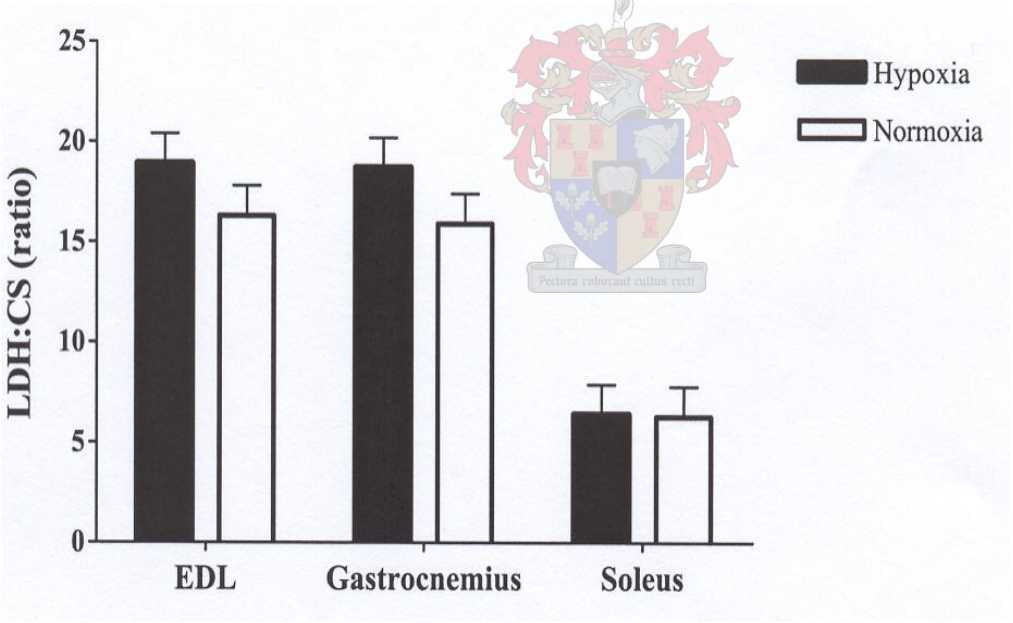


Figure 6. LDH : CS activity for skeletal muscle exposed to hypoxia (means ± SE)

✶ indicates significant difference between soleus and other muscles ($p < 0.0001$)

2.3. Potential for carbohydrate glycolysis vs. fatty acid oxidation (LDH:HAD)

Soleus exhibited the lowest glycolysis to fat β-oxidation ratio (Fig.7). This effectively means that soleus has a considerably greater potential for fatty acid metabolism, this being significantly different from the fat utilisation potential of EDL and gastrocnemius. (Conversely, EDL and gastrocnemius have a significantly greater ability to anaerobically break down glucose to lactate than does soleus muscle.)

Acute hypobaric hypoxic exposure did not alter the existing substrate utilisation potential in the different muscle types (Tables 7.1 - 7.3 in Appendix, page 50).

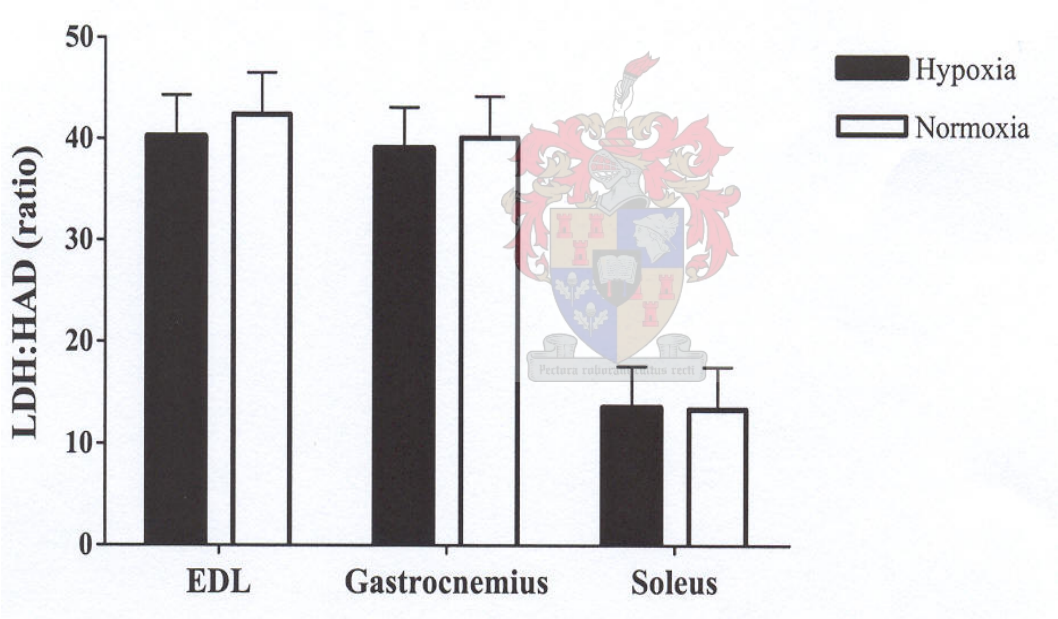


Figure 7. LDH : HAD activity for skeletal muscle exposed to hypoxia (means ± SE)

✶ indicates significant difference between soleus and other muscles ($p < 0.0001$)

2.4. Potential for anaerobic glycolysis vs. total carbohydrate metabolism (PFK:LDH)

The PFK:LDH permutation determines the proportion of glucose broken down to lactate by lactate dehydrogenase (anaerobic glycolysis) relative to glucose destined for aerobic, oxidative pathways (total glycolytic commitment).

Gastrocnemius has the lowest PFK:LDH ratio of normoxic tissue (Fig. 8) and this was statistically different from the PFK:LDH ratio of soleus ($p = 0.05$) (Tables 8.1 – 8.3 in the Appendix, page 51). These data should be seen in context with the absolute enzyme activities (pages 18,19) and infer an enhanced LDH activity relative to PFK in gastrocnemius and thus a greater ability for anaerobic glycolysis (lactate generation).

Following oxygen restriction, PFK:LDH is elevated in the gastrocnemius indicating, rather curiously, a decreased anaerobic turnover of lactate and increased activity of PFK. Statistics, however, confirm that hypoxia of short duration has no significant effect on anaerobic glycolysis in preference to aerobic glucose metabolism in the gastrocnemius or other muscles.

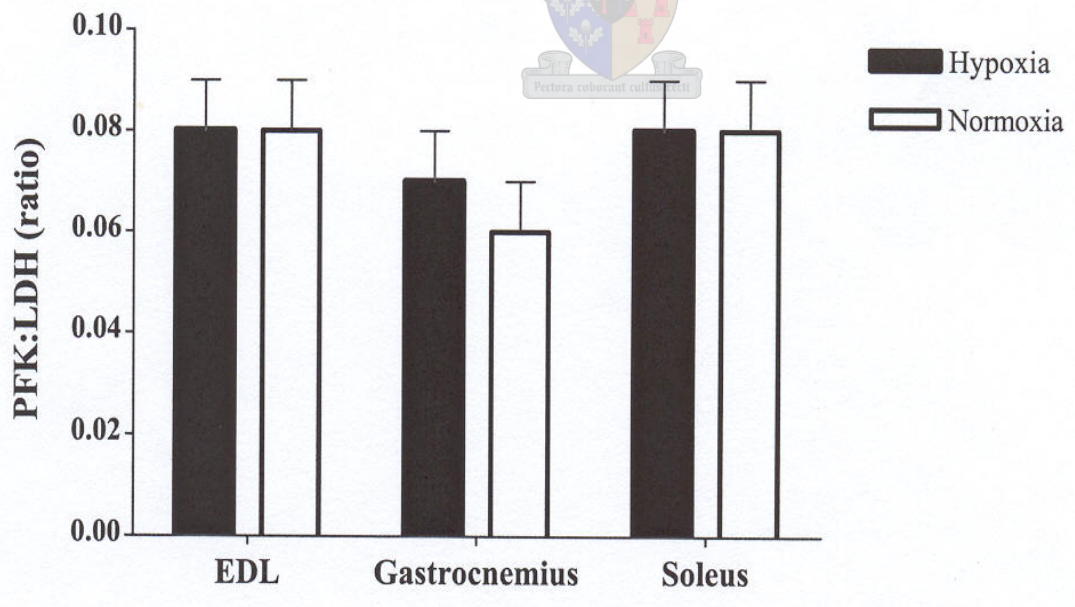


Figure 8. PFK : LDH activity for skeletal muscle exposed to hypoxia (means ± SE)

✶ indicates significant difference between gastrocnemius and soleus (normoxia)

Table of summary and comparative enzyme analyses

Enzyme activity data concluded from this study are tabulated with those published for similar studies on several other species (Table 9). Direct comparisons should be avoided since conditions of hypoxia, prior exposure, muscles tested or species lifestyle are not equivalent. For example, our data are generated from laboratory rats exposed to acute hypoxia while those for Andean natives are generated from high altitude dwellers acclimated to chronic hypoxia (Desplanches et al. 1996). Seal data are generated from mammals that have evolved to forage in conditions of transient but intense hypoxia (Kanatous et al. 1999, 2002).

	HAD	CS	CS:HAD	PFK	LDH
gastrocnemius (hypoxia)	17.3 ± 2.1	37.9 ± 3.9	2.2 ± 0.3	48.9 ± 3.9	660.4 ± 28.9
(normoxia)	17.9 ± 2.1	45.7 ± 3.9	2.6 ± 0.3	38.2 ± 3.9*	645.5 ± 28.9
EDL (hypoxia)	19.6 ± 2.1	38.7 ± 3.9	2.2 ± 0.3	52.3 ± 3.9	692.0 ± 28.9
(normoxia)	17.2 ± 2.1	43.5 ± 3.9	2.6 ± 0.3	53.4 ± 3.9*	705.2 ± 28.9
soleus (hypoxia)	23.3 ± 2.1	48.6 ± 3.9	2.2 ± 0.3	24.9 ± 3.9	304.4 ± 28.9$
(normoxia)	22.3 ± 2.1	46.7 ± 3.9	2.1 ± 0.3	21.8 ± 3.9*	282.8 ± 28.9$
v. medialis◆ (cotton rat)	11.9 ± 1.7	24.0 ± 1.4	2.0 ± 0.3	ND	ND
v. lateralis◆◆ (highlanders)	7.3 ± 0.9	10.0 ± 0.7	ND	43.9 ± 5.7	ND
v. lateralis ◆◆◆ (normoxia)	24.5 ± 6.0	16.5 ± 3.2	ND	48.3 ± 7.9	161.0 ± 36.0
(hypoxia)	18.0 ± 3.9#	12.7 ± 2.1#	ND	52.6 ± 9.6	162 ± 53.0
pectoralis >> (non swim)	38.9 ± 7.5	12.4 ± 0.8	0.3	ND	739 ± 66
long dorsi>> (swim)	48.5 ± 9.0	12.0 ± 1.3	0.3	ND	563 ± 89%
hindlimb >> (dog)	16.6 ± 2.8	24.8 ± 3.3	ND	ND	396 ± 38

Table 9. Metabolic enzyme activities of skeletal muscles exposed to hypoxia
Activity expressed as means ± SE or SD>> and µmol product /min/g dry wt or wet wt◆◆◆
ND is not determined

* significant variance between EDL, soleus, gastrocnemius (normoxia) $p \leq 0.05$
$ significant variance between soleus and other muscles (normoxia, hypoxia) $p \leq 0.05$
\# significant effect of hypoxia
% significant decrease in swim muscles

◆ Cotton Rat data from Kanatous et al. (1999)
◆◆ Andean native data from Desplanches et al. (1996)
◆◆◆ Mountain climber data (before and after Mt.Everest climb) from Howald et al. (1990)
>> Wedell Seal and dog data from Kanatous et al. (2002)

3. Food Consumption and Body Weight changes: Effect on Enzyme Activity

Our data indicate a mean weight loss of 8.3 g (± 6.1) in rats exposed to hypoxia for 48 hours (Table 10). Oxygen depleted animals consumed considerably less food than normoxic controls (33.3 g ± 4.4 vs. 46.2 ± 1.9) (Table 10). These data corroborate reports that body weight of adult rats exposed to normobaric hypoxia decreased by about 10 % during the first two days of exposure (Daneshrad et al. 2000). The same investigators reported that hypoxia-induced diet restriction over a prolonged period is *per se* responsible for affecting the activity of certain enzymes. (We made an effort to control for this by pair-feeding our animals)

N		Food consumed (g)	Body wt change (g)
1	hypoxia	30	-18.2
2		36.2	-7
3		33.2	-3
4		25.5	-17
5		37.7	-3.9
6		35.8	-5.1
7		37.7	-9.3
	mean (± SD)	**33.3 ± 4.4**	**- 8.3 ± 6.1**
1	normoxia	30	-3◊
2		50	13.5
3		45	9.5
4		45	1.2
5		46	12
6		45.5	12.6
7		45.6	1.7
	mean (± SD)	**46.2 ± 1.9**	**8.4 ± 5.6**

Table 10. Food consumption and respective body weight changes for all trial animals
◊ weight loss in this animal on account of inadequate food supply

Covariant analysis determined no correlation between the amount of food (substrate) consumed and activity of any of the metabolic enzymes under investigation. Although, as could be expected, there is a very close correlation between food consumption and weight change, this did not translate into a correlated effect on enzyme activity. Statistical analyses are listed in Tables B.1 - B.4 in Appendix (page 52).

DISCUSSION

The aim of this study was to investigate the effect of acute hypoxia, *per se*, on the potential of various pathways for substrate utilisation in different skeletal muscles. Material was also collected for a twin-study investigating the effect of ambient oxygen reduction on expression of AMP kinase, an enzyme profoundly involved with energy sensing and signalling.

Inter-Muscular Variance

Muscles of different fibre composition demonstrated variance in glycolytic capacity. Comparative analysis of PFK and LDH activities confirmed that EDL (flexor muscle) has the highest capacity for glycolysis while soleus (extensor) demonstrated considerably decreased activity of these key glycolytic enzymes. These findings are in accordance with those of Spamer & Pette (1977, 1979), Takekura et al. (1994) and Tikkanen et al. (1995) and confirm the status of EDL as a fast, glycolytic muscle. Soleus has the least active glycolytic pathway in general and additionally it has considerably less capacity than EDL and mixed fibre gastrocnemius to anaerobically generate lactate.

As for inter-muscular differences in oxidative capacity, no meaningful variance ($p > 0.05$) in activity levels of the oxidative enzymes CS and HAD was demonstrated between the muscle groups. It was somewhat unexpected to find that CS activity was not elevated in soleus, being a muscle made up almost entirely of oxidative fibres. In contrast, Takekura (1994) reported elevated oxidative capacity (by analysis of succinate dehydrogenase) in soleus relative to EDL. It should be remembered however, that laboratory animals have little access to the activities which are likely to stimulate expression of the latent potential.

All muscle tissues exhibited CS:HAD ratios within the range 2.1 - 2.6 indicating that fat oxidation does not account for the majority of aerobic metabolism in Wistars. Nevertheless, as determined by the LDH:HAD permutation, soleus has a significantly greater potential for fatty acid utilisation than either gastrocnemius or EDL as well as greater capacity for aerobic metabolism (LDH:CS) than the other muscles.

Substrate Utilisation

Our investigation examined the immediate consequences of exposure to an oxygen-deficient environment as opposed to the effects of chronic exposure, or adaptations, which have received more attention. Also, while we report on the influence of hypoxia *per se*, the majority of published research has focussed on the combined stresses of exercise training and hypoxia.

Several studies have shown *chronic* hypoxia to induce metabolic pathways in skeletal muscle that favour the most efficient use of oxygen. Thus, acclimatisation to hypoxic environments causes upregulation of glycolytic pathways in favour of oxidative pathways and increased reliance of muscle cells on carbohydrate substrate instead of lipids (Desplanches et al. 1996; Hoppeler et al. 2001). Kennedy et al. (2001) in point of fact, reported a downregulation of HAD activity (fat oxidation) in cardiac and quadriceps muscle of rats after chronic (5 weeks) exposure.

Paradoxically, Ou & Leiter (2004) found no evidence that anaerobic metabolic processes were upregulated to sustain energy consumption during chronic hypoxia. The lack of major changes in the capacities of anaerobic glycolytic pathways was consistent with the maintenance of normal aerobic metabolism of rats at 5500 m.

Strenuous exercise, as in climbing Mount Everest, was revealed to intensify the stress of hypoxic exposure and induce an aerobic to anaerobic shift of muscle energy metabolism. This reflects that, in skeletal muscle, glycolytic enzyme activities were increased and oxidative enzyme activities were decreased (Howald et al. 1990). These changes oppose those observed with endurance training in normoxia and in simulated altitude where oxidative capacity increased in fast-twitch muscles (Bigard et al. 1991; Melissa et al. 1997) and LDH activity decreased (Bigard et al. 1991). This should perhaps caution against direct comparisons of observations made from controlled laboratory situations and those from authentic altitude experience.

Uniquely, swimming muscles of seals appear to be adapted for aerobic lipid metabolism under the hypoxic conditions that occur during diving. Kanatous et al. (1999; 2002) demonstrated increased activity levels of CS and HAD in swimming muscles and in addition, the anaerobic capacity as determined by LDH activity, was significantly lower in swimming muscles than in non swimming muscles.

Results from our investigation reveal that hypobaric hypoxia of short term duration induced no meaningful responses in either the glycolytic or oxidative biochemical pathways. A discussion of our findings follows.

Oxidative Enzymes

HAD: Although, in our investigation, HAD activity (lipid oxidation) was somewhat elevated in hypoxic EDL and soleus and slightly depressed in hypoxic gastrocnemius, these differences from normoxic controls were not significant ($p > 0.05$). The results prompt us to conclude that fatty acid oxidation pathways in skeletal muscle of various fibre types are neither upregulated nor downregulated in response to hypoxia of acute (48 hours) duration.

Similarly, Kennedy et al. (2001) report that HAD activity was unchanged in soleus, EDL, quadriceps and gastrocnemius muscles after acute (24 hours) altitude exposure. However, the results they obtained for chronic exposure suggest that acclimatisation to high altitude is key in selectively decreasing enzymes for fat utilisation and oxidation. As evidenced by our results, 48 hours of hypoxia is insufficient to induce peripheral tissue acclimatisation.

Studies of exercise training in hypoxia offer inconsistent results with regards to lipid metabolism. High-altitude natives subjected to training programmes in hypoxia showed no significant increase in HAD activity of the vastus lateralis muscle and thus no increased capacity to oxidise fat which is the usual response to endurance training *per se* (Desplanches et al 1996). However, unadapted rats trained at high altitude, demonstrated that HAD activity levels in the EDL increased up to 36 % versus sedentary controls and versus sea level trained rats (Bigard et al. 1991). These data also seem to support the important role of acclimatisation or adaptation.

CS: Acute hypoxia effected a reduction in CS activity within EDL and gastrocnemius and slightly increased CS activity levels within soleus. However, after subjection to statistical scrutiny, we established that CS activity in all skeletal muscle groups was non significantly altered and conclude that the citric acid cycle - as a reference for total oxidative capacity - is not affected by short exposures to hypobaric hypoxia. Affirming our evidence, Kennedy et al. (2001) established that CS activity in different skeletal muscles is unaffected by acute (24 hours) altitude exposure.

Oxidative responses to chronic hypoxia are inconclusive and hard to assess. Kennedy et al. (2001) found CS activity in skeletal muscle unaltered by chronic (5 weeks) altitude exposure, prompting their assertion that mitochondrial content remains unchanged. However, Hochachka (1992) reported a very low absolute CS activity in Quechuas and Sherpas compared to lowlanders while Desplanches et al. (1996) reported reduced muscle oxidative capacity (CS activity) in native highland populations, as well as for lowland populations after prolonged exposure to hypoxia. Interestingly, chronic hypoxia did not affect CS activity levels in cardiac muscle (Daneshrad et al. 2000).

Exercise training in hypoxia enhanced CS activity in human quadriceps muscle (Melissa et al. 1997) and likewise increased CS levels in EDL (rat) about 24% above those of sedentary controls and sea level trained controls (Bigard et al. 1991). While these data may suggest that hypoxia is responsible for increased oxidative capacity, Desplanches et al. (1996) demonstrated that acclimated subjects who trained in hypoxia as well as in normoxia induced a 45 % increase in vastus lateralis CS activity, and concluded that exercise training rather than hypoxia is the reason for increased oxidative enzyme activity. On the contrary, Howald et al. (1990) report that muscle oxidative capacity is moderately reduced after severe exercise at altitude; Mount Everest climbers demonstrated that CS activities in the vastus lateralis muscle were reduced by 20 % after completion of the expedition and a return to sea level.

Glycolytic Enzymes

PFK: We found that PFK levels were increased in gastrocnemius and soleus in response to acute hypoxia but were more or less unaltered in EDL. Only the increase in activity of gastrocnemius muscle tended towards statistical significance ($p = 0.06$). Our results therefore do not conclude an upregulation of the glycolytic pathway and we were unable to establish increased reliance on glucose substrate in any of the muscles as a consequence of acute oxygen deprivation.

Once more, a literature review reveals inconsistencies with respect to the effect of oxygen reduction on metabolic pathways. Yoshino et al. (1990) suggest that acute hypoxic exposure shifts metabolism to anaerobic glycolysis; tissue hypoxia causes a decrease in ATP and an increase in AMP and this results in the activation of PFK. High intensity training in hypoxia and in normoxia was revealed to increase levels of PFK mRNA in vastus lateralis muscle, with the larger changes attributable to a hypoxic stimulus (Hoppeler & Vogt, 2001). On the contrary, native Highlanders trained in either hypoxia or acute normoxia demonstrated no significant increase in activity of PFK in the vastus lateralis (Desplanches et al. 1996). Once again, this points to acclimatisation playing a role in the response to hypoxia yet Green et al. (1989) established that maximal activities of glycolytic enzymes (as well as those of the citric acid cycle and β oxidation) were unchanged in human vastus lateralis after 40 days of progressive altitude to a simulated summit of Mount Everest.

LDH: Lactate is a metabolic intermediate which is directed to mitochondrial oxidation by a proposed lactate shuttle (Brooks in McClelland et al. 2002). LDH is purported to be involved in the shuttling of lactate between cells (Brooks in McClelland et al. 2002).

LDH activity was reduced in skeletal muscle of Wistar rats that exercised in hypoxia (Bigard et al. 1991) but remained unchanged in soleus muscle (and myocardium) of chronically exposed, unexercised rats (Ou & Leiter, 2004). Although Daneshrad et al. (2000) demonstrated enhanced LDH levels in cardiac muscle chronically (3 weeks) exposed to oxygen deficiency, this elevation was found to result more from the diet restriction which accompanies hypoxia. While dietary restriction was evident in our study, the short duration exposure was insufficient to

induce such a change in skeletal muscle. McClelland & Brooks (2002) reported that longterm hypoxia influenced lactate metabolism and LDH expression in rat soleus and plantaris muscles to a greater extent than in red or white gastrocnemius, which showed no significant response. These data and our results confirm the heterogeneity between muscle groups in small mammals.

Our experiments showed LDH activity to be unaffected by acute hypoxia, meaning that reduced levels of ambient oxygen in the short term did not promote breakdown of glucose to lactate via the anaerobic pathway. These results, in conjunction with those obtained for PFK, prompt the assertion that glycolytic pathways in skeletal muscle were not altered by an acute exposure to hypobaric hypoxia. The implications in so far as substrate utilisation is concerned will be further examined in the following segment on metabolic potentials.

Metabolic Potentials

Enzyme activity ratios may yield better insight into metabolic organisation than absolute activities (Hochachka 1992) and when changes in absolute enzyme activities are not evidenced, these may become apparent in the ratios (Green et al. 1989).

CS:HAD Although hypoxia appeared to reduce this ratio (and by inference the contribution of fatty acid oxidation relative to total oxidative capacity) in gastrocnemius and EDL these changes were not proven statistically meaningful. The relative amount of fat metabolism in soleus muscle remained almost unaltered by hypoxia. These data infer that hypoxia of short duration does not alter the levels of fat acid oxidation in so far as it contributes to total oxidative capacity. Similarly, Green et al. (1989) found HAD / CS ratios to be unaffected after several days of exposure to progressive hypobaric hypoxia.

LDH:CS Had the observed increase in LDH:CS in EDL and gastrocnemius after exposure to hypoxia proved significant, then this would have inferred increased utilisation of the anaerobic component of the glycolytic pathway at the expense of the aerobic and oxidative component of the glycolytic pathway. However, this was not the case and we report no effect on anaerobic metabolism in any muscle types following acute hypoxic exposure. While our data are in

keeping with unchanged LDH / CS, as described by Green et al. (1989), after progressive altitude, Tanaka et al. (1997) contrastingly found that this potential tended to be lower in skeletal muscle exposed to hypobaric hypoxia.

LDH:HAD Exposure to an acute hypoxic environment did not alter the potential preference for substrate in the different skeletal muscles. Soleus inherently has considerably more potential for fatty acid (oxidative) metabolism than either gastrocnemius or EDL but oxygen depletion for a period of 48 hours was not sufficient to 'upregulate' glycolytic pathways in this or the other muscles.

PFK:LDH This permutation allows one to examine that proportion of carbohydrate which is anaerobically metabolised to lactate as a proportion of total carbohydrate utilisation potential. Hochacka et al. (1983) reported a correlation with PFK / LDH ratios (in conjunction with PFK and LDH maximal activities) and the way the glycolytic pathway is used. A high ratio is indicative of muscle enzyme organisation geared for aerobic glucose catabolism while a low ratio is indicative either of preferential fat catabolism or high anaerobic glycolytic capacity (Hochachka 1992).

While we report that all ratios were low (≤ 0.08), these data need to be cautiously assessed in the context of maximal PFK and LDH activities. In the case of EDL and gastrocnemius these ratios corroborate high anaerobic glycolytic capacity in comparison to soleus. The derived ratio of 0.08 for soleus, examined in conjunction with PFK and LDH levels, is indicative of significantly low, yet proportional maximal enzyme activities in this muscle, as well as a preferential fat utilisation capacity (determined by LDH:HAD).

We report that oxygen deprivation of short duration did not significantly influence either aspect of carbohydrate metabolism and did not increase the reliance of any of the tested muscles on anaerobic pathways in preference to potential carbohydrate oxidation. On the other hand , after chronic exposure to simulated altitude, Green et al. (1989) found LDH / PFK to be elevated and attributed this alteration to a reduction in PFK, which indicated improved ability of the muscle cell for pyruvate disposal via lactate formation relative to glycolysis.

Effect of Nutritional Status

Altitude can lead to dietary imbalance and weight loss - often due to reduced energy intake as well as elevated basal metabolic rate (Kennedy et al. 2001) - and possibly to substrate depletion in muscle cells (Desplanche et al. 1996; Kennedy et al. 2001).

Although, in our experience, rats that were exposed to acute hypobaric hypoxia lost on average 8.3 grams body mass and consumed significantly less food (33.3 g) than counterparts kept in normoxia (46.2 g), this weight loss and reduced energy intake did not affect the maximal activities of any of the metabolic enzymes tested and consequently did not alter substrate utilisation. Voluntary activity of rats in hypoxia was noticeably reduced, however this remains an observation as we did not have the technology available to quantify movement.

CONCLUSION

In conclusion, this research reveals the robustness of skeletal muscle of any fibre composition in response to hypoxia of acute duration. This starkly contrasts with cardiac muscle which responds very rapidly (within 48 hours) by changes in gene regulation and substrate utilisation (Daneshrad et al. 2000; Essop et al. 2004; Ngumbela et al. 2003; Sharma et al. 2004). We propose that hypoxic exposure of an acute nature, is insufficient to elicit the changes in skeletal muscle that have been documented after chronic exposure or adaptation, albeit these research findings are confounding. Kennedy et al. (2001) lend support to this by proposing that short duration studies may not allow time for mitogenesis or other mitochondrial adaptations.

The lack of consensus concerning the effects of oxygen-deficiency on skeletal muscle metabolism, may be due to a combination of factors such as duration of exposure, degree of hypoxia, degree of activity and species variations (Kennedy et al. 2001). Care should be exercised when extrapolating trans-specific findings, for example, rat skeletal muscle may be less (or more) responsive to both acute and chronic hypoxia than human muscle and this may be related to the demand for oxygen rather than the limited supply *per se*. While mountaineering expeditions have been the models used for some human observations, factors such as low temperatures, dietary imbalance and extremes of exercise are each potentially capable of modifying or interacting with the hypoxic stressor (Green et al. 1989).

Muscle sampling procedures and fluorometry-based assays were proven statistically reproducible and attested to both the reliability of the equipment and the procedures used for the assessment of enzyme activity. Although it may have been useful to measure concomitant changes in cardiac muscle we neglected to collect heart from our experimental animals. It may also have been informative to monitor each individual rat's voluntary activity had the technology been available.

I speculate that data being gathered in the twin-study will reveal that AMPK expression adapts to short duration hypoxia, even in skeletal muscle which is less responsive than myocardium.

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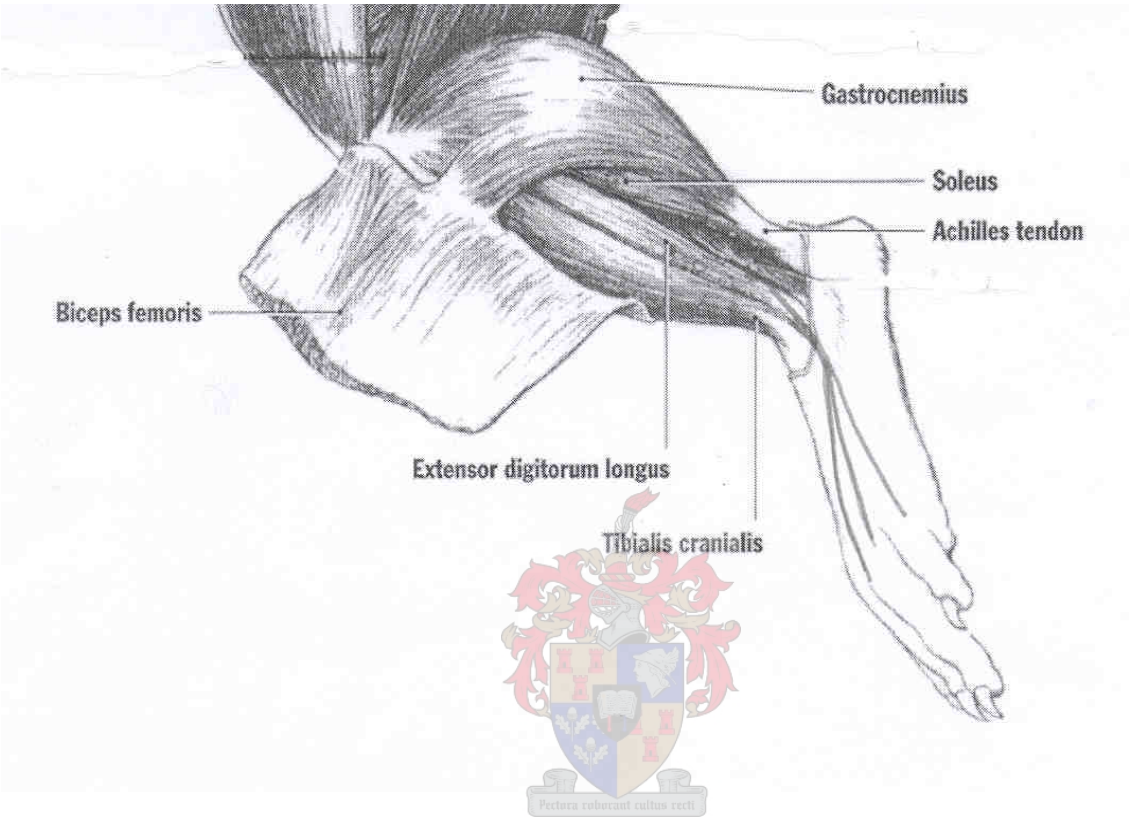
APPENDIX Anatomy of Hindlimb

Rat lower hindlimb (lateral aspect) showing the location of soleus, EDL and gastrocnemius muscles



At dissection: the EDL was identified by tracing it to the associated tendons which insert on the outer four toes. The muscle was snipped off at the site where the tendons begin branching and again where the EDL originates at the condyle of the fibula. The EDL is a small muscle requiring skill in its extraction.

The soleus is strap-like, deep red in colour and easily identified by cutting the achilles tendon and peeling back the gastrocnemius. It was cut at the achilles tendon and at the tibial head.

The gastrocnemius is conspicuously large and consists of two bellies. The muscle bellies were separated and each was preserved for enzyme assays.

* In dissecting gastrocnemius I consistently sampled across the middle of the belly but was perhaps less consistent with the area (deep vs. superficial) I attained. This may have increased the chances for sampling error. EDL and soleus are mostly homogenous with negligible fibre regionalisation (Wang et al. 2001) and since each muscle was cross-sectioned across the midpoint, sampling error was minimised.

APPENDIX Reagents and Protocols

1. Homogenising Potassium Buffer (pH 7.3)

A. KH_2PO_4	0.1M	To 30 ml buffer B, in a small beaker with a pH probe, add buffer A to adjust the pH to 7.3. Homogenising buffer can be stored at -20°C
B. K_2HPO_4 anhydrous	0.1M	

2. Phosphofructokinase (PFK) Reaction Buffer

	Stock	Final Concentration (FC)
TRIS (pH 8)	0.5 M	0.05 M
ATP (disodium salt)	0.1 M	1 mM
AMP	0.1 M	1 mM
$MgCl_2$	1 M	2 mM
Na_2HPO_4	0.25 M	1.25 mM
Fructose 6 Phosphate	0.1 M	1 mM
BSA	10 %	0.05 %
Mercaptoethanol	14.2 M	1 mM
NADH	0.1 M	10 µM
Aldolase	10 mg/ml	12 µg/ml
Triosephosphate isomerase (TPI) + glyceraldehyde phosphate dehydrogenase (GDH)	10 mg/ml	15 µg/ml

3. Lactate dehydrogenase (LDH) Reaction Buffer

	Stock	FC
TRIS (pH 8)	0.5 M	50 mM
EDTA	0.1 M	5 mM
NADH	0.1 M	30 µM
Pyruvate	0.1 M	2 mM

4. Hydroxyacyl CoA dehydrogenase (HAD) Reaction Buffer

	Stock	FC
EDTA	0.1 M	4 mM
TRIS (pH 8)	0.5 M	50 mM
NADH	0.1 M	30 µM
Acetoacetyl CoA	1 mM	0.02 mM

5. Citrate synthase (CS) Reaction Buffer

	Stock	FC
TRIS (pH 8)	0.5 M	0.1 M
EDTA	0.1 M	2.5 mM
Malate	0.1 M	1 mM
NAD	0.1 M	0.5 mM
Acetyl CoA	3 mM	0.06 mM
Malate dehydrogenase	5 mg/ml	8 µg/ml

6. Operations: 'KC JUNIOR' software program

Open MODIFY PROTOCOL and PROTOCOL DEFINITION in order to ascertain that:

- excitation wavelength is at 340/30 and emission wavelength is at 460/40
- optics is set at TOP and sensitivity is set at 100
- 'Shaking' and 'Incubation' are disabled (not options with this program)
- 'Read Intervals' are specific for each enzyme assay

Select READ METHOD and TEMPLATE to specify the wells to be read

Select *Kinetics* for enzyme assays (*Endpoint* for standard curve)

Select READ PLATE and READ and go to final dialog box before loading samples

- Load samples into microtitre plate (For standard curve, read an empty multiwell plate to determine background fluorescence)

Select RESULTS once readings complete and EXPORT DATA to empty Excel worksheet

Arrange raw data horizontally, then COPY to specifically formulated worksheet

Insert the slope value obtained from NADH standard curve in the F/min row of the spreadsheet

APPENDIX Assessment of Assay Reliability

Reliability and reproducibility of the fluorometric equipment was assessed on duplicate muscle preparations. For each assay, ANOVA was determined and results were represented graphically (Figs. A -12). In all instances Y axis values are µmol / min / g dry wt.

In none of the assays were there significantly variant absorbancy readings. These data suggest that the equipment was reliable and that the fluorometric recordings were reproducible.

HAD assay

Although there were slight variations in recordings for normoxic as well as hypoxic gastrocnemius and EDL samples (Fig.A), these differences were not significant.

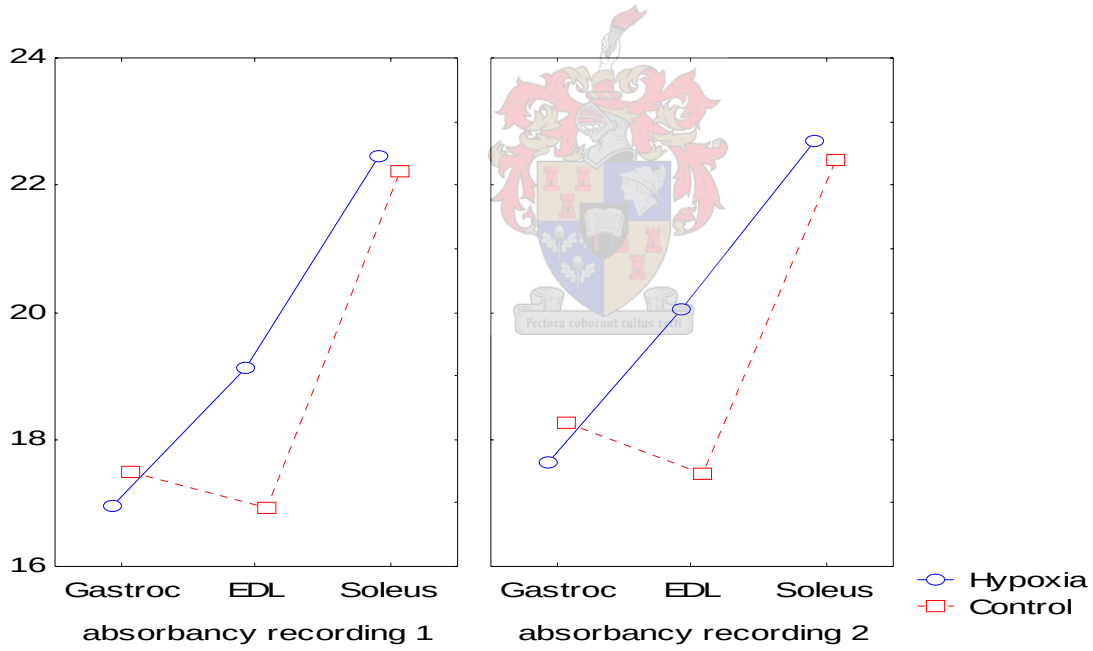


Figure A. HAD assay: repetitive fluorometric readings

CS assay

Although the graphs in Fig.B show some variation in the duplicity of the fluorometric recordings, more especially those readings of hypoxic muscle tissue, ANOVA determined the variance insignificance.

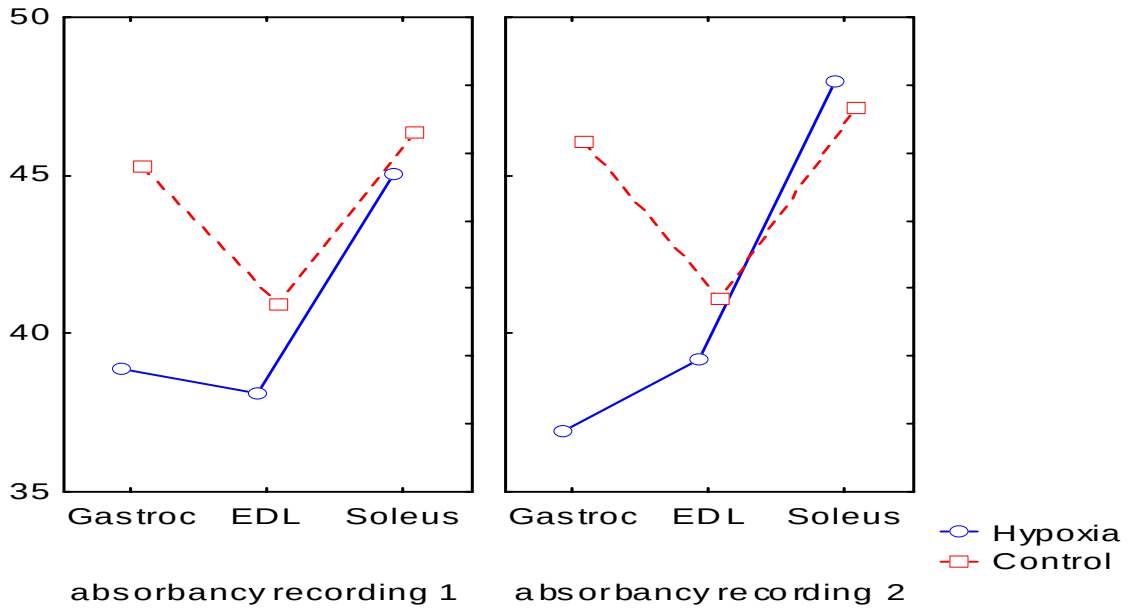


Figure B. CS assay: repetitive fluorometric readings

LDH assay

Figure C shows that there is some variation between fluorometric recordings with respect to hypoxic gastrocnemius and EDL muscles. These were found to be non significant.

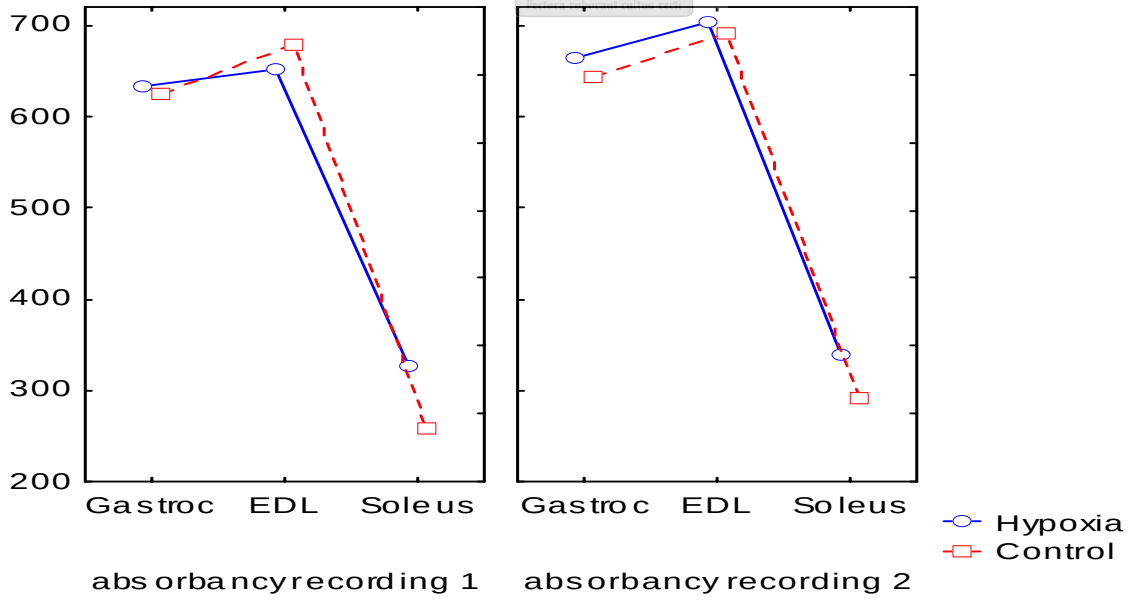


Figure C. LDH assay: repetitive fluorometric readings

PFK assay

No variations between replicate absorbancy recordings (Fig. D) were of any significance.

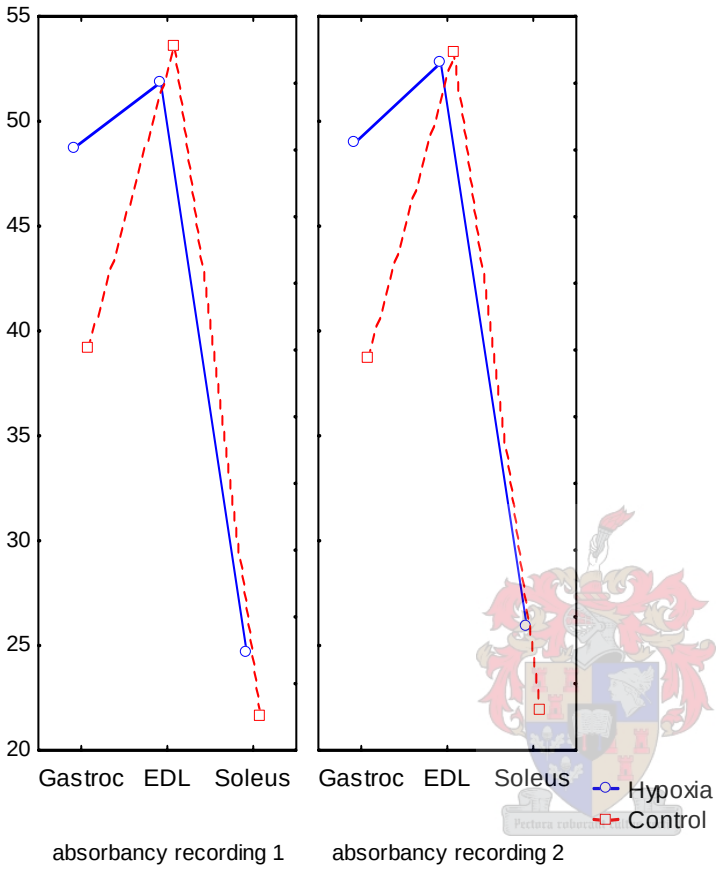


Figure D. PFK assay: repetitive fluorometric readings

APPENDIX Statistical Analyses of Enzyme Activities

Maximal Enzyme Activities

In all statistical tables the following abbreviations apply: *SS* for sum of squares; *DF* for degrees of freedom; *MS* for mean of squares; *SD* for standard deviation; *SE* for standard error.

1. Hydroxyacyl CoA Dehydrogenase

Table 1.1 HAD activity (µmol/ min/ g dry wt) within various experimental / control muscle groups

Muscle	Condition	HAD activity means	SD	SE
EDL	hypoxia	19.58	8.03	2.08
	normoxia	17.19	3.26	
Gastrocnemius	hypoxia	17.30	2.95	2.08
	normoxia	17.87	5.96	
Soleus	hypoxia	23.27	6.41	2.08
	normoxia	22.32	4.53	

Table 1.2 MANOVA for HAD activity

	SS	DF	MS	F test	probability
hypoxia / normoxia	8.96	1	8.96	0.2973	0.588943
muscle type	220.77	2	110.39	3.6611	0.035698
hypox normox*muscle	15.32	2	7.66	0.2541	0.777001
Error	1085.44	36	30.15		

Table 1.3 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	HAD	1	2	3	4	5	6
1	hypoxic EDL		0.441908	0.216163	0.421172	0.563355	0.356576
2	hypoxic gastroc	0.441908		0.049109	0.971378	0.847032	0.095600
3	hypoxic soleus	0.216163	◊ 0.049109		0.045428	0.073698	0.747052
4	normoxic EDL	0.421172	0.971378	◊ 0.045428		0.819061	0.089060
5	normoxic gastroc	0.563355	0.847032	0.073698	0.819061		0.137959
6	normoxic soleus	0.356576	0.095600	0.747052	0.089060	0.137959	

2. Citrate Synthase

Table 2.1 CS activity (µmol/ min/ g dry wt) within various experimental / control muscle groups

Muscle	Condition	CS activity means	SD	SE
EDL	hypoxia	38.67	10.47	3.94
	normoxia	43.46	5.08	
Gastrocnemius	hypoxia	37.90	11.44	3.94
	normoxia	45.65	15.68	
Soleus	hypoxia	48.60	7.70	3.94
	normoxia	46.74	9.03	

Table 2.2 MANOVA for CS activity

	SS	DF	MS	F test	probability
hypoxia / normoxia	133.30	1	133.30	1.2248	0.275763
muscle type	368.35	2	184.18	1.6922	0.198423
hypox ctrl*muscle	169.82	2	84.91	0.7802	0.465921
Error	3918.10	36	108.84		

Table 2.3 Fisher *post hoc* test (LSD) ($p \leq 0.05$)

	CS	1	2	3	4	5	6
1	hypoxic EDL		0.890643	0.083270	0.395626	0.218488	0.156346
2	hypoxic gastroc	0.890643		0.062814	0.324846	0.172794	0.121441
3	hypoxic soleus	0.083270	0.062814		0.362789	0.600010	0.740494
4	normoxic EDL	0.395626	0.324846	0.362789		0.696856	0.560204
5	normoxic gastroc	0.218488	0.172794	0.600010	0.696856		0.846259
6	normoxic soleus	0.156346	0.121441	0.740494	0.560204	0.846259	

3. Phosphofructokinase

Table 3.1 PFK activity (µmol/ min/ g dry wt) within various experimental / control muscle groups

muscle	condition	PFK activity means	SD	SE
EDL	hypoxia	52.33	11.52	3.86
	normoxia	53.40	6.56	
Gastrocnemius	hypoxia	48.86	15.32	3.86
	normoxia	38.23	6.58	
Soleus	hypoxia	24.94	12.88	3.86
	normoxia	21.77	2.56	

Table 3.2 MANOVA for PFK activity ◊ denotes significance

	SS	DF	MS	F test	probability
hypoxia / normoxia	189.04	1	189.04	1.8118	0.186710
muscle type	6369.47	2	3184.74	30.5232	◊0.000000
hypox ctrl*muscle	245.90	2	122.95	1.1784	0.319368
Error	3756.18	36	104.34		

Table 3.2 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	PFK	1	2	3	4	5	6
1	hypoxic EDL		0.529734	0.000014	0.845878	0.014019	0.000002
2	hypoxic gastroc	0.529734		0.000098	0.411821	0.059242	0.000017
3	hypoxic soleus	◊ 0.000014	◊ 0.000098		0.000008	0.020031	0.566096
4	normoxic EDL	0.845878	0.411821	◊ 0.000008		0.008625	0.000001
5	normoxic gastroc	◊ 0.014019	0.059242	◊ 0.020031	◊ 0.008625		0.004714
6	normoxic soleus	◊ 0.000002	◊ 0.000017	0.566096	◊ 0.000001	◊ 0.004714	

4. Lactate dehydrogenase

Table 4.1 LDH activity (µmol/ min/ g dry wt) within various experimental / control muscle groups

muscle	condition	LDH activity means	SD	SE
EDL	hypoxia	691.98	62.61	28.92
	normoxia	705.17	70.51	
Gastrocnemius	hypoxia	660.38	81.92	28.92
	normoxia	645.55	84.41	
Soleus	hypoxia	304.41	105.12	28.92
	normoxia	282.75	36.63	

Table 4.2 MANOVA for LDH activity ◊ denotes significance

	SS	DF	MS	F test	probability
hypoxia / normoxia	633	1	633	0.108	0.744128
muscle type	1377885	2	688943	117.701	◊ 0.000000
hypox ctrl*muscle	2386	2	1193	0.204	0.816549
Error	210720	36	5853		

Table 4.3 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	LDH	1	2	3	4	5	6
1	hypoxic EDL		0.444685	0.000000	0.748987	0.263668	0.000000
2	hypoxic gastroc	0.444685		0.000000	0.280687	0.718947	0.000000
3	hypoxic soleus	◊ 0.000000	◊ 0.000000		0.000000	0.000000	0.599760
4	normoxic EDL	0.748987	0.280687	◊ 0.000000		0.153526	0.000000
5	normoxic gastroc	0.263668	0.718947	◊ 0.000000	0.153526		0.000000
6	normoxic soleus	◊ 0.000000	◊ 0.000000	0.599760	◊ 0.000000	◊ 0.000000	

Metabolic Potentials (Enzyme Activity Ratios)

A univariate test of significance was performed before the Fisher test, on each occasion.

5. CS:HAD

Table 5.1 Ratio CS:HAD means, standard deviations and standard error

muscle	condition	Mean	SD	SE
EDL	hypoxia	2.23	1.00	0.25
	normoxia	2.59	0.47	
gastrocnemius	hypoxia	2.23	0.67	0.25
	normoxia	2.62	0.72	
soleus	hypoxia	2.18	0.54	0.25
	normoxia	2.13	0.38	

Table 5.2 Fisher *post hoc* test (LSD) ($p \leq 0.05$)

	CS:HAD	1	2	3	4	5	6
1	hypoxic EDL		0.997126	0.903615	0.310551	0.270808	0.793678
2	hypoxic gastroc	0.997126		0.906467	0.308872	0.269283	0.796453
3	hypoxic soleus	0.903615	0.906467		0.257520	0.222872	0.888238
4	normoxic EDL	0.310551	0.308872	0.257520		0.928915	0.204576
5	normoxic gastroc	0.270808	0.269283	0.222872	0.928915		0.175525
6	normoxic soleus	0.793678	0.796453	0.888238	0.204576	0.175525	

6. LDH:CS

Table 6.1 Ratio LDH:CS means, standard deviations and standard error

Muscle	condition	Mean	SD	SE
EDL	hypoxia	18.9	4.66	1.5
	normoxia	16.3	1.87	
gastrocnemius	hypoxia	18.7	5.08	1.5
	normoxia	15.9	6.28	
soleus	hypoxia	6.4	2.15	1.5
	normoxia	6.3	1.72	

Table 6.2 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	LDH:CS	1	2	3	4	5	6
1	hypoxic EDL		0.913498	0.000001	0.23807	0.171083	0.000001
2	hypoxic gastroc	0.913498		0.000002	0.282797	0.206234	0.000002
3	hypoxic soleus	◊0.000001	◊0.000002		0.00005	0.00009	0.97306
4	normoxic EDL	0.238070	0.282797	◊0.00005		0.845051	0.000045
5	normoxic gastroc	0.171083	0.206234	◊0.00009	0.845051		0.000081
6	normoxic soleus	◊0.000001	◊0.000002	0.97306	◊0.000045	◊0.00008	

7. LDH:HAD

Table 7.1 Ratio LDH:HAD means, standard deviations and standard error

muscle	condition	Mean	SD	SE
EDL	hypoxic	40.2	15.63	4.1
	normoxic	42.4	9.66	
gastrocnemius	hypoxic	39.0	7.50	4.1
	normoxic	40.1	16.76	
soleus	hypoxic	13.5	4.07	4.1
	normoxic	13.4	4.51	

Table 7.2 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	LDH:HAD	1	2	3	4	5	6
1	hypoxic EDL		0.839201	0.000053	0.713388	0.987631	0.00005
2	hypoxic gastroc	0.839201		0.000098	0.56913	0.851327	0.000092
3	hypoxic soleus	◊0.000053	◊0.000098		0.000017	0.000056	0.983069
4	normoxic EDL	0.713388	0.56913	◊0.000017		0.701893	0.000016
5	normoxic gastroc	0.987631	0.851327	◊0.000056	0.701893		0.000052
6	normoxic soleus	◊0.00005	◊0.000092	0.983069	◊0.000016	◊0.000052	

8. PFK:LDH

Table 8.1 Ratio PFK:LDH means, standard deviations and standard error

muscle	condition	Mean	SD	SE
EDL	hypoxia	0.08	0.02	0.01
	normoxia	0.08	0.02	
gastrocnemius	hypoxia	0.07	0.02	0.01
	normoxia	0.06	0.01	
soleus	hypoxia	0.08	0.02	0.01
	normoxia	0.08	0.02	

Table 8.2 Fisher *post hoc* test (LSD) ($p \leq 0.05$) ◊ denotes significance

	PFK:LDH	1	2	3	4	5	6
1	hypoxic EDL		0.750624	0.658287	0.978805	0.087887	0.819835
2	hypoxic gastroc	0.750624		0.448534	0.730603	0.160187	0.585929
3	hypoxic soleus	0.658287	0.448534		0.677546	0.034288	0.829784
4	normoxic EDL	0.978805	0.730603	0.677546		0.083342	0.840531
5	normoxic gastroc	0.087887	0.160187	◊ 0.034288	0.083342		0.054951
6	normoxic soleus	0.819835	0.585929	0.829784	0.840531	◊ 0.05495	

APPENDIX Effect of Food on Enzyme Activity

Table B1. HAD activity: Effect of food consumption/weight loss (ANOVA)

	SS	DF	MS	F	p
food consumpt (g)	47.567	1	47.5665	1.55825	0.220449
wt change (g)	25.595	1	25.5948	0.83847	0.366283
hypox / normox	2.926	1	2.9257	0.09584	0.758765
Error	1037.868	34	30.5255		

Table B2. CS activity: Effect of food consumption/weight loss (ANOVA)

	SS	DF	MS	F	p
food consumpt (g)	164.428	1	164.4282	1.491157	0.230438
wt change (g)	118.654	1	118.6536	1.076039	0.306907
hypox / normox	72.763	1	72.7630	0.659869	0.422258
Error	3749.141	34	110.2689		

Table B3. PFK activity: Effect of food consumption/weight loss (ANOVA)

	SS	DF	MS	F	p
food consumpt (g)	167.817	1	167.817	1.59045	0.215847
wt change (g)	79.890	1	79.890	0.75714	0.390328
hypox / normox	55.104	1	55.104	0.52223	0.474833
Error	3587.535	34	105.516		

Table B4. LDH activity: Effect of food consumption/weight loss (ANOVA)

	SS	DF	MS	F	p
food consumpt (g)	9623	1	9622.7	1.6474	0.207994
wt change (g)	1489	1	1489.3	0.2550	0.616863
hypox / normox	3119	1	3119.3	0.5340	0.469931
Error	198601	34	5841.2		