

CHARACTERISTICS AND ADAPTATION OF SKELETAL MUSCLE TO ENDURANCE EXERCISE



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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature

Date



SUMMARY

Skeletal muscle adapts to stimuli by modifying structural and metabolic protein expression. Furthermore, a muscle group may vary within itself to accommodate specialisation in regions. Structural and metabolic characteristics of an individual are regulated partly by genotype, but contraction duration and intensity may play a greater role in muscle phenotype. The aims of this dissertation were to investigate: structural and metabolic regionalisation in a muscle group, possible relationships between training volume and intensity and hybrid fibres, muscle characteristics of athletes from two different ethnic groups, and muscle adaptation in already well-trained athletes subjected to high intensity interval training.

Myosin heavy chain (MHC) isoform content and citrate synthase (CS) activities were measured in the *Quadriceps femoris* (QF) muscle of 18 female rats. Muscle was divided into superficial, middle and deep, distal, central and proximal parts. MHC IIb and IIx were more abundant in superficial regions ($P < 0.05$) with low CS activities compared to deeper parts. Isoform content varied along the length of deep regions. This study showed that the QF has regional specialisation. Therefore, standardisation of sampling site is important.

Hybrid fibre proportions in muscle biopsies of 12 middle distance runners and 12 non-runners were investigated. MHC IIa/IIx correlated with training volume/week in runners ($r = -0.66$, $P < 0.05$) and MHC IIa/IIx correlated with exercise hours/week in non-runners ($r = -0.72$, $P < 0.01$). Average preferred racing distance (PRD_A) correlated better with MHC IIa/IIx in runners ($r = -0.85$, $P < 0.001$). MHC IIa/IIx may therefore be more closely related to exercise intensity than previously thought.

Fibre type characteristics and performance markers were investigated in 13 Xhosa and 13 Caucasian distance runners, matched for performance, training volume and PRD_A . Xhosa runners had less MHC I and more MHC IIa fibres in muscle biopsies than Caucasian runners ($P < 0.05$). Xhosa runners had lower plasma lactate at 80% peak treadmill speed (PTS) ($P < 0.05$), but higher lactate dehydrogenase (LDH) ($P < 0.01$) and phosphofructokinase ($P = 0.07$) activities in homogenate muscle samples. LDH activities in MHC I ($P = 0.05$) and IIa ($P < 0.05$) fibre pools were higher in Xhosa runners. Xhosa athletes may thus have a genetic advantage or they may have adapted to running at a higher intensity.

Six weeks of individually standardised high intensity interval treadmill training (HIIT) were investigated in 15 well-trained runners. PTS increased after HIIT ($P < 0.01$), while maximum oxygen consumption (VO_{2max}) only showed a tendency to have increased as a result of HIIT ($P =$

0.06). Sub-maximal tests showed lower plasma lactate at 64% PTS ($P = 0.06$), with lower heart rates at workloads from 64% to 80% PTS ($P < 0.01$) after HIIT. No changes were observed for cross-sectional area, capillary supply and enzyme activities in homogenates muscle samples. LDH activity showed a trend ($P = 0.06$) to have increased in MHC IIa pools after HIIT. Higher HIIT speed was related to decreases in MHC I fibres, but increases in MHC IIa/IIx fibres ($r = -0.70$ and $r = 0.68$, respectively, $P < 0.05$). Therefore, HIIT may alter muscle fibre composition in well-trained runners, with a concomitant improvement in performance markers.



Skeletspier kan adapteer deur strukturele en metaboliese proteïen ekspresie te verander as gevolg van stimulant. 'n Spiergroep kan ook intern verskil om spesialisering in spierdele toe te laat. Strukturele en metaboliese karaktereïenskappe van 'n individu word deels gereguleer deur gene, maar kontraksie tydperk en intensiteit mag 'n groter rol speel in spierfenotipe. Die doelwitte van hierdie tesis was om ondersoek in te stel in: strukturele en metaboliese eienskappe in spiergroepstreke, moontlike verhoudings tussen oefeningsvolume of intensiteit en baster vesels, spier eienskappe in atlete van twee etniese groepe, en spier adaptasie in goed geoefende atlete blootgestel aan hoë intensiteit interval oefening.

Miosien swaar ketting (MSK) isovorm inhoud en sitraat sintase (SS) aktiwiteit is gemeet in die *Quadriceps femoris* (QF) spier van 18 wyfies rotte. Spiere was opgedeel in oppervlakkig, middel en diep, asook distaal, sentraal en proksimale dele. MSK Iib en Iix was meer oorvloedig in oppervlakkige dele ($P < 0.05$) met lae SS aktiwiteit in vergelyking met dieper dele. Isovorm inhoud het ook verskil oor die lengte van diep dele. Dus bevat die QF gespesialiseerde streke en is die area van monsterneming belangrik.

Baster vesel proporsies is ondersoek in spiermonsters van 12 middel afstand hardlopers en 12 nie-hardlopers. MSK Iia/Iix van hardlopers het met oefeningsvolume/week gekorreleer ($r = -0.66$, $P < 0.05$), asook MSK Iia/Iix van nie-hardlopers met oefeningsure/week ($r = -0.72$, $P < 0.01$). Gemiddelde voorkeur wedloop afstand (VW_{AG}) het beter met MSK Iia/Iix gekorreleer in hardlopers ($r = -0.85$, $P < 0.001$). MSK Iia/Iix mag dus meer verwant wees aan oefeningsintensiteit.

Veseltipe eienskappe en prestasie merkers was ondersoek in 13 Xhosa en 13 Caucasian langafstand atlete, geëwেকnie vir prestasie, oefeningsvolume en VMA_G . Xhosa hardlopers het minder tipe I en meer tipe IIA vesels in hul spiermonsters gehad as die Caucasian hardlopers ($P < 0.05$). Xhosa hardlopers het laer plasma laktaat by 80% van hul maksimale trapmeul spoed (MTS) ($P < 0.05$), maar hoër laktaat dihidrogenase (LDH) ($P < 0.01$) en fosfofruktokinase ($P = 0.07$) aktiwiteit in homogene spiermonsters gehad. LDH aktiwiteit in MSK I ($P = 0.05$) en Iia ($P < 0.05$) veselbondels was hoër in Xhosa hardlopers. Xhosa atlete mag dus 'n genetiese voorsprong geniet, of hulle het geadapteer om by hoër intensiteite te hardloop.

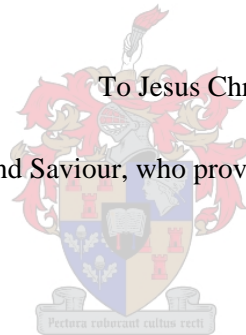
Ses weke van geïndividualiseerde gestandaardiseerde hoë intensiteit interval trapmeul oefening (HIIT) was ondersoek in 15 goed geoefende hardlopers. MTS het verhoog na HIIT ($P < 0.01$), en maksimale suurstof verbruik (VO_{2max}) het 'n neiging getoon om te verhoog het na HIIT ($P = 0.07$). Submaksimale toetse het laer plasma laktaat by 64% MTS getoon ($P = 0.06$), met laer harttempos

by werkladings 64% tot 80% MTS ($P < 0.01$). Geen veranderings was gemerk vir deursnit area, kapillêre toevoer en ensiem aktiwiteite in homogene spiermonsters nie. LDH aktiwiteit het 'n neiging getoon om te verhoog het ($P = 0.06$) in MSK IIa veselbondels na HIIT. Hoër HIIT snelhede was verwant aan 'n daling in MSK I vesels, maar 'n verhoging in MSK IIa/IIx vesels ($r = -0.70$ en $r = 0.68$, respektiwelik, $P < 0.05$). HIIT mag dus spier veseltipe verander in goed geoefende hardlopers, met gevolglike verbetering in prestasie merkers.



To Jesus Christ ...

... my Lord and Saviour, who provides in my everyday needs.



ACKNOWLEDGEMENTS **5**

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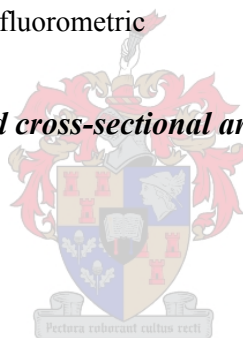
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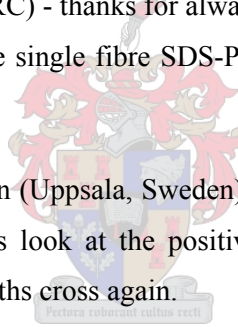
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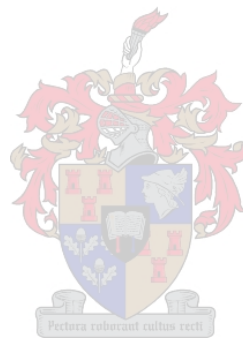
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ABBREVIATIONS

(NH ₄) ₂ S	ammonium sulphide	EDTA	ethylenediaminetetraacetic acid
°C	degrees Celsius	ETC	electron transport chain
μ	micro	F-1-6-BP	fructose 1,6 bisphosphate
3HAD	3-hydroxyacyl CoA dehydrogenase	F-6-P	fructose 6 phosphate
acetyl CoA	acetyl Coenzyme A	FD	fibre diameter
ADP	adenosine diphosphate	FFA	free fatty acids
AMP	adenosine monophosphate	g	gram
AMPK	AMP-activated protein kinase	GDH	glyceraldehyde-3-phosphate dehydrogenase
ANOVA	analysis of variance	GLUT	glucose transporter
APS	ammonium persulfate	GM	gastrocnemius medialis
AQM	acute quadriplegic myopathy	h	hour
ATP	adenosine triphosphate	H ⁺	hydrogen ions
ATPase	adenosine triphosphatase	H ₂ O	water
BMI	body mass index	HCl	hydrochloric acid
BSA	bovine serum albumin	HIF-1	hypoxia inducible factor 1
bts	beats	HIIT	high intensity interval training
Ca ²⁺	calcium ions	HK	hexokinase
CaCl ₂	calcium chloride	HR _{max}	maximal heart rate
CaM	calmodulin	Hz	hertz
CaMK	CaM-dependent protein kinase	ITS	interval training speed
CaMKK	CAMK kinase	K ⁺	potassium ions
CK	creatine kinase	K ₂ HPO ₄	di-potassium hydrogen phosphate
CKMM	muscle-specific creatine kinase	K ₂ SO ₄	potassium sulphate
cm	centimetres	KCl	potassium chloride
CO ₂	carbon dioxide	kDa	kilo Dalton
CoA	coenzyme A	kg	kilogram
CoCl ₂	cobalt chloride	KH ₂ PO ₄	potassium dihydrogen phosphate
COX	cytochrome oxidase	km	kilometres
Cr	creatine	LDH	lactate dehydrogenase
CS	citrate synthase	M	molar
CSA	cross-sectional area	m	metre
DMD	Duchene muscular dystrophy	MAPK	mitogen-activated protein kinase
DNA	deoxyribunucleic acid	MCIP	modulatory calcineurin interacting protein
DTNB	5,5' dithiobis 2-nitrobenzoic acid	MCT	monocarboxylate transporters
dw	dry weight	MDH	malate dehydrogenase
EDL	extensor digitorum longus	MEF2	myocyte-specific enhancer protein
Mg ²⁺	magnesium ions	PRD _A	average preferred racing distance
MgCl ₂	magnesium chloride	PTS	peak treadmill speed
MGF	mechano growth factor	QF	Quadriceps femoris

MHC	myosin heavy chain	RER	respiratory exchange ratio
min	minute	RNA	ribonucleic acid
MLC	myosin light chain	rpm	revolutions per minute
Mr	molecular weight	SD	standard deviation
mRNA	messenger RNA	SDH	succinate dehydrogenase
mTOR	mammalian target of rapamycin	SDS	sodium dodecyl sulfate
N	number of subjects	sec	second
NA	neurogenic atrophy	SERCA	sarcoplasmic reticulum calcium-ATPase
Na ⁺	sodium ions	TA	tibialis anterior
Na ₂ HPO ₄	di-sodium hydrogen phosphate	TCA	tricarboxylic acid cycle / Krebs cycle
NaCl	sodium chloride	TEMED	N,N,N',N'-tetramethylethylenediamine
NAD ⁺	oxidised nicotinamide adenine dinucleotide	TG	triglycerides
NADH	reduced nicotinamide adenine dinucleotide	Tmax	maximum time at Vmax
NaOH	sodium hydroxide	Tn	troponin
NF-AT	nuclear factor for T cells	TPI	triose phosphate isomerase
nm	nanometres	Tris	tris(hydroxymethyl)aminomethane
no.	number	TSH	thyroid stimulating hormone
ns	non-significant	TTF	time to fatigue
O ₂	oxygen molecule	UK	United Kingdom
PAGE	polyacrylamide gel electrophoresis	USA	United States of America
PAS	periodic acid stain	V ₀	shortening velocity
PCr	phosphocreatine	VCO ₂	volume of carbon dioxide exhalation
PDC	pyruvate dehydrogenase complex	VEGF	vascular endothelial growth factor
PFK	phosphofructokinase	VE _{max}	maximum ventilation volume per minute
P _i	inorganic phosphate	Vmax	initial speed associated with reaching VO ₂ max
PK	pyruvate kinase	VO ₂	volume of oxygen consumption
PO ₄ ²⁻	phosphate ions	VO ₂ max	maximal oxygen consumption
PPAR	peroxisome proliferator-activated receptors	wk	week
PPO	peak power output	ww	wet weight
PRD	preferred racing distance		

I “Are you born great, or made great?”

In the field of sport performance, the above question can be rephrased. “What determines if an individual will be the best in their chosen event?” For the past few decades, sport scientists have tried to answer this question by approaching it from various angles.

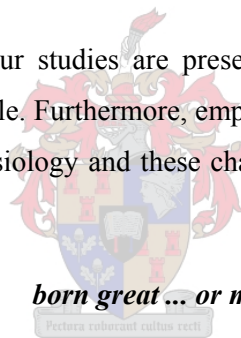
Considering the importance of individual determination and motivation, it could be speculated that many persons should be able to perform at Olympic level if they chose to apply themselves to this goal. Why then are there so few elite athletes in the world? Some favour the view that genetic factors influence performance significantly. Bouchard *et al.* (1992) speculated that up to 40% of endurance performance may be related to genetic factors, but that this value may in fact be lower. Scientists are currently seeking for those genes that influence sports performance.

Since the requirements for excellent performance are different in different sports, a genetic basis for Olympic success must be approached from the point of view of searching for genotypes related to specific phenotypes. Some gene polymorphisms have been found to be associated with certain phenotypes that are advantageous to exercise performance (Rankinen *et al.*, 2004; Rivera *et al.*, 1997a; Rivera *et al.*, 1997b). However, many experiments have indicated that the human body is very adaptable to exercise stimuli. Muscle is the machinery that generates motion, and is also one of those tissues that adapts very well to exercise. Structural and metabolic adaptations have been investigated in various animal and human exercise intervention studies, which include diverse stimuli e.g. from weight training to ultra endurance training. Therefore, where the search for exercise performance continues, it is still important to have a thorough understanding of the extent of adaptability of the intramuscular phenotype, as well as what effect this might have on whole body performance.

Specialisation in running (either sprinting, middle distance running or endurance) may be influenced by genetics. A few years ago, an article published in a local South African newspaper (Arlidge, 2000) claimed that East African black runners have a genetic advantage compared to others across the world when it comes to endurance events. The journalist quoted studies conducted by Saltin and co-workers (Saltin *et al.*, 1995b; Saltin *et al.*, 1995a). His interpretation of the scientific studies was that Kenyans are better at running events because of their slender build. Furthermore, studies comparing South African black runners with white runners also stated that black runners have better running economy, are more resistant to fatigue, have some different muscle characteristics as well as the ability to perform better at high environmental temperatures (Bosch *et al.*, 1990; Coetzer *et al.*, 1993; Marino *et al.*, 2004; Weston *et al.*, 1999). Does this mean that other nations will not be able to perform well or win the gold medal in endurance running events?

At the recent Olympic Games (Athens, Greece, 2004), very few of the final events from 800 m to the marathon were won by Kenyan runners. In fact, the 800 m and the marathon were won by a Russian and an Italian, respectively. Furthermore, the 5000 m and 10 000 m finals were won by a Moroccan and an Ethiopian, respectively. However, despite the poor performances of Kenyan endurance runners at the Olympic Games, they have dominated World Championship events in cross-country, where the depth of talent is important, for the past decades. Once again, the circumstantial evidence seems to justify on the one hand a genetic basis for elite athletic performance, and on the other hand that training may be the key to becoming a champion. Some gene polymorphisms have been found to be associated with certain phenotypes that are advantageous to exercise performance (Rankinen *et al.*, 2004; Rivera *et al.*, 1997a; Rivera *et al.*, 1997b). On the other hand, these genes that are associated with performance, may only be activated as a result of extreme training – training representing an environmental factor. This latter statement represents gene-environment interaction. While the search for exercise performance genotypes continues, it is still important to have a thorough understanding of the extent of adaptability of the intramuscular phenotype, as well as what effect this might have on whole body performance.

In the present dissertation, four studies are presented, focusing on structural and metabolic characteristics in skeletal muscle. Furthermore, emphasis is also placed on relationships between performance, whole body physiology and these characteristics, in the quest to unravel whether athletes are



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Literature review

1.1 Introduction

Adaptation is defined as modification or alteration to enhance or inhibit functionality of a system, either directly or indirectly. Many systems in living organisms can undergo adaptation as a result of the effects of signalling molecules and pathways activated by internal or external stimuli. This may involve the regulation of enzyme activities with almost immediate effect or longer term regulation, or both. Longer term regulation occurs by transcription of DNA to messenger RNA, followed by translation to protein with a concomitant change in protein concentration. One system in mammalian species particularly prone to adaptation is skeletal muscle. This plasticity can be attributed to the fact that skeletal muscle is the only system that can produce movement, and the latter is crucial to the survival of humans and animals. Up- and down-regulation of genes resulting in activation or inhibition of protein expression, respectively, are both directly responsible for changes in muscle structure and metabolism. Changes include on the one hand the contractile properties and on the other hand the supply and utilisation of fuel by muscle.

The two main proteins responsible for contraction are actin and myosin. This latter protein is a dimer constructed of two myosin heavy chains (MHC), with two myosin light chains bound to each MHC monomer (reviewed by Cooke (1995)). The MHC is the most important contractile protein in determining contraction speed of the muscle, as it contains the ATPase activity responsible for ATP hydrolysis, a crucial step for contraction. The faster hydrolysis occurs, the faster the velocity of contraction (He *et al.*, 2000). Anatomical position of skeletal muscle plays an important role in endogenous muscle protein expression, giving rise to specialised muscle groups, such as eye muscles compared to leg muscles (Staron, 1997). Furthermore, the same muscle at birth may vary substantially in structural and metabolic characteristics compared to the adult form (Swynghedauw, 1986). However, both of these aforementioned are closely related to function, and can adapt to physical requirements (Punkt, 2002). This adaptation contributes to muscle fibre type.

Muscle fibre type is also characterised by a capacity to supply ATP that must parallel the demand thereof. Therefore, with a change in contraction properties, there is usually a parallel change in metabolic properties. For example, with an increase in contraction frequency, the demand for ATP is increased. In order to maintain the ATP supply and prevent contraction failure, metabolic pathways adapt by increasing the flux of substrates through the pathway by

mass action, direct activation of key enzymes in the pathway and/or increasing enzyme concentrations (increase in protein expression) in relevant pathways.

This dissertation is aimed at investigating the adaptation of particular proteins influencing muscle contraction, namely the MHC and selected metabolic enzymes in pathways responsible for ATP supply. A second aim is to investigate these biochemical factors in the context of whole body physiological functioning during movement.

In order to generate motion under any circumstance requires some sort of a motility system. Bacteria, invertebrates and vertebrates all have some sort of motility or contractile (muscular) system, each designed to serve a specific function. Mammals have three types of muscle tissue, namely cardiac, smooth and skeletal muscle. Each of these has distinct properties and locations in the body related to functionality. However, these functions are interlinked in higher organisms.

Cardiac muscle is only found in the heart and does not have the ability to contract voluntarily. The cells are 50 to 100 μm in length, 14 μm in diameter, striated (have sarcomeres) and branched, with a single nucleus in each cell (Ross *et al.*, 1989). The main function of cardiac muscle is to line the chambers of the heart, to contract in order to drive blood through the body, which in turn delivers oxygen and nutrients to organs and transports carbon dioxide and waste products to locations where they can be disposed of. The heart can also adapt in the short term and the longer term in response to an increased demand for blood flow, by increasing contraction rate (heart rate) or by cellular hypertrophy, respectively.

Smooth muscle, found in most visceral organs and blood vessels, is unable to contract voluntarily. The cells are between 30 and 200 μm in length, have diameters between 3 and 8 μm , have no sarcomeres and are spindle shaped with a single central nucleus (Ross *et al.*, 1989). Contraction is very slow compared to cardiac muscle and hence the demand for ATP is low (Sieck and Regnier, 2001). The main function is to regulate blood flow within the circulatory system to alter supply to a specific tissue. This regulation is a short term adaptation and is achieved by vasoconstriction and vasodilatation in a coordinated fashion and in response to local tissue demands (Joyner and Thomas, 2003). Parts of this muscular system are also subject to longer term adaptation e.g. the capillaries (Hudlicka, 1985).

Muscle attached to the skeleton is termed skeletal muscle and allows for voluntary contraction and relaxation. Because of the macroscopic and microscopic structure of skeletal muscle, such contraction and relaxation can result in movement around the bony joints. Demand for such movement results in short- and long term regulation of skeletal muscle itself, as well as of the functionally linked cardiac muscle and closely related aspects of smooth muscle.

1.2 Skeletal Muscle

1.2.1 Macroscopic composition

In the mammalian species, skeletal muscle is the only tissue that allows the organism to generate work, in the form of locomotion. Skeletal muscle is structurally organised into distinct sections, each section consisting of numerous muscle cells, with extracellular matrix and connective tissue culminating in tendons on each end of the whole muscle. The tendons attach the muscle cells to the skeleton. Muscle groups contract or relax in a coordinated fashion to allow the skeleton to perform rotations, extensions or flexions.

Muscle cells are innervated by motor neurons, which allow for voluntary contractions. A single nerve axon is usually branched and interacts with many single muscle cells, forming a motor unit. When this single neuron fires, only those fibres innervated by the neuron, will contract. However, especially in larger muscles, those cells may not necessarily be directly situated next to each other and can be distributed within the muscle (Saltin and Gollnick, 1983). Depending on the nerve firing frequency, nerves play a significant role in determining if the fibre will eventually resemble a slow twitch or fast twitch fibre (Pette, 2001). This will be discussed in more detail in section 1.6.2.

In order for the muscle cells and consequently the muscle (as a whole) to function properly, a constant supply of nutrients and oxygen, as well as the capacity to remove waste products and carbon dioxide are needed. These functions are maintained by a capillary network surrounding each muscle fibre. This network sprouts from arteries and ends in veins. The number of capillaries surrounding a muscle fibre may vary in relation to the muscle fibre type and depends on muscular demand for oxygen (Hepple, 2000). Section 1.6 will focus more on the detail of capillarisation in muscle and the significance in active muscle.

1.2.2 Microscopic composition

Skeletal muscle fibres can vary in length from as little as 100 μm up to 50 cm depending on the muscle group and species (Ross *et al.*, 1989). On the other hand, the diameter of fibres is much smaller, ranging between 60 to 100 μm (Ross *et al.*, 1989). Visualising muscle fibres under the light microscope can be accomplished in longitudinal- or cross-sections by utilising different histological staining methods (discussed in section 1.4.2 and Appendix B).

The most common characteristics in the longitudinal view (excluding the length of the fibres) are that fibres lie parallel to each other and have a striated appearance. This latter characteristic is due to the organisation of the myofibrils that lie parallel to each other with a strict pattern that more or less overlap of different fibrillar proteins. Myofibrils (with a diameter ranging between 1 to 2 μm) are constructed of serially joined sarcomeres, with the latter being constructed of

actin and myosin, two proteins that are the contraction “hardware” of muscle fibres. Section 1.3 will elaborate on the proteins forming a sarcomere and the myofibrils. Another characteristic is multiple nuclei and multiple mitochondria, dispersed across the length of the fibre. Nuclei are arranged to have control over a specific range called the nuclear domain (Pavlath *et al.*, 1989), whereas the mitochondria may be situated just under the sarcolemma or within the myofibrillar spaces (Brooks *et al.*, 1999). These structural characteristics combined with the long length of skeletal muscle fibres result in heterogeneity within single cells.

Muscle fibres are most commonly viewed in cross-sectional sections. Characteristics of this view are large cells and nuclei situated close to the sarcolemma. The latter characteristic is very important in identifying myopathy such as central core disease and certain types of muscular dystrophy in which the nucleus is abnormally situated amongst the myofibrils (Bornemann and Goebel, 2001). Cross-sectional views also allow the visualisation of the capillary supply of each muscle fibre. The significance of the cellular diameter in muscle function will be discussed in more detail in section 1.4.4.

Other organelles situated in muscle fibres are the sarcoplasmic reticulum (responsible for Ca²⁺ release and re-uptake) and mitochondria. The former will not be discussed in this dissertation, although it is acknowledged here that it is integrally important to contraction and relaxation (Carroll *et al.*, 1997), is different between fibre types and is subject to adaptation (Green *et al.*, 2003). The number of mitochondria in muscle fibres may vary substantially, depending on the fibre type and the frequency and duration of activation of the muscle (Saltin and Gollnick, 1983). In addition, muscle fibres also have large glycogen stores, capable of providing substrates at a rapid pace. Although not strictly speaking an organelle, recent information implies structural complexity in glycogen macromolecules (Marchand *et al.*, 2002). Figure 1.2.1 depicts the structure and organisation of a muscle from the muscle group to myofibrils. Section 1.4.3 will discuss mitochondria and metabolism of muscle fibres in more detail.

1.2.3 Fibre types

Early in the 1900s, scientists already noticed that there was a difference in contraction speed of muscle groups. In addition, they also found that some muscle groups were darker in colour than others. Later it was discovered that the darker muscle groups had a higher concentration of myoglobin, a protein similar to haemoglobin found in red blood cells, than those appearing lighter and even white. Ranvier, in 1874, showed that the contraction speed of a dark red muscle was significantly slower than those of paler colour and that the latter type of muscle had a lower blood capillary supply. These characteristics led to the division of muscle groups into red slow twitch muscle and white fast twitch muscle. Since then, methods of identifying fibre type have advanced, using histological staining, antibodies and biochemical separation techniques (see section 1.4.2). Contraction characteristics of muscle fibres will be discussed in sections 1.4.1.

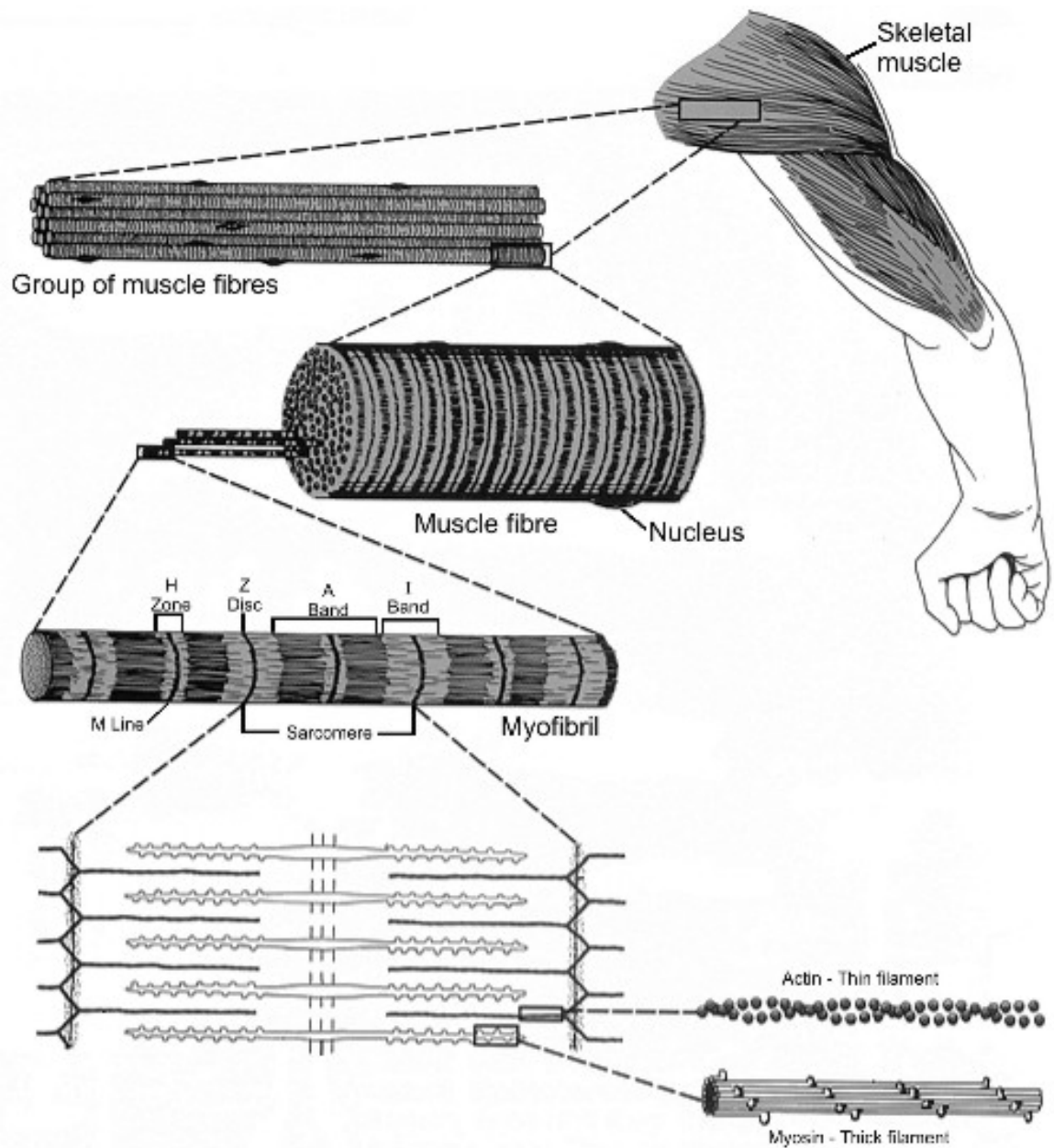


Figure 1.2.1 Schematic diagram of skeletal muscle organisation from a muscle group to filament level. From Bloom and Fawcett (1975).

1.3 Muscle proteins

1.3.1 Proteins characteristic of muscle cells

Muscle cells consist of unique proteins, many of which are not commonly found in other types of cells, or are found, but in another isoform. They may serve more than one purpose, such as providing structural support, hydrolysing ATP and producing force. This section will focus on some of the major proteins identified in skeletal muscle cells.

Examples of proteins important for contraction or metabolism, but not associated with the myofibrils

Dystrophin

Dystrophin is a 3685-residue protein (~427 kDa) and may have numerous isoforms. The protein is associated with the inner sarcolemma and may serve the purpose of anchoring specific membrane glycoproteins. Although it has a low concentration in muscle (approximately 0.002%), mutations in the gene coding for dystrophin result in muscular dystrophy, such as the lethal Duchenne muscular dystrophy (DMD) (Betto *et al.*, 1999). It was shown that adult chicken muscle with this disease, lacks the ability to completely mature to adult muscle, and expresses a mixture of adult and neonatal myosin heavy chain isoforms (Rushbrook *et al.*, 1987). Not only do mutations in the dystrophin gene result in DMD, but recently, transgenic mice over-expressing the signal transduction protein, caveolin-3, had inhibition of dystrophin expression and had typical DMD characteristics in their muscle (Galbiati *et al.*, 2000).

One characteristic of DMD muscle is that force production capacity declines. Dystrophin has been highlighted to indicate that, although the focus of this dissertation will be on the force-producing myofibrillar protein myosin, the latter cannot be considered to function in isolation.

Myoglobin

As was previously mentioned, myoglobin is analogous to haemoglobin. The general function of myoglobin was thought to be a reserve for oxygen molecules to be used when muscle was deprived of oxygen either by blood flow restriction, or very early in intense contractions before short-term increases in circulatory oxygen supply. A study conducted by Terrados *et al.* (1990) showed that myoglobin content increased in response to training under hypobaric conditions, but that it stayed the same when the intervention was performed under normobaric conditions. However, it was recently shown that myoglobin *-/-* knockout mice had a normal ventilatory response to hypoxic conditions, normal heart function and could maintain the same level of sub-maximal exercise compared to wild type mice. The only difference was that the heart and skeletal muscle of the knockout mice were colourless (Garry *et al.*, 1998). This finding raises new questions on the function of myoglobin in muscle and needs further investigation. This protein has been highlighted to indicate that, although the metabolic focus of this dissertation will be on oxidative enzyme capacity, the latter cannot be considered to function in isolation (and also that the mitochondria in which they are situated, do not provide the red colour typically associated with oxidative fibre types).

Proteins responsible for contraction – the myofibrils

In Figure 1.3.1 the main proteins found in a single contractile unit, namely the sarcomere, are depicted. Each of these proteins has two or more isoforms. Some isoforms are specific to certain fibre types as well as tissue specific, e.g. only found in skeletal muscle and not in cardiac muscle. It must also be noted that there are species variations in the amino acid sequences of

some of the protein isoforms. Table 1.3.1 summarises the molecular mass and functionality of these proteins (Schiaffino and Reggiani, 1996). Because these proteins have multiple isoforms, each isoform may have a distinct impact on the structure, contractile and metabolic properties of a muscle fibre. Myosin is directly involved in generating motion and is the essential protein in determining fibre type. In the next section, the myosin molecule will be discussed in detail.

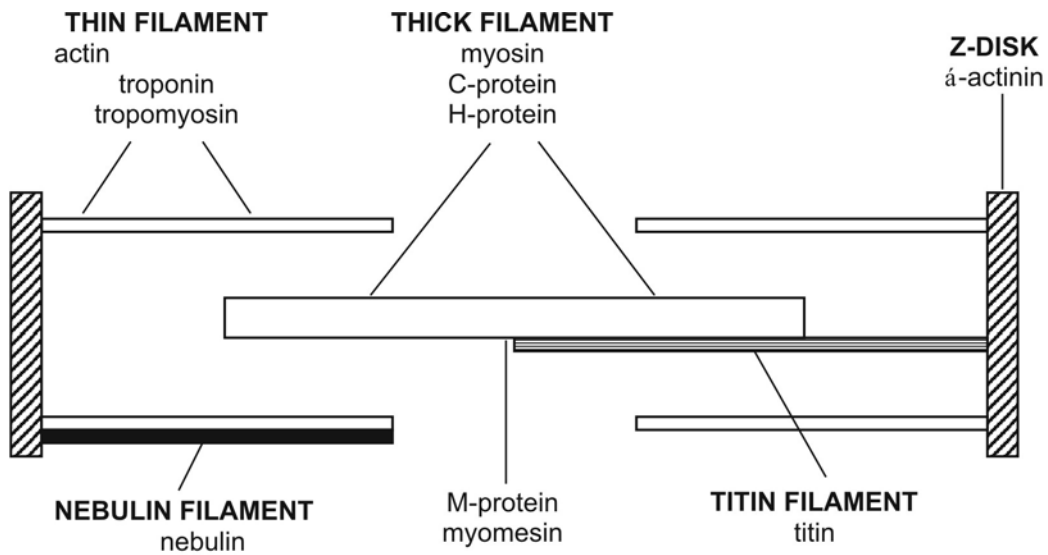


Figure 1.3.1 Structural scheme of a sarcomere and its main proteins. From Schiaffino & Reggiani (1996).

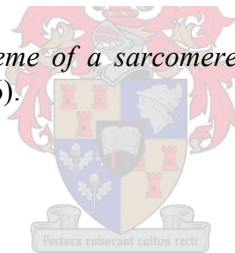


Table 1.3.1 Summary of the molecular mass and functionality of the main proteins found in thick- and thin filaments and the Z-disk. Subunit proteins are listed in italics underneath the complex protein name. From Schiaffino & Reggiani (1996).

Protein or complex	Mw (kDa)	Functionality
Thick filament		
Myosin heavy chain	~200 - 230	Contains myosin ATPase enzyme; binds to actin; protein responsible for motility generation
Myosin essential light chain	~17 - 25	Essential for actin binding and myosin ATPase activity
Myosin regulatory light chain	~16 - 25	May increase force production at low Ca ²⁺ concentrations
Titin	~3000	Determines the thick filament length; Protein-C binding sites; binds M-protein and myomesin
C-Protein	~140	Binds to myosin; probable stabilising protein
H-Protein	~74	Interacts with myosin
M-Protein	~100	Interacts with titin
Thin filament		
Actin	~41	Contains binding site for myosin; attached to Z-disks and pulls two Z-disks closer together
Tropomyosin	~34 - 36	Protein that shields the actin binding sites from myosin
Troponin	~72	Globular protein consisting of three subgroups
<i>TnC</i>	~17	Binds Ca ²⁺ ions
<i>TnI</i>	~27 - 31	Binds actin and inhibits actomyosin ATPase activity
<i>TnT</i>	~36 - 39	Binds tropomyosin
Nebulin	~700 - 900	Absent in cardiac muscle; determines thin filament length
Z-disks		Binds thin filaments in a barbed fashion forming the Z-disk

kDa, kilo Daltons; Mr, molecular weight; Tn, troponin

1.3.2 More about the myosin proteins

Biochemical structure

Presently, there are 18 classes of myosin molecules identified in eukaryotic cells, falling under the global term of molecular motor proteins. Mammalian skeletal muscle myosin forms a small part of this classification and falls under the class II myosins (Schiaffino and Reggiani, 1996). In this section, emphasis will be placed on the myosin from mammalian skeletal muscle.

Myosin is a globular hexamer protein consisting of two heavy chain subunits (~200 - 230 kDa each) and four light chain subunits (~16 - 25 kDa each) (Moss *et al.*, 1995). The number and sizes of these subunits make myosin one of the largest known protein complexes. The heavy chain consists of a globular head, neck and a long tail region. The head region contains the actin binding site, which is in the form of a cleft, the ATP binding site and ATPase enzyme. This

ATPase enzyme forms part of the heavy chain and is not an enzyme which covalently binds to the heavy chain. Two types of light chains exist, namely an essential (alkali) light chain and a regulatory (phosphorylatable) light chain.

Figures 1.3.2 and 1.3.3 show a ball model and a simplified schematic drawing of the myosin molecule, respectively.

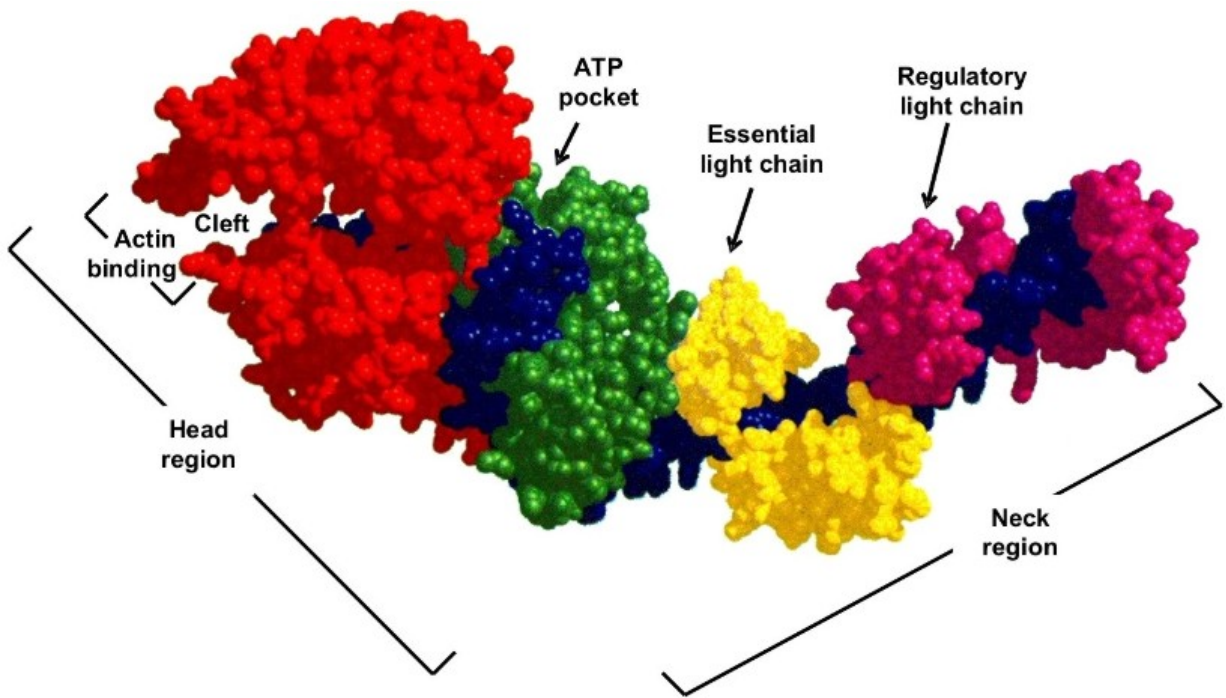


Figure 1.3.2 Biochemical structure of subfragment-1 of the myosin molecule. From Rayment *et al.* (1993).

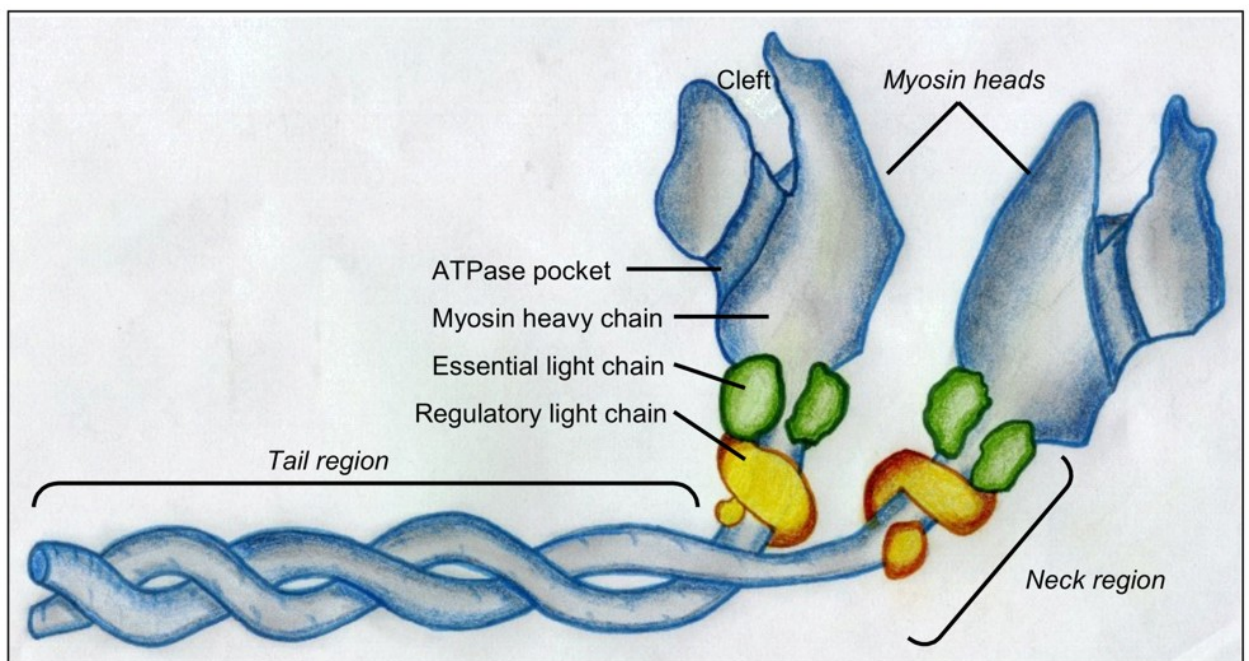


Figure 1.3.3 A simplified drawing of the myosin molecule based on Figure 1.3.2 and Schiaffino & Reggiani (1996) showing the head, neck and tail region and the locations of the heavy chains, light chains, ATPase pocket and cleft.

Myosin light chains – isoforms and genes

The various myosin light chain (MLC) and myosin heavy chain (MHC) isoforms are of great importance in determining the contractile properties of a muscle. However, isoforms also exist of the other muscle proteins, such as troponin and tropomyosin, which can also contribute significantly to the muscle fibre properties.

The light chains are located close to the neck region of the MHC with the essential light chain attached to the neck region and in direct contact with the head region of the MHC. The regulatory light chain is located on the neck region in close proximity to the essential light chain. Both essential and regulatory light chains play no role in the activity of the ATPase, but may play a significant role in determining the contractile properties of muscle fibres (Barton and Buckingham, 1985) by regulating interactions of myosin and actin (reviewed by Cooke (1997)). For instance, phosphorylation of the regulatory light chain has been shown to increase the force production of the actin-myosin complex in an environment of low Ca^{2+} concentrations (reviewed by Schiaffino and Reggiani (1996)).

Various isoforms have been identified for the MLCs in mammalian skeletal muscle and are classified in Table 1.3.2. Each isoform differs in molecular mass which may range between ~16 to 25 kDa. These isoforms were detected in muscle groups having distinct contractile properties, hence the nomenclature fast and slow isoforms (Barton and Buckingham, 1985; Moss *et al.*, 1995; Schiaffino and Reggiani, 1996; Staron and Johnson, 1993). Two further isoforms were detected, the MLC_{1emb} found in embryonic muscle (which has also been identified in adult masseter muscle) and a second isoform, MLC_{2m} , expressed in carnivorous mandibular muscles, which is similar to a light chain expressed in monkey skeletal muscle (Schiaffino and Reggiani, 1996). All these isoforms are not restricted to a specific muscle and may be expressed in other muscle types (Schiaffino and Reggiani, 1994). The role of these isoforms in determining contractile speed of fibres will be discussed in section 1.4.1.

Table 1.3.2 Summary of the myosin light chain isoforms identified in mammalian skeletal muscle. Compiled from Schiaffino & Reggiani (1994) and Staron (1993).

Type of MLC	Developmental muscle	Fast fibres	Slow fibres
Essential light chains	MLC_{1emb}	MLC_{1f}	MLC_{1sa}
		MLC_{3f}	MLC_{1sb}
Regulatory light chains		MLC_{2fa}	MLC_{2sa}
		MLC_{2fb}	MLC_{2sb}
		MLC_{2m}	

Myosin light chain genes

All the MLCs of human skeletal muscle, except MLC_{1f} and MLC_{3f}, are coded by distinct genes located on different chromosomes (Schiaffino and Reggiani, 1994). The MLC_{1f} and MLC_{3f} gene is located on chromosome 2q33-q34 and these isoforms are derived from two distinct promoter regions and alternative splicing of the first four exons. The MLC_{1f} transcript contains exons 1 and 4, while the MLC_{3f} transcript contains exons 2 and 3 (Schiaffino and Reggiani, 1996). The light chain isoform expression is not determined by the heavy chain isoform expression, which means that e.g. slow isoforms as well as fast isoforms may be expressed in slow fibres and *visa versa* (Barton and Buckingham, 1985).

Myosin heavy chains – isoforms and genes

There are a number of MHC isoforms identified in mammalian muscle and the number is still increasing (Table 1.3.3). Of these, only a few are actively expressed in adult skeletal muscle. Most of these isoforms have been identified using gel electrophoresis, with antibodies directed at the specific isoforms, or by mRNA analysis of e.g. single muscle fibres (Pereira Sant'Ana *et al.*, 1997).

Of the nine isoforms, only MHCs I_β, MHC IIa, MHC IIx and MHC IIb are predominantly expressed in adult skeletal muscle (Schiaffino and Reggiani, 1996). However, Galler *et al.* (1997) identified that the MHC I_α isoform, usually found in cardiac muscle, is also expressed in rabbit skeletal muscle.

In human skeletal muscle, three isoforms are commonly expressed, namely MHC I, MHC IIa and MHC IIx. Previously it was thought that no MHC IIb isoform was expressed in human skeletal muscle. However, recently, Andersen and co-workers (2002) showed with Western blotting and immunohistochemistry that the MHC IIb isoform does indeed exist in human skeletal muscle, but that it is mostly restricted to specialised muscles of the eye and larynx.

Most animals from the large animal kingdom express the MHC I, MHC IIa and MHC IIx isoforms (Duris *et al.*, 2000; Kohn *et al.*, 2005). Recently it was shown, using nucleotide sequencing, that bovine skeletal muscle indeed only expressed MHC I, MHC IIa and MHC IIx and no MHC IIb isoforms in large muscle groups known to be used for meat production (Chikuni *et al.*, 2004). Kohn *et al.* (2005) also showed that the three MHC isoforms corresponding to MHC I, MHC IIa and MHC IIx are expressed in impala (*Aepyceros melampus*) skeletal muscle, a non-domesticated animal.

Smaller animals such as rats, rabbits, guinea pigs and mice, commonly express the MHC IIb isoform in conjunction with the other three (Lucas *et al.*, 2000). However, recently, Lefaucheur *et al.* (2002) showed that fast pig muscle also expresses MHC IIb in conjunction with the other three. This raises the possibility that all four isoforms may be found in the large muscle groups

of large animals. Figure 1.3.4 shows examples of the MHC isoforms, separated by gel electrophoresis, of human, rat and impala skeletal muscle.

The significance of expressing different isoforms in muscle is that each isoform differs in myosin ATPase activity and gives specific properties to muscle fibres (Rivero *et al.*, 1999). This topic will be further elaborated on in section 1.4.

Table 1.3.3 *Myosin heavy chain isoforms expressed in mammalian muscle.* From Schiaffino & Reggiani (1996).

Isoform	Location of expression
MHC I _α	Mandible muscle; extraocular muscle; heart muscle
MHC I _β	Slow skeletal muscle; heart ventricles
MHC IIa	Fast skeletal muscle
MHC IIx	Fast skeletal muscle
MHC IIb	Fast skeletal muscle
MHC _{emb}	Developing skeletal muscle; mandible muscle; extraocular muscle
MHC _{neo}	Developing skeletal muscle; mandible muscle; extraocular muscle
MHC _{eo}	Extraocular muscle
MHC _m	Mandible muscle (carnivores)

Previously, the human MHC IIx isoform was mistakenly classified as MHC IIb, but recent evidence suggested that this isoform is 94% homologous to the rat MHC IIx isoform and less homologous to rat MHC IIb, and was renamed thereafter (Schiaffino and Reggiani, 1996).

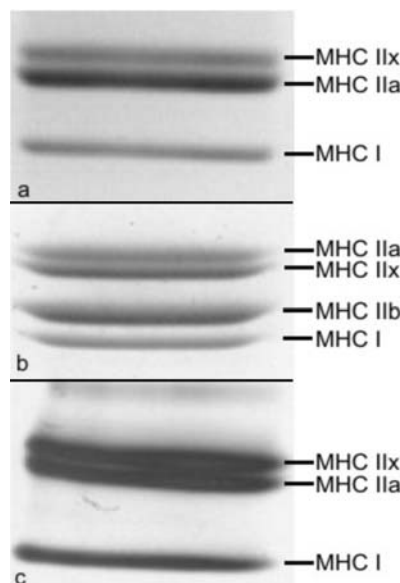


Figure 1.3.4 *Myosin heavy chain isoforms in skeletal muscle of three species separated by gel electrophoresis (SDS-PAGE).* a. Human (Andersen *et al.*, 1994b) b. Rat

(Talmadge and Roy, 1993) c. Impala (Kohn *et al.*, 2005). Note that the separation order of the various isoforms from different species may differ.

Myosin heavy chain genes

The MHC genes in mammalian muscle originate from multigene families. In human muscle, the MHC I_α, MHC I_β, MHC IIa, MHC IIx, MHC IIb, MHC_{emb}, MHC_{neo} and MHC_{eo} isoform genes are found on chromosomes 14 and 17, whereas mouse and rat genes are found on chromosomes 11 and 14, and 10 and 14, respectively (Weiss *et al.*, 1999). Some lower species, like the scallop and *Drosophila melanogaster*, derive their isoforms from alternative RNA splice variants (Niyitray *et al.*, 1994).

Sequence analyses of the genes show that there is more conservation between specific isoforms of different species than between the isoforms within a specie. According to Weiss *et al.* (Weiss *et al.*, 1999), it is suggested that the MHC isoforms do not tolerate vast sequence mutations and this was supported by Vikstrom & Leinwand (1996) who showed that more than fifty different residue mutations (occurring as point mutations) in the human MHC I_β isoform each correlated with hypertrophic cardiomyopathy. Therefore, the sequence conservation of the genes responsible for the MHC isoforms is crucial for proper muscular function. The MHC isoforms also play a significant role in determining an organisms overall phenotype. Acakpo-Satchivi *et al.* (1997) showed that MHC IIb and MHC IIx knockout mice had different physiological characteristics compared to wild type, such as kyphosis (hunch back), muscle weakness and a decreased body mass. However, the MHC IIx knockout mice showed the most phenotypic change in whole body and muscular characteristics. On account of this study, new insights were revealed regarding the importance of the expression of the various MHC isoforms.

The genes coding for the various MHCs are homologous to one another, with some being more homologous than others. High homology was found between the MHC I_α and MHC I_β sequences (collectively termed MHC I isoforms), with similar trends for the MHC IIa, IIx and IIb isoforms (collectively MHC II isoforms). However, between the MHC I and II isoforms there seems to be a variation of ~20% in the sequence. These findings by Weiss *et al.* (1999) may indicate that the isoforms share a common ancestral gene and sub-isoforms may be to optimise the sequential MHC isoform functionality. Figure 1.3.5 shows the homology of the different isoforms in human skeletal muscle.

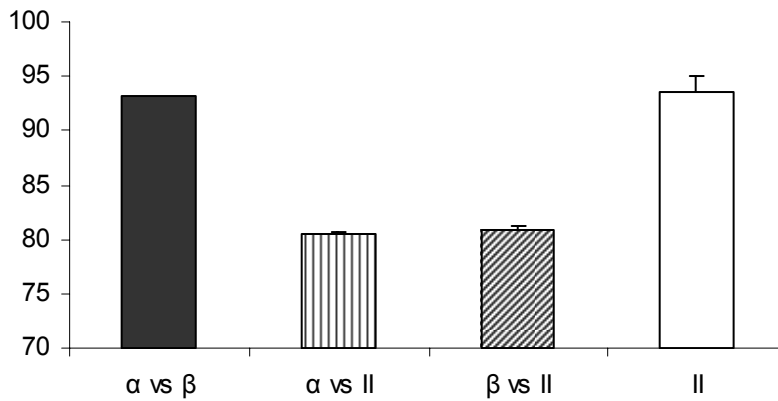


Figure 1.3.5 Homology in gene sequences between MHC I_{α} and MHC I_{β} (α vs β), MHC I_{α} and the MHC II isoforms (α vs II), MHC I_{β} and the MHC II isoforms (β vs II) and within MHC IIa, IIx and IIb isoforms (II). From Weiss *et al.* (1999).

Regulation of the MHC genes and what may influence expression will be discussed in sections 1.5 and 1.6.

1.3.3 The mechanism of protein-protein interaction of cross-bridge cycle

Although Figure 1.3.6 is a simplified model of the cross-bridge cycle (and this cycle is much more complex than depicted), it is necessary to briefly discuss this model in order to relate the different components to the properties of muscle fibre types.

In short, the myosin (M) is strongly bound to the actin (A) binding site and forms the myosin-actin complex (AM). When ATP (1, Figure 1.3.6) binds to the ATP pocket on the myosin head, the head is released from the actin site (2, Figure 1.3.6). ATP is still bound to the head (M.ATP) and the myosin head is free to bind with any other actin site available. The ATPase enzyme hydrolyses the bound ATP to ADP and P_i (3, Figure 1.3.6), which are still bound to the myosin head (M.ADP. P_i). This hydrolysis results in a conformational change in the myosin head, swinging the head away from the tail region. Once this isomerisation has occurred, the myosin head weakly attaches itself to a new actin binding site (4, Figure 1.3.6) forming a new complex (AM.ADP. P_i). The release of the bound P_i (5, Figure 1.3.6) initiates the power stroke, dragging the actin filament closer to the M-line. When ADP is released from the AM.ADP complex (6, Figure 1.3.6), it once again forms a strong binding between myosin and actin (AM) (Cooke, 1995; De la Cruz and Ostap, 2004).

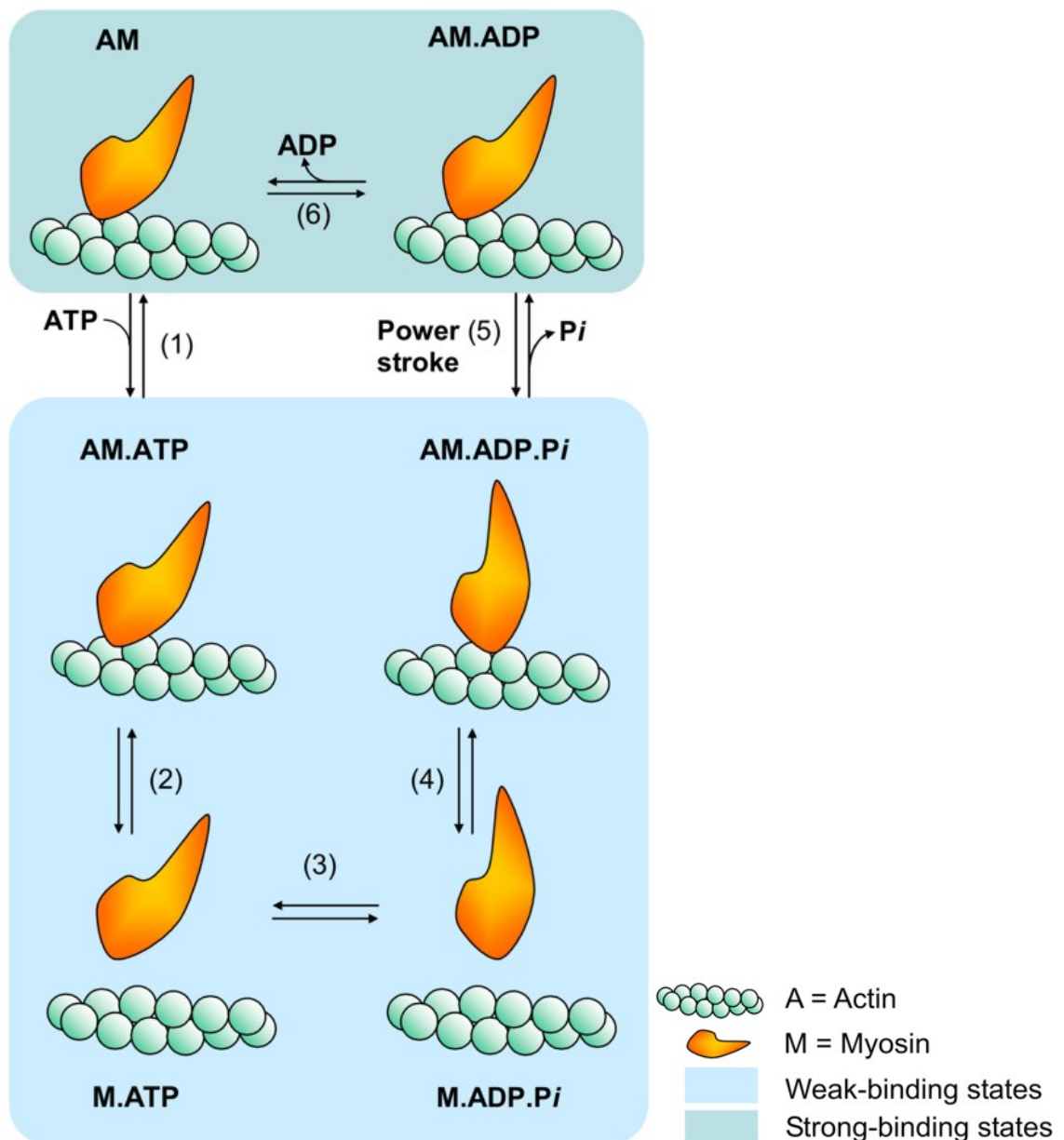


Figure 1.3.6 Schematic representation of the cross-bridge cycle in skeletal muscle. Steps 1 – 5 are described in the text. From De la Cruz & Ostap (2004).

The cross-bridge cycle is under stringent regulatory control. Troponin and tropomyosin aid in this regulation (Voet and Voet, 1995). The tropomyosin molecule forms an elongated chain along actin and shields the myosin binding sites. Troponin C (part of the troponin complex) is bound to tropomyosin and contains Ca^{2+} binding sites. Once a nerve impulse stimulates the release of Ca^{2+} from the sarcoplasmic reticulum, Ca^{2+} binds to troponin C. Troponin C undergoes a conformational change and this changes the orientation of tropomyosin, revealing the myosin binding sites on actin and the cross-bridge cycle may be initiated (Voet and Voet, 1995). In living cells, the Ca^{2+} is rapidly removed from the cytosol by ATP driven transporters back into the sarcoplasmic reticulum (Cooke, 1995; De la Cruz and Ostap, 2004). Both the force and distance produced by one cross-bridge cycle have been determined and were calculated as 3 – 5 pN and 12 nm, respectively (Cooke, 1995).

To relate this section to those that follow, it is clear that for normal contraction to occur, many molecules of ATP are required for cross-bridge cycling and active re-uptake of Ca^{2+} . Further, ATP is also required for the $\text{Na}^+ - \text{K}^+$ ATPase to restore the resting membrane potential for the following activation to commence. This process requires metabolic pathways that will endure the supply of ATP to meet the demand of ATP consumption. Section 1.4.3 will focus more on this scenario.

Ca^{2+} is not only the initiator of the cross-bridge cycle, but also plays an important role in cell signalling, being an important upstream signal for activating specific proteins responsible for gene transcription, that may regulate both structural and metabolic components of muscle cells. More on cell signalling will be discussed in section 1.5.

1.4 Muscle fibre types

The classification of muscle into various fibre types can be dated back by almost a hundred years when it was first observed that a colour difference between muscle groups existed. However, over the past years it was shown that it is the myosin molecule itself that plays an important role in determining fibre type. In conjunction with this, the adaptation of various metabolic pathways supplying ATP to the muscle fibres also plays a significant role. In a later section (1.4.2), the various ways of expressing muscle fibre types will be summarised.

1.4.1 Role of myosin in fibre types

Myosin has been discussed on the basis that it binds to actin and with ATP hydrolysis via the myosin ATPase enzyme, brings forth contraction. Classic work done by Michael Barany and his co-workers in 1965 and 1967 showed that the ATPase activity of myosin extracted from fast and slow muscles was different with fast muscle myosin having the highest activity (Barany *et al.*, 1965; Barany, 1967). This discovery was the foundation for linking fibre contractile velocity and other parameters to the chemical properties of the myosin molecules. Two components of myosin have the main influence on contractile properties and will be discussed in this section, namely the MHC isoforms and myosin essential light chain isoforms.

Myosin heavy chain isoforms

The MHC isoforms have been shown to be the main determinant of the myosin ATPase activity and contractile speed. Each heavy chain isoform has a unique ATPase activity, and is ranked from slowest to fastest in contractile activity: MHC I, MHC IIa, MHC IIx and MHC IIb. However, it may not only be the hydrolysis speed of the ATPase enzymes that is important. Studies on fast and slow muscle showed that MHC I binds ATP twice as fast as those with fast isoforms, but that the dissociation of myosin from actin is six times higher in fast muscle compared to slow muscle (Schiaffino and Reggiani, 1996). Recently, Han *et al.* (2003) showed that there is a difference in ATP consumption rate within distinct fibre types of the diaphragm

muscle of rats. This research group also showed that the ATP consumption rate is dependent on the MHC isoform expressed and follows the same pattern as the ATPase activity. All four these properties have a direct influence on the contraction velocity (Larsson and Moss, 1993); however, the MLCs may also play a role (see below).

Two studies by independent groups (Harridge *et al.*, 1996; Larsson and Moss, 1993) showed that maximum shortening velocity (V_0) was four times lower in human single muscle fibres expressing pure MHC I compared to those expressing pure MHC IIa. Larsson and Moss (1993) also showed that the V_0 of single fibres containing pure MHC IIx was three and eleven times greater than pure MHC IIa and pure MHC I fibres, respectively.

It is possible for a single muscle fibre to express more than one myosin heavy chain isoform simultaneously. These fibres are commonly referred to as hybrid fibres and the most common combination found in adult skeletal muscle is that of MHC IIa and MHC IIx isoforms (MHC IIa/IIx hybrid) (Andersen *et al.*, 1994b; Stephenson, 2001). These hybrid fibres differ from pure fibres in contractile properties. In the same two studies, Larsson *et al.* (1993) and Harridge *et al.* (1996) showed that the V_0 of MHC IIa/IIx hybrid fibres lay between those of pure MHC IIx and MHC IIa fibres. The next most common hybrid fibre is the I/IIa hybrid (Andersen *et al.*, 1994b; Stephenson, 2001). The same pattern was observed for the V_0 of MHC I/IIa hybrids (i.e. between the V_0 of each pure fibre type), but the mean V_0 values of MHC I/IIa hybrids did not correspond between the two authors. This can be explained by the percentage expression of each isoform, which may have a significant effect on the V_0 . Larsson and Moss (1993) clearly showed in their experiment that the V_0 follows an exponential increase with an increase in the percentage MHC IIx in MHC IIa/IIx hybrid fibres (see Figure 1.4.1).

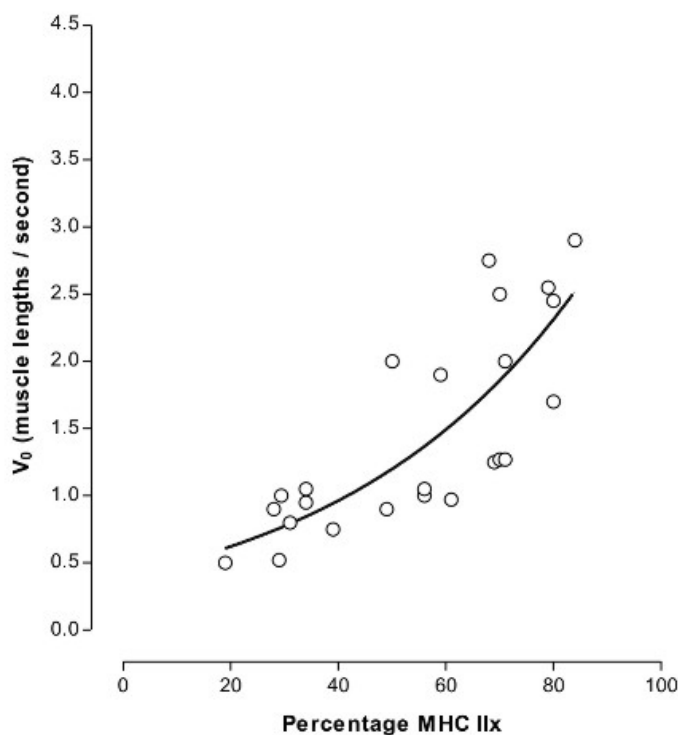


Figure 1.4.1 Relationship between maximum shortening velocity (V_0) and the proportion of MHC IIx in MHC IIa/IIx hybrid fibres. From Larsson & Moss (1993).

Myosin light chains

In 1982, Sivaramakrishnan and Burke (1982) showed that removal of the MLCs from the S1 sub-fragment of the myosin molecule had no effect on the actin activated ATPase activity of the MHC. This was shown at a physiological ionic strength, but at a low ionic strength, the influence was evident (Schiaffino and Reggiani, 1996).

Over the years, many isoforms of the MLCs have been identified in both human and animals (Schiaffino and Reggiani, 1996) and various combinations are evident, which may not be restricted to the fibres expressing a certain MHC isoform (Schiaffino and Reggiani, 1996; Staron and Pette, 1987). Evidence suggested that the light chain isoforms played a significant role in modifying the myosin ATPase kinetics. However, this has been questioned in a recent review by Timson (2003), who suggested that ATPase activity itself may not be affected but that the transduction of the force may be altered, thus altering the contractile properties.

The role of the MLCs seems to be more focussed on regulating the contractile velocity of a fibre. Studies conducted on rat MLC isoforms and the influence on V_0 showed that V_0 is proportional to the relative content of the MLC_{3f} isoform in fast fibre types (Bottinelli *et al.*, 1994). This can be explained by the fact that myosin with the MLC_{1f} isoform binds actin more strongly than those with the MLC_{3f} isoform (Schiaffino and Reggiani, 1996; Timson, 2003), thus slowing the cross-bridge cycle. It can also be argued that with a stronger binding of the

myosin molecule to actin (evident from the MLC studies), longer time can be spent generating tension and force. However, this statement is still under investigation.

To conclude this section, it is mostly the MHC isoforms that determine how fast a single fibre will contract. Contraction velocity is also influenced by the co-expression of these isoforms. However, the function of the hybrid fibres is still being debated e.g. whether they are functional adaptations or merely transitional from one pure isoform to another, or even both (Stephenson, 2001). The MLCs do play a role, but mainly in modulating the shortening velocity of a fibre. In this dissertation, Chapter 3 will focus specifically on hybrid fibres. An aspect that will not be addressed, is that other proteins may contribute to the muscle fibre characteristics, such as the troponins and myosin binding protein C (Bottinelli, 2001).

With the variations in the myosin ATPase activities, the supply of ATP to the motor head and to other ATPases, especially with sarcoplasmic reticulum ATPase used to actively remove Ca^{2+} from the cytoplasm, is crucial. The muscle cells must therefore have metabolic systems that can endure ATP hydrolysis (section 1.4.3).

1.4.2 Experimental classification of skeletal muscle fibre types

During the past few decades, many fibre type classifications have been developed using contractile speed of the fibres, ATPase stability, oxidative potential of fibres, antibodies directed against the various MHC isoforms, probes directed against MHC mRNA and using methods that analyse whole muscles, muscle sections or single fibres. All these systems have different advantages and disadvantages. Some are very time consuming, whereas other techniques only give a moderate indication of the fibre type. For the past ten years, identifying the exact fibre type has become crucial in understanding skeletal muscle phenotype and various regulatory mechanisms, its capacity to adapt as well as e.g. the identification of hybrid fibres and the proportions of the different MHC isoforms expressed within that fibre. In this dissertation, two classification techniques was used to determine the fibre type of human muscle, namely myosin ATPase staining (utilising the stability of the ATPase at acidic and alkaline pH) and separation of the MHC isoforms of homogenates or single fibres using SDS-PAGE. The latter technique is very time consuming, but results in exact quantification of the relative proportions of the MHC isoforms expressed in hybrid fibres, whereas the former technique has the advantage that it allows for morphometric assessment and co-analysis of e.g. capillary supply. The nomenclature that will be followed in this dissertation is that of Andersen *et al.* (1994b), namely that fibres typed histochemically will be referred to as e.g. types IIA or IIX, whereas equivalent fibres typed using SDS-PAGE will be referred to as types Iia or Iix. Furthermore, conventional ATPase fibre type classification in humans refer to the type IIX fibre as type IIB, but because the type IIB fibre type is more related to the rat type IIX, this dissertation will refer to human

type IIB fibres as type IIX (Schiaffino and Reggiani, 1996). Table 1.4.2 summarises the fibre type classification identifiable with ATPase histochemistry and SDS-PAGE of single fibres.

Table 1.4.2 Classification of fibre types in human skeletal muscle. Note that the rat has an extra MHC IIB isoform, not included in this table, which will be referred to as type IIB. From Andersen *et al.* (1994b).

ATPase	Pure fibres			Hybrid fibres			
	I	IIA	IIX	IC	IIC	IAC	IAX
SDS-PAGE	MHC I	MHC IIa	MHC IIx	MHC I/IIa		MHC IIax	

1.4.3 Metabolism and fibre types

As mentioned before, for contraction and relaxation to occur in muscle fibres, a vast amount of ATP is required. It is required for the active transportation of Ca^{2+} back into the sarcoplasmic reticulum and for the restoration of the resting membrane potential by active sarcoplasmic reticulum SERCA pumps and Na^+/K^+ , ATP-dependent membrane pumps, respectively. The maximal rate of ATP consumption varies greatly between fibre types. Therefore, the muscle fibre must have metabolic systems that can adequately supply ATP molecules equal to the demand for ATP. If this co-adaptation did not occur (taking into consideration the low quantity of stored ATP), muscle would go into a state of rigor within seconds of exercise.

Substrates and products

The fed state or activity patterns of the organism, or both, influence the concentrations of various substrates and metabolites in any tissue, especially in muscle. Mammalian species have five common substrate sources for ATP production, with some stored in high quantities within the muscle and/or other organs. These substrates are phosphocreatine (PCr), glucose, glycogen and triglycerides (TG) (including free fatty acids (FFA)). The fifth substrate is protein from which amino acids are mobilised in order to provide fuel, or to replenish carbon skeleton substrates in particular pathways, but protein oxidation occurs in substantial amounts only in extreme cases of low nutrition. Glucose is either obtained from dietary intake or produced *de novo* in the liver and circulates in the blood stream, whereas glycogen is stored in the liver and muscle cells. Fat or TG is stored in adipose tissue, is mobilised to FFA and glycerol which can then enter the muscle cells and be metabolised. Muscle cells also have TG stores in the form of droplets, but the content in the muscle cells is much less than in adipose tissue (Hoppeler, 1999a).

PCr is a “high energy” phosphate mainly found in skeletal muscle. The transfer of a phosphate from PCr to ADP is the fastest ATP replenishment compared to the other fuel sources, but PCr is also the substrate with the lowest concentration in muscle. Concentrations of PCr may vary

between species and also in muscles from the same species (Snow *et al.*, 1998). Brannon *et al.* (1997) showed that rat *Plantaris* muscle, consisting mainly of type IIX and IIB fibres, had 2.5 times more PCr than rat *Soleus* muscle, containing mostly type I fibres. In a recent human study, it was shown that type I fibres had significantly less PCr stores than type IIAX hybrid fibres, with no difference between the type I and type IIA fibres (Karatzaferi *et al.*, 2001). They also showed that PCr stores are nearly depleted in all fibres during a 25 second all-out power test, but after 1.5 minutes of rest, the PCr stores were already two thirds replenished. The creatine content of muscles can be increased by supplementation with exogenous creatine products, but only to a certain extent (~ 25% more) (Greenhaff *et al.*, 1994). Furthermore, creatine supplementation improves recovery between high intensity exercise bouts (Brannon *et al.*, 1997).

Full oxidation of glucose and glycogen (following the glycolytic, tricarboxylic acid cycle (TCA) and electron transport chain (ETC) route) is the second fastest replenishment system of ATP. However, oxidation of carbohydrates only through glycolysis (end product being lactate), is the faster, but depletes this substrate source more rapidly with less ATP generation (refer to the section on metabolic pathways in muscle fibres). Glycogen stores are larger than those of PCr, with quickly available glucose being “stored” as blood glucose. Glycogen stores are tightly regulated and muscle does not preferentially mobilise stored glycogen under non-exercising conditions. For instance, in the muscle of 27-hour fasted humans there was no change in glycogen content (Nieman *et al.*, 1987). There are also concentration differences in glycogen storage between species and even fibre types. In a review by Karlsson *et al.* (1999), the authors pointed out that laboratory animals, such as rats and mice, contain more glycogen in the type IIX and IIB fibres compared to type IIA and I fibres, but that type IIA fibres of the sheep contain more glycogen than their type IIX fibres. This is likely a result of differences in fibre type recruitment pattern. Humans also may vary in resting glycogen content in either type I and II fibres, which depends mainly on physical activity habits. Ingestion of carbohydrates prior to exercise, have also shown to improve endurance performance, increasing blood glucose availability (Hargreaves *et al.*, 2004).

The last major source of ATP replenishment, namely fat, is the slowest system, but there is an abundance of fat stores (Hoppeler *et al.*, 1999). Fat is also mobilised during periods of fasting or long duration exercise when it is released from adipose tissue into the blood as free fatty acids (FFA). For instance, in 27-hour fasted humans, blood FFA concentration was approximately three times higher than in non-fasted individuals (Nieman *et al.*, 1987). Similarly, four hours of endurance cycling increased blood FFA concentration 40-fold (Meyer *et al.*, 2003).

Protein and amino acids are not considered to be fuels, but do play an important role in the maintenance of cellular process systems, such as maintenance of the enzyme pathways for

breakdown of the four aforementioned substrates. Low intensity exercise does not increase overall protein breakdown in muscle, but at higher intensities, amino acids derived from protein degradation have been shown to aid in the synthesis of TCA cycle intermediates and glutamine (Shimomura *et al.*, 2004). This increase is needed especially during exercise in order to increase the flux through the TCA pathway. Only leucine and part of the isoleucine molecule can be converted to acetyl-CoA to be oxidized (Wagenmakers, 1998), but other amino acids can enter the TCA cycle (e.g. the malate – aspartate shuttle).

All these substrates play significant roles in supplying energy to the muscle. In the next section, focus will be on the enzyme pathways and selected representative enzymes reflecting that pathway, and recent advances in the regulation of these pathways, specifically during an increase in muscular activity.

Metabolic pathways in muscle fibres

Utilising the various substrates discussed above, requires pathways in order to transfer the chemical energy stored in them to fulfil the ATP demand. Each pathway has unique characteristics and therefore the demand for ATP in the muscle will determine substrate utilisation during muscle contraction. Firstly, each pathway varies in the rate at which the breakdown of the substrates take place (mostly enzyme activity dependent), as well as the number of enzyme steps. However, with a decrease in steps, there is also a decrease in the number of ATP molecules produced. The next section on enzymatic pathways will focus on their substrates, selected enzymes and the number of ATPs produced. In addition, this will be brought into the context of the various fibre types found in skeletal muscle, thus relating metabolic ATP provision capacity to the MHC ATPase activity. It should be mentioned that all the pathways and substrates exist in each fibre type, but may vary both in substrate concentration and reaction capacity. Figure 1.4.2 shows a simplified schematic representation of the four substrates and their association with the various pathways.

The enzyme creatine kinase (CK) catalyses the transfer of the phosphate group from PCr to ADP in order to produce one molecule of ATP. There are two isoforms present in muscle cells, namely a cytosolic and mitochondrial CK. The former is concentrated at the M-line of the sarcomere as well as associated with the Ca²⁺ pumps of the sarcoplasmic reticulum, whereas the latter is situated in the inter-membrane space of mitochondria (Bruton *et al.*, 2003). The replenishment of ATP from PCr is very fast compared to the other pathways (partly because of the few steps required) and quickly depletes the PCr stores. Fibres expressing mostly MHC IIX and MHC IIb are commonly associated with a high capacity in this pathway and exhibit a greater CK activity than slow type I fibres (Saltin *et al.*, 1977). Recently it was speculated by Bruton *et al.* (2003) that this pathway may not be as important in generating ATP as previously believed. Comparing CK knockout (CK *-/-*) with wild type mice, the authors showed that the

knockout mice had normal muscular function, but that the knockout was associated with a possible compensatory adaptation, namely an increase in mitochondrial Ca^{2+} concentration (Bruton *et al.*, 2003). The increase in mitochondrial Ca^{2+} concentration stimulates mitochondrial respiration, and overshadows the lack of CK. Greenhaff (2001) proposed the following three functions for the PCr system: first, it functions as a temporal energy buffer for the myofibrils; second, it acts as an energy carrier between the mitochondria and the cytosol and third, it maintains $[\text{ATP}]/[\text{ADP}]$ ratios within the mitochondrial membrane.

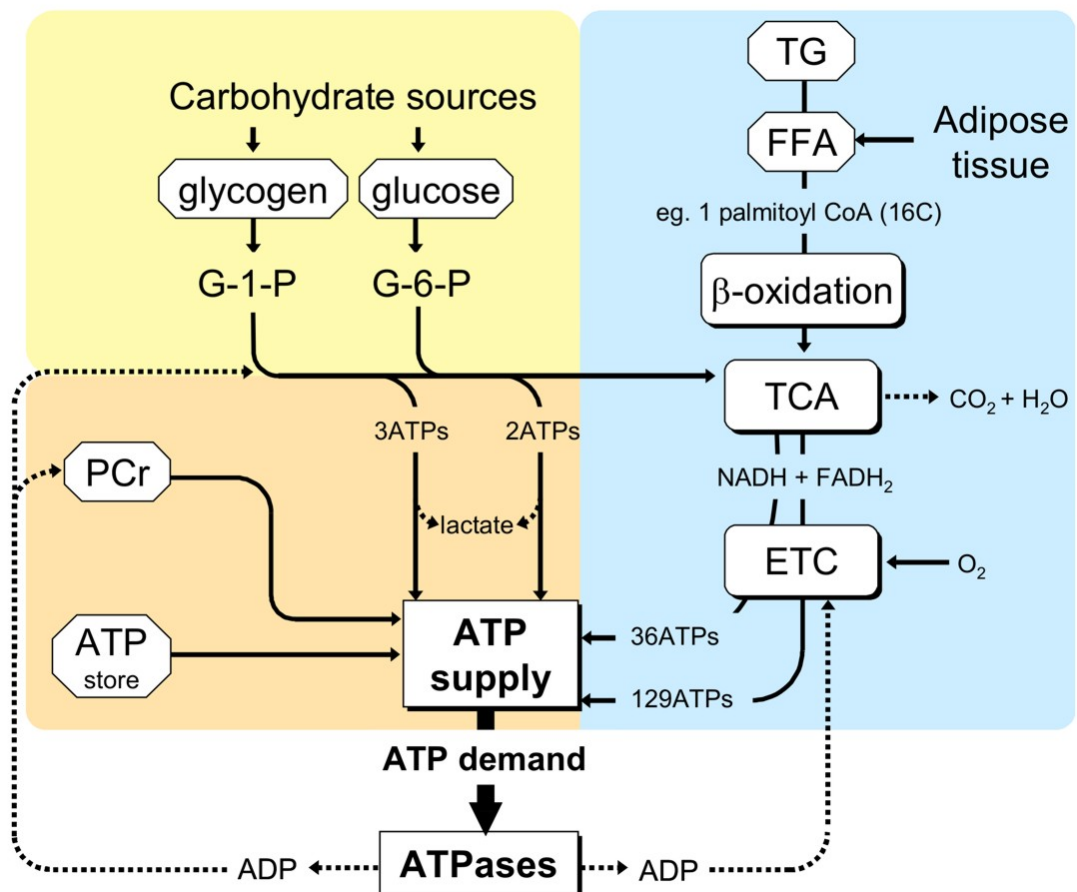


Figure 1.4.2 Simplified schematic representation of the major substrates, their metabolic pathways in skeletal muscle, and the number of ATPs produced per molecule of substrate. TG, triglycerides; FFA, free fatty acids; TCA, tricarboxylic acid cycle; ETC, electron transport chain; PCr, phosphocreatine. Anaerobic metabolism: yellow (glycolysis) and orange block; aerobic metabolism: blue block. Model adapted from Voet and Voet (1995).

Glycolysis and glycogenolysis are the starting points of carbohydrate catabolism to produce ATP. Glucose and glycogen are metabolised through various steps to pyruvate, where the latter can either enter the TCA cycle, located in the mitochondria, or be metabolised to lactate. Generating ATP with the end product as lactate, only produces 2 to 3 ATPs, depending on which substrate was used. However, when pyruvate is metabolised to ATP through the TCA cycle and ETC, 36 ATPs are generated from one glucose molecule. This latter pathway is slow compared to that of glycolysis (Hochachka, 1985), but has a greater capacity to adapt (see section 1.6).

β -oxidation is responsible for the metabolism of FFA to acetyl coenzyme A (acetyl CoA) molecules, takes place in the mitochondria and requires a number of enzymes. Although this pathway is the slowest in generating ATPs, it nevertheless produces the highest number of ATPs from one molecule of substrate (FFA).

Determining fuel preference at rest or during activity can be accomplished using a non-invasive technique that requires the measurement of the volume of CO₂ produced and the volume of O₂ utilised during muscle activity. The respiratory exchange ratio (RER: VCO₂/VO₂) supplies an estimate of the percentage of carbohydrate and fat utilised at a given intensity of muscle contraction and has been used for many years in exercise physiology. Oxidising 100% carbohydrate at a given intensity results in an RER value of 1.00. This is because the complete metabolism of a glucose molecule results in utilisation of 6 O₂ molecules and a production of 6 CO₂ molecules. However, during the metabolism of fat, more O₂ is used by the mitochondria than CO₂ produced, resulting in a RER value of less than 1.00. For example, biochemical calculation (not shown) reveals that an RER value of 0.80 gives rise to a utilisation of 33% carbohydrate and 67% fat by the body at a particular intensity (Bergman and Brooks, 1999). RER values greater than 1.00 are associated with excessive lactate production, but are actually due to an increase in expired CO₂, as a result of the carbonate buffering mechanism in the blood required to neutralise the increased H⁺ formation that occurs during very high intensity exercise (Cox and Jenkins, 1994; Kowalchuk and Scheuermann, 1995). Therefore, the RER value is a useful measurement to indirectly determine fuel preference during exercise (Chapters 4 and 5).

There are a large number of enzymes taking part in glycolysis, β -oxidation, the TCA cycle and ETC. Measuring all the activities can be expensive and some enzyme activities are experimentally difficult to measure. Through the years, researchers in muscle biochemistry have identified enzymes that are reliable in giving an estimate of the capacity of the various pathways and are summarised in Table 1.4.1. A variety of cross-sectional studies have provided insight into how the enzyme capacities may vary depending on factors such as species, muscle regionalisation and fibre type (Essen-Gustavsson and Henriksson, 1984; Kernell, 1998; Kohn *et al.*, 2005; Punkt, 2002).

Enzyme activities and the number of mitochondria can vary substantially between species, between individuals within a species, between muscle groups, between fibre types and even along the length of the fibres. Most of these differences are related to the type of muscular activity for which the muscle is used most, as power generator or for endurance, which will have a direct influence on the fuel preference and a concomitant influence on the metabolic pathways. For instance, the cheetah, known to be the fastest land animal over short distances, but having poor endurance capability, has very low muscle oxidative enzyme activities and low mitochondrial numbers, but very high glycolytic capacities (Williams *et al.*, 1997). On the other

hand, animals known to be able to sustain longer periods of distance running, such as antelope, and those known for extreme endurance capability (horses), have been shown to contain both high glycolytic and oxidative capacity in their muscles (Essen-Gustavsson and Rehbinder, 1985; Kohn *et al.*, 2005; Lopez-Rivero *et al.*, 1992). Muscle groups also tend to have variations in enzyme activities, but this can be explained mainly by their functionality which is also highly correlated with the muscle fibre type (Bass *et al.*, 1969; Spamer and Pette, 1977; Spamer and Pette, 1979).

Table 1.4.1 Summary of the key enzymes commonly measured in skeletal muscle of each metabolic pathway. Abbreviations of each enzyme are given in parenthesis. TCA, tricarboxylic acid cycle; ETC, electron transport chain. Enzymes measured in the present dissertation are underlined.

Creatine system	Glycolysis	TCA	β-oxidation	ETC
Creatine kinase (CK)	Hexokinase (HK)	<u>Citrate synthase (CS)</u>	<u>3-hydroxyacetyl CoA dehydrogenase (3HAD)</u>	Cytochrome oxidase (COX)
	<u>Phosphofructokinase (PFK)</u>	Malate dehydrogenase (MDH)		
	<u>Lactate dehydrogenase (LDH)</u>	Succinate dehydrogenase (SDH)		
	Pyruvate kinase (PK)			
	Pyruvate dehydrogenase complex (PDC)			

Single fibre studies confirmed that fast twitch fibres have lower oxidative capacities and less mitochondria compared to slow twitch fibres (Chi *et al.*, 1983; Essen *et al.*, 1975; Essen-Gustavsson and Henriksson, 1984). However, sometimes it may occur that the enzyme activities of the fibre types overlap. The concept that variation in enzyme activities along muscle fibres may exist, was first discarded when Pette *et al.* (1980) found no difference. However, Lowry's group did find variations along the fibres (Hintz *et al.*, 1984), which was later confirmed by Reichmann (1992) who showed variations in enzyme activities in patients suffering from a mitochondrial myopathy. There has been renewed focus on this issue regarding localisation of enzyme activities and muscle fibre type (Korfage and Van Eijden, 1999; Punkt *et al.*, 1998). In short, there exists a large variation in enzyme activities within a muscle group (e.g. the *Vastus* muscle) with concomitant contractile variations. These regional differences may indicate muscle specialisation, and therefore contribute significantly to the overall functionality of the muscle (see Chapter 2 for further detailed literature review).

Three substrate transport proteins in skeletal muscle fibres may also indirectly affect the functionality of the pathways and they are: glucose transporters (GLUT 4), monocarboxylate transporters (MCT 1 and MCT 4), and carnitine palmitoyltransferase (Coles *et al.*, 2004;

Hildebrandt and Neuffer, 2000; Ryder *et al.*, 1999), but they were not measured in this dissertation.

Each pathway contributes in providing energy in the form of ATPs to the working muscle. Figure 1.4.3 shows a schematic hypothesis of the contribution of the various pathways during exercise at a moderate intensity over time. This clearly shows that the activation and contribution of the pathways are time dependent. However, an increase in contraction intensity may change the way this graph looks, as muscular fatigue may set in before the later pathways are activated (Hawley and Hopkins, 1995). For example, adaptation of the muscular and cardio-respiratory system to training may also have a large impact on the outlook of this graph and will be brought into context in section 1.6. Training intensity (directly related to the speed of muscle contraction) has been shown to shift fuel preference, e.g. glycolytic and oxidative enzyme activities were higher after high intensity training compared to endurance training, since the latter only showed an increase in TCA cycle enzymes (Tremblay *et al.*, 1994). However, controversy still remains on these adaptation of the pathways when looking at various training regimes and intensities, but will be further discussed in section 1.6 and Chapters 4 and 5.

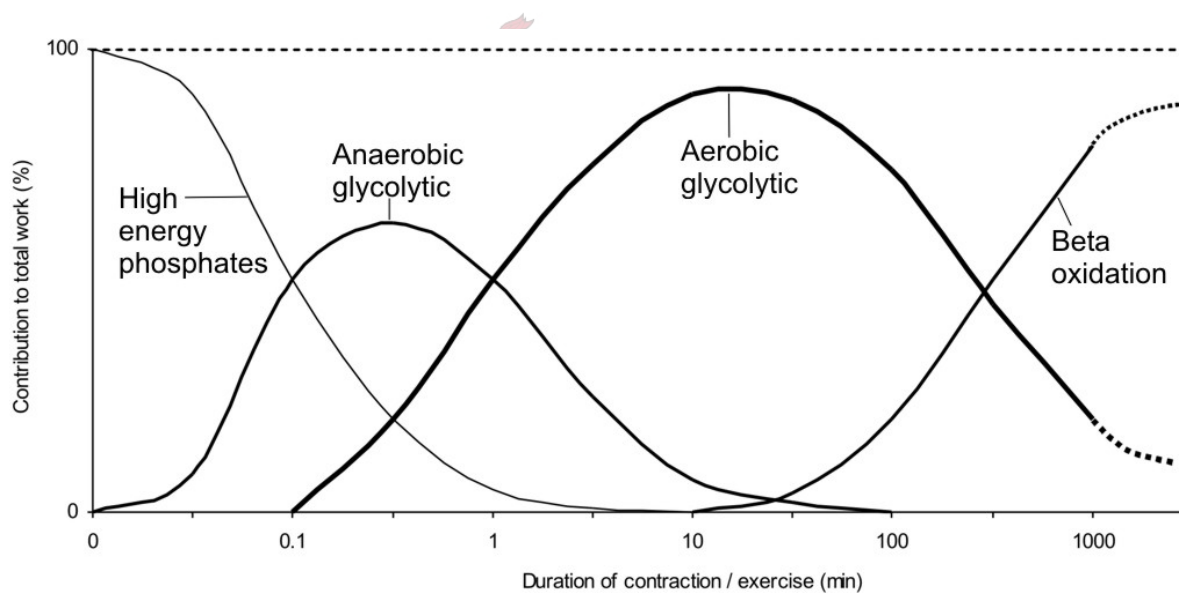


Figure 1.4.3 Graph representing the contribution of the four pathways in providing energy. From Hawley and Hopkins (1995).

Consequence of contraction on metabolism

Regulation of the pathways can be mediated by a change in enzyme concentration, allowing for a greater or reduced flux through the pathways, but also by other mechanisms. Scientists have investigated exogenous substrate supplementation such as carbohydrate, protein and fat, as well as hormonal influences and the effect of drugs. However, the main factor governing metabolism in skeletal muscle is contraction itself, with the aforementioned mechanisms playing a modulating role.

It is well established that training of previously untrained subjects or animals results in an increase in mitochondria, therefore increasing the oxidative enzyme concentration in the muscle fibres, which is directly responsible for the enhanced oxidative capacity (Gollnick and Saltin, 1982; Saltin and Gollnick, 1983; Tonkonogi and Sahlin, 2002). Increasing the concentration of the enzymes results in a typical Michaelis–Menton increase in reaction velocity, which results in a higher flux of substrate through the pathways allowing for an increase in product before the reaction is saturated (Gollnick and Saltin, 1982). Similarly, inhibition of the various pathways by substrates or products has been determined and may be exerted directly on the enzyme in question or on enzymes up– or downstream from it.

Although the topic of this dissertation is not focussed on the regulation or activation of metabolic pathways, but rather on differences in the potential of pathways associated with endurance training (Chapters 4 and 5), it seems necessary to mention some aspects of muscle metabolism.

Activation of metabolic pathways goes hand in hand with mass action. However, it is not supply but rather demand that influences flux (Hochachka, 1985). A decrease in product usually shifts the reaction towards the end product, resulting in a greater demand for the substrate. Skeletal muscle has intracellular carbohydrate and fat stores, but during prolonged exercise, also uses circulating supplies. The transportation and uptake of blood glucose and FFA are crucial for maintaining relatively high intramuscular substrate levels as long as possible. For example, the main transporter for glucose in skeletal muscle is GLUT4, but the GLUT1 transporter is also expressed in skeletal muscle. During exercise, stored GLUT4 transporters are translocated to the sarcolemma, where they assist in the uptake of blood glucose (Furtado *et al.*, 2003). GLUT4 expression is more related to activity level of the muscle, rather than the muscle fibre type, as was shown by Dugaard *et al.* (2000). Activation and translocation of GLUT4 transporters may be under separate control. It is suggested that GLUT4 activation is mediated by the activation of mitogen-activated protein kinase (MAPK), with the latter being activated by muscle contraction (Furtado *et al.*, 2003). Furthermore, AMP-activated protein kinase (AMPK) has also been shown to be involved in an increase in glucose uptake during exercise, with the level of AMPK activation directly proportional to the intensity of the exercise (Musi and Goodyear, 2003). However, the mechanism for increased translocation to the sarcolemma is still unknown. Interestingly, it seems that the GLUT4 protein is not essential for post-exercise glucose uptake, as Ryder *et al.* (1999) showed that in GLUT4-deficient mice, muscle glycogen was restored after 24 hours, similar to wild-type mice. More research needs to be done to evaluate the importance of this transporter protein.

Fat is the most economical fuel in producing large quantities of ATP. With an increase in mitochondria within the cells as a result of exercise training, an increase in carnitine

palmitoyltransferase, β -oxidation and TCA cycle enzymes occurs. These increases result in the ability to increase FFA substrate utilisation, and therefore have a sparing effect on muscle glycogen (Gollnick and Saltin, 1982).

However, the complexity of the regulatory mechanisms involved in carbohydrate and fat metabolism at rest and as a result of endurance exercise is not well understood (Smekal *et al.*, 2003). Most of the controversy in fat metabolism in animals and humans arise from methodological differences. For example, when measuring fat oxidation in animals, nutritional state is important. Whether the animals (or humans) were fasted or not prior to the tests, changes hormonal influences on fuel preference and can change the overall outcome of the results (Smekal *et al.*, 2003; Watt *et al.*, 2002). More controlled research needs to be done to elucidate the controversy.

Another controversy that is important in muscle metabolism (and will be discussed in greater detail in Chapter 4), especially during intense exercise, is the fate of lactate. Brooks and his research group (2000) have proposed the now controversial lactate shuttle system. This involves a mechanism where lactate may be re-converted to pyruvate and subsequently metabolised by the TCA cycle, therefore reusing the lactate as a substrate to produce ATP (van Hall, 2000). It has been proposed that this shuttle may be found within the fibre producing the lactate, or between adjacent muscle fibres. However, new evidence suggests that the proposed mitochondrial LDH responsible for the conversion of lactate to pyruvate, does not exist (Rasmussen *et al.*, 2002; Sahlin *et al.*, 2002), but this topic needs more research.

There exist many more regulatory aspects or proposed mechanisms regulating muscle metabolism, such as cellular signalling pathways that regulate or activate metabolism. However, the aim of this section was to show that, despite the fact that this dissertation will focus on the required enzyme pathways and their adaptations due to exercise training, muscle metabolism is not that simple (more detail in section 1.6).

1.4.4 Fibre size

It is well known that muscle fibres can increase (hypertrophy) or decrease (atrophy) in size, depending on the contraction frequency. Furthermore, a direct relationship exists between force generation and cross-sectional area (CSA) (Widrick *et al.*, 1996). Hypertrophy is associated with an increase in individual muscle fibres both in area and diameter by incorporation of more myofibrils (actin and myosin). However, the opposite effect, namely atrophy, results in a loss in myofibril content of cells, also reducing power output and is mostly observed in inactive muscle (or spaceflight) (Baldwin, 1996). CSA of a fibre type may also respond differently to different stimuli (Wilmore and Costill, 1999), e.g. the stimulus frequency. The average CSA of fibre types in the *Vastus lateralis* muscle of sedentary men between the ages of 23 and 30 years are

5310 ± 1210, 6110 ± 1200 and 5600 ± 1450 μm² for type I, type IIA and type IIX fibres, respectively (Saltin *et al.*, 1977). In Chapters 4 and 5, CSA was measured to determine whether this has a significant effect on performance in already well-trained athletes.

1.5 Cellular signalling pathways – regulation of muscle characteristics

Modification in gene transcription requires some sort of signal. These signals are usually the result of a shift in homeostasis, such as muscle contraction or hormonal influence. From the previous sections and later in section 1.6, it is clear that muscle structure and metabolism, directly influencing contractile properties, can be influenced by signals, whether it is during contraction itself or leading to adaptation. This section briefly discusses recent advances in cellular signals related to muscle adaptation. Although a multitude of signalling proteins exist, only a few are focussed on, merely for relevance to the chapters following in this dissertation.

Intracellular Ca²⁺ has been proven to mediate a vast number of cellular processes in addition to its role in regulation of skeletal muscle contraction (Stull, 2001). Calcium rarely acts on its own, and usually binds to mediator proteins, such as calmodulin (CaM). CaM has four Ca²⁺ binding sites and can regulate other signal transduction proteins such as calcineurin and CaM-dependent protein kinase (CaMK) I, II and IV.

Calcineurin (also known as protein phosphatase 2B) is located in the cytoplasm and has a CaM binding site, as well as Ca²⁺ binding sites. Once Ca²⁺/CaM binds, calcineurin itself can then bind directly to other molecules such as Nuclear Factor of Activated T cells (NF-AT)c, and allowing dephosphorylation of the latter molecule, which renders it active (Olson and Williams, 2000). This activated form can then translocate to the nucleus to allow transcription (Ikura *et al.*, 2003), by binding directly to DNA, or to other transcription factors such as myocyte-specific enhancer factor 2 (MEF2) and AP1 (Crabtree, 2001; Musaro *et al.*, 1999; Olson and Williams, 2000; Rothermel *et al.*, 2003). The localisation of calcineurin may also be an important factor. The protein calsarcin binds calcineurin and targets it to the Z-disk of the sarcomere. Three calsarcin isoforms have been identified, namely calsarcin 1, expressed only in slow skeletal muscle fibres and cardiac muscle, and calsarcin 2 and 3, which is only expressed in fast skeletal muscle fibres (Rothermel *et al.*, 2003; Schiaffino and Serrano, 2002). This seems to indicate that calsarcins directly link calcineurin to the contractile apparatus, which in turn implies that muscle fibre activation may influence calcineurin activation, and may therefore have a direct influence on fibre type.

To maintain activated NF-ATc in the nucleus, there must be a persistent elevation of intracellular Ca²⁺ levels, therefore mediating continuous calcineurin activity. This is because NF-ATc proteins are rapidly exported from the nucleus by re-phosphorylation of the same residue on that molecule (Crabtree, 2001). Therefore, this is a good mechanistic explanation for

why the duration of exposure to training (volume) might influence gene expression, consequently altering fibre type and metabolism. Whether intensity of contraction plays a role, still needs to be elucidated. It was, however, recently proposed that another signalling protein, namely mammalian target of rapamycin (mTOR), is responsible for muscle fibre size, independent of calcineurin activity (Schiaffino and Serrano, 2002). This increase in muscle fibre size may be directly influenced by the intensity of muscle contraction.

The activated the NF-AT complex, already translocated to the nucleus, may also activate the expression of modulatory calcineurin interacting protein (MCIP), specifically MCIP1, which has 4 variants MCIP1.1 to 1.4 (Rothermel *et al.*, 2003). MCIP1 proteins bind directly to the calcineurin A subunit and inhibit phosphatase activity. It has been postulated that it might only bind to the activated form of the calcineurin A subunit. This proposes a negative feedback regulatory mechanism for calcineurin (Rothermel *et al.*, 2003). MCIP1.4 is preferentially expressed in muscle groups containing slow muscle fibres and it is also reported that endurance exercise may increase MCIP1.4 transcription in skeletal muscle (Norrbon *et al.*, 2004; Rothermel *et al.*, 2003). Figure 1.5.1 depicts a schematic representation of the activation and inhibition of calcineurin activity.

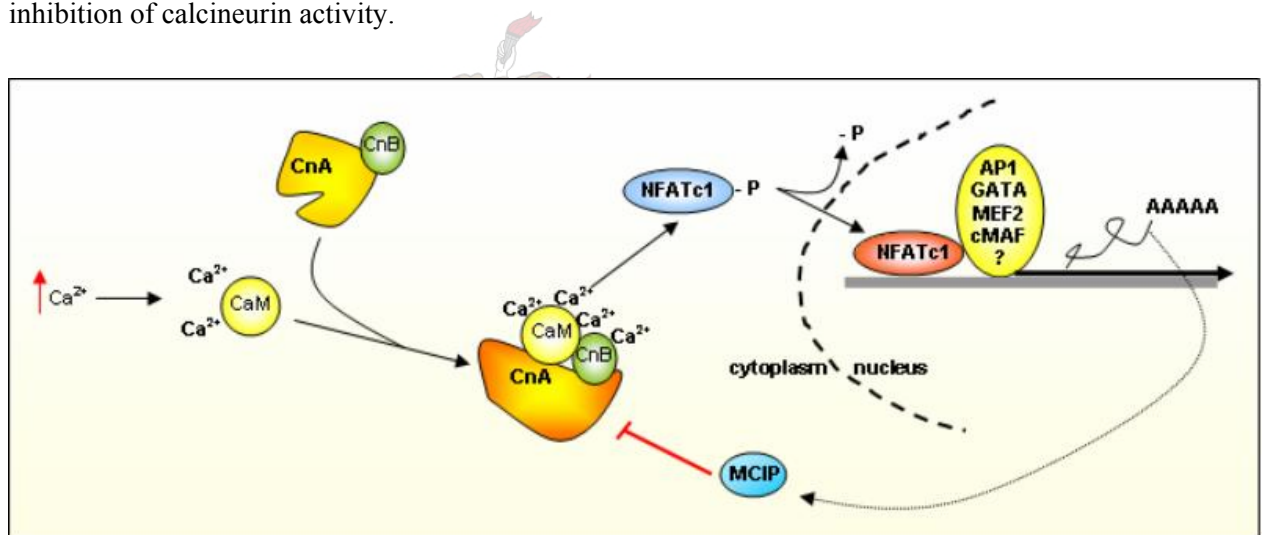


Figure 1.5.1 Calcineurin activation and inhibition pathway in skeletal muscle. From Rothermel *et al.* (2003). See text for abbreviations.

In contrast to calcineurin that dephosphorylates target proteins, CaMK belongs to a family of proteins that phosphorylates a target protein. There are three types of CaMKs, namely CaMKI, CaMKII, and CaMKIV. An extra upstream protein, CaMK kinase (CaMKK) is also included in this family. CaMKK is activated by Ca^{2+} /CaM binding and subsequently may phosphorylate CaMKI and CaMKIV. CaMKI expression is found in all types of cells, whereas CaMKIV is not expressed in skeletal muscle, but is found in cardiac muscle (Chin, 2004; Corcoran and Means, 2001). The role of CaMKII has only recently been investigated in skeletal muscle and has been found to be down-regulated in atrophying skeletal muscle (Chin, 2004) and up-regulated in response to muscle contraction (exercise) (Rose and Hargreaves, 2003). Similarly, these kinases

have also been shown to be involved in mitochondrial biogenesis. However, more research is needed in order to show the specific mechanisms involved, whether it plays a significant role in muscle fibre type switching and metabolism.

Besides the calcium-activated signal transduction pathways, three other possible mechanisms have been proposed: cell surface receptor activation by extracellular signalling molecules released from the motor nerve, direct sensing of mechanical forces generated by loading conditions, and sensing of intracellular metabolite concentrations that change as a consequence of muscle contraction (Olson and Williams, 2000).

Mechanical factors influencing gene expression

Studies done on muscle that was electrically stimulated with repeated contractions, showed that this intervention led to the expression of type I fibres. This was also true for the passive stretch of muscle, where type I sarcomeres were added to the type II fibres. This led to the identification of the mechano growth factor (MGF) that has the ability to activate the expression of MHC I. This factor has no effect on switching of MHC IIx to MHC IIa. What the specific pathway of this factor is, is still unclear and whether exercise plays a role, still needs to be investigated. However, it may be postulated that MGF may be influenced by exercise, as this involves contraction and stretching of the muscle (Goldspink, 2003).

Motoneuronal activity and sensing

Studies conducted on increased calcineurin activity or the inhibition of calcineurin and Ca^{2+} , led to the conclusion that calcineurin activity promotes type I fibre expression (Serrano *et al.*, 2001). It has been shown that different patterns of motoneuronal activity can alter the fibre type. Type I fibres usually have an almost continuous basis of activation, resulting in an oscillation of intracellular Ca^{2+} concentration ranging between 100 to 300 nM. This continued activity might just be necessary for continuous calcineurin activation to activate NF-ATc. On the other hand, motoneurons that innervate type IIA and IIX fibres, fire only intermittently (Olson and Williams, 2000). However, it has been shown that calcineurin activity played no role in the conversion of IIX to IIA fibre types in both cultured muscle cells and *in vivo* rat and mouse muscle (Schiaffino and Serrano, 2002; Serrano *et al.*, 2001), indicating another mechanism or signalling protein involvement.

NF-AT activation by calcineurin also appears to be involved in gene activation in subsets of fast muscle fibres. It was shown that calcineurin controls the amount of e.g. myoglobin content in both type I and IIA fibres. In cultured muscle cells, NF-AT over-expression or constitutively active calcineurin preferentially activated MHC IIa, but not MHC IIx or MHC IIb genes in mice (Schiaffino and Serrano, 2002).

Calcineurin inhibition by Cain (also known as Cabin) down-regulates MHC I and MHC IIa gene expression, but up-regulates MHC IIx and MHC IIb expression in adult slow skeletal muscle. This suggests that calcineurin controls the induction and maintenance of fibre type specific gene expression programmes (Schiaffino and Serrano, 2002). However, Cain is only found in very low quantities in skeletal muscle compared to the brain (Olson and Williams, 2000) and whether this molecule plays a significant role in adaptation due to muscle contraction, still needs to be investigated. Because inactive calcineurin promotes slow to fast muscle fibre type transitions, it may also explain why paralysis and detraining result in fibre type switching to faster isoforms, as intracellular Ca^{2+} is required to activate calcineurin.

Mitochondrial biogenesis

Many studies have focused on mitochondrial biogenesis mechanisms not only in muscle, but also in other tissues (for a review see Lee and Wei (2005)). Since this dissertation is directed at analysis on protein level rather than the mechanisms involved, only a few transgenic studies will be highlighted.

It has long been known that mitochondrial density increases with muscle activity, for instance endurance exercise increases the amount of mitochondria per fibre, but also stimulates fibre transitions. Recently, the focus of signalling protein research has shifted to the peroxisome proliferator-activated receptors (PPAR). One of the major effects of these proteins is the up-regulation of fat oxidation in adipose tissue and skeletal muscle. Specifically, the delta (δ) form is the predominant isoform found in skeletal muscle (Luquet *et al.*, 2004). The role of PPAR δ was further elucidated when Wang *et al.* (2004) showed that transgenic mice over-expressing this protein, could run twice the distance than wild type mice, therefore having a greater resistance to fatigue – typical scenario when comparing trained with untrained individuals. Furthermore, these mice had an abundance of type I fibres and an increase in mitochondrial numbers in their skeletal muscle (Luquet *et al.*, 2003). The data suggest that new ways or drugs may be developed to control obesity. However, this also opens the door for drugs to enhance performance in sport, but whether this will be effective, still needs to be investigated.

The few signalling proteins mentioned above are only the tip of the ice berg of cellular signals involved in fibre type maintenance and mitochondrial biogenesis. Many more exist and may be activated by hormones, such as thyroid hormone, which may be independent of muscle activation. However, these signals may play important roles during exercise. Furthermore, specific exercise (e.g. endurance vs. resistance exercise) may activate other signals, and may therefore also have different effects on skeletal muscle, but more research needs to be performed to elucidate specificity.

1.6 Adaptation of muscle fibres

For adaptation to occur, tissue's homeostasis must first be perturbed. Voluntary contraction (e.g. exercise) is one of the main factors that perturbs skeletal muscle's homeostasis and drives changes in MHC isoform expression and alters metabolism, causing adaptation in fibre type. Other factors that can modulate or mediate adaptation are genetics, circulating agents, such as hormones and cytokines, alterations in the usual muscular activation which include paralysis, detraining, added physical training or electrical stimulation. External factors that could be called environmental factors that have a direct effect on the organism, including the muscular system are altitude, temperature, nutritional status and socio-economic status. Furthermore, these factors or stimuli trigger internal cellular signals that transfer the external stimuli to the nuclei where transcription of specific genes are up- or down regulated.

1.6.1 Search for genes contributing to muscle characteristics

The influence of genetics on skeletal muscle is a very broad field. In animals, there are large variations in genetic makeup between species and this contributes extensively to the overall structure and function of skeletal muscle. For example, studies have shown that hind limb muscles of the cheetah and antelope varies significantly in structural and metabolism, therefore affecting functionality (Essen-Gustavsson and Rehbinder, 1985; Kohn *et al.*, 2005; Williams *et al.*, 1997). Comparing the MHC isoform distribution in hind limbs of a larger variety of animals, it is clear that smaller animals (such as rats, mice and rabbits) regularly express the fast MHC IIb isoform, whereas in larger animal species (horses and cattle), including humans, the MHC IIb isoform is a rare phenomenon and is restricted to specialised muscle groups (Allen *et al.*, 2001; Andersen *et al.*, 2002; Lucas *et al.*, 2000; Rivero *et al.*, 1997; Talmadge and Roy, 1993).

Heritability of muscle characteristics

Heritage may be defined as the transfer of phenotypic characteristics from parent to descendent. In living organisms, including bacteria, this is accomplished through the genes inherited from both parents that will comprise the genotype of the organism and eventually influence the phenotype. Typical examples of phenotypic variation in mammals are hair and eye colour. Humans have a much broader phenotype influenced by genotype that includes i.e. body stature, skin colour, the amount of fat around the eyes, or nose length. An organism's genotype also influences the phenotype of internal tissues such as its muscular characteristics, as muscle usually comprises a large proportion of the total organism.

Throughout the years, researchers have tried to identify genetic determinants of muscle characteristics. Most of these studies have related muscular characteristics to performance such as the ability to perform work in the form of exercise. In a review by Bouchard *et al.* (1986), they made the statement, based on the findings in previous studies and other studies on

characteristics of mono- and dizygotic twins and brothers, that 45% of the variability of type I fibres may be attributed to genotype. The remaining percentage would be attributed to muscular activity and environmental influences. Simoneau and Bouchard (1989) further investigated the variability in muscle fibre type and enzyme activities in 418 non-related human muscle biopsy samples, concluding that the coefficient of variation of the parameters measured ranged between 21 to 71%, indicating a large inter-individual variation. Bouchard and his group have thereafter been involved in the Familial Heritage study, focussing on several aspects of inheritance and how this may influence muscle characteristics and overall performance (Bouchard *et al.*, 1995). Some of the findings will be discussed here.

Bouchard's group focussed on the muscle-specific creatine kinase (CKMM) genes in elite endurance runners and found that there was no association between the genes and $VO_2\text{max}$ (a measurement of physical fitness level) of the athletes (Rivera *et al.*, 1997b). However, when they endurance trained 160 unrelated sedentary parents and 80 unrelated adult offspring for 20 weeks, a significant association was observed between the CKMM polymorphism and the change in $VO_2\text{max}$ (Rivera *et al.*, 1997a). However, the authors were sceptical about whether there was a direct connection between the two measurements, or if CKMM only co-varied with another parameter, or other parameters not measured. This scenario was strengthened by a recent publication by Bruton and co-workers (2003) where they showed that CK knockout mice had normal muscular function, and in some cases, more superior contractile properties, compared to wild type mice. Therefore, a knockout of a specific gene may be compensated for by another not assessed. Recently, the Bouchard group published a gene map relating 109 autosomal genes to specific performance markers (Rankinen *et al.*, 2004). Furthermore, 15 mitochondrial genes had been identified where sequence variants may influence fitness and performance phenotypes. Since this thesis deals more with phenotype than specific genotypes, these will not be discussed here. The search for polymorphisms in putatively key protein genes is not the only way to study the influence of genetics on skeletal muscle and exercise performance. Bouchard himself describes the "bottom-up vs. top-down" approaches as both being relevant and necessary (Bouchard *et al.*, 1992). Indeed over the past 15 years, whilst gene mapping was progressing, population and familial studies were also making progress in delineating what phenotypes can be described as different in distinct populations. These studies were generally performed in small laboratory animals and in human subjects, although in the latter case, with fairly small sample sizes.

The genetic effects in small animals were first investigated in 1989 by a group investigating voluntary running in rats. The authors divided the animals in three categories, high, medium and low activity (km/day), but could not find any difference in cytochrome oxidase and HK activities (Rodnick *et al.*, 1989). The group of Sieck selectively bred mice for 10 generations

with high voluntary running capability and the authors postulated a significant genetic effect on activity level and aerobic capacity (VO_{2max}) (Swallow *et al.*, 1998), and activity and body mass (Swallow *et al.*, 1999). However, no relationship was found between muscle characteristics (fibre type and SDH enzyme activity in the medial *Gastrocnemius*) and aerobic capacity, indicating that performance is not necessarily reflected in muscle composition (Zhan *et al.*, 1999). However, Houle-Leroy *et al.* (2000) showed that there were significant differences in enzyme activities between mice from a 14 generation inbreeding programme and randomly selected control mice, but that the enzyme response to training was similar in both lines. To complicate matters more, the authors suggest that gender plays an important role in the activities of enzymes. This is somehow contradictory to the literature, where it is commonly accepted that fibre type is influenced and oxidative capacity is enhanced by training. However, possible explanations for the poor relationships may be large inter-individual variations. Furthermore, it may be that gene transcription varies significantly between individuals, as was discussed in the CK knockout mice (Bruton *et al.*, 2003). This implies that other gene products not measured may be activated and may therefore contribute to the poor relationships found.

Finally, Harrison *et al.* (2002) compared two strains of mice lacking either the MHC Iib gene or the MHC Iix gene. In both groups, voluntary running performance was reduced significantly, but CS activity was increased in MHC Iib null mice. The latter result can be explained by the fact that the MHC isoform distribution differed significantly in the muscle analysed. MHC Iix fibres tend to have similar or higher oxidative capacity than MHC Iib fibres and in this study, MHC Iib null muscle had a compensatory higher expression of MHC Iix and therefore gained in overall CS activity, whereas MHC Iix null mice showed lower CS activity and a higher expression of MHC Iib.

Scientists, however, are gradually closing in on finding at least some of the specific genes and activators responsible for enhanced performance and muscle characteristics by investigating the role and regulation of cellular signalling pathways in muscle (section 1.5). However, as it was proposed by Andersen *et al.* (2000), it may be that an individuals' genes are not the only important factor in determining muscular characteristics for enhanced performance, and that the external factors, including trainability, may indeed play a crucial role.

Do some human populations have a genetic advantage?

It is well known that East African runners dominate the world of endurance running and African-American athletes the sprinting events. Recent world road running rankings for 2005 listed 77 and 8 Kenyan and Ethiopian athletes, respectively, under the top 100 in the world (source: International Association of Athletics Federations). This led scientists to explore the possibility that these athletes might have a genetic advantage. But not only in the elite athletes

per se, but that ethnic background plays a role in performance and that physiological and biochemical differences may exist between races.

Few studies have investigated both physiological and biochemical parameters between races, specifically between black/African and white/European groups, but it must be kept in mind that cultural and social influences may also play significant roles. In 1986, Ama *et al.* (1986) compared white (North American) and black (originating and living in West and Central Africa) sedentary subjects and found that black subjects had a higher proportion of fast twitch fibres with concomitant higher glycolytic enzyme activities (CK, HK, PFK and LDH). This led the authors to conclude that black subjects from West African origin may have an advantage when competing in short sprinting events. This finding can be brought into context when results of the previous Olympic Games in Athens (2004) are considered. Taking the first 3 positions in the 100 m, 200 m and 400 m final male sprinting events, only one out of the nine athletes was from white European descent, with the remainder from black African descent. For women competing in the same events, three were from white European descent, with the remaining six from black African populations. Furthermore, Hickner *et al.* (2001) showed that lean black African-American women had lower fat oxidation capacity compared to their white counterparts. One possible explanation for lower fat oxidation in the black women may be the influence of lower levels of thyroid stimulating hormone (TSH). TSH activates the release of thyroid hormone resulting in an up-regulation of resting metabolism, and therefore fat oxidation. This was confirmed in a study by Schectman *et al.* (1991), who found that black subjects had significantly lower TSH levels than white subjects. However, the results of these aforementioned studies should be interpreted with caution, as cultural influences, such as diet and physical habits (i.e. recreational activities, means of transportation, or type of work) may have a significant influence on physiological parameters.

Ama *et al.* (1990) showed that maximal force production, peak power output (PPO), and total work output during a 10, 30 and 90 second maximal voluntary knee extensor test was not different between black subjects from West and Central Africa compared to their white counterparts. However, the authors did show that the black subjects had a lower resistance to fatigue during the same tests compared to their white counterparts. These findings could therefore not strengthen the hypothesis for an advantage in sprinting events in black athletes originating from the Western parts of Africa. However, one aspect that may have had an influence on the findings is that the black subjects were significantly older than their white counterparts, although they were matched for body height and weight. This suggests that the black subjects may have been subjected to a longer period of inactivity than their white counterparts, which may have had a direct influence on fatigue resistance. Similarly, it was

reported that the subjects were sedentary at the time of testing, but previous sporting encounters (e.g. at school level) were not reported.

In contrast to the finding of higher type II fibre proportions in black subjects than white subjects in the study by Ama *et al.* (1986), Duey *et al.* (1997) found no difference between fibre type proportions and capillary density in muscle biopsies in black and white recreationally active subjects (born and living in the USA). However, once again, methodological problems may have contributed to the outcome of the results as the white subjects were older and heavier than their black counterparts.

If a genetic advantage exists in population groups, then the possibility may arise that some populations may respond differently to training. However, Skinner *et al.* (2001) showed no difference in the response (VO_2 and $\text{VO}_{2\text{max}}$) to a training regime between black and white subjects, with both groups including individuals with a low, medium or high response to training. It may be that individual responses may have an influence, as Bouchard (1995) commented that large variations exist between subjects subjected to the same training regime.

Results from these studies do not specifically indicate whether a significant genetic influence for performance exist in the populations in question. The studies discussed so far have been conducted in groups of sedentary individuals. Therefore, on the one hand, the confounding influence of physical activity / training was not present. On the other hand, any genotype-environment interaction such as trainability was also not assessed.

The lay press has sensationalised the success of African athletes by ascribing it to a racial advantage, when scientists are not clear about this issue. A newspaper article in the South African Mail and Guardian (Arlidge, 2000), quoting papers by Saltin's research group (Saltin *et al.*, 1995b; Saltin *et al.*, 1995a), stated that black runners are genetically programmed to run faster than their white counterparts. Saltin's group investigated Scandinavian and Kenyan runners who trained and lived together during a training camp in Kenya. During this time, the researchers performed physiological exercise tests, as well as muscle biopsies for analysis of fibre type and enzyme activities. No differences were found for fibre type, capillary density, and $\text{VO}_{2\text{max}}$, but the marker enzyme for fat oxidation, 3HAD activity, was significantly higher in Kenyan runners. Also, Kenyans were more economical when running, and had lower plasma ammonia concentrations during sub-maximal and maximal exercise tests. However, Saltin *et al.* (1995b) reported that the average diet of the Kenyan runners were high in protein content, but higher dietary protein is also associated with higher ammonia production during exercise (MacLean *et al.*, 1994). The lower ammonia concentration may therefore be related to the higher fat metabolism in Kenyan muscles.

Similarly, plasma lactate concentrations at the same absolute workloads were lower in the Kenyan compared to the Scandinavian runners, and may be related to the higher fat metabolism of the Kenyan runners. Although total LDH activity was not different between the two groups, it was found that Kenyan runners had a higher ratio of LDH₁₋₂/LDH₄₋₅ in their *Gastrocnemius* muscle compared to the Scandinavian runners prior to the training camp. The LDH₁₋₂ isoforms, predominantly found in heart muscle, favours the conversion of lactate to pyruvate, whereas the LDH₄₋₅ isoforms favour pyruvate to lactate conversion (van Hall, 2000). After the training camp at altitude, the LDH ratio increased in the Scandinavian runners to similar values to the Kenyan runners (Saltin *et al.*, 1995a). Similarly, plasma lactate concentrations after the training camp was also significantly reduced (Saltin *et al.*, 1995b). Whether the decrease in plasma concentration was because of the altitude effect or because of an increase in training intensity, was not established, but both may have had an influence. However, these results do not imply that Kenyans' phenotypic differences are based on a genetic advantage. Life style (daily walking and running from a young age) may also have played a significant role in setting phenotypic differences (Larsen, 2003).

Work by Bosch *et al.* (1990), Coetzer *et al.* (1993) and Weston *et al.* (1999) also investigated the physiological and biochemical characteristics in South African black and white athletes. These studies were more controlled as in each study, both groups came from the same area at or close to sea level, eliminating altitude exposure as a confounding factor. Bosch *et al.* (1990) showed that there was no difference in VO₂max between black and white athletes, but that black athletes ran a simulated marathon at a higher percentage of their VO₂max. Similarly, black athletes ran at a higher RER, which is an indication of preference for carbohydrate above fat as fuel. No muscle biopsies were taken, thus it is difficult to determine if the higher RER was related to the higher intensity at which they ran, or to differences in muscle enzyme activities. Nonetheless, the finding of higher fractional utilisation of their maximal oxygen consumption capacity, was significant. Fractional utilisation of VO₂max is related to training volume, but whether it is related to training intensity, is still unclear (Scrimgeour *et al.*, 1986). However, this may be related to muscle fibre type and oxidative enzyme capacity of the muscle.

Coetzer *et al.* (1993) found that, although both black and white groups had similar training volumes per week, the same VO₂max values and percentage type I fibres, black athletes trained at a higher intensity than white athletes. However, the training intensity was assessed from questionnaires and may be questionable whether this method was adequate. Plasma lactate concentrations were also lower in black athletes during a sub-maximal exercise test. During a maximal voluntary force production test of the quadriceps muscle, although lower force production, black athletes could withstand the test for a longer time until isometric force was reduced to 70% of maximal voluntary contraction. However, it could be argued that, because the

force produced in this test was lower in the black athletes, they would have been able to withstand the test for a longer period of time, therefore, once again, may have been more related to a methodological problem with the test itself.

Weston *et al.* (1999) found similar results to the above two studies, but in addition showed that CS and 3-HAD activities were elevated significantly in muscle biopsies from the black athletes compared to the white athletes. On the other hand, though not significant, black athletes also tended to have a lower percentage of type I fibres (therefore more type II fibres), as well as a tendency to have higher PFK activity in their muscle. This is in accordance with the literature that shows that fast twitch fibres have higher PFK activities (Essen *et al.*, 1975). However, since type II fibres are also associated with a lower CS activity than type I fibres, it is interesting that the black runners had the higher CS and PFK activities as well as type II fibres. It therefore would have been valuable to have determined exact fibre type proportions, dividing the type II fibres into the type IIA and IIX sub-populations. Furthermore, it has also been reported that the oxidative potential of type IIA fibres may be equal or even higher than type I fibres (Saltin and Gollnick, 1983), and that this may be related to external factors, such as training intensity (more adaptable), or because of genotype.

In a recent study, Marino *et al.* (2004) reported that black endurance runners could run a self-paced 8 km time trial in a shorter duration at 35 °C than their white counterparts, with no difference observed at cooler (15 °C) conditions. Sweat rate was also lower in these black runners. The authors attribute these findings to the smaller body size of the black runners, thus indicating an advantage when running at higher temperatures. There may be a possibility that living conditions (socio-economic) and training intensity may contribute significantly to the better running capability of the black runners in the heat.

The studies above could not determine exact racial differences on muscle characteristics and lacked statistical power in subject numbers. However, one possible explanation did arise from these studies that training intensity may be a more crucial determinant of muscle characteristics and performance than racial genetic variation. Therefore, Chapter 3 investigates specialisation distance (as a measure of training intensity) on muscle fibre type in endurance trained athletes as well as recreational active subjects. Furthermore, Chapter 4 continues the search for racial genetic variation, and has been structured to include extensive muscle fibre type characterisation and enzyme analyses, as well as analyses of enzyme activities in pools of type I and type IIA fibres. Table 1.6.1 summarises selected studies involving black and white endurance runners, most importantly, those on whom muscle biopsies were performed.

Table 1.6.1 Selected studies on black and white endurance runners and possible factors that may have influenced the overall outcome of the results.

References	Subject no.		Selection criteria	Muscle biopsies	Possible confounding factors
	White	Black			
Bosch <i>et al.</i> , 1990	10	9	Recent marathon time matched between groups (<165 min)	None	Training volume and intensity may have been different; age difference not reported
Saltin <i>et al.</i> , 1995a	5	4	None reported	Yes, type I, IIA, IIX fibres and enzyme activities determined	Altitude, diet, training volume & intensity, preferred racing distance. Kenyan athletes were older than the Scandinavians. Low subject numbers.
Coetzer <i>et al.</i> , 1993	5	6	1 mile (<4 min), 3 km (<8,3 min), 5 km (<14 min)	Yes, type I fibres determined, no enzyme activities	Black athletes were smaller in stature and lighter, reported faster 10 and 21.1 km times; age not reported; only percentage type I fibres measured; not matched for performance; low subject numbers
Weston <i>et al.</i> , 1999	5	7	Performance matched	Yes, type I fibres and enzymes activities determined	Blacks were smaller in stature and lighter; tended to be younger; no preferred racing distance reported; low subject numbers

1.6.2 Muscle activation

In order for muscle to adapt to the various external or internal signals, the relative genes must be activated and transcribed to mRNA, where after translation follows of the mRNA to protein. Andersen and Schiaffino (1997) compared two techniques for determining fibre type, the one utilising RNA probes directed at the three MHC isoforms (therefore investigating gene activation) and the other, the use of antibodies directed at the slow and fast isoforms. The samples used were from pre- and post-trained subjects. In some of the fibres analysed, there was a mismatch between the specific MHC mRNA and its resultant protein. The effect was more pronounced after the training period. The same authors proposed a model between mRNA and protein expression that, during the early stages of fibre type transition, mRNA of both MHC isoforms may be present, with only the existing protein being expressed, but during the later stage, the mRNA of the existing MHC isoform is switched off with no detection there after, but that both isoforms will be found on protein level. This hypothesis may therefore explain the construction and existence of hybrid fibre types in skeletal muscle in response to stimuli.

Neural input including electrical stimulation and re-innervation

Muscular activity in itself is probably the most important factor contributing to muscle adaptation, both structurally and metabolically. This might even overshadow genetics and environmental conditions for some muscle characteristics, i.e. paralysis and electrical stimulation (see section below).

Muscular activity is dependent on neural input, e.g. the neurons that innervate a specific muscle group. Neural input, chronic activation or lack thereof, may therefore change fibre type expression and metabolism. The most classic examples of the influence of neural input are electrical stimulation, de- and re-innervation. Studies showed that by electrically stimulating skeletal muscle, muscle fibre type switches can be accomplished (Pette and Vrbova, 1992). By applying high frequency electrical stimulation, fibres can be stimulated to undergo shifts from type I to type IIA fibres, where low frequency stimulation resulted in the transformation of fibres from type IIA to type I (Martin *et al.*, 1992a). In addition, the duration of electrical stimulation may also play a key role in which direction fibre type will transform. For instance, eight hours per day of electrical stimulation (20 Hz pulses) in spinal chord injured subjects for 24 weeks resulted in a significant increase in type I fibre proportions of the TA muscle (Martin *et al.*, 1992b). Greve *et al.* (1993), on the other hand, although showing a transformation of type IIB to type IIA fibres, showed no change in type I fibre proportions after 3 months of 30 minutes per day (at 20 Hz) electrical stimulation in the *Quadriceps femoris* muscle of spinal chord injured patients. Therefore, the duration of stimulation time may be crucial for an increase in type I fibre proportions. Endurance runners are usually associated with a high proportion of type I fibres (Saltin *et al.*, 1977), and part of this reason may be because of the time spent running (e.g. completing a marathon requires continuous running of up to two and a half hours in well-trained endurance runners).

In contrast to electrical stimulation duration at low frequencies, higher frequencies may elicit different results, as Andersen *et al.* (1996) showed in a year long electrical stimulation of the *Vastus lateralis* muscle of paralysed subjects. The stimulation was carried out three times per week for 30 minutes per day at a frequency of 60 Hz. After a year, no increase in type I fibres were observed, but a significant decrease in type IIB and IIA/IIB hybrid fibres with a concomitant increase in type IIA fibre proportions occurred. This type of stimulation is typical in sports requiring fast contraction and relaxation of muscles, such as in sprinting events. In a three month study on sprint training adaptation in sprint runners, it was shown that type IIA fibres significantly increased after the training period (Andersen *et al.*, 1994b). However, what the effect of an increase in contraction intensity will be in already trained endurance athletes, has not yet been investigated. Based on the findings reported above, it may be hypothesised that a further conversion to type IIA fibres will be observed, but that the CSA of the fibres may also increase.

Some extreme conditions of muscular activation and immobilisation will be discussed below, each having a significant influence on muscle morphometry and metabolism. Paralysis, spaceflight, and training, specifically low intensity endurance, high intensity endurance,

resistance and sprint training also significantly alter neural activity patterns. In each case, sedentary models will be used as comparison.

Paralysis

The inability of signals from the brain and spine reaching the muscles *via* the neurons to stimulate muscle contraction may be caused by neuronal degenerative diseases or from spinal chord injury. This results in paralysis or the inability to voluntarily contract muscle. Although paralysis itself is not studied in this thesis, it is a good extreme model to illustrate and discuss control of muscle protein expression in addition to contraction.

One of the main phenotypic observations of muscle in response to paralysis is the reduction in CSA of the fibres (atrophy) (Talmadge *et al.*, 2002b). It seems clear that the quantity of the two most abundant proteins, actin and myosin (which are directly involved in the cross-bridge cycle), decrease in response to paralysis. The latter loss was also shown by Matsumoto *et al.* (2000), where they found a significant decrease in the MHC / actin ratio in response to acute quadriplegic myopathy (AQM). However, it seems that neural input plays an even more important role, as it was recently shown by Di Giovanni *et al.* (2004), that the activation of signalling pathways are different between AQM and neurogenic atrophy (NA). For instance, the MAPK cascade was activated in AQM, but not in NA. Therefore, more research needs to be conducted in order to elucidate the mechanisms involved in protein catabolism in these two extreme cases.

It has been reported by various authors that there is also a slow to fast transformation in fibre types, especially all the way on the continuum to type IIX fibres as a result of no nerve stimulation (Andersen *et al.*, 1996; Furnsinn *et al.*, 1999; Talmadge *et al.*, 2002a). Overall analysis of fibres also showed a substantial increase in hybrid fibres containing two or more isoforms (Andersen *et al.*, 1996). These two facts indicate, in the case of proteins with various isoforms, muscle activation and the exposure to mechanical strain will also affect fibre type. In addition, another signal may be the absence of neurally derived factors that influence gene expression. For instance, calcineurin has been associated with the expression of MHC I isoforms (section 1.5). Because calcineurin is readily inhibited by MCIP, in order to induce a large calcineurin response, a long duration of constant elevation of intracellular Ca^{2+} is required. Therefore, with no neural activation of the muscle fibres, calcineurin activity is inhibited and do not stimulate MHC I expression.

However, electrical stimulation of paralysed muscle showed that this process of slow to fast fibre type conversion can be reversed (see section under Neural Input), therefore indicating that neural enervation and activity is important in regulating muscle fibre type.

The above discussion indicates that paralysis alters both the quantity and isoform content of myosin and this has distinct effects on the whole muscle. With the increase in fast twitch fibre types, Talmadge *et al.* (2002b) showed that spinal chord injury resulted in a significant decline in fatigue resistance in rat *Soleus* muscle. In addition, muscle enzyme activities, especially the oxidative enzymes, may decrease in activity. After only 7 days of paralysis, rat *Soleus* muscle showed a significant decline in SDH activity, resulting in a decline in fatigue resistance (Jasmin *et al.*, 1995). It is therefore possible that the same factors controlling contractile protein quantity and myosin isoform expression also control the genes regulating the quantity of mitochondria. In section 1.5, it was discussed that activated PPAR δ resulted in an increase in mitochondria and type I fibres in transgenic mice. However, the mechanisms involved in both these phenotypes are not yet well understood. Whether PPAR δ alone can modulate both, or whether it also activates calcineurin needs to be elucidated. Furthermore, it is well known that type I fibres contain more mitochondria than type IIa fibres, the latter also known to exhibit high oxidative potential, but whether type IIa fibres may be stimulated to contain more mitochondria than type I fibres, still needs to be investigated.

Spaceflight and detraining

Two less extreme models, compared to paralysis, are detraining and spaceflight. These two models go hand in hand in that they both reflect a decrease in muscle activity and work. However, they cannot be considered the same, since during spaceflight there is an added major lack of mechanical stimulus from gravity, even at rest. Nevertheless, significant changes have been observed for both spaceflight and detraining on muscle morphometry and metabolism. Eleven days of spaceflight revealed a significant decrease in CSA of all fibre types in human *Vastus lateralis* muscle, and a shift from slow to fast twitch fibre types (Baldwin, 1996; Edgerton *et al.*, 1995). The number of capillaries per fibre also decreased for all the fibre types measured. Although not statistically significant, it was noted that oxidative enzyme capacity decreased. These data seem to indicate that gravity requires the constant activation of muscle groups (e.g. stabilisers), or specific motor units in most muscle groups, therefore being able to induce muscle fibre type transformation and adaptation of metabolism.

Detraining has been suggested to have similar effects as spaceflight on fibre type and oxidative capacity in trained athletes. For instance, three months of detraining, after a high intensity training period of three months, showed a significant increase in MHC IIx and a decrease in MHC IIa expression in the *Vastus lateralis* muscle of humans (Andersen and Aagaard, 2000). These authors did not investigate enzyme activities, but a number of studies have been performed on both humans and animals. Detraining may result in decreases in oxidative enzyme capacities and these effects can be observed within six weeks (Chi *et al.*, 1983; Henriksson and

Reitman, 1977). Changes in the glycolytic capacity are not evident, but may be related to the fitness level of the subject (Chi *et al.*, 1983).

The relevance of these observations discussed in this section to studies on athletes, is the effect illness or injury might have on the muscle characteristics. The risk of injury in sport is much greater than in a sedentary / recreationally active life style. Therefore, athletes in Chapters 3, 4 and 5 were carefully screened for injury or illness before included in the studies.

Continuous sub-maximum, intermittent high intensity endurance or sprint training

This section will focus on three topics, namely muscle structural and metabolic differences between trained and untrained individuals, adaptations of muscle to various types of exercise in untrained subjects and added training effects in already well-trained athletes.

For the past four decades, scientists have investigated fibre type and enzyme activities in muscle samples of untrained and trained subjects. These cross-sectional studies have revealed that there is a distinct difference between the two groups. For instance, the pioneering study by Gollnick *et al.* (1973) showed that skeletal muscle of trained subjects have significantly more type I fibres and higher oxidative capacity, especially those that are endurance trained (cycling or running) compared to untrained subjects. Other studies have confirmed these findings, but have also incorporated other types of training, such as athletes performing resistance training or runners with different specialisation distances (e.g. Essen-Gustavsson and Henriksson, 1984; Harber *et al.*, 2002; Harber *et al.*, 2004; Jurimae *et al.*, 1997; Williamson *et al.*, 2001). These studies further showed that the less active the individual, the more the occurrence of type IIX fibres.

Not only does the fibre type of these athletes differ, but metabolic enzyme activities and VO₂max may vary substantially. Figures 1.6.1, 1.6.2 and 1.6.3 represent the VO₂max, fibre type proportions and enzyme activities relative to untrained individuals, respectively, in various sports, ranging from sprinters to extreme endurance athletes and will be discussed subsequently.

VO₂max has for years been a measure of fitness level, especially for endurance type sports. Events requiring little endurance capability (e.g. weight lifting and sprinting), indicate that these athletes have a low VO₂max, and may even be similar to untrained individuals. On the other hand, events requiring muscular activity over extended periods of time (e.g. endurance runners, cyclists and skiers), requires additional energy sources and is usually derived from the oxidative metabolism of carbohydrate and fat. The muscle therefore requires a large amount of oxygen to allow these metabolic pathways to properly function. Figure 1.6.1 shows that athletes requiring short, but powerful muscle contractions have a low VO₂max, whereas athletes performing exercise over an extended period of time, have a high oxygen consumption rate. These findings were further strengthened by the investigation of the muscle fibre type and selected enzymes

representing each metabolic pathway (recall from section 1.4.3) and are presented in Figures 1.6.2 and 1.6.3.

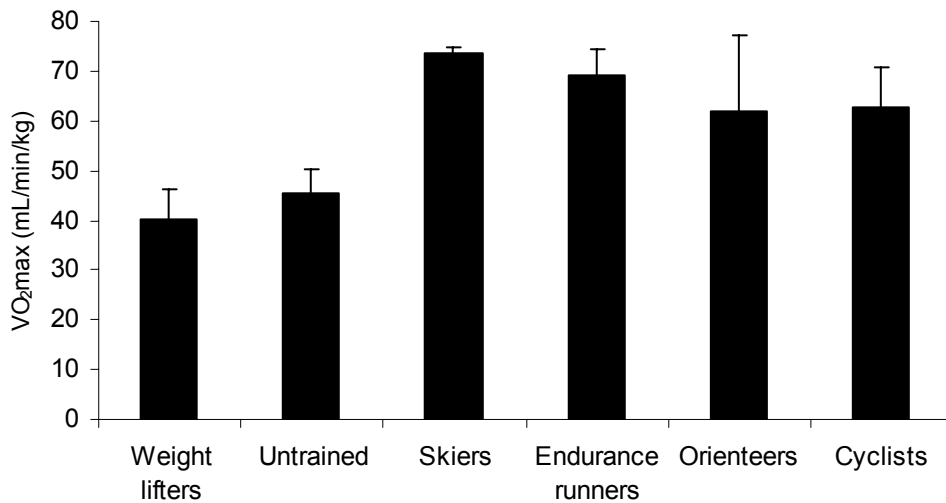


Figure 1.6.1 Maximal oxygen consumption (VO_2max) of athletes and untrained individuals. Data compiled from Chi *et al.*, 1983; Coggan *et al.*, 1990; Duey *et al.*, 1997; Essen-Gustavsson and Henriksson, 1984; Evertsen *et al.*, 1999; Gollnick *et al.*, 1972; Green *et al.*, 1991; Harber *et al.*, 2002; Jansson and Kaijser, 1977; Klitgaard *et al.*, 1990; Prince *et al.*, 1976; Proctor *et al.*, 1995; Saltin *et al.*, 1995a; Shepley *et al.*, 1992; Weston *et al.*, 1997; Weston *et al.*, 1999.

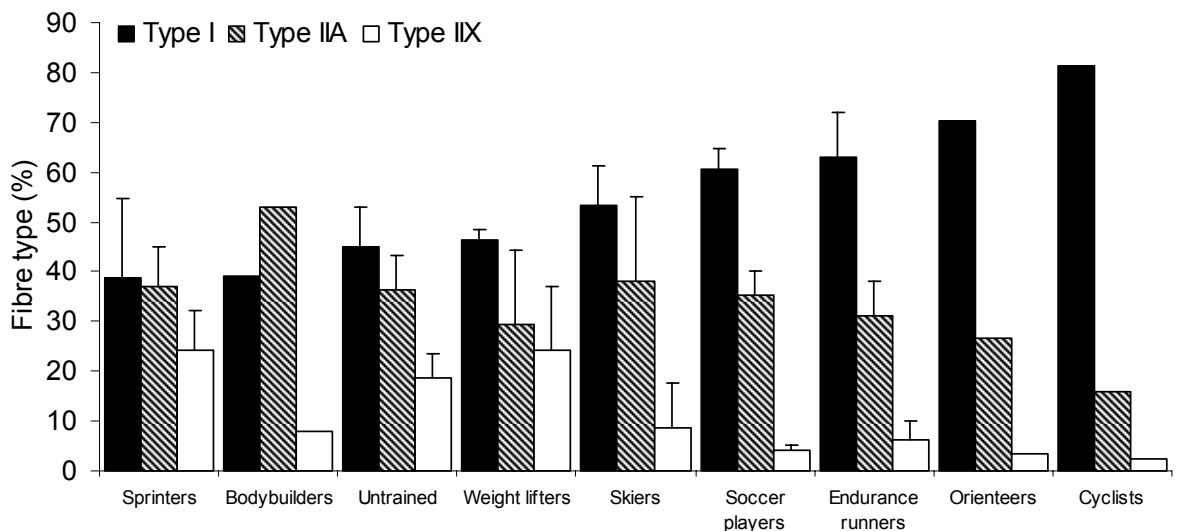


Figure 1.6.2 Muscle fibre type in untrained and trained athletes. Data compiled from Ama *et al.*, 1986; Andersen *et al.*, 1994a; Andersen *et al.*, 1994b; Coggan *et al.*, 1990; Duey *et al.*, 1997; Essen-Gustavsson and Henriksson, 1984; Evertsen *et al.*, 1999; Green *et al.*, 1991; Harber *et al.*, 2002; Jansson and Kaijser, 1977; Klitgaard *et al.*, 1990; Prince *et al.*, 1976; Proctor *et al.*, 1995; Saltin *et al.*, 1995a; Tesch *et al.*, 1989.

There are large variations in muscle fibre type between trained and untrained individuals, with some studies showing that these differences are statistically significant. Type I muscle fibres have large oxidative capacities, with concomitant high oxidative enzyme concentrations, are the predominant fibre type in endurance trained athletes and range between 55 – 85% of the fibre

proportions (Figure 1.6.2). On the other hand, athletes requiring power have lower type I and higher type IIA and IIX fibre proportions (refer to Figure 1.6.2 for references).

On enzymatic level, the four enzymes that are markers for glycolysis (PFK), lactate production (LDH), the TCA (CS) and fat oxidation (3HAD), all vary substantially. Sprinters have low oxidative, but higher glycolytic and lactate production capacities than untrained subjects (Figure 1.6.3). On the other hand, endurance runners and cyclists both have large oxidative, but lower lactate production capacities. Both fibre type and metabolism are therefore related to one another, but it seems more that the choice of metabolism will be largely dependent on neural activity, and consequently muscle activation and contraction speed.

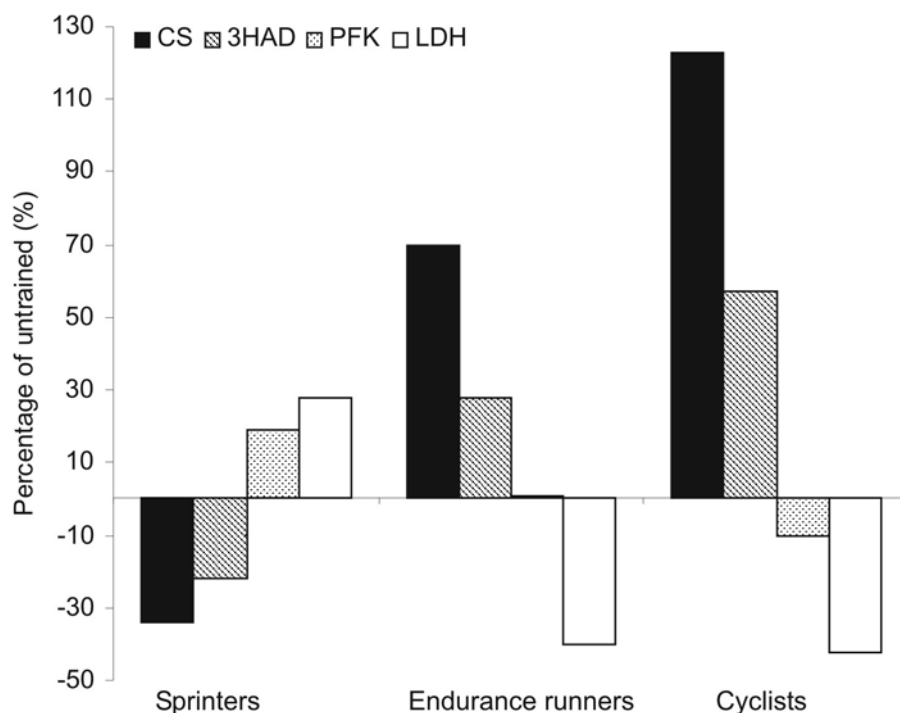


Figure 1.6.3 Variation in enzyme activities in trained athletes from various events, normalised to untrained values. CS, citrate synthase; 3HAD, 3-hydroxyacetyl Co-enzyme A dehydrogenase; PFK, phosphofructokinase; LDH, lactate dehydrogenase. Data compiled from Chi *et al.*, 1983; Essen-Gustavsson and Henriksson, 1984; Gollnick *et al.*, 1972; Harber *et al.*, 2002; Jansson and Kaijser, 1977; Proctor *et al.*, 1995; Weston *et al.*, 1999.

Whole body physiology (i.e. $VO_2\max$), muscle fibre type and metabolism are different between untrained and trained individuals, each of these characteristics contributing to the performance of the athlete. Furthermore, the intensity of contraction (i.e. the sporting event) must have a direct influence on these characteristics. The alternative is also that an individual's fibre type is pre-programmed, meaning that the genetic composition of the athlete plays a more crucial role. To determine whether fibre type and metabolism can be altered, training interventions of both endurance and short duration contractions were conducted on untrained individuals. A summary of selected studies are presented in Table 1.6.2. Important factors that may play crucial roles in

muscle and whole body adaptation, are training duration (e.g. 6 weeks), session duration (e.g. 1 hour) and the intensity at which the session was performed (e.g. 67% VO₂max).

For many years it was believed that man was born with a set fibre type, which will directly affect performance ability, as was discussed above. However, this hypothesis has been discarded as a result of longitudinal training studies, investigating muscle fibre characteristics and metabolism (for example Andersen and Henriksson, 1977; Gollnick *et al.*, 1973; Holloszy and Coyle, 1984). Therefore, exercise indeed stimulates muscle adaptation, but the intensity and duration of exercise may play a significant role in regulating to what extent the muscle adapts. Fibre type and metabolic adaptations seen in these studies are comparable to those obtained from electrical stimulation. Many training intervention studies exist on both humans and animals, but for the purpose of this dissertation, only selected human studies will be highlighted.

Using untrained volunteers to participate in training interventions has shed light on fibre type transformations and metabolism. Common traits in sprint training (powerful muscle contractions for short durations) are that type I fibre proportions seem to reduce, with a concomitant increase in type II fibre types (Dawson *et al.*, 1998; Liljedahl *et al.*, 1996). Similarly, a clear tendency is that LDH and PFK increase in activities as a result of this type of training. However, the latter may only occur because of a larger proportion of type II fibres in the muscle. Essen-Gustavsson and Henriksson (1984) have shown that type II fibres have a higher glycolytic capacity than type I fibres, but that type I fibres have a higher oxidative capacity than type II fibres. On the other hand, the possibility arises that the CSA of type II fibres may increase as a result of the training with no change in the CSA of type I fibres, resulting in the higher glycolytic enzyme activities and the reduced oxidative activities. Two of the listed studies (Linossier *et al.*, 1997; Macdougall *et al.*, 1998) have shown an increase in CS and 3HAD activities, as well as an increase in LDH and PFK activities, as a result of sprint training. No fibre type proportions were determined and therefore make discussion limited. On the contrary, all these studies listed show an increase in VO₂max, thus pointing out that sprint training (running and cycling) may also have an endurance component.

The differences between endurance and the above sprinting events are two-fold: muscle contractions last longer and the intensity of contractions is lower in endurance activities. To illustrate the above statement, two extreme examples would be the speed at which a 100 m sprint and a 10 km endurance event take place. The world's fastest 100 m (9.9 seconds) sprints and 10 km (26.5 minutes) races are run at an average speed of 36.5 and 22.7 km/h, respectively.

All the studies presented in Table 1.6.2 reported a significant increase in VO₂max after endurance training. Even as little as 10 days of cycling for two hours per day elicited a significant increase (Green *et al.*, 1991). Consistent in these studies (with some not statistically

significant) were a reduction in the glycolytic type IIX fibres, with a concomitant increase in the more oxidative type IIA fibres. The main finding in these studies, however, is that none showed a significant change in type I fibre proportions. In fact, four studies show a reduction with only one showing an increase (5%). CS, the enzyme in the TCA, is the only enzyme that showed a consistent increase in activity after endurance training in all the presented studies. LDH activity did not change as a result of endurance training, but the tendency was to have decreased. The remaining two enzymes, 3HAD and PFK, despite one study showing the opposite (although not significant) tend to increase and decrease in activity, respectively. One shortcoming of the presented endurance studies was the lack of reporting performance changes, either as a measure of endurance or fatigue resistance at a specified workload. An increase in performance is usually the end goal of all training protocols. Only one reported a decrease in 4 km race time after training, but the remaining seven failed to report it.

Resistance training usually involves training muscle groups with weights (added load). By increasing the load, the demand for more power is required. Many resistance training protocols are based upon the one repetition maximum (RM) of an individual. In other words, it is the load (in kg) that could be lifted only once. Depending on the load (which will evidently determine the number of repetitions accomplished) should therefore have different effects on the muscle. The studies presented in Table 1.6.2 all involved weight training, each varying in load, the number of repetitions and the duration of the training intervention. Only three of the studies reported an improvement in power output / strength after 8 to 12 weeks of resistance training (Andersen and Aagaard, 2000; Masuda *et al.*, 1999; Williamson *et al.*, 2001). Reported VO_2max seems to decrease as a result of weight training. This attribute may be explained by the fact that muscle hypertrophy may occur, which may increase total body mass and hence decrease VO_2max expressed relative to body mass. Interestingly, all the studies showed a tendency for an increase in the proportion of type I fibres. Williamson *et al.* (2000) have shown that after 12 weeks of resistance training in older men (~74 years old), a 33% increase in type I fibre proportions were observed ($P < 0.05$), with no statistical significant change in type IIA (+24%) and type IIX (-15%) fibre proportions. On the other hand, two studies showed a significant increase in type IIA fibre proportions after 12 weeks of resistance training (Andersen and Aagaard, 2000; Williamson *et al.*, 2001). Three of the five studies that determined fibre type, showed a significant decrease in the type IIX fibre type after 8 to 12 weeks of resistance training (Andersen and Aagaard, 2000; Campos *et al.*, 2002). Although the type IIX fibre proportions were not changed in the two studies by Williamson *et al.* (2000; 2001), the authors did show a significant decrease in the type IIA/IIX hybrid fibre proportions, indicating a switch to type IIA. The activities of the enzymes determined showed a significant reduction only in CS activity (Masuda *et al.*, 1999). In the study by Green *et al.* (1999), 3HAD, PFK and LDH activity showed a tendency to increase. Although fibre type was not measured in this study, the resulting

fibre type of the previous studies may explain these findings, but accurate conclusions cannot be drawn.

To summarise the findings of the presented training intervention studies (Table 1.6.2) on untrained individuals, the main conclusions are that both sprint and endurance training increases $VO_2\text{max}$, whereas resistance training has no effect on this parameter. No clear cut picture could be drawn for fibre type and oxidative enzyme changes as a result of sprint training, but the glycolytic enzyme activities (LDH and PFK) seem to increase. Studies conducted on untrained subjects subjected to endurance training have indicated that a decrease in type IIX fibres is evident, with a possible increase in type IIA fibres. Oxidative capacity (CS and 3HAD) increases, while the glycolytic capacity may or may not change. Resistance training may elicit a decrease in $VO_2\text{max}$ (as a result of an increase in body weight), but may also increase the proportion of type I fibres, with a concomitant decrease in type IIX fibre types. In contrast to the fibre type change, glycolytic capacity may increase.

Few studies have investigated the effect of additional training, whether it is resistance, endurance or interval training, on the muscle characteristics of already well-trained athletes (Table 1.6.3). Andersen *et al.* (1994b) showed that the inclusion of high resistance strength training in already well-trained sprinters resulted in a significant increase in PPO, with significant muscle fibre type adaptations, increasing the proportion of type IIA fibres, with a concomitant decrease in type I and IIX fibre types. Although no controls were used in this study, it may be speculated that the sprint training has contributed to these results.

Army recruits may fall into the same category as athletes, as endurance and strength training forms a substantial portion of their physical preparation. Twelve weeks of weight training resulted in an increase and decrease in type IIA and type IIX fibre proportions, respectively in these recruits (Kraemer *et al.*, 1995). This study made use of army control subjects, and the effect was therefore directly a result of the added training.

Increasing the volume of endurance training may increase $VO_2\text{max}$, improve economy and muscle enzyme activities in already well trained subjects. Sjodin *et al.* (1982) showed that PFK activity was significantly reduced after 14 weeks of treadmill training at the speed associated with the onset of blood lactate accumulation in well-trained runners. These athletes only trained once a week in the laboratory, and continued with their normal daily training throughout the intervention. Although not significant, CS activity showed a tendency to have increased by 11%. On the other hand, Neary *et al.* (1995) showed that 8 weeks of endurance cycling increased $VO_2\text{max}$ and power output at the same percentage of $VO_2\text{max}$ in already well-trained cyclists.

Incorporating high intensity interval training (HIIT) has for a long time been used by coaches around the world to improve performance, especially in runners (Martin and Coe, 1997). HIIT

usually consist of intervals at a relatively high intensity, with small rest periods in between. One such study by Shepley *et al.* (1992) showed that only one week of HIIT (at 115-120% VO_2max) performed by endurance runners improved their time to fatigue (22%) at a relative high speed, but also that CS activity was significantly increased. Weston *et al.* (1997), on the other hand, showed no change in CS or 3HAD activity in well-trained cyclists after four weeks of HIIT (at 80-85% PPO), but that PFK activity tended to have increased by 13% (not significant). The studies by these authors and Acevedo and Goldfarb (1989) all showed that performance can be increased by incorporating HIIT in the training schedule of already well-trained athletes. However, determining the correct intensity and duration of the intervals may become problematic.

Hill and Rowell (1997) were the first authors to investigate training at the velocity associated with VO_2max (V_{max}). Later, work by Billat and her research group (Billat, 2001a; Billat, 2001b; Billat *et al.*, 1999), as well as Smith *et al.* (1999; 2003) showed that performance of elite athletes can be improved at this velocity proposed by Hill and Rowell (1997). In an initial investigation, Smith *et al.* (1999) showed that running intervals at V_{max} are dependent on the duration of the intervals. The authors concluded that setting the interval time to 60% of the maximal time spent by each individual at V_{max} (T_{max}), was optimum to elicit improvements in performance after four weeks of the programme. Therefore, this method also allows for a scientific approach to increase performance in already well-trained runners. Unfortunately, none of the authors who used this approach investigated possible muscle adaptations. Therefore, Chapter 5 focussed on muscle and physiological adaptations in already well-trained runners utilising the protocols proposed by Smith *et al.* (1999; 2003).

One aspect that is certainly very difficult to control for, and may influence the outcomes of training intervention studies or even cross-sectional comparative studies, is the amount of baseline training performed by athletes prior to the intervention. Similarly, no two studies are alike in subject recruitment and methodological approach. These two factors make comparisons between groups as well as between studies, very difficult. However, most of the studies conducted on humans have shown similar findings for both changes in fibre type and metabolism, regardless of baseline training or training type, and points out that training induces adaptation of muscle. In the three studies on human athletes (Chapters 3 to 5), baseline training was always reported in order to either explain physiological or biochemical characteristics, or during the matching process.

Another factor contributing to possible physiological and muscular adaptations is the intensity of exercise. Harber *et al.* (2002) compared distance runners (3 000 m to 10 000 m) and middle distance runners (800 m – 1 500 m) and showed that the latter group had more type IIA fibres compared to distance runners. No differences were found between the oxidative enzyme

capacities. The difference between the athletes' fibre type may be attributed to the selected specialisation distance of athletes and is also further investigated in Chapter 3.

To conclude this section, muscle activation, the duration and intensity of contraction plays a significant role in determining muscle characteristics, and may be seen as the primary contributing factors. However, the next section will focus on external factors / stimuli that may also contribute to the overall phenotype.

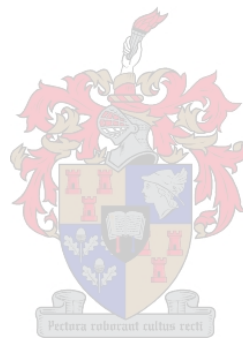


Table 1.6.2 Effect of sprint, resistance and endurance training on performance, maximal oxygen consumption (VO_2max), fibre type and muscle enzyme activities in untrained human subjects. Fibre type was primarily determined histologically (ATPase).

	N	Intensity	Description of training	Performance	VO_2max	Fibre type			Enzymes			
						I	IIA	IIX	CS	3HAD	PFK	LDH
SPRINT TRAINING												
Liljedahl <i>et al.</i> , 1996	11xM	0.75N/kg	C: 3x30 sec, 20 min rest, 3 x wk, 4 wks	nc PPO		-10%	+3%	+75%	-3%	+2%	+14%	+17%*
Allemeier <i>et al.</i> , 1994	11xM	NR	C: 3x30 sec, 20 min rest, 2-3 x wk, 6 wks	nc PPO	+13%	-5%	+6%	-44%				
Dawson <i>et al.</i> , 1998	9xM	90-100% PS	R: 4-8x30-80 m, 1:6 rest, 3 x wk, 6 wks	↓ 40 m time	+6%*	-21%*			-32%*		+2%	
Harridge <i>et al.</i> , 1998	7xM	6% BW	C: 3x8-16-3 sec, 30 sec rest, 4 x wk, 6 wks	↑ PPO		-8%	+23%	+36%				
Macdougall <i>et al.</i> , 1998	12xM	100% Effort	C: 4-10x30 sec, 4 min rest, 3 x wk, 7 wks	↑ PPO	+8%*				+36%*	+39%	+49%*	+7%
Linossier <i>et al.</i> , 1997	8xM	8% BW	C: 2x15x5 sec(55 sec rest), 15 min rest, 4 x wk, 9 wks	↑ PPO	+3%*				+7%	+1%	+17%*	+31%*
RESISTANCE TRAINING												
Campos <i>et al.</i> , 2002	9xM	RM	W: 4x3-5RM, 3 min rest, 2-3 x wk, 8 wks	nc PPO	-4%	+12%	-7%	-61%*				
Campos <i>et al.</i> , 2002	11xM	RM	W: 3x9-11RM, 2 min rest, 2-3 x wk, 8 wks	nc PPO	-5%	+5%	+3%	-51%*				
Masuda <i>et al.</i> , 1999	6xM	90% 1 RM	W: 5x6-10 reps, 3 min rest, 2 x wk, 8 wks	↑ PPO		+5%	-5%	+7%	-9%*			
Green <i>et al.</i> , 1999	9x	RM	W: 3x6-8RM, 2 min rest, 3 x wk, 12 wks	NR	-7%					+8%	+15%	+27%
Andersen and Aagaard, 2000	9xM	High	W: 3-4x6-15 reps, 3 x wk, 12 wks	↑ PPO		+4%	+23%*	-60%*				
Williamson <i>et al.</i> , 2001†	6xM	80% 1RM	W: 3x10 reps, 2-3min rest, 3 x wk, 12 wks	↑ Strength	+34%*	+9%	+85%*	ND				
ENDURANCE TRAINING												
Green <i>et al.</i> , 1991	8xM	59% VO_2max	C: 2 hours, 10-12 days	NR	+4%*	-1%	+5%	-11%	+23%	+7%	-12%	-6%
Andersen and Henriksson, 1977	12xM	81% VO_2max	C: 30 min, 4 x wk, 8 wks	NR	+18%*	+5%	+14%*	-26%*				
Baumann <i>et al.</i> , 1987	4xM	~90% HRmax	C: 30 min, 5 x wk, 8 wks	NR	+13%*	-1%	+13%	-38%*				
LeBlanc <i>et al.</i> , 2004	8xM	75% VO_2max	C: 1 hours, 5 x wk, 8 wks	NR	+15%*				+40%*			
Bylund <i>et al.</i> , 1977	20xM	80-90% HR	V: Running, jogging, basket ball, X-country, 8 wks	↓ 4 km time	+13%*	-3%			+45%*	+3%	+4%	-6%
Green <i>et al.</i> , 1999	7xM	68% VO_2max	C: 2 hours, 6 x wk, 11 wks	NR	+15%*					+66%*	-6%	-7%
Tremblay <i>et al.</i> , 1994	17xM&F	60-85% HRmax	C: 30-45 min, 4 x wk, 20 wks	NR	+32%*					+18%	-10%	
Bylund <i>et al.</i> , 1977	20xM	80-90% HR	C: Running, jogging, basket ball, X-country, 24 wks	↓ 4 km time	+26%*	-1%			+46%*	-13%	-4%	-3%

3HAD, 3-hydroxyacetyl Co A dehydrogenase; BW, body weight; C, cycle; CS, citrate synthase; F, females; HR, heart rate; LDH, lactate dehydrogenase; M, males; nc, no change; ND, not detected; NR, not reported; PFK, phosphofructokinase; PPO, peak power output; PS, peak speed; R, run; RM, repetition maximum; V, various; VO_2max , maximum oxygen consumption; W, Weights; wk, week. †, fibre type determined from MHC content of single fibres, * indicates significant training effect ($P < 0.05$).

Table 1.6.3 Effect of sprint, resistance, endurance, high intensity interval and combination training on performance, maximal oxygen consumption (VO_{2max}), fibre type and muscle enzyme activities in already well-trained athletes. Fibre type was primarily determined histologically (ATPase).

	N	Athletes	Description of training	VO_{2max}	Performance	Fibre type			Enzymes			
						I	IIA	IIX	CS	3HAD	PFK	LDH
SPRINT TRAINING												
Andersen <i>et al.</i> , 1994b	6xM	Sprinters	Sprint, resistance, uphill sprints, interval running, 6 x wk, 12 wks		↑ PPO*	-15%*	+50%*	-44%*				
RESISTANCE TRAINING												
Kraemer <i>et al.</i> , 1995	9xM	Army males	Weight training, 4 x wk, 12 wks	-1%	↑ Strength*	0%	+74%*	-90%*				
ENDURANCE TRAINING												
Sjodin <i>et al.</i> , 1982	8xM	Runners	20 min @ V_{OBLA} , 1 x week, 14 wks	+2%					+11%		-30%*	-3%
Neary <i>et al.</i> , 1995	8x	Cyclists	80-85% VO_{2max} , 60 min, 5 x wk, 8 wks	+12%*	↑ sub-max PO*							
HIGH INTENSITY INTERVAL TRAINING												
Shepley <i>et al.</i> , 1992	9xM	Runners	5 x 500 m @ 115-120% VO_{2max} , 5 x wk, 1 wk	0%	↑ TTF*				+22%*			
Weston <i>et al.</i> , 1997	6xM	Cyclists	6-8 min @ 80% PPO, 1 min rest, 6 sessions in 4 wks		↑ PPO & ↓ 40 min TT*				+3%	+2%	+13%	
Kraemer <i>et al.</i> , 1995	8xM	Army males	(Max distance in 40 min @ 80-85% VO_{2max} + 200-800 m intervals @ 95-100+% VO_{2max} 1:4-0.5 rest)x2 x wk, 12 wks	+12%*	nc in PPO*	+1%	+32%*	-54%*				
Smith <i>et al.</i> , 2003	9xM	Runners	60% T_{max} @ V_{max} , 6 intervals, 1:2 rest, 2 x wk, 4 wks	+6%	↓ 3000 m time*							
Acevedo and Goldfarb, 1989	7xM	Runners	(intervals @ 90-95% HR_{max} , 2xFartlek sessions), 3 x wk, 8 wks	+1%	↓ 10 km time & TTF*							
Smith <i>et al.</i> , 1999	5xM	Runners	6 x intervals @ 60-75% T_{max} + 30 min @ 60% V_{max} , 3 x wk, 4 wks	nc	↓ 3000 m time, ↑ Peak V_{max} *							
COMBINATION TRAINING												
Kraemer <i>et al.</i> , 1995	9xM	Army males	Combined interval, endurance and resistance, 4 x wk, 12 wks	+8%*	↑ Strength*	+4%	+39%*	-87%*				

M, males; nc, no change; PPO, peak power output; TT, time trial; TTF, time to fatigue; V_{max} , initial velocity associated with VO_{2max} ; V_{OBLA} , velocity at onset of blood lactate accumulation; VO_{2max} , maximal oxygen consumption; wk, week; * indicates significant training effect ($P < 0.05$).

1.6.3 Environmental factors

Temperature

Environmental temperature has a significant influence on mammalian physiology, but it may also have an influence on fibre type and metabolism of skeletal muscle. Although no study has investigated the influence of environmental temperature on muscle fibre type in humans, there are a vast number of studies that investigated the effects of temperature in different species of animals, both hibernating and non-hibernating. In rat *Soleus* muscle, 19 weeks of cold exposure significantly increased type IIA fibres with a concomitant decrease in type I fibres (Walters and Constable, 1993). However, pigs exposed to 12 °C revealed an increase in type I fibres, with a concomitant decrease in type IIA and IIB fibres in the *Semispinalis* muscle (Lefaucheur *et al.*, 1991). Rats exposed to 4 – 5 °C for 8 weeks, showed no change in fibre type in the TA muscle, whereas the hamster showed a reduction in type I fibres (Deveci and Egginton, 2002). Also in the latter study, maximal oxygen consumption increased in rats after cold acclimation.

Acclimation may also be muscle specific. It was reported by Walters and Constable (1993) that the EDL muscle of the rat showed no change in fibre type after cold exposure. The same was observed in pig muscle, where the *Longissimus* muscle showed no change in fibre type (Lefaucheur *et al.*, 1991). Other components of the muscle may also change, such as capillary-to-fibre ratio, mean cross-sectional areas of the fibres, and oxidative and glycolytic enzyme activities as a result of variations in temperature (Deveci and Egginton, 2002; Lefaucheur *et al.*, 1991).

Thyroid hormone has been proposed as the main regulator of metabolic rate and is directly influenced by environmental temperature. The study by Lefaucheur *et al.* (1991) showed that pigs acclimated to cold had a larger thyroid gland than controls. Studies mostly on rats have shown that hypothyroidism or hyperthyroidism may have significant influences on muscle fibre type and metabolism (Caiozzo *et al.*, 1998; Li and Larsson, 1997; Winder *et al.*, 1975). Briefly, hyperthyroidism has been shown to up-regulate fast MHC II mRNA, but chronically low thyroid levels were associated with an increase in MHC I expression (Moss *et al.*, 1995).

Altitude

With an increase in altitude, barometric pressure decreases, and the direct effect is a decrease in oxygen diffusion across the alveolar membrane of the lungs into the blood. This results in a lower oxygen concentration in arterial blood. Ambient oxygen concentration is ~21% at sea level, and remains the same with an increase in altitude. In the laboratory, altitude can be simulated by the inspiration of a lower oxygen concentration or by decreasing the pressure in a hypobaric chamber, or both.

Many studies have investigated the effect of exercise at altitude on the whole body and muscle, both in animals and humans residing at altitude (Hochachka *et al.*, 1992; Jackson *et al.*, 1987; Matheson *et al.*, 1991; Rosser and Hochachka, 1993; Young *et al.*, 1984). However, there are some methodological problems when comparing studies. The most important factor is that the altitudes at which the various studies have been conducted, varies considerably and the results obtained from these studies makes interpretation difficult (Terrados, 1992). However, some conclusions may be drawn. Increases in haemoglobin content of red blood cells, as well as an increase in hematocrit have been shown in humans exposed to hypoxic conditions (Vogt *et al.*, 2001). Maximal oxygen uptake decreases significantly even in well-trained athletes at altitudes higher than 900 meters above sea level (Terrados, 1992). Therefore, altitude or hypoxia has a significant influence on the physiology of mammalian organisms.

Altitude also has a significant effect on muscle morphometry and metabolism. There seem to be discrepancies regarding myoglobin content of muscle in subjects subjected to altitude (Terrados, 1992). According to Terrados (1992), some researchers have showed an increase in myoglobin content of muscle, with others showing no increase or even a decrease.

Guinea pigs subjected to 5 days of hypoxia (13% oxygen) showed a decrease in type IIA fibres and an increase in type IIB fibre proportions compared to controls (Jackson *et al.*, 1987). Humans, on the other hand, may respond differently. However, most of the studies conducted on humans also included training (Parolin *et al.*, 2000b; Terrados, 1992; Vogt *et al.*, 2001), but studies conducted on humans residing at altitude showed that muscle and physiological parameters are significantly influenced by altitude (Hochachka *et al.*, 1982; Rosser and Hochachka, 1993). Cross-country skiers who trained at 2 700m above sea-level for two weeks showed a significant increase in type IIA fibres in the *Triceps brachii* with no changes in the *Gastrocnemius* muscle (Mizuno *et al.*, 1990). Therefore, fibre type and metabolism is influenced by exposure to altitude and may be a consequence of a direct or indirect effect on muscle. The exact mechanism is still under investigation.

Capillary supply may also be altered by altitude due to the lack of oxygen supply. Altitude training showed an increase in the number of capillaries around type IIA fibres in the *Triceps brachii* muscles of cross-country skiers, but no change for type I fibres (Mizuno *et al.*, 1990). However, studies conducted in the Himalayas on sea level subjects showed a decrease in muscle mass and muscle fibre size, with a concomitant increase in capillary density (Hoppeler, 1999b). The latter finding could be explained by the loss in fibre cross-sectional area, therefore no new capillaries developed. It was however found that the hypoxia inducible factor 1 (HIF-1), a downstream activator of vascular endothelial growth factor (VEGF) was elevated in muscle subjected to training at altitude, independent of training intensity (Hoppeler, 1999b; Vogt *et al.*, 2001).

Because altitude results in a decrease in oxygen carrying capacity in arterial blood, a definitive effect is also observed on metabolism in muscle. Various studies have shown changes in metabolic enzyme activities for both oxidative and glycolytic pathways. However, once again, it should be stressed that there are discrepancies with regards to these findings. Table 1.6.4 summarises selected investigations of the effects of altitude on muscle enzyme activities.

The findings of these studies show the controversy regarding altitude exposure. The differences observed in the response to altitude might be attributed to study design and methodology in enzyme analysis. Hypoxia may also be muscle specific such as in the case of cold acclimation. The other factor might be that hypoxia only have an influence on muscle in the presence of muscle contraction (such as exercise). For instance, the heart is constantly stimulated to undergo contraction. Daneshrad *et al.* (2000) showed that exposure to 10% hypoxic conditions of rats for three weeks, significantly increased HK and LDH activities, and a decrease in 3HAD activity in both the left and right ventricle, with no change in enzyme activities in the *Soleus*.

The concentration of muscle metabolites for the enzymes may also be influenced by the altitude effect. A study conducted by Parolin *et al.* (2000a) showed that pyruvate production, pyruvate oxidation and lactate accumulation was significantly increased under hypoxic (11% oxygen) conditions compared to the same intensity under normoxic conditions. Therefore, taking into account the effects that temperature and altitude may have on both muscle biochemistry and physiology, both of these factors were controlled for in Chapters 2 to 5.

Table 1.6.4 Comparison of enzyme activities from longitudinal studies investigating the influence of altitude acclimation with or without training.

Description	Muscle	Training	HK	CS	3-HAD	LDH	PFK	Reference
Human: X-country skiers. Altitude: stay at 2100m, train at 2700m	<i>Gastrocnemius</i>	2 weeks		↓	↓	↔	↓	Mizuno <i>et al.</i> , 1990
Human: X-country skiers. Altitude: stay at 2100m, train at 2700m	<i>Triceps</i>	2 weeks		↔	↔	↔	↔	Mizuno <i>et al.</i> , 1990
Human: Hypobaric simulating 2 300m above sea-level. Training	<i>Vastus lateralis</i>	4 weeks		↑	↑	↓	↔	Terrados <i>et al.</i> , 1990
Rat: Hypoxia (10%), no training	<i>Soleus</i>	3 weeks		↔	↔	↔	↔	Daneshrad <i>et al.</i> , 2000
Rat: Hypoxia (10%), no training	<i>Gastrocnemius</i>	3 weeks	↑	↔				Daneshrad <i>et al.</i> , 2000

Socio-economical environment

All living creatures must obtain energy from food sources. In the animal kingdom, this usually is not a persistent problem. However, humans are different. The aspect of socio-economic impact is a much debatable issue. In some animals, it can be seen that once they have fed, they will not be bothered about where their next meal will come from. On the other hand, humans rely on money to purchase their daily meals. Poverty in South Africa and the African continent, especially in the black communities, may exist in more than three quarters of the population. With the lack of funds, these people may not be able to improve their education or experience in order to uplift themselves from poverty. Participating in sport on a professional level is one way of uplifting oneself from poverty. The motivation for participating in a certain field of sport may be due to status, financial needs or the mere glory thereof. However, each of these mentioned has a psychological impact on performance, and may also have an impact on the physiology of the body. The motivation to train harder to eventually obtain that goal may vary between people. The same may be justified for the Kenyan athletes, where it is more valuable to run well and train hard to gain honour, than for mere pleasure. However, assessment of motivation can be a difficult task, but cannot be excluded as a factor that may influence the physiological and biochemical outcome of the results in Chapters 4 and 5.

1.7 Study objectives

The objectives of this dissertation are as follows:

- Chapter 2 – to determine the MHC isoform distribution and CS activities in different sections of the *Quadriceps* muscle of the rat and relate the two parameters to one another.
- Chapter 3 – to determine the occurrence of hybrid fibres in muscle biopsies from well-trained and sedentary humans.
- Chapter 4 – to investigate whole body physiology and muscle characteristics in muscle biopsy samples from well-trained endurance athletes of two distinct ethnic descents.
- Chapter 5 – to investigate whole body physiology and biochemical adaptations in muscle biopsy samples from well-trained endurance athletes subjected to a six week HIIT protocol.

Each chapter is presented as a separate entity (Chapters 2 – 5) and summarised in Chapter 6.

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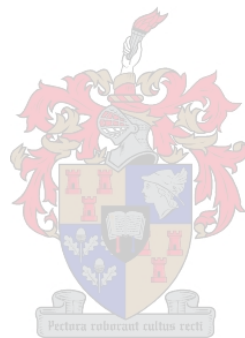
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Myosin heavy chain isoforms and citrate synthase activity in different sections of rat Quadriceps muscle

2.1 Introduction

Skeletal muscle is heterogeneous with respect to fibre types and enzyme activities, both of which have been investigated in various muscle groups from various species (Elder *et al.*, 1982; Hitomi *et al.*, 2005; Kohn *et al.*, 2005; Maltin *et al.*, 1989). A large variation exists between some of these muscle groups, e.g. the *Soleus* vs. the *Gastrocnemius* muscle. A muscle group itself may also vary substantially in fibre type and metabolic capacity in different areas, an observation researchers have termed muscle regionalisation (Kernell, 1998; Punkt, 2002). This variation within a muscle may allow the muscle to function as a slow or a fast contracting muscle, depending on the motor units utilised. Furthermore, a single muscle fibre may, or may not, vary in myosin heavy chain (MHC) isoform expression and metabolic characteristics, which would also affect the contractile properties of that fibre (Edman *et al.*, 1985; Reichmann, 1992; Staron and Pette, 1987).

Contractile speed of fibres is mainly determined by the type and relative quantities of MHC isoforms expressed, but may also be further modulated by other factors such as the myosin light chain isoform content (Larsson and Moss, 1993; Moss *et al.*, 1995; Schiaffino and Reggiani, 1994). Four MHC isoforms, namely MHC I, MHC IIa, MHC IIx and MHC IIb have been identified in rat skeletal muscle (Talmadge and Roy, 1993) with the first mentioned having the slowest ATPase activity and the last mentioned, the fastest (Schiaffino and Reggiani, 1994). The contractile property of fatigability is influenced by oxidative enzyme capacity (Nemeth *et al.*, 1981). Citrate synthase activity (CS) has been used as an indicator of oxidative potential in skeletal muscle (Bouchard *et al.*, 1992; Gollnick and Saltin, 1982) and is associated with fatigue resistance in single muscle fibres (Essen-Gustavsson and Henriksson, 1984; Nemeth *et al.*, 1981). These properties seem to be associated with the MHC isoforms expressed in the muscle, as fast contracting fibres have lower oxidative enzyme activities and *vice versa* (Pette, 1985). However, oxidative capacity can increase or decrease depending on the stimulus, without a change in the MHC isoform content (Gollnick *et al.*, 1985).

In the rat, the *Quadriceps femoris* (QF) muscles, which consist of the *Vastus lateralis*, *Vastus medialis*, *Vastus intermedius* and *Rectus femoris*, play an important role in both sprinting and endurance type behaviour, thus serving a dual purpose. Delp and Duan (Delp and Duan, 1996) characterised seventy-six rat muscle groups according to both fibre type and CS activity and found that deep regions of the *Vastus lateralis* and *Vastus medialis* had significantly higher CS

activities and type I fibre proportions than superficial regions, thus clearly indicating muscle regionalisation. Although clear differences in contractile and metabolic properties exist when comparing fibres far from each other on the fibre type continuum (e.g. type I vs. IIB), how closely these properties are regulated is not that clear since both fibre types expressing pure MHC I and IIa are associated with high oxidative capacity (Pette and Staron, 1993), and even fibre types expressing pure MHC IIb from different regions may vary in oxidative capacity (Larsson *et al.*, 1991).

Nakatani *et al.* (2000) investigated both fibre type distribution and succinate dehydrogenase (SDH) activity in cross-sections of the *Plantaris* and *Tibialis anterior* (TA) muscles of the rat at levels ranging from superficial to deep. They concluded that type IIB fibres had much higher SDH activity in deep parts compared to superficial parts. Furthermore, although it is generally accepted that slow twitch fibres have greater oxidative capacity than fast twitch fibres (Chi *et al.*, 1986; Essen *et al.*, 1975), Nakatani *et al.* (2000) found that in rat *Soleus* muscle, the type IIA fibres had a higher SDH activity than the type I fibres. Therefore, the heterogeneity of skeletal muscle is not as predictable as previously thought (Bass *et al.*, 1969; Pette, 1985).

A number of studies have investigated the distribution of fibre types in a specific muscle group, not only superficial to deep, but also along the length of the muscle. Recently, Wang and Kernell (2000) investigated the proximal to distal organisation of fibre types in five muscle groups of the rat hind limb (*Extensor digitorum longus* (EDL), *Flexor digitorum* and *Hallucis longus*, *Gastrocnemius medialis* (GM), *Peroneus longus* (PE) and TA) and concluded that there is a significant difference in the distribution of the type of fibres along the length of the muscle. They further concluded that in most of the muscles analysed, type I fibres were predominantly located in the proximal vicinity of the muscles analysed. However, Lexell *et al.* (1994), in a study investigating the fibre type proportions in rabbit TA and EDL muscles, found that EDL muscle contained significantly more type I fibres in the distal parts. Therefore, muscle regionalisation may also be related to species. Wang and Kernell (2001a; 2001b) also concluded that fibre type regionalisation in muscle groups follows a general and graded pattern from superficial to deep, and from proximal to distal, but may vary between species e.g. rat, rabbit and mouse. Furthermore, Torrella and co-workers (2000) showed that lateral to medial differences in fibre type exist in rat TA muscle, but that a large inter-individual variation exists. None of these studies investigated the regionalisation of metabolic properties.

As mentioned earlier, enzyme activities may vary from superficial to deep regions of a muscle, but recent studies also indicate that they may vary along the length of the muscle (Punkt *et al.*, 1998; Reichmann, 1992), although not all studies are in agreement (Pette *et al.*, 1980). In a review, Punkt (2002) discussed how the regions of the EDL and the *Soleus* muscles differed for both metabolic enzyme profiles and fibre type distribution. For EDL, a decrease in slow

oxidative and fast oxidative fibre types was observed, with a concomitant increase in fast glycolytic fibre types from proximal to distal. The opposite was observed for the *Soleus* muscle. Similarly, Sakuma *et al.* (1995) compared fibre types in proximal, middle and distal regions of rat *Soleus* and *Plantaris* muscles and found that these muscles differed in regionalisation. Therefore, it seems necessary to characterise regionalisation of each specific muscle or muscle group of interest, since general conclusions may not apply.

Finally, although several studies have been performed on rats (Table 2.1), rat skeletal muscle expresses four MHC isoforms, and in most of the studies, inadequate identification of the isoforms was performed. Of these studies, only one (Delp and Duan, 1996) did not focus exclusively on the lower hind limb muscle, despite the fact that the upper hind limb is frequently used to assess other properties that could be influenced by fibre type. Furthermore, only a few studies have investigated enzyme activities in different regions of the muscle groups. Therefore, the purpose of the present study was:

- (1) to characterise the QF in terms of distribution of all four MHC isoforms and CS activity,
- (2) to determine if, in addition to differences from superficial to deep, there are also differences from proximal to distal regions, and
- (3) to assess whether or not there was an association between a specific MHC isoform and CS activity within the different regions of the QF.

2.2 Methodology

2.2.1 Animals

The ethics committee of sub-committee B of Research Administration at the University of Stellenbosch approved the study. Eighteen healthy female Sprague-Dawley rats, four months of age, were selected randomly from litters and were given normal rat chow and water *ad lib*. Rats were sacrificed by decapitation and the QF (*Vastus lateralis*, *Vastus medialis*, *Vastus intermedius* and *Rectus femoris*) muscles were carefully dissected out as a whole group. A small piece of string was tied with the knot located at the superficial distal region to identify *in vivo* orientation of the muscles. Muscle was frozen in liquid nitrogen and stored at -87 °C.

2.2.2 Division of muscle

The muscle was allowed to thaw briefly at 4 °C. The QF was divided into three regions (superficial, middle and deep), and each of these regions was divided into three parts (distal, centre and proximal) as depicted in Figure 2.1.

2.2.3 Homogenisation of samples

A small piece from each section was weighed and transferred to a glass homogeniser. A 1:19 ratio of a 100 mM phosphate buffer, pH 7.4, containing 0.02% bovine serum albumin was added. The section was thoroughly homogenised with a glass rod on ice and sonicated with an ultrasound disintegrator (Virtis Sonicators, USA) three times for ten seconds on ice. After sonication, any connective tissue was removed, patted dry, weighed and subtracted from the original muscle weight. Homogenates were stored at -87 °C until analyses.

2.2.4 MHC isoform determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Talmadge and Roy (1993). β -mercaptoethanol was added to the upper running buffer to a final concentration of 0.16%. The homogenate sample was diluted with a sample buffer containing 10% glycerol, 5% β -mercaptoethanol, 2.3% SDS and 8 mM Tris base. Samples were heated for ten minutes at 60 °C and stored at -87 °C until electrophoresis. Before electrophoresis, samples were briefly boiled for two minutes, allowed to cool and loaded on to the gels. Gels were run for 28 hours at constant 70 volts at 4 °C, stained with Coomassie R250 and scanned using a computer scanner. Band densities were analysed using a software package (CREAM 1-D, KEM-EN-TEC, Denmark). Values are expressed as a percentage of the total number of bands distinguishable for each sample (see appendix B for details).

2.2.5 Citrate synthase activity

CS activity was measured using a modified Srere (1969) protocol. Briefly, the assay reagent contained 85 mM Tris buffer, pH 8.3, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.2 mM acetyl-coenzyme A, 0.5 mM oxaloacetate and 10 μ L of the homogenate sample. CS activity was measured for five minutes at 412 nm in a spectrophotometer (Cary-50) at room temperature and expressed as μ mol/min/g wet weight (see appendix B for details).

2.3.6 Statistical analysis

All values are presented as mean \pm standard deviation (SD). Data were analysed using a repeated measures ANOVA with a Bonferroni correction for unequal variance for each MHC isoform in all nine sections. The $P < 0.05$ confidence level was used to indicate statistical significance. Correlation coefficients between each MHC isoform and CS activity were calculated using the two-tailed Pearson's correlation test.

2.3 Results

MHC isoform contents and CS activities of nine sections of the QF muscle of the rat were determined. The nine sections were named S, M or D according to superficial, middle or deep region, and d, c or p from distal through central to proximal parts.

Figure 2.2 shows an example of the MHC isoforms separated by SDS-PAGE in three different sections (dS, cM and pD). Qualitatively, this figure indicates that superficial regions expressed mostly MHC IIb and IIx, whereas MHC IIa began to appear in middle regions and was more abundant in deep regions. The expression of MHC I was only clearly detected in the deep region, in this example. Where no trace was found of an isoform, the value was included in the statistical analysis as zero.

The percentages of each of the MHC isoforms expressed in the different sections are reported in Figure 2.3 (panels A, B, C) and Figure 2.4 (panels A, B, C). In Figure 2.3, the comparison is specifically made from superficial to deep in the proximal (A), central (B) and distal (C) regions, separately. In all three regions from proximal to distal, the MHC IIb decreased significantly from superficial to middle and more significantly from superficial to deep parts. However, even in the deep portion, the quantity of MHC IIb expression was approximately 30%. The concomitant increase was not in MHC IIx, but in MHC IIa and MHC I expression (Figure 2.3). The increase in the latter two isoforms was more pronounced in the proximal part and less in the distal part. Although the MHC IIx isoform expression was significantly different in some parts, there was no pattern from superficial to deep regions in all three parts.

No difference was observed between the percentages of any particular MHC isoform in any part of the superficial region of the QF muscle (proximal, centre or distal) (Figure 2.4A). In the middle region of the muscle, section dM expressed significantly more MHC IIb than sections cM and pM ($P < 0.05$), but again the concomitant lower expression was not MHC IIx, but MHC IIa ($P < 0.05$) (Figure 2.4B). For MHC IIx, expression was similar from the proximal through the centre to the distal parts of the superficial, middle and deep regions. However, the deep region showed the most variation in MHC I isoform expression (Figure 2.4C). MHC I, MHC IIa and MHC IIb were significantly different between all three parts of the deep region with less MHC I and MHC IIa in the distal-deep part than either the central-deep ($P < 0.01$) or the proximal-deep ($P < 0.001$) parts. However, MHC IIb isoform expression had the opposite distribution: higher in the distal-deep part than the central-deep part ($P < 0.001$), which in turn was higher than the proximal-deep part ($P < 0.05$). On the contrary, no difference in MHC IIx expression was observed between any of the parts of the deep region.

CS activities were determined as a marker for oxidative capacity in the different regions and parts of the QF. The CS activities were similarly low in the three superficial regions but

increased in both middle and deep regions (Table 2.2). The deep region had approximately twice the activity of the superficial region. Statistical analysis revealed that middle and deep regions, with the exception of the distal-middle section, had significantly higher CS activities than the corresponding part of the superficial region (all $P < 0.05$), with no differences in activity between the middle and deep regions.

Figure 2.5 illustrates the relationship between MHC IIb proportions and CS activities of the proximal-middle section ($r = -0.54$, $P < 0.05$). No relationships were observed between any of the remaining isoforms and CS activities of the remaining 8 sections (e.g. Figures 2.6 and 2.7). In some parts, this was because of an absence of the isoform (e.g. Figure 2.6) and in other parts, there was simply no relationship (e.g. Figure 2.7), despite a range in MHC I expression from 5 to 30% and in CS activity from ~15 to 30 $\mu\text{mol}/\text{min}/\text{g ww}$.

2.4 Discussion

Comparison of the expression of four MHC isoforms and CS activities in nine sections of the QF in rat skeletal muscle was studied by investigating these parameters from superficial to deep regions, and within each region from proximal to distal parts. The main finding was that MHC I, MHC IIa and MHC IIb expression was significantly different across the length of the deep region with the proximal portion having more slow twitch MHC and the distal portion more fast ($P < 0.05$, Figure 2.4C). A similar finding was also apparent in the middle region, but only for MHC IIa and MHC IIb (proximal, central and distal, Figure 2.4B). In contrast, no difference in CS activities was observed across the length of the muscle in any region (Table 2.2). However, from superficial to deep, both the MHC isoform expression and CS activities were significantly different ($P < 0.05$). Although the change in MHC isoform showed a graded pattern, with a high expression of MHC IIb in the superficial region (with low CS activities), and as the level of depth increases, MHC IIb expression decreased gradually with a concomitant increase in MHC IIa and I expression, the CS activities already increased markedly in the middle region (Figures 2.3 and Table 2.2).

Previous studies indicated that a muscle group could have large differences both in fibre type and oxidative capacity (Punkt, 2002). In addition, some fibre types may also be absent, such as type IIb in the rat *Soleus* muscle (Delp and Duan, 1996; Talmadge and Roy, 1993). In the present study, all four MHC isoforms commonly expressed in rat skeletal muscle were found in the QF. However, when different sections were investigated, it was found that some isoforms were not present. The two most commonly expressed isoforms were MHC IIb and MHC IIx and were detected in all nine sections, including those in the deep region (Figure 2.3).

The pattern of MHC isoform expression showed a gradual increase in MHC I and IIa from superficial to deep, with a concomitant decrease in MHC IIb expression. However, the MHC IIx

isoform only showed an increase in expression in the central superficial and middle sections. This finding of more type I fibres in deeper sections correlates with the fact that this is a common phenomenon in other muscle groups of the rat (Delp and Duan, 1996; Punkt, 2002). Despite these patterns of decreasing MHC IIb expression, the deep region still had substantial proportions of this fast isoform.

The current data confirm the observation of Wang and Kernell (2000) in terms of the increased expression of type I fibres in the deep region, but that in the middle region, the significant difference was actually found in the MHC IIa fibres rather than the MHC I fibres. The present study, however, expands on their findings by indicating which of the subdivisions of the type II fibres show the concomitant opposite tendency (i.e. a decrement). This was not distributed between the fast isoform types, but was restricted to the MHC IIb isoform (Figures 2.3 and 2.4).

Significant differences in MHC isoform content were observed from proximal to distal in the QF muscle. Specifically, the regions closer to the hip had more oxidative fibre types whereas closer to the knee, there were more fibres expressing MHC IIb. However, this significance was more pronounced in the deep than the middle region and was not apparent in the superficial region. Although the fastest fibre type's MHC expression differed significantly, the variation along the length of the deep region was not observed for the MHC IIx isoform, the second fastest of the isoforms (Figure 2.4).

Sakuma *et al.* (1995) also compared fibre types in proximal, central and distal regions of rat *Soleus* and *Plantaris* muscles and found higher proportions of type I fibres in the proximal region of the *Soleus*, but higher proportions of type I and IIA in the middle region of the *Plantaris*. In the present study, the QF muscle followed a similar pattern to that reported by the latter authors. However, it may be that these patterns vary between muscle groups of various species and should not be taken as a general phenomenon (Wang and Kernell, 2000; Wang and Kernell, 2001b). For example, two studies have attempted to determine the mechanisms underlying such regional differences in fibre type. Campbell *et al.* (1996) determined that the proximity of a region to the synapse may influence SDH activity. These authors showed that there are large differences in SDH activities between regions at the motor endplate, subsarcolemmal and inter-myofibrillar portions. However, they also showed that the activities of SDH in these regions remained the same in muscle subjected to six weeks of muscle overload, despite a significant increase in overall muscle mass and cross-sectional area of the *Soleus* muscle. Thus, a coordinated adaptation of both cell size and oxidative capacity was observed, without the relatively higher expression of oxidative capacity usually seen with endurance training (Sugiura *et al.*, 1992). Maturation may also influence muscle fibre type in muscle groups, as was shown by Maltin *et al.* (1989), where nine muscle groups were investigated in rats from the ages of 19 (postnatal), 50 (young), 100 (adult) and 360 days (aged).

Fibre type in those muscles analysed varied significantly, but most of the variation occurred between the ages of 19 to 100 days, whereafter no further variation was observed. Therefore, it may be concluded that neuromuscular activity after birth plays an important role in determining muscle fibre type in adulthood.

In a study by Torella *et al.* (2000), significant differences in fibre type distribution were found not only for superficial to deep and proximal to distal, but also lateral to medial in rat TA muscle. These authors also reported that large variations in fibre type distribution exist between rats. In the present study, the question of whether such variations are mirrored by variations in CS activities was investigated. The activities of CS did not follow a graded increase from superficial to deep, except in the distal region. In the proximal and central parts, there were no differences in CS activity between middle and deep regions. However, no differences in activities were observed from proximal to distal, despite the observed differences in fibre type. Only the proximal-middle section showed an inverse relationship between MHC IIB proportions and CS activities (Figure 2.5). No relationships were observed between any of the remaining isoforms and CS activities in any of the remaining eight sections. This could be explained, only in part, by the lack of expression of e.g. MHC I in superficial regions (Figure 2.6 for central-superficial). In the central-deep region, where both CS activity and MHC I expression was high, there was also no correlation, despite variation in both parameters between different rats (Figure 2.7).

It is generally accepted that slow twitch fibres have greater oxidative capacity than fast twitch fibres. However, Nakatani *et al.* (2000) found that in rat *Soleus* muscle, the type IIA fibres had a higher SDH activity than the type I fibres. Similarly, the authors also reported higher SDH activity in type IIB fibres from deep regions of the *Plantaris* and TA muscles compared to more superficial parts. Pette (1985) claimed that a large variation in enzyme activities exists between and within fibre types. Therefore, the possibility arises that in rodents, anatomical position has a stronger influence on fibre type than physical activity, which is reflected in CS activity, and that this contributes to the poor relationships between the MHC isoforms and CS activities of the present study. A question that remains unanswered from this study is whether fibre type is more closely related to postural activation (or lack thereof) or a pre-programmed anatomical position.

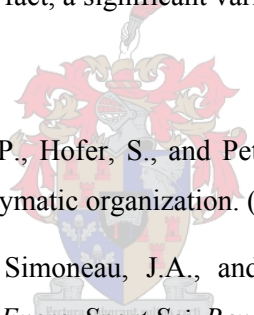
2.5 Conclusion

This investigation revealed that there is high diversity in MHC isoform expression across the QF muscle of the rat. The QF is an important muscle in any activity concerning mobility of mammals. In the case of the rat, it seems that the QF muscle has a much greater variety of uses than e.g. the *Soleus* because of the diversity of fibre type distribution and oxidative capacity. It was observed that vertical levels of the muscle show the most differences concerning MHC

isoform expression and oxidative potential, but a novel finding is that these differences also appear horizontally. The biochemical data also seem to imply that the superficial part is the centre for short exploding bursts, using anaerobic metabolism as the main fuel source. Moving deeper and more proximal, it appears that these regions would be the most active in endurance activities, with high oxidative capacities and the expression of slow MHC I. It is not clear whether these activities are related to posture (frequent, low power activation) or physical activity (less frequent, but more powerful). Possibly, the deep region closest to the hip may be involved in postural activities (having both the highest CS activity and high MHC I content). Differences in exercise habits between these laboratory rats only seemed to co-influence the MHC IIB content and CS activities in the mid-region closest to the knee and hip. In other regions it is not clear whether CS activities were related to exercise and fibre type to posture or pre-programmed anatomical position, or not.

The findings of this study stress the concern for accurate reporting of exact sampling site when investigating muscle characteristics, or adaptations to stimuli. Many researchers do not report exact sampling site, or merely distinguish between “red” or “white” *Vastus* or “superficial” or “deep”, where there may be, in fact, a significant variation between adjacent regions.

2.6 References

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Table 2.1 Summary of selected literature that investigated muscle regionalisation in various species and muscle groups.

Authors	Species	Muscles	Regions	Fibre types	Enzymes
Torrella <i>et al.</i> , 2000	Rat	TA	Proximal, equatorial, distal, anterior, posterior, medial, lateral	SO, FOG, FG	None
Punkt <i>et al.</i> , 1998	Rat	Soleus, EDL	Insertion, middle, origin, deep, central, superficial	SO, FOG, FG	SDH, GPDH, ATPase
Sakuma <i>et al.</i> , 1995	Rat	Soleus, Plantaris	Proximal, middle, distal	I, IIC, IIA, IIB	None
Delp and Duan, 1996	Rat	76 muscle groups	Various	I, IIA, IIX, IIB	CS
Wang and Kernell, 2001a	Rat, rabbit, mouse	Soleus, EDL, FD, GM, PL, TA	Proximal, middle, distal	I	None
Wang and Kernell, 2000	Rat	EDL, FD, GM, PL, TA	Proximal, middle, distal	I	None
Nakatani <i>et al.</i> , 2000	Rat	Plantaris, TA	Superficial, middle, deep	I, IIA, IIB	SDH

ATPase, adenosine triphosphatase; CS, citrate synthase; EDL, *Extensor digitorum longus*; FD, *Flexor digitorum*; FG, fast glycolytic; FOG, fast oxidative glycolytic; GM, *Gastrocnemius medialis*; GPDH, glycerol-3-phosphate dehydrogenase; PL, *Peroneus longus*; SO, slow oxidative; SDH, succinate dehydrogenase; TA, *Tibialis anterior*

Table 2.2 Citrate synthase activities of nine sections in rat *Quadriceps* muscle ($\mu\text{mol}/\text{min}/\text{g}$ wet weight) ($N = 18$).

	p	c	d
S	12.0 \pm 3.4	12.4 \pm 3.0	12.2 \pm 2.3
M	23.5 \pm 9.6*	23.1 \pm 4.9*	18.8 \pm 4.9
D	28.0 \pm 9.5*	24.8 \pm 8.0*	22.5 \pm 5.2*

Data are presented as mean \pm SD. Statistical analysis was performed using repeated measures ANOVA with a Bonferroni post-hoc test. * Different from S ($P < 0.05$).

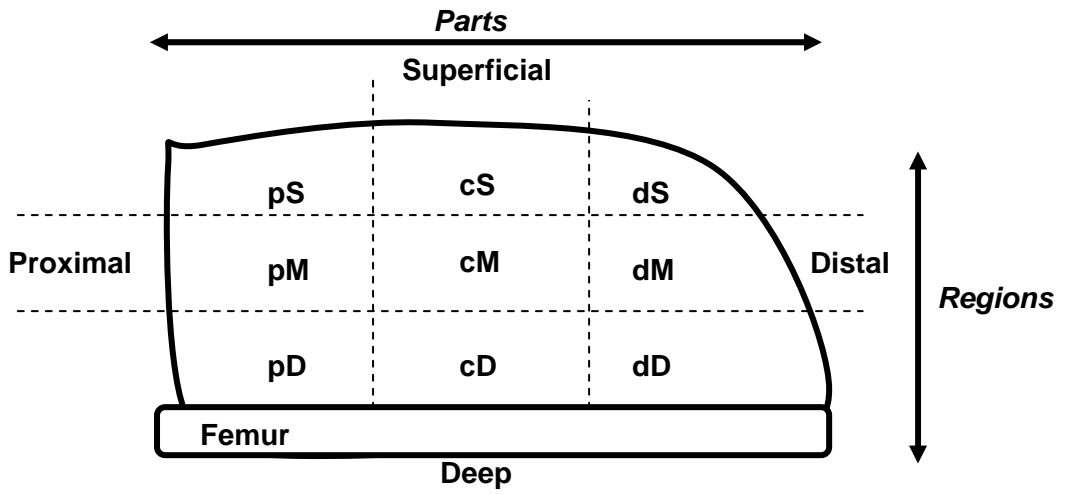


Figure 2.1 Layout of nine sections of Quadriceps muscle of rat upper hind limb.

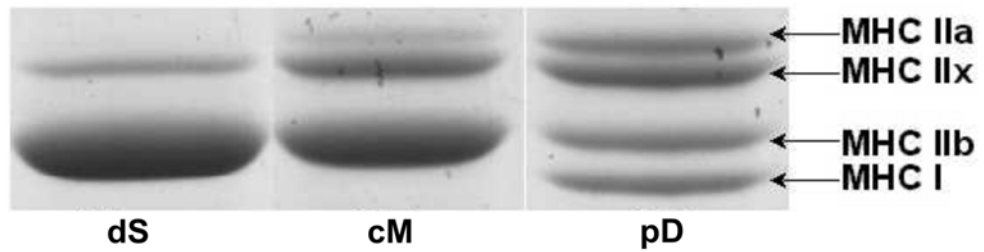


Figure 2.2 An example of the MHC isoforms expressed in three different sections of the Quadriceps muscle of rat upper hind limb.



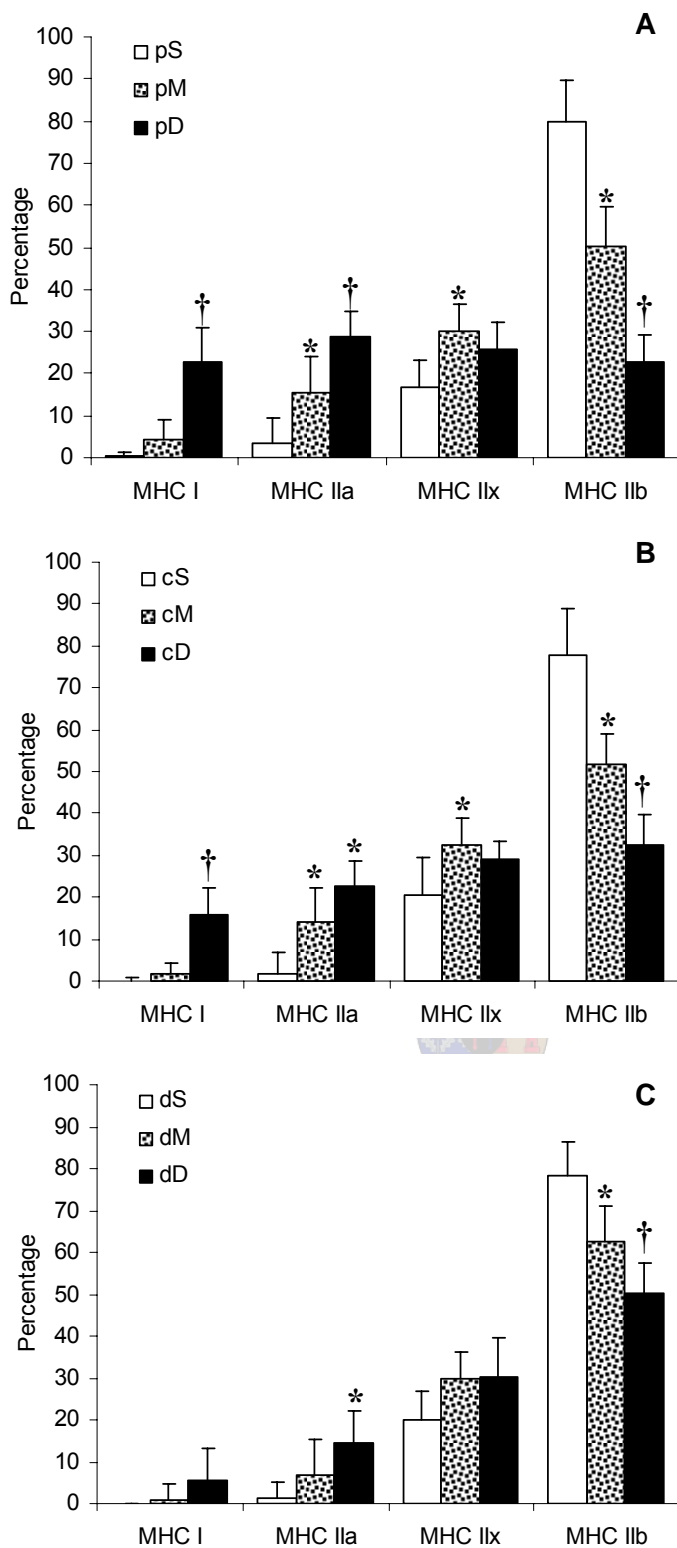


Figure 2.3 *Myosin heavy chain (MHC) isoform distribution in the nine sections of rat Quadriceps muscle.* Panel A. proximal (p), panel B. central (c), panel C. distal (d) regions, each ranging from superficial (S) to middle (M) to deep (D) portions (N = 18). Data are presented as mean \pm SD. Statistical analyses were performed using repeated measures ANOVA with a Bonferroni correction. Different ($P < 0.05$): * from section S; † from sections S and M.

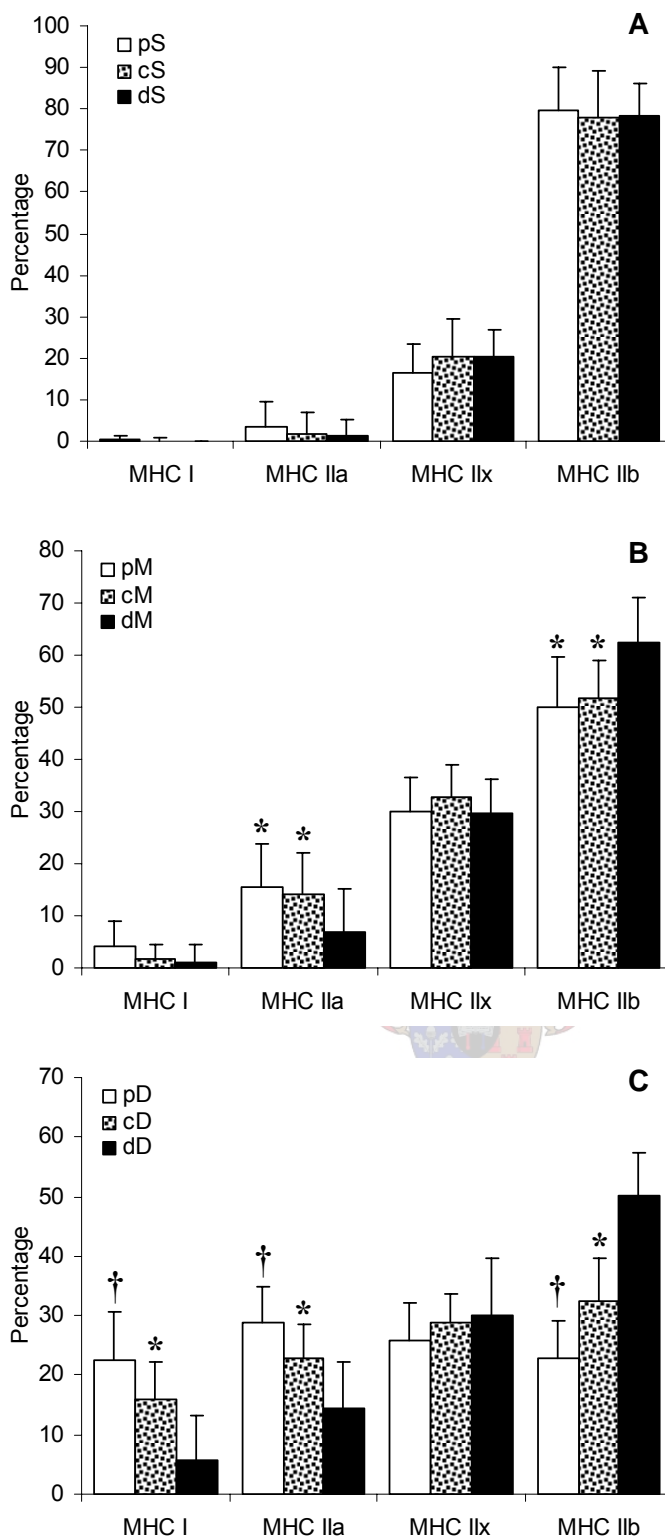


Figure 2.4 Myosin heavy chain (MHC) isoform distribution in rat *Quadriceps* muscle. Panel A. superficial (S), panel B. middle (M), panel C. deep (D) region, each ranging from proximal (p) to central (c) to distal (d) portions (N = 18). Data are presented as mean \pm SD. Statistical analyses were performed using repeated measures ANOVA with a Bonferroni correction. Different ($P < 0.05$): * from section d; † from sections d and c.

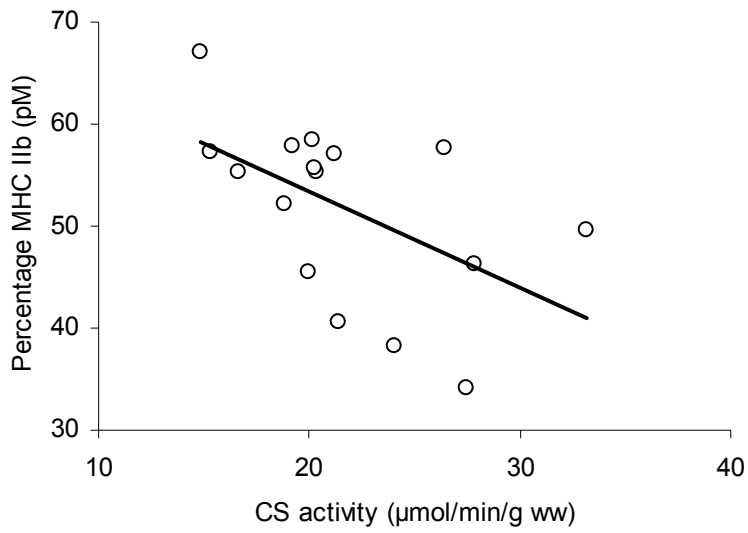


Figure 2.5 Relationship between MHC IIb and citrate synthase activity within the proximal, mid-portion of rat *Quadriceps* muscle (section pM). Pearson's r and significance: $r = -0.54$, $P < 0.05$.

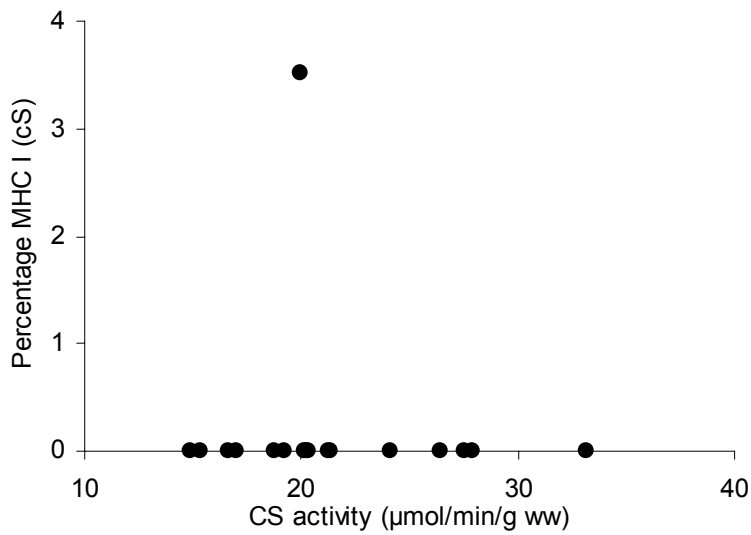


Figure 2.6 Relationship between MHC I and citrate synthase activity within the superficial mid-portion of rat *Quadriceps* muscle (section cS). Pearson's r and significance: $r = 0.13$, non-significant).

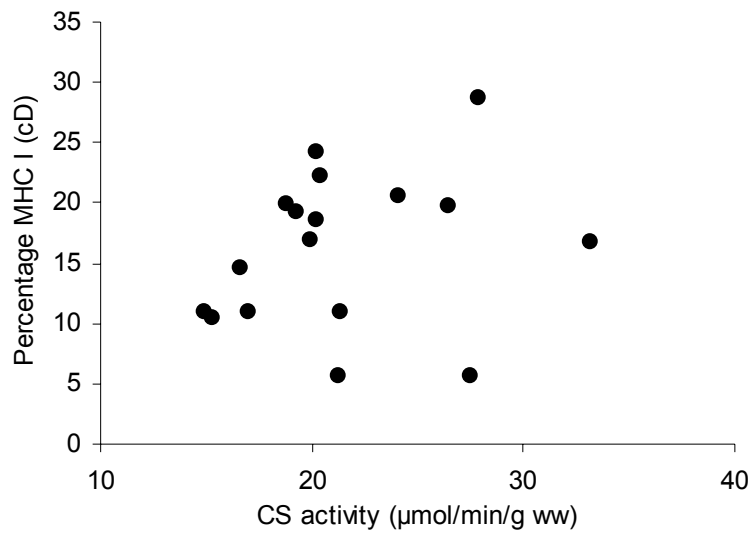
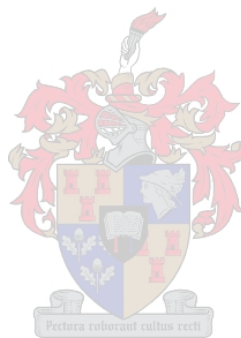


Figure 2.7 Relationship between MHC I and citrate synthase activity within the proximal, mid-portion of rat *Quadriceps* muscle (section cD). Pearson's r and significance: $r = 0.04$, non-significant).



Single fibre analysis of human skeletal muscle

3.1 Introduction

Human skeletal muscle mainly expresses three myosin heavy chain isoforms, namely MHC I, MHC IIa and MHC IIx, with each isoform giving rise to special and various contractile properties for the individual fibres and the muscle as a whole, respectively (Larsson and Moss, 1993). Fibres may express only one isoform (pure fibres) or multiple combinations of the three isoforms, with the latter commonly referred to as hybrid fibres (Staron, 1997).

It is accepted that endurance athletes have a predominance of fibres expressing mostly MHC I and MHC IIa (Gollnick *et al.*, 1972; Saltin and Gollnick, 1983). However, both the type and volume of training may play significant roles in determining how many hybrid fibres exist. Few studies have thoroughly investigated the effect of specific exercise types and volumes on muscle hybridity in humans. Harber *et al.* (2002) showed that distance runners (3000 – 10 000 m) had 6% MHC I/IIa and no MHC IIa/IIx hybrid fibres. In contrast, cross-country skiers had a high incidence of MHC I/IIa hybrid fibres (~36%) (Klitgaard *et al.*, 1990). It is at present unclear why these two studies, both of endurance athletes, differ so much in the incidence of type I/IIa hybrid fibres. Track and field athletes participating in events not longer than 400 m had 34% hybrid fibres of which 12% were MHC I/IIa and 6% MHC I/IIa/IIx hybrid fibres (Parcell *et al.*, 2003). There are two ways to interpret the findings in this study. It is possible that the volume of a particular type of training was not sufficient for fibres to convert to the required phenotype, or that the overall volume of training was too low. An alternative interpretation is that the fibres were responding to different stimuli (different intensities) and that this aspect of training promotes the existence of hybrid fibres.

The role of hybrid fibres in skeletal muscle is still unclear. In a recent review, Stephenson (2001) suggested that a hybrid fibre might be a “fine tuned” fibre to optimise levels of endurance, power output or fatigue resistance within a broad range and a continuum. However, other researchers still support the idea of hybrid fibres being transitional and influenced by training interventions (Andersen *et al.*, 1994; Putman *et al.*, 2004; Williamson *et al.*, 2001).

The purpose of this study therefore was to investigate the occurrence of hybrid fibres in human subjects who varied in training volume and type. In the study design, both endurance athletes and recreationally active subjects, with a wide variety of preferred racing distances or recreational sports participation, respectively, were included. Given the above, it is also hypothesised that hybrid fibre occurrence may be directly related to both training volume and intensity.

3.2 Methodology

3.2.1 Subjects

Twelve healthy male middle distance runners (age, 22 ± 3 years; weight, 62 ± 9 kg; height, 174 ± 10 cm) and 12 healthy male recreationally active subjects, performing no systematic running (non-runners) (age, 24 ± 2 years; weight, 68 ± 13 kg; height, 179 ± 9 cm), were recruited, and signed an Informed Consent Form. The study was approved by the University of Stellenbosch ethics committee for research on human subjects (Sub-Committee C). Inclusion criteria for runners were as follows: ability to complete a 10 km road race in under 35 minutes (mean 10 km personal best in previous 3 months: 32.8 ± 1.5 min); training more than 50 km per week; no additional sport participation; must not race distances less than 1 500 m as preferred track distance or longer than 21.1 km as preferred off-road racing distance.

Each individual completed a detailed questionnaire on the amount of training or exercise performed in a typical period of four weeks. Other questions included e.g. preferred racing distance (runners) and type of exercise for non-runners. Runners were requested to report preferred racing distances for track, cross-country and road races. In case of two or more preferred racing distances reported, the average was calculated and reported as average preferred racing distance (PRD_A) (refer to Table 3.1). Exercise quantity was calculated per week and the 4-week average was expressed either as kilometres per week (runners) or hours of exercise per week (non-runners).

3.2.2 Procedures

Subjects completed an incremental test on a treadmill (RunRace, Technogym, Italy) until exhaustion. The initial speed of 14 km/h and 7 km/h for runners and non-runners, respectively, was increased every 30 seconds with 0.5 km/h. Breath-by-breath samples were analysed for volume, oxygen and carbon dioxide contents (Jaeger OxyCon Pro, Germany) and heart rate was monitored throughout the test (Polar, Finland) (see appendix A for details).

A medical doctor experienced in the technique, performed the muscle biopsies. Local anesthetic (Xylotox, Adcock Ingram) was administered to the *Vastus lateralis* and a small cut was made using a scalpel blade. A sterile trephine needle (Stille, Sweden) was inserted into the mid-portion of the muscle and with the addition of suction, $\pm 100 - 150$ mg tissue was removed with a quick cutting action of the needle (Bergström, 1962). Muscle specimens were rapidly frozen in liquid nitrogen and stored at -87 °C until single fibre analysis (see appendix A for details).

Muscle samples were freeze-dried overnight and individual muscle fibres dissected in a humidity controlled room. A total of 2608 fibres (mean 109 ± 38 per subject) were dissected. Each fibre was transferred to a capillary tube containing 30 μ L of a denaturing buffer (10%

glycerol, 5% β -mercaptoethanol, 2.3% sodium dodecyl sulphate (SDS) and 62.5 mM Tris-HCl, pH 6.80). Fibres were allowed to denature overnight at room temperature (Biral *et al.*, 1988). Gel electrophoresis was carried out according to the method of Talmadge and Roy (1993), with β -mercaptoethanol added to the upper running buffer to a concentration of 0.03 M prior to electrophoresis (Blough *et al.*, 1996). Electrophoretic conditions were constant 70 V for 24 hours at 4 °C. Gels were subsequently silver stained (PlusOne silver stain kit, Amersham, Sweden). Bands were identified according to Pereira Sant'Ana *et al.* (1997). For details, see legend to Figure 3.1.

3.2.3 Statistics

All values are reported as mean \pm standard deviation. The Mann-Whitney U test was applied for statistical comparison between runners and non-runners. The $P < 0.05$ confidence level was used to indicate statistical significance. Correlations were performed using the Pearson's correlation coefficient, in each group, separately. Where appropriate, one phase exponential curve-fitting was applied using non-linear regression analysis that minimized the sum of squares of actual distance of points from the curve (not weighted; not forced through 0). Goodness of fit is reported as R^2 values. Iterations proceeded until the change in the sum of squares between two consecutive iterations was less than 0.01%.

3.3 Results

3.3.1 Training data and maximum oxygen consumption

Runners trained a distance of 82.9 ± 23.9 km/wk (range 55 – 120 km/wk) and non-runners exercised on average 4.1 ± 4.5 h/wk (range 0 – 13 h/wk). Runners had a PRD_A of 11.8 ± 6.4 km (range 2.3 – 21.1 km). Furthermore, runners had a significantly higher peak treadmill velocity (21.6 ± 1.1 vs. 13.7 ± 0.8 km/h, $P < 0.05$) and maximum oxygen consumption capacity (68.7 ± 4.7 vs. 42.6 ± 3.6 mL/min/kg, $P < 0.05$) than non-runners. (See Table 3.1 for details)

3.3.2 Single fibre electrophoresis

Clear distinctions were evident in the mobility of the MHC isoforms of each single fibre (Figure 3.1). Fibres expressing pure MHC I, MHC IIa and MHC IIx, as well as hybrid fibres expressing both MHC I and MHC IIa (MHC I/IIa) and MHC IIa and MHC IIx (MHC IIa/IIx) in both groups, were identified and expressed as a percentage of total number of fibres (Figure 3.2). Runners had more fibres expressing pure MHC I than non-runners ($P < 0.01$) and no significant difference in fibres expressing pure MHC IIa. Fibres expressing pure MHC IIx and MHC IIa/IIx hybrids were higher in non-runners than in runners ($P < 0.01$). The percentage total hybrids were higher in non-runners compared to runners ($P < 0.05$).

The amount of pure fibres did not correlate, using Pearson's correlation, with the volume of exercise in either group and also not with preferred racing distance in runners (Table 3.2). However, significant correlation coefficients were observed between the amount of training (Figure 3.3A: $r = -0.66$, $P < 0.05$) or exercise (Figure 3.3C: $r = -0.72$, $P < 0.01$) performed per week and MHC IIa/IIx proportions. Exponential curve fitting showed a better relationship between the MHC IIa/IIx hybrids and volume of exercise (Figures 3.3B and 3.3D). Exponential curve fitting (Figure 3.4) of MHC IIa/IIx hybrids and PRD_A in runners showed an exponential decrease in the hybrids with an increase in racing distance (Pearson's correlation $r = -0.85$, $P < 0.001$) whereas no relationship was observed for MHC I/IIa hybrid fibres and PRD_A .

The highest PRD_A for the athlete group was 21.1 km. This value was halved in order to separate athletes with short and long PRD_A s. This value was rounded off to 12 km in order to include enough subjects per group for statistical power. Figure 3.5 shows that runners who preferred racing distances on average less than 12 km had significantly more MHC IIa/IIx hybrids ($P < 0.01$) than those preferring to race on average over distances more than 12 km. These two sub-groups of runners also differed significantly for training distance (67 ± 19 km/wk vs. 94 ± 21 km/wk) although less significantly ($P < 0.05$) than for the proportion of MHC IIa/IIx hybrids. The proportion of MHC I/IIa hybrid fibres did not differ between these two sub-groups of runners.

3.4 Discussion

The present study agrees with other studies showing that distance runners have more fibres expressing pure MHC I and fewer fibres expressing pure MHC IIx and both MHC IIa and IIx than recreationally active subjects (Essen-Gustavsson and Henriksson, 1984; Harber *et al.*, 2002). However, the novel issue that the current study addressed is the question of what type and amount of exercise is required to decrease these MHC IIx-containing fibres. For this purpose, endurance runners with varying training distances and varying preferred racing distance, as well as a second group of subjects ranging from sedentary to recreationally active in a variety of sports, were recruited. Despite significant differences between runners and non-runners for the mean proportions of MHC IIx fibres and MHC IIa/IIx hybrid fibres, the main finding of this study was that exercise volume (km/wk and h/wk) of both groups correlated with MHC IIa/IIx hybrid fibre proportions as well as the total proportion of hybrid fibres. However, PRD_A of runners also correlated with MHC IIa/IIx hybrid fibre proportions and the proportion of total hybrid fibres. This study also showed that there was no correlation between training / exercise volume and MHC I/IIa hybrid fibre proportions in either of the groups.

In the present study, single fibre classification with SDS-PAGE was used rather than the conventional ATPase histochemical method and showed that, despite significant differences in

the proportion of type I fibres in runners compared to non-runners, runners only had $50 \pm 14\%$ slow twitch fibres. This is less than previously reported for distance runners (Gollnick *et al.*, 1972; Harber *et al.*, 2002; Tesch and Karlsson, 1985), but is similar to the findings reported by Weston *et al.* (1999) for runners recruited from the same geographical region. This latter difference may be attributed to large variations in running and specialisation distance.

An important finding from the relation between the proportions of MHC IIA/IIX hybrid fibres to exercise volume was that a number of data points fell outside the 95% confidence limits of the linear regression line (runners: Figure 3.3A; non-runners: Figure 3.3C). However, reanalysis of the data showed that with an increase in exercise volume, there was an exponential decrease in MHC IIA/IIX hybrid fibre proportions in both runners and non-runners (Figures 3.3B and 3.3D, respectively). R^2 values for the exponential fits were much higher than those for the poorer fitting linear regressions (although there were statistically significant correlations with the latter). The data suggested that there is a critical volume of exercise necessary to induce hybrid fibres to decrease, but only for MHC IIA/IIX hybrid fibres within the volumes of exercise performed by the subjects in the current study. The data further suggests that MHC IIA/IIX hybrid fibres may only exist due to inactivity, which can be supported by the effect of paralysis, weightlessness and detraining (Andersen *et al.*, 1996; Andersen and Aagaard, 2000; Baldwin, 1996; Oishi *et al.*, 1998). However, the current data shows an even better exponential fit with PRD_A than with training volume in runners, and this finding is more in support of “fine tuning” through multiple expression of MHC isoforms to accommodate both endurance and high intensity demands. The present study provides indirect evidence that hybrid fibres might not be transitional.

Another finding in this study was that both runners and non-runners had similar proportions of MHC I/IIA hybrid fibres, which may also suggest that these fibres might serve a functional purpose (Figure 3.2). For runners identified as sub-elite, the runners in the present study had a personal best for a 10 km road race of 32.8 ± 1.5 minutes. This may, in part, explain the relatively low proportion of type I fibres, if it is considered that a more elite level may have higher type I proportions. But, as was shown in various interventions using different training methods, training intensity may shift fibre proportions to type IIA (Andersen *et al.*, 1994; Williamson *et al.*, 2001). These two studies used extreme intensities (resistance training), but may be extrapolated to high intensity endurance events differing in time.

3.5 Conclusion

On evaluation of the current literature and the present study, we hypothesise that hybrid fibres might have a dual function: being both transitional and “fine tuning” fibres for effectiveness. The latter part of this statement is supported by the significant correlation between MHC IIA/IIX

hybrid fibres and preferred racing distance (Figure 3.4A), whereas the first is supported by the relationship with training volume (Figures 3.3A and C). This study is the first to show an exponential decrease in MHC IIA/IIX hybrids as exercise volume increases irrespective of exercise type (running vs. non-running) (Figures 3.3B and D). It is also the first to use average preferred racing distance as an indirect indication of training intensity and a direct indication of racing intensity and the first to show that runners also have an exponential relationship with MHC IIA/IIX hybrids (Figures 3.4A). In addition, runners that preferred a racing distance of less than 12 km, showed higher MHC IIA/IIX hybrid fibre proportions than runners preferring more than 12 km (Figure 3.5). However, no relationship was found between MHC I/IIA hybrid fibre proportions and PRD_A, or exercise volume in either group (Figure 3.4B). These observations suggest that exercise volume, at least within the range of the subjects in the present study, will decrease MHC IIA/IIX hybrid fibre proportions, but not MHC I/IIA hybrid fibres. More research needs to be conducted to determine the relative influence of training volume vs. intensity of training on hybrid fibre proportions.

3.6 References

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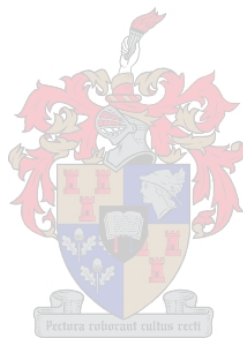


Table 3.1 Total training and participation in specific categories of exercise in runners (R) and non-runners (NR).

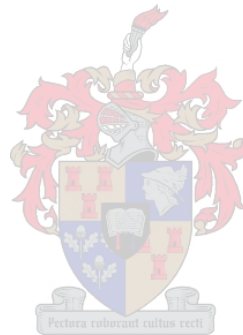
	Exercise	Categories of participation	PRD	PRD_A
	<i>km/week</i>			<i>km</i>
R1	80	Track & road	5 000 m, 21.1 km	13.1
R2	110	Road	21.1 km	21.1
R3	60	Track & road	800 m, 10 km	5.8
R4	80	Road	10 km, 21.1 km	15.6
R5	60	Track	3 000 m, 5 000 m	4.0
R6	75	Cross-Country & road	4 km, 21.1 km	12.6
R7	60	Track & Cross-country	3 000 m, 12 km	7.5
R8	120	Road	10 km, 21.1 km	15.5
R9	100	Track & road	800 m, 21.1 km	11.0
R10	55	Track	1 500 m, 3 000 m	2.3
R11	120	Road	21.1 km	21.1
R12	75	Track & road	5 000 m, 21.1 km	13.1
	<i>hours/week</i>			
NR1	1	Active walking		
NR2	0	None		
NR3	2	Recreational soccer		
NR4	8	Gymnastics & coaching		
NR5	0	None		
NR6	13	Gymnastics & coaching		
NR7	0	None		
NR8	0	None		
NR9	10	Recreational soccer		
NR10	5	Recreational volleyball		
NR11	7	Recreational soccer		
NR12	3	Recreational soccer		

PRD, preferred racing distance; PRD_A, average preferred racing distance

Table 3.2 Relationships between pure fibre type (%) and training volume, average preferred racing distance (PRD_A) and exercise in runners and non-runners.

		Type I	Type IIa	Type IIx
Runners				
Training volume (km/week)	r	0.24	0.18	-0.43
	P	ns	ns	ns
PRD_A (km)	r	0.40	0.01	-0.39
	P	ns	ns	ns
Non-runners				
Exercise (hours/week)	r	0.30	0.41	-0.34
	P	ns	ns	ns

Pearson's correlation was applied to the data. Values are correlation coefficients.



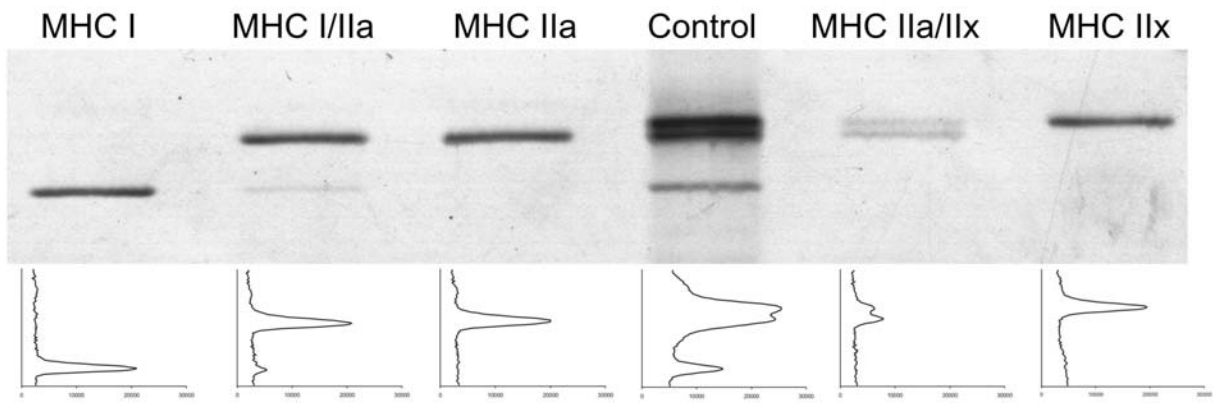


Figure 3.1 Myosin heavy chain isoform mobility in single muscle fibres. A homogenate muscle sample containing all three MHC isoforms was run as the control.

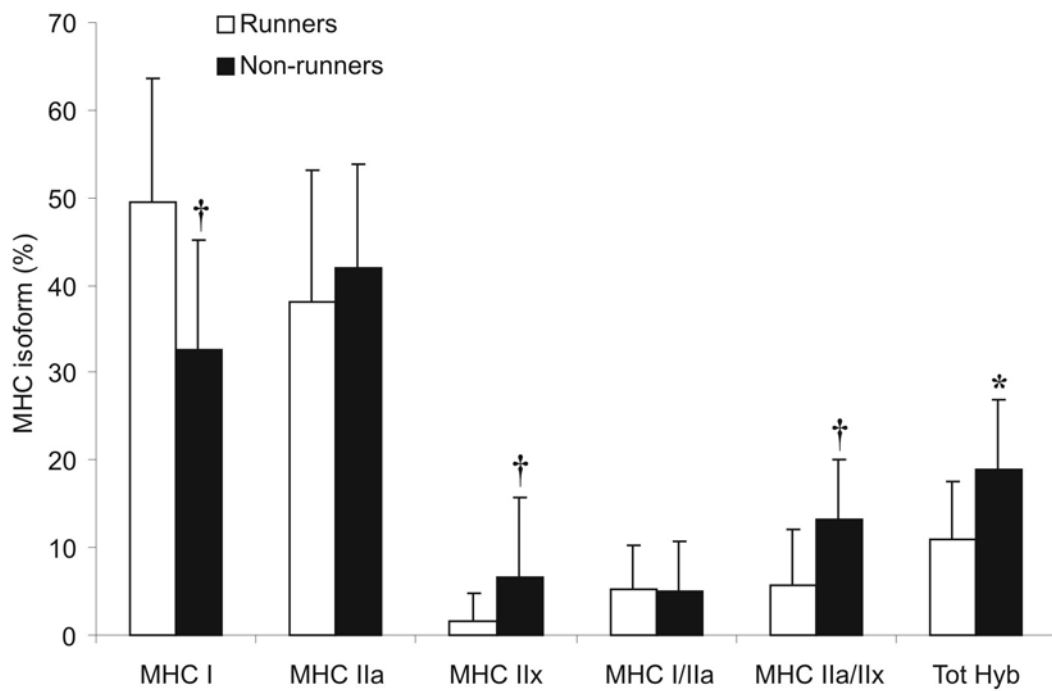


Figure 3.2 Muscle fibre type percentages (%) determined by single fibre electrophoresis of runners and non-runners. Values are means \pm SD. Tot Hyb, Total hybrids. Different from runners: * $P < 0.05$; † $P < 0.01$.

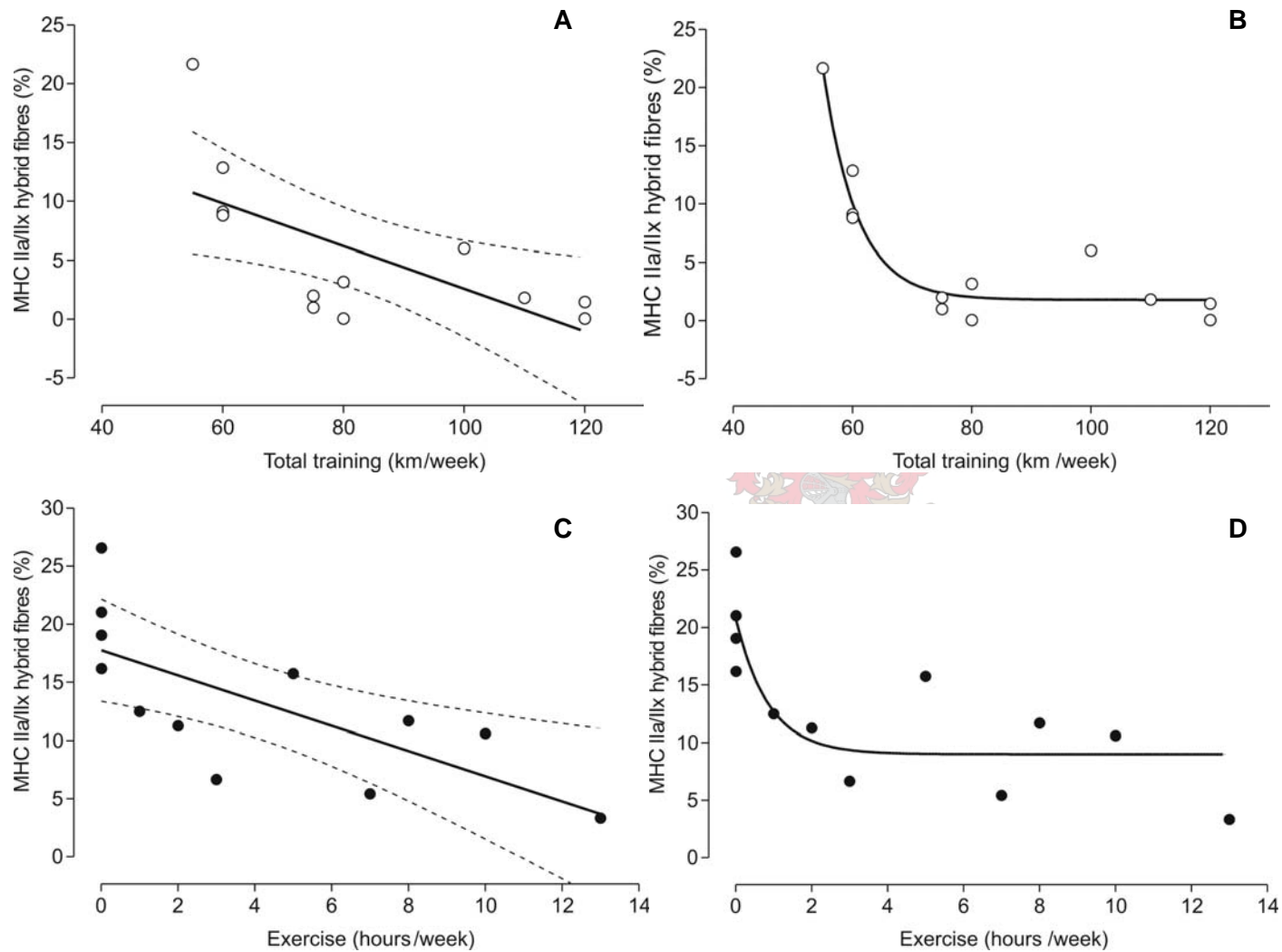


Figure 3.3 Relationships between percentage MHC IIa/IIx hybrid fibres and training volume (runners, A and B) and exercise volume (non-runners, C and D). Graphs A and C represent linear regression analysis with 95% confidence limits. Graphs B and D represents exponential curve fitting: B: $y = 342200e^{-0.18x} + 1.8$, $R^2 = 0.92$; D: $y = 11.8e^{-1.2x} + 9$, $R^2 = 0.67$.

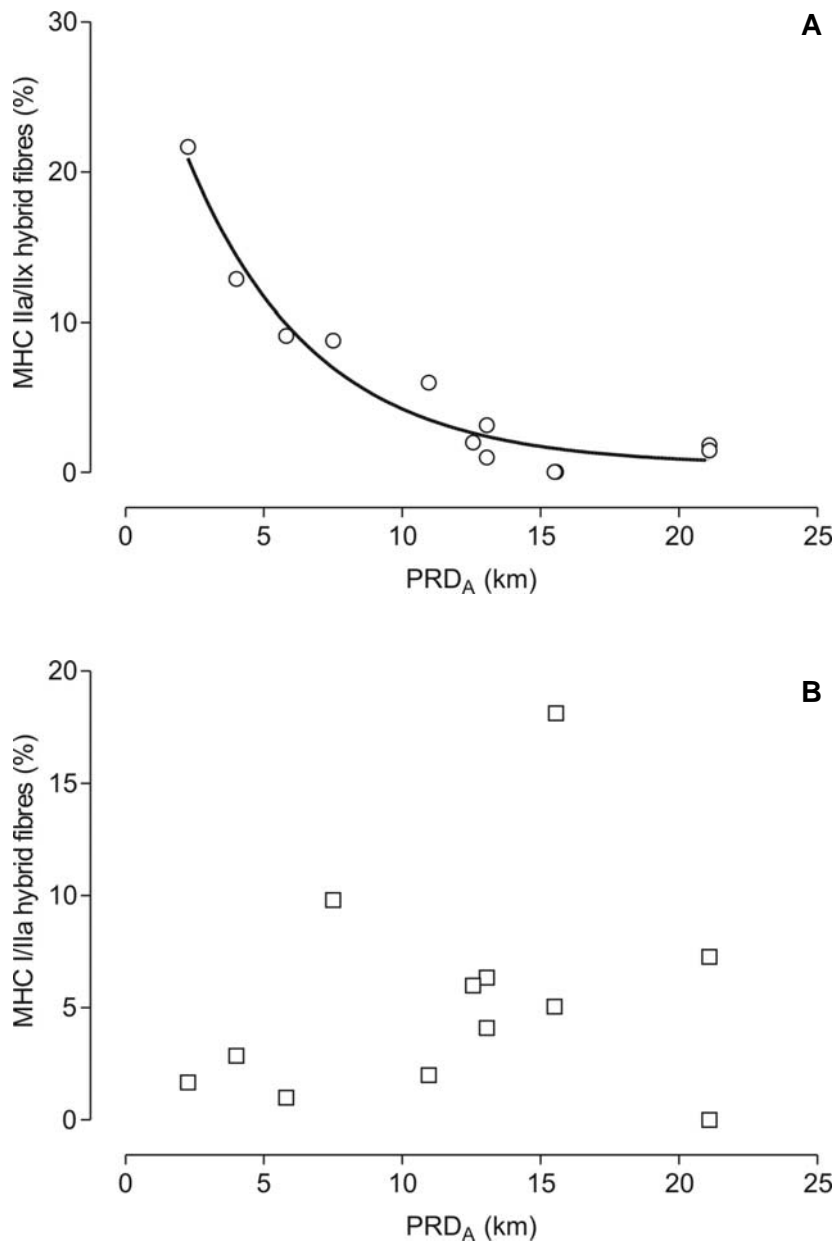


Figure 3.4 Relationship between (A) MHC IIa/IIx hybrid fibres and average preferred racing distance (PRD_A), (B) MHC I/IIa hybrid fibres and PRD_A . Exponential curve fitting for A: $y = 33.4e^{0.22x} + 0.47$, $R^2 = 0.95$.

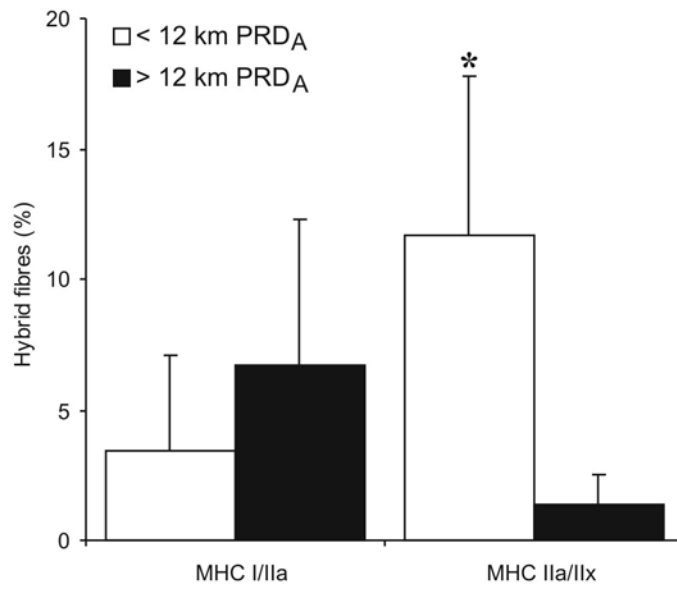


Figure 3.5 Percentage hybrid fibres in runners preferring a racing distance < 12 km and > 12 km. Values are means \pm SD. Statistical analysis was performed using the unpaired Mann-Whitney U test. * Different from > 12 km ($P < 0.01$).



Do Xhosa and Caucasian endurance athletes really differ?

4.1 Introduction

The search for genetic promoters for human sporting performance and health-related fitness phenotypes is still continuing (Scott *et al.*, 2005). Genes that are potential markers for exercise performance have been linked to either a population group, or groups tending to have the same phenotype, such as body size and proportions or high oxygen consumption (for recent reviews see Rankinen *et al.* (2004) and Beunen and Thomis (2004)). Some genes have been linked to endurance performance, others to sprinting events (Calvo *et al.*, 2002) and it has been found that genes may be involved in the response of an individual (Bouchard, 1995) or population to training (Chagnon *et al.*, 2001).

Exercise performance capacity is usually determined by standardised tests, such as those for maximal oxygen consumption (VO₂max), fatigue resistance at a specific workload, heart rate and economy of movement (to name but a few). Studies on the effect of heritage on most of these parameters are currently in progress and it seems that certain genes may only contribute in specific populations (Chagnon *et al.*, 2001; Rankinen *et al.*, 2004). For example, blood pressure was related to the angiogenin gene (*Avall*) polymorphism in black but not white individuals (Rivera *et al.*, 2001). Similarly, in populations specified by exercise phenotype, the creatine kinase gene polymorphisms (CK-NcoI and CK-TaqI) have been associated with the change in VO₂max in sedentary subjects who underwent training, but these polymorphisms were not related to performance in elite endurance athletes (Rivera *et al.*, 1997a; Rivera *et al.*, 1997b). Other genes have been shown to be related to both endurance capacity and body composition, such as peroxisome proliferator activated receptor delta (PPAR δ), the over expression of which has a direct influence on both phenotypes in mice (Wang *et al.*, 2004).

Other phenotypes previously associated with exercise performance include biochemical markers in skeletal muscle and blood. Muscle fibre type, enzyme activities and plasma lactate concentrations have been shown to vary significantly between sedentary individuals and endurance runners (Essen-Gustavsson and Henriksson, 1984; Gollnick *et al.*, 1972; Hurley *et al.*, 1984). For example, endurance runners generally have a high proportion of type I fibres, and a high oxidative potential in their skeletal muscle, compared to sedentary subjects (Gollnick *et al.*, 1972). These parameters may be even more enhanced in competitive and elite endurance athletes (Costill, 1967). Unless genotypic variability contributes to a phenotype known to be related to performance, it may not be relevant.

Hence, the identification of phenotypic and genotypic variability between individuals and even populations should both continue to be the focus of research. Several investigators have searched for phenotypic differences, especially between black and white endurance athletes (Bosch *et al.*, 1990; Coetzer *et al.*, 1993; Saltin *et al.*, 1995a; Weston *et al.*, 1999; Weston *et al.*, 2000).

Running events from middle distances (800 to 10 000 m) to long distances (half and full marathon) are dominated by East African black runners (Larsen, 2003; Weston *et al.*, 2000). Recent world road running rankings for 2005 listed 77 and 8 Kenyan and Ethiopian athletes, respectively, under the top 100 in the world (source: International Association of Athletics Federations). The possibility therefore may exist that these populations have a genetic advantage when it comes to endurance running. For the past ten years, researchers have investigated the physiological and biochemical markers, as well as the training habits in Kenyan and South African black distance runners and their counterparts of European descent. However, whether the dominance of African runners is due to genetic inheritance or is as a result of other factors, still remains unanswered. Even whether or not phenotypic differences appear consistently, is still a matter of debate (Saltin *et al.*, 1995a; Weston *et al.*, 1999).

The study by Bosch *et al.* (1990) was one of the first to show that black marathon runners were smaller in body size and ran at a higher percentage of their VO_2 max during a simulated treadmill marathon compared to their white counterparts. No differences were observed for VO_2 max and respiratory exchange ratio (RER). On the other hand, Coetzer *et al.* (1993) showed that black endurance runners, who had longer preferred racing distances than their white counterparts, had a lower RER at maximum intensity, but no difference in running economy assessed as oxygen consumption during sub-maximal tests at absolute velocities (17 and 21 km/h). A consistent finding of both Bosch *et al.* (1990) and Coetzer *et al.* (1993) was that black endurance runners had lower blood lactate concentrations during the sub-maximal tests. The lower blood lactate was not related to the “typical” fibre type profile of endurance runners, as Coetzer *et al.* (1993) showed that black runners, although specialising in longer distances, tended to have a lower proportion of type I muscle fibres (not significantly different), compared to white endurance runners (white: 63 ± 13.3 ; black: 53 ± 5).

Weston *et al.* (1999) expanded on the phenotypic assessment of the previous two studies by incorporating the analysis of enzyme activities in the muscle biopsies. The authors also developed a different approach to the sub-maximal testing, incorporating more workloads at relative intensities (72, 80, 88 and 92% of peak treadmill speed (PTS)) and a time to fatigue test (at 92% of PTS). Black athletes could run longer at the 92% workload, but plasma lactate concentration was lower

only at 88% with no statistically significant differences at the other workloads. This was attributed to the higher citrate synthase (CS) and 3-hydroxyacyl CoA dehydrogenase (3HAD) activities found in the muscle biopsies of the black endurance runners. On the other hand, although not statistically different because of low subject numbers, black endurance runners, once again, showed that there was a tendency to have less type I fibres compared to white endurance runners (17% difference, *P* value not reported). This finding seemed difficult to explain given the literature showing that type I fibres are associated with higher CS and 3HAD activities (Essen-Gustavsson and Henriksson, 1984). In all three the aforementioned studies, black endurance runners were significantly shorter and lighter than their white counterparts. Although these studies advanced our scientific understanding of differences in the endurance phenotype of black and white distance runners, they were each flawed in some respect. For example, they did not properly characterise the various distance events in which the black and white endurance runners competed. Similarly, both training volume and intensity were parameters poorly assessed. Coetzer *et al.* (1993) reported no difference in training volume between black and white endurance runners, but suggested that black athletes might have been training longer at a level greater than 80% of their VO_2max . However, their method for assessment of these aspects was not described.

In another study by Weston *et al.* (2000), the authors compared the running economy at a fixed workload and the fractional utilisation of VO_2max at race pace of 8 black and 8 white endurance runners who preferred 10 km races, but who matched fairly well for body mass. Unfortunately, the black endurance runners were still shorter in stature compared to their white counterparts, and although not statistically different, the mean body mass was 3.5 kg lighter. The black endurance runners had lower VO_2max and PTS values (not significant) than their white counterparts, and this might explain the higher fractional utilisation of VO_2max at their 10 km race pace assessed on the treadmill. No difference was found for plasma lactate concentrations at sub-maximal intensity.

Lactate dehydrogenase (LDH) is an enzyme that produces lactate from pyruvate. There are several isozymes of LDH (van Hall, 2000). LDH is a tetramer protein, consisting of either the M (muscle) or H (heart) type subunits. These subunits may combine in different ratios to form five LDH isozymes, numbered from 1 to 5. LDH_1 and LDH_2 are predominantly found in heart muscle, whereas LDH_4 and LDH_5 are found in skeletal muscle and liver. The heart isozymes (LDH_{1-2}) favour conversion of lactate to pyruvate, whereas skeletal muscle isozymes (LDH_{4-5}) favour pyruvate to lactate conversion. All five isozymes may be expressed in skeletal muscle in various amounts, but it has also been suggested that the LDH isozymes may be compartmentalised with those favouring lactate to pyruvate conversion situated close to the mitochondria (van Hall, 2000).

Lactate is no longer considered to be merely an end product of anaerobic glycolysis, rather consensus is that lactate can be utilised as a fuel by other muscles or organs during exercise (Gladden, 2004; van Hall, 2000). Brooks and his research group (2000) have proposed an intracellular lactate shuttle that allows lactate produced during glycolysis to be shuttled immediately to the mitochondria in the same fibre where it can be converted back to pyruvate and subsequently metabolised. However, the existence of this intracellular shuttle is still under debate and data from other laboratories do not confirm that a mitochondrial LDH exists (Rasmussen *et al.*, 2002; Sahlin *et al.*, 2002). However, it may be that the problem is more related to methodological irregularities (i.e. sample preparation) between research groups and that this topic needs further investigation (Brooks, 2002).

Saltin *et al.* (1995a) investigated muscle and performance characteristics in 13 Kenyan and 12 Scandinavian runners. Kenyans had higher mean 3HAD activity in their *Gastrocnemius* muscle biopsies and the ratio of LDH isozymes was different between the two groups. Kenyans had a higher ratio of LDH_{1,2}:LDH_{4,5}, but after the Scandinavians trained for 14 days at altitude, the difference between the groups for these ratios became non-significant. However, these findings might have been complicated by the low subject numbers (5 Kenyans and 6 Scandinavians) as some athletes were unwilling to freely give a muscle biopsy. Also, women were included in the Scandinavian group and the Kenyan subject group consisted of senior and junior runners. In addition, the effects that altitude may have on the physiology and biochemistry could also have complicated this study. Nevertheless, in a separate report by the same authors, it was shown that the Kenyans had lower plasma lactate levels at sub-maximal intensities compared to Scandinavian runners (Saltin *et al.*, 1995b). No differences were found between the two groups for VO₂max and haemoglobin concentrations, suggesting a peripheral, rather than a central cause, for the lower lactate accumulation during exercise. Once again, there may have been difficulties matching the subjects for prior altitude exposure.

Two crucial factors which have been linked to both physiological and biochemical adaptations in many studies, are training volume and the intensity of training. Although these variables are difficult to quantify in the field setting (Hopkins, 1991), studies using electrical stimulation on rats have shown that both fibre type and enzymes may respond differently to different stimulating frequencies (Pette and Vrbova, 1992; Windisch *et al.*, 1998). These data suggest that differences in cross-sectional studies of endurance vs. sprint athletes may be related not only to a natural selection into the events based on pre-training phenotype, but are also related to training-induced adaptations. In contrast to the well known endurance training-induced shift in type IIX to type IIA fibres (Baumann *et al.*, 1987; Jansson and Kaijser, 1977), Andersen *et al.* (1994) showed that short distance athletes

undergoing three months of sprint and strength training significantly reduced their type I fibres with a concomitant increase in the proportions of type IIA fibres. Therefore, the studies conducted on black and white athletes might have been complicated by training volume and intensity, both of which may be directly related to preferred racing distance.

The aim of the present study was to do a comprehensive phenotypic comparison between black endurance runners from distinct ethnic origin (Xhosa) and white endurance runners of Caucasian descent who were closely matched for average preferred specialisation distance (PRD_A), training volume and recent best 10 km race time. This study investigated possible differences in whole body physiology and muscle biopsy characteristics, including fibre type and single muscle fibre analyses. Focus was also placed on increasing the sample size compared with previous studies.

4.2 Methodology

4.2.1 Subject recruitment and training volume assessment

The Stellenbosch University Sub-Committee C for research on human subjects approved this study. Twenty-six healthy male athletes (13 Caucasian and 13 ethnic Xhosa) were recruited from local athletic clubs. Each athlete signed a written informed consent. Athletes were informed about all the tests and possible risks involved. Where a subject was unfamiliar with the language, an interpreter was used.

Subjects were excluded if they were not competing in races, if they had a 10 km road race time of more than 37 minutes, if they had experienced any illness or injury for the previous six months, or if they trained less than 45 km per week. To ensure Xhosa ethnic origin, all athletes had to report familial heritage of both parents and grandparents. Caucasian athletes were defined as being from European descent excluding Scandinavian and Latin countries, and Xhosa athletes were from Xhosa speaking family lineage. South Africa has two major, large black ethnic groups, namely Xhosa and Zulu. For centuries, these populations lived in distinct regions of South Africa. Cultural differences result in these tribes rarely intermarrying with each other. The Xhosa ethnicity of the subjects was based on the stated first language reported and for both parents and grandparents although, for the latter, it should be acknowledged that blacks of Sotho origin lived geographically close to the majority of the Xhosa population for centuries. Grandchildren may not be fully aware of the ethnicity of the grandparents, especially not the grandmothers who take on the cultural identity of the group into whom they marry. However, from an anthropological perspective, it is regarded that Xhosa people do not actively intermarry with other African tribes, and this is supported by genetic evidence from Lane *et al.* (2002).

Each athlete completed a detailed questionnaire reporting favourite race distance for road, track and cross-country competitions, recent 10 km personal best time (PB) and typical training volume per week specifically for the previous three months. PRD_A was calculated for each athlete by taking the average of the three favourite racing distances, one for each of the three disciplines mentioned above. In some cases, athletes participated in only two of these disciplines, but no athletes competed in only one. Athletes were matched for weekly training volume, 10 km PB and PRD_A (Table 4.1).

4.2.2 *Laboratory exercise testing*

Exercise tests and muscle biopsies were performed on separate days, allowing recovery from previous running tests for at least two days. Athletes were encouraged to be well rested and to abstain from races and only perform very low intensity training the day prior to testing. All athletes were thoroughly familiarised on the treadmill (including exposure to low and high intensity running on the treadmill) prior to running tests.

VO₂max testing and peak treadmill speed

Athletes performed two incremental maximal exercise tests to fatigue on a treadmill (RunRace, TechnoGym, Italy), with continuous measurement of heart rate (Polar, Finland), oxygen consumption (VO_2), RER and minute ventilation (V_E) (Jaeger Oxycon Pro, Germany) throughout the test. Athletes were allowed a 5 minute warm-up on the treadmill. All athletes started the test at 14 km/h (flat gradient) for 30 seconds, whereafter the intensity was increased by 0.5 km/h every 30 seconds until fatigue set in. Athletes were said to have attained their maximal ability when two of the following criteria were fulfilled: (a) heart rate within 5 beats/min of theoretical maximum heart rate ($220 - \text{age}$) (b) RER value greater than 1.10 and (c) a plateau in VO_2 (Staab *et al.*, 2003). Whenever these criteria were not fulfilled, athletes had to perform the same test on the next visit and were encouraged verbally during the test to perform better (see appendix A for details). PTS in km/h was calculated as follows taking every second into account:

$$PTS = \text{Completed full intensity (km/h)} + \frac{(\text{seconds at final intensity})}{30 \text{ seconds}} \times 0.5 \text{ km/h}$$

Sub-maximal exercise test and blood sampling

Prior to the sub-maximal test, athletes were fitted with an intravenous catheter (Jelco 22G, Johnson & Johnson) and a three-way stopcock, which were flushed with saline containing 0.04% heparin (Heparin Novo, Novo Nordisk, South Africa). Athletes were then allowed a 5 minute treadmill running warm-up and brief stretching.

The sub-maximal workloads corresponded to 64, 72 and 80% of each individual's PTS (the highest attained during one of the two maximal tests). Athletes ran for 5 minutes at each workload and breath-by-breath measurements were recorded as described for the incremental test. After each

workload, the athlete used the railings to lift himself off the treadmill and placed his feet on opposite sides of the belt. Three millilitres of blood were collected in a sealed test tube containing fluoride oxalate (Vacutainer, BD, UK), mixed and stored on ice. After a period of 1 minute of rest, the intensity was increased to the next workload and the athlete commenced running. Blood was centrifuged directly after the test at 3 000 rpm and the plasma stored at -87 °C until analysis (see appendix A and B for details). Running economy was determined for the speed, 16.1 km/h, by plotting VO₂ and treadmill speed.

Muscle biopsy

A needle biopsy was obtained from the *Vastus lateralis* muscle using the suction-assisted technique described by Evans *et al.* (1982). The biopsy site was at the same depth (2 cm) and in a similar position for all athletes, corresponding to one third along the total length of the upper leg, distal to the hip joint. The biopsy was split into three parts, two were frozen in liquid nitrogen for subsequent homogenate and single fibre analyses, and the third was mounted in embedding medium (Jung Tissue Freezing Medium, Leica Instruments, Germany) and rapidly frozen in iso-pentane, pre-cooled with liquid nitrogen. All biopsy samples were stored at -87 °C (see appendix A for details).

4.2.3 Biochemical analyses

Plasma lactate concentration

Plasma lactate concentrations (mmol/L) were determined using a commercially available kit (Lactate PAP, bioMérieux sa, France) and a spectrophotometer (Bio-Tek Instruments, USA) set at 505 nm. The kit relies on the principle of an enzymatic conversion of plasma lactate, resulting in a detectable colour that is concentration dependent. Values are expressed as mean ± SD (see appendix B for details).

Morphology of fibres

Fibre typing of muscle samples was based on the method by Brooke & Kaiser (1970). Three serial cross-sections (10 µm) were cut onto glass slides and placed into pre-incubation medium set at exactly pH 4.30, 4.60 and 10.30, whereafter the samples were visualised and photographed (Nikon CoolPix Microscope system, Japan). Fibres were identified as either types I, IC, IIC, IIAC, IIA, IIAX or IIX according to the staining intensities described by Staron (1997), and expressed as a percentage of the total number of fibres counted. In this study, fibre types IC, IIC and IIAC numbers were pooled and termed type I/IIA because of low counts in each of the aforementioned subgroup.

Cross-sectional area (CSA, µm²) and fibre diameter (FD, µm) were determined using a computer software programme (SimplePCI ver 1.0, Nikon, Japan) on the same slides photographed for the

fibre typing. Fibres were divided into two groups, namely type I (pure type I fibres only) and II, the latter comprising of fibre types I/IIA, IIA, IIAX and IIX.

Enzyme activities and myosin heavy chain composition in homogenate samples

Muscle biopsy samples, previously frozen in liquid nitrogen, were freeze-dried overnight. A small piece was weighed, crushed in a test tube and a ratio of 1 mg:400 μ L, chilled 100 mM potassium phosphate buffer, pH 7.30, was added. Samples were kept on ice and sonicated (Virtis Sonicators, USA) three times for ten seconds on ice, with a ten second delay between intervals.

Phosphofructokinase (PFK), CS, LDH and 3HAD activities were determined using the fluorometric methods described by Essen-Gustavsson and Henriksson (1984), with slight modifications. Reagent and sample volumes were decreased to accommodate the microplate reader (Bio-Tek instruments, USA). The enzyme reagent was always 250 μ L and sample volumes for PFK, CS and 3HAD were 5 μ L, and 3 μ L for the LDH assay. The emission at 460 nm was recorded for 5 minutes with 30 second intervals using an excitation wavelength of 340 nm. (Refer to appendix B for protocols and calculation details). Enzyme activities are expressed as μ mol/min/g dry weight (dw).

Myosin heavy chain (MHC) isoform contents of homogenate samples were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Talmadge and Roy (1993) with β -mercaptoethanol added to the upper running buffer to a concentration of 0.03 M prior to electrophoresis (Blough *et al.*, 1996). Electrophoresis was carried out using a mini-gel system (Bio-Rad, USA) for 16 hours at constant 70 volts at 4 °C. Gels were stained with Coomassie Blue R250 and subsequently scanned using a computer scanner. Relative percentages of the bands were quantified using a software package (CREAM 1D, KEM-EN-TEC, Denmark). (Refer to appendix B for details on SDS-PAGE, staining and relative band intensity quantification.)

Single fibre identification and enzyme activities.

Single muscle fibres were dissected from freeze-dried samples in a humidity controlled room (40% humidity, 20 °C). A total of 2857 (mean of ~130 fibres per sample) fibres were dissected. A small piece of each fibre was cut off, transferred to a capillary tube containing SDS denaturing buffer and left overnight to dissolve. The remaining piece was sealed and stored in a labelled glass capillary tube at -87 °C. Identification of the fibre types was carried out electrophoretically on the dissolved fragment, using the same protocol as described for the determination of MHC content in homogenates. However, gels were silver stained (Amersham, Sweden) and fibres identified as expressing either pure fibres containing MHC I, IIA or IIX, or hybrid fibres expressing both MHC I and MHC IIA (I/IIA), or MHC IIA and MHC IIX (IIA/IIX).

LDH activities in pools of type I and pools of type IIa fibres were determined for each subject. The pooled fibres were weighed on a microbalance (to 3 decimals of a milligram), calibrated with known weights. Pool weights ranged between ~40 and ~100 μg . After weighing on a Cahn 25 electro-balance, the sample was transferred to a micro tube, and 400 μL chilled 100 mM potassium phosphate buffer (pH 7.30) was added per 1 mg sample. Sonication was only carried out once for 10 seconds to prevent enzyme activity loss. Enzyme activity determination was carried out in the same way as for the homogenate samples. Enzyme activities are expressed as $\mu\text{mol}/\text{min}/\text{g dw}$. (Refer to appendix B for detail on the single fibre dissection, determinations of fibre type and enzyme activities.)

4.2.4 *Statistical analysis*

Statistical comparisons between population groups were performed using the Wilcoxon signed rank test for non-parametric matched pair data. However, due to lower sample numbers in the enzyme pools, statistical significance was determined with the Mann-Whitney U test for non-parametric unpaired data. Significance for all was set at $P < 0.05$. Correlation coefficients were calculated using the two-tailed Pearson's correlation test to assess specific associations.

4.3 **Results**

Good matching of athletes for training volume, 10 km PB and PRD_A was obtained. As can be seen in Table 4.1, athletes were coded according to matching pairs and for matching, none of the three variables considered was given first priority; rather all three were taken into account. A final consideration was whether or not the muscle biopsies of the pairs contained complete data sets for both physiological and biochemical analyses (see ‡, Table 4.1).

Table 4.2 reports the subject characteristics as well as the maximal exercise test results, the latter taken from the highest PTS of the two tests. Xhosa athletes were lighter and shorter than their matched counterparts ($P < 0.01$). VO_2max expressed relative to body weight was similar in the recruited athletes. However, when values were not corrected for body weight, significant differences were apparent (see VO_2max and $V_{E\text{max}}$ expressed in L/min, Table 4.2). These differences are seen because of both height and weight that directly influence oxygen consumption and hence also minute ventilation. Both groups reached similarly high peak treadmill speeds and RERmax , as would be expected for subjects matched for performance and indicated that both groups were similarly familiar with treadmill running at maximal capacity.

At all the sub-maximal workloads, no differences were observed for RER, heart rate and VO_2 expressed relative to body mass (Table 4.3), with the exception of VO_2 at 80% PTS, which showed

a trend to be higher in Xhosa compared to Caucasian athletes ($P = 0.09$). V_E was significantly lower in Xhosa athletes at 64 and 72% PTS workloads, but became non-significant at the higher workload (80% PTS). Economy at 16.1 km/h expressed relative to body mass and relative to body mass scaled to $0.75 \text{ (kg}^{0.75}\text{)}$, was similar for both groups.

During the sub-maximal test, there were no differences in the mean plasma lactate concentrations between the groups at the 64 and 72% PTS workloads (Figure 4.1). However, at 80% PTS, the Xhosa athletes had lower mean plasma lactate concentrations than their Caucasian counterparts ($P < 0.05$).

Morphometry of type I and type II muscle fibres of nine pairs of Caucasian and Xhosa biopsies was compared. No differences in CSA (μm^2) and FD (μm) were observed for either fibre type between groups (Table 4.4). A large variability is apparent for both fibre types in both groups.

Muscle fibre type proportions, determined with ATPase histochemistry in nine pairs, and using MHC isoform content in homogenate samples in 13 pairs, are reported in Figure 4.2. Xhosa athletes had less type I fibres with a concomitant higher proportion of type IIA fibres than their Caucasian counterparts ($P < 0.05$). This was further confirmed with the MHC isoform analysis that showed lower MHC I and higher MHC Iia expression in Xhosa athletes ($P < 0.05$). However, there was no difference between Xhosa and Caucasian athletes for the proportions of type I/IIA, IIA/X and IIX fibres.

The four enzymes, PFK, CS, 3HAD and LDH, were selected to represent the capacity of glycolysis, the Krebs's cycle, β -oxidation and the capacity to produce lactate from pyruvate, respectively. Enzyme activities were analysed in homogenate muscle samples of nine pairs and were similar in range to those reported by Essen-Gustavsson and Henriksson (1984). No differences were observed for CS, PFK and 3HAD activities (Figure 4.3). However, LDH activity was higher in Xhosa athletes compared to Caucasian athletes ($P < 0.01$), with PFK activity showing a trend to be higher in Xhosa athletes ($P = 0.07$).

LDH activities in distinct typed fibre pools and homogenate samples are represented graphically in Figure 4.4. Only subjects with activities for both pools and homogenates were used in this figure (Caucasian: $N = 6$; Xhosa: $N = 7$). The homogenate LDH activity was higher in Xhosa compared to Caucasian athletes. The range of activities between the two fibre types were similar in range to that reported by Essen-Gustavsson and Henriksson (1984). Statistical analysis between fibre types within each group showed that the mean LDH activity of type I fibre pools was significantly lower than the mean for the type Iia pools for both Caucasian and Xhosa athletes ($P < 0.05$). However, the

mean LDH activities in type I and type IIa pools of Caucasian athletes were significantly lower than the mean for type I and type IIa pools in Xhosa athletes ($P < 0.05$).

Relationships were observed between LDH activity and either the MHC I or MHC IIa contents of muscle samples (MHC I: $r = -0.57$, $P < 0.05$; MHC IIa: $r = 0.63$, $P < 0.01$, Figure 4.5A) and PFK (MHC I: $r = -0.63$, $P < 0.01$; MHC IIa: $r = 0.58$, $P < 0.05$) in homogenate samples. No relationship was observed between plasma lactate at 80% PTS and LDH ($r = -0.31$, ns, Figure 4.5B), or MHC IIa ($r = 0.23$, ns, Figure 4.5C) content of muscle samples. However, there was a significant relationship between the ratio of LDH activity and MHC IIa in homogenate and plasma lactate at 80% PTS ($r = -0.56$, $P < 0.05$, Figure 4.5D).

4.4 Discussion

The present study confirms the main finding of previous related studies, namely that there were lower plasma lactate concentrations during the sub-maximal exercise tests in black endurance athletes compared to white athletes, particularly at higher relative intensities. Furthermore, this is the first study comparing the phenotypes of athletes from two distinctly different groups that included comprehensive analysis of skeletal muscle. Specifically, the present study is the first to do complete fibre type analyses using two separate methods and to measure enzyme activities in homogenate samples and in pools of pure type I or pure type IIa fibres. A major finding was that Xhosa athletes had lower type I fibre proportions, something that had been suggested before but not conclusively proved. The most novel finding was that Xhosa athletes had higher LDH activities in muscle samples analysed as homogenates, but also in pools of both type I or pools of type IIa fibres, indicating that the higher activity in the homogenate sample was not simply a result of higher proportions of fast twitch fibres. Finally, unlike other studies, running economy did not differ and neither did oxidative enzyme activities, findings that possibly confirm the close matching of the athletes.

Athletes' physical and physiological characteristics

Past research on black and white athletes was not able to match the two groups of athletes for body size. Studies on South African black runners (Bosch *et al.*, 1990; Coetzer *et al.*, 1993; Marino *et al.*, 2004; Weston *et al.*, 1999) all reported that black athletes are shorter and lighter than their Caucasian counterparts. Body size does play a significant role in lung volume and has been shown to differ extensively between population groups (Yap *et al.*, 2001). Correcting for weight should balance out the size effect, but according to Svedenhag (1995) it is more correct to express oxygen uptake as $\text{mL}/\text{min}/\text{kg}^{0.75}$ than $\text{mL}/\text{min}/\text{kg}$. Both calculation methods were applied, but neither resulted in a difference in maximal oxygen consumption between Xhosa and Caucasian athletes.

This is in accordance with previous studies on South African black and white athletes, as well as Caucasians and Kenyan runners (Coetzer *et al.*, 1993; Saltin *et al.*, 1995a; Weston *et al.*, 1999). However, $VO_2\text{max}$ is only one of many factors contributing to elite endurance performance capacity (Myburgh, 2003).

Muscle fibre type, CSA, FD and MHC

One factor proposed by Costill (1967) that may contribute to elite endurance performance on the road, is a high percentage of type I fibres. However, Xhosa athletes had less type I fibres and more type IIA fibres (Figure 4.2A) than their Caucasian counterparts. This was confirmed by MHC isoform content analysis (which also takes into account any MHC I or IIA co-expression in hybrid fibres) which showed that they had lower MHC I and higher MHC IIA expression (Figure 4.2B). In the studies by Coetzer *et al.* (1993) and Weston *et al.* (1999), black runners also showed tendencies to have less type I fibres, but those findings were not statistically significant. This might have been because of low subject numbers or less stringent matching. However, even in the present study with higher subject numbers and more accurate matching, two possible explanations arise for the low type I fibre proportions in Xhosa athletes. First, Xhosa athletes may have trained longer at a higher intensity than their Caucasian counterparts as was suggested by Coetzer *et al.* (1993) who reported that black South African runners trained for a longer period of time per week above 80% of their $VO_2\text{max}$. Higher training intensity (as well as high frequency electrical stimulation in models) may convert type I fibres to faster fibre types (Andersen *et al.*, 1994; Pette and Vrbova, 1992). Alternatively, it may be that Xhosa athletes genetically have more type II fibres. Ama *et al.* (1986) reported that black sedentary people from Central and West Africa had less type I fibres (33 vs. 41%) than sedentary Caucasians and speculated that this finding may explain why the sprinting events are dominated by African-American black people originating from that part of Africa. In contrast, Klitgaard *et al.* (1990) showed that Scandinavian elite cross-country skiers had less type I fibres than recreationally active subjects, suggesting that the type of training may affect fibre type proportions. Unfortunately, the present study did not include any sedentary subjects, and should be considered for future investigation.

However, fibre type composition in elite Kenyan runners was not different from elite Scandinavian runners with both groups having a large proportion of type I fibres ($\pm 70\%$) (Saltin *et al.*, 1995a). Saltin *et al.* (1995a) did not report any specialised racing distances for either the Kenyan or Scandinavian runners and this therefore may have played an important role in the observed fibre type distribution. While the Kenyans had a small range in type I fibre proportions (62 – 76%), the Scandinavians had a very large variation (43 – 84%). The Xhosa and Caucasian athletes in the present study both had large variations in type I fibre distribution within the groups (Xhosa: 31 to 68

%; Caucasian: 48 to 81%), but this was still shown to be significantly different because the subjects were well matched for PRD_A . Similar ranges were observed for the MHC I isoform distribution within homogenate muscle samples of the Xhosa and Caucasian groups (Xhosa: 34 to 66 %; Caucasian: 43 to 85%).

The present study had the advantage that it also reports more fibre type subdivisions than the previous studies performed on South African athletes. Interestingly, both the current data and the Kenyan-Scandinavian study by Saltin *et al.* (1995a) indicate the presence of some pure type IIX fibres within the fibre type distribution in the muscles of both groups. This finding is surprising as it is commonly accepted that endurance athletes convert their type IIX fibres fairly easy into type IIA (Andersen and Henriksson, 1977; Baumann *et al.*, 1987).

The fibre type proportions of the Xhosa and Caucasian athletes seem to differ from other studies, specifically investigating endurance runners. Harber *et al.* (2002) analysed the muscle fibre type in middle distance (800 m – 1 500 m) and distance runners (3 000 m to 10 000 m). On comparing the findings of Harber *et al.* (2002) to those of the Kenyan athletes by Saltin *et al.* (1995a), the Kenyan athletes had similar fibre type distribution to the longer distance runners of Harber *et al.* (2002) (mean type I fibre proportion \pm 72%). However, the type I fibres of Xhosa athletes in the present study were more related to that of the middle distance runners of Harber *et al.* (2002) with the Caucasian athletes ranging between middle and distance runners, despite the PRD_A of both groups averaging 11.6 ± 5.2 km. However, the Xhosa and Caucasian athletes were matched for PRD_A and it may be that the difference in fibre type distribution between these athletes might be partly related to training intensity, which was not assessed directly.

Another factor playing an important role in power generation is the CSA of muscle fibres (Gollnick *et al.*, 1972; Trappe *et al.*, 2003). No differences in CSA and FD were observed between Xhosa and Caucasian athletes, which is similar to the findings of Saltin *et al.* (1995a). Neither were the CSA of the fibres associated with PRD_A ($r = 0.16$ and $r = 0.23$ for type I and II, respectively).

Lactate and the contributing enzymes

Two of the main findings in the present study both involved observed differences in variables related to lactate metabolism. During the sub-maximal test, Xhosa athletes had lower plasma lactate concentrations at 80% PTS. Weston *et al.* (1999) and Coetzer *et al.* (1993) both reported lower plasma lactate concentrations during sub-maximal exercise tests (~88% of PTS) in black runners compared to their white counterparts. Bosch *et al.* (1990) also showed lower plasma lactate levels for black athletes during a simulated marathon on the treadmill, but questioned the physiological importance of that finding due to the relatively low values. Saltin *et al.* (1995b) showed similar

observations in Kenyan runners during sub-maximal exercise tests, but proposed that this finding may be more related to the effect of altitude, despite the higher fat oxidation capacities in the *Gastrocnemius* muscle of Kenyans that could have explained this. Weston *et al.* (1999) explained the lower plasma lactate in their study by correlations with the higher CS activity found in the muscle samples of the black runners compared to white runners. No relationship was found for the aforementioned parameters in the present study.

Enzyme analysis of the Xhosa and Caucasian muscle samples in homogenates revealed higher mean LDH activity in Xhosa athletes with no difference in the activities of CS or 3HAD (Figure 4.3). PFK activity only tended to be higher in the Xhosa athletes compared to their Caucasian counterparts. Furthermore, LDH activity was significantly higher in type I and IIa fibre pools of Xhosa athletes compared to the Caucasian athletes, indicating that this was not a fibre type related phenomenon (Figure 4.4). The Xhosa athletes did have more type IIa fibres (which in general have higher LDH and PFK activities than type I fibres), and this may therefore partly explain the higher LDH and PFK activities observed in homogenates (Essen-Gustavsson and Henriksson, 1984). However, as mentioned before, the LDH activity in both the pools was higher in Xhosa athletes, thus showing that the higher LDH activity in the homogenate samples is not only due to the fibre type. Regression analysis revealed that the proportion of MHC IIa could explain only approximately 36% of the variation in LDH activity (Figure 4.5A). Because only total LDH activity was measured, it can only be speculated that the Xhosa athletes may have different ratios of LDH isozymes, which may influence total LDH activity. An increase in isozymes 1 and 2 favours lactate oxidation (van Hall, 2000) and these were also the isozymes shown to be higher in Kenyan athletes (Saltin *et al.*, 1995a). Despite not having analysed the LDH isozyme ratios, plasma lactate at 80% PTS was inversely related to LDH activity corrected for fibre type (LDH/MHC IIa). Hence, these muscle characteristics influenced the whole body phenotype, although only by approximately 30% (Figure 4.5D).

Weston *et al.* (1999) found that the CS and 3HAD activities were higher in black runners compared to whites, and Saltin *et al.* (1995a) showed higher 3HAD activity in Kenyan runners. These enzymes may explain part of the remaining unexplained variability in lactate accumulation, as CS and 3HAD promote the oxidation of carbohydrates and FFA, respectively, and theoretically, lower lactate production (van Hall, 2000). However, the current data showed no consistent differences in CS and 3HAD activities between Xhosa and Caucasian runners in homogenates, and these enzyme activities did not correlate with plasma lactate accumulation at 80% PTS. The tendency for the PFK activity to be higher in Xhosa athletes suggests a greater carbohydrate flux capacity through the glycolytic pathway. This could support the notion that more plasma lactate should be produced at

higher exercise intensities in the Xhosa athletes, as the CS activities did not differ between the two groups. In contrast to this, plasma lactate concentrations at the same relative intensity (at 80% PTS) was lower in the Xhosa athletes compared to Caucasian. Therefore, the data strongly suggest that Xhosa athletes utilised their carbohydrate fuel in a better way at high intensity sub-maximal workloads. A possible mechanism is that lactate could be metabolised in another fashion (such as the proposed lactate shuttle system Brooks (2002)) or that a greater control over the glycolytic pathway may exist. One interesting finding was that neither the MHC IIa nor LDH activity showed a relationship with plasma lactate at 80% PTS (Figures 4.5B and C). However, once the LDH activity and the MHC IIa content were expressed as a ratio (LDH/MHC IIa), a significant relationship was observed ($P < 0.05$, Figure 4.5D), indicating that the higher the LDH activity, despite normalised for fibre type, the lower the accumulation of plasma lactate. This may indicate indirectly that the function of LDH might be more complex than merely a non-oxidative enzyme producing lactate in fast twitch fibres. It also implies that any additional LDH activity, over and above that explained by fibre type, may reduce the plasma lactate concentration (at least at 80% PTS) (Figure 4.1). Therefore, the only possible mechanism would be promoting lactate oxidation.

Despite the discussion above providing a plausible explanation for the observed lower lactate accumulation in African athletes of East and South African origin, it does not explain whether the higher LDH activity is a genetically determined trait or not. The cumulative data of the previous studies may support the notion that intensity of training is a factor influencing this and possibly other muscle characteristics. Unfortunately, no studies have compared the response of Xhosa and Caucasian sedentary subjects during sub-maximal exercise. Recently, Billat *et al.* (2003) compared male Kenyan runners' training and found that those performing training at higher speeds had a significantly higher VO_2 max and better 10 km performance time than athletes training at lower speeds. Similarly, Coetzer *et al.* (1993) reported that the training per week of black runners consisted of more high intensity training than their white counterparts (~40% vs. 20%). Although these two studies, particularly that of Billat *et al.* (2003), quantified training intensity, neither could relate it to muscle enzyme characteristics. To fully explain the origin of phenotypic differences between populations, it would be necessary in the future to assess training intensity in detail.

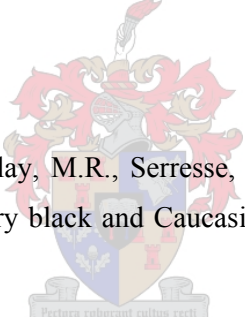
4.5 Conclusions

In conclusion, Xhosa athletes had higher type IIA- and lower type I fibre proportions and lower plasma lactate concentrations at a high sub-maximal exercise intensity than their Caucasian counterparts. Furthermore, Xhosa athletes had no difference in muscle oxidative capacity, but had higher LDH activities in homogenate samples and in type I and IIA fibre pools. These data support

the formulation of two hypotheses: 1) that Xhosa athletes may have a genotypic advantage influencing their phenotypic intramuscular profile and racing ability when it comes to the current speeds at which distance running events (such as 5 – 15 km races) are run or 2) that Xhosa athletes may train at a higher intensity than their Caucasian counterparts, explaining both the low lactate phenotype and the dominance of black runners in endurance running events over these distances. Whether fibre type and enzymatic differences in skeletal muscle are genotype- or training-dependent, remains to be proven. Furthermore, another question that arises, is how lower plasma lactate concentrations during exercise can be beneficial to enhance performance.

More studies need to be conducted to elucidate the effect of high intensity training on already well-trained athletes' muscle characteristics to determine definitively if the findings of this study are related to inherent fibre type differences or a difference in training response in black and white athletes. Although only LDH activity differed significantly in the present study, both PFK and LDH activities correlated with fibre type, but LDH activity was still higher in pools of type I or IIa fibres of Xhosa athletes, which suggest that either training intensity or genotype may have an influence on muscle characteristics.

4.6 References

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Table 4.1 Matching of Caucasian (C) and Xhosa (X) athletes according to usual training volume, recent 10 km personal best race time (PB), and average preferred racing distance (PRD_A).

Subjects	Training <i>km/wk</i>	10 km PB <i>min</i>	PRD _A <i>km</i>		Subjects	Training <i>km/wk</i>	10 km PB <i>min</i>	PRD _A <i>km</i>
C1 [§]	120	33.5	15.6	‡	X1 [§]	110	32.3	7.9
C2 [§]	110	30.1	15.5	‡	X2 [§]	115	31.6	16.6
C3	110	32.5	15.6		X3 [§]	100	34.1	11.0
C4	110	31.8	15.0		X4	120	30.4	21.1
C5 [§]	100	36.2	15.6	‡	X5	75	34.3	13.1
C6	100	32.1	7.5	‡	X6 [§]	120	31.5	13.1
C7	80	31.2	11.0		X7 [§]	65	30.8	15.6
C8 [§]	70	34.0	21.1	‡	X8 [§]	65	33.3	15.0
C9 [§]	60	33.2	10.0	‡	X9 [§]	60	33.5	7.50
C10	60	33.7	5.8	‡	X10	50	33.5	6.8
C11 [§]	60	36.5	5.8	‡	X11 [§]	60	36.4	5.4
C12	60	33.6	1.5		X12	60	33.0	3.0
C13	45	34.0	15.6	‡	X13 [§]	60	33.7	10.0
Mean ± SD	83 ± 26	33.3 ± 1.8	12.0 ± 5.5			82 ± 27	33.0 ± 1.6	11.2 ± 5.1
Significance						<i>ns</i>	<i>ns</i>	<i>ns</i>

Values are presented as mean ± SD. Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. Racing times in minutes and seconds were converted to minutes and decimals. ‡ indicates a pair with a complete data set of all subsequent physiological tests and muscle biopsy analyses (excluding pools of enzymes). § indicates a subject for whom pools of single fibres were analysed. ns, not significant; PB, personal best; PRD_A, average preferred racing distance

Table 4.2 Subject characteristics and maximal exercise test results of Caucasian and Xhosa athletes.

	Caucasian	Xhosa	Significance
Age (years)	21.9 ± 2.0	22.3 ± 2.9	<i>ns</i>
Weight (kg)	67.8 ± 6.6	59.6 ± 7.4	<i>P < 0.01</i>
Height (cm)	182 ± 8	172 ± 6	<i>P < 0.01</i>
BMI (kg/m ²)	20.5 ± 1.1	20.1 ± 1.5	<i>ns</i>
VO ₂ max (mL/min/kg)	68.2 ± 4.5	68.7 ± 5.9	<i>ns</i>
VO ₂ max (mL/min/kg ^{0.75})	144.3 ± 9.6	145.5 ± 12.5	<i>ns</i>
VO ₂ max (L/min)	4.6 ± 0.4	4.1 ± 0.3	<i>P < 0.01</i>
V _E max (L/min)	158 ± 14	145 ± 17	<i>P = 0.07</i>
V _E /VO ₂ at max	34.4 ± 2.2	35.7 ± 4.4	<i>ns</i>
RERmax	1.17 ± 0.06	1.17 ± 0.05	<i>ns</i>
HRmax (beats/min)	192 ± 8	191 ± 9	<i>ns</i>
PTS (km/h)	22.0 ± 0.9	21.6 ± 1.1	<i>ns</i>

Values are presented as mean ± SD (N = 13). Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. BMI, body mass index; HR, heart rate; PTS, peak treadmill speed; RER, respiratory exchange ratio; V_E, minute ventilation; VO₂, oxygen consumption

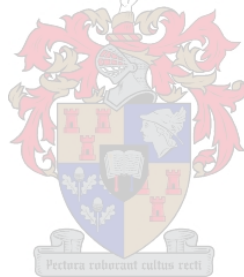


Table 4.3 A comparison of Caucasian and Xhosa athletes' physiological parameters, including running economy and metabolic profile relative to peak treadmill speed (PTS).

	Caucasian	Xhosa	Significance
<i>Economy at 16.1 km/h</i>			
VO ₂ (mL/min/kg)	55.5 ± 3.8	56.9 ± 3.1	ns
VO ₂ (mL/min/kg ^{0.75})	118 ± 8	120 ± 7	ns
<i>64% of PTS</i>			
Treadmill speed (km/h)	13.9 ± 0.6	14.0 ± 0.8	ns
VO ₂ (mL/min/kg)	49.8 ± 3.0	50.0 ± 2.9	ns
RER	0.91 ± 0.02	0.91 ± 0.04	ns
HR (beats/min)	156 ± 8	159 ± 11	ns
V _E (L/min)	83 ± 7	72 ± 9	<i>P</i> < 0.05
<i>72% of PTS</i>			
Treadmill speed (km/h)	15.7 ± 0.7	15.8 ± 0.9	ns
VO ₂ (mL/min/kg)	54.7 ± 3.5	55.8 ± 2.6	ns
RER	0.94 ± 0.02	0.96 ± 0.04	ns
HR (beats/min)	169 ± 8	170 ± 13	ns
V _E (L/min)	98 ± 8	86 ± 10	<i>P</i> < 0.05
<i>80% of PTS</i>			
Treadmill speed (km/h)	17.4 ± 0.8	17.5 ± 1.0	ns
VO ₂ (mL/min/kg)	58.6 ± 3.4	61.6 ± 3.1	ns
RER	0.99 ± 0.04	1.00 ± 0.05	ns
HR (beats/min)	180 ± 5	183 ± 13	ns
V _E (L/min)	116 ± 13	106 ± 14	ns

Values are means ± SD (N = 10). Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. HR, heart rate; RER, respiratory exchange ratio; V_E, minute ventilation; VO₂, oxygen consumption

Table 4.4 Cross-sectional area and fibre diameter of Caucasian and Xhosa muscle biopsy samples.

	Caucasian	Xhosa	Significance
<i>Cross-sectional area (µm²)</i>			
Type I	5316 ± 1610	5588 ± 1592	ns
Type II	6233 ± 2646	6722 ± 1420	ns
<i>Fibre diameter (µm)</i>			
Type I	80.2 ± 11.4	81.2 ± 13.1	ns
Type II	86.2 ± 18.0	91.1 ± 9.2	ns

Values are means ± SD (N =9). Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data.

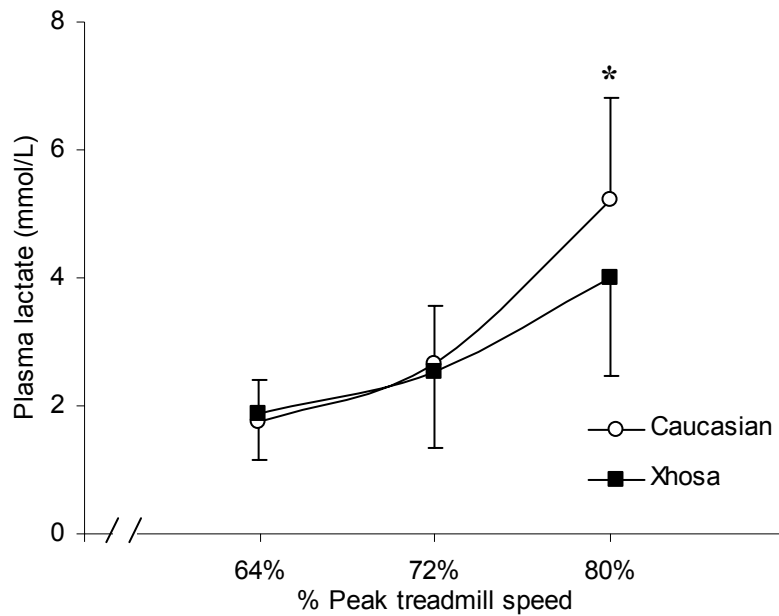


Figure 4.1 Plasma lactate concentration during the sub-maximal exercise test in Caucasian and Xhosa athletes. Values are means \pm SD (N = 12 pairs). Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. * different from Caucasian ($P < 0.05$).

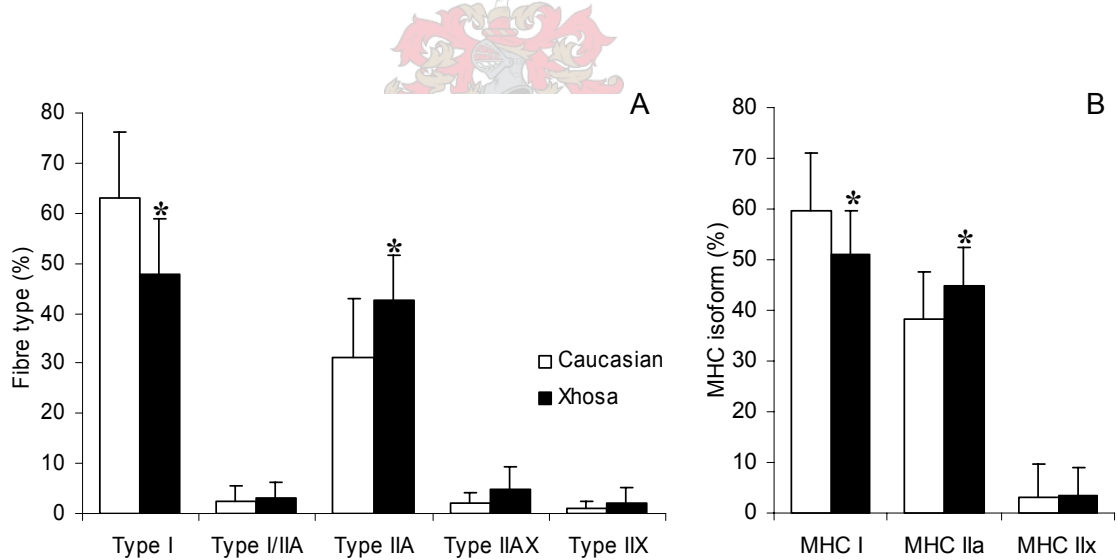


Figure 4.2 Fibre types (A, N = 9 pairs) and myosin heavy chain (MHC) isoform content (B, N = 13) in homogenate muscle samples of Caucasian and Xhosa athletes. Values are means \pm SD. Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. * different from Caucasian ($P < 0.05$).

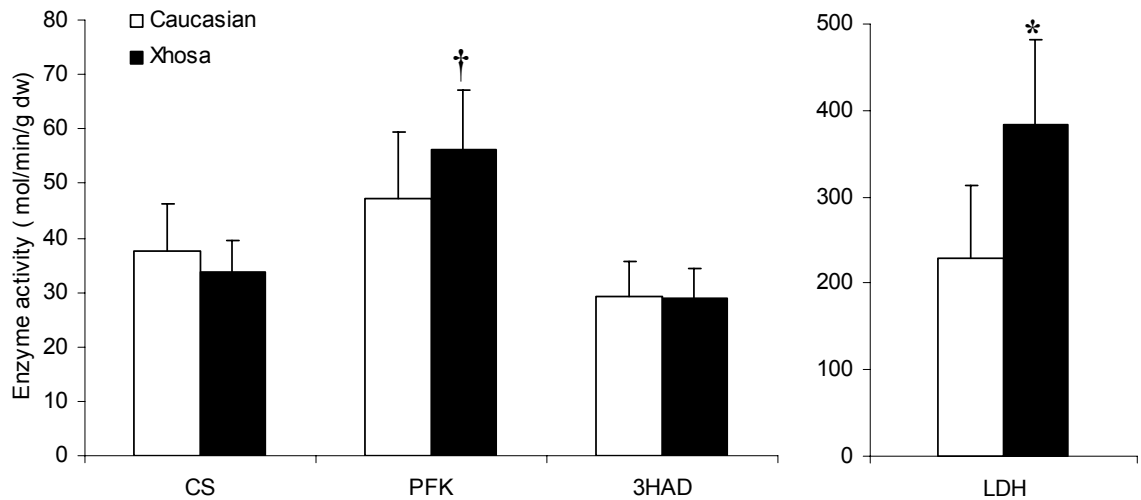


Figure 4.3 Enzyme activities in homogenate samples ($N = 9$ pairs) of Caucasian and Xhosa athletes. Values are means \pm SD. Statistical analyses were performed using the Wilcoxon signed rank test for non-parametric paired data. * different from Caucasian ($P < 0.01$); † different from Caucasian ($P = 0.07$). CS, citrate synthase; PFK, phosphofruktokinase; 3HAD, 3-hydroxyacyl-Co A dehydrogenase; LDH, lactate dehydrogenase.

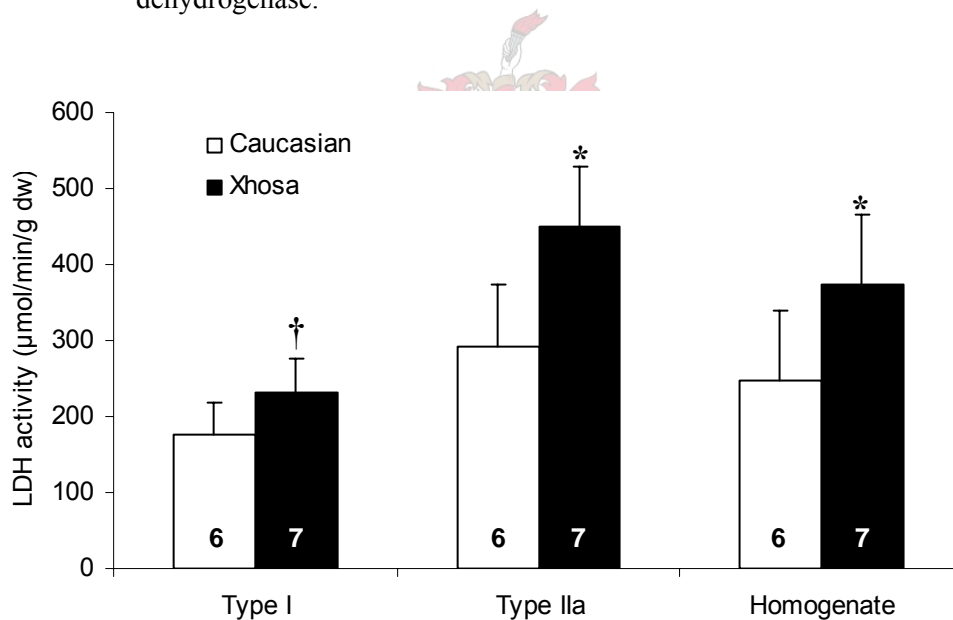


Figure 4.4 LDH activities in pools of single fibres and homogenates of Caucasian and Xhosa athletes who could not be paired for this analysis. Values are means \pm SD. Mann-Whitney U test for non-parametric unpaired data were used for statistical comparison between Xhosa and Caucasian groups. * different from Caucasian ($P < 0.05$); † different from Caucasian ($P = 0.05$). Sample size is indicated within each bar. LDH, lactate dehydrogenase.

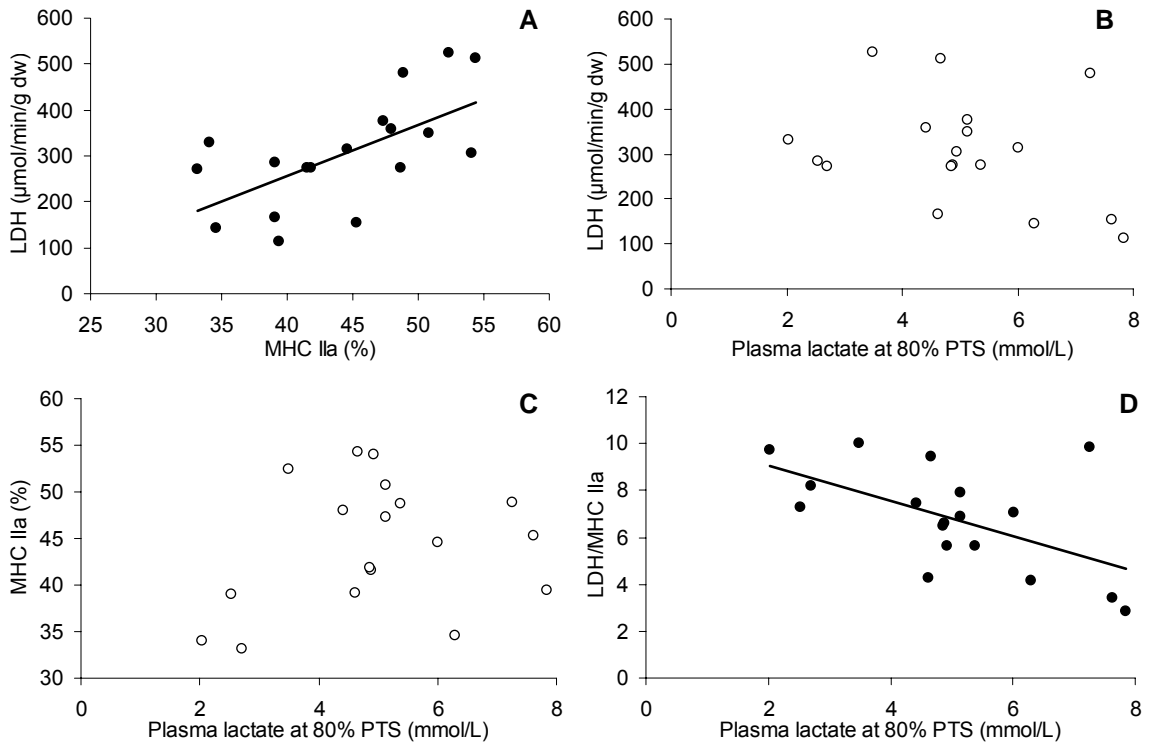
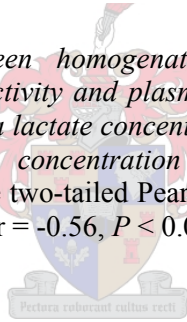


Figure 4.5 Relationships between homogenate LDH activity and MHC IIa (panel A), homogenate LDH activity and plasma lactate concentration at 80% PTS (panel B), MHC IIa and plasma lactate concentration at 80% PTS (panel C) and LDH/MHC IIa and plasma lactate concentration at 80% PTS (panel D). Relationships were determined using the two-tailed Pearson's correlation test (see text). Pearson's r : A: $r = 0.63$, $P < 0.01$; D: $r = -0.56$, $P < 0.05$.



High intensity training intervention in endurance trained athletes

5.1 Introduction

High intensity training has been used in conjunction with endurance training for almost a hundred years (Billat, 2001a). The world's best endurance runners and cyclists all have some sort of high intensity session incorporated in their weekly training schedule. However, there is little consistency in the structure of these sessions. Coaches around the world tend to develop their own programmes from experience rather than using scientific backing (Martin and Coe, 1997). High intensity sessions could be continuous e.g. short time trials, or could consist of high intensity interval training (HIIT), but the latter is more popular among top athletes.

What is HIIT?

According to Saltin *et al.* (1976), a HIIT session consists of five basic characteristics, namely the intensity of the intervals (e.g. speed), the time-ratio for high and low intensities (e.g. 3:2 HIIT: rest or low intensity recovery), the duration (or distance) at high and low intensities, the difference between these intensities (amplitude), and finally, the number of repetitions. Scientists have investigated the effects of various HIIT protocols on performance, physiological and biochemical markers (for a recent review, see (Kubukeli *et al.*, 2002)). The HIIT protocols for research studies are set up according to the aforementioned characteristics, but still vary from moderate to high intensity (70 to 100% of maximal oxygen consumption (VO_{2max})) to supra-maximal intensity (> 100% VO_{2max}) (Billat, 2001a). Part of the reason for the variation in protocols is because of the various types of sporting events that may have different demands, but also because the scientists may have proposed that a particular intensity could affect a particular physiological or biochemical parameter of interest (i.e. VO_{2max} or fibre type, respectively) (Acevedo and Goldfarb, 1989; Smith *et al.*, 2003; Stepto *et al.*, 2002; Weston *et al.*, 1997).

Typical training protocols of endurance athletes

Interval training has been studied in endurance runners and cyclists (Billat, 2001a; Neary *et al.*, 1995; Smith *et al.*, 1999; Weston *et al.*, 1997). It has been suggested that HIIT protocols should be developed around the initial velocity associated with VO_{2max} (V_{max}) in order to improve the resistance to fatigue of already well-trained athletes performing at their VO_{2max} (Billat, 2001a; Hill and Rowell, 1997). The maximum time (T_{max}) that can be sustained at V_{max} before the intervention begins was also suggested to be an important parameter in setting up the interval duration in an individualised way (see below for test). Smith *et al.* (1999) investigated the effect of

the duration of intervals at V_{max} on performance of five middle distance runners who performed individualised HIIT on a treadmill for four weeks. Athletes were required to complete six intervals per day, twice a week with the interval times varying on a daily basis. The time of each running interval was set between 60 and 75% of T_{max} , with the rest interval at half the running interval time. After the training, all athletes significantly improved their 3000 m time (from 616.6 to 599.6 seconds, $P < 0.05$). Furthermore, there was also an improvement in VO_{2max} , V_{max} and T_{max} . It was further concluded, in another generalised, statement that setting the interval time between 60 and 75% of T_{max} at an intensity of V_{max} was sufficient to elicit improvements in performance. Later, Smith *et al.* (2003) improved on their previous study by comparing two specific interval durations rather than using a range. Well-trained runners performed HIIT on the treadmill for four weeks. One group was required to run 6 intervals lasting 60% of T_{max} , and a second group five intervals lasting 70% of T_{max} . The rest:work ratio was set at 1:2. Only the group training at 60% T_{max} improved overall 3000 m time after the four weeks with no change in VO_{2max} or V_{max} in either of the groups. It was further suggested by the authors, that the 70% T_{max} interval duration was too long for their athletes to maintain, since athletes were prone not to finish the training sessions. It was therefore suggested that 60% T_{max} was the maximum required interval time for a HIIT programme associated with V_{max} . In the present study, this training protocol was applied, but V_{max} was set at 94% of maximal treadmill speed achieved during the VO_{2max} test.

Physiological and biochemical differences between trained and untrained individuals

It is well known that there are distinct differences between trained and untrained individuals, in performance and at the physiological and muscular levels (Gollnick *et al.*, 1972; Holloszy and Coyle, 1984; Jansson and Kaijser, 1977; Jurimae *et al.*, 1997; Klitgaard *et al.*, 1990; Parcell *et al.*, 2003; Prince *et al.*, 1976; Saltin *et al.*, 1977; Simoneau, 1995; Tesch and Karlsson, 1985). Not only do untrained individuals have a lower VO_{2max} (Saltin *et al.*, 1977), worse economy during sub-maximal exercise (Maughan *et al.*, 1986) and highly fatigable (MacRae *et al.*, 1992), they also may have less type I fibres with a concomitantly higher proportion of type IIA and IIX fibres (Saltin *et al.*, 1977), with low oxidative potential in their muscles compared to trained individuals (Bylund *et al.*, 1977).

Training effects on untrained individuals

The effects of endurance, sprint, resistance and HIIT training on the physiological and muscular adaptations in untrained humans and animals have been well documented. Both cycling or running endurance training have been shown to result in an increase in oxidative potential of muscle (e.g. citrate synthase (CS) activity, malic enzyme activity, succinate dehydrogenase (SDH) activity and cytochrome c concentrations) (Dudley *et al.*, 1982; Gollnick *et al.*, 1973; LeBlanc *et al.*, 2004; Mole

et al., 1973; Siu *et al.*, 2003; Stone *et al.*, 1996) with a concomitant increase in VO_2max (Bylund *et al.*, 1977; Gollnick *et al.*, 1973; MacRae *et al.*, 1992), a decrease in lactate production at the same absolute intensity of exercise (Favier *et al.*, 1986; Hurley *et al.*, 1984; MacRae *et al.*, 1992; Spina *et al.*, 1996) and improved economy (Franch *et al.*, 1998). Hexokinase, an enzyme responsible for the phosphorylation of glucose before it can be metabolised by the glycolytic pathway, has been shown to increase in activity after endurance training in both humans and rats, therefore increasing the ability to metabolise carbohydrates from the circulating pool (Baldwin *et al.*, 1973; Bylund *et al.*, 1977; Spina *et al.*, 1996). However, controversy still remains whether glycolytic enzyme activities in muscle change due to endurance training (such as phosphofructokinase (PFK) or lactate dehydrogenase (LDH) activity) (Baldwin *et al.*, 1973; Bylund *et al.*, 1977; Gollnick *et al.*, 1973; Mole *et al.*, 1973). For example, Sjodin *et al.* (1982) showed that, in already well-trained runners, 14 weeks of endurance training at an intensity related to the onset of blood lactate accumulation resulted in a significant decrease in PFK activity, with no change in LDH activity. Most of the literature shows no change in glycolytic capacity (PFK and LDH activities) as a result of endurance training (Bylund *et al.*, 1977; Green *et al.*, 1999; Green *et al.*, 1991; Tremblay *et al.*, 1994).

On the other hand, short sprint exercise or resistance training may have different effects on muscle enzyme activities because of a greater need to produce energy very quickly. MacDougall *et al.* (1998) concluded that both glycolytic and oxidative enzyme capacities may increase or remain the same with sprint training. However, Dawson *et al.* (1998) reported a decrease in CS activity with no change in PFK activity after six weeks of ten second sprints performed in sets of four to eight, three times per week. Although these findings remain unexplained, a weakness of training studies is sometimes that the subjects' pre-study training status is not well described and poorly controlled, which may result in differences between studies in muscle adaptation.

Both sprint and endurance training have significant effects on the muscle fibre type composition in untrained individuals, especially on the proportions of fast oxidative type IIA vs. very fast glycolytic type IIX fibres. It has been shown that endurance training results in a significant decrease in type IIX fibres, with a concomitant increase in type IIA fibres (Andersen and Henriksson, 1977b). However, some studies that investigated the effect of endurance training have reported a significant decrease in type IIX fibres, with a statistically insignificant change in either type I or type IIA fibre proportions (Baumann *et al.*, 1987; O'Neill *et al.*, 1999; Putman *et al.*, 2004). This could be explained if subjects responded differently, with some increasing in type IIA and others in type I fibres. Studies on chronic low frequency electrical stimulation of rat muscle (stimulation duration > 3 months) have shown that it is possible to increase type I fibre proportions (Pette and Vrbova, 1992; Windisch *et al.*, 1998). It may therefore be that the training duration (exposure per week and

training weeks) in most human studies was too short for significant conversion of type IIA to type I fibres. Sprint training may cause similar changes in fibre type as the aforementioned endurance training studies because of the oxidative requirements during the recovery phases between sprints. Studies showed that progressive resistance training or sprint cycling decreased type IIX fibres, with a concomitant increase in type IIA fibres (Allemeier *et al.*, 1994; Andersen and Aagaard, 2000; Williamson *et al.*, 2001). Other studies even showed a significant increase in type I fibres with resistance training (Trappe *et al.*, 2000; Williamson *et al.*, 2000). However, change in training may result in no change in all the fibre types (Harridge *et al.*, 1998; Trappe *et al.*, 2001; Trappe *et al.*, 2004). Once again, subject selection and pre-study training regime may be the cause of these differing results. Furthermore, an increase in cross-sectional area and capillary supply have also been associated with greater power output and oxidative capacity of muscle, respectively, as a result of training (Dawson *et al.*, 1998; Gollnick *et al.*, 1973; Green *et al.*, 1991; Jensen *et al.*, 2004; Putman *et al.*, 2004; Trappe *et al.*, 2001).

Effect of HIIT in already well-trained athletes

Despite the well-established adaptations in performance, physiological and biochemical markers of previously untrained individuals exposed to systematic training, the literature is still unclear what the effects of HIIT may be in already well-trained endurance runners, both physiological and biochemical (the latter including fibre type and metabolism). More studies exist on physiological adaptations in runners, than muscle adaptations. Hence, the former will be discussed only from results of studies in runners whereas the latter will be discussed from results of studies in other well-trained athletes.

In well-trained runners, HIIT resulted in improved economy and reduced plasma lactate concentrations at the same absolute sub-maximal intensity (Acevedo and Goldfarb, 1989). Acevedo and Goldfarb (1989) only performed whole body physiological assessment, and no muscle biopsies were obtained. Only a few studies have investigated the effect of HIIT on muscle adaptation of already well-trained athletes, but with large variation in results. Andersen *et al.* (1994) showed that in elite sprinters, three months of heavy resistance training in conjunction with normal sprint training improved power output, and significantly decreased both type I and type IIX fibre proportions, with a concomitant increase in type IIA fibres. Evertsen *et al.* (1999), on the other hand, showed no change in fibre type proportions in well-trained cross-country skiers after five months of HIIT, but showed that SDH activity and performance were increased and PFK activity was reduced after the intervention. In contrast, four weeks of HIIT in well-trained cyclists resulted in an increase in performance, but no change in enzyme activities (Weston *et al.*, 1997). An interesting finding by Sjodin *et al.* (1982) was that 14 weeks of an additional 20 minute HIIT

training per week at an intensity associated with the onset of blood lactate accumulation in well-trained distance runners, decreased PFK activity, but increased the ratio of the heart specific LDH isoform relative to skeletal muscle LDH isoform. This change in LDH isoform expression promotes lactate to pyruvate conversion (van Hall, 2000), which may be indirect support for the lactate shuttle (Brooks, 2000). In a recent review by Kubukeli *et al.* (2002), the authors suggested that more training intervention studies need to be performed on already well-trained athletes to understand the biochemical adaptations in muscle and changes in whole-body physiology. Furthermore, no study has thoroughly investigated the effect of HIIT intervention on both muscle fibre type and enzyme activities in already well-trained endurance runners.

Therefore, the aim of the present study was to thoroughly investigate physiological markers of performance and muscle characteristics, which included enzyme activities in homogenates and pools of single fibres before and after a six week HIIT training intervention in well-trained endurance runners. The HIIT intervention was based on the training programmes described by Billat (2001a) and Smith *et al.* (2003) (see methods section for a discussion of the HIIT protocol). Furthermore, this study aimed to shed light on the controversies in the literature regarding adaptations in physiology and muscle in already well-trained athletes by including a larger group of athletes than typically used in previous studies.

5.2 Methodology

5.2.1 *Subject recruitment and training volume assessment*

The study was approved by the Ethics committee of Sub-Committee C of Research Administration of the University of Stellenbosch. Fifteen endurance runners were recruited from local athletic clubs. Athletes were informed about the possible risks of the study. Each athlete signed a written informed consent. Athletes were excluded if they were not actively competing in races, had a 10 km road race personal best time (PB) of more than 39 minutes, and any illness or injury for the past six months.

Each athlete completed a detailed questionnaire including various subject characteristics and reported favourite race distance for the following three running disciplines: road, track and cross-country. A recent 10 km PB was reported for the previous three months before testing commenced. An average preferred race specialisation distance (PRD_A) was calculated for each athlete by taking the average of the three favourite distances, one for each of the three disciplines mentioned above. In some cases, athletes participated in only two of these disciplines, but no athletes competed in only one.

Athletes recorded race distances and times, the type of training, total distance run and the duration of training sessions in training diaries provided. The recording period started two weeks before the training intervention (before HIIT) and included the laboratory exercise tests. Training was also recorded during the HIIT programme and after HIIT until the end of the testing period, and included laboratory training and testing. The average training for the two training periods was calculated and expressed as distance per week.

All athletes took part in a 10 km field test prior to the commencement of the HIIT programme. All athletes performed the field test on the same day to assess their current 10 km performance status under the same racing conditions. The simulated race was run in the evening and athletes were verbally encouraged to perform their best. Water was supplied during the race. Due to a staggered entry into the actual laboratory training phase (with up to 4 weeks between subjects, no 10 km field test was held after the HIIT intervention. Within 4 weeks, the first subjects may have started to detrain and the last subjects would have had a different level of tapering compared to the subjects who started in the middle.

5.2.2 *Laboratory exercise testing*

All athletes were familiarised with treadmill running before any testing was performed. Exercise tests and muscle biopsies were performed on separate days, allowing recovery from previous testing for at least two days. Athletes were encouraged to be well rested and to abstain from races and only perform short duration low intensity training prior to the day of testing.

VO₂max testing and peak treadmill speed

A maximal exercise test was performed before and after the HIIT programme. Athletes performed an incremental exercise test to fatigue on a treadmill (RunRace, TechnoGym, Italy), with continuous measurement of heart rate (Polar, Finland), oxygen consumption (VO₂), respiratory exchange ratio (RER) and minute ventilation (V_E) (Jaeger Oxycon Pro, Germany) throughout the test. Athletes were allowed a five minute warm-up on the treadmill before testing started. All athletes started the test at 14 km/h (flat gradient) for 30 seconds, whereafter the intensity was increased by 0.5 km/h every 30 seconds until fatigue set in. Athletes were said to have attained their maximal ability when two of the following criteria were fulfilled: (a) heart rate within 5 beats/min of theoretical age-predicted maximal heart rate (220 – age) (b) RER value greater than 1.10 and (c) a plateau in VO₂ (Staab *et al.*, 2003). Whenever two of these criteria were not fulfilled, athletes had to perform the same test on the next visit and were encouraged verbally to perform better. (Refer to appendix A for details.) Peak treadmill speed in km/h (PTS) was calculated as follow taking every second into account:

$$\text{PTS} = \text{Completed full workload (km/h)} + \frac{(\text{seconds at final workload})}{30 \text{ seconds}} \times 0.5 \text{ km/h}$$

Sub-maximal exercise test and blood sampling

Prior to the sub-maximal test, athletes were fitted with an intravenous catheter (Jelco 22G, Johnson & Johnson) and a three-way stopcock that were flushed with saline containing 0.04% heparin (Heparin Novo, Novo Nordisk, South Africa). Athletes were then allowed five minutes of treadmill running warm-up and brief stretching.

The sub-maximal workloads corresponded to 64, 72 and 80% of each individual's baseline PTS and were termed workload 1, workload 2 and workload 3, respectively. An absolute workload 4 was added to the test, where all athletes ran at 20 km/h. Athletes ran for five minutes at each workload and breath-by-breath measurements were recorded. After each workload, the athlete used the railings of the treadmill to lift himself off the treadmill and placed his feet on opposite sides of the belt. Three millilitres of blood were collected in a sealed test tube containing fluoride oxalate (Vacutainer, BD, UK), mixed and stored on ice. After a period of 1 minute of rest, the intensity was increased to the next workload and the athlete commenced running. Directly after the final workload was completed and the blood sample taken, additional blood samples were collected at 3, 6, 12, 15 and 18 minute time-points, post running. Blood was centrifuged directly after the test at 3 000 rpm and the plasma stored at -87 °C until analysis. The same absolute workloads were used for the sub-maximal test after the HIIT programme. (Refer to appendix A for details.)

Muscle biopsies

A needle biopsy was obtained from the *Vastus lateralis* muscle in a rested condition using the suction-assisted technique described by Evans *et al.* (Evans *et al.*, 1982) at baseline and after the HIIT programme. The biopsy site was at the same depth (2 cm) and in a similar position for all athletes, corresponding to one third along the total length of the upper leg, distal to the hip joint. The biopsy was split into three parts, two frozen in liquid nitrogen for homogenate and single fibre analyses, and the third was mounted in embedding medium (Jung Tissue Freezing Medium, Leica Instruments, Germany) and rapidly frozen in iso-pentane, pre-cooled with liquid nitrogen. All biopsy samples were stored at -87 °C.

5.2.3 High intensity training intervention

The HIIT programme was based on those described by Billat (2001a; 2001b) and Smith *et al.* (1999; 2003), with some modifications. Interval training speed (ITS) was calculated as 94% of the PTS from the maximal exercise test of each athlete, instead of the initial speed associated with the onset of maximal VO₂, as PTS has been shown to correlate better with 10 km performance than VO₂max

(Noakes *et al.*, 1990). To determine the duration of intervals, athletes visited the laboratory on a separate day from testing and warmed up at a low intensity on the treadmill for five minutes. After adequate rest was allowed, athletes were instructed to run as long as possible at their calculated ITS. Athletes were verbally encouraged to run as long as possible and the time was recorded (T_{\max}). T_{\max} at 94% PTS was 290 ± 58 seconds, with a mean speed at 94% PTS of 20.1 ± 0.7 km/h.

Training interval duration was calculated as follows: $60\% \times T_{\max}$. The minimum interval duration was 114 seconds and the maximum was 213 seconds. The duration of the recovery interval was set at half of the calculated high intensity interval time. Therefore, the HIIT programme was defined as:

6 x intervals at ITS for $60\% T_{\max}$ with $\frac{1}{2}$ ($60\% T_{\max}$) as recovery

Athletes visited the laboratory twice a week (minimum one day rest in between sessions) for six weeks and performed the HIIT programme on a treadmill. No adjustments were made to the ITS or the recovery duration. The projected number of intervals to be completed per athlete was 6 intervals per session x 2 sessions per week x 6 weeks = 72 intervals.

5.2.3 *Biochemical analyses*

Plasma lactate concentration

Plasma lactate concentrations (mmol/L) were determined using a commercially available kit (Lactate PAP, bioMérieux sa, France) and a spectrophotometer (Biotek Instruments, USA) set at 505 nm. The kit relies on the principle of an enzymatic conversion of plasma lactate, resulting in a detectable colour that is lactate concentration dependent. Values are expressed as mean \pm SD.

Muscle morphology

Fibre typing of muscle samples was based on the method by Brooke & Kaiser (Brooke and Kaiser, 1970). Three serial cross-sections (10 μm) were cut onto glass slides and placed into pre-incubation medium set at exactly pH 4.30, 4.60 and 10.30, whereafter the samples were visualised and photographed (Nikon CoolPix Microscope system, Japan). Fibres were identified as either types I, IC, IIC, IIAC, IIA, IIAX or IIX according to the staining intensities described by Staron (1997), and expressed as a percentage of the total number of fibres counted. In this study, fibre types IC, IIC and IIAC proportions were pooled and termed type I/IIA because of low counts in each of the aforementioned subgroups. An average of 271 ± 113 fibres were analysed per sample. Values are expressed as mean percentage \pm SD.

Cross-sectional area (CSA, μm^2) and fibre diameter (FD, μm) were determined using a computer software programme (SimplePCI ver 1.0, Nikon, Japan) on the same slides photographed for fibre

typing. Fibres were divided into two groups, namely type I (pure type I fibres only) and II, the latter comprising of fibre types I/IIA, IIA, IIAx and IIX. An average of 87 ± 40 type I and 68 ± 27 type II fibres were analysed per sample.

Capillary count

A capillary count for each biopsy was determined on histological slides using the amylose-PAS stain of Andersen and Henriksson (1977a). Slides were photographed and capillaries surrounding an average of 82 ± 32 fibres were counted. Values are expressed as the average number of capillaries around a fibre without taking into account fibre type or size.

Enzyme activities in homogenate muscle samples

Muscle biopsy samples previously frozen in liquid nitrogen, were freeze-dried overnight. A small piece was weighed, crushed in a test tube and 400 μ L chilled 100 mM potassium phosphate buffer (pH 7.30) was added per 1 mg sample. Samples were kept on ice and sonicated (Virtis Sonicators, USA) three times for ten seconds on ice, with a ten second delay between intervals.

LDH and CS activities were determined in homogenate using the fluorometric methods described by Essen-Gustavsson and Henriksson (1984), with slight modifications. These modifications were mainly a reduction in reagent and sample volumes to accommodate the microplate reader (Biotek Instruments, USA). The enzyme reagent was always 250 μ L and the sample volume 5 μ L and 3 μ L for the CS and LDH assay, respectively. Enzyme assay reactions were performed for five minutes at room temperature with readings taken at 30 second intervals (refer to Appendix B for protocol and calculation details). Enzyme activities are expressed as μ mol/min/g dry weight (dw).

Single fibre identification and enzyme activities

Single muscle fibres were dissected from freeze-dried samples in a humidity controlled room (40% humidity, 20 °C). A total of 3009 (mean of 150 ± 26 fibres per sample) fibres were dissected. A small piece of each fibre was cut off, transferred to a capillary tube containing sodium dodecyl sulfate (SDS) denaturing buffer and left overnight to dissolve. The remaining piece was sealed and stored in a labelled glass capillary tube at -87 °C. Identification of the fibre types was carried out electrophoretically. Myosin heavy chain (MHC) isoform content of each fibre was determined using SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Talmadge and Roy (1993) with β -mercaptoethanol added to the upper running buffer to a concentration of 0.03 M prior to electrophoresis (Blough *et al.*, 1996). Electrophoresis was carried out using a mini-gel system (Bio-Rad, USA) for 16 hours at constant 70 volts at 4 °C. Gels were silver stained (Amersham, Sweden) and fibres identified as expressing either pure MHC I, IIA or IIX, or hybrid fibres expressing both MHC I and MHC IIA (I/IIA), or MHC IIA and MHC IIX (IIA/IIX).

LDH activities in pools of type I and pools of type IIa fibres were determined for each subject. The pooled fibres were weighed on a microbalance (Cahn 25 electro-balance), calibrated with known weights. Pool weights ranged between ~40 and ~100 µg. After weighing, the samples were transferred to a micro tube, and 400 µL chilled 100 mM potassium phosphate buffer (pH 7.30) was added per 1 mg sample. Sonication was only carried out once for 10 seconds to prevent enzyme activity loss. Enzyme activity determinations were carried out in the same way as for the homogenate samples. (Refer to Appendix B for detail on the single fibre dissection, determination of fibre type and enzyme activities.) Enzyme activities are expressed as µmol/min/g dw.

5.2.5 Statistical analyses

Statistical comparisons between data from before and after the HIIT programme were performed using the Wilcoxon signed rank test for non-parametric paired data. A one-way ANOVA with a Bonferroni correction was performed on the plasma lactate results obtained during the recovery phase after the sub-maximal exercise tests. Significance for all analyses was set at $P < 0.05$. Correlation coefficients were calculated using the two-tailed Pearson's correlation test.

5.3 Results

The number of intervals completed varied during the initial weeks of the HIIT intervention, as athletes found it difficult to complete all six intervals. However, as the weeks progressed, the athletes were able to complete all intervals. The athletes completed 55 ± 15 intervals (average interval time lasting 2.7 ± 0.8 minutes) at an average ITS of 20.1 ± 0.7 km/h. Both total running time and total distance for the HIIT intervention were calculated and means were 160 ± 46 minutes and 54 ± 17 km, respectively.

Body mass and BMI did not change after the HIIT programme (Table 5.1). Training distance per week was also unchanged between the two testing periods. HIIT replaced certain daily training sessions of the total training performed per week, but amounted to only ~17 % of the total training.

PTS increased significantly ($P < 0.01$), and $VO_2\text{max}$ expressed relative to body mass, only showed a trend to have increased ($P = 0.07$) after the HIIT intervention (Table 5.2). No changes in $RER\text{max}$, $HR\text{max}$, $VO_2\text{max}$ (expressed as mL/min) or $V_E\text{max}$ were observed.

Table 5.3 and Figures 5.1 and 5.2 report the sub-maximal exercise testing before and after the HIIT programme at workloads 1, 2 and 3. Before HIIT, workloads 1 to 3 were set relative to individual PTS. After the HIIT intervention, the same absolute speeds were used despite a change in PTS in order to assess improvement in physiological measurements at the same workloads. VO_2 (mL/min)

and VO_2 expressed per body mass ($\text{mL}/\text{min}/\text{kg}$) did not change after the HIIT intervention, but VO_2 expressed as percentage of $\text{VO}_{2\text{max}}$ was significantly reduced at all three workloads (Table 5.3 and Figure 5.1). Similarly, heart rate at each workload was also significantly reduced after the HIIT intervention (Figure 5.2). Plasma lactate showed a trend to be lower at workload 1, but was significantly increased at workload 2, with no change at workload 3. No changes in RER or V_E were observed after the intervention.

Some athletes ($N = 6$) were able to complete 20 km/h (workload 4) before and after the HIIT programme, whereas the remaining nine athletes were unable to finish this workload before the training intervention. Therefore, the six athletes' results at workload 4 were analysed separately from the remaining nine. Those who completed both time periods, showed a significant decrease in their heart rate and V_E , with no change in RER, VO_2 ($\text{mL}/\text{min}/\text{kg}$) or plasma lactate concentration (Table 5.4A). However, this group of athletes showed a reduction in their plasma lactate at 3 and 6 minutes during the recovery phase, with no change at the later time points (Figure 5.3A).

The remaining nine athletes had no change in VO_2 , heart rate or RER, but V_E and the duration at workload 4 were significantly higher after the HIIT intervention (Table 5.4B). No change in recovery plasma lactate was observed after the HIIT intervention (Figure 5.3B).

Analyses of histological sections revealed that only the proportion of type I/IIA fibres significantly decreased after the HIIT intervention ($P < 0.05$) (Table 5.5). No change was observed for the remainder of the fibre types. CSA, FD and capillary counts were not altered by the HIIT programme.

The fibre type proportions calculated from single fibres identified by electrophoresis were slightly different from those identified in histological sections (Table 5.6). No statistical difference was observed between the proportions of these fibre types before and after the intervention. The change in fibre type proportion (After – Before HIIT) determined from MHC content of single fibres was calculated for each athlete and is presented in Figure 5.4 as delta values reported as mean \pm SD.

Figures 5.5 and 5.6 show the significant relationships ($P < 0.05$) between training speeds of the HIIT programme and the change in type I fibres and change in type IIa/IIx hybrid fibres, respectively. Although there was a linear correlation between delta I and training speed, the relationship may be more closely described as a threshold effect, which decreases in type I proportions occurring only with ITS $>$ than 20 km/h. There was a clearer continuum for the delta IIa/IIx.

No change in CS activity was observed before ($28 \pm 6 \mu\text{mol}/\text{min}/\text{g dw}$) and after ($22 \pm 9 \mu\text{mol}/\text{min}/\text{g dw}$) the HIIT intervention. The activity of LDH in pools of type I and IIa fibres, as well as in homogenate, is reported in Figure 5.7. A trend was observed for LDH activity to have increased after the HIIT intervention in type IIa fibre pools ($P = 0.06$), with no change in type I pools or homogenate samples.

5.4 Discussion

The main findings of this study were that systematic, supervised HIIT in already well-trained athletes altered performance, metabolism and some intramuscular characteristics. The most significant changes were increased maximum performance (PTS and time to fatigue at 20 km/h) and lower heart rate at sub-maximal exercise intensities after the HIIT programme. Although neither VO_2max nor economy improved significantly, the VO_2 as a percentage of maximum at sub-maximal workloads declined significantly. Significant adaptations in skeletal muscle were not seen for the whole group, however type I/IIa hybrid fibres declined in histological samples. Furthermore, a tendency was observed for LDH activity to have increased in type IIa fibre pools after the HIIT intervention.

Smith *et al.* (1999) showed that four weeks of HIIT significantly increased VO_2max in trained runners. Similar increases in VO_2max were observed in runners after six weeks (Franch *et al.*, 1998) and four months (Tanaka *et al.*, 1986) of HIIT. HIIT significantly increased the PTS, with VO_2max only showing a trend to have increased after the HIIT intervention in the current study (Table 5.2). The weight of the athletes did not change (Table 5.1), indicating that the effect of HIIT was directly on oxygen consumption. In contrast, other studies have shown that HIIT may not improve VO_2max or PTS, but may only improve field test performance as was shown by Acevedo and Goldfarb (1989), Billat *et al.* (1999) and Sjodin *et al.* (1982). Differences between studies may be due to pre-intervention training status. Demarle *et al.* (2003) showed that VO_2max and PTS only increased in subjects who had a relatively low training volume, whereas no change was observed in well-trained athletes. The fitness level of the athletes in the present study varied and there was also a wide variety of 10 km times during the field test (range between 31.0 to 39.0 minutes). This may partly explain why VO_2max did not increase significantly, but PTS did.

Although maximal heart rate showed no change (Table 5.2), the sub-maximal exercise tests revealed that after the HIIT programme, athletes were able to run at a lower heart rate at the same absolute workload (Figure 5.2). Franch *et al.* (1998) and Billat *et al.* (1999) both showed a reduced heart rate at sub-maximal intensity after HIIT, and this may therefore be one of the major physiological adaptations associated with HIIT. Similarly, since VO_2max showed a tendency to have increased,

the %VO₂max values at workloads 1 to 3 were lower after HIIT. Sjodin *et al.* (1982) also showed that after 14 weeks of HIIT, athletes ran at a lower percentage of their VO₂max at the same speed. These adaptations to HIIT in the current study may have contributed to the improved performance at 20 km/h and to the higher PTS.

Plasma lactate concentration is known to decrease in response to training (Acevedo and Goldfarb, 1989; MacRae *et al.*, 1992). However, in the present study, the plasma lactate concentration was only reduced at the lowest workload, but was higher at workload 2 with no difference at workload 3 (Table 5.3). It may be that the fitness level and training distance per week of the athletes were too widespread. As a result of this possibility, the athletes from the present study were divided into two groups: those who were able to finish workload 4 at both time points (Table 5.4A) and those who could not (Table 5.4B). The group who were able to finish both time points at workload 4, showed a significant decrease in their heart rate and V_E after the training intervention. Plasma lactate concentration after this workload, although not statistically different, seemed to be lower (7.1 ± 1.5 vs. 5.3 ± 1.9 ; $P = 0.21$) and lack of significance may be attributed to the low subject number. However, during the recovery phase, plasma lactate was significantly reduced at 3 and 6 minutes (Figure 5.3A). These findings indicate that the training intervention in these athletes elicited a significant change in lactate disappearance.

On the other hand, the group that was unable to finish workload 4, showed no change in heart rate. V_E was higher after the training intervention, with plasma lactate concentrations showing a trend to have increased. The explanation for this may merely be a result of the significantly longer time spent at this workload. Plasma lactate concentrations were also not reduced during the recovery phase (Figure 5.3B).

Overall, no change in fibre type proportions were observed for type I, IIA, IIAX and IIX, with the exception of type I/IIA hybrid fibres (Table 5.5). It is possible that the latter finding may be merely a methodological error, as no change was observed when using fibre proportions identified by the single fibre method (Table 5.6). However, more fibres are counted during histological analysis and there is evidence for decreases in hybrid fibre proportions with training interventions. Recently, Putman *et al.* (2004) investigated the effect of 12 weeks of combined strength and endurance training in humans and found that fibres co-expressing more than one isoform (hybrid fibres) decreased for type I/IIa and type IIa/IIx hybrid fibres. Also, the MHC isoform IIa was increased and MHC IIx was decreased. It may therefore be argued that the training period of the current study was too short to elicit significant fibre type alterations. However, the training speed itself may also have played a role in determining whether fibre types switched or not (from slow to fast or *vice versa*), as

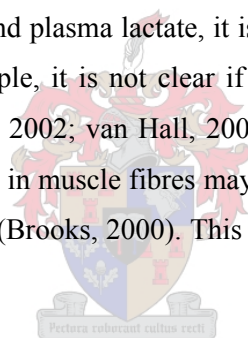
was shown in Figures 5.5 and 5.6. Although not measured, the possibility also arises that the mRNA transcripts for the switch in MHC isoforms were already present, but that on protein level, the transformation was not detected yet. Andersen and Schiaffino (1997) reported that a large variation in mRNA and the relevant protein levels exist as a result of training or detraining. Although not significant, a pattern was observed when considering the change in fibre type (after – before) (Figure 5.4). The resultant picture gives the impression that with HIIT, a possible pattern for fibre type switch may be a decrease in type I with a concomitant increase in type IIa and IIa/IIx fibres. This pattern is similar to those found in studies investigating resistance or sprint training (Andersen *et al.*, 1994; Putman *et al.*, 2004). Progressive resistance and sprint training for three months have both resulted in an increase in the number of type IIA fibres with a concomitant decrease in type IIX fibres (Andersen *et al.*, 1994; Williamson *et al.*, 2001).

The function of hybrid fibres is not yet established, but in a review by Stephenson (Stephenson, 2001), it was proposed that these fibres may be transitional (caught in the act of converting to a pure fibre type) or that they may serve a purpose of “fine tuning” for optimal muscle performance. Andersen *et al.* (1994) reported that after three months of intense strength and interval training, both pure type I and type IIa/IIx hybrid fibres showed a tendency to convert to type IIa. The same pattern was observed by Williamson *et al.* (2001) and Putman *et al.* (2004) after the same period of progressive resistance training. The present data, however, suggest that, in trained endurance runners, the speed of the interval training is crucial for predicting the change in type I and type IIa/IIx fibres (Figures 5.5 and 5.6). These data show that with an increase in training intensity, type I fibres may be converted to a faster type. Similarly, with an increase in training intensity, type IIa/IIx hybrid fibre populations might increase. These latter findings strengthen the possible fibre type conversion pattern proposed in Figure 5.4. Careful consideration of data in Figures 5.5 and 5.6 shows that the actual speed of interval training may be more important in determining the direction of fibre type change than a speed set relative to capacity. Athletes for whom 94% of PTS fell below 20 km/h tended toward no change in type I and a decrease in type IIa/IIx proportions, whereas the clearer changes occurred in those athletes who performed HIIT at ≥ 20 km/h. No relationships were found between the remaining fibre types.

Fibre CSA and capillary supply can improve muscle power and the supply of oxygen to the muscle, respectively (Jensen *et al.*, 2004; Putman *et al.*, 2004). These adaptations were recently demonstrated in response to high intensity strength training. Putman *et al.* (2004) showed a significant increase in CSA after 12 weeks of strength training in both type I and IIA fibres, but only in type IIA fibres of subjects performing a combination of strength and endurance training. Jensen *et al.* (2004) showed an increase in muscle capillary to fibre ratio after four weeks of two high

intensity interval training programmes at workloads corresponding to 90% and 150% $\text{VO}_{2\text{max}}$. However, the present study showed no change in CSA or capillary supply as a result of HIIT (Table 5.5). The reason for this may be that the studies by Putman *et al.* (2004) and Jensen *et al.* (2004) used untrained subjects, whereas in the present study, the subjects were already well-trained. The CSA and the number of capillaries around a fibre in the current study were high when compared to data from previous studies (Coggan *et al.*, 1990; Saltin *et al.*, 1977). The possibility arises that well-trained athletes already have adapted their CSA and capillary supply.

Similarly, CS activity was not altered after the HIIT intervention. The present study is the first to have investigated LDH activity in pools of identified fibre types before and after HIIT. LDH activity in type IIa fibre pools showed a tendency to have increased in response to the HIIT intervention, with no change in either type I fibre pools or homogenate LDH activities. Sjodin *et al.* (1982) found no change in LDH activity in homogenate samples after 14 weeks of HIIT in well-trained marathon runners. The present data suggest that as a result of the HIIT, more type IIa fibres need to be recruited, therefore increasing the demand for ATP production. However, as no relationships were found between LDH activities and plasma lactate, it is not clear what the physiological effect might be of this adaptation. For example, it is not clear if the much debated lactate shuttle system was affected (Brooks, 2000; Brooks, 2002; van Hall, 2000). The lactate shuttle system is based on the hypothesis that lactate, produced in muscle fibres may be re-converted to pyruvate, either within the same fibre or by adjacent fibres (Brooks, 2000). This pyruvate may then enter the tricarboxylic acid cycle to be metabolised.



5.5 Conclusion

Six weeks of HIIT elicited significant enhancements in both performance, physiological and biochemical variables in trained endurance athletes. It is proposed that HIIT of high enough absolute speed might significantly decrease the number of type I fibres, and even increase type IIa/IIx hybrid fibres. Furthermore, training at high intensity relative to each athlete's capacity may result in a decrease in type IIa/IIx fibre proportions if the absolute speed is not sufficient for the opposite effect. HIIT may also enhance metabolic pathways, especially the metabolism of lactate. However, longer training periods are proposed with larger subject numbers. Moreover, more studies are needed to elicit the finding of higher LDH activity in type IIa fibres, focussing on the metabolic adaptations associated with HIIT.

Recent studies on the cellular signalling pathways responsible for muscle adaptations may elicit more information on the adaptation to HIIT in future studies. Schiaffino and Serrano (2002) have suggested that fibre type may be regulated by calcineurin activity, whereas the cell size is regulated

by mTOR, the latter being a crucial required signalling step for the activation of protein synthesis. Similarly, other pathways involving peroxisome proliferator-activated receptor δ (PPAR δ) have been shown to be actively involved in fibre type, metabolism and overall improvement in endurance performance albeit in rodents (Suwa *et al.*, 2003; Wang *et al.*, 2004). It may therefore be that some of these activators are enhanced because of HIIT, and needs to be included in future studies.

5.6 References

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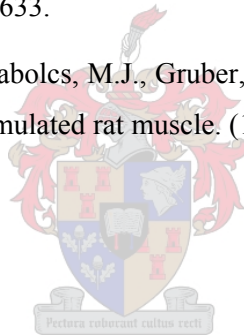


Table 5.1 Subject characteristics of athletes: before and after high intensity interval training (HIIT).

	Before HIIT	After HIIT	Significance
Age (yrs)	22.3 ± 2.1		
Height (cm)	177.4 ± 8.2		
Weight (kg)	65.0 ± 9.5	64.6 ± 9.1	<i>ns</i>
BMI	20.5 ± 1.7	20.4 ± 1.5	<i>ns</i>
PRD _A (km)*	10.4 ± 4.2		
10 km time field test (min)	35.2 ± 2.7		
Average training (km/wk)	50.7 ± 23.8	52.7 ± 25.8	<i>ns</i>
HIIT (km/wk)		9.0 ± 2.7	

Data are presented as mean ± SD (N = 15). Statistical analyses were performed with the Wilcoxon signed rank test for non-parametric paired data. BMI, body mass index. Training per week before the HIIT programme was for two weeks, which also included the exercise tests. Training during the HIIT intervention included both treadmill training and exercise tests. * average preferred racing distance: average of the two or three race distances, one for each discipline (track, road, cross-country) in which each athlete competed.

Table 5.2 Maximal exercise tests: before and after high intensity interval training (HIIT).

	Pre HIIT	Post HIIT	Significance
PTS (km/h)	21.2 ± 0.7	22.3 ± 1.1	<i>P < 0.01</i>
VO ₂ max (mL/min/kg)	67.2 ± 4.9	69.5 ± 2.6	<i>P = 0.07</i>
VO ₂ max (mL/min)	4327 ± 491	4461 ± 575	<i>ns</i>
RERmax	1.15 ± 0.03	1.15 ± 0.03	<i>ns</i>
HRmax (beats/min)	190 ± 7	187 ± 7	<i>ns</i>
V _E max (L/min)	155 ± 17	159 ± 20	<i>ns</i>

Data are presented as mean ± SD (N = 15). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. HR, heart rate; RER, respiratory exchange ratio; PTS, peak treadmill speed; V_E, minute ventilation; VO₂, oxygen consumption

Table 5.3 Sub-maximal exercise tests: before and after high intensity interval training (HIIT).

	Before HIIT	After HIIT	Significance
<i>Workload 1: 13.5 ± 0.5 km/h</i>			
% VO ₂ max	75.0 ± 5.1	71.4 ± 4.3	<i>P</i> < 0.01
VO ₂ (mL/min)	3243 ± 397	3190 ± 488	<i>ns</i>
RER	0.90 ± 0.03	0.90 ± 0.02	<i>ns</i>
V _E (L/min)	81 ± 11	81 ± 11	<i>ns</i>
Plasma lactate (mmol/L)	2.0 ± 0.7	1.7 ± 0.6	<i>P</i> = 0.06
<i>Workload 2: 15.2 ± 0.5 km/h</i>			
% VO ₂ max	82.8 ± 5.6	79.7 ± 4.8	<i>P</i> < 0.05
VO ₂ (mL/min)	3582 ± 470	3557 ± 514	<i>ns</i>
RER	0.94 ± 0.03	0.94 ± 0.03	<i>ns</i>
V _E (L/min)	97 ± 12	98 ± 13	<i>ns</i>
Plasma lactate (mmol/L)	2.1 ± 0.9	2.5 ± 1.0	<i>P</i> < 0.05
<i>Workload 3: 16.9 ± 0.6 km/h</i>			
% VO ₂ max	89.7 ± 5.5	86.9 ± 5.3	<i>P</i> < 0.05
VO ₂ (mL/min)	3879 ± 481	3874 ± 542	<i>ns</i>
RER	0.98 ± 0.04	0.96 ± 0.03	<i>ns</i>
V _E (L/min)	115 ± 15	114 ± 17	<i>ns</i>
Plasma lactate (mmol/L)	4.6 ± 1.8	4.2 ± 2.0	<i>ns</i>

Data are presented as mean ± SD (N = 15). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. Each data point (except lactate) was calculated as the average from the last minute data set at each workload. HR, heart rate; RER, respiratory exchange ratio; V_E, minute ventilation; VO₂, oxygen consumption; % VO₂, percentage of maximum oxygen consumption

Table 5.4A Physiological response during a set absolute workload in athletes who were able to complete 5 minutes: before and after high intensity interval training (HIIT).

Workload 4: 20 km/h			
	Before HIIT	After HIIT	Significance
VO ₂ (ml/min/kg)	65.2 ± 4.7	66.6 ± 4.3	ns
HR (bts/min)	187 ± 11	178 ± 10	<i>P</i> < 0.05
RER	1.06 ± 0.03	1.03 ± 0.03	ns
V _E (l/min)	146 ± 20	138 ± 20	<i>P</i> < 0.05
Plasma lactate (mmol/L)	7.1 ± 1.5	5.3 ± 1.9	ns

Data are presented as mean ± SD (N = 6). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. Each data point (except lactate) was calculated as the average from the last minute data set at each workload. HR, heart rate; RER, respiratory exchange ratio; V_E, minute ventilation; VO₂, oxygen consumption

Table 5.4B Physiological response during a set absolute workload in athletes who were unable to complete 5 minutes: before and after high intensity interval training (HIIT).

Workload 4: 20 km/h			
	Before HIIT	After HIIT	
Time (min) at workload	1.8 ± 0.8	3.3 ± 1.2	<i>P</i> < 0.05
VO ₂ (ml/min/kg)	61.2 ± 6.7	65.6 ± 2.2	ns
HR (bts/min)	189 ± 8	186 ± 6	ns
RER	1.02 ± 0.10	1.08 ± 0.03	ns
V _E (l/min)	135 ± 29	148 ± 16	<i>P</i> < 0.05
Plasma lactate (mmol/L)	7.4 ± 2.6	8.5 ± 2.4	<i>P</i> = 0.07

Data are presented as mean ± SD (N = 9). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. Each data point (except lactate) was calculated as the average from the last minute data set at each workload. The time parameter includes 3 x 5 minute workloads performed prior to the workload at 20 km/h. Only two athletes were able to complete the 5 minute workload after the HIIT intervention. HR, heart rate; RER, respiratory exchange ratio; V_E, minute ventilation; VO₂, oxygen consumption

Table 5.5 *Histological analyses of skeletal muscle biopsies for fibre type, cross-sectional area, fibre diameter and the number of capillaries around a fibre: before and after high intensity interval training (HIIT).*

	Before HIIT	After HIIT	Significance
Fibre type (%)			
Type I	55.5 ± 15.9	52.1 ± 15.3	<i>ns</i>
Type I/IIA	8.5 ± 10.4	1.3 ± 1.6	<i>P < 0.05</i>
Type IIA	31.7 ± 8.6	36.1 ± 15.3	<i>ns</i>
Type IIAX	2.4 ± 2.3	3.6 ± 4.1	<i>ns</i>
Type IIX	1.9 ± 2.3	6.8 ± 11.4	<i>ns</i>
CSA (µm ²)			
Type I	4463 ± 1125	3893 ± 870	<i>ns</i>
Type II	5366 ± 1579	4643 ± 1165	<i>ns</i>
FD (µm)			
Type I	73.9 ± 8.7	69.1 ± 8.1	<i>ns</i>
Type II	80.6 ± 11.6	75.5 ± 9.5	<i>ns</i>
Number of capillaries around a fibre	5.9 ± 0.9	5.6 ± 1.2	<i>ns</i>

Data are presented as mean ± SD. Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. Sample size for all was N = 12, except capillaries with N = 13. CSA, cross-sectional area; FD, fibre diameter

Table 5.6 *Fibre type determined from MHC isoform content in single fibres: before and after high intensity interval training (HIIT).*

Fibre type	Before HIIT	After HIIT	Significance
Type I	49.0 ± 19.7	41.6 ± 13.4	<i>ns</i>
Type I/IIa	7.3 ± 4.7	6.9 ± 4.2	<i>ns</i>
Type IIa	32.6 ± 15.0	35.7 ± 17.8	<i>ns</i>
Type IIa/IIx	6.3 ± 5.7	10.6 ± 8.9	<i>ns</i>
Type IIx	3.8 ± 6.1	4.3 ± 5.7	<i>ns</i>
Type Iax	0.5 ± 0.8	0.6 ± 0.7	<i>ns</i>
Type Ix	0.9 ± 2.1	0.3 ± 0.8	<i>ns</i>
Total Hybrids	14.9 ± 7.5	18.4 ± 9.5	<i>ns</i>

Data are presented as mean ± SD (N = 10). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. A total of 3009 fibres were dissected. MHC, myosin heavy chain

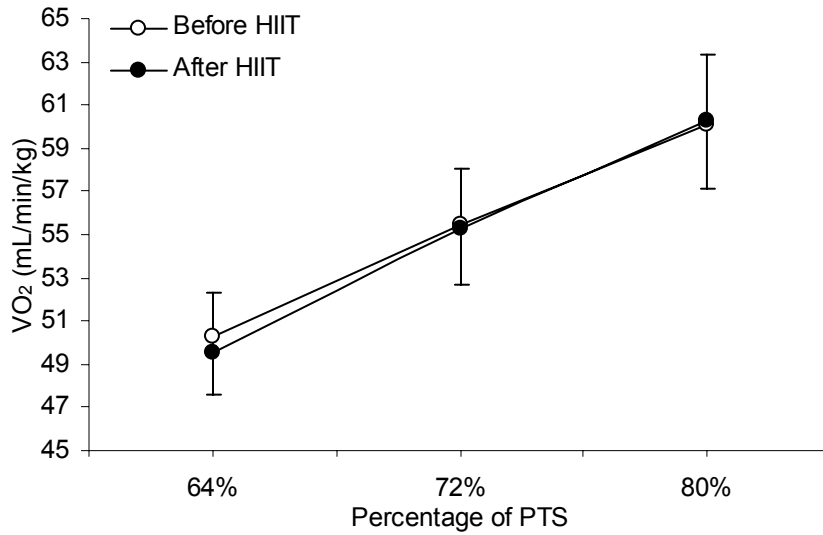


Figure 5.1 Oxygen consumption during the sub-maximal exercise test: before and after high intensity interval training (HIIT). Data are presented as mean \pm SD (N = 15). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. * Different from Before HIIT ($P < 0.05$).

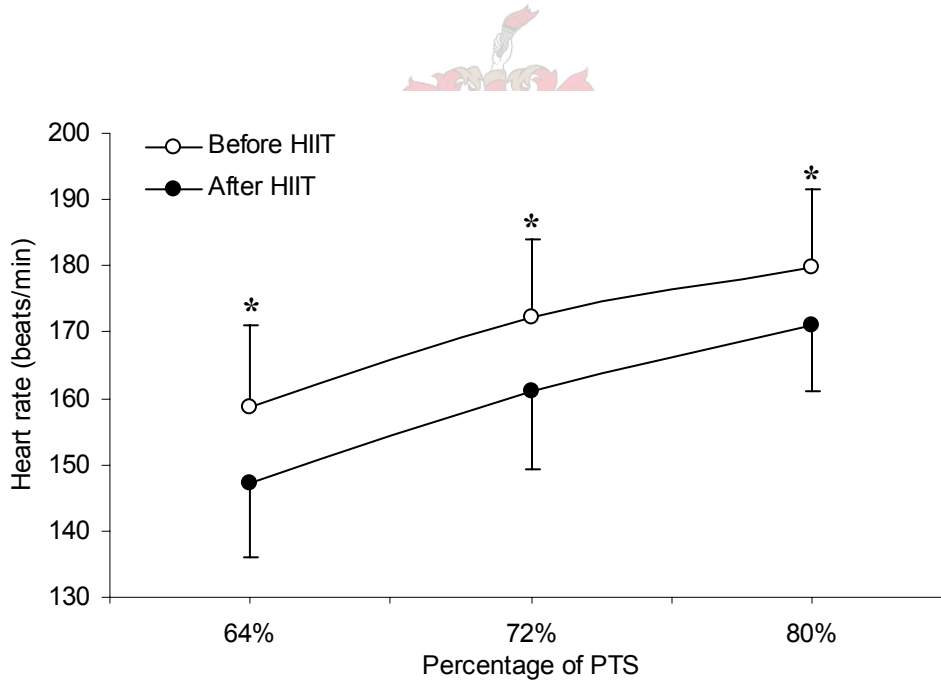


Figure 5.2 Heart rate during the sub-maximal exercise test: before and after high intensity interval training (HIIT). Data are presented as mean \pm SD (N = 15). Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. * Different from Before HIIT ($P < 0.01$).

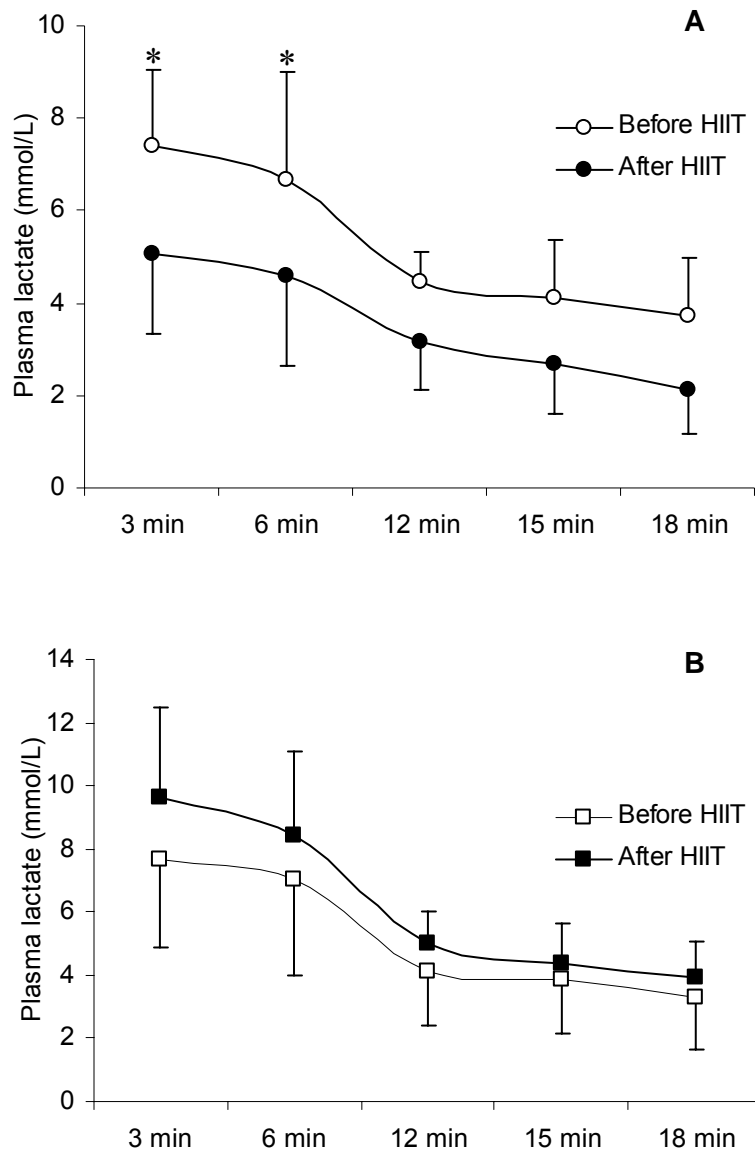


Figure 5.3 Plasma lactate concentration during the recovery phase after the sub-maximal exercise test in: A. athletes that completed 5 minutes at 20 km/h before and after HIIT ($N = 6$), B. athletes that was unable to complete 5 minutes at 20 km/h before HIIT ($N = 9$). Data are presented as mean \pm SD. Statistical analysis was performed by one-way ANOVA with a Bonferroni correction. * Different from Before HIIT ($P < 0.05$).

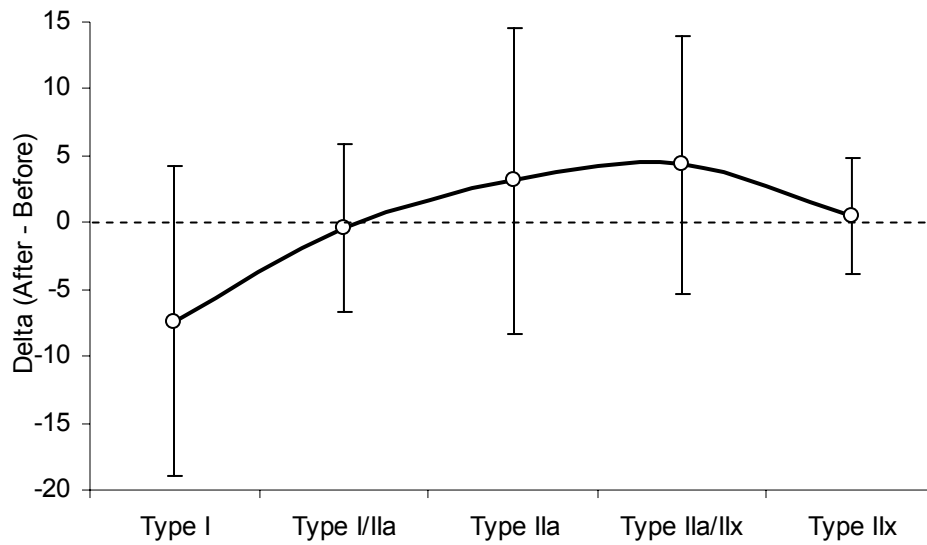


Figure 5.4 Change in fibre type composition due to the effect of high intensity interval training. Data are presented as mean \pm SD (N = 10). Change (delta) was calculated by subtracting the before values from the after HIIT for each athlete. Connection line only included for visual aid.

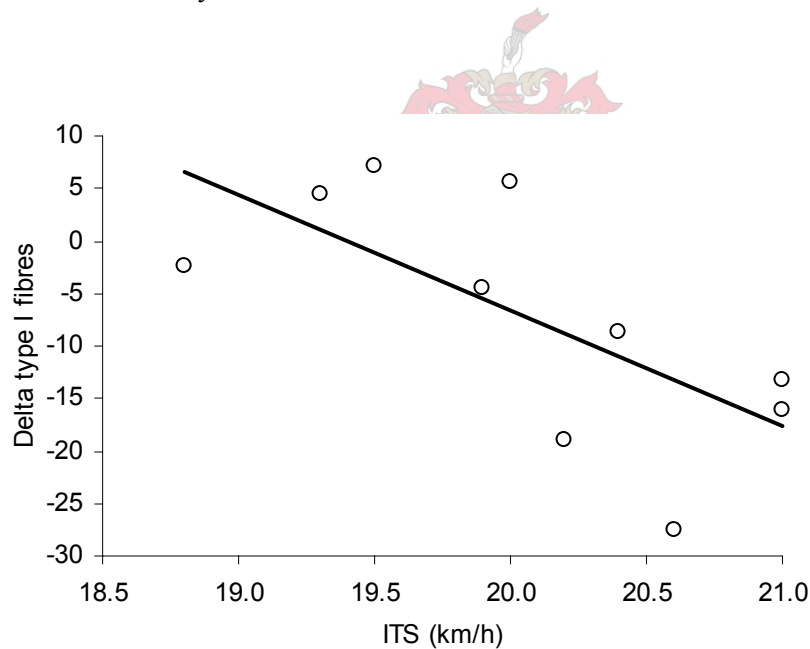


Figure 5.5 Relationship between change in fibre type I distribution and speed of training on the treadmill. Pearson's r and significance: $r = -0.70$, $P < 0.05$.

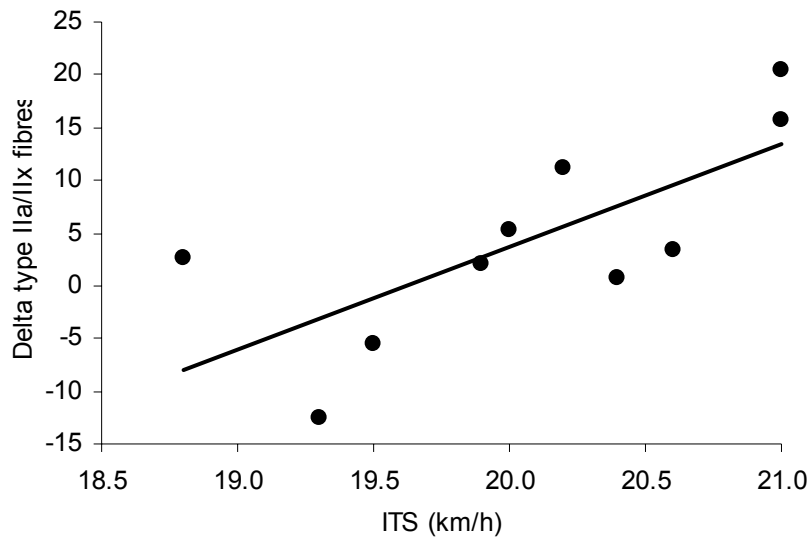


Figure 5.6 Relationship between change in fibre type IIa/IIx distribution and speed of training on the treadmill. Pearson's r and significance: $r = 0.68$, $P < 0.05$.

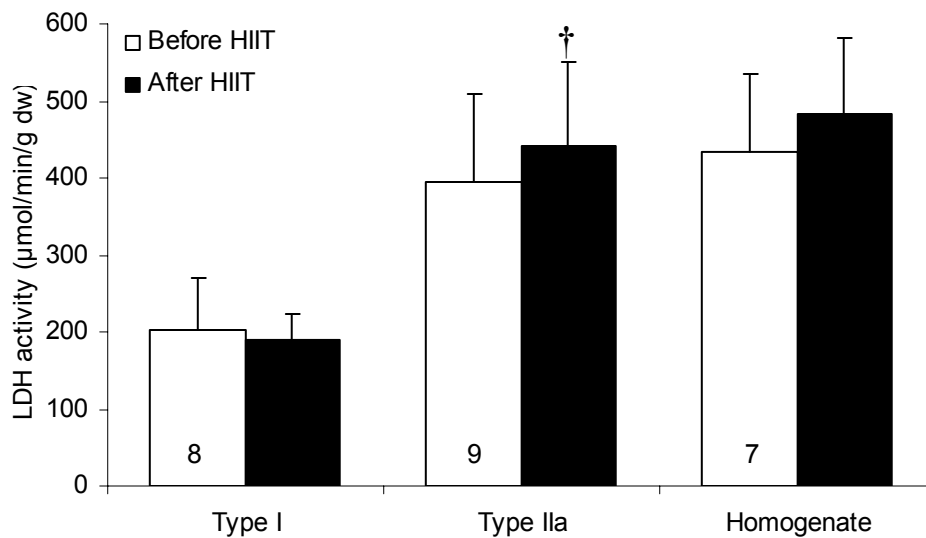


Figure 5.6 Lactate dehydrogenase activities in pools of single fibres and homogenate muscle samples: before and after high intensity interval training (HIIT). Data are presented as mean \pm SD. Values in white boxes represent sample number. Statistical analysis was performed with the Wilcoxon signed rank test for non-parametric paired data. † $P = 0.06$. dw, dry weight.

Final conclusion and future studies

6.1 Summary, general interpretation and conclusions

The main focus of this study was on the plasticity of skeletal muscle enzyme activity and MHC isoforms in the context of normal physiological activity, as opposed to extreme models such as paralysis or 24 hour stimulation which assess the boundaries of plasticity. It was therefore necessary to link the tissue characteristics to functional performance in order to investigate associations and possible adaptations.

The main finding of Chapter 2 was that there are large regional variations in the muscle characteristics, of representative proteins involved in contraction (MHC) and metabolism (CS activities) within a muscle group localised in one area of the body (upper hind-limb *Quadriceps*). When analysing the three middle sections (superficial, middle and deep), as expected, the superficial regions were more associated with fast contracting fibres, with a concomitantly low oxidative potential. However, the deep region of the QF muscle contained both fast and slow contractile capabilities, reflected by the expression of all four MHC isoforms, despite high CS activity. Longitudinal variation is seldom investigated, especially not assessing both MHC and CS activity to determine the extent of co-adaptation. A novel finding for this muscle group was that the variation in MHC isoform expression also varied significantly across the length of the muscle, but that CS remained the same, also indicated by the poor relationships observed between oxidative capacity and the MHC isoforms. These findings highlight the importance of proper standardisation of the sampling site for comparative studies, and indicate that CS activity is not tightly linked to slow contractile protein expression. The latter statement could be explained that mitochondria content of fibres is partly independent of fibre type, and may largely be influenced by muscle activity. It may also imply that the regulation of slow twitch MHC expression is independent of mitochondrial biogenesis. The two signalling proteins, namely calcineurin and PPAR δ have been discussed in section 1.5. An increase in calcineurin activity has been shown to elicit MHC I isoform expression. Whether this signalling protein is involved in mitochondrial biogenesis, is still under investigation. The muscle of transgenic mice over expressing PPAR δ revealed a high quantity of type I fibres as well as the number of mitochondria compared to wild type mice (Wang *et al.*, 2004). Whether they function independently or in conjunction with each other, are still under investigation. However, these molecules may also play a significant role in fibre type regionalisation.

Chapter 3 focused on comparing the muscle characteristics of human recreationally active subjects to those of runners. This study was specifically designed so that each group consisted of subjects with a range of habitual activities to enable the assessment of relationships. As expected, there was a negative relationship between the number of hybrid fibres co-expressing MHC IIa and IIx and training volume in runners. Similarly, training hours in recreationally active subjects also showed the same relationship. Although linear relationships might have been expected, a novel finding was that exponential curve fitting resulted in a higher R^2 value than linear regression. This indicates that a critical amount of training (volume) or intensity level (PRD_A) is required to be sufficient to activate or inhibit MHC IIa or MHC IIx gene transcription in the same fibre. Another main finding was that the exponential relationship between MHC IIa/IIx hybrid fibres and PRD_A in runners was even better than training volume. These relationships were not found for the hybrid fibres co-expressing MHC I and MHC IIa in either of the groups. It therefore seems that the intensity of exercise and the volume of contraction both play an important role in the occurrence of MHC IIa/IIx hybrid fibres, whereas the proportion of the MHC I/IIa hybrid fibres remains unexplained. This finding indicates that MHC IIx or MHC IIa gene regulation is more sensitive to muscle contraction intensity, rather than the duration of muscle activation at a lower intensity. In order for calcineurin activation to promote MHC I gene expression, it is required that a constant concentration of Ca^{2+} be present in the fibres (Olson and Williams, 2000). It may therefore be argued that high intensity force contractions, with longer periods of relaxation, do not have the ability to activate calcineurin, but to rather promote fast isoform expression.

The genotype of an individual has been proposed to play a significant role in the phenotypic characteristics of skeletal muscle. Chapter 4 focussed on athletes from two distinct ethnic backgrounds (Xhosa and Caucasian athletes). Matching closely for training volume, performance and relative exercise intensity (PRD_A), it was found that Xhosa athletes had significantly more type IIa fibres, with concomitantly lower type I fibre proportions. Plasma lactate was lower in the Xhosa athletes at 80% of their peak treadmill speed, but muscle analysis showed higher LDH activity in the Xhosa athletes, a finding that was confirmed by single fibre pool enzyme analyses. This can be partly explained by the lactate shuttle model proposed by Brooks (Brooks, 2000). Three hypotheses have been proposed namely: (1) that lactate produced in the muscle is transported to other organs or tissues and metabolised, or (2) that lactate produced in i.e. type II muscle fibres is transported to adjacent type I fibres who may have the ability to process the acquired lactate, or (3) that the fibre has the ability to metabolise its own lactate during contraction. All three hypotheses can be applied to the results obtained in Chapter 4, but additional analyses of i.e. monocarboxylate transporters would be required. Alternatively, Xhosa athletes may have a genetic advantage in fuel metabolism,

resulting in the lower plasma lactate accumulation, secondary to enhanced muscle adaptation despite similar training. However, an alternative hypothesis is that the Xhosa athletes trained at a higher intensity and that the findings are an adaptive response. Therefore, the case is not closed regarding black and white athletes. The following chapter therefore investigated the effect on muscle of an increase in the intensity of training.

High intensity interval training (HIIT) has previously been shown to increase performance, but also to elicit distinct adaptations in muscle and metabolic fuel utilisation. In Chapter 5, the main findings were that the training resulted in an improvement of physiological markers for performance, such as peak treadmill speed and $VO_2\text{max}$. Similarly, sub-maximal indicators of significant adaptations included a plasma lactate decrease at the lowest workload of the sub-maximal exercise test, with heart rate that decreased at all sub-maximal workloads after HIIT. Muscle fibre type did not change significantly after the HIIT programme. However, the general pattern for the change in fibre type indicated that a possible switch from type I to type IIa may become evident with more subjects or a longer intervention duration at the higher HIIT speeds. This was confirmed by a significant negative relationship between the change in type I fibre proportions and absolute interval training speed, as well as a positive relationship between interval training speed and type IIa/IIx hybrid fibres. Enzyme activities in pools of single fibres also showed only a trend to have increased in LDH activity in type IIa fibre pools ($P = 0.06$), with no significant increase in the homogenate LDH activity or the type I pools. This adaptation indicates that the capacity to produce lactate from carbohydrate may be increased only in type IIa fibres as a result of HIIT. This finding does not shed light on the higher LDH activity found in homogenate samples and type IIa and type I fibre pools of Xhosa athletes, which may indicate a gene-environment interaction influencing LDH expression in black endurance runners. Alternatively, training intensity was responsible for the difference in Chapter 4, but Chapter 5 could not prove this conclusively.

Final thought...

One aspect that distinguishes humans from animals is competitive **motivation**, outside of the areas of mating, nutrition or life-and-death situations. This aspect is very difficult to quantify, but is very important. In fact, the human brain is so powerful that it may override pain or let muscles generate great power. Legend has it that a man was able to lift an 800 kg steel door from his child's leg – afterwards it took 10 men to lift that same door! Of course, in this example, stress hormones may inhibit pain receptors, and the signal for hormone release came from the external situation which was unlike sporting competition. What drives the human to ultimate sporting performance may also not be based only on genetic, physiological or biochemical advantage, but may start in the athlete's head. As Kayser (Kayser, 2003) stated – “Exercise starts and ends in the brain”. Nonetheless,

despite current trends to study e.g. neurobiology in sport, it remains relevant to have a full understanding of muscle adaptations to modern training methods, and even to influence training methods based on scientific evidence.

6.2 Limitations

There are various methods of identifying the exact fibre type of muscle samples, e.g. ATPase histochemistry, antibodies directed against the MHC isoforms, homogenate and single fibre SDS-PAGE. Each method has limitations and advantages, but the most crucial for all these methods is the question of “how much is enough”? How many total fibres need to be counted to represent an accurate measure of the fibre type of a muscle? At a recent workshop on muscle analytical techniques (ECSS 2003, Salzburg, Austria), J.L. Andersen commented that counting 200 ± 20 fibres per biopsy sample for ATPase histochemistry and immunohistochemistry, should give an appropriate account of the fibre type in humans. However, it was also pointed out that these methods mentioned do not correspond to each other when two or more methods were used on the same biopsy. This problem mainly seems to occur when hybrid fibres are misclassified. Harber *et al.* (Harber *et al.*, 2002) showed that by using only 80 – 85 single fibres per sample (identified by single fibre gel electrophoresis), the fibre type proportions resembled similar proportions to those calculated from ATPase histochemistry in long distance endurance athletes. However, the relationships between the two methods started to decline, especially in type IIX / MHC IIX proportions, as the preferred racing distance of the athletes decreased. Similar comparisons were made by Andersen *et al.* (Andersen *et al.*, 1994) in well-trained sprint athletes. It almost seems that the more hybrid fibres and IIX fibres are expected, the more fibres should be counted and this may become a problem as the sample sizes of human biopsies are very small ($\pm 50 - 80$ mg).

Single fibre dissection and electrophoresis are extremely time-consuming. Dissecting of 100 – 150 fibres may take up to 8 hours (for the experienced hand). Furthermore, many gel electrophoresis systems are necessary to analyse many samples simultaneously and to complicate matters further, adequate separation of the MHC isoforms requires an electrophoresis time of at least 16 hours. However, this technique allows multiple experiments to be performed on the same fibre, such as subsequent analysis of the enzyme activities in identified pure pools. The enzyme activities in pools of identified fibre type may be a more accurate way of assessing metabolic capacity, as homogenate samples may contain more connective tissue, which can be misleading when analysing the end results. The influence of fibre type may also obscure the influence of training.

The variation between these two methods is depicted in Figure 6.1. The data was compiled from those in Chapters 4 and 5 and clearly shows a significant difference between fibres identified as type

I and the two hybrid fibre populations. Emphasis is once again placed on the misclassification of fibre populations using the ATPase method, but low fibre numbers (single fibre SDS-PAGE method) can also contribute to these variations. Biopsy samples may also vary substantially in the number of fibres it contains, and would generally result in a Type II error. Staron (Staron, 1997) pointed out that this type of error may contribute as much as 10% of overall analyses.

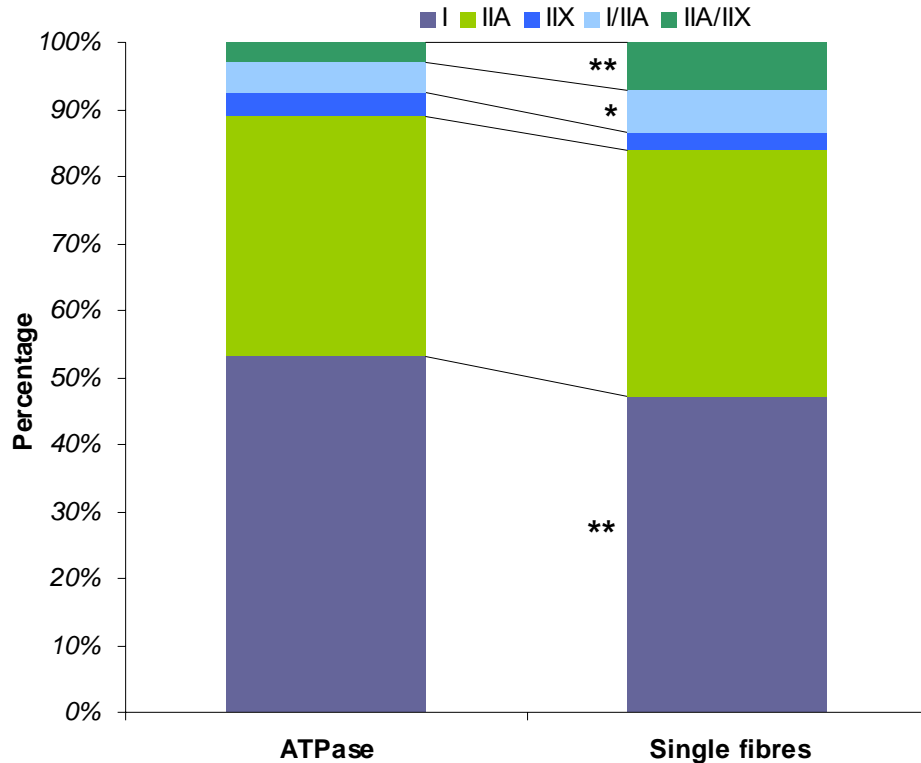


Figure 6.1. Comparison of fibre type proportions obtained from ATPase histochemistry vs. single fibre electrophoresis. Graphic was compiled from the data of Chapters 4 and 5. Different: * $P < 0.05$; ** $P < 0.01$.

One of the major limitations investigating training models in humans is the number of subjects. Investigating already well-trained athletes, fulfilling specific criteria and willing to participate, can be a major challenge even before the experiment commences, especially when the population of these athletes are low. Training individuals under the same controlled conditions and at a specific intensity may also be limited by the training equipment available. One of the possible explanations for the small variations in muscle fibre type and enzyme activities (Chapter 5) can be attributed to the duration of the training intervention. Six weeks may have been insufficient to elicit significant adaptations in the muscle. Therefore, a longer training period is suggested. However, performance markers did change significantly, which shows that the protocol itself was successful. Furthermore,

a control sedentary group in Chapter 4, consisting of Xhosa and Caucasian subjects, would have strengthened the results found the enzyme and fibre type profiles. Therefore, it is strongly recommended that a control group be included in future studies.

6.3 References

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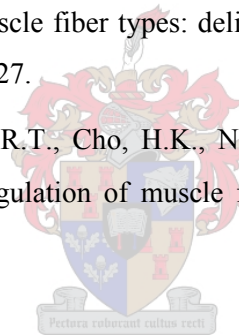
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Physiological assessment

A.1 Maximal oxygen consumption in humans

Maximum oxygen consumption (VO_{2max}) is performed using a treadmill (TechnoGym, Italy), heart rate monitor (Polar, Finland), oxygen (O_2) and carbon dioxide (CO_2) analyser (Jaeger OxyCon Pro, Germany). Before any treadmill test, subjects must be familiarized with treadmill running. This usually occurs on a separate day. Subjects are allowed a warm up period of 5 minutes and a 5 minute stretching session before commencing the test. Subjects must be instructed to always give their best and during the test, they must be verbally encouraged in order to reach their absolute maximum.

A.1.1 VO_{2max} test

The method was adapted from Weston *et al.* (1999). Thirty minutes prior to the test, the OxyCon must be switched on to allow the machine to warm up. This step is crucial as the internal components are sensitive to heat and can lead to errors in the concentration calculations of O_2 and CO_2 . Ambient conditions (air pressure and ambient temperature) are calibrated using the automatic function provided by the OxyCon software. A set volume of air is calibrated by using the automatic function. Because ambient air contains $\pm 21\%$ O_2 and the expired air of an individual contains less O_2 , calibrating with a known O_2 is not recommended (manufacturer's instructions). However, expired CO_2 of an individual is always more than ambient air, and a calibration gas containing 5% CO_2 , balanced with nitrogen (Afrox, South Africa) is used to calibrate for CO_2 . However, the CO_2 concentration is very important for accurate measurement and it is recommended that the calibration gas be analysed to two decimal places (e.g. 4.96% CO_2).

Before the test is initiated, the subject is fitted with a heart rate monitor (Polar, Finland) to measure heart rate and a mask fitted with a turbine. The mask should fit comfortably with no air leakage between the mask and skin. Connected to the turbine are infrared sensors which allows for the registration of the fins of the turbine to calculate the volume of air. The sensors relay the signal to the OxyCon. Also connected to the turbine is a small tube that allows expired air to be collected by the OxyCon for analyses of O_2 and CO_2 content.

The starting velocity for all subjects is set to 14 km/h. The subjects must be instructed to stand with their feet straddled over the treadmill, and when instructed to start, the subject must lower himself

on to the treadmill and start running. This is to ensure that the subject still has control over his movement. Similarly, the OxyCon software is started to record the data. After 30 seconds of running, the speed of the treadmill is increased with 0.5 km/h. This cycle continues until exhaustion sets in. Expired gas concentrations and volume are measured every 10 seconds throughout the test. Peak treadmill speed (PTS) and VO_2max , respiratory exchange ratio (RER), minute ventilation (V_E) and heart rate are calculated automatically by the OxyCon.

A.1.2 Criteria for obtaining VO_2max

The following criteria are used to obtain a true estimate of VO_2max for each subject: (Staab *et al.*, 2003)

- 1) was the RER greater than 1.10?
- 2) did the VO_2 plateau?
- 3) and was the heart rate at maximum for the subject within 5 beats per minute of the theoretical maximum heart rate ($220 - \text{age}$)?

At least two of the above criteria must be fulfilled to obtain a true VO_2max . If this is not the case, the test must be repeated on a separate day.

A.2 Sub-maximal exercise test in humans

The purpose of this test is to determine the efficiency of utilising fuel at sub-maximal running intensities of each athlete. The parameters include VO_2 , RER, heart rate, V_E and plasma lactate concentrations. The method was adapted from Coetzer *et al.* (1993) and Weston *et al.* (1999; 2000).

For each subject, intensities are calculated according to the PTS obtained during the VO_2max test (see section A.1). The intensities are 64%, 72% and 80% of the PTS. An additional workload was added during the HIIT programme (Chapter 5) of 20 km/h.

Prior to the warm-up and test, a venous catheter is inserted into the forearm and a resting blood sample obtained (see section B.2). Subjects are allowed a 5 minute warm up and 5 minute stretching period whereafter the mask, oxygen analyser and heart rate monitor are fitted. The mask should fit comfortably with no air leakage between the mask and skin. Subjects start the test at 64% and run for 5 minutes. After the intensity, a blood sample is collected in a fluoride oxalate test tube (Vacutainer, BD, UK) and stored on ice. For more details on blood sampling and lactate analyses, see section B.2. The procedure is repeated until the final workload has been completed.

A.3 Training logs and questionnaires

Subjects supplied the type of training completed over a period of 7 months in a normal diary (Chapter 5). This included races performed, kilometres and time during training and relative intensities. In order to assess in the analyses of the muscle biopsies and fitness level of the subject, training history assessment was also included (Chapters 3 and 4).

A.4 10 km field test

For recruitment of subjects for the study in Chapter 5, a 10 km field test was conducted. All potential subjects participated in this race. The route was carefully measured using a standard measuring wheel and markings were placed at each kilometre. Subjects ran together and during the race, they were verbally encouraged. Water was supplied at the 3, 5 and 7 km mark. Race times were recorded and logged.

A.5 High intensity interval training (HIIT) programme

The training intervention programme is based on studies conducted by Smith *et al.* (1999; 2003), Hill and Rowell (1997) and Billat (2001a; 2001b), but with modifications. The principle is as follows: each subject has a distinct speed where maximum VO_2 is reached, but the subject may increase the speed with no further increase in VO_2 . The initial speed associated with the $\text{VO}_{2\text{max}}$, is termed V_{max} . However, this value was found to correspond to $\pm 94\%$ of the subjects PTS. Therefore, the intensity of the training was set at 94% PTS for each athlete.

In order to determine training time, the subject must perform an additional test on a separate day and run as long as possible at V_{max} . This is termed T_{max} . Smith *et al.* (2003) determined that 60% of T_{max} is the minimal interval training time, with a session containing 6 intervals per day. Therefore, a session was:

6 x 60% T_{max} at 94% PTS, with $\frac{1}{2}(60\% T_{\text{max}})$ as recovery period

This is performed twice a week for 6 weeks.

A.6 Muscle biopsy – site, cutting and freezing

Each human subject underwent a muscle biopsy. The method was based on those from Bergström (1962) and Evans *et al.* (1982). A qualified medical practitioner must perform the biopsies. Local anesthetic (Xylotox: Lignocaine HCl 2% (m/v) containing Noradrenaline 1:80 000, Adcock Ingram) is injected at the site of the biopsy whereafter a cut is made with a sterile scalpel blade. A sterilised biopsy needle (Stille, Sweden) is inserted into the cut; suction applied and with a quick thrust of the

inner part of the biopsy needle, a piece of muscle is removed. The wound is attended to by the doctor and subjects must be advised not to train for at least 2 consecutive days. Applying a quick thrust ensures that the piece of muscle do not slip out from the needle. Cutting too slow also causes internal bruising and delays the healing process.

The specific site of each biopsy is determined by allowing the subject to stand up straight with arms adducted down the side. A mark is made on the *Vastus lateralis* muscle, directly beneath the thumb. This ensures that the site for each subject was always at the same ratio.

Part of the muscle sample is mounted on a wetted piece of cork marked with the sample identification and date on the opposite side. The muscle piece is covered with tissue embedding medium (Jung Tissue Freezing Medium, Leica Instruments, Germany) and frozen in *iso*-pentane pre-cooled by liquid nitrogen. The remaining piece of muscle is divided into two parts and frozen in liquid nitrogen. Samples must be stored at -87 °C until analyses.

A.7 Muscle sampling and processing of rat skeletal muscle

Rats are decapitated and the quadriceps muscle excised. The muscle is then oriented to reveal proximal, distal, superficial and deep regions. A string is tied around the base with the knot facing towards the superficial-distal part. The whole muscle is then submerged into liquid nitrogen for at least 2 minutes to allow complete freezing and stored at -87 °C until analyses.

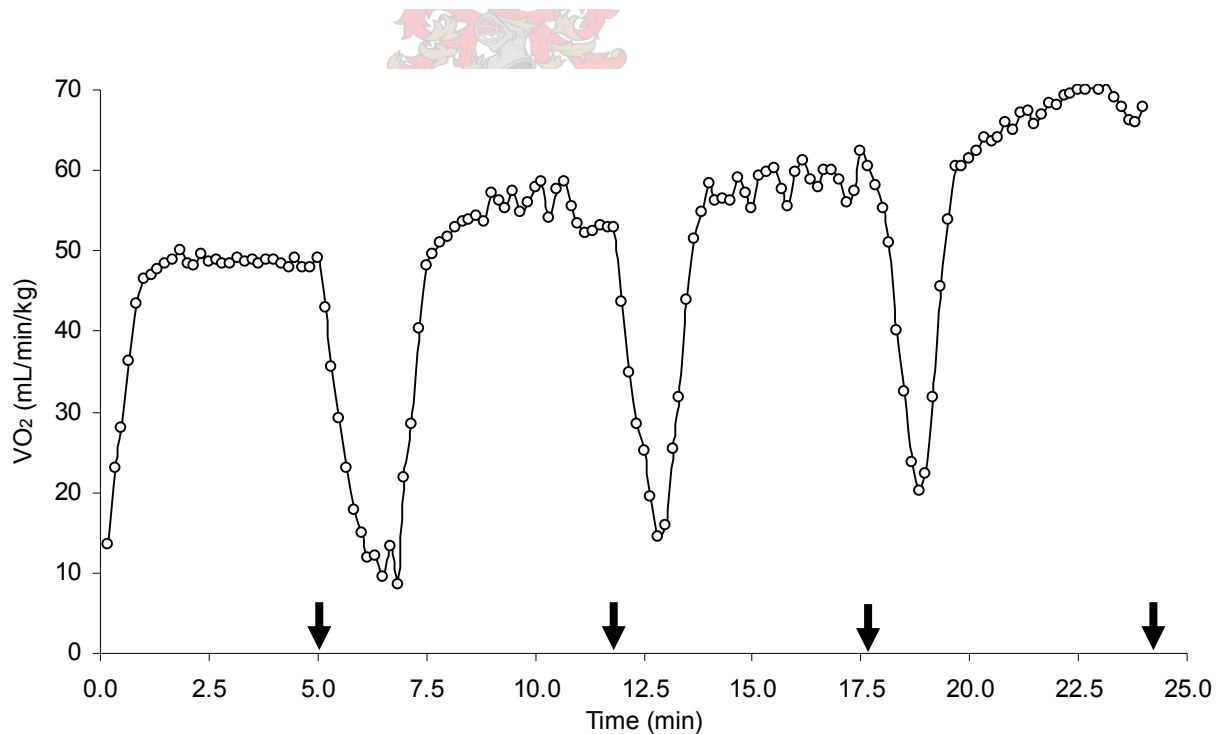
To divide the muscle into the 9 sections, allow the muscle to thaw slightly (see Figure 2.1 of Chapter 2). This makes cutting easier. Divide the muscle into the 9 sections, whereafter processing the muscle for enzyme analyses and SDS-PAGE can be performed.

A.8 Examples

Example A.8.1 Partial table of a maximum oxygen consumption exercise test (VO_{2max}) results obtained from the Jaeger OxyCon Pro.

Mpho Yanah		VO2max		Pre-Training							
Identification:	MYX200301	Date of Birth:	16/04/1983								
Last Name:	Yanah	First Name:	Mpho								
Race:	Xhosa	Sex:	male								
Height:	171 cm	Weight:	56 kg								
Age:	20 Years	Rel. Weight:	78%								
BSA:	1.65 m ²	Date:	13/08/2003								
FEV 1:	4.14 l	FVC:	4.86 l								
Temperature:	21 °C	Baro. pressure:	1009 hPa								
PTS:	21.7										

Time [min]	V'E [l/min]	BF [l/min]	VO ₂ /kg [ml/min/kg]	V'O ₂ [ml/min]	V'CO ₂ [ml/min]	RER	HR [1/min]	FEO ₂ [%]	FECO ₂ [%]	PTS
00:10	32	51	19.3	1073	932	0.87	90	17.16	3.39	13.5
00:20	42	40	29.7	1646	1336	0.81	126	16.36	3.88	
00:30	41	31	38.5	2135	1427	0.67	136	15.05	4.24	14.0
....
07:20	131	66	75.8	4207	4934	1.17	190	16.83	4.65	20.8
07:30	129	67	74.6	4140	4891	1.18	190	16.85	4.66	21.0
07:40	138	67	76.1	4222	5069	1.20	190	17.01	4.53	21.2
07:50	141	68	75.8	4209	5105	1.21	187	17.09	4.47	21.3
08:00	141	69	75.4	4184	5125	1.22	190	17.12	4.47	21.5
08:10	142	69	75.4	4185	5174	1.24	196	17.12	4.50	21.7



Example A.8.2 Graph showing the oxygen consumption per kilogram (mL/min/kg) of a sub-maximal exercise test (Chapters 4 and 5). Arrows indicate the time points where blood withdrawal took place for lactate analyses. Each workload was set to 64, 72 and 80% PTS with the final workload set to 20 km/h.

A.9 References

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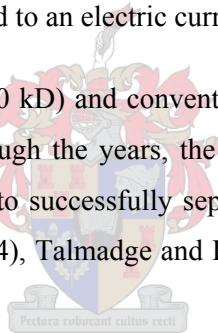
Biochemical analyses of muscle and blood samples

B.1 Myosin heavy chain (MHC) isoform separation of human and rat skeletal muscle with SDS-PAGE

B.1.1 Introduction

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an effective protein separation technique. SDS contains a hydrophobic tail and a polar head region. The principle of this technique is that combining SDS with proteins, the quaternary structure of proteins is disrupted and proteins denature. Because of the hydrophobic tail of SDS, it incorporates itself into the hydrophobic parts of the denatured protein. Furthermore, the polar negative head region gives the protein an all over negative charge allowing the protein to migrate according to size when loaded onto a polyacrylamide gel subjected to an electric current.

The MHC is a large protein (± 220 kD) and conventional electrophoresis is unable to separate the isoforms into distinct bands. Through the years, the compositions of the gels and electrophoresis conditions have changed in order to successfully separate the isoforms. The procedure below was adapted from Andersen *et al.* (1994), Talmadge and Roy (1993) and Blough *et al.* (1996) and were used in Chapters 2 to 5.



B.1.2 SDS-sample preparation

Muscle samples are placed in a sample buffer containing 10% glycerol, 5% β -mercap-toethanol, 2.3% SDS, 62.5 mM Tris buffer pH 6.80, and 0.02% bromophenol blue. Samples are mixed and heated for 10 minutes in a water bath at 60 °C. After heating, the samples are allowed to cool down and stored at -87 °C. Prior to loading onto the gel, thaw and boil the samples again for 2 minutes at 100 °C.

In order to save on human tissue, muscle homogenates prepared for enzyme analysis may be used for MHC isoform determination (see section B.3). The protein content of each sample is first measured in the homogenate samples using the Bradford method (see section B.7). For the proper band intensity on the gels, samples are then diluted with SDS-buffer according to the staining intensity table in section B.1.8.

To prepare single fibres for SDS-PAGE, see section B.6.

B.1.3 Chemicals and solutions used

Separating gel stock solutions	Stacking gel
100% Glycerol	100% Glycerol
30% Acrylamide- <i>bis</i> -acrylamide (50:1)	30% Acrylamide- <i>bis</i> -acrylamide (50:1)
1.5 M Lower Tris buffer pH 8.80	0.5 M Upper Tris buffer pH 6.80
10% SDS	10% SDS
1 M Glycine	0.1 M EDTA pH 7.0
10% Ammonium persulfate (APS)	10% Ammonium persulfate (APS)
TEMED	TEMED

Electrophoresis running buffer

Inside running buffer	Outer running buffer
150 mM Glycine	75 mM Glycine
100 mM Tris	50 mM Tris
0.1% SDS	0.05% SDS

B.1.4 Gel electrophoresis equipment

All equipment was purchased from Bio-Rad Laboratories (USA).

B.1.5 Separating gel

The separating gel had a final concentration of 8% acrylamide, 0.16% *bis*-acrylamide, 0.4% SDS, 0.2 M Tris (pH 8.8), 0.1 M glycine and 30% glycerol. The table below depicts the volumes of stock solutions used to make the separating gel for one large (16 cm in length, 1 mm spacers) or two mini gels (0.75 mm spacers).

Solution	1 x Large gel	2 x Mini gels
100% Glycerol	7.50 mL	3.00 mL
30% Acrylamide- <i>bis</i> -acrylamide (50:1)	6.70 mL	2.70 mL
1.5 M Lower Tris buffer pH 8.8	3.35 mL	1.33 mL
10% SDS	1.00 mL	0.40 mL
1 M Glycine	2.50 mL	1.00 mL
dH ₂ O	3.75 mL	1.50 mL
* 10% APS	250 µL	100 µL
* TEMED	20 µL	5 µL
Total volume	25.07 mL	10.05 mL

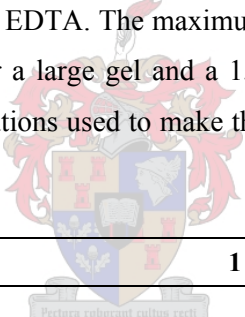
* Should be added last after degassing of solution

Procedure:

1. Clean the glass plates and spacers with dH₂O followed ethanol.
2. Assemble gel sandwich as described in the manufacturer's manual.
3. Use a permanent marker to indicate the top of the separating gel (use the comb as a guide).
4. Add the stock solutions together in a glass beaker and add the glycerol last.
5. Mix well.
6. Degas the separating gel solution for 10 minutes.
7. Add the APS and stir for 30 seconds.
8. Add TEMED and stir for 10 seconds
9. Rapidly transfer the separating gel solution between the glass plates using a pipette.
10. Add *iso*-butanol on top of the separating gel and allow polymerisation for 30 minutes.

B.1.6 Stacking gel

The stacking gel has a final concentration of 4% acrylamide, 0.08% *bis*-acrylamide, 0.46% SDS, 0.125 M Tris (pH 6.8) and 4 mM EDTA. The maximum number of wells per gel for adequate MHC separation is a 20-well comb for a large gel and a 15-well comb for a mini gel. The table below depicts the volumes of stock solutions used to make the stacking gel for one large gel and two mini gels.



Solution	1 x Large gel	2 x Mini gels
100% Glycerol	1.50 mL	500 µL
30% Acrylamide- <i>bis</i> -acrylamide (50:1)	667 µL	220 µL
0.5 M Upper Tris buffer pH 6.8	700 µL	235 µL
10% SDS	200 µL	65 µL
0.1 M EDTA pH 7.0	200 µL	65 µL
dH ₂ O	1.68 mL	560 µL
* 10% Ammonium persulfate (APS)	50 µL	15 µL
* TEMED	10 µL	3 µL
Total volume	5.01 mL	1.66 mL

* Should be added last

Procedure:

1. Add the stock solutions to a beaker (or test tube) and mix well.
2. After the 30 minutes polymerisation time for the separating gel, remove the *iso*-butanol and rinse with dH₂O. Remove excess water with strips of filter paper.

- Clean the combs with dH₂O followed by ethanol and pat dry. Insert the combs between the two glass plates.
- Add the APS to the stacking gel solution and mix for 30 seconds.
- Finally, add the TEMED and mix for 10 seconds.
- Using a pipette, transfer the stacking gel solution between the glass plates. Make sure that no bubbles form under the well.
- Allow to set for 1 hour.
- Prepare the electrophoresis running buffers as follow:

	Final concentration	gram / 1 000 mL
Inside running buffer	150 mM glycine	11.25 g
	100 mM Tris	12.1 g
	0.1% SDS	1 g
Outer running buffer	Dilute 1 part inside running buffer with 1 part dH ₂ O	

Cool buffers to 4 °C. The pH of the buffers can be disregarded.

- After 1 hour, gently remove the combs.
- Number the bottom of the wells with a permanent marker. If more than one gel is to be run, mark each gel too.
- Clamp the gel sandwiches to the cooling core of the electrophoresis apparatus (as shown in the manual).
- Fill the inside buffer dam with inside running buffer.

B.1.7 Loading and electrophoresis

Prepare samples as described under *SDS-sample preparation*.

- Use a syringe (e.g. Hamilton), and transfer the required volume to each well. NB. Use the guide in section B.1.8 for a reference of MHC band intensities. For single fibres, use the following volumes:

System	No of wells / gel	Volume
Large gel system	15	25 µL
	20	20 µL
Mini gel system	10	15 µL
	15	10 µL

- Between each sample loading, rinse the syringe three times with dH₂O.

3. After all samples have been loaded, fill the outer running buffer chamber with outer running buffer and gently insert the gel core into this chamber.
4. Add 400 μL (for large gel system) or 150 μL (for mini gel system) β -mercaptoethanol to the inside running buffer and fill completely to the top with inside running buffer.
5. Close the lid and place the gel unit in a cold room (4 $^{\circ}\text{C}$). Alternatively, a Styrofoam box filled with ice can be used.
6. Apply constant 70 V and run the mini gels for 16 hours (or over night) and large gel system for not less than 24 hours.

B.1.8 Staining and analysis

Gels are stained using a silver staining kit (PlusOne, Pharmacia) in the case of single fibres or Coomassie Brilliant Blue R250 in the case of homogenate samples. After staining, the gels are scanned using a transparency scanner (or densitometer) and images stored on a computer. For adequate scanning, resolution of the scanning software should be set between 400 and 600 dots per inch (dpi).

The following chart was developed in order to project good band resolution and to avoid under- or overloading of the SDS-samples (Kohn *et al.*, unpublished data)

Total Protein (ng) loaded	Silver stain	Coomassie R250
5	Yellow	
10	Yellow	
15	Orange	
20	Orange	
100	Orange	Yellow
500	Red	Orange
1 000	Red	Orange
5 000	Purple	Red
15 000	Black	Purple

Key to table 1

Not detected	Seen with eye, but not detected by scanner	Relatively scanned	Perfect intensity	Relatively overloaded	Overloaded
	Yellow	Orange	Red	Purple	Black

B.1.9 MHC content of homogenate samples

Homogenate samples containing two or three isoforms (showing two or three distinct bands), are further analysed using a densitometry software package (Cream 1D, KEM-EN-TEC, Denmark). The bands are expressed as a percentage of the total of two or three bands. For further detail, see the example in section B.1.11.

B.1.10 Factors influencing MHC separation

Because of the long electrophoresis duration, two issues arise that may influence band resolution, namely denaturing of proteins and heat generation. The first problem is overcome by the addition of β -mercaptoethanol to the inside running buffer prior to electrophoresis (Blough *et al.*, 1996). Uneven heat distribution across the thickness of the gel results in slanted bands (also known as ghost bands or the Venetian blind effect). Because of the MHC IIa and MHC IIx bands that are close together, this effect may result in misinterpretation of the overall MHC distribution in the sample. Figure B.1.10.1 illustrates the Venetian blind effect.

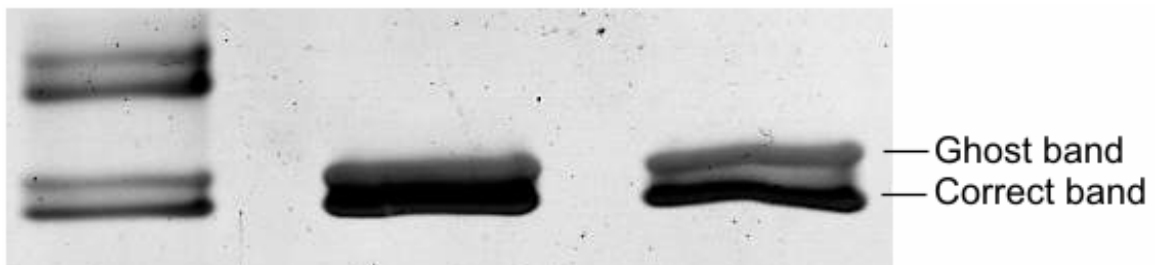
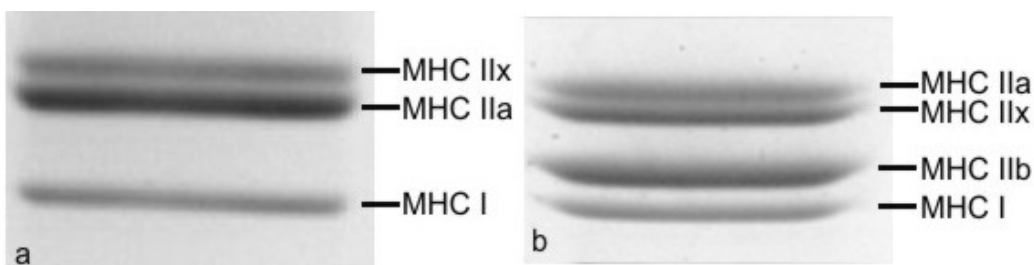


Figure B.1.10.1 The Venetian blind effect (also known as ghost bands)

A final remark on MHC separation is that enough time must be allowed for adequate polymerization of the separating gel. It was observed that if polymerization occurs too fast, the separation of all the MHC isoforms are poor.

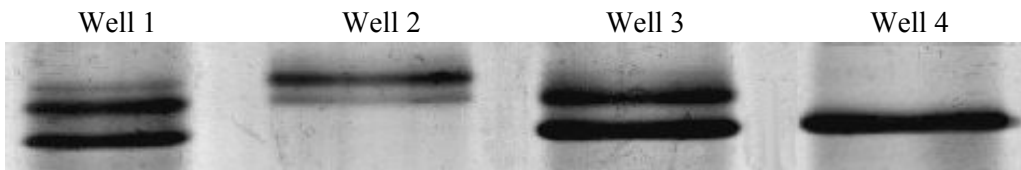
B.1.11 Examples

Example B.1.11.1 shows a human muscle sample containing all three isoforms after electrophoresis and silver staining. The lower band represents MHC I, the middle band MHC IIa and the top band MHC IIx, as confirmed by Pereira Sant'Ana *et al.* (1997).

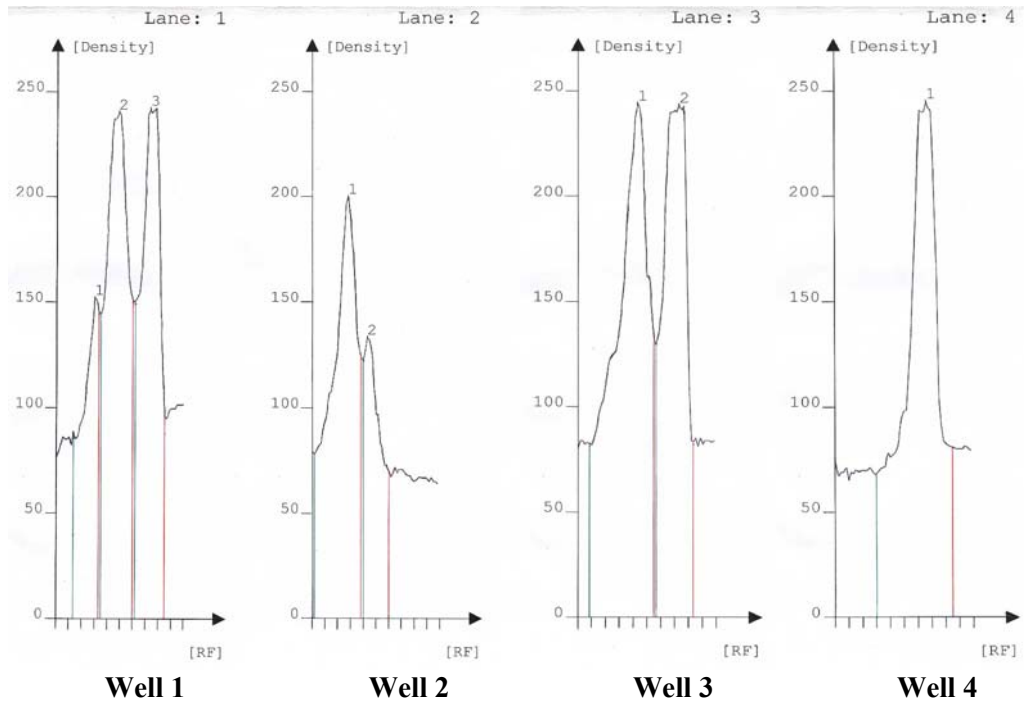


Example B.1.11.1 A muscle homogenate (a. human; b. rat) sample containing all the MHC isoforms, and indicating the location of the isoforms.

Single muscle fibre (section B.6) and homogenate MHC isoform content are shown in example B.1.11.2. These gels were scanned and analysed using the CREAM 1D software package. Example B.1.11.3 and 4 shows the histogram of relative band intensities and the percentage of each isoform, respectively.



Example B.1.11.2 Example of a gel containing 4 lanes, each with varying amounts of MHC isoforms.



Example B.1.11.3 Histogram of the bands in Example B.1.11.2. analysed with the CREAM 1D software package.

Example B.1.11.4 Results calculated by the CREAM ID software package. Area % is taken as the percentage of MHC content.

Date	: 11 Nov 2000				
Time	: 1:22:20 PM				
Sample Name	: c:\mydocu~1\tertius\experi~1\labora~1\myosin~1\gel1.bmp				
No. Lanes	: 4				
Unit	: [RF]				
Lane	Band	Height	Area	Area %	RF
1	1	152	1338	19.19	30.51 MHC IIx
1	2	240	2952	42.33	49.15 MHC IIa
1	3	242	2683	38.48	74.58 MHC I
2	1	200	2861	69.42	28.81 MHC IIx
2	2	133	1260	30.58	44.07 MHC IIa
3	1	244	4245	58.10	44.07 MHC IIa
3	2	243	3061	41.90	74.58 MHC I
4	1	245	4304	100.00	67.80 MHC I

B.2 Micro assay for plasma lactate concentration determination

B.2.1 Blood sampling and preparation

Blood is obtained from a sterile intravenous catheter (Jelco 22G, Johnson & Johnson). A sterile 3-way stop cock (Brittan Healthcare, South Africa) is attached to the catheter (refer to section B.2.3 for sequence of catheterisation). To avoid blood clotting after sampling, the catheter is flushed with a saline solution containing 0.04% heparin (Heparin Novo, Novo Nordisk, South Africa). Blood (3 mL per sample) is collected in pre-marked fluoride oxalate (as anti-coagulant) test tubes (Vacutainer, BD, UK) and stored on ice. After all blood samples are obtained, centrifuge the blood at 3 000 rpm for 10 minutes, decant plasma into clean micro-tubes and store at -87 °C.

B.2.2 Micro assay in determining plasma lactate concentration

The plasma lactate concentration of blood is determined with the aid of a kit (Lactate PAP, bioMérieux sa, France). However, the protocol was slightly modified so that samples could be analysed using a micro-plate reader. This consisted of reducing the final volume of reagent from 1 mL to 300 µL. A pilot study showed that accurate results are obtained when 3 µL of sample is used and if the reagent is diluted twice (examples B.2.3.2 and 3). The table below shows the protocol and modifications.

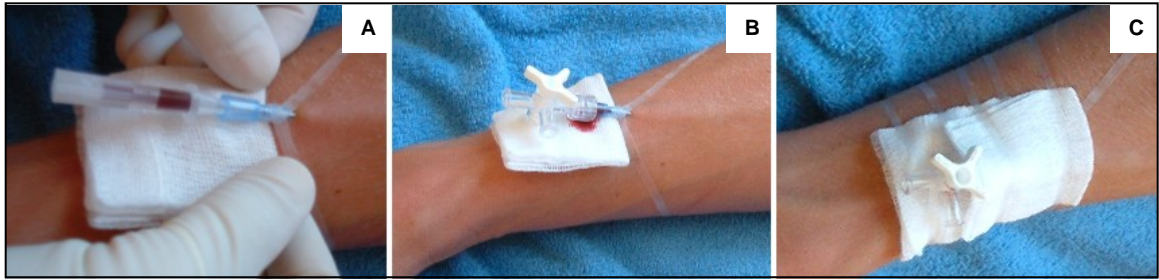
	Original assay	Modification
Lactate standard volume	10 µL	3 µL
Unknown sample volume	10 µL	3 µL
Reagent	-	Diluted twice
Reagent volume	1000 µL	300 µL

B.2.3 Examples

B.2.3.1 Catheterisation procedure

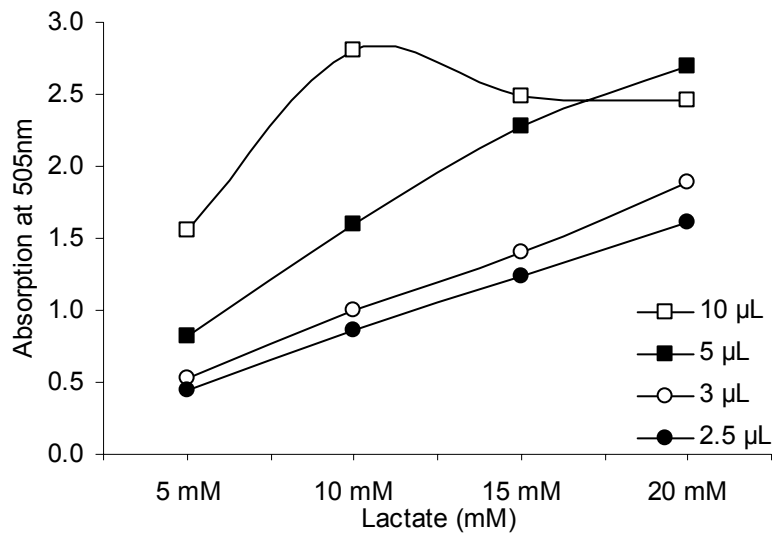
A qualified nurse, medical doctor or a qualification in phlebotomy is necessary to perform this procedure.

1. Make sure that all consumables and tubes are ready. Keep the saline-heparin solution on ice and wear gloves at all times.
2. Make sure the subject is in a comfortable position. In cold weather, it is advisable to place the forearm in a bucket of warm water for 10 minutes to dilate the veins. Dry the arm after water submersion.
3. Tie the tourniquet around the arm and tighten slightly. Search for a suitable vein and avoid valves. Also keep in mind that the veins higher up in the arm are more stable and do not constrict as much as in the lower part of the arm.
4. Shave the area where the catheter will be inserted and wipe with an alcohol swab.
5. Remove the stop cock and catheter from its sterilised wrapper. Make sure the stop cock is closed by turning the tap.
6. Gently insert the catheter into the vein and allow for the blood to flush back. Slightly pull the needle backwards, gently push the remaining part of the catheter into the vein, and place a clean piece of gauze under the catheter-end. Undo the tourniquet.
7. Tie the catheter to the forearm with a strip of TransPore, remove the needle and attach the stop cock. Fasten the stop cock to the forearm with TransPore.
8. Draw a blood sample with a clean syringe and inject \pm 1 mL saline-heparin solution. Example B.2.3.1 shows the catheterisation procedure graphically.
9. Repeat step 8 until the end of the testing procedure.
10. When blood sampling is very poor, three factors might be playing a role. The first might be vasoconstriction and to overcome this problem, allow the subject to make slow fist contractions with the catheterized arm. The second factor might be that the stop cock has not been opened completely. The third factor might be that the wall of the vein might be blocking the catheter. Slightly pull back on the catheter without applying suction from the syringe.

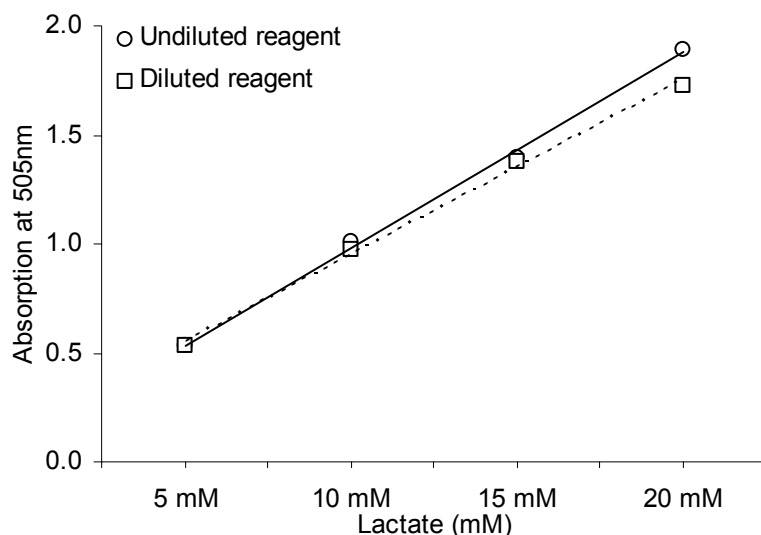


Example B.2.3.1 Main steps in catheterization. A. Insertion, blood back flush and tying of catheter to forearm, B. Attaching the stop cock, C. Tying the stop cock to the forearm.

11. When all blood sampling is completed, cut the TransPore with scissors and gently remove the catheter. Quickly wipe the site of the needle penetration with an alcohol swab and apply pressure with clean gauze.



Example B.2.3.2 Volume curves of standard lactate concentrations using 300 µL undiluted enzyme reagent from the Lactate PAP kit. Volumes 2.5 and 3.0 µL resulted in a straight line across the concentration gradient (Kohn *et al.*, unpublished data).



Example B.2.3.3 Standard curves of 3 μL standard lactate concentrations using 300 μL undiluted and twice diluted enzyme reagent of the Lactate PAP kit. (Kohn *et al.*, unpublished data).

B.3 Miniaturisation of enzyme assays for fluorometer

B.3.1 Introduction

Two different approaches were followed to determine the enzyme activities in muscle samples. In the rat study (Chapter 2), citrate synthase (CS) activity was determined spectrophotometrically, and the activity expressed as $\mu\text{mol}/\text{min}/\text{gram}$ wet weight. In the human studies, activities of four enzymes were measured namely CS, phosphofructokinase (PFK), 3-hydroxyacetyl CoA dehydrogenase (3HAD) and lactate dehydrogenase (LDH) activities. These enzymes were measured fluorometrically and their activities expressed as $\mu\text{mol}/\text{min}/\text{gram}$ dry weight.

B.3.2 Sample preparation

The homogenising buffer for the preparation of the muscle was the same for the rat and human muscle.

Homogenising buffer stock solutions (store at +4 °C):

	Chemical	Mw (mol/g)	Final concentration
A	KH_2PO_4	136.1	0.1 M
B	K_2HPO_4	228.2	0.1 M

1. Add 30 mL of buffer B to a small beaker and place the pH probe into that solution.
2. Use buffer A and adjust the buffer to pH 7.30. This buffer can be stored at $-20\text{ }^\circ\text{C}$

Rat muscle

1. Dissect the sample free of any remaining connective tissue or blood. This should preferably be done at $-20\text{ }^{\circ}\text{C}$.
2. Weigh the sample.
3. Chop sample fine with scalpel and add homogenising buffer:
1:19 for wet weight (e.g. $25\text{ mg} \times 19 = 475\text{ }\mu\text{L}$)
4. Homogenise by hand using a glass homogeniser on ice.
5. Sonicate the sample 3 x 10 seconds on ice.
6. Decant into micro-tubes. These may be stored at $-87\text{ }^{\circ}\text{C}$.
7. If connective tissue is detected in the sample, carefully remove it, pat dry and weigh. This value should be subtracted from the original weight.

Human muscle

Frozen biopsy samples are freeze dried overnight in a freeze dryer. Samples are stored in a vacuumed bottle at $-87\text{ }^{\circ}\text{C}$.

The same sample is used for all four fluorometric assays listed below. The samples are prepared as follow:

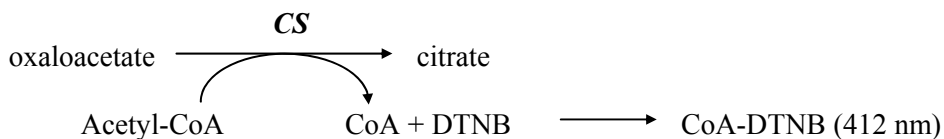
1. The freeze dried sample is allowed to reach room temperature in a temperature ($+21\text{ }^{\circ}\text{C}$) and humidity (40% humidity) controlled room.
2. Under a stereo microscope, cut a small piece off from the cross-sectional side of the biopsy in order to include all the fibres.
3. Before weighing, turn the scale on and allow warming up for at least 30 minutes. After this period, calibrate the scale using the appropriate calibration weights. During the weighing process, recheck the calibration.
4. Place the sample in a weighing pan and record the weight as mg dry weight.
5. Transfer this piece to a micro-tube. Crush the sample with a steel needle and store at $-20\text{ }^{\circ}\text{C}$ until further use.
6. Use a dilution ratio of 1:400, thus for every 1 mg of tissue, add $400\text{ }\mu\text{L}$ chilled 0.1 M potassium phosphate buffer, pH 7.30.
7. Centrifuge for 30 seconds in a bench centrifuge.
8. Sonicate sample for 3 x 10 seconds while keeping the sample on ice.
9. Vortex sample for 5 seconds and keep the sample on ice.

NB: For the PFK assay, samples can not be frozen. For the remaining three, samples may be stored at $-87\text{ }^{\circ}\text{C}$ until use.

B.3.3 Citrate synthase assay – spectrophotometrically

B.3.3.1 Principle

The assay incorporates the binding of a chemical DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) to co-enzyme A, which in turn undergoes a conformational change and is detectable at 412 nm. The formula below shows the reactions, which was based on the method by Srere (1969):



B.3.3.2 Enzyme reagent solutions

Stock solution	Comments
0.1 M Tris buffer pH 8.30	
1 mM DTNB (Sigma D8130)	Make up with 0.1 M Tris buffer. Light sensitive – store dark
4 mM Acetyl CoA (Roche 101 907)	Make up with dH ₂ O. Can be stored at -87 °C
10 mM Oxaloacetate (Sigma O 4126)	Make up with Tris buffer. Can be stored at -87 °C

B.3.3.3 Conduct of assay

1. Add 10 μL sample, 50 μL acetyl CoA, 100 μL DTNB and 795 μL Tris to a cuvette.
2. Add 50 μL oxaloacetate, invert cuvette, and read every 30 seconds for 5 min at 412 nm.
3. Determine slope as absorption per minute.
4. Calculation: $A = \epsilon cl$ where $A = \text{absorption} / \text{minute}$
 $\epsilon = \text{Extinction coefficient of } 13\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$
 $l = \text{path length (1 cm)}$

Thus

$$\frac{\Delta \text{Abs} / \text{min} \times 100}{\epsilon \times [\text{muscle}] \text{g/L}} = \text{mol/min/g wet weight}$$

B.3.4 NADH standard and standard curve generation - fluorometric

In order to use the fluorometric procedures, a standard NADH curve must be generated on the specific day of the assay. The following procedures explain how to prepare an NADH standard, how to determine the true concentration using a spectrophotometer and to generate the standard curve:

B.3.4.1 NADH standard

1. Make a sodium carbonate buffer (0.85 g Na₂CO₃ + 0.17 g NaHCO₃; make up to 100 mL with dH₂O).

2. Weigh off ± 36 mg NADH in a test tube with a screw top, add 10 mL sodium carbonate buffer, and mix well.
3. Heat the NADH solution for 10 minutes in a water bath at 60 °C to destroy NAD⁺.
4. Aliquot NADH into micro-tubes and store at -87 °C (Do not store at -20 °C).

B.3.4.2 Standardisation of NADH

1. Add 2 mL 0.1 M Tris buffer pH 8.0 to 4 quartz cuvettes (use the stock solution from the fluorometric CS method).
2. Zero the spectrophotometer at 340 nm using one of the cuvettes.
3. Pipette 40 μ L of the NADH standard to the remaining cuvettes, mix well and read at 340 nm.
4. Determine the true NADH concentration by using the following equation:

$$\frac{\text{NADH absorption}}{6270 \text{ M}^{-1}} \times \frac{\text{Total volume} \times 1000}{\text{Volume NADH}} = \text{mM NADH}$$

B.3.4.3 Generating NADH standard curve

1. Use black or white plates, depending on the sensitivity of the assay (determined by the concentration of the muscle sample used). Make sure the fluorometer is switched on for at least 20 minutes. Set the excitation wavelength to 340 nm and the emission wavelength to 460 nm.
2. Read the background fluorescence of each well.
3. Dilute the original NADH standard 11 times (1:10) with dH₂O.
4. By using the table below, pipette the required volumes in duplicate into each well. The table is marked according to the micro-plate:

	Well 1	
	NADH (μ L)	dH ₂ O (μ L)
A	0	10
B	2	8
C	4	6
D	6	4
E	8	2
F	10	0

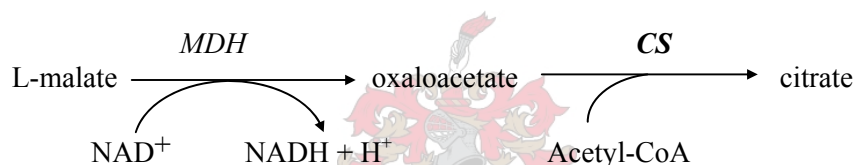
5. Add 250 μ L 0.1 M Tris buffer pH 8.0 to each well and read.
6. Subtract each background value from each NADH read.
7. Subtract the blank value from the rest of the measurements.

8. Draw a graph with the fluorescence values on the Y-axis and NADH concentration (μM) on the X-axis.
9. Determine the slope, expressed as fluorescent units / μM
10. See sections B.3.5 to B.3.8 for the assays and examples.

B.3.5 Citrate synthase – fluorometric

B.3.5.1 Principle

With fluorometry, the fluorescence is measured which comes from reduced forms of NAD and NADP. The reaction can either in itself cause the increase or decrease in fluorescence e.g. $\text{NADH} + \text{H}^+$, or be coupled to a multiple enzyme reaction, as in this case. During analysis, the fluorescence is measured at known time intervals and the difference per minute is calculated. Knowing the weight of the sample and its dilution, the enzyme activity, expressed as $\mu\text{mol}/\text{min}/\text{g}$ dry weight, is calculated. The method below is based on the methods by Essen-Gustavsson and Henriksson (1984) and Essen *et al.* (1975).



B.3.5.2 Enzyme reagent solution

Stock solution	Company	Volume	Final concentration
1 M Tris buffer pH 8.0	Sigma T-1503	5 mL	100 mM
0.1 M EDTA	Sigma ED 2 SS	1.25 mL	2.5 mM
0.1 M NAD^+	Roche 127 965	0.25 mL	0.5 mM
0.1 M L-Malate	Sigma M-1125	0.5 mL	1 mM
5 mg/mL MDH	Roche 127 256	80 μL	8 $\mu\text{g}/\text{mL}$
Make up to 50 mL with dH_2O and keep on ice.			
* 3 mM Acetyl CoA	Roche 101 907	1 mL	0.06 mM

* Add just before use

B.3.5.3 Conduct of assay

1. Set-up fluorometer software and spreadsheets before commencing the assay. (Excitation wavelength: 340 nm; emission wavelength: 460 nm)
2. Perform an NADH standard curve as described in section B.3.4.
3. Thaw the muscle homogenate samples, mix and keep on ice. Allow enzyme reagent solution to reach room temperature.

4. Pipette 5 μL sample in duplicate into the corner of each well of a black fluorescence plate (FluoroNunc plates).
5. After completion of sample transfer, add 250 μL of enzyme reagent solution to each well, using a multi-channel pipette.
6. Rapidly transfer the plate to the fluorometer and read for 3 minutes with 15 second intervals. Determine the slope of the reaction in fluorescence units / minute.
7. Repeat until all samples are completed.
8. Use the following equation to determine the enzyme activity of citrate synthase (keep in mind that the dilution factor may vary between assays).

Slope of enzyme activity: Fluorescence / minute
 Slope of standard NADH curve: Fluorescence / μM
 Muscle concentration: grams / litre dry weight or protein

Thus

$$\frac{\text{Slope of enzyme activity}}{\text{Slope of standard NADH curve} \times \text{Muscle concentration}}$$

(in units)

$$\frac{\text{Fluorescence} \times \mu\text{mol} \times \text{dilution factor} \times \text{liter}}{\text{Fluorescence} \times \text{liter} \times \text{gram} \times \text{minute}}$$

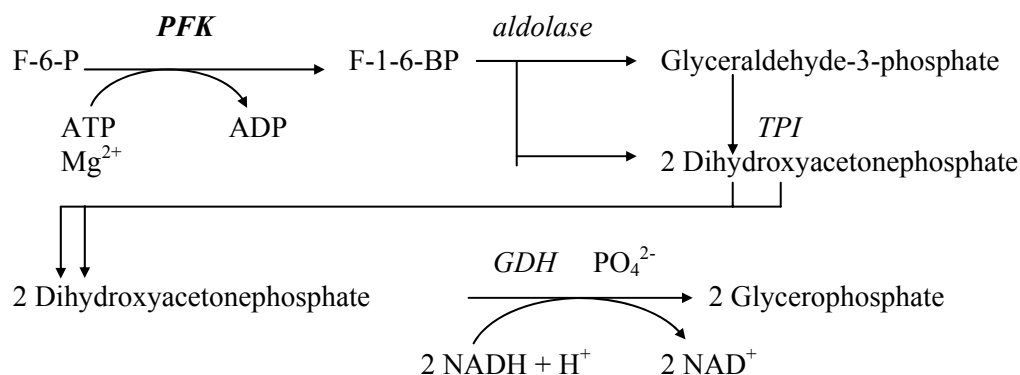
$$= \mu\text{mol}/\text{min}/\text{g muscle (dry weight)}$$

9. Refer to section B.3.9 for an example of the calculations.

B.3.6 Phosphofructokinase – fluorometric

B.3.6.1 Principle

The principle of this assay is the same as the citrate synthase assay. This assay uses a multiple combination of enzymes linked to each other in order to generate a fluorescence signal. However, during the reaction, one molecule of the substrate generates two NAD^+ molecules from NADH, therefore the final reading should be divided by two. The method below is based on the methods by Essen-Gustavsson and Henriksson (1984) and Essen *et al.* (1975).



B.3.6.2 Enzyme reagent solution

Stock solution	Company	Volume	Final concentration
1 M Tris buffer pH 8.0	Sigma T-1503	2.5 mL	50 mM
0.1 M ATP	Sigma A-5394	500 μL	1 mM
0.1 M AMP	Sigma A-1877	500 μL	1 mM
1 M MgCl_2	Merck 5833	100 μL	2 mM
0.25 M Na_2HPO_4	Merck 6580	250 μL	1.25 mM
0.1 M F-6-P	Sigma F-3627	500 μL	1 mM
0.1 M NADH	Roche 107 735	5 μL	10 μM
10% BSA	Sigma A-2153	250 μL	0.05%
14.3 M β -mercaptoethanol	Sigma M-6250	4 μL	1 mM
10 mg/ml Aldolase	Roche 102 644	60 μL	12 $\mu\text{g/mL}$
10 mg/ml TPI / G-3-P-dh	Roche 127787	40 μL	8 $\mu\text{g/mL}$

Make up to 50 mL with dH_2O and keep on ice.

B.3.6.3 Conduct of assay

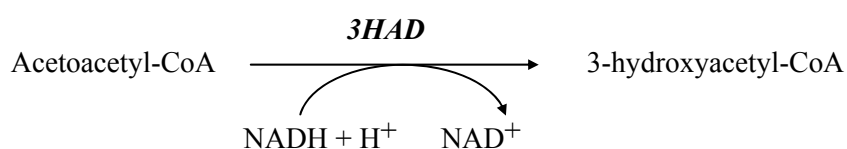
1. Set-up fluorometer software and spreadsheets before commencing assay. (Excitation wavelength: 340 nm; emission wavelength: 460 nm)
2. Perform an NADH standard curve as described in section B.3.4.
3. Prepare the homogenate muscle samples. Allow the enzyme reagent solution to reach room temperature.
4. Pipette 3 μL sample in duplicate into the corner of each well of a black fluorescence plate (FluoroNunc plates).
5. After completion of sample transfer, add 250 μL of enzyme reagent solution to each well, using a multi-channel pipette.
6. Transfer the micro-plate to the fluorometer and read for 3 minutes with 15 second intervals. Determine the slope of the reaction in fluorescence / minute.

7. Repeat until all samples are completed.
8. To determine enzyme activity, refer to section B.3.5.3 point 8. Remember to divide the enzyme activity for PFK by 2.
9. Refer to section B.3.9 for an example of the calculations.

B.3.7 3-Hydroxyacyl CoA dehydrogenase – fluorometric

B.3.7.1 Principle

The principle of this assay is the same as the citrate synthase assay. This assay uses only one reaction with 3HAD as the enzyme to generate a fluorescence signal. The method below is based on the methods by Essen-Gustavsson and Henriksson (1984) and Essen *et al.* (1975).



B.3.7.2 Enzyme reagent solution

Stock solution	Company	Volume	Final concentration
1 M Tris buffer pH 8.0	Sigma T-1503	2.5 mL	50 mM
0.1 M EDTA	Sigma ED2SS	2 mL	4 mM
0.1 M NADH	Roche 107 735	15 μ L	30 μ M
Make up to 50 mL with dH ₂ O and keep on ice.			
*1 mM Acetoacetyl-CoA	Sigma A-1625	1 mL	20 μ M

B.3.7.3 Conduct of assay

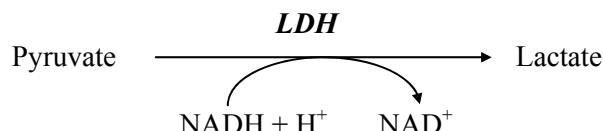
1. Set-up fluorometer software and spreadsheets before commencing the assay. (Excitation wavelength: 340 nm; emission wavelength: 460 nm)
2. Perform an NADH standard curve as described in section B.3.4.
3. Thaw the muscle homogenate samples, mix and keep on ice. Allow the enzyme reagent solution to reach room temperature.
4. Pipette 5 μ L sample in duplicate into the corner of each well of a black fluorescence plate (FluoroNunc plates).
5. After completion of sample transfer, add 250 μ L of enzyme reagent solution to each well, using a multi-channel pipette.
6. Transfer the micro-plate to the fluorometer and read for 3 minutes with 30 second intervals. Determine the slope of the reaction in fluorescence / minute.
7. Repeat until all samples are completed.

8. To determine enzyme activity, refer to section B.3.5.3 point 8.

B.3.8 Lactate dehydrogenase – fluorometric

B.3.8.1 Principle

The principle of this assay is the same as the citrate synthase assay. This assay uses only one reaction with LDH as the enzyme to generate a fluorescence signal. This is a rapid assay and it is advisable that the sample be diluted twice. The method below is based on the methods by Essen-Gustavsson and Henriksson (1984) and Essen *et al.* (1975).



B.3.8.2 Enzyme reagent solution

Stock solution	Company	Volume	Final concentration
1 M Tris buffer pH 8.0	Sigma T-1503	2.5 mL	50 mM
0.1 M EDTA	Sigma ED2SS	2.5 mL	5 mM
0.1 M NADH	Roche 107 735	15 μ L	30 μ M
Make up to 50 mL with dH ₂ O and keep on ice.			
*1 mM Sodium pyruvate	Sigma P-2256	1 mL	20 μ M

B.3.8.3 Conduct of assay

1. Set-up fluorometer software and spreadsheets before commencing assay. Excitation wavelength: 340 nm; emission wavelength: 460 nm)
2. Perform an NADH standard curve as described in section B.3.4.
3. Thaw the muscle homogenate samples, mix and keep on ice. Allow the enzyme reagent solution to reach room temperature.
4. Pipette between 2 to 3 μ L sample in duplicate into the corner of each well of a black fluorescence plate (FluoroNunc plates).
5. After completion of sample transfer, add 250 μ L of enzyme reagent solution to each well, using a multi-channel pipette.
6. Transfer the micro-plate to the fluorometer and read for 3 minutes with 15 second intervals. Determine the slope of the reaction in fluorescence / minute.
7. Repeat until all samples are completed.
8. To determine enzyme activity, refer to section B.3.5.3 point 8.
9. Refer to section B.3.9 for an example of the calculations.

B.3.9 Examples

B.3.9.1 NADH Standard curve

In order to generate a standard curve for NADH, the following procedures should be followed (must be assayed in duplicate as in example below):

1. Read the background fluorescence of the wells where the standard NADH will be transferred to. (Readings T1 and T2)
2. Pipette 0, 2, 4, 6, 8 and 10 μL of NADH into each well followed by 250 μL of 0.1 M Tris buffer, pH 8.0. Record the readings (Rd 1 and Rd 2)
3. Subtract the background fluorescence from the NADH readings (Rd 1 – T1; Rd 2- T2).
4. Average the values (Average).
5. Subtract the blank reading (containing no NADH) from the rest of the averaged values (Avg – blk).
6. Calculate the concentrations of the NADH standards as follow:

Original NADH concentration that was diluted 11x: 467 μM

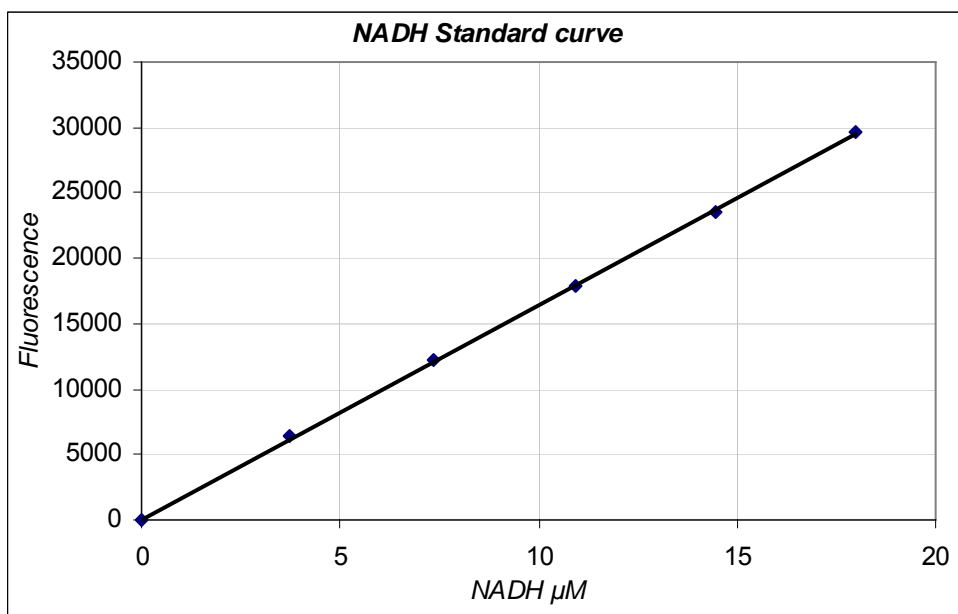
thus $467 \mu\text{M} \times 2 \mu\text{L} = \text{final concentration} \times 252 \mu\text{L}$

final concentration = $(467 \mu\text{M} \times 2 \mu\text{L})/252 \mu\text{L} = 3.71 \mu\text{M NADH}$

Repeat calculations for all.

$\mu\text{L NADH}$	T1	T2	Rd 1	Rd 2	Rd 1 - T1	Rd 2 - T2	Average	Avg - blk	NADH μM	
0	383	360	3003	3053	2620	2693	2657	0	0	
2	379	350	9356	9651	8977	9301	9139	6483	3.71	
4	345	348	15602	14728	15257	14380	14819	12162	7.35	
6	381	333	21138	20520	20757	20187	20472	17816	10.95	
8	367	345	27172	25889	26805	25544	26175	23518	14.48	
10	386	349	32275	33100	31889	32751	32320	29664	17.96	
								Slope	1631	

The slope has units that are fluorescence/ μM (FI/ μM).



Example B.3.9.1 NADH standard curve

Time	Read 1	Read 2
0.0	37205	37268
0.5	35108	35533
1.0	33405	33407
1.5	32085	32149
2.0	30290	30267
2.5	28727	28435
3.0	26974	26797
Fl/min	-3326	-3482
	-3404	
	156	
%Error	-4.57	
NADH Standard curve Fl/ μM	1631	1631
Muscle g/l	2.5	2.5
dilution	51	51
$\mu\text{mol/min/g dw}$	-41.61	-43.55
Average	42.58	

Example B.3.9.2 Enzyme activity calculations (3HAD)

B.4 ATPase histochemistry and cross-sectional area of fibres

B.4.1 Principle of method

The myosin heavy chain consists of a tale and a head region. The latter contains the ATPase enzyme and the actin binding site. Human skeletal muscle has the ability to express three myosin ATPase isoforms, each differing in enzyme activity. These different activities are directly related to the speed of muscle fibre contraction. Each ATPase isoform possesses the ability to be active or inactive under acidic or alkaline conditions. This characteristic of the myosin ATPases are utilised by prior pre-incubation of cryosections at various pH levels, followed by ATP exposure, and histochemical visualisation. Fibres containing active ATPases after pre-incubation at a specific pH will stain black, whereas those containing inactive ATPases, will remain unstained. The method is based on Brooke and Kaiser (1970a; 1970b) and Staron (1997). However, fibres termed IIB has been shown to be more closely related to the rat IIX and in this dissertation, fibres will be termed Type I, IC, IIC, IIAC, IIA, IIAX and IIX (Schiaffino and Reggiani, 1996). Figure B.4.1 indicates the various pH values and staining profile characteristics of human skeletal muscle.

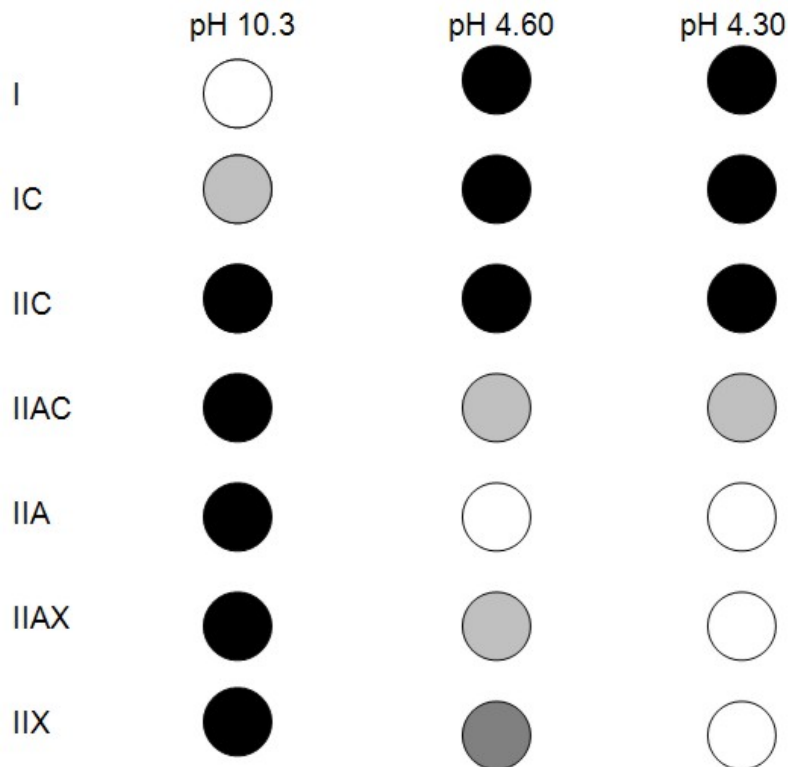


Figure B.4.1 Schematic representation of the colour intensities produced by the different stabilities of the myosin ATPase enzymes.

B.4.2 Working solutions

Chemical	Weight	Final concentration
Solution 1 pH 10.30		
Glycine	2.25 g	100 mM
CaCl ₂	2.40 g	54 mM
NaCl	1.76 g	100 mM
NaOH	1.08 g	90 mM
Make up to 270 mL with dH ₂ O. Adjust pH with 32% HCl or 5 M NaOH to pH 10.30. Fill to 300 mL.		
Solution 2		
Sodium acetate	3.90 g	100 mM
KCl	3.70 g	100 mM
Fill to 500 mL with dH ₂ O.		
Solution 2A (pH 4.30)	Adjust 100 mL of solution 2 to pH 4.30 with glacial acetic acid	
Solution 2B (pH 4.60)	Adjust 100 mL of solution 2 to pH 4.60 with glacial acetic acid	
Solution 3		
ATP (Sigma A-5394)	0.10 g	3.1 mM
Add ATP to 60 mL of Solution 1. Adjust pH with HCl to 9.40		

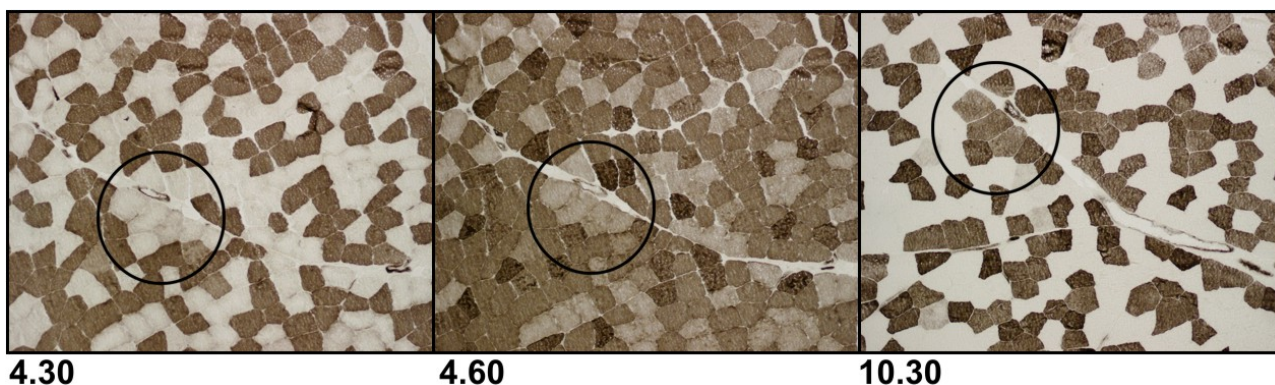
B.4.3 Procedure

1. In order to perform the ATPase staining, three muscle cryosections of 10 µm mounted on glass slides are needed. Mark each with the sample code and the pre-incubation pH. To save on muscle and time, cut 6 slides for each sample, saving the slides at 4 °C until use.
2. Pre-incubate the cryosections as follow:

Solution 1 pH 10.30	9 minutes in a shaking water bath at 37 °C
Solution 2A pH 4.30	1 minute at room temperature
Solution 2B pH 4.60	1 minute at room temperature
3. Rinse in dH₂O ten times.
4. Incubate all slides for 30 minutes at 37 °C in Solution 3.
5. Rinse in dH₂O ten times.
6. Incubate all the slides for 3 minutes at room temperature in a 1% CaCl₂ solution.
7. Rinse in dH₂O ten times.
8. Incubate all the slides for 3 minutes at room temperature in a 2% CoCl₂ solution.
9. Rinse in dH₂O ten times.
10. Incubate all the slides for 1 minute at room temperature in a 1% (NH₄)₂S solution.
11. Rinse well in dH₂O and mount with glycerine gelatine mounting medium.

12. Identify the areas in each slide using a microscope which show the same fibre patterns. pH 10.30 should be a negative image of pH 4.30 (see example B.4.4). When available, take pictures of each slide.
13. Cross-sectional area is determined on the same slides using the SimplePCI software (Nikon Instruments, Japan).

B.4.4 Example



Example B.4.1 An example of the staining intensities acquired by the ATPase staining method. (Photographs were taken with the Nikon CoolPix Microscope system, Japan.) Values below each photograph indicate the pre-incubation pH. The circle in each photograph indicates an area of identical representation of the muscle, but with different staining intensities, thus different fibre type.

B.5 Visualising capillaries

The protocol listed below utilises the periodic acid Schiff's reaction and was adapted from Andersen (1975).

B.5.1 Amylase PAS staining

B.5.1.1 Working solutions

Carnoy's fixing solution		Periodine acid solution	
Acetic acid	0.5 mL	Periodine acid (Merk 524)	0.1 g
Chloroform	1.5 mL	dH ₂ O	make up to 10 mL
95% Ethanol	8 mL	*Coleman's Feulgen reagent	
Amylase solution		Fuchsin (Merck 15937)	5 g
α -amylase (Sigma A-6880)	50 mg	K ₂ SO ₄	10 g
dH ₂ O	make up to 10 mL	1 M HCl	50 ml
		dH ₂ O	make up to 1 litre

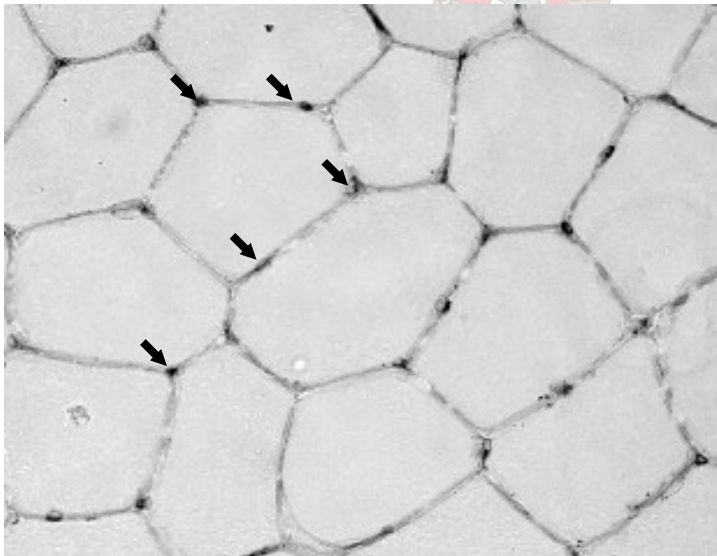
* This substance is carcinogenic.

B.5.1.2 Procedure

1. Cut 20 μm thick slices and place on glass slides.
2. Place the slides overnight in fixing solution at $-20\text{ }^{\circ}\text{C}$.
3. The following day, allow the slides to equilibrate to room temperature for 10 minutes.
4. Wash the slides well with dH_2O .
5. Incubate the slides for 10 minutes in amylase solution in a shaking water bath ($37\text{ }^{\circ}\text{C}$).
6. Rinse with dH_2O .
7. Incubate at room temperature in periodine acid solution for 12 minutes.
8. Rinse with dH_2O .
9. Incubate the slides in Coleman's Feulgen reagent for 12 minutes in a shaking water bath ($37\text{ }^{\circ}\text{C}$). After this period, make sure to discard the reagent in the proper manner for carcinogenic substances.
10. Wash the slides under running water for 10 minutes.
11. Dehydrate slides and mount with DPX (BDH, UK).

Refer to Example B.5.2.

B.5.2 Example



Example B.5.2.1 Amylase PAS staining for capillaries in human skeletal muscle. Black arrows indicate capillaries.

B.6 Single fibre preparation

B.6.1 Dissection of single fibres

The process of dissecting single fibres is very delicate and time consuming. It is therefore crucial that the muscle sample be adequately freeze-dried and had no exposure to thawing during that process. Single muscle fibres are dissected under a stereo microscope, situated in a temperature (21 °C) and humidity (< 40% relative humidity) controlled room. These conditions are essential so that freeze-dried samples absorb the minimum amount of water. Dissect using scalpel blades or dissection needles.

B.6.2 Preparation for SDS-PAGE

After an adequate number of fibres have been dissected, start transferring pieces of the fibres to capillary tubes (Vitrex, Denmark), which one end is fused and filled with SDS-sample buffer. Allow the piece of fibre to dissolve into the solution and after \pm 20 minutes, cover the end with a piece of Parafilm. After repeating this process for all the fibres, store at -87 °C until gel electrophoresis.

B.6.2 Pools of single fibres

During the preparation of the fibres for SDS-PAGE, the other half of the fibre is stored in a fused capillary tube, and closed with Parafilm. Store these fibres at -87 °C. After classification of each single fibre by SDS-PAGE, pool the fibres of the same type. Carefully weight the pools on a balance that is capable of recording 7 decimal places. Transfer the pools to 0.5 mL micro-tubes. Add homogenising buffer (ratio 1:400) to the pools using the same buffer as described in section B.3.2. Allow the pools to absorb the buffer for at least an hour, therefore keep the pools on ice. Briefly centrifuge the pools for 30 seconds to allow the pools to move to the bottom of the tube. Sonicate the pools on ice at a low frequency for 10 seconds, twice. Check if the pools have dissolved, and if not, re-sonicate. Pools can be stored at -87 °C, but not for measuring PFK activity. This enzyme needs to be measured directly after homogenisation.

B.7 Muscle protein concentration determination

B.7.1 Principle of assay

The protein assay is based on the Bradford dye binding procedure Bradford (1976), measuring the colour change of Coomassie Brilliant Blue G-250 dye, when binding to proteins. When the dye binds to protein, it undergoes a conformational change and shifts its absorption maximum to 595 nm. These bindings can be quantified and by using a standard curve, concentrations of protein can

be measured. For the assay described below, the protein concentration range is accurate between 0.05 – 0.5 g/L protein.

B.7.2 Bradford reagent

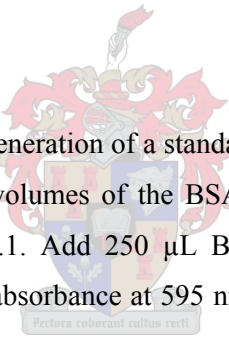
The reagent to conduct the protein assay is commonly known as Bradford reagent and is commercially available. Alternatively, it can be prepared in the laboratory. The following section describes the preparation of 500 mL of this reagent:

1. Weigh off 0.1 g Coomassie Brilliant Blue G-250 in a 200 mL beaker.
2. Add 25 mL 100% ethanol and stir.
3. Slowly add 50 ml 85% phosphoric acid and stir with a glass rod.
4. Transfer the above solution to a 500 mL volumetric flask containing approximately 100 mL dH₂O.
5. Make up to 500 mL with dH₂O.
6. Filter through Whatman no 1 filter paper and store.

B.7.3 Conduct of assay

B.7.3.1 BSA Standard curve

The Bradford assay requires the generation of a standard protein curve and a 0.5g/L BSA standard is used for this purpose. Standard volumes of the BSA standard are transferred to a micro-plate in duplicate according to table B.7.1. Add 250 µL Bradford reagent to each and allow 5 minute incubation before measuring the absorbance at 595 nm in a micro-plate reader. For more details on calculations, see section B.7.4.



	0.5 g/L BSA	dH ₂ O	Bradford reagent	[BSA] g/L
Blank	–	10 µL	250 µL	0.0
Standard 1	2 µL	8 µL	250 µL	0.1
Standard 2	4 µL	6 µL	250 µL	0.2
Standard 3	6 µL	4 µL	250 µL	0.3
Standard 4	8 µL	2 µL	250 µL	0.4
Standard 5	10 µL	–	250 µL	0.5

Table B.7.1 Standard volumes added to a micro-plate to generate the standard curve for the Bradford assay.

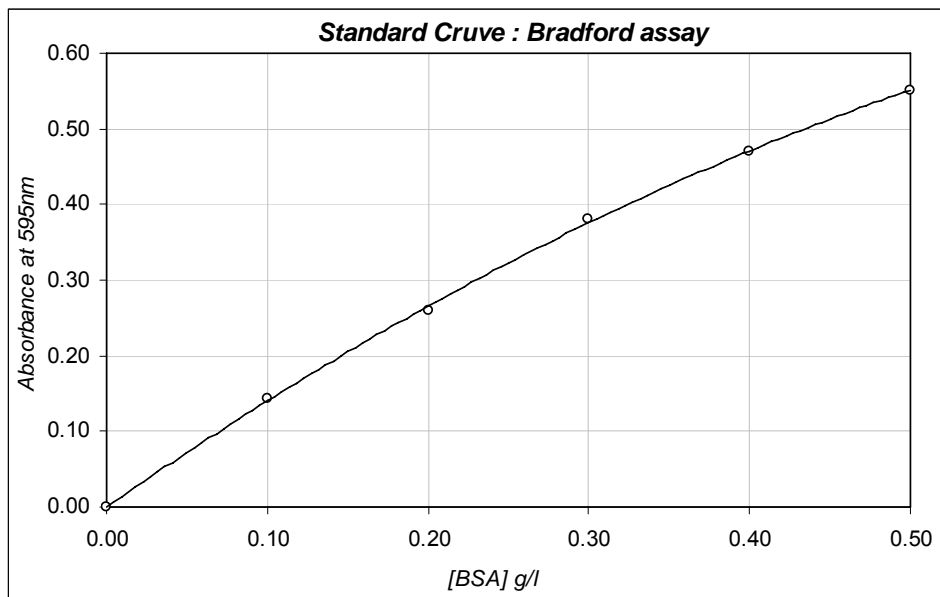
B.7.3.2 Unknown sample

Pipette 10 µL sample in duplicate followed by 250 µL Bradford reagent. When it is suspected that the protein concentration might be above the allowed range, dilute the sample (e.g. 5x).

B.7.4 Example

Standard curve

[BSA] g/L	Absorption reading 1	Absorption reading 2	Average
0.0	0.00	0.00	0.00
0.1	0.13	0.15	0.14
0.2	0.24	0.28	0.26
0.3	0.38	0.38	0.38
0.4	0.47	0.47	0.47
0.5	0.55	0.55	0.55



Equation: $y = -0.75x^2 + 1.48x$

Use the following equation to calculate the concentration of the unknown sample:

$$x = -b \pm \frac{\sqrt{b^2 - 4ac}}{2a}$$

Read 1	Read 2	Average	SQRT($b^2 - 4ac$)	$(-b \pm X)/2a$	dilution 1x	[Protein] g/L
0.325	0.330	0.328	1.78	0.42	1	2.2

B.8 References

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