Training induced adaptations in horse skeletal muscle

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Training geïnduceerde adaptatie in skeletspieren van paarden
(Met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

Various human and equine studies have been carried out to unravel the mechanisms behind adaptation towards training and mal-adaptation due to periods of intensified training, the latter also known as overtraining[5, 10, 19, 26, 33, 46-48]. The search for markers of overtraining has been subject of a number of scientific studies. The major difficulty in finding such markers is related to the differences of the model used and the constancy of the parameters investigated. To date, the (over)training models used have either focused on orthopedic, metabolic, psychologic or patho-physiological aspects as separate entities. Furthermore, it may be questioned whether the training programs used in the scientific studies published coincide with the normal work of equine trainers. Another aspect that has not received proper scientific attention is the use of human derived parameters in equine tissue. Finally, the major drawback in most equine studies is the fact that the horses used have a veterinary medical history that is often unknown and have experienced different training regimens under different trainers in the past.
Therefore, in this thesis we will focus on the development of an equine model of (intensified) training in conjunction with the verification of markers in equine tissue that, previously, have been shown to play a role in the (mal)adaptation process in human training studies.

Throughout history horses have been acknowledged for their supreme power, speed, endurance and work capacity. Once domesticated, about 6000 years ago, horses were mainly kept for meat and milk. Shortly after, humans started riding horses, and the horse was used as pack animal by nomadic tribes. Horses soon became the favourite draft animal for wagons and with the development of lighter wheels and chariots the combination of horse and wagon became a powerful weapon in warfare and hunting. Due to the increased physical demands of hunting and warfare, training and skills development became an important issue. The first recordings of systematic training of warhorses dates back to around 1350 B.C., as the Hittite horse master Kikkuli recorded his training methods on four clay tablets. He describes a 7 month training regimen that involves systematic conditioning, grain feeding and introduces the precursor of interval training. When these methods were reproduced at the University of New England, Australia (1993)[34] it resulted in extremely fit horses while remaining sound. The first text on training horses for mounted cavalry is written by a Greek general, Xenophon (400 B.C.). He introduced a sensible understanding of the horse with great emphasis on the horses' wellbeing and reasonable introduction to new skills. Although warfare has been a recurring stimulus for horsemanship, horses have also been used throughout history as sports animals. The use of horses in racing events dates back to the classic Olympic games. The origins of modern horse racing, and thus performance training, lie in the 12th century, when the English crusaders returned with swift Arabian horses.

Beneficial effects of training for health and wellbeing

Beneficial effects of regular exercise and physiological training are widely known in human medicine. These effects vary from increased quality of life, extended life expectancy, reduced mental health problems and a positive effect on metabolic disorders[4, 6, 9]. On the basis of physiological similarities between men and horse, beneficial effects of regular exercise on health and well-being are also expected for horses. Evolutionary horses wandered for miles on the great plains in Eurasia, ready for flight from any danger. Regular exercise is therefore not only beneficial; it is one of the main necessities of life for equids. It is therefore remarkable to notice that modern equine training practice focuses mainly on technical skills rather than conditioning exercises. Scientists from the field of human and equine exercise physiology agree that basic training principles based on physical conditioning apply to both humans and horses. We must bear in mind, however, that equine and human physiology is not similar.

The major objective in exercise and training is to cause physiologic adaptations to improve performance in specific tasks. Therefore, the effects of training are largely dependent on the type of exercise applied. This requires carefully planned and executed activities, and adherence to the training program.

Independent of the objective of a training program, two general principles must be fulfilled, i.e. the overload principle and the specificity principle.

Overload principle:

A specific exercise intensity or overload must be applied in a training session in order to force the body to adapt. If the exercise intensity is too low, no gain in performance can be anticipated[26, 32]. The exercise intensity is the most important factor to increase performance, and should be well above 'normal'. The appropriate overload for each subject or animal can be achieved by manipulating the training frequency, intensity and duration, both within one training session, as well as between training sessions. If a training program is well constructed on an individual basis, recovery and adaptation will occur within 3-7 days. If, however, insufficient rest is taken between the training loads, the athlete enters a situation of chronic overload, which results in a delayed adaptation process[26, 30].

Specificity principle:

The training response is highly specific. In aerobic or endurance training for example the mode of training must overload the appropriate muscles, as well as the cardiovascular system. In jumping events, lasting around one minute, anaerobic power, strength of particular muscles and neuromuscular coordination determine performance. If the principle of specificity is not accomplished, performance enhancement can not be anticipated[32].

Malicious effects of (over)training

Despite the beneficial effects on health, training is also associated with health problems in humans and horses. Horses may develop tendon or bone injuries, muscular problems or even mental problems[36, 50]. Especially when the training load (exercise intensity times duration) exceeds exercise capacity, horses are prone to problems. If this repeated overload persists for a period of time, an athlete may enter a state of chronic overload, also known as overreaching or in a more severe situation overtraining. If recovery takes place within 2-3 weeks, the athlete is considered to be overreached, whereas when fatigue and performance remains decreased for more than 6 weeks, the athlete is considered to be overtrained[10, 26, 47, 48]. The symptomatology of overreaching and overtraining is very complex, which prompted researchers to speak of the 'overtraining syndrome'[5, 10, 26]. For the past decades much research has been dedicated to unravel the mechanisms behind the occurrence and development of chronic overload. Various stages of chronic overload can be distinguished, e.g., mechanical overload, metabolic overload, overreaching and overtraining or staleness. Mechanical overload results from too much mechanical stress on connective tissue, tendons, articulations and bone and is reflected in orthopedic problems in horses. Metabolic overload is observed mainly in skeletal muscle when substrate availability during exercise is insufficient and leads to early fatigue despite unchanged, or even decreased, exercise intensity[27]. When rest periods are too short to replenish substrate storage and protein synthesis in skeletal muscle, which is very important to prevent, among others, orthopaedic problems, overreaching may occur. If sufficient rest is taken, these problems may disappear within a period of two weeks. If the imbalance between rest and exercise is maintained however, the total amount of physiological stress results in far-reaching dysfunction of both central (neuro-endocrine, central nervous system) and peripheral (skeletal muscle damage, high susceptibility for inflammation) systems. It may take several months to a year to recover from this long-term overtraining or staleness[26, 29, 48].

Skeletal muscle structure and contraction

As mentioned before, skeletal muscles are among the most sensitive organs for adaptive changes induced by exercise and training. Therefore, knowledge of functional anatomy is essential for the understanding of the effects of chronic overload.

A muscle consists of a large series of parallel muscle fibres which generates force for contraction. Each muscle fibre is essentially an individual muscle cell. It is 30-100µm in diameter and can have a length of several millimetres up to several centimetres. Each muscle fibre is enclosed by a plasma membrane (sarcolemma) and a thin layer of connective tissue, the endomysium. A bundle of muscle fibres is called a muscle fascicle and is also encircled by a layer of connective tissue and collagen called the perimysium. The muscle compartments that make up the muscle are held together by a

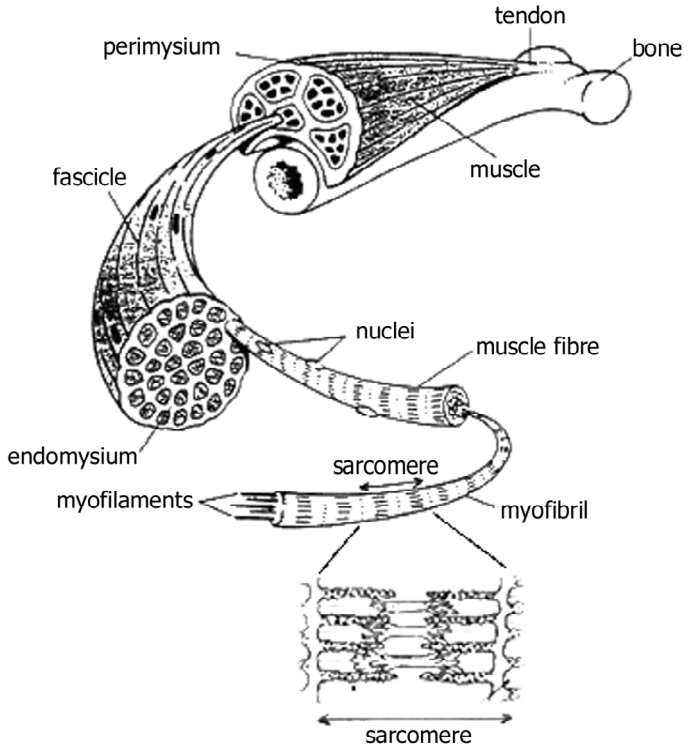
dense layer of connective tissue, the epimysium, which extends into the tendon of a muscle. These connective tissue layers are interconnected which makes it essential in the mechanical transmission of force from the muscle fibre (figure 1.1).

Muscle fibres originate in the embryonic phase as myoblasts that fuse into multinucleated myofibers and eventually form skeletal muscle tissue. The core of the mature muscle cell is filled with organelles, of which the myofibril is the most abundant. Other important organelles are the sarcoplasmatic reticulum, the mitochondria and the nuclei. The myofibril contains the contractile proteins actin, troponin, tropomyosin and myosin. Myosin and actin together represent 55% of total protein in skeletal muscle. These proteins are organized into sarcomeres with a length of approximately 2 µm that results in the striated appearance of skeletal muscle[25].

Figure 1.1

Schematic representation of skeletal muscle anatomical makeup.

The muscle is composed of muscle fascicles that contain muscle fibres. The myofibrils in the muscle fibre are responsible for muscle contraction.



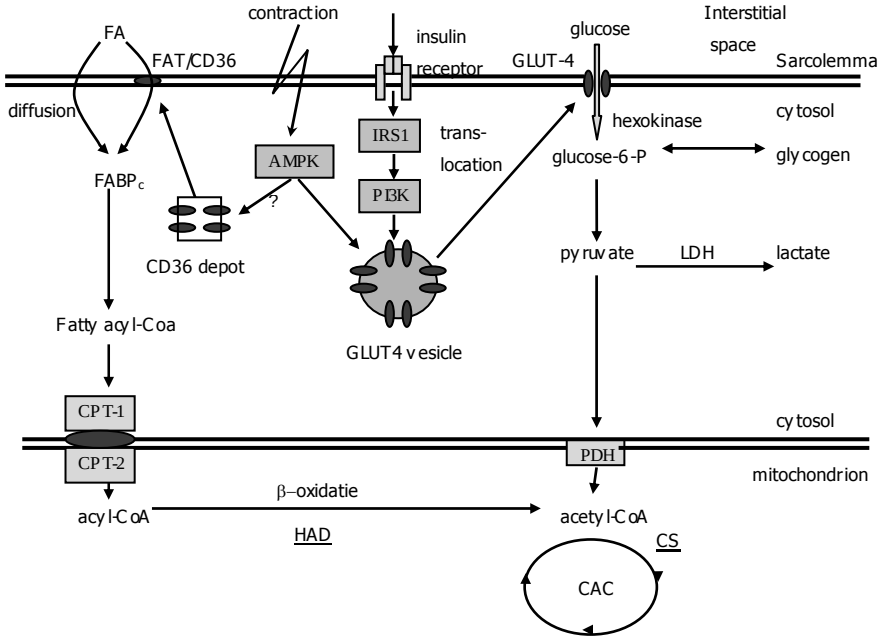
A muscle contraction is initialized when the muscle fibres' sarcolemma is depolarized by an electrical stimulus from the nervous system. The depolarization of the sarcolemma results in a release of calcium ions from the sarcoplasmic reticulum. Calcium ions are necessary to initiate the interaction between actin and myosin. The energy source for contraction is provided by adenosine tri-phosphate (ATP) that binds to the myosin ATP-ase. The binding of the ATP to the myosin molecule initiates a conformational change in the protein that is responsible for the contraction. The transport of calcium ions back into the sarcoplasmic reticulum is also ATP dependent[32].

Skeletal muscles contract due to the innervation by motor neurons from the spinal cord. The neuron leaves the spinal cord through the ventral root and is peripherally divided into many branches. Each branch innervates a single muscle fibre, and the neuron (with branches) and the fibres innervated by the neuron is called the motor unit. In order to increase muscle force, an increased amount of motor units can be recruited by the central nervous system[11]. Alternatively, the firing rate of the motor unit can be increased, leading to fiercer contractions of the particular motor unit[41]. The depolarization of the sarcolemma induced by the motor neuron spreads throughout the muscle fibre and the interstitial fluid surrounding the muscle fibre. These voltage differences from a single motor unit can be measured by needle electromyography (EMG) and is called a motor unit potential (MUP). The shape of the MUP can provide valuable information about neuromuscular properties[51].
Not all muscle fibres are the same however. Based on morphology, histochemistry and biochemistry muscle fibres can be classified in several fibre types. The two main classes are slow-twitch, or type 1 fibres, and fast-twitch, or type 2 fibres. In general, type 1 fibres have a higher oxidative capacity and are therefore more fatigue resistant. Type 2 fibres are less fatigue resistant, but can generate more power due to their faster contraction speed. Type 2 fibres can be sub-divided in 2a, 2x and 2b fibres, of which the type 2a fibres have the highest, 2b the lowest and 2x intermediate oxidative capacity. Equine skeletal muscle does not contain type 2b muscle fibres however[39]. It has been shown in several species that fibres with characteristics of two or more different muscle fibre types exist, so-called hybrid fibres[7, 8, 37].
In a skeletal muscle the fibre type distribution is mainly determined genetically in the developmental stages, however, fibre type distribution can be significantly changed by neuromuscular activity[37], exercise[15, 43], age[8] and disease[35].

Substrate metabolism in skeletal muscle

The muscle contraction depends largely on the energy supplied by ATP. The concentration of ATP present in the muscle fibre, about 6 millimolar (mM) in horses, is sufficient to maintain a full contraction for 2 seconds. After the ATP is split into ADP and P_i, the ADP is rephosphorylized into ATP in a fraction of a second. For this purpose, several energy stores are present in skeletal muscle cells. Important energy stores are phosphocreatine, glycogen and triglycerides. The phosphocreatine supply in skeletal muscle is exhausted after 10 seconds of maximal contraction[31]. During rest and exercise, glucose and triglycerides provide the most energy needed. Both substrates are stored in skeletal muscle, glucose as glycogen and triglycerides as lipid droplets. Glucose from the blood can not pass the sarcolemma of skeletal muscle itself because of the lipophobic nature of the molecule. A family of glucose transport proteins has been discovered that transports glucose across the cell membrane. In skeletal muscle, the most abundant glucose transport proteins are GLUT-1 and GLUT-4[13, 38]. These proteins form pores in the plasma membrane that selectively allows glucose to pass. GLUT-4 is a protein that resides in an intracellular compartment and translocates to the plasma membrane after distinct stimuli among which insulin and contraction are the most potent. These stimuli act through independent pathways in skeletal muscle. Intracellular, glucose is immediately phosphorylized by the enzyme hexokinase. This traps glucose inside the cell and it is stored intracellular as glycogen granules (figure 1.2).
For a long time, it has been thought that fatty acids can pass the plasma membrane effortlessly due to their lipophilic nature. To date, we know that long chain fatty acids use a facilitating transport mechanism[1, 12, 21]. At least three transport proteins have been associated with facilitated fatty acid transport, fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP) and plasma membrane fatty acid binding protein (FABPpm). FAT/CD36 is mainly expressed in low abundance in oxidative muscle fibres and in sarcolemmal aggregates all fibre types (figure 1.3). It has also been shown to translocate to the plasma membrane in a similar way as GLUT-4[2]. Intracellular, fatty acids are stored in lipid droplets as triglycerides near the mitochondria.
There are two main pathways for the metabolism of these energy stores, and the biochemical conversion of these energy stores is catalyzed by a number of specialized key metabolic enzymes.

Figure 1.2
Schematic overview of skeletal muscle metabolism
This figure provides a schematic representation of glucose and fatty acid metabolism in skeletal muscle. The right hand side represents a summary of glucose metabolic pathways and the left hand side a summary of fatty acid metabolic pathways. These pathways converge at the citric acid cycle (CAC).

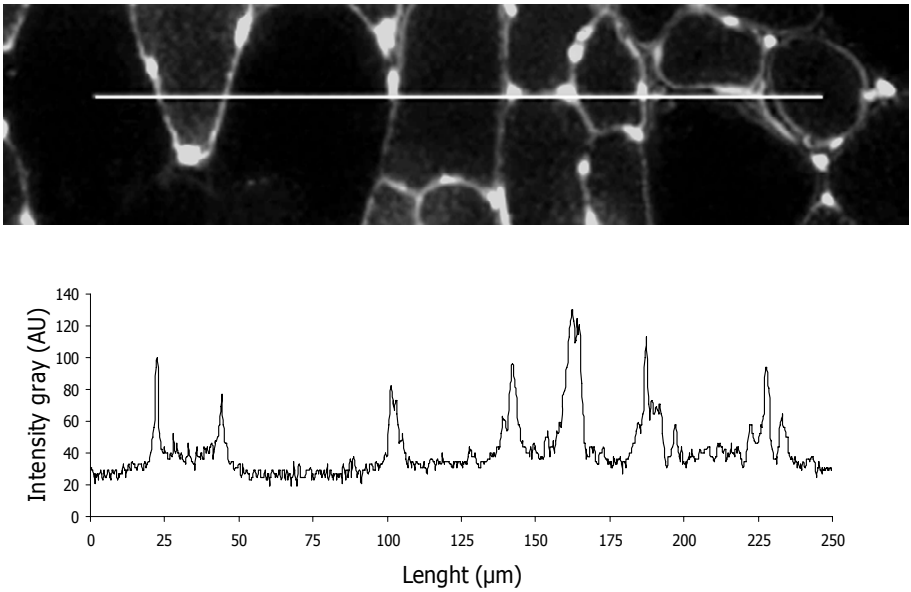


Abbreviations: FAT/CD36, fatty acid translocase; FABPc, fatty acid binding protein cytosol; CPT, carnitine palmytoyl transferase; AMPK, adenosine monophosphate activated protein kinase; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; HAD, 3-hydroxyacyl-CoA dehydrogenase; GLUT-4, glucose transporter 4; PDH, pyruvate dehydrogenase; CAC, citric acid cycle; CS, citrate synthase; LDH, lactate dehydrogenase.

The first pathway involves the anaerobic breakdown of glucose, glycolysis. In this pathway glucose from glycogen or plasma is broken down into pyruvaat (figure 1.2). In the absence of sufficient intracellular oxygen pyruvaat is converted to lactate that is removed from the cell by specific transporters, monocarboxylate transporters (MCT). These MCT proteins promote transport of lactate anions and protons from production sites (anaerobic metabolism, mainly in fast glycolytic muscle fibres) to oxidation sites (heart, brain and oxidative muscle fibres).
A large family of these transport proteins has been identified in various species and tissues[3, 18, 17]. In human and rodent skeletal muscle the main MCT isoforms present are MCT1 and MCT4 and a small amount of MCT2[3, 20].

In porcine skeletal muscle also evidence of MCT2 expression has been established[42]. These different isoforms have a specific function in inward or outward transport of monocarboxylates, however they are not capable of transporting lactate against a concentration gradient. Because MCT1 is mainly expressed in red oxidative muscle fibres, and has a low equilibrium constant (K_m) and high transport capacity for lactate anions, it has been proposed to function mainly as lactate anion importer. On the other hand, MCT4 is mainly expressed in white glycolytic muscle fibres and has a high K_m and lower lactate anion affinity, and therefore it is probably related to lactate anion efflux[18]. Much less is known about the exact role of MCT2 in skeletal muscle[18, 20, 42]. In equine skeletal muscles these proteins have not yet been described however.

Figure .1.3
FAT/CD36 expression in skeletal muscle sarcolemma
Fluorescence labelling of FAT/CD36 (upper panel) is shown in mice skeletal muscle. Gray intensity, representing FAT/CD36 expression, is measured along the horizontal line, and is shown in the graph in the lower panel. FAT/CD36 is primary located near the plasma membrane and in sarcolemmal aggregates as is shown in this figure.



If sufficient oxygen is available, pyruvaat is transported into the mitochondria and is converted into acetyl-CoA that is further metabolized in the citric acid cycle (CA-cycle). The second pathway is the aerobic breakdown of fatty acids, a metabolic favourable pathway because fatty

acids provide over three times more ATP per gram than glucose. Fatty acids have to be transported into the mitochondria first by a specific transport protein, carnitine palmytoyl-CoA transferase 1 (CPT1)[44]. Once inside the mitochondrial matrix the β-oxidation pathway splices two carbons from the carbon skeleton of the fatty acid. These are converted in acetyl-CoA, and the cycle repeats itself until all carbon units are converted to acetyl-CoA. The rate limiting enzyme in β-oxidation is 3-hydroxyacyl-CoA dehydrogenase (HAD). The acetyl-CoA then enters the CA-cycle at the same point as acetyl-CoA from pyruvaat. Citrate synthase (CS), a key enzyme in the CA-cycle, is considered a good measure for mitochondrial density and the maximal speed of aerobic metabolism[32].

Metabolic training adaptation

During exercise the energy demand is acutely increased and this has to be met by a concomitant increase in ATP production. Enzyme activity increases due to stimulatory factors as decreased ATP/ADP ratio, increased cellular calcium concentration and removal of allosteric inhibition factors. Beside these acute effects, also adaptation to the chronic exercise stress and increased energy demand occurs. These adaptations involve increases in energy stores, glycogen and triglycerides[16, 43], but also an increase in gene transcription and protein synthesis[22]. If the exercise stimulus is repeated, as happens in training, long term adaptations occur in skeletal muscle to cope with the chronic increased energy expenditure. Most of these changes have been observed in a number of mammalian species. One of the most universal adaptations to training are changes in cardiovascular function[32]. An increase in cardiac output and capillarization in skeletal muscle allow an increase in substrate and oxygen supply to skeletal muscle. Increased capillarization in skeletal muscle does not only increase substrate and oxygen supply to skeletal muscle, but also provides an increase in surface area for substrate and oxygen transport[32]. These increases of substrate supply and capillary surface area result in a higher flux of substrates across the sarcolemma. This is illustrated by a increase in protein expression after training of both GLUT-4[38], for glucose transport, and FAT/CD36[45], for fatty acid transport. The signalling events responsible for the translocation of GLUT-4 to the plasma membrane are also up regulated due to training[52]. Therefore a larger increase in membrane bound GLUT-4 is observed with an unchanged stimulus after training[14]. Inside the muscle cell, energy storage capacity is increased, resulting in higher glycogen concentrations in trained subjects and higher intramuscular lipid droplets in trained subjects[16, 43]. After exercise these intracellular substrate sources are faster replenished in trained skeletal muscle. Dependent on training strategy, increases in glycolytic or oxidative enzyme activity or both are observed. After endurance training the increase in energy expenditure during exercise at the same sub-maximal relative workload as untrained subjects, is almost totally accounted for by an increase in fatty acid oxidation[49]. This is reflected by increases in oxidative enzymatic activity and increased mitochondrial density[23]. High

intensity training on the other hand, results in increases in glycolytic enzyme activity[40]. This leads to a higher endurance during high intensity performance. Dependent of the aim of the training, several forms of training can be applied, each with its specific physiological adaptations. The sum of these physiological adaptations however, leads to an increase in performance.

Outline of the thesis

It appears that the physiological and biochemical adaptation of skeletal muscle to training in equine species shows a lot of similarities with human and rodent physiological adaptation. On the other hand it is becoming increasingly clear that intra-cellular mechanisms of adaptation (substrate transport, enzyme activity, etc) differ considerably between species. To date, our knowledge about adaptation mechanisms to training and acute exercise in horses still trails behind our knowledge in humans and rodents. The major drawbacks in equine training physiological research are the lack of an appropriate training model and the lack of control of sensitivity and specificity of parameters used in human and rodent research. Furthermore, scientists become aware of the fact that the coupling of biochemical properties of skeletal muscle and other physiological systems play a role in the whole body adaptation during training adaptation.

The present thesis focuses on the development of a scientific based controlled equine training model and the adaptation processes in equine skeletal muscle physiological, biochemical pathways based on human and rodent training models.

The two major aims of this thesis are:

1. To develop a controlled training program based on training that is being used in equine sports, in particular Standardbred trotters.
2. To evaluate adaptation in equine skeletal muscle to exercise and training in substrate transport proteins, enzyme activity and electromyographic properties

For this purpose, in chapter 2 we will describe the general research design of the study. Here we apply a standardized training protocol based on practical Standardbred trotter training regimens.

In chapter 3 we will determine the immunohistochemical expression and localization of GLUT-4 and FAT/CD36 in equine skeletal muscle as has been successfully demonstrated in human and rodent models.

Chapter 4 describes the effects of training on the expression of specific membrane transport proteins for lactic acid. During exercise, one of the principal causes of fatigue is lactate accumulation in skeletal muscle. In humans and rodents specific lactate transporters (monocarboxylate transporters) have been identified. We hypothesized that the equine Standardbred trotter expresses MCT proteins in skeletal muscle in the same way as humans and rodents.

In chapter five the metabolic effects of acute exercise are studied before and after a period of 18 weeks of training. Acute exercise leads to activation

of a broad spectrum of systems, including metabolic key enzymatic activity in skeletal muscle. Whether such changes also apply to horses is still a matter of debate.
In chapter 6 we will focus on the effects of training and intensified training on the EMG signals in Standardbred horses. Recently it has been shown that neuromuscular excitation is improved in well trained, non fatigued endurance athletes but deteriorates after prolonged heavy exercise and high volume training, leading to low amplitude EMG[28]. This may be related to the exercise-induced symptoms of fatigue[10, 24, 26, 48]. Therefore, in the study reported in chapter six, EMG and metabolic key enzymatic activity are measured after a period of normal and intensified training in order to evaluate the role of EMG in healthy trained and intensified trained Standardbred trotters.
Chapter 7 describes the use of a pathological state in horses (lower motor neuron disease [LMND]) as a denervation model for metabolic research. LMND is diagnosed based on histopathological and electromyographical (EMG) analysis. The disease is characterized by degeneration of neurons innervating type 1 and type 2 fibres[35]. This leads to muscle atrophy, fasciculations, muscle weakness and an abnormal high whole body glucose metabolism. We hypothesize that EMG signals are coupled to changes in skeletal muscle intracellular carbohydrate metabolism.
Finally, in chapter eight, the overall results of this thesis will be discussed in the light of present literature regarding acute exercise and periods of normal and intensified training.

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Chapter 2

General research design of training and intensified training

The main interventions in this thesis consist of prolonged training and intensified training. The training regimen used for these experiments is based on experiences in Standardbred trotter training practice and a previously performed study in Standardbred trotters[2]. This training is specifically designed to induce changes in a wide range of physiological parameters that are influenced by training and intensified training. Physical exercise brings about major adaptations in the metabolic profile of skeletal muscle parallel to physiological muscular parameters like increases in capillarization, strength and hypertrophy[8]. It is hypothesized that the training protocol applied in this thesis induced measurable metabolic adaptations in skeletal muscle. Exercise and training induced changes in behaviorlogical, endocrinological and muscular signal transduction and muscle proteome are currently investigated in parallel studies. This chapter provides a broader insight in the main training interventions applied in the studies described in upcoming chapters.

Research design

Horses

A total of twelve healthy Standardbred geldings were included in the study after a two-month quarantine period at the research centre during which they walked daily 45 minutes at the walking machine. At the start of the experiment, horses were aged 20 ± 2 (mean ± SD) months and had a body weight of 368 ± 45 kg. We chose to use young Standardbred trotters because these horses had no known history of health and exercise problems and had not been involved in any kind of organized exercise or training regimen previously. Furthermore, at this age regular training and conditioning usually starts for Standardbred trotters. We could however not prevent that due to injury, two horses were not able to complete the training. Horses were housed individually in boxes and their diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance (58 MJ NE (range 54-66). Water and salt blocks were provided ad libitum. In phase three and four of the training experiment, horses received vitamin E and selenium (Pavo-E-Seleen) supplement and vitamin and mineral supplements (Equitop Forte). For organisational purposes, the study was performed in two different groups of six horses in two successive years. At the start of each year, the horses were divided in three pairs based on age. Of every pair, one horse was randomly selected for the intensified training program in phase three. Therefore, each year three horses were subjected to an intensified training program, whereas the other three horses served as controls. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Training

In order to standardize training to the individual exercise capacity of the horses, training and exercise intensity were adapted to the maximal individual heart frequency. For this purpose, an incremental exercise test was performed at the end of phase 1 of the training. After thirty minutes walking in a horse-walker, horses performed an incremental exercise test on a high speed treadmill (Mustang 2000, Kagra, Graber HG, Switzerland). The incremental exercise test started with a warming up and thereafter horses trotted for 2 minutes at 5 m/s, followed by 2 minutes at 6 m/s. Intensity was further increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed where horses could not keep up with the treadmill. Heart frequency was monitored with a Polar S.610i (Polar Electro Oy, Kempele, Finland) and on-line ECG recording (Cardio Perfect Stress 4.0; Cardio Perfect Inc, Atlanta, GA, USA). In the first year, all six horses performed this test at the end of phase one and phase two, and reached a mean maximal heart frequency of 221 ± 17 beats per minute (bpm). A plateau in heart frequency at maximal exercise intensity was, however, not observed in all animals. Therefore the measured maximal heart frequency can only be considered as average peak heart frequency. Based on the maximal heart frequency of horses that did reach a plateau in maximal heart frequency during the test, and on measurements in a previous study with 2-year old standardbred stallions[3], an estimated maximal heart frequency ($HF_{est\text{-}max}$) of 240 bpm was predicted. This $HF_{est\text{-}max}$ was used to guide training intensity (speed and inclination) on the treadmill, and was adjusted on a weekly basis to the measured peak heart frequencies during training. The incremental exercise test was difficult to perform for the young, relatively untrained horses however. They had trouble with the coordination on the treadmill during the high speeds necessary to obtain maximal heart frequencies. Therefore, and also to minimize the risk of injuries during the incremental exercise test, the test was not performed in the second year group.

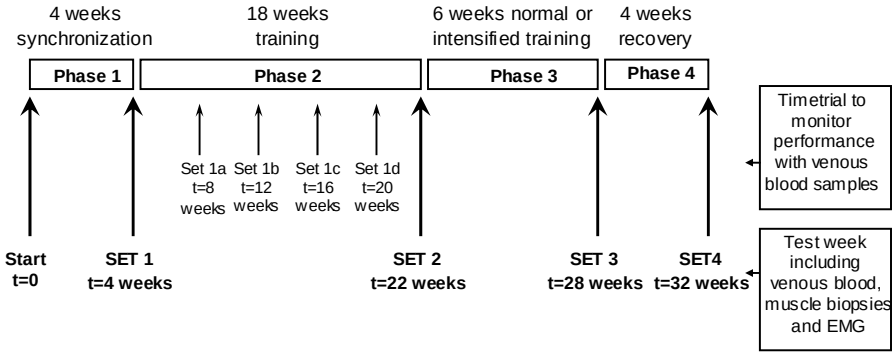
The training period consisted of a total of 32 weeks divided in four phases. A schematic representation of these phases is provided in figure 2.1. In the first phase of four weeks, the horses were introduced to the high speed treadmill. Each training session was preceded by 30 minute warming-up at the walking machine followed by an 8 minute warming-up at the treadmill, which consisted of 4 minutes walking at a speed of 1.6 m/s and 4 minutes slow trotting at a speed of 3.0-4.0 m/s, no incline.

The training program during phase 1 consisted of four endurance training sessions a week: week 1, 30% $HF_{est\text{-}max}$ for 20-30 minutes/session; week 2, 30% $HF_{est\text{-}max}$ for 25-45 minutes/session; week 3, 40% $HF_{est\text{-}max}$ for 30-45 minutes/session; and week 4, 50% $HF_{est\text{-}max}$ for 35-45 minutes/session. Each training session ended with a cooling down that consisted of a 5 minute walk at the treadmill followed by 30 minute walk at the walking machine.

In the second phase, the horses received an 18 week training program of mixed endurance training (ET) and high intensity training (HIT). Days of ET

were alternated with HIT. Each training session started with 30 minute walking in the horse-walker and an 8 minute warming up (4 minutes 1.6 m/s and 4 minutes 4.5 m/s) at the treadmill. An ET session consisted further of continuous trotting for 20-24 minutes at 60% HFest-max or trotting for 16-18 minutes at 75% HFest-max. Each HIT session contained either three 3 minute bouts or four 2 minute bouts of exercise at 80%-85% HR peak interspersed by 3 or 2 minute recovery bouts at 60% HFest-max. Each training session ended with a cooling down consisted of a 5 minute walk at the treadmill followed by 30 minutes walk at the walking machine. The horses exercised 4 days/wk throughout the entire training period of phase 2. On the resting days the horses walked for 60 minutes at the walking machine.

Figure 2.1
Outline of the training program
A total of 12 horses entered the study and were subdivided in pairs. One pair of horses could not finish the training program due to injuries and were taken out of the study after SET2. Exercise tests (SET) were performed to monitor performance.



In the third phase, one horse of each pair was randomly selected and subjected to an intensified training program whereas the other horse of each pair continued training at the volume and intensity they received in the second phase. The intensified training regimen in this phase consisted of alternating days of HIT and ET as described in phase 2 for 6 days/week during the first three weeks. Exercise intensity during the ET was gradually increased to 75% $HF_{est\text{-}max}$. The last three weeks horses were trained 7 days a week, four times HIT and three times ET. The HIT consisted of three bouts of 75% or 80 % $HF_{est\text{-}max}$ for 3 minutes interspersed with 2 minutes rest at 60% $HF_{est\text{-}max}$. Endurance running was performed three times a week according to the first three weeks of this period.
The aim of these six weeks intensified training was to induce an increased amount of stress on skeletal muscle. In this thesis, analysis of muscle biopsies is used to provide insights in metabolic adaptation of skeletal

muscle, and possibly metabolic mal-adaptation due to these increased stress levels.
In phase 4, all horses performed endurance training for 20 minute at 60% $HF_{est\text{-}max}$ for 3 days and 70% $HF_{est\text{-}max}$ for 1 day a week. On rest days horses walked in a horse-walker for 60 minutes.

Experimental procedures

Time trial

Every four weeks a time-trial (or standardized exercise test (SET)) was performed to evaluate the level of performance. Muscle biopsies were only taken at the last SET in a training phase. Before the SET started, horses walked for 30 minutes in the horse-walker followed by a warming up at the treadmill that consisted of 4 minutes walking (1.6 m/s) and four minutes slow trotting (4.5 m/s) and one minute walking (1.6 m/s). This was immediately followed by the time trial procedure where the horses trotted for 20 minutes at a speed and inclination that elicited a heart frequency of approximately 80% of the maximal heart frequency. The cooling down consisted of walking on the treadmill for 5 minutes (1.6 m/s) followed by 30 minutes of walking in the horse walker. Heart frequency was monitored constantly with a Polar S.610i, and during tests with muscle biopsy sampling also with on-line ECG measurement. Speed and inclination of the treadmill could be adjusted to archive the desired heart frequency. In phase three and four, the speed and inclination were not further increased. This made comparison between the tests possible. Venous blood was drawn from the jugular vein before the test (t=0 minutes), after the warming up (t=9 minutes), and every 5 minutes during the 20-minute run during the SET (t=14, 19, 24, 29, 34 minutes) and 1, 3, 6 and 24 hours after the SET. Samples were kept on ice until whole blood lactate and pH had been analysed (ABL-605; Radiometer Copenhagen, Westlake, Ohio). Subsequently the samples were centrifuged and plasma and serum were recovered and stored at -20°C for additional analysis.

Muscle biopsies

Muscle biopsies were taken under local anaesthesia (lidocain hydrochlorine (2%) without adrenalin) using a modified Bergström biopsy needle (Maastricht instruments, Maastricht, The Netherlands) with a diameter of 7 mm. Approximately 60 minutes before the standardized exercise test, a 5 cm deep biopsy of the M. Vastus Lateralis (VL) was taken at a point 15 cm ventral to the centre of the tuber coxae and 7 cm caudal to the cranial border of the VL muscle. Also a 4 cm deep biopsy was taken from the M. Pectoralis Descendens (PD) at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle. These muscles were selected for their easy accessibility for biopsy procedures and EMG analysis. Furthermore, each of these muscles has a pronounced contribution in propulsion[9, 10, 13]. Within thirty minutes after cessation of the SET a second

muscle biopsy was obtained from a new incision 3 cm caudal to the point of the first biopsy. Blood and fat tissue were carefully removed from the biopsy and the biopsy was thereafter split in two. One part was frozen in isopentane cooled to its freezing point in liquid nitrogen for immuno-histochemical procedures and another part of the biopsy was immediately frozen in liquid nitrogen for biochemical procedures. Frozen muscle tissue was stored at -80ºC.

EMG

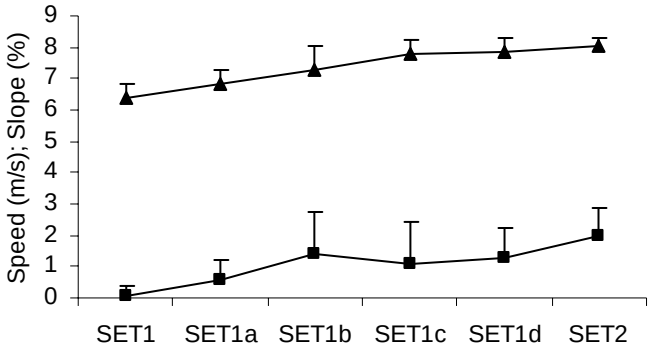
In needle electromyography (EMG) examination the electrical activity generated by the motor unit is recorded and analysed. As neurological adaptations and motor unit firing patterns are among the first adaptations after training[5], needle EMG can provide valuable information on neuromuscular adaptations to training. Furthermore, needle EMG provides one means of discriminating between neurogenic and myogenic (mal)adaptation that might occur with an intensified training regimen[7, 14]. At the end of each training phase, the day before performing a SET, quantitative electromyographic (EMG) analysis was performed of the vastus lateralis (VL), pectoralis descendens (PD) and subclavian (SC) muscle. After applying a small amount of local anaesthetic (lidocain hydrochlorine (2%) without adrenalin) a probe connected to a thermometer was inserted through a 16 gauge, 60 mm needle into a muscle to record intramuscular temperature. EMG recordings were made using a disposable concentric needle (50-100 mm) (Nicolet Biomedical Inc., Madison, WI, USA) with a sampling area of 0.068 mm^2. A portable EMG apparatus (Nicolet Meridian, Nicolet Biomedical Inc., Madison, WI, USA) was used for the recording of EMG. Amplifier gain was 50-100 µV for spontaneous activity and 10-500 µV for MUP recording. At least 3 insertions and three directions per insertion were made per investigated muscle. Insertional activity, pathological spontaneous activity, motor unit action potentials (MUPs) and satellite potentials were recorded. Spontaneous activity was assessed outside the endplate region in the same regions in which MUPs were obtained. Per muscle 20-30 MUPs were analysed. The waveform of a MUP can be described by amplitude, duration, number of phases and number of turns. MUPs with a high amplitude and short duration are in close proximity of the electrode tip and are suitable for analysis[1, 11]. The amplitude is defined as the maximal positive and negative deflection from the baseline. Only the MUPs closest to the recording tip contribute the spike amplitude. The duration is defined as the time from the initial deflection to the final return back to the baseline. It depends on the degree of synchrony among individual muscle fibre action potentials and their conduction velocity. A phase is defined as departure from and return to baseline, and can be counted as number of baseline crossings plus one. Under normal circumstances a MUP consists of four or less phases. Finally, a turn is defined as a change in the direction of the signal, independent of baseline crossing. An increase in the number of turns indicates loss of

synchronisation of discharging muscle fibres[14]. To be analysed, a MUP had to have a rise time of maximally 0.80 ms and had to occur repeatedly at least four times. For each semi automatically analysed MUP, reproducibility was checked by superimposition of at least four waveforms. Analysis of waveform was checked manually. EMG examination in conscious horses took place in stocks.

Observations and evaluation

The training resulted in an increase in fitness and endurance as horses were able to complete the SET at a higher speed and inclination of the treadmill during SET 2 compared to SET 1 (figure 2.2). The aim of the current study was to evaluate the metabolic effects of training and intensified training in horse skeletal muscle. Therefore, we compared a group of Standardbred trotters with unchanged training load (controls) with a group of Standardbred trotters subjected to an increase in training volume and training intensity (intensified). Overreaching and overtraining are associated with deterioration of performance, decreased body weight, increased oxygen consumption at sub-maximal exercise and a decrease in plasma lactic acid accumulation during sub-maximal exercise[4, 6, 12]. At SET 3 no significant differences between control and intensified trained group in performance, body weight, or lactic acid accumulation during exercise were observed. Other signs that may indicate overreaching, are found in horse gait. Some horses of the intensified group were unable to maintain trot at high speeds during training or the SET and started

Figure 2.2
Speed and slope of the treadmill during the standardized exercise test
Both speed (triangles) and slope (squares) increased during the 18 week training program indicating an increase in performance. Statistical ANOVA analysis ($p<0.05$) was performed to calculate differences between SET1 and SET2. Speed and slope were adjusted to elicit a heart frequency of approximately 80% HFest-max. After SET 2 there were no further adjustments of speed and incline in order to make comparisons between the control group and intensified group possible.



galloping. One horse was not able to complete the SET after the intensified training period and ceased trotting after 10 minutes. In addition, changes in behaviour and endocrinal function were observed between the control and intensified trained Standardbred trotters (M. van Dierendonck; E. de Graaf-Roelfsema; personal communication).

The goal of training in this thesis is to increase performance and measure metabolic adaptations in skeletal muscle. Performance, defined by run speed and inclination of the treadmill during the SET, was increased after training. A period of intensified training is applied to increase stress levels in horses. Biochemical and neuromuscular analysis are used to determine adaptation to training and intensified training. Intensified training implies further increases in skeletal muscle metabolic parameters compared to the control training. If stress levels increase above the maximal threshold, mal-adaptation resulting in signs of overreaching may develop. The amount of stress needed to induce metabolic mal-adaptation is not well defined however. The well-controlled standardized training protocol used in this thesis, serves to gain further insights in metabolic adaptation of skeletal muscle to training and intensified training.

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Chapter 3

Investigation of the expression and localization of glucose transporter 4 (GLUT-4) and fatty acid translocase (FAT/CD36) in equine skeletal muscle

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Abstract

Objective
To investigate of the expression and localization of glucose transporter 4 (GLUT-4) and fatty acid translocase (FAT/CD36) in equine skeletal muscle.
Sample Population
Muscle biopsy specimens obtained from 5 healthy Dutch Warmblood horses.
Procedures
Percutaneous biopsy specimens were obtained from the vastus lateralis, pectoralis descendens, and triceps brachii muscles. Cryosections were stained with combinations of GLUT-4 and myosin heavy chain (MHC) specific antibodies or FAT/CD36 and MHC antibodies to assess the fibre specific expression of GLUT-4 and FAT/CD36 in equine skeletal muscle via indirect immunofluorescent microscopy.
Results
Immunofluorescent staining revealed that GLUT-4 was predominantly expressed in the cytosol of fast type-2B fibres of equine skeletal muscle although several type-1 fibres in the vastus lateralis muscle were GLUT-4-positive. In all muscle fibres examined microscopically, FAT/CD36 was strongly expressed in the sarcolemma and capillaries. Type-1 muscle fibres also expressed small intracellular amounts of FAT/CD36 but no intracellular FAT/CD36 expression was be detected in type-2 fibres.
Conclusions and Clinical Relevance
In equine skeletal muscle, GLUT-4 and FAT/CD36 are expressed in a fibre type selective manner.

Introduction

Glucose and fatty acids are the main metabolic fuels for skeletal muscle; however, their use by skeletal muscle is contingent on their respective transport into the muscle cells. Glucose transport in mammalian skeletal muscle is almost exclusively mediated by the glucose transporter 4 (GLUT-4) protein[36, 42]. In the soleus muscle of rats, GLUT-4 proteins reside in intracellular sub-sarcolemmal groups of vesicles, Golgilike structures at the nuclear poles, and between mitochondria and the myofibrillar region[6, 35]. Insulin and muscle contraction are 2 potent stimuli for GLUT-4 translocation and both recruit GLUT-4 from these intracellular storage sites. The exercise sensitive GLUT-4 pool is associated with an endosomal transferrin receptor and the insulin sensitive GLUT-4 pool does not co-translocate with this protein[2, 35]. There is still much debate about the exact nature of the GLUT-4 pool subdivisions and translocation mechanism. McCutcheon et al[31] determined the GLUT-4 protein content in isolated muscle membrane fractions of homogenates of equine gluteal medius muscle. In rodents, GLUT-4 protein content is higher in muscles that consist predominantly of myosin heavy chain (MHC) I (type-1, oxidative) fibres, compared with muscles that express predominantly MHC IIA and IIB (type-2, glycolytic) in their fibres[19, 30]. In biopsy specimens of human skeletal muscle, inconsistent results have been reported regarding the extent of GLUT-4 protein expression per muscle fibre type[5, 10, 16], and it has been suggested that alternative factors such as motor unit firing rate, training status, and glycolytic activity may influence GLUT-4 expression in skeletal muscle[5, 11].
Transsarcolemmal membrane transport of fatty acids consists of passive diffusion (primarily for short and medium chain fatty acids) and a facilitated transport mechanism (for long chain fatty acids [LCFA]). Findings of studies in rodents have provided firm evidence that membrane-associated proteins are involved in the cellular uptake of LCFA. Five different membrane-associated proteins have been identified as potential fatty acid receptors or transporters[1, 15, 38-41, 43]. Of these, an 88-kd integral membrane protein has been determined to be involved in fatty acid transport across the plasma membrane[1]. This putative membrane fatty acid translocase (FAT) appears to be highly homologous (85%) to the human leukocyte differentiation antigen CD36 (glycoprotein IV)[1, 14]. and shall be designated FAT/CD36 in this report. The putative role of FAT/CD36 on LCFA uptake in rodent skeletal muscle has been studied extensively. The FAT/CD36 resides in intracellular membrane fractions and translocates to the plasma membrane of skeletal muscle cells after insulin or contraction stimuli[4, 29]. In rodent skeletal muscle, the expression of FAT/CD36 is higher in type-1 fibres, compared with its expression in type-2 fibres[3].
To our knowledge, no previous studies have been performed to investigate the fibre type selective expression of GLUT-4 in the skeletal muscle of horses. The purpose of the study reported here was to investigate of the

expression and localization of GLUT-4 and FAT/CD36 in equine skeletal muscle. It was our hypothesis that the fibre type specific expression of GLUT-4 in different skeletal muscles (the vastus lateralis, triceps brachii, and pectoralis descendens muscles) of horses is comparable to that reported in human skeletal muscle (i.e., higher expression of GLUT-4 in glycolytic type-2 fibres, compared with that in oxidative type-1 fibres)[5]. Furthermore, we hypothesized that FAT/CD36 is expressed in equine skeletal muscle in a fibre type specific manner, supporting a functional role in fatty acid metabolism[18].

Materials and Methods

Animals

Five adult healthy Dutch Warmblood horses (4 mares and 1 gelding) were used in this study. Horses were 4.5 to 9.5 years old (mean ± SD , 7.1 ± 1.9 y) and weighed 563 to 717 kg (mean weight, 632 ± 66 kg). The horses were owned by the Faculty of Veterinary Medicine of Utrecht University, The Netherlands. They did not have a history of myopathies, and none of the horses was involved in strenuous exercise in the 48 hours prior to the study. The horses were housed in tied standings and accustomed to frequent handling. The diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance. The total diet contained 10% ash, 14.5% crude protein, 1.3% crude fat, 20% crude fibre and 56.2% other carbohydrates. Water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

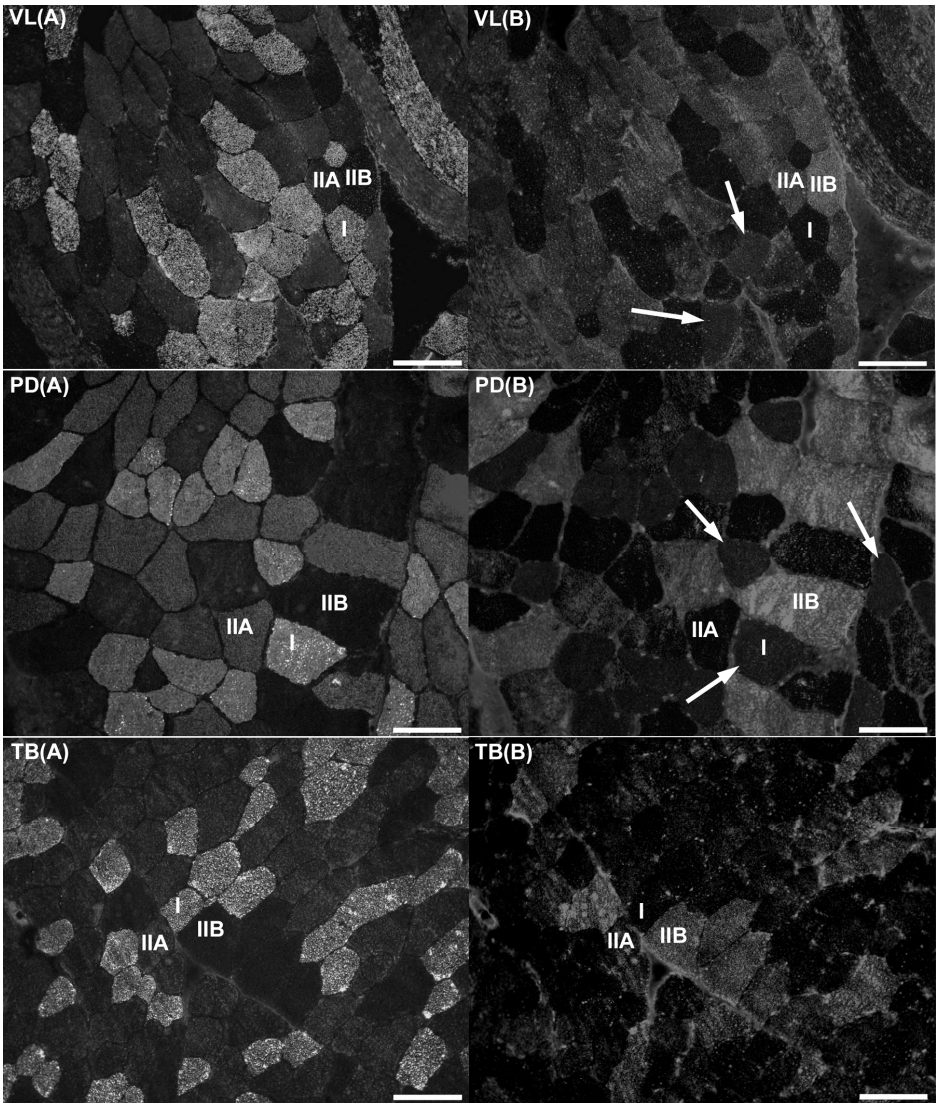
Muscle biopsies

Muscle biopsy specimens were obtained after application of local anaesthesia by use of a modified Bergström needle (Maastricht Instruments, Maastricht, The Netherlands) with a diameter of 7 mm. A 5-cm deep biopsy specimen of the vastus lateralis muscle and 4-cm deep biopsy specimens of the triceps brachii and the pectoralis descendens muscles were obtained from each of the 5 horses. Blood was carefully removed from the biopsy specimens before they were embedded in embedding medium for frozen tissue specimens (Tissue-Tek, Sakura Finetek Europe bv, Zoeterwoude, The Netherlands) and frozen in isopentane cooled to the freezing point in liquid nitrogen for immunohistochemical analysis. Muscle samples were stored at –80^{o}C until analyzed.

Immunofluorescence staining for GLUT-4, FAT/CD36, and muscle fibre type

Frozen muscle tissue was cryosectioned (CM3050, Leica, Nussloch, Germany) at -20ºC. Transverse sections (5 μm) were thaw-mounted on uncoated glass slides and air-dried. Sections were stored at -80°C and air-dried prior to staining procedures. For labelling, sections were fixed in methanol for 5 minutes followed by acetone fixation for 1 minute, and air-dried. Sections were pre-incubated for 20 minutes with 10% normal horse serum in PBS solution. Some sections were subsequently incubated overnight (approximately 16 hours) at 4°C with primary mouse monoclonal antibodies against MHC I (A4.840[9]; diluted 1:25) and MHC IIA (N2.261[45]; diluted 1:25) (both Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa) and the rabbit polyclonal antibody GLUT-4 (GLUT-4-BW[5]; diluted 1:40), all diluted in PBS solution. Other sections were incubated overnight at 4ºC with primary mouse monoclonal antibodies against MHC 1(A4.840; diluted 1:25) and FAT/CD36 (131.4[33]; diluted 1:25; provided by Dr. N.N. Tandon, Otsuka Pharmaceutical Co, Rockville, Md) in PBS solution. In serial sections, primary antibodies were omitted as a negative control. Sections were subsequently rinsed 3 times for a 5-minute period in PBS solution and incubated for 30 minutes at room temperature (±18ºC) with secondary Alexa conjugated antibodies (goat anti-mouse IgM Alexa 488 [diluted 1:200], goat anti-mouse IgG Alexa 350 [diluted 1:130], goat anti-rabbit IgG Alexa 555 [diluted 1:400]for GLUT-4 sections or goat anti-mouse IgM Alexa 488 [diluted 1:200] and goat anti-mouse IgG Alexa 555 [diluted 1:400] (Alexa Fluor 488, 350, and 555, Molecular Probes Europe bv, Leiden, The Netherlands) for FAT/CD36 sections) diluted in PBS solution. Finally, sections were rinsed 3 times for a 5-minute period in PBS solution. Sections prepared for investigation of GLUT-4 expression were mounted in Mowiol-Tris-HCl (pH, 8.5), whereas sections prepared for investigation of FAT/CD36 expression were mounted in Mowiol with 4'-6'-diamino-2-phenylindole (DAPI; 0.5 μg/ml) to stain nuclei. Sections were examined by use of a fluorescence microscope (Nikon E800 fluorescence microscope, Uvikon, Bunnik, The Netherlands) coupled to a progressive scan colour charge-coupled device camera (Basler A113 C progressive scan color CCD camera, Ahrensburg, Germany) with a "Bayer" colour filter at an output picture resolution of 1,300 X 1,030 pixels (horizontal X vertical) and a pixel size of 6.7 X 6.7 μm. Digitally captured images were processed and analyzed with computer software (Lucia 5.49 software, Nikon, Düsseldorf, Germany).

Figure 3.1
Results of immunofluorescence GLUT-4 staining
In the photomicrographs designated VL(A), PD(A), and TB(A), muscle fibre myosin heavy chain (MHC) isoforms are identified: MCH I-positive fibres appear lightest, MCH IIA-positive fibres appear intermediate, and MCH IIB-positive fibres have no staining (black). In the photomicrographs VL(B), PD(B), and TB(B), GLUT-4 protein staining appears gray; in all muscles examined, GLUT-4 protein expression is highest in MHC IIB-positive fibres. Arrows indicate MHC I-positive fibres that also express GLUT-4 protein. In all panels, bar = 100 μm.



Results

Fibre type distribution

Skeletal muscle fibres are classified according to the expression of the dominant MHC isoform. In the study of this report, no functional analysis of myosin isoforms was performed and muscle sections were stained with MHC I (type-1) and IIA (type-2A) antibodies, such that the MHC IIB or IIX fibres remained unstained. For purposes of this report, the unstained fraction of muscle fibres will be referred to as type-2B muscle fibres.
Specimens of the vastus lateralis muscle contained a slightly higher portion of type-1 than type-2A muscle fibres and a minority of type-2B muscle fibres. The pectoralis descendens muscle also had few type-2B muscle fibres, whereas type-2A muscle fibres were most abundant. The triceps brachii muscle contained a relatively large portion of type-2B fibres, whereas type-1 and -2A fibres were equally distributed. Via fluorescence microscopy, a minimum of 200 fibres/biopsy specimen were manually and randomly selected and counted; mean percentages of each muscle fibre per muscle were calculated (table 3.1).

Table 3.1

Distribution of myosin heavy chain (MHC) isoforms (mean ± SD)

Distribution of fiber type/muscle are expressed as percentages and were assessed via immunohistochemical staining in specimens of 3 skeletal muscles obtained from each of 5 horses

Muscle	% MHC I	% MHC IIA	% MHC IIB
Vastus lateralis	48 ± 3.5	38 ± 2.2	14 ± 4.0
Pectoralis descendens	34 ± 6.4	52 ± 3.7	14 ± 7.0
Triceps brachii	35 ± 4.1	37 ± 8.3	28 ± 12.1

Content of GLUT-4 in equine muscle

Evaluation of immunofluorescence staining of sections of equine skeletal muscle revealed a distinct chessboard pattern of fibres stained either strongly or weakly for GLUT-4 (figure 3.1). Results of triple immunofluorescence staining of sections indicated that type-1 and -2A fibres had a low expression of GLUT-4, compared with type-2B fibres in all 3 muscles (figure 3.1).In control slides, no substantial background staining was observed. In fibres that were incidentally cut longitudinally to the muscle fibre axis, a pattern of strings of GLUT-4 aggregates distributed parallel to the fibre axis was detected (figure 3.2), as has been observed previously in rat and human skeletal muscle[35].In sections of vastus lateralis muscle that had a high abundance of type-1 muscle fibres, a small portion of

oxidative type-1 fibres expressed GLUT-4 protein (figure 3.1). In the pectoralis descendens muscle, GLUT-4 was expressed predominantly in type-2B muscle fibres, but GLUT-4 was expressed in all 3 muscle fibre types. Sections of triceps brachii muscle had low amounts of GLUT-4 in type-1 and -2A muscle fibres and had a high expression in type-2B fibres as well.

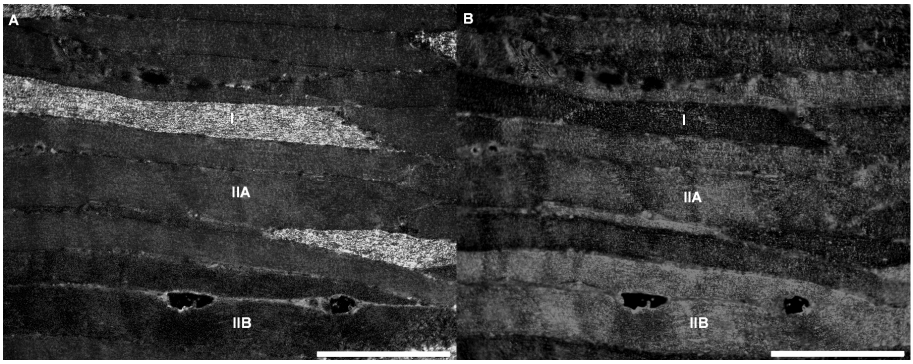
Content of FAT/CD36 in equine muscle

Immunofluorescent labelling of sections with antibodies against FAT/CD36 revealed strong expression of this protein at the sarcolemma of all equine skeletal muscle fibres and no substantial staining in control slides. A low intracellular expression of the FAT/CD36 protein was observed in type-1 fibres, whereas no intracellular FAT/CD36 expression could be detected in type-2 fibres (figure 3.3). In both type-1 and type-2 fibres, FAT/CD36 aggregates were observed in close relation to the sarcolemma. These are most likely positioned near capillaries to aid in the uptake of LCFA from the plasma. In the triceps brachii, pectoralis descendens, and vastus lateralis muscles, slight intracellular FAT/CD36 expression was limited to type-1 muscle fibres, although the sarcolemmal aggregates were observed in all muscle fibre types (figure 3.3 and 3.4).

Figure 3.2

Photomicrographs of longitudinal sections of equine pectoralis descendens muscle after staining for MCH isoforms and GLUT-4 protein

A—Results of staining for MCH isoforms. The MHC I-positive muscle fibres appear lightest, MCH IIA-positive fibres appear intermediate, and MCH IIB-positive fibres are not stained. B—Results of staining for GLUT-4 protein. The GLUT-4 protein (in gray) is distributed as dotted strings along the fibre axis. In both panels, bar = 100 µm.



Discussion

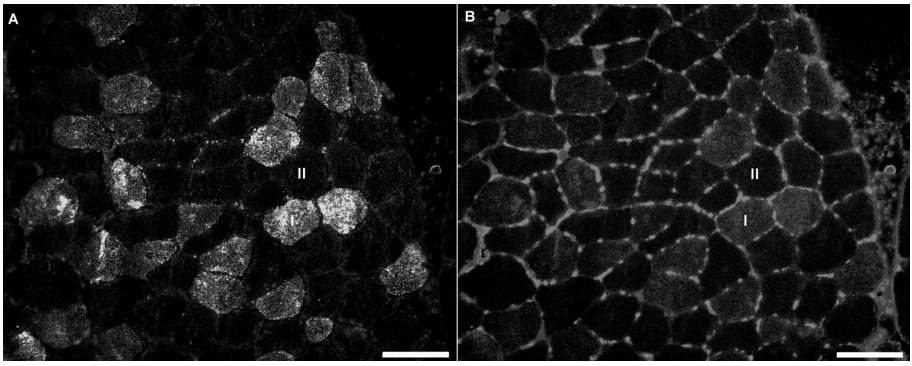
Results of the indirect immunofluorescence assays used in the study reported here, indicated that GLUT-4 and FAT/CD36 protein expression in equine skeletal muscle is fibre type selective. Our findings indicated that equine skeletal muscle fibre type-2B has a higher expression of GLUT-4 protein, compared with that of type-1 and -2A muscle fibres. In type-1 muscle fibres, FAT/CD36 is expressed intracellular, but is mainly clustered in aggregates near muscle capillaries in all muscle fibre types. In general, results of the GLUT-4 immunofluorescence staining performed in our study are dissimilar to those of other studies involving rodent skeletal muscle extracts that indicated higher GLUT-4 protein content in muscle which contained predominantly type-1 muscle fibres versus muscle which contained predominantly type-2 muscle fibres[19, 30], but are consistent with data obtained from skeletal muscles of humans and rodents in a previous investigation in our laboratory[5]. Species specific differences in GLUT-4 expression have been reported[21] in goat and calf muscles, in which skeletal muscle GLUT-4 content decreased with increasing oxidative capacity in different muscle groups. In a study by Katsumata et al. [24], an upregulation of GLUT-4 was detected in the skeletal muscle of pigs after a period of mildly inadequate nutrition; concurrently, there was a decrease in insulin sensitivity in these pigs, compared with pigs receiving twice as much nutrition. These results were all obtained by analysis of mixed muscle

Figure 3.3

Photomicrographs of sections of equine pectoralis descendens muscle after staining for FAT/CD36 expression

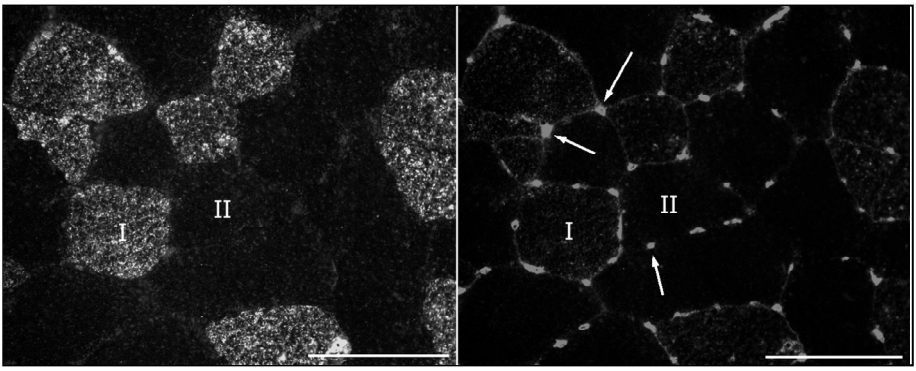
A—The MHC I-positive muscle fibres (I) appear lightest, MHC 2 (A and B) muscle fibres are unstained (II)

B—Distribution of FAT/CD36 in muscle fibres. Notice that MHC I-positive fibres have a low intracellular expression of FAT/CD36 (I), whereas MHC 2 (A and B) have no detectable intracellular expression of FAT/CD36 (II). FAT/CD36 is mainly clustered in sarcolemmal aggregates near capillaries in all fibre types. In both panels, bar = 100 µm.



homogenates and although rodent skeletal muscle often contains a predominance of either MHC I or II fibres (thereby justifying its division into slow-twitch and fast-twitch muscle), other species exhibit a more mixed muscle fibre pattern. Similar to findings in human skeletal muscle, skeletal muscle of horses has a heterogeneous fibre type distribution and different fibre type distributions have been reported between biopsy specimens obtained at sites only 2 cm apart[28, 37]. On the basis of these data, we selected a more direct method of assessment of the relation between GLUT-4 and fibre type distributions; this method involved immunohistochemical triple staining of muscle sections for GLUT-4 with MHC specific antibodies which, to our knowledge, had not been applied before in equine muscle studies. This technique has been used in investigations involving rodent and human skeletal muscle; results of a study[9] involving this technique by Borghouts et al. confirmed higher GLUT-4 expression in type-1 muscle fibres compared with that in other fibre types only in rat gastrocnemius muscle; in the soleus, extensor digitorum longus, tibialis cranialis, and vastus lateralis muscles, GLUT-4 expression was higher in type-2 muscle fibres[5]. In human skeletal muscle, some researchers have detected higher GLUT-4 protein content in type-1 muscle fibres[10, 11, 16], whereas others identified higher GLUT-4 expression in type-2 skeletal muscle fibres[5]. From these findings and results of our study, in which GLUT-4 was detected in some type-1 muscle fibres in vastus lateralis muscle of horses but most commonly in type-2B muscle fibres, it seems unlikely that a strict coupling between GLUT-4 protein expression and muscle fibre type composition exists in mammalian skeletal muscle, and alternative factors contribute to GLUT-4 expression in

Figure 3.4
Photomicrograph of a section of vastus lateralis muscle illustrating FAT/CD36 expression in equine muscle fibres
The type-1 fibres (lightest; A) express low quantities of FAT/CD36 (gray; B) intracellularly but FAT/CD36 is mainly expressed in sarcolemmal aggregates in all fibre types, presumably near capillaries (example indicated by arrows). Also notice that sarcolemmal staining of FAT/CD36 is visible in type-1 muscle fibres. In both panels, bar = 100 µm.



skeletal muscle[5, 11].

Expression and localization of GLUT-4 is not only dependent on muscle innervation and fibre type, but is also influenced by different stimuli. Insulin is a potent stimulus for GLUT-4 translocation and transcription, and it increases glucose uptake and GLUT-4 expression in rats[22]. Muscular contraction is another stimulus for GLUT-4 translocation and exercise training also increases GLUT-4 gene transcription and expression[26, 34]. In exercise-trained rats, primarily fast twitch muscle fibres increase insulin-stimulated glucose uptake[34]. In rats, stimulation of AMP-activated protein kinase (an exercise linked activator of GLUT-4 translocation) with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) for 5 days increased GLUT-4 protein in type-2 muscle fibres but not in type-1 muscle fibres[7]. Finally, diet can influence GLUT-4 expression. A high carbohydrate diet is associated with increases in GLUT-4 expression in all muscle fibre types in rats[27], whereas a high fat diet is associated with a decrease in GLUT-4 expression in rats, compared with control values[23]. These results also indicate that skeletal muscle can adapt fibre specific glucose uptake and GLUT-4 expression to different stimuli.

By application of triple indirect immunofluorescence assays, our data have indicated that FAT/CD36 protein is expressed in equine skeletal muscle in a fibre type selective manner. The FAT/CD36 protein is expressed in sarcolemmal aggregates in all muscle fibre types and intracellularly in small amounts in type-1 skeletal muscle fibre types. This is in accordance with results of a study[3] involving rat skeletal muscle that indicated higher expression of FAT/CD36 protein in red, oxidative skeletal muscle homogenates, compared with that in white, glycolytic skeletal muscle homogenates. Recently, this relation was also confirmed in human skeletal muscle with experimental procedures similar to those used in the study of this report[25]. The FAT/CD36 is a 88-kd protein that is redistributed to the plasma membrane of muscle fibres after insulin stimulation[29] or contraction[4] in rats and correlates with an increased rate of palmitate uptake, compared with uptake in unstimulated cells[4, 3, 29]. In the same species, oxidative skeletal muscles have the highest potential for oxidation of fatty acids and the uptake (incorporation and oxidation) of LCFA is greatest in oxidative muscle strips[13]. In rats, palmitate uptake in giant vesicles obtained from red, oxidative (type-1) skeletal muscle was increased, compared with uptake in giant vesicles obtained from white, glycolytic (type-2) skeletal muscle[3]. Moreover, intramuscular lipid content (assessed by oil red O staining) is higher in human type-1 skeletal muscle fibres than in other muscle fibre types[20]. These observations suggest a higher capacity for uptake of LCFA in oxidative skeletal muscle fibres and therefore the amount of FAT/CD36 is expected to be higher in these muscle fibre types. However, horses have a different dietary pattern than that of rodents and humans and derive more nutrients from fermentation of cellulose which results in a larger metabolic dependence on plasma volatile fatty acids[32]. These quantities of short and medium chain fatty acids make it questionable if horses need a facilitative

LCFA transport system. Nevertheless, provision of supplemental fat in the diet increases the oxidative power of skeletal muscle in horses[12] and horses have a diurnal rhythm in plasma LCFA concentrations[32]. In endurance trained humans, a high fat diet resulted in an increase in FAT/CD36 expression and a marked increase in fat oxidation, compared with a high carbohydrate diet[8]. Geelen et al. reported an increase in carnitine palmitoyl transferase-1 activity in oxidative (masseter) skeletal muscle of ponies fed a diet supplemented with soyabean oil[17]; in humans, an increase in carnitine palmitoyl transferase-1 m-RNA detected after training was correlated with an increase in FAT/CD36 mRNA in skeletal muscle[44]. Thus, the identification of FAT/CD36 in equine skeletal muscle in our study suggests a functional role of this transport protein in lipid metabolism in horses.

The findings of the study of this report indicated that GLUT-4 protein is expressed in equine muscle in a fibre type specific manner that is analogous to GLUT-4 protein expression in human muscle fibres, specifically with regard to the higher expression in type-2B muscle fibres, compared with that in type-1 muscle fibres. Intracellular FAT/CD36 is expressed primarily in type-1 muscle fibres in the skeletal muscle of horses, but FAT/CD36 is expressed in sarcolemmal aggregates in all muscle fibre types. Among the different muscle fibre types, these findings suggest a higher glucose uptake capacity in type-2B muscle fibres and a higher fatty acid uptake capacity in type-1 muscle fibres.

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Chapter 4

Monocarboxylate transporter-1, -2 and -4 (MCT1, MCT2 and MCT4) in Vastus Lateralis muscle of Standardbred horses

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Abstract

During high intensity exercise or during the later stages of endurance exercise, lactate accumulates. This is associated with fatigue. In several species a family of monocarboxylate transporters (MCT) have been identified that seem to be involved in lactic acid homeostasis. Although lactic acid accumulation is a well known phenomenon in these proteins have not been identified in equines. Furthermore, MCT proteins may also be involved in volatile fatty acid transport, an important energy source in the non-ruminant herbivore, the horse.

Methods

Muscle biopsy specimens were obtained from 6 Standardbred trotters. Next horses performed a standardized exercise test and plasma lactate accumulation was measured. MCT1, MCT2 and MCT4 isoforms were identified by Western blotting. Muscle LDH activity and isoenzyme distribution were also determined. The same analysis was performed after a 18-week period of training.

Results

MCT1, 2 and 4 were identified by Western blotting. MCT2 had a relative high protein expression compared to a positive control of rat skeletal muscle. LDH isoenzyme 5 was the most pronounced LDH isoenzyme with low abundance of the other isoenzymes. Plasma lactate during the exercise test was increased after training compared to pre-training values. No other effects of training were found.

Clinical Relevance

The identification of MCT proteins implicates that lactate homeostasis in equines is regulated in a similar pattern as in other mammals. The relative high expression of MCT2 compared to rat skeletal muscle may be involved in a regulatory role in volatile fatty acid transport in equine skeletal muscle.

Introduction

Exercise and training have a major impact on muscle metabolic function. During high intensity exercise or during the later stages of endurance exercise, lactate accumulates [24]. At physiological pH, in the muscle cell (between 6 and 8), lactate is dissociated in a lactate anion (La^-) and a proton. Accumulation of the La^- and the protons during exercise in the muscle cell lead to inhibition of metabolic enzymes and the development of fatigue [9]. The La^-, however, can not diffuse out of the cell because of the electric charge of the molecule. Removal of lactic acid from the exercising muscle cells is facilitated by a family of monocarboxylate transporters (MCT's) [12, 11]. MCT's mediate membrane transport with an obligatory 1:1 coupling between monocarboxylates (among which the La^-) and a proton [16]. Therefore, MCT's have an important function in the maintenance of intracellular pH homeostasis.

To date, a family of 14 MCT isoforms have been identified that are involved in the transport of lactate, pyruvate, ketone bodies, and branched-chain ketoacids [12, 11]. Of all the isoforms discovered so far, MCT1, MCT2 and MCT4 are most abundantly expressed in skeletal muscle [4, 7, 13, 25]. MCT1 is predominantly found in the oxidative fibre types, and has a high correlation with lactate uptake and oxidative metabolism [14, 15] . The expression of MCT1 is therefore associated with the influx of lactate for oxidation in skeletal muscle. MCT4 on the other hand, is mainly expressed in glycolytic muscle fibres and is, despite a relative low affinity for lactate, associated with lactate efflux from the muscle cell [12].

The properties of MCT2 have not been described in detail yet. The protein has been shown to be expressed in sarcolemma of oxidative muscle fibres [7, 13] and its expression during developmental stages in rat brain suggest a function in the influx of monocarboxylates in oxidative tissue [23].

It has been shown that in humans and rats MCT1 and MCT4 expression are increased in response to either endurance or high intensity training [1, 5, 20]. Additionally, plasma lactate and skeletal muscle lactate dehydrogenase (LDH) activity and LDH-isoenzyme expression have been shown to correlate positively with MCT proteins [10, 29, 30].

To the best of our knowledge, MCT proteins have not previously been studied in equine skeletal muscle. This is odd in the light of the tremendous aerobic and anaerobic capacity of horses in relation to its non-ruminant herbivore nature [19]. Among others, horses derive their energy from the volatile fatty acids (acetic acid, propionic acid and butyric acid), which are all monocarboxylates, produced by hindgut fermatation.

Therefore, the aim of the present study is:1) to explore the expression of the most important muscle based MCT's in Standardbred trotter muscle biopsies and 2) to evaluate the level of expression after a period of training.

Material and Methods

Horses

In this study, 6 Standardbred geldings were used. Horses were aged 20 ± 2 months in this study and were owned by the Faculty of Veterinary Medicine of the University of Utrecht, The Netherlands. The horses were individually housed and their diet consisted of grass silage supplemented with concentrate feed and vitamin supplements and met nutrient requirements for maintenance and performance (58 MJ NE (range 54-66). Salt blocks and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Training

The training period consisted of a total of 22 weeks divided in 2 phases as described in phase 1 and phase 2 in Chapter 2. In short, the horses received an initial training of 4 weeks of light endurance training to get accustomed to trotting on a high speed treadmill (Mustang 2000, Kagra, Graber HG, Switzerland). The training phase (18 weeks) consisted of two types of exercise, endurance running and interval running. The days of interval running were alternated with days of endurance running. The endurance running consisted of 20-24 min of continuous level running at 60% HR_{peak} or 16-18 min at 75% HR_{peak}. The interval training consisted of three 3-min bouts at 80-90% HR_{peak} or four 2-min bouts at 80-90% HR_{peak} and interspersed with 3-min or 2-min periods at 60% HR_{peak}. Each training session ended with a cooling down consisted of a 5 min walk at the treadmill followed by 30 min walk at the walking machine. The horses exercised 4 days/wk throughout the entire training period of the training phase. On the resting days the horses walked for 60 minutes at the walking machine.

Exercise test

A standardised exercise test (SET) was performed in all horses before and after the training phase (phase 2). The SET started with a 4 min warming up period of walking at 1.5 m/s followed by 4 minutes of trot at 4.5 m/s. Next, after 1 minute of additional walking at 1.5 m/s horses trotted for 20 min at approximately 80% HR_{peak}. Finally horses were allowed to cool down for 5 min at 1.5 m/s. Heart rate was measured using a Polar S610i heart rate meter (Polar S610i, Polar Electro, Kempele, Finland) and continuous ECG monitoring (Cardio Perfect Stress 4.0; Cardio Perfect Inc, Atlanta, GA, USA). Venous blood was drawn from the jugular vein before the test (t=0 minutes), after the warming up (t=9 minutes), and every 5 minutes during the SET (t=14, 19, 24, 29, 34). Samples were kept on ice until whole blood lactate and pH had been analysed (ABL-605 Radiometer Copenhagen, Westlake, Ohio).

Muscle biopsies were taken approximately 60 minutes before the SET. A 5 cm deep biopsy of the M. Vastus Lateralis (VL) taken under local anaesthesia (lidocain hydrochlorine (2%) without adrenalin) using a modified Bergström biopsy needle (Maastricht instruments, Maastricht, The Netherlands) with a diameter of 7 mm. Blood and fat tissue were removed from the biopsy and the biopsy was there after immediately frozen in liquid nitrogen for biochemical analysis. Frozen muscle tissue was stored at -80ºC.

Biochemical Analysis

For enzymatic analysis 50 mg of frozen muscle tissue was homogenised in 1 ml SET buffer (250 mM sucrose; 2 mM EDTA; 10 mM Tris-HCL) with a Ultraturrax homogeniser. Homogenates were subsequently sonificated 3 times and centrifuged at 15.000g for 10 min. Supernatants were stored at -80ºC until analysed. All chemicals used were of analytical grade.
Total LDH in muscle homogenates was determined using a commercially available assay (ABX diagnostics; Radiometer Nederland BV, Zoetermeer, The Netherlands). Distribution of different LDH iso-enzymes in muscle homogenates was analysed with a commercially available kit (Sebia Benelux S.A., Brussels, Belgium).

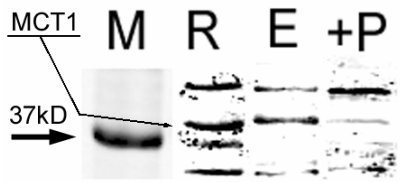
Western blotting of MCT1, 2 and 4 proteins

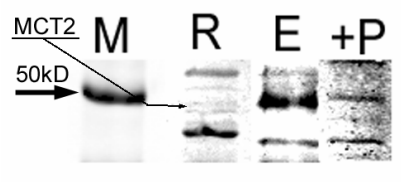
Approximately 50 mg of muscle tissue was homogenized with potter tubes in ice-cold buffer containing 210 mM Sucrose, 30 mM HEPES, 5 mM EDTA, 2 mM EGTA and 1 minitablet protease inhibitors (Roche Applied Science, Almere, The Netherlands). The sample was diluted with a buffer containing 1.17 M KCl, 58.3 mM Na-pyrophosphate and 1 mM DTT. Samples were centrifuged at 150,000g for 90 minutes and the supernatant (cytosol fraction) was stored at -80ºC. The pellet was resuspended in 50 µl buffer containing 10 mM Tris, 1 mM EDTA and 0.1% Triton X-100 (membrane fraction), and stored at -80ºC until blotting procedures. Polyacrylamide sodium dodecysulphate (SDS) gel electrophoresis was performed according to Laemmli [18]. In short, 50 µl homogenate was boiled 5 minutes in an equal volume of SDS sample buffer containing 2.3% SDS and 5% β-mercapto-ethanol and subsequently centrifuged for 5 minutes. Equal amounts of protein (~25 µg) were loaded on 10% polyacrylamide gels and electrophorized at 200 V for 55 minutes. After electrophoresis protein was transferred to a nitrocellulose membrane by blotting for 60 minutes at 100 V.
For detection of MCT protein, nitrocellulose sheets were pre-treated with Odysey blocking buffer (Licor Biosciences; Westburg b.v., Leusden The Netherlands) diluted 1:1 in PBS for 60 minutes. Incubation with MCT1, 2 and 4 antibodies (Santa Cruz Biotechnology; Tebu-bio, Heerhugowaard, The Netherlands), diluted 1:10000 in blocking buffer, was carried out overnight (16 hours) at room temperature with gentle shaking. After three washing steps with 0.1% Tween-20 in PBS blots were incubated for 60 minutes with fluorescent conjugated donkey anti goat secondary antibody (Rockland;

Tebu-bio, Heerhugowaard, The Netherlands). Blots were scanned with an odyssey IR scanner (Licor Biosciences; Westburg b.v., Leusden The Netherlands) and results were expressed as integral intensities and as relative intensities to a positive control sample obtained from rat gastrocnemius muscle.

Statistical analysis

All data are shown as mean ± SD and were tested for significance with one way ANOVA analysis (post hoc: Bonferoni) or a paired samples students t-test, level of significance was set at p=0.05.





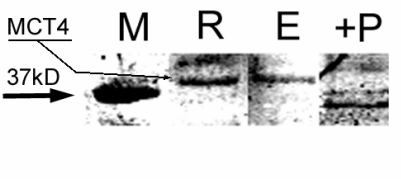


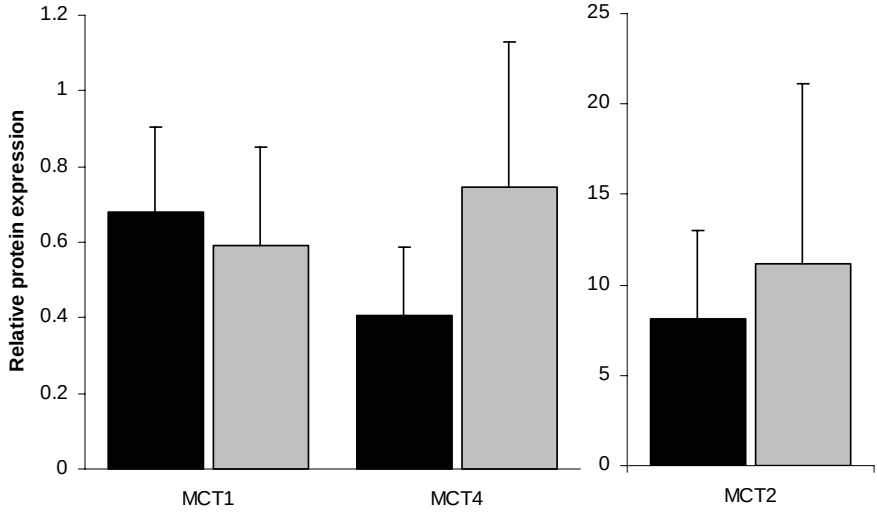
Figure 4.1
This figure shows representative blots of MCT1, 2 and 4
Lane M represents protein weight marker, R represents rat gastronemius control muscle sample, E represents equine vastus lateralis muscle and +P represents a example of pre-incubation with control peptide.

Results

MCT expression

A representative Western blot showing MCT1, MCT2 and MCT4 in VL membranous fraction is shown in figure 4.1. MCT1, MCT2 and MCT4 are all present in the M-fraction of Standardbred muscles. Semi quantative densitometric analysis of the blots showed that MCT1, MCT2 and MCT4 expression in membranous fractions remain unaltered upon training (P>0.05). MCT-2 however showed 60% increase and a large inter-individual variation (figure 4.2).

Figure 4.2
Protein expression of MCT protein
Densitometric analysis of MCT protein is expressed relative to a rat gastrocnemius muscle sample, which is set at 1. Black bars represent protein expression before training, and gray bars represent protein expression after training. No significant effect of training was observed. Another remarkable feature is the relative high expression of MCT2 compared to rat muscle.



Plasma Lactate

Training resulted in an increase in the total plasma lactate accumulation during exercise as measured as the area under the curve during the 20-minute endurance run in the SET (figure 4.3).Trained horses exercised at an increased absolute intensity, while relative intensity (~80% HR_{peak}) was the same in untrained and trained horses.

Total LDH activity and LDH-isoenzymes

Five LDH-isoenzymes were detected in the vastus lateralis muscle homogenates of the Standardbred trotter. LDH-5 was the major LDH isoenzyme found followed by LDH-3, LHD-2 and LDH-4 and LDH-1 (84.2, 7.3, 3.5, 2.8 and 2.2% respectively). The training applied in this study did not lead to significant changes in LDH isoenzyme distribution in VL muscle (figure 4.4).
Also LDH activity expressed as µmol/gram muscle/minute showed no significant differences after 18 weeks of training (223.9 ± 58.8 vs 187.1 ± 36.7 for SET 1 and SET 2, respectively) (figure 4.5).

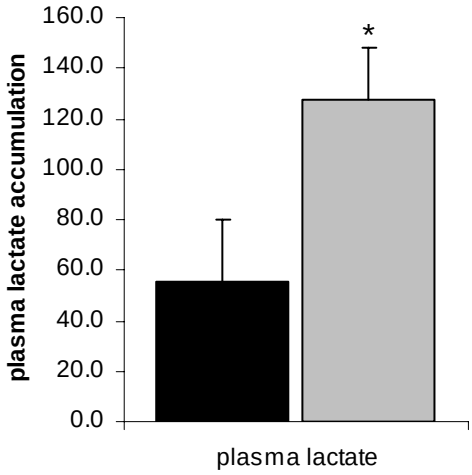


Figure 4.3
Plasma lactate (Area Under Curve) accumulation during the SET before (SET1; black bar) and after (SET2; gray bar) training. At SET2 lactate accumulation in pheripheral blood is significantly higher ($p<0.05$) compared to SET1.

Discussion

Here we report, to the best of our knowledge for the first time, the expression of lactate transport proteins MCT1, MCT2 and MCT4 in skeletal muscle of Standardbred trotters. The relative high expression of MCT2 compared to rat gastrocnemius muscle was, at first unexpected, but may fit well with the gastrointestinal function and high volatile fatty acid metabolism in the horse. Based on similarities in muscle fibre type distribution, enzymatic activity and protein expression in horses and human skeletal muscle, we propose similar roles for MCT proteins in equine skeletal muscle. As mentioned above a major difference between human and horse metabolism is the large availability of volatile (short chain) fatty acids (SCFA) derived from hindgut fermentation in horses. This is a particularly interesting and probably underestimated energy source of horse skeletal muscle. The SCFA acetate, propionate, and butyrate are monocarboxylates which represent the most abundant anions in the colonic lumen. Stein et al. (2000) showed that the uptake of short-chain fatty acids uptake into the Caco–2 cell line is initiated by a pH-dependent and carrier mediated transport mechanism involving the MCT's [28]. These findings are in line with the findings of Gill et al. (2005) who reported an imported role for MCT's in the uptake of short-chain fatty acids [6]. Furthermore, Koho et al. (2005) reported MCT2 and MCT1 as isoforms with the greatest affinity for SCFA in reindeer liver tissue [25]. Therefore, we propose that MCT2 functions as a monocarboxylate transporter with special emphasis on SCFA in horse skeletal muscle.
Antibodies used to identify MCT proteins in this study, were raised against human (MCT1 and 4) and mouse (MCT2) MCT proteins, and the question

remains whether these antibodies cross-react with equine MCT. The function and structure of MCT proteins seems to be highly conserved among species [12]. Nevertheless, in our Western blottings multiple bands were visible, also in a positive control sample of rat gastrocnemius muscle. Pre-incubation with control peptide showed selective diminishment of one specific band in the region of interest (30-60 kD) however. This band could therefore be designated as the specific MCT protein.

Figure 4.4
LDH-isoenzyme distribution in vastus lateralis muscle before (SET1; black bar) and after (SET2; gray bar) training. No significant differences were observed after training in LDH-isenzyme distribution.

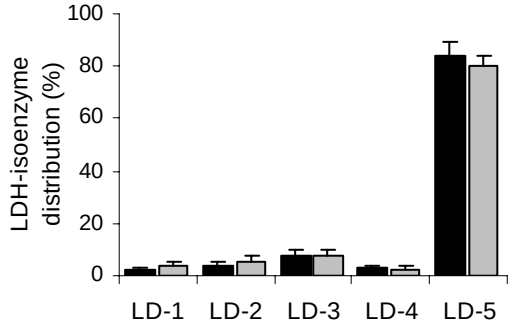
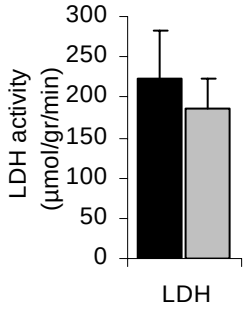


Figure 4.5
Total LDH activity in vastus lateralis muscle at SET1 and SET2. No significant differences were observed between these timepoints.



Plasma lactate concentrations during the SET were significantly higher after a period of training. This result seems to contrast to other reports indicating a decrease in lactate accumulation after training [8, 19, 21]. The higher plasma lactate concentration observed in trained horses could be related to the exercise intensity of the SET. Both SETs were performed at the same relative workload. Therefore energy expenditure was increased during SET 2 compared to SET 1, and this can lead to increases in muscular lactate production [27]. Parallel to increased in plasma lactate, heart frequency during the second test was also increased. Therefore, workload may have been underestimated in the first SET and may have been of too moderate intensity to produce significant amounts of lactate.
Additionally, it must be realized that equines have the unique ability of increasing red blood cell volume during exercise. It has previously been shown that red blood cells express MCT proteins with a high affinity in equines and have a large part in lactate clearance from plasma, especially during exercise [22, 26].
Previous studies show that endurance training results in a significant upregulation of the expression of both MCT1 and MCT4 [5] in human skeletal muscle, or selectively MCT1 [3] in rodents. In this study we could not observe significant changes in the expression of MCT1, MCT2 and MCT4 after training. It has been suggested that adaptations in expression of MCT1 due

to exercise and training are intensity dependent. In line with this hypothesis, Baker et al. (1998) showed no increase in rat muscle MCT1 expression after moderate intensity training but did find an increase in some muscles after high intensity training [1]. Pilegaard et al. (1999) showed increase in MCT1 and MCT4 during high intensity training[20]. In contrast, Juel et al. (2004) and Bickham et al. (2006) were not able to detect increases in MCT4 protein expression after sprint training in human subjects [2, 17]. Compared to these studies, the training load in our study were relatively low. This could be one explanation for the absence of an increase in the expression of MCT1 and MCT4 in equine skeletal muscle.

In conclusion, we report, for the first time, that MCT1, MCT2 and MCT4 proteins are abundantly expressed in skeletal muscle of Standardbred trotters. This implicates that lactate homeostasis in equines is regulated in a similar pattern as in other mammals. The relative high abundance of MCT2 compared to rat gastrocnemius muscle might implicate a regulatory role in short chain fatty acid transport in equine skeletal muscle for this isoform. We suggest therefore that hindgut formation and the subsequent production of short-chain fatty acids play a more dominant role in equine fatty acid metabolism.

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Chapter 5

Metabolic effects of training and acute sub-maximal exercise in Vastus Lateralis muscle and Pectoralis Descendens Muscle in Standardbred trotters

Submitted for publication

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Abstract

Objective:
Adaptation of skeletal muscle to acute exercise and training is, among other modifications, caused by changes in enzyme activities. Insights in adaptation to acute exercise and training in different muscles is of major importance in understanding the equine athlete's physiological capacities. The aim of this study is to evaluate the effects of training and sub-maximal exercise on enzymatic activity in two different skeletal muscles

Animals:
Six (6) untrained Standardbred trotters that had not been involved in any kind of exercise or training regimen previously.

Procedures:
Before and after a training period of 18 weeks, horses completed a sub-maximal standardized exercise test (SET). Before and after each SET muscle biopsies were taken from vastus lateralis muscle (VL) and pectoralis descendens muscle (PD). Enzymatic activity of hexokinase, citrate synthase (CS), and 3-hydroxyacyl-CoA dehydrogenase (HAD) was determined as well as glycogen concentration.

Results:
Hexokinase activity increased during acute exercise, whereas glycogen concentration decreased. Significant differences between VL and PD were observed in glycogen content and enzymatic activity. Enzymatic activity of CS and HAD did not change with exercise or training.

Conclusions and clinical relevance:
Increases in hexokinase and decreases in glycogen concentration suggest increased glucose flux after acute sub-maximal exercise. PD muscles had an increased glycogen concentration and an increase in enzymatic activity of CS and HAD compared to VL muscles. Data on metabolic effects of acute sub-maximal exercise in equines is scarce and this study addresses this issue.

Introduction

Activity of key metabolic skeletal muscle enzymes is often used as reflection of muscle metabolic capacity[31]. For instance, increasing metabolic demands during exercise results in depletion of muscular adenosine triphosphate (ATP) sources and requires activation of metabolic pathways. The speed of ATP resynthesis depends on the activity of key enzymes of glycolytic and oxidative metabolism. To study changes in enzymatic activity in skeletal muscle in vivo, acute exercise bouts and periods of training are often used as intervention strategies[6, 8, 11].

It is well known that training, among other, brings along many metabolic changes in skeletal muscle. For instance, increases in intramuscular energy stores (carbohydrates and lipids), increased capillarization and mitochondrial density and concomitant adaptations in key metabolic enzymes have been shown to occur after training in different species[4, 7, 11, 24, 32]. Data on the effects of acute sub-maximal exercise on enzymatic activity in untrained and trained skeletal muscle in horses is scarce however. Although decrements of glycogen concentrations post-exercise have well been described, less is known about the effects of acute sub-maximal exercise on enzymatic activity of key enzymes in horses. This is somewhat surprising since several metabolic adaptations in enzyme activities and metabolic gene transcription have been reported after acute exercise in several species[17, 29, 31].

Differences in metabolic profile between skeletal muscles, or even within a single muscle, have been described for horses[4, 7, 14, 22]. This implicates that responses to acute exercise are not necessarily similar between muscles. Interestingly, Kim et al. (2005) recently showed that 10 weeks of physical training resulted in increases in citrate synthase and 3-OH-acyl-CoA-dehydrogenase activity in equine triceps muscle but not in semimembranosus muscle[14].

In the present study, we selected the vastus lateralis muscle (VL) and pectoralis descendens muscle (PD) because these muscles have an explicit function in locomotion, and display a different muscle fibre type distribution[34]. The VL muscle is a knee extensor in the hind limb and functions primarily as power generator for propulsion whereas the role of the PD is suggested to be more anti-gravitational than propulsive but is actively contracted during locomotor activity as well[19, 36].

The first aim of the present study was to investigate the effects of an acute bout of exercise and the second aim was to investigate the effects of long-term training on key enzymatic activity and glycogen storage in two different muscles that play an important role during propulsion (VL and PD)[19, 20].

Material and Methods

Animals

Six (6) untrained Standardbred geldings were used in this study. Horses were aged 18 ± 2 months at the start of this study and weighed 374 ± 23kg (mean ±SD). They had not been involved in any kind of exercise or training regimen previously. The diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance. Water was provided ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Training

Horses were trained according to phase one and phase two of the training protocol described in Chapter 2 of this thesis. In short, the horses received an initial training of 4 weeks to get accustomed to trotting on a high speed treadmill (Mustang 2000, Graber AG., Fahrwanger, Switzerland). This "synchronisation" period consisted of endurance training. In the succeeding period of 18 weeks the horses were also trained on a high speed treadmill. The training program consisted of two types of exercise, endurance running and interval running for 4 days/week. The days of interval running were alternated with days of endurance running. On the resting days, all horses walked for 60 min in the horse walker.

Exercise test

A standardised exercise test (SET) was performed before and after the 18 week training period. The SET started with a 4 min warming up period of walking at 1.5 m/s followed by 4 min of trot at 4.5 m/s. Next, after 1 min of additional walking at 1.5 m/s, horses trotted continuously for 20 min. During the 20 min SET, speed and inclination of the treadmill were adapted to elicit a heart rate of 180-190 BPM (approximately 80% of maximal heart rate). Finally horses were allowed to cool down for 5 min at 1.5 m/s. Heart rate was measured using a Polar S.610i heart rate meter (Polar S.610i, Polar Electro, Kempele, Finland). Horses were only allowed to trot. In the untrained group this matched with a speed of 6.5-7.0m/s whereas the trained group trotted at a speed of 8.0-8.5m/s with a treadmill inclination of 1-2.5%.

Muscle biopsies

Muscle biopsies were taken under local anaesthesia (lidocain hydrochlorine (2%) without adrenaline) using a modified Bergström biopsy needle (Maastricht Instruments, Maastricht, The Netherlands) with a diameter of 7 mm. Approximately 60 minutes before the standardized exercise test, a 5 cm deep biopsy of the M. Vastus Lateralis (VL) was taken at a point 15 cm

ventral to the centre of the tuber coxae and 7 cm caudal to the cranial border of the VL muscle. Also a 4 cm deep biopsy was taken at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle from the M. Pectoralis Descendens (PD). Fifteen (15) minutes after cessation of the SET a second muscle biopsy was obtained from a new incision 3 cm caudal to the point of the first biopsy. Blood and fat tissue were carefully removed from the biopsy and the biopsy was thereafter immediately frozen in liquid nitrogen. Frozen muscle tissue was stored at -80ºC.

Assays

For enzymatic analysis 50 mg of frozen muscle tissue was homogenised in 1 ml SET buffer (250 mM sucrose; 2 mM EDTA; 10 mM Tris-HCL) with a Ultraturrax homogeniser. Homogenates were subsequently sonificated 3 times and centrifuged at 15.000g for 10 min. Supernatants were stored at -80ºC until analysed. All chemicals used were of analytical grade.

Glycogen

Approximately 50 mg of frozen muscle tissue was freeze-dried overnight (16 hours). Dry tissue was boiled in 1 N HCl for three hours and equilibrated with 1 N NaOH. Samples were centrifuged and stored at -20ºC. Glucose concentration in the supernatant was assayed with a commercially available glucose assay (Glucose (Hexokinase) assay, Radiometer Nederland BV., Zoetermeer, The Netherlands) on an automated analyser (Cobas Fara automated analyser, Roche Diagnostics, Basel, Switzerland).

Hexokinase

Hexokinase was assayed according to a modified protocol from Bergmeyer et al.[1]. The assay mixture contained 50 mM Tris, 1 mM glucose, 2 mM ATP, 5 mM $MgCl_2$, 0.65 mM NADP and 600 U/ml Glucose-6-Phosphate dehydrogenase. Appearance of NADPH was measured photometrical at 340 nm and 37ºC on an automated analyser.

Citrate Synthase (CS)

CS was assayed according to Sheppard and Garland[25]. The assay mixture contained 100 µM dithiobis-(-2nitrobenzoicacid) (DTNB), 50 µM acetyl-CoA and 50 µM oxaloacetic acid. Appearance of DTNB-CoA was measured spectophotometrical at 412 nm at 37ºC on an automated analyser.

3-Hydroxy-acyl dehydrogenase (HAD)

Activity of HAD was assayed by measuring the amount of NAD^+ that was formed in the reaction Acetoacetyl-CoA + NADH → 3-hydroxy-butyryl-CoA + NAD^+ on an automated analyser. The reagent used contained 10 mM NADH solution which was diluted 50 times in a tetrasodiumpyrophosphate (100 mM) buffer. The reaction was started by adding 2 mM acetoacetyl-CoA and the formation of NAD^+ was measured spectophotometrical at 340 nm at 37ºC.

Statistics

All data are shown as mean ± SD and were tested for significance with one way ANOVA analysis (post hoc: Bonferoni) or a paired samples students t-test, level of significance was set at p=0.05.

Results

Exercise training resulted in a higher exercise load during the SET in the trained horses at a fixed heart rate. In the trained horses this was accomplished by increasing the slope of the treadmill by 1% to 2.5%. Higher speeds were not possible for the intended pace: trotting. Body weight increased significantly ($p<0.05$) from 347±22 kg (mean ± SD) in untrained horses to 389±19 kg after training.

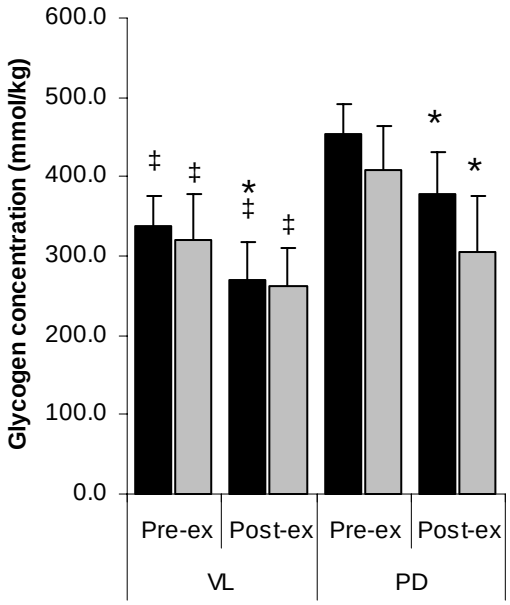

Figure 5.1
Glycogen concentration
Glycogen concentrations (mmol/kg dry weight) in vastus lateralis (VL) and pectoralis descendens (PD) muscle before (Pre-ex) and after (Post-ex) acute exercise in untrained (black bars) and trained (gray bars) horses. Significant ($p<0.05$) effects of acute exercise (*) and between VL and PD (‡) are indicated. No significant effects of training were observed in basal glycogen concentrations.

Glycogen values were significantly decreased after acute exercise in untrained muscles (figure 5.1). In VL muscle glycogen content decreased significantly ($p<0.05$) with 19.8% (±14.0%) after acute exercise in untrained horses while the decrease was not significant ($p=0.07$) 16.4% (±18.7%) in trained horses. In PD muscle the decrease was significant ($p<0.05$) with 16.5% (±14.3%) and 24.9% (±14.3%) for untrained and trained horses, respectively. In PD muscles the decrease in glycogen concentration after acute exercise was also apparent after the training period. Pre-exercise glycogen values were significantly higher in PD muscles

compared to VL muscles before and after training (figure 5.1). The, glycogen content of both muscles was not increased after the 18 week training period.
Citrate synthase activity was not affected by acute exercise. In VL muscle the activity of CS was significantly increased after a period of training, but also in trained muscles no effects of acute exercise were observed (figure 5.2). CS activity was significantly higher in PD muscles before training compared to VL muscles (figure 5.2). After training this effect was not apparent (p=0.06) due to increased variation in CS activity.

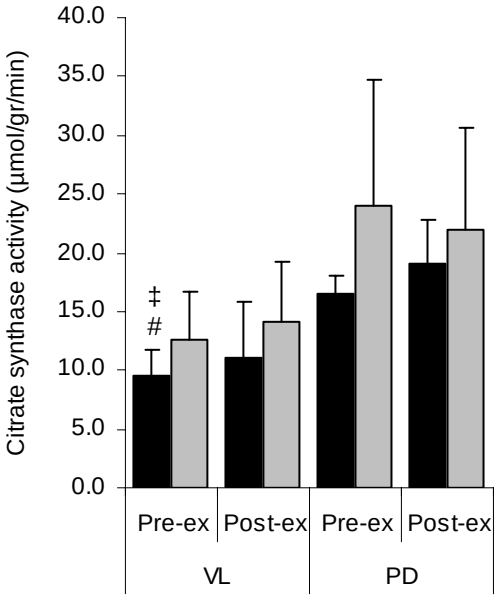

Figure 5.2
Citrate synthase (CS)
CS activity (µmol/gr/min wet weight) in vastus lateralis (VL) and pectoralis descendens (PD) muscle before (Pre-ex) and after (Post-ex) acute exercise in untrained (SET1; black bars) and trained (SET2; gray bars) horses. Significant ($p<0.05$) effects of training (#) are and differences between VL and PD (‡) are indicated. No significant effects of acute exercise were observed in CS activity.

Basal HAD activity was significantly higher in PD muscles irrespective of training and exercise (figure 5.3). No other effects of acute exercise or training were observed in this study.

The activity of hexokinase did not change after acute sub-maximal exercise in untrained horses. Training resulted in a significant decrease in pre-exercise hexokinase activity compared to pre-exercise values before training. In trained muscles hexokinase activity was increased after acute exercise (figure 5.4).

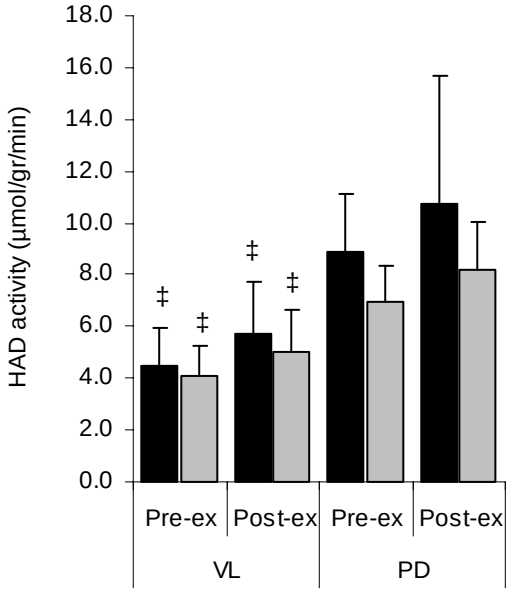

Figure 5.3
3-Hydroxy-acyl dehydrogenase (HAD) activity
HAD activity (µmol/gr/min wet weight) in vastus lateralis (VL) and pectoralis descendens (PD) muscle before (Pre-ex) and after (Post-ex) acute exercise in untrained (SET1; black bars) and trained (SET2; gray bars) horses. Significant ($p<0.05$) differences between VL and PD (‡) are indicated. No effects of training and acute exercise were observed in HAD activity.

Discussion

The results of the present study showed that acute sub-maximal exercise in Standardbred horses has no significant effects on enzymatic activity of CS and HAD. Sub-maximal exercise for 20 minutes reduced intramuscular glycogen stores in this study. This, in combination with a post-exercise increase in hexokinase activity, indicates an increase in glucose metabolism and an increase in glucose phosphorylation in skeletal muscle during exercise. Basal activities of CS and HAD and glycogen concentration are higher in PD than in VL muscles. Intramuscular glycogen concentration and enzymatic activity of CS and HAD measured enzymes were not increased after a period of training whereas hexokinase activity was significantly decreased after training.

Glycogen

Glucose is one of the major fuel sources during high intensity exercise[16]. In the present study, glycogen concentrations decreased by 19.8% (±14.0%) and 16.5% (±14.3%) in untrained VL and PD respectively ($p<0.05$), despite the sub-maximal exercise load. During sub-maximal exercise with high rates of fatty acid oxidation a small amount of glucose metabolism is always needed to provide intermediates for citric acid cycle functioning. Furthermore, glycolysis provides an important source for cytosolic ATP that

is needed for maintenance of electrolyte balance[28]. Therefore, it has been postulated that "fatty acids burn in a flame of carbohydrate.

Some authors have reported a glycogen sparing effect upon a period of training[6, 11]. We were not able to detect such an effect. A plausible explanation for this difference may be attributed to the same relative workload (both groups exercised at approximately 80% of maximal heart-rate resulting in higher absolute workloads in trained horses) applied to trained and untrained horses. Van Loon et al. (1999) showed that increased energy expenditure in trained human subjects is completely accounted for by an increased fat oxidation[35]. As a result, this would yield similar rates of glycogen degradation in both groups. Furthermore, the decrease in glycogen concentration in our study is somewhat lower than reported in previous studies[16, 31], which might be caused by the fact that during sub-maximal exercise fatty acid oxidation provides a substantial contribution to total energy expenditure[27].

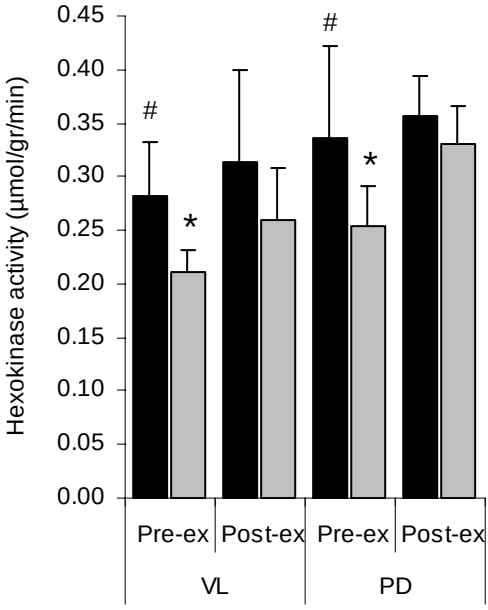

Figure 5.4
Hexokinase activity
Hexokinase activity (µmol/gr/min wet weight) in vastus lateralis (VL) and pectoralis descendens (PD) muscle before (Pre-ex) and after (Post-ex) acute exercise in untrained (SET1;black bars) and trained (SET2; gray bars) horses. Significant ($p<0.05$) effects of acute exercise (*) and training (#) are indicated. No significant effects between VL and PD were observed in hexokinase activity.

In previous studies, it has been shown that exercise training results in increases in basal glycogen content[18, 24]. In the present study, basal glycogen concentrations were not changed after 18 weeks of training. The most likely explanation for these findings is the fact that in our horses glycogen resynthesis may not have been completed since the last training was 24 hours before the SET. Human and rodent research revealed that glycogen resynthesis is complete within 24 hours[16]. In horses it may, dependent on diet, take up to 72 hours for complete restoration of glycogen

stores after exhaustive exercise[12]. Furthermore, it may be possible that the applied training stimulus for VL and PD muscles was not sufficient to elicit the supercompensation increase in glycogen content.

Citrate Syntase (CS)

In the present study acute exercise had no effect on CS activity. In human subjects CS activity increased after exercise both in trained and untrained subjects[17, 29]. In these studies, however, the total amount of exercise exceeded the amount of exercise performed by our horses. After 18 weeks training CS activity was increased in the VL muscle. No significant increase in CS activity was observed in the PD muscle after training. Most studies in horses with prolonged training reported increases in maximal CS activity[7, 11, 23, 24]. Although Geor et al. (1999) also found no increase in CS activity in a 10 day consecutive training study[6]. The lack of an increase in CS activity after training in the PD in the present study is probably due to the large individual standard deviation in post-training samples. Factors that may bias CS activity analysis are time elapsed between exercise and biopsy sampling, exercise intensity and within muscle differences in muscle fibre typing[17]. A significant higher CS activity in PD compared to VL muscles was found in the untrained horses, in the trained horses this difference disappeared. As CS is mainly used as a marker for mitochondrial density, we conclude that mitochondrial density is higher in PD muscle compared to VL muscle. Furthermore, mitochondrial density is increased after training in VL muscle, but not in PD muscle. This may indicate that VL contributes more to propulsion than PD, which may in turn act as an anti-gravitational muscle.

3-Hydroxy-acyl dehydrogenase (HAD)

In the present study, HAD activity was not influenced by acute sub-maximal exercise in VL muscles and PD muscles. Basal HAD activity did not change after training in VL and PD. There was however a significant higher basal activity of HAD in PD muscles compared to VL muscle. Kim et al. (2005) found an increase in HAD activity after training in equine triceps brachii muscles, but not in semimembranosus muscle[14]. Other results in horses report contradictory results as well. For instance, Hodgson et al. (1985) and Serrano et al. (2000) report a significant increase in HAD activity after a period of training[11, 24], whereas Geor et al. (1999), Gottlieb et al. (1989) and Roneus et al. (1992) reported no differences in HAD activity after training[6, 7, 23]. These differences in HAD activity may be attributed to the differences in training intensity and duration applied in these studies. Furthermore it has been suggested that HAD is not the rate limiting enzyme in total fatty acid metabolism[37]. HAD does reflect mitochondrial potential for fatty acid oxidation however, fatty acid oxidation is dependent on more factors than HAD activity, especially during exercise[27, 33]. Therefore, exercise related changes in fatty acid metabolism may not be reflected in adaptation of HAD activity. For instance, carnitine palmitoyl transferase 1 (CPT-1) regulates the entry of fatty acids into mitochondrion and is rate-limiting in this process[37].

Furthermore, Tunstall et al. (2002) found increases in CPT-1 gene expression that was not accompanied by changes in HAD gene expression after 9 days of exercise training in humans[30]. This may indicate that fatty acid availability for β-oxidation is tightly regulated and that downstream enzymes for oxidation such as HAD are in sufficient excess for the increased flux of fatty acids during exercise and training. Finally, diet could also be a potential confounder of fatty acid metabolism measurements, although diet was standard in this study. Helge and Kiens (1997) found that HAD activity is increased after 7 weeks of interval training in combination with a high fat diet in humans whereas 7 weeks of interval training in combination with a high glucose diet did not result in an increase in HAD activity[9]. In addition, Geelen et al. (2001) identified increases in CPT-1 as most effective in upgrading fatty acid oxidation in ponies with different dietary interventions[5]. These and our results indicate that exercise and training are not the sole factors that influence fat oxidation and activity of regulatory enzymes. Therefore, we can conclude that 18 weeks of training according to the protocol employed in this study does not increase mitochondrial activity of fatty acid β-oxidation. We can not exclude that other changes in fatty acid metabolism occurred in skeletal muscle after training.

Hexokinase

Hexokinase activity was decreased after training in this study. In other studies hexokinase activity was increased after training in horses[3] and human subjects[9, 15]. A decrease in hexokinase activity has been reported with growth in horses that may account for the decrease seen in the young Standardbred trotters in this study[10]. In trained horses, hexokinase activity was increased after acute sub-maximal exercise. These results contrast to a previous study in horses that did not find an increase in total hexokinase activity after maximal and sub-maximal exercise[2]. In human subjects however, acute exercise has been shown to increase both hexokinase activity and hexokinase protein transcription[15]. The increase in hexokinase can be attributed to the increase in muscle glucose transport and GLUT-4 translocation towards the plasma membrane that results in an increase in glucose availability for phosphorylation by hexokinase[21].

General overview enzymatic activities and muscle biopsies

Exercise intensity in the "synchronisation" period was very light, and therefore it can not be excluded that training adaptations had already occurred in this four week period preceding the first SET. The 20 month old trotters had not been involved in any training of exercise regimen, and training adaptations can occur within several weeks in untrained horses and humans[7, 26].

Most studies on muscle metabolism in horses have been performed in muscle biopsies from a single muscle, mostly the gluteal muscle[6, 18, 24, 31]. Such results have been suggested to provide whole body adaptation towards exercise and/or training. Several researchers, however, have reported

muscle specific differences in key enzymatic basal activity at rest, with ageing and after training[4, 14, 23]. For instance, Kim et al. (2005) reported that triceps muscle, but not semimembranosus muscle, showed an increase in enzymatic activity (CS and HAD) after 10 weeks training[14]. These results suggest that training-induced changes in one skeletal muscle are not uniformly applicable to other muscles and most likely to whole body adaptation.
This could be related to the fact that skeletal muscle is a heterogeneous tissue that is composed of several muscle fibre types that are either oxidative in nature (type 1) or rely more on anaerobic metabolism (type 2a and 2x)[31]. Fibre type composition of each muscle may show large intra individual variation, even with sampling depth, and may lead to different activities of key metabolic enzymes and muscle fibre types[4, 13, 22]. Research in our laboratory found no effects of training in these horses on muscle fibre type. VL muscle expressed 56% type 1, 26% type 2a and 18 % type 2x muscle fibres and PD muscle 20%, 29%, 51% respectively[34]. The higher proportion of type II muscle fibres in PD muscle may explain the higher glycogen values observed in this muscle. Oxidative enzyme activity, however, was also increased in PD muscles, which is paradoxical to this fibre distribution, as VL muscles have a higher ratio of oxidative (type I and type IIa) muscle fibres. Therefore, not all differences in metabolic properties between VL and PD muscles can be accounted for by differences in muscle fibre type.

In conclusion, in the current study, acute sub-maximal treadmill exercise resulted in an increase in muscle glucose metabolism reflected by decreases in muscular glycogen concentration and a concomitant increase in hexokinase activity. The present study failed to identify other metabolic adaptations in skeletal muscle. This is probably due to the exercise protocol used. For standardization purposes horses were only allowed to trot, an unnatural gait at high speeds. Furthermore, the duration of 20 minutes is probably too short exercise duration at sub-maximal exercise intensity to provoke enough acute adaptations in skeletal muscle. Additionally, metabolic properties of skeletal muscle may have returned to baseline values 15 minutes after exercise, the moment the biopsy was taken.
An 18 week training period had no effects on the activity of metabolic key enzymes CS and HAD and glycogen concentration, and resulted in a decrease in hexokinase activity. This may be due to the training protocol used in this study. This probably did not put sufficient stress on the selected muscles to induce pronounced changes in mitochondrial density.
Finally, PD muscles had an increased glycogen concentration and an increase in enzymatic activity of CS and HAD compared to VL muscles. This, in combination with previous results[4, 14, 22] shows that diagnostic and/or scientific results obtained in a single skeletal muscle are not generally applicable to the horses' physiological adaptation in general.

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Chapter 6

Effect of training and intensified training on quantitative electromyographic and biochemical parameters in Standardbreds

Submitted for publication

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Abstract

Adaptation of skeletal muscle to training can be measured by both quantitative electromyography (EMG) and biochemical analysis of muscle biopsies. An adverse effect of too intense training without adequate recovery may be lead to overreaching or overtraining. No information is available regarding the threshold for maximal training stress.

Methods

In this study, 12 standardbred geldings were subjected to an 18 week training program. After this period, a group of six horses were subjected to an intensified training protocol in order to increase training stress, whereas the other six animals continued normal training. Before and at the end of each training phase, quantitative electromyography (EMG) analysis and biochemical enzyme activity in muscle biopsies was performed in order to identify (mal)adaptation of skeletal muscle to training and intensified training.

Results

After 18 weeks of training, quantitative EMG parameters showed a significant adaptation to training whereas in muscle biopsies only hexokinase activity was decreased significantly. Intensified training induced a stronger training adaptation in quantitative EMG variables compared to controls. Enzyme activities of HAD and CS displayed increased activity after a period of both normal and intensified training. No quantitative differences between the two training regimens were observed.

Conclusions

We conclude that quantitative EMG analysis provides a sensitive tool to monitor training adaptation. Furthermore, we suggest that our intensified training lead to a normal training adaptation rather than mal-adaptation in skeletal muscle, that was visible in both quantitative EMG and biochemical analysis.

Introduction

The aim of training is to induce improvement of athletic performance. In the previous chapter, however, we have shown that 18 weeks of increasing training loads elicited almost no changes in metabolic properties of vastus lateralis and pectoralis muscle of equines compared to control animals that trained with unchanged training loads. This unexpected finding might be due to an inadequate training load or insensitivity of the parameters used to measure training adaptation.

Several studies have shown that muscle excitation and neuromuscular parameters change according to the training load[16, 18, 19]. Therefore, in this study, we chose to evaluate both neuromuscular and biochemical adaptations induced by training followed by a period of intensified training as described in Chapter 2.

In needle electromyography (EMG), electrical activity generated by muscle fibres is recorded, analysed, and interpreted. Pathological spontaneous activity, insertional activity and motor unit action potentials (MUP) are most commonly analysed EMG variables. Several factors influence the recorded signal, among these are temperature, age and examined muscle and training[4, 6, 7, 13, 16, 26, 30, 34, 35]. Weight, height, and sex have little correlation with motor unit action potentials (MUPs) variables[4, 6, 10, 30, 36]. Since it appears from former EMG studies in the horse that the horse follows the general principles of motor units alterations as described in man[2, 33, 37], it seems reasonable to assume that training effects as described in EMG in man, are applicable to the horse.

Neural adaptation seems to play a greater role in strength gain due to training than hypertrophy[18]. An increase in muscle strength without noticeable hypertrophy is considered the result of neural involvement in acquisition of muscular strength[16]. Another mechanism for increase in muscle force is motor unit synchronization[15]. MUP amplitude is positively influenced by both hypertrophy and increased neuromuscular excitability, whereas MUP variables as duration, phase and turns are influenced by synchronization of the MU.

An early sign of overreaching due to training is a deterioration of neuromuscular excitation, leading to low amplitude EMG[24, 23]. Therefore, quantitative EMG analysis is a possible tool for the early identification of an overload of training induced stress. Additionally, quantitative EMG analysis is used to detect pathology in muscle or nerve[2, 31, 36]. During exercise, glucose and fatty acids are primarily metabolized to provide energy for the contraction process. Training has been shown to a pronounced effect on maximal enzyme activities[12, 17, 28]. Several researchers report adaptation of associated enzyme activities in response to training in horses[8, 20, 28]. Increased oxygen consumption, decreased glycogen concentration and decreased lactic acid accumulation have been identified as metabolic

markers for overreaching and overtraining[14, 22]. This suggests that metabolic parameters sensitive for increases in training induced stress.
The aim of the current study is to elucidate neuromuscular and biochemical adaptations in skeletal muscle induce by training. Furthermore, we investigated whether a higher than normal training load could be analysed by means of quantitative EMG and biochemical analysis of enzyme activity.

Material and Methods

Horses

12 Standardbred geldings were used in this study. Horses were aged 20 ± 2 months in this study and were owned by the Faculty of Veterinary Medicine of the University of Utrecht, The Netherlands. The horses were individually housed and their diet consisted of grass silage supplemented with concentrate feed and vitamin supplements and met nutrient requirements for maintenance and performance (58 MJ NE (range 54-66). Salt blocks and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Medical Ethical Committee of the Utrecht University, and complied with the principles of laboratory animal care.

Training

The Standardbred trotters were trained according to the protocol described in Chapter 2. In short, all training sessions and exercise tests were performed on a high speed treadmill (Mustang 2000, Graber AG. Switzerland). The exercise intensity during the training was based on fixed percentages of the peak heart rate, as obtained in a previous study[5]. The training intensity was adjusted according to the heart rate measurements with a Polar heart rate monitor (Polar S610i, Polar Electro, Kempele, Finland) during the training sessions on a weekly basis.
The horses were randomly divided into 2 groups: control horses (subject to basic training) and intensified trained horses (subject to increased workload). Training was divided into four phases: 1) acclimatisation to exercise on the treadmill for 4 weeks with endurance training (40-50% HR_{max}); 2) training period for 18 weeks with alternating both endurance (~ 60-75% HR_{max}) and high intensity training (~ 80-90 % HR_{max}); 3) increased training volume and intensity for 6 weeks, and 4) detraining for 4 weeks (~60% HR_{max}).

Exercise test

A standardised exercise test (SET) was performed in all horses every four weeks and on the final day of each phase to monitor performance. SET 1, 2, 3 and 4 corresponded to the exercise test at the end of each phase of the training. The SET started with a 4 min warming up period of walking at 1.5m/s followed by 4 minutes of trot at 4.5m/s. Next, after 1 minute of

additional walking at 1.5m/s horses trotted for 20 min at approximately 80% HRmax. Finally horses were allowed to cool down for 5 min at 1.5 m/s. Heart rate was measured using a Polar S610i heart rate meter and continuous ECG monitoring (Cardio Perfect Stress 4.0; Cardio Perfect Inc, Atlanta, GA, USA).

Muscle biopies

Muscle biopsies were taken approximately 60 minutes before SET1, 2, 3 and 4. A 5 cm deep biopsy of the M. Vastus Lateralis (VL) and 4 cm deep biopsy the M. Pectoralis Descendens (PD) were taken under local anaesthesia (lidocain hydrochlorine (2%)) using a modified Bergström biopsy needle (Maastricht instruments, Maastricht, The Netherlands) with a diameter of 7 mm. Blood and fat tissue were removed as fast as possible from the biopsy and the biopsy was there after immediately frozen in liquid nitrogen for biochemical analysis. Frozen muscle tissue was stored at -80ºC.

EMG examination

At the end of each training phase, the day before performing the SET, quantitative electromyographic (EMG) analysis was performed in the VL, PD and subclavian (SC) muscle. Measuring left and right muscles was alternated. Insertional activity, pathological spontaneous activity (PSA), MUPs and satellite potentials were recorded. Spontaneous activity was assessed outside the endplate region in the same regions in which MUPs were obtained. Pathological spontaneous activity was considered indicative of pathology if present in two or more locations.
Details on definition, materials and methods of EMG examination can be found in Chapter 2. In short, EMG signals were recorded using a portable apparatus (Nicolet Meridian, Nicolet Biomedical Inc., Madison, WI, USA) and concentric needles (Nicolet Biomedical Inc., Madison, WI, USA). Band pass was between 5 Hz and 10 kHz. Sweep speed was 10-20 ms/division. Amplifier gain was 50-100 µV for spontaneous activity and 10-500 µV for MUP recording. At least 3 insertions and three directions per insertion were made per investigated muscle. Insertional activity, pathological spontaneous activity and motor unit action potentials were recorded. 20-30 MUPS per muscle were analysed.

Biochemical assays

For assessment of glycogen concentration approximately 50mg of frozen muscle tissue was freeze-dried overnight (16 hours). Dry tissue was boiled in 1N HCl for three hours and equilibrated with 1N NaOH. Samples were centrifuged and stored at -20ºC. Glucose concentration in the supernatant was assayed with a commercially available glucose assay (Glucose (Hexokinase) assay, Radiometer Nederland BV., Zoetermeer, The Netherlands) on an automated analyser (Cobas Fara automated analyser, Roche Diagnostics, Basel, Switzerland).

For enzymatic analysis 50 mg of muscle tissue was homogenised in 1 ml SET buffer (250 mM sucrose; 2 mM EDTA; 10 mM Tris-HCL) with a Ultraturrax homogeniser. Homogenates were subsequently sonificated 3 times and centrifuged at 15.000g for 10 min. Supernatants were stored at -80ºC until analysed. All chemicals used were of analytical grade.
Hexokinase was assayed according to a modified protocol from Bergmeyer et al.[3]. The assay mixture contained 50 mM Tris, 1 mM glucose, 2 M ATP, 5 mM $MgCl_2$, 0.65 mM NADP and 600 U/ml Glucose-6-Phosphate dehydrogenase. Appearance of NADPH was measured photometrical at 340 nm and 37ºC on an automated analyser.
Citrate Synthase (CS) was assayed according to Sheppard and Garland[29]. The assay mixture contained 100 µM dithiobis-(-2nitrobenzoicacid) (DTNB), 50 µM acetyl-CoA and 50 µM oxaloacetic acid. Appearance of DTNB-CoA was measured spectophotometrical at 412 nm at 37ºC on an automated analyser.
Activity of 3-Hydroxy-acyl dehydrogenase (HAD) was assayed by measuring the amount of NAD^+ that was formed in the reaction Acetoacetyl-CoA + NADH → 3-hydroxy-butyryl-CoA + NAD^+ on an automated analyser. The reagent we used was 10mM NADH solution that was diluted 50 times in a tetrasodiumpyrophosphate (100 mM) buffer. Adding 2 mM acetoacetyl-CoA started the reaction and the formation of NAD^+ was measured spectophotometrical at 340 nm at 37ºC.

Statistical analysis

The EMG were statistically analysed as described before[33, 37] using SPSS (SPSS; Headquarters 2133S. Wacker drive, Chicago Illinois, 60606 USA) as statistical program. The positively skewed data on MUP variables were transformed into natural logarithm (ln) to enable statistical analysis. To facilitate the interpretation, the mean ln transformed data are represented as geometric means (gmean) that were derived from back transformation of the ln values. Descriptive statistics were used to calculate gmean, standard deviation (SD) and 95 % confidence intervals (95 CI).
Univariate analysis was performed to determine differences between control and intensified trained horses (group difference), horse couples, muscle, time points (SET) and to determine if group difference were the result of the (over) training. Contrast and post hoc testing according to Bonferroni of the transformed data were used to localise differences. Significance was set at 0.05, two tailed.
For statistical analysis of all biochemical essays and physiological characteristics, one way ANOVA were used. Post hoc tests according to Bonferroni were performed. Significance was set at 0.05, two tailed.

Results

Due to injury, two horses (one control and one intensified trained) were not able to complete the training. Analysis is therefore bases on the remaining 10 horses. Body weight (392 ± 40 to 427 ± 44 kg) and height at withers (150.5 ± 7.4 to 154.6 ± 5.1 cm) increased significantly during the training period. There were no significant differences between the two training groups. Body temperatures were not different at the four SETs, between different muscles or between both training groups.

EMG analysis

The insertional activity was not prolonged in both groups. In 1 control horse, the VL showed fibrillation potentials at SET 4, the PD showed fibrillation potentials and positive sharp waves at SET 4 and in the SC fibrillation potentials and neuromyotonic discharges were found occasionally. All intensified trained horses showed at some point pathological spontaneous activity (PSA). In 4 out of 5 intensified trained horses this was present in the VL, in 2 out of 5 in the PD and in no horses in the SC. In the VL fibrillation potentials, positive sharp waves or doublets were recorded, and in the PD fibrillation potentials or neuromyotonia. At SET 2 the presence of PSA was present in 2 out of 5 horses, at SET 3 and 4 this was present in 3out of 5 horses.
Satellite potentials were present in both groups, however no significant differences were found between groups and the four SETs.
Semi quantitative analysis indicated abnormally high MUP variables in 1 out of 5 couples. In the intensified trained horse of this couple high MUP variables were observed in PD and SCL muscle at all SETs, and in VL muscle at SET 3. In the control horse this was the case in the VL and SCL horse at SET 3 and SET 4. At SET1, these two horses showed significant higher MUP values ($p<0.001$) compared to the additional control horses ($p< 0.001$) and the control horses of the first training year ($p<0.001$). In the intensified trained horse this was measured in all 3 muscles and in the control horse in the SC and VL muscle.

- Ln duration of the MUP (table 6.1): The ln duration in the intensified trained horses was higher than in the controls ($p<0.004$) during the training period. The ln duration at SET1 was higher than at SET 3 and 4 ($p<0.0019$ in all muscles. Ln duration in the lateral vastus muscle was lower than in the SC muscle ($p<0.004$). The effect of training was highest for the SC muscle ($p<0.006$). The muscles showed different changes in time ($p<0.006$). The effect of training was the highest at SET 3 and 4 ($p<0.001$).
- Ln amplitude (table 6.2): The ln amplitude was significant higher in the intensified trained group during the training period ($P<0.001$). The ln amplitude at SET 2 was higher than at SET 1 ($p<0.019$) and higher than at SET 4 ($P<0.002$). At SET 2 and 3 the ln amplitude was significant highest

($p<0.034$) and was still higher at SET 4 than at SET 1 ($p<0.034$). This effect was most obvious in the VL muscle and the PD muscle because the ln amplitude was higher in the PD and VL muscle than in the SC muscle ($p<0.001$).
The effect of the difference in training was present in all three muscles ($p<0.001$) and not the same for all three muscles ($p<0.002$). The Ln amplitude was significantly the highest at SET 2 ($p<0.002$) with a difference between the control and intensified trained group. In the intensified trained group the ln amplitude was not only higher at SET 2, but also at SET 3, and decreased at SET 4.
Ln phase of the MUP (table 6.3): The ln phase was significant higher in the intensified trained group ($P<0.007$) during the training period. The ln phase at SET 1 was higher than at SET 2 ($p<0.025$), SET 3 ($p<0.001$) and SET 4 ($P<0.035$) (table 3). The ln phase in the SC muscle was lower than in the PD

Table 6.1:

Results of univariate analysis for Ln amplitude

The control group was subjected to the normal training and the test group was subjected to intensified training in phase 3 of the training. "Time" number indicates the SET number, and "muscle" the analysed muscle. * Indicates a statistical difference at this level.

	Test-group	Variable	gmean	SD*	95 CI
Group	Control	amplitude (μV)	217	1.01	210-225
	Test *		219	1.01	213-228
Time	1*	amplitude (μV)	213	1.03	203-223
	2*		230	1.03	219-242
	3		218	1.02	208-229
	4		214	1.02	204-224
Muscle	sc*	amplitude (μV)	192	1.02	184-200
	pd		238	1.02	226-248
	vl		230	1.02	221-240

Abbreviations

Gmean: geometric mean; μV: microvolt; CI: confidence interval; Ln: natural logarithm, SD; standard deviation; sc: subclavian muscle; pd; descending pectoral muscle; vl: lateral vastus muscle.

(p<0.002) and VL muscle (p<0.0015). An effect of difference in training was not present.
- Ln turn of the MUP (table 6.4): The ln turn was significant higher in the intensified trained group (P<0.006) during the training period. The ln turn in the SC muscle was lower than in the PD muscle (p<0.001) and VL muscle (p<0.0001). There is an effect of training during the whole period with a lower number of turns at SET 3 in VL and PD (p<0.019) in the intensified trained group. In the SC the number of phases is significant lower than in the VL and PD (p<0.001), with at SET 2 the lowest value.

Biochemical assays

Results of the training phase (phase 1 and phase 2) are extensively described and discussed in Chapter 5. In short, we observed a significant decreased hexokinase activity (p<0.05) after 18 weeks of combined interval and endurance training in VL muscle (differences between SET 1 and SET

Table 6.2:

Results of univariate analysis for Ln duration

The control group was subjected to the normal training and the test group was subjected to intensified training in phase 3 of the training. "Time" number indicates the SET number, and "muscle" the analysed muscle. * Indicates a statistical difference at this level.

	Test-group	Variable	gmean	SD	95 CI gmean
Group	Control	duration (ms)	4.8	1.01	4.7-5.1
	Test*		5.0	1.01	4.9-5.2
Time	1	duration (ms)	5.1	1.01	5.0-5.3
	2		5.1	1.01	4.9-5.3
	3*		4.6	1.01	4.5-5.2
	4*		4.8	1.01	4.7-4.9
Muscle	sc	duration (ms)	5.0	1.01	4.9-5.2
	pd		4.9	1.01	4.9-5.0
	vl*		4.8	1.01	4.6-4.9

Abbreviations

Gmean: geometric mean; μV: microvolt; CI: confidence interval; Ln: natural logarithm, SD; standard deviation; sc: subclavian muscle; pd; descending pectoral muscle; vl: lateral vastus muscle.

2). No further effects of this training period were observed in enzymatic activity of CS and HAD and glycogen concentration in VL or PD muscle (see also Chapter 5).

Intensified training, as applied in this part of the study, resulted in a significant increase in CS activity compared to control training in VL muscle (figure 6.1). No other differences were observed between intensified and control training. Activity of HAD was significantly increased after 6 weeks of intensified training in VL and PD, but HAD activity also increased in controls. Therefore, no differences between the two training regimens were observed (figure 6.2). This was also the case for hexokinase activity that increased significantly in VL in both control and intensified trained group. No effects in PD muscles were observed however (figure 6.3). No effects of training or intensified training were observed in glycogen concentrations in VL and PD muscles either (figure 6.4).

Table 6.3

Results of univariate analysis for phase

The control group was subjected to the normal training and the test group was subjected to intensified training in phase 3 of the training. "Time" number indicates the SET number, and "muscle" the analysed muscle. * Indicates a statistical difference at this level.

	Test-group	Variable	gMean	SD*	95 CI gmean
Group	Control	Phase	2.2	1.0	2.2-2.3
	Test*		2.3	1.01	2.3-2.4
Time	1*	Phase	2.4	1.01	2.3-2.4
	2		2.3	1.01	2.2-2.3
	3		2.2	1.01	2.2-2.3
	4		2.2	1.01	2.2-2.3
Muscle	sc*	Phase	2.2	1.01	2.2-2.3
	pd		2.3	1.01	2.3-2.4
	vl		2.3	1.01	2.3-2.4

Abbreviations

Gmean: geometric mean; CI: confidence interval; Ln: natural logarithm, SD; standard deviation; sc: subclavian muscle; pd; descending pectoral muscle; vl: lateral vastus muscle.

Discussion

The aim of the current study is to elucidate neuromuscular and biochemical adaptations in horse skeletal muscle to training in the equine athlete. The training protocol used in this study resulted in changes in quantitative EMG values associated with training. A period of intensified training resulted in an increase in training associated adaptations in skeletal muscles. No evidence of adverse adaptation could be observed after the period intensified training. Biochemical analysis of skeletal muscle biopsies resulted in less pronounced training adaptation than quantitative EMG.
An increase of muscle force as a result of training can, among other factors, be the result of increased hypertrophy of muscle fibres and/or motor unit synchronisation[16, 24]. The significant increase in MUP amplitude at SET 2 compared to SET 1 in the VL and PD muscle can be the result of an increase in muscle fibre diameter, recruitment of larger (type 2) motor units, and

Table 6.4

Results of univariate analysis for number of turns

The control group was subjected to the normal training and the test group was subjected to intensified training in phase 3 of the training. "Time" number indicates the SET number, and "muscle" the analysed muscle. * Indicates a statistical difference at this level.

	Test-group	Variable	gmean	SD	95 CI gmean
Group	Control	Ln turn	2.5	1.01	2.5-2.6
	Test*		2.6	1.01	2.6-2.7
Time	1*	Ln turn	2.7	1.01	2.6-2.7
	2		2.6	1.01	2.5-2.6
	3		2.5	1.01	2.4-2.5
	4		2.6	1.01	2.5-2.6
Muscle	sc*	Ln turn	2.5	1.01	2.4-2.5
	pd		2.6	1.01	2.5-2.7
	vl		2.7	1.01	2.6-2.7

Abbreviations

Gmean: geometric mean; CI: confidence interval; Ln: natural logarithm, SD; standard deviation; sc: subclavian muscle; pd; descending pectoral muscle; vl: lateral vastus muscle.

Figure 6.1
Citrate synthase (CS) activity
Black bars represent normal trained horses and gray bars represent intensified trained horses. CS activity in vastus lateralis (VL) muscle is significantly ($p<0.05$) increased at SET4 compared to SET2 (*) in the control group. A significant ($p<0.05$) difference is apparent between control and intensified group in VL muscle at SET3 (+). No significant differences were observed in

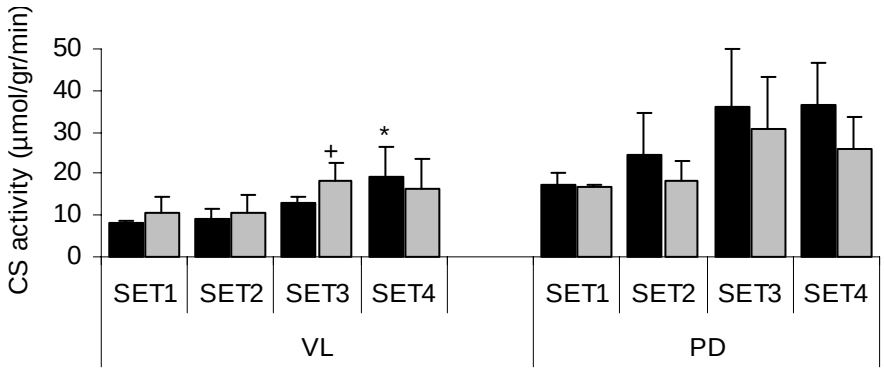


Figure 6.2
3-Hydroxy-acyl dehydrogenase (HAD) activity
HAD activity in vastus lateralis (VL) and pectralis descendens (PD) muscle at SET 1,2,3 and 4. No effects of training were observed between SET1 and SET2. In the VL of the intensified trained group, HAD activity was significantly ($p<0.05$) increased after the intensified training period and was also increased at SET4 in the control group (*). A similar image was observed in PD muscle.

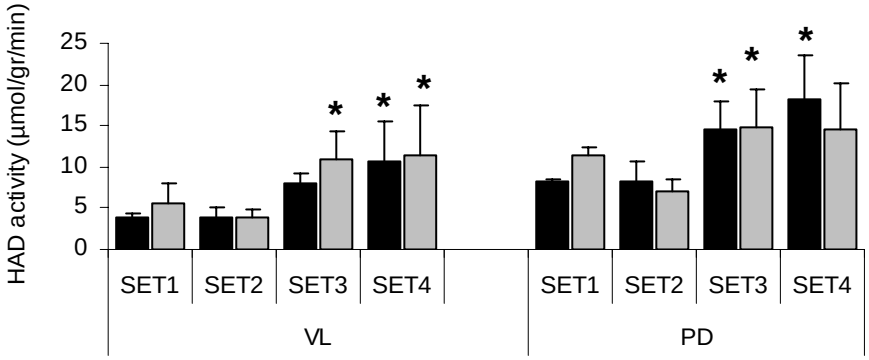


Figure 6.3
Hexokinase activity
Black bars represent normal trained horses and gray bars represent intensified trained horses. Hexokinase activity was significantly decreased in vastus lateralis (VL) muscle after training (#; $p<0.05$). A significant increase ($p<0.05$) in hexokinase activity was observed between SET2 and SET3 in intensified trained and control group (*). No group differences were observed.

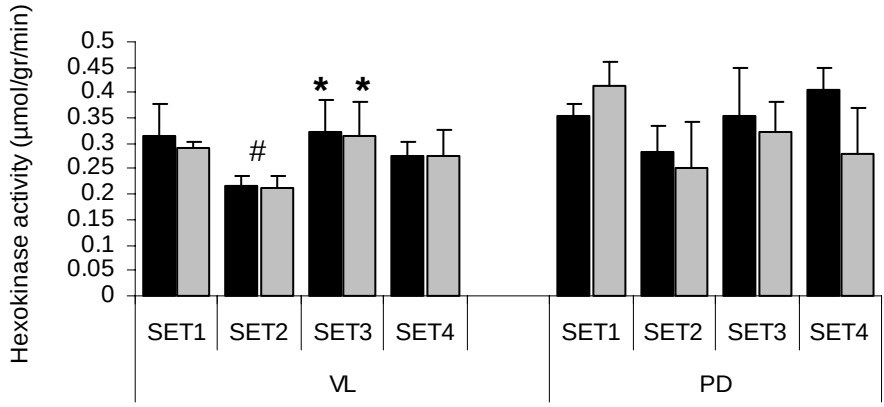
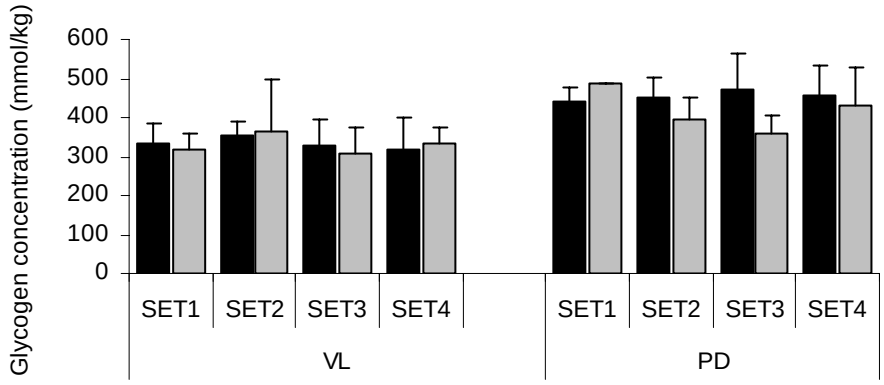


Figure 6. 4
Glycogen concentration
Black bars represent normal trained horses and gray bars represent intensified trained horses. Resting glycogen concentration (mmol/gr dry weight) in VL and PD at SET1,2,3 and 4. No differences in glycogen concentration were observed.



increased synchronisation of the muscle fibre action potentials[16, 19, 23]. Fibre diameters were not increased according to the results of the six horses trained in the first year of the study (M. v. d. Burg; personal communication). Therefore, the higher amplitude can be interpreted as recruitment of larger motor units and synchronisation due to training.
The adaptations in biochemical properties of skeletal muscle during the training period of 18 weeks are described in Chapter 5. The limited number of biochemical adaptations, in combination with the observed changes in MUP parameters, indicates that needle EMG analysis is potentially a sensitive tool in measuring effects of training in equine skeletal muscle.
Studies concerning the use of neuromuscular excitability to recognize overtraining in athletes, report that an increase in neural stimulus was required to produce a single contraction of reference muscle[24, 23]. Since EMG patterns in horses follow an identical adaptation process to training as in man, it is reasonable to assume that effects of intensified training are also applicable to the horse.
The significant lower duration, and lower number of phases and number of turns at SET 3 compared to SET 2, in both controls and intensified trained horses, can be interpreted as synchronisation of the individual muscle fibre action potentials contributing to the MUP[9, 30]. Only Ln duration of the MUP was significantly lower in intensified trained horses compared to controls. Motor unit synchronisation is a possible mechanism for increase in muscle strength due to training[15]. As an alternative explanation, myopathy can lead to a reduction in duration in MUPs[9, 26, 36], but without evidence of decrease in amplitude and increased presence of pathological spontaneous activity, this is a less likely explanation. The neuropathies detected in the two individual horses might be the explanation for the irregularities in their gait. It has to be considered that potentially, this might influence other outcomes from this study with regard to biochemical, behavioural or hormonal stress effects. Factors such as body temperature, intramuscular temperature and growth in general, can also, influence MUP variables[7, 13, 16]. However, these variables were not different among the groups and therefore temperature and growth do not account for the differences found between the groups at SET3.
Increased motor unit synchronisation in this period was accompanied by increases in mitochondrial density (measured by CS activity), increases in mitochondrial β-oxidation (HAD) and increases in glucose phosphorylation (hexokinase), as observed previously after training[11, 17, 20]. Only CS activity was significantly increased in the intensified trained horses compared to controls, indicating an increased mitochondrial density due to intensified training. The increases in enzymatic activity can be explained by the fact that intensified trained horses recruited more muscle force as a training adaptation according to the EMG. Therefore, both EMG and biochemical results in this study indicate an effect of training, in controls and intensified trained horses, rather than evidence of "overtraining".
In this study we analysed different muscles (EMG: VL, PD and SC, biochemistry: VL and PD) because EMG values and biochemical enzyme

activities can vary significantly between muscles as we have seen in Chapter 5[7, 12, 21]. The differences between muscles observed in this study, might be a function of differences in muscle fibre type. Muscle fibre type influences all MUP variables[7, 13, 30, 34, 35] and recruitment of larger type II motor units results in high amplitude MUPs[9]. Additionally, type I fibres usually have a higher mitochondrial density and higher oxidative metabolism compared to type II muscle fibres.

Quantitative EMG data indicate that the training effect is lowest for the SC muscle and highest for the VL muscle. This is to be expected regarding the different role these muscles play in propulsion with the most important role for the VL muscle. The fact that the SC muscle has the lowest values of the majority of MUP variables is in agreement with former studies on MUP analysis in horses, and is explained by its higher percentage of type I fibres [34]. The values of the VL muscle and PD muscle are less different from each other. This might be reflected by the fact that the VL muscle contained a unexpected high percentage type I fibres in horses in this study[32]. Previous studies in different horse breeds showed a high abundance of type 2 muscle fibres in VL muscles[1, 25]. Differences in muscle fibre type distribution may also explain the differences in biochemical properties between VL and PD[12, 27]. In Chapter 5 however, we could not explain all biochemical differences on basis of different muscle fibre type in the same horses.

In this study, we used quantitative EMG and biochemical analysis of skeletal muscle to evaluate changes induced by training and intensified training. EMG analysis showed clear signs of training adaptation after 18 weeks of training whereas biochemical assays were less sensitive for training induced adaptations. EMG analysis therefore provides a powerful tool to for the identification of small improvements in fitness in horses.

Quantitative EMG and biochemical analysis both showed no signs of mal-adaptation after intensified training compared to controls. There was however, a stronger training associated adaptation in both quantitative EMG parameters and biochemical parameters observed in this study in the intensified trained horses compared to controls. Therefore, we conclude that the intensified training protocol used in this study does not increase stress levels above the maximal threshold for skeletal muscle. It can however not be excluded that overtraining at the neuromuscular level is preceded by endocrine and other parameters. Such studies are currently being executed. Finally, we think that sarcolemmal substrate transport proteins for fatty acids (FAT/CD36), glucose (GLUT-4) and monocarboxylates (MCT), as identified in Chapters 3 and 4, may have an important function in skeletal muscle metabolism. It may very well be possible that these proteins determine the threshold for substrate oxidation after prolonged intense training. Due to technical problems we were not able to perform such analyses.

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Chapter 7

Lateral vastus muscle phenotype and associated glucose metabolism in hoses with lower motor neuron disease

Submitted for publication

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Abstract

Background

The purpose of the study reported here was to investigate whether the increased whole body basal glucose metabolism as seen in equine LMND (resembling the sporadic form of human progressive spinal muscle atrophy) is associated with enhanced muscle glucose metabolism and GLUT-4 protein expression in vastus lateralis muscle secondary to denervation and reinnervation processes.

Methods

Glycogen concentration, enzyme activity of citrate synthase (CS) and 3-Hydroxy-acyl dehydrogenase (HAD), immunofluorescent and membrane bound GLUT-4 expression and muscle fibre type was studied in vastus lateralis muscle biopsies from 5 LMND patients and 5 healthy controls. In addition, the EMG and the euglycemic hyperinsulinemic clamp techniques were performed in12 LMND patients and 15 healthy controls.

Findings

LMND patients had significantly decreased muscle glycogen concentrations as well as CS and HAD activities. A significant shift in muscle fibre type from I to II as based on both morphometry and metabolic analysis was found combined with a non-significant increase in GLUT-4 expression ($p=0.07$). EMG revealed neurogenic abnormalities associated with denervation as well as reinnervation and it assessed neurogenic changes before histopathological abnormalities were visible.

Interpretation

The anaerobic changes in affected lateral vastus muscle are in agreement with a shift in muscle fibre type from I to II. On the basis of EMG analysis we conclude that both denervation and reinnervation cause this shift in muscle fibre type, although no direct morphometric evidence was found for reinnervation. GLUT-4 protein expression in lateral vastus muscle in LMND-affected horses in the current study obviously did not comply with the increased whole body basal glucose metabolism, thereby possibly facilitating net muscular glycogen loss. Neurogenic changes in skeletal muscle seem to precede myogenic changes and therefore EMG-analysis is seen as an attractive tool for early identification of neuromuscular disease.

Introduction

Contractile and neural activity directly modulate skeletal muscle glucose transporter 4 (GLUT-4) protein concentrations independent of insulin action. GLUT-4 expression in rat soleus muscle was decreased by 50% as soon as after 3 days of sciatic nerve sectioning[6, 12]. However, soleus muscle from hindlimb-suspended rats, develops an enhanced capacity for insulin-stimulated glucose transport and displays substantial increases in GLUT-4 protein, but showed muscle atrophy similar to denervated soleus but, in contrast[12]. Thus, it seems that increasing GLUT4 expression in intact animals may be an effective strategy with which to alleviate insulin resistance.
Equine lower motor neuron disease (LMND) is a neurodegenerative disease that affects the lower motor neurones in the brainstem and spinal cord and was first described by Cummings et al. in 1990. Equine LMND is characterized by weight loss, trembling, and muscle weakness combined with a good or ravenous appetite[4]. In addition, increased whole body basal glucose metabolism was shown in horses suffering from LMND using the euglycemic hyperinsulinemic clamp technique[24]. In agreement with the increased whole body basal glucose metabolism a significant transition from type I (slow/oxidative) to type II (fast/glycolytic) muscle fibre in equine LMND was reported[17]. Glucose transporter 4 (GLUT-4) is the main glucose transport protein in skeletal muscle and it was suggested that an increased translocation of this protein to the plasma membrane is responsible for the increase in whole body basal glucose uptake[24]. Increased protein expression of GLUT-4 was shown in fast glycolytic muscle fibres compared to slow oxidative muscle fibres in horses[23].
Based on the similarity in epidemiological, clinical and neuropathological findings, equine LMND resembles the sporadic form of progressive spinal muscle atrophy (PSMA) in humans rather than human amyotrophic lateral sclerosis (ALS). Equine LMND differs from classical ALS in that upper motor neuron pyramidal tracts are not involved[22].
Similar to ALS, oxidative injury to the lower motor neuron appears to have an important role in the pathogenesis of the disease[7]. Furthermore, some human ALS and PSMA patients also experience a similar clinical scenario to that previously described for equine LMND[22]. As a consequence, equine LMND is a spontaneously occurring animal motor neuron disease that could provide clues to the pathogenesis of ALS and PSMA.
Electromyography (EMG) analysis is a powerful tool in the ante-mortem detection of both neurogenic and myogenic disorders and can discriminate neurogenic from myogenic disease[25]. It has also been suggested that neurogenic changes as measured by EMG precede myogenic changes[11]. In addition, in Chapter 6, we have shown that neuromuscular changes reflected biochemical adaptation and seemed to precede biochemical training adaptations.

The purpose of the study reported here was to investigate whether the increased whole body basal glucose metabolism as seen in equine LMND is associated with enhanced glucose metabolism and GLUT-4 protein expression, as observed in immobilized rats[12] in vastus lateralis muscle. A second aim was to test the hypothesis that this increase in glucose metabolism is secondary to denervation and reinnervation processes. Therefore, EMG analysis was applied in this study for the early detection of such changes. To our knowledge this is the first time that these phenomena have been studied in an integrated manner.

Material and Methods

Patient group

Twelve adult horses (10 Dutch Warmbloods and 2 crossbreds) with clinical symptoms of LMND that were evaluated at the Faculty of Veterinary Medicine, Utrecht University, were used in this study. The 12 animals comprised 6 geldings, 5 mares and 1 stallion. The mean age of the horses with LMND was 7.3 ± 3.6 years (range 1 to 15 years), and their mean weight was 467 ± 95 kg (range 300 to 557 kg). The ante mortem diagnosis of LMND was made on the basis of history, clinical signs, low plasma vitamin E concentration, increased plasma activity of muscle enzymes, and quantitative EMG analysis. In all twelve cases, a tentative diagnosis of LMND was initially obtained by quantitative EMG analysis of motor unit action potentials (MUPs).

Control group

Fifteen clinically healthy Dutch Warmbloods (6 geldings and 9 mares) were used as the matched control horses. The mean age of these horses was 7.4 ± 1.8 years (range, 4 to 10 years), and their mean weight was 630 ± 45 kg (range, 563 to 690 kg).

Diagnostic procedures

The EMG procedures were performed as described in Chapter 2[25] and the euglycemic hyperinsulinemic clamp technique was performed as described previously[1, 24]. Muscle biopsies were taken under local anaesthesia using a modified Bergström needle (Maastricht Instruments, Maastricht, The Netherlands) with a diameter of 7 mm. A 5 cm deep biopsy of the lateral vastus muscle (VL) was taken on the crossing of a line 15 cm ventrally from the tuber coxae and 10 cm caudally from the cranial border. If the horses were euthanized a surgical biopsy was taken from the same location. After positioning the muscle fibres in longitudinal position and cross section, the tissue was frozen in isopentane that was precooled to just above its freezing point in liquid nitrogen. Another part of the muscle biopsy was trimmed free

of fat and connective tissue and directly frozen in liquid nitrogen for biochemical analysis.

Muscle histopathology

Haematoxyline Eosin and Gomori tri-chrome staining was routinely applied to reveal morphological abnormalities. Abnormalities categorized as neurogenic atrophy includes group atrophy, elongation of atrophic muscle fibres and type grouping. Type grouping was defined as the complete encircling of a fibre from the least predominant fibre type by fibres of the same type. NADH, Cytochrome C oxidase, Succinate Dehydrogenase, Acid phosphatase, Periodic Acid Schiff and ATP-ase (pH 4.3 and 9.4) staining were used to discern enzymatic abnormalities. Sudan Black B and Oil red O stains were used to identify possible disturbances in lipid metabolism.
The ATP-ase 9.4 staining was used for measurement of muscle fibre diameter (morphometry). Per field, one hundred muscle fibres in cross section were counted and cross sectional fibre diameter of type I fibres and type II fibres were measured. Additionally the percentage of type I fibres was calculated. This examination was performed in three fields at 400x magnification. From 20 randomly chosen type I fibres in the three fields, the distance to the 5 closest surrounding type I fibres, termed the mean nearest neighbour distance (NND), was measured (magnification 400x). From these 20 mean nearest neighbour distances of type I fibres, the overall mean neighbour distance was calculated for each horse. In addition, the number of single type I fibres and the number of groups of type I fibres consisting of two adjacent type I fibres, 3, 4 or up to 8 and more than 8 adjacent type I fibres, were counted in four different fields (magnification 100x).

Muscle fibre type

Immunohistochemical analysis of myosin heavy chain (MyHC) expression was performed in a subpopulation of five out of twelve LMND horses and 5 controls with monoclonal antibodies (Mab) specific to MyHC isoforms in order to differentiate various MyHC isoforms and especially hybrid muscle fibres. Mab Slow (1:2000, clone NOQ7.5.4D) reacts with type I, Mab Fast (1:2000, clone MY-32) (both Sigma, Missouri, USA) with type IIa and IId and Mab A4.74 (1:50) (Alexis Biochemicals, Axxora Deutschland GmbH, Grunberg, Germany) with type IIa. Mab 412-R1D5 (1:25) (Gift from Prof. Moorman, Academic Medical Centre, Amsterdam, The Netherlands) reacts with type I and IId. Transverse serial sections (5 μm) were made and slides were rinsed in PBS, blocked in Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25 % gelatine and 0.05 % Tween-20; pH 8.0) for 15 minutes, followed by rinsing in PBS. After incubation overnight at room temperature with the Mab's sections were rinsed in PBS and incubated with secondary antibody goat anti mouse, highly cross-adsorbed whole antibody conjugate Alexa Fluor 568 (Molecular Probes, Invitrogen, Breda, The Netherlands), at a dilution of 1:200 for 45 minutes (dark). Finally, sections were rinsed in large volumes of PBS, mounted in Fluorsave Reagent (Calbiochem, EMD

Biosciences, Inc, Darmstadt, Germany), and left to dry at 37 °C (dark). Double staining of fibre perimeter was performed with a Wheat Glutamin Antibody (dilution 1:500) directly coupled to Alexa Fluor 350 (both Molecular Probes, Invitrogen, Breda, The Netherlands). Incubation was performed together with secondary antibody.

Immunofluorescent GLUT-4 protein analysis

Five µm thick sections of skeletal muscle biopsies from a subpopulation of five out of twelve LMND horses and 5 controls were cryo-sectioned at –20 ºC and thaw mounted on uncoated glass slides, air dried and stored at -20ºC. Serial sections were stained for MyHC distributions and GLUT-4 protein as previously described[23]. Serial slides stained either for GLUT-4 and Caveolin-3 or MyHC1, 2A, and laminin were evaluated using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan colour CCD camera, with a Bayer color filter, an output picture resolution of 1300x1030 pixels (HxV) and a pixel size of 6.7x6.7 µm. Digitally captured images were processed and analyzed using Lucia 5.49 software (Nikon, Düsseldorf, Germany).
Caveolin-3 was used as cell membrane marker in sections stained for GLUT-4 expression and laminin was used as cell membrane marker in combination with MyHC I and IIa staining for muscle fibre identification. Unstained muscle fibres in MyHC sections were identified as type IId fibres. Mean density of GLUT-4 signal was determined for all complete cells and muscle fibre type was identified in serial sections. Background staining as observed in control slides in which primary antibody was omitted was subtracted from the mean density signal.

Biochemistry

Approximately 50 mg of frozen muscle tissue from a subpopulation of five out of twelve LMND horses and 5 controls was freeze dried overnight (±16 hours) in order to assess glycogen content. Dry tissue was boiled in 1 N hydrochloric acid (HCl) for three hours and equilibrated with 1 N sodiumhydroxide (NaOH). Samples were centrifuged and stored at -20ºC. Glucose content was assayed in the supernatant with a glucose assay (Radiometer Copenhagen; Zoetermeer, The Netherlands) on an automated analyzer (Cobas Fara; Roche Diagnostics Netherlands BV, Almere, The Netherlands)
For enzymatic analysis another 50 mg of muscle tissue was homogenized in 1 ml SET buffer (250 mM sucrose; 2 mM EDTA; 10 mM Tris-HCL) with a Ultraturrax homogenizer. Homogenates were subsequently sonicated and centrifuged at 15,000g for 10 minutes. Supernatants were stored at -80ºC until analysis. All chemicals used were of analytical grade.
Citrate Synthase (CS) was assayed according to Sheppard and Garland[20]. The assay mixture contained 100 µM dithiobis-(-2nitrobenzoicacid) (DTNB), 50 µM acetyl-CoA and 50 µM oxaloacetic acid. Appearance of DTNB-CoA was

measured specto-photometrically at 412 nm at 37ºC on an automated analyzer.
Activity of 3-Hydroxy-acyl dehydrogenase (HAD) was assayed by measuring the amount of NAD^+ that was formed in the reaction Acetoacetyl-CoA + NADH → 3-hydroxy-butyryl-CoA + NAD^+ on an automated analyzer. The reagent consisted of 10 mM NADH solution which was diluted 50 times in a tetra-sodiumpyrophosphate (100mM) buffer. The reaction was started by adding 2 mM acetoacetyl-CoA and the formation of NAD^+ was measured spectophotometrically at 340nm at 37ºC.

Western blotting of GLUT-4 protein
Approximately 30-40 20 µm sections of muscle biopsies from a subpopulation of five out of twelve LMND horses and 5 controls were homogenized with potter tubes in ice-cold buffer containing 210 mM Sucrose, 30 mM HEPES, 5 mM EDTA, 2 mM EGTA and 1 minitablet protease inhibitors (Roche Applied Science, Almere, The Netherlands). The sample was diluted with a buffer containing 1.17 M KCl, 58.3 mM NaPyrophosphate and 1 mM DTT. Samples were centrifuged at 150,000g for 90 minutes and the supernatant was discarded. The pellet was resuspended in 50 µl buffer containing 10 mM Tris, 1 mM EDTA and 0.1% Triton X-100 and is referred to as total membrane fraction (M-fraction).
Polyacrylamide sodium dodecysulphate (SDS) gel electrophoresis was performed according to Laemmli[15]. In short, 50 µl M-fraction was boiled 5 minutes in an equal volume of SDS sample buffer containing 2.3% SDS and 5% β-mercaptoethanol and subsequently centrifuged for 5 minutes. Equal amounts of protein (25 µg) were loaded on 12% polyacrylamide gels and electrophorized at 200 V for 55 minutes. After electrophoresis protein was transferred to a nitrocellulose membrane by blotting for 60 minutes at 100 V.
For detection of GLUT-4 protein nitrocellulose sheets were pre-treated with Odysey blocking buffer (Licor Biosciences; Westburg b.v., Leusden The Netherlands) diluted 1:1 in PBS for 60 minutes. Incubation with GLUT-4 antibody[2], diluted 1:10000 in blocking buffer, was carried out overnight (16 hours) at room temperature with gentle shaking. After three washing steps with 0.1% Tween-20 in PBS blots were incubated for 60 minutes with fluorescent conjugated goat anti rabbit secondary antibody (Rockland; Tebu-bio, Heerhugowaard, The Netherlands). Blots were scanned with an odyssey IR scanner (Licor Biosciences; Westburg b.v., Leusden The Netherlands) and results were expressed as integral intensities and as relative intensities to a positive control sample obtained from rat gastrocnemius muscle.

Statistical analysis
Because all MUP variables (namely the duration, amplitude, number of phases and number of turns) were positively skewed in the LMND and control group these were transformed into natural logarithm (ln) to enable statistical analysis. Group differences (LMND vs control), muscle differences

and interactions between the two groups were determined using univariate analysis. The ln transformed variables of individual patients were compared with the control group, and values of pooled data of the patient group with values of pooled the data of the control group. Independent t-test was used to determine differences between the control group and LMND group. Paired t-test was used to determine differences within horses and Pearson's correlation coefficients were calculated between these variables. Significance was set at 0.05, two-tailed. The results of quantitative EMG of the LMND group were compared with the 95 CI reference ranges for neuropathy as established previously[24, 25]. One way ANOVA (post-hoc Bonferroni) was used to determine differences in biochemical and muscle fibre type distribution between LMND and controls.

Results

EMG analysis

Insertional activity did not differ significantly between patients and controls, nor between the muscles studied (subclavian, triceps and lateral vastus muscles). Needle insertion frequently induced pathological spontaneous activity such as fibrillation potentials or positive sharp waves in the patients. In all muscles of the LMND-affected horses, fibrillation potentials and positive sharp waves were recorded (table 7.1). The mean duration and amplitude of fibrillation potentials was 4.0 ms (SD 1.4) and 52 µV (SD 22), respectively (95 CI: 3.8-4.1 ms and 50-55 µV, respectively). The mean duration and amplitude of positive sharp waves was 5.1 ms (SD 2.0) and 70 µV (SD 53), respectively (95 CI: 4.8-5.3 ms and 64-75 µV, respectively). Furthermore, doublets, triplets or multiplets, neuromyotonia (mean firing frequency 168 Hz, SD 67.1) and CRDs (mean firing frequency 138 Hz, SD 14.4) could be recorded. The quantitative EMG analysis in horses with LMND revealed neurogenic abnormalities like an increase in MUP duration, an increase in number of phases and number of turns (Table 1) as well as an increase in prevalence of polyphasic and complex MUPs consistent with motor neuron or axon loss. These findings indicated neurogenic abnormalities.

Ante-mortem histopathological examination of the lateral vastus muscle using the conventional criteria revealed only minor neurogenic atrophy in six horses out of twelve LMND-affected horses, whereas EMG-analysis revealed abnormalities in all horses. No correlations were found between rectal and intramuscular temperatures, or between temperatures and MUP variables.

Basal glucose metabolism

The euglycemic hyperinsulinemic clamp technique performed in eight of the 12 LMND-affected horses revealed a 2.7 times (± 1.2) increased whole body

glucose uptake as compared with controls equivalent with 28.7 ± 1.3 (range, 12.5 to 50.3) µmol glucose/kg BW/min.

Table 7.1
Motor unit potential variables in lower motor neuron disease horses and control horses
Duration, amplitude, no. of phases, no. of turns, polyphasic MUPs and complex MUPs are all significantly higher in LMND compared to controls.

		Controls		LMND	
Variable	**Muscle**	**mean (SD)**	**95 CI**	**mean (SD)**	**95 CI**
Duration	sc	6.9 (4.5)	6.4- 7.4	11.6 (7.9)	10.5-12.7
(ms)	tb	8.8 (9.1)	7.6- 10.0	17.4 (14.0)	15.2-19.6
	vl	9.0 (9.8)	7.8- 10.2	14.3 (12.2)	12.7-16.0
Amplitude	sc	300 (302)	266- 334	558 (869)	438- 679
(µV)	tb	880 (1366)	702-1058	1611 (2124)	1281-1942
	vl	704 (1126)	571- 836	1336 (1881)	1077-1596
No. of	sc	2.7 (1.1)	2.6- 2.9	3.7 (2.0)	3.4-4.0
phases	tb	2.5 (0.8)	2.4- 2.6	3.3 (1.3)	3.1-3.5
	vl	2.7 (1.0)	2.6- 2.9	3.3 (2.3)	3.1-3.5
No. of turns	sc	3.3 (1.8)	3.1- 3.5	5.1 (3.9)	4.6-5.7
	tb	3.0 (1.5)	2.8- 3.2	4.7 (2.6)	4.3-5.1
	vl	3.4 (1.7)	3.2- 3.6	4.6 (2.9)	4.2-5.0
Percentage	sc	4.0 (2.2)	2.0- 6.0	27.6 (18.2)	8.6-46.7
of Polyphasic	tb	5.9 (2.8)	13.0-11.2	26.4 (9.8)	16.2-36.7
MUPs	vl	4.0 (7.8)	10.2-14.0	24.2 (20.7)	5.1-43.4
Percentage	sc	4.0 (7.9)	3.2- 8.5	33.1 (10.3)	22.3-44.0
of complex	tb	6.9 (2.9)	3.1-11.2	31.3 (16.7)	12.8-53.9
MUPs	vl	9.8 (2.7)	3.2-16.4	25.4 (12.2)	14.6-47.5

LMND = lower motor neuron disease. MUP = motor unit action potential. SD =Standard Deviation. 95 CI=95 % confidence interval of the mean.investigated muscles: subclavian muscle (sc), triceps muscle (tb), lateral vastus muscle (vl)

Postmortem examination

The diagnosis of LMND was later confirmed by post mortem microscopic examination of the spinal cord and peripheral nerves according to Valentine et al. in nine of the twelve cases[22]. The other three horses were not available for necropsy.
Histological, the cell bodies of the motor neurones in the ventral horns of the spinal cord and in the medulla oblongata consistently had chromatolysis and acidophilic necrosis. Lesions consistent with dysautonomia were found also in four of the horses necropsied after histological examination of the autonomic ganglia.

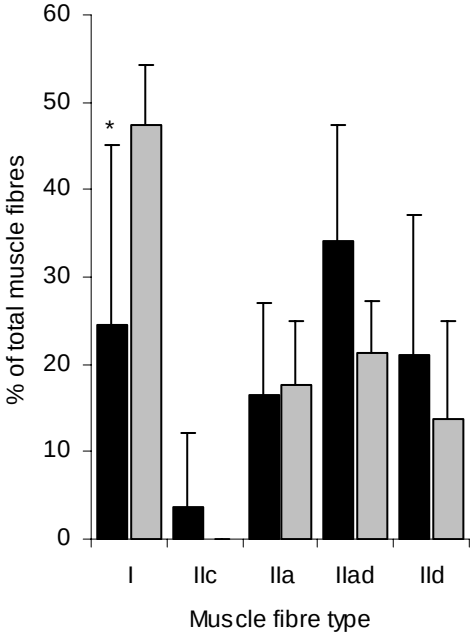


Figure 7.1
Muscle fibre type distribution in percentages in LMND patients and control horses
Black bars represent LMND patients and gray bars represent healthy controls. A significant ($p>0.05$) decrease was observed in the percentage of type I fibres. The unique expression of hybrid type IIc (combination of type I and type IIa) and the increase (NS; $p=0.08$) in hybrid IIad muscle fibres is one indication that processes like denervation and reinervation may occur in skeletal muscle of LMND patients.

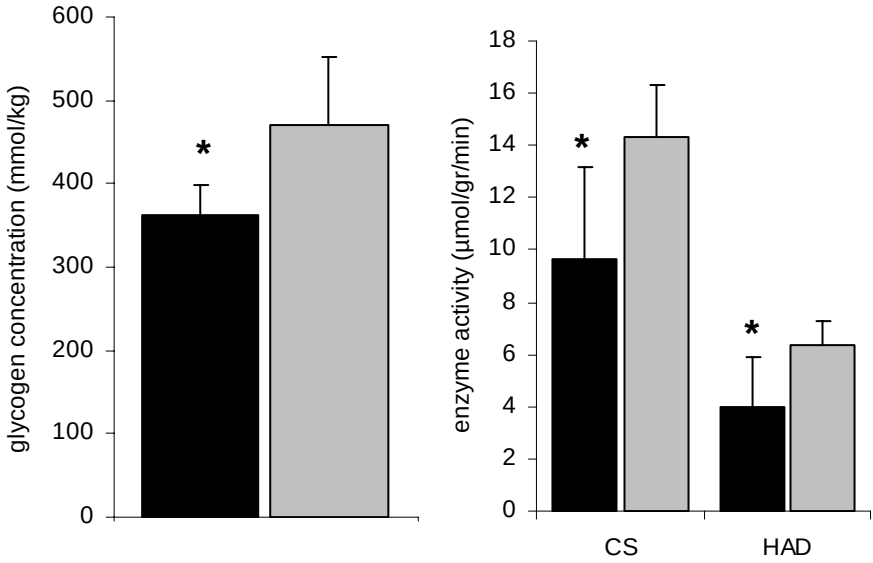
Muscle histopathology

Application of additional staining to sections of vastus lateralis muscle tissues did neither reveal the presence of polysaccharide storage myopathy (PSSM) nor other myopathic abnormalities. None of the examined LMND-affected horses showed type grouping, whereas 4 horses had elongation or angular atrophy of muscle fibres, which is a characteristic of neuropathy. Muscle fibre type distribution was significantly changed in LMND-affected horses compared to controls (figure 7.1). The relative abundance of type I muscle fibres was decreased in LMND patients compared to controls. As far as the other fibre types were concerned no significant changes were found. In control horses, the mean diameter of type II muscle fibres was significantly ($p<0.01$) larger than the mean diameter of type I fibres, whereas in the LMND group no significant differences were found. The mean

diameter of type II fibres and type I fibres were significantly smaller in LMND-affected horses than in controls ($p<0.03$). The mean number of solitary type I fibres was significantly higher in LMND-affected horses than in controls (66 ± 28 and 33 ± 14, respectively; $p<0.01$). Morphometry indicated no significant differences in the NND of type I fibres of the LMND-affected horses compared with the controls. In the controls, a significant negative correlation was found between the percentage type I fibres and the NND of type I fibres ($r= -0.86$, $p<0.0001$). However, in the LMND-affected horses, no such correlation was found. This finding was illustrated by a low NND of type I fibres in association with a low percentage type I fibres in five of 7 LMND-affected horses examined by means of morphometry.

Figure 7.2
Glycogen concentration (mmol/kg dry weight)
Glycogen concentration is significantly ($p<0.05$)(*) decreased in LMND compared to control vastus lateralis muscle (VL).

Figure 7.3
Cs and HAD activity
Enzymatic activities of citrate synthase (CS) and 3-Hydroxy-acyl dehydrogenase (HAD) (µmol/gram/min) are significantly ($p<0.05$)(*) decreased in LMND vastus lateralis muscle compared to controls.



In 9 of the LMND-affected horses, small groups of atrophied type I and type II fibres were seen as well as slight to moderate angular atrophy. Gomori trichrome staining revealed ragged red fibres in 2 horses, fibre splitting in 2 horses and grouped atrophy in 3 horses. In three LMND-affected horses, NADH staining showed minor to moderate central elucidation resembling core like lesions, whereas in the Cytochrome C staining this was present in 2 horses. Abnormalities with reference to the fat staining were not present.

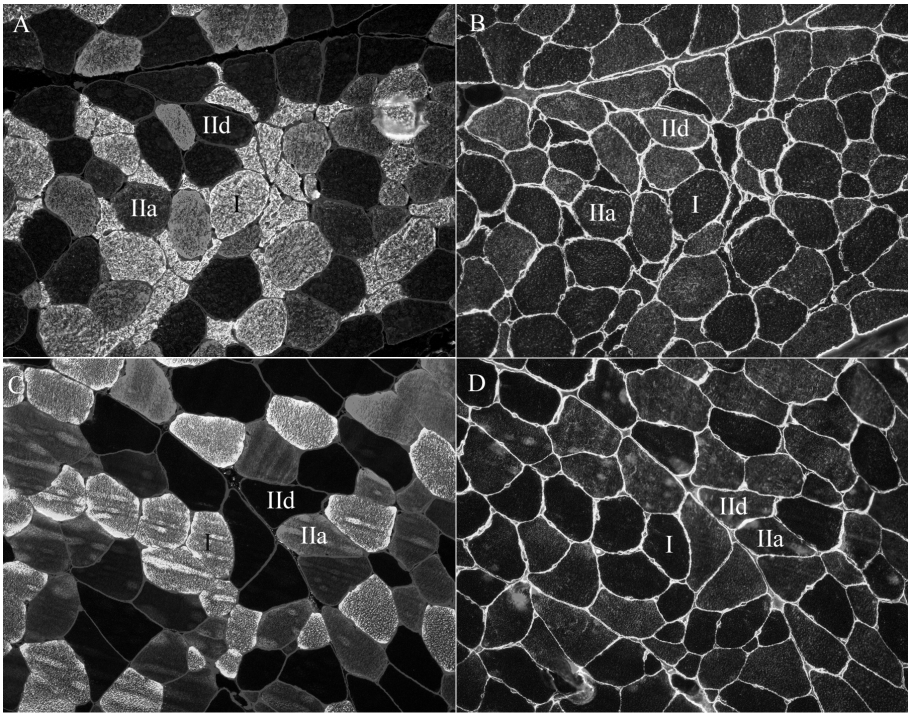
Biochemistry

Lateral vastus muscle glycogen concentrations were significantly ($p<0.05$) lower in LMND-affected horses compared to healthy controls (362 and 471 mmol/kg, respectively (figure 7.2). CS (9.6 and 14.3 µmol/g/min, respectively) and HAD (4.0 and 6.3 µmol/g/min, respectively) activities were both significantly ($p<0.05$) lower in VL muscle of LMND-affected horses compared to healthy controls (figure 7.3).

Figure 7.4a

GLUT-4 staining and muscle fibre type in LMND and control

MyHC is stained in image A and C and GLUT-4 is stained in image B and D. Representative examples of LMND patients are shown in the upper panels (A and B) and healthy controls in the lower panels (C and D). MyHC I is stained lightest, MHC IIa is stained intermediate and MyHC IId is unstained (darkest) in panel A and C. Serial sections of Glut-4 staining (panel B and D) is shown in gray, the fierce membrane associated staining is caused by the membrane marker laminin. Type I muscle fibres show angular atrophy and a small general atrophy in type II muscle fibres in LMND patients. As shown in Chapter 3, type IId fibres show the strongest GLUT-4 staining and type I fibres the lowest. No differences in fibre specific GLUT-4 staining were observed.



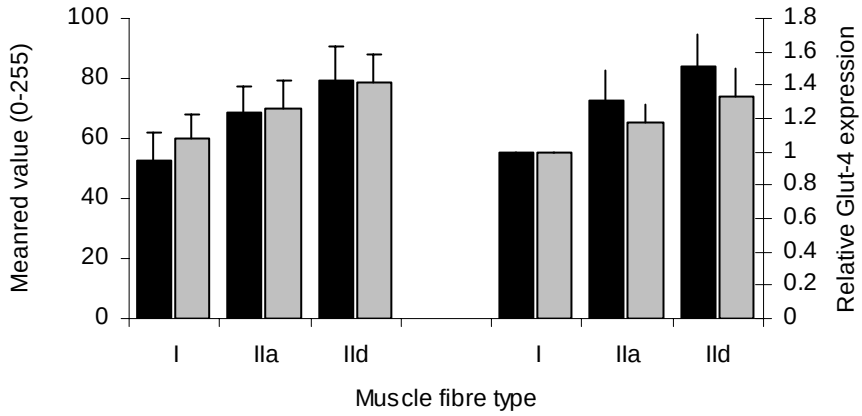
Immunofluorescent GLUT-4 protein analysis

Both in lateral vastus muscles of LMND-affected horses and control horses type IId fibres displayed the strongest signal for GLUT-4 protein expression and type I fibres had the lowest staining signal (figure 7.4a). With reference to various fibre types however, no differences were found between LMND-affected and control lateral vastus muscle. Even if the GLUT-4 protein content was expressed as percentage of type I muscle fibre expression of GLUT-4 no differences were observed (figure 7.4b).

Figure 7.4b

Semi quantitative analysis of GLUT-4 immuno-fluorescent staining

No significant (P>0.05) changes in fibre type specific GLUT-4 expression between LMND affected animals (black bars) and healthy controls (gray bars) were observed. In the left side of the figure, the mean intensity of the GLUT-4 signal was measured per fibre type. In the right side of the figure, the intensity of the GLUT-4 signal is expressed relative to the expression in type I muscle fibres



Glut-4 protein expression

Western blotting for GLUT-4 protein revealed bands at ~ 43 kD as previously reported[2]. Although lateral vastus muscle of LMND-affected horses expressed almost twice as much GLUT-4 protein in M-fraction compared with controls the differences were not significant (p=0.07)(figure 7.5).

Discussion

One aim of the study reported here was to investigate whether the increased whole body basal glucose metabolism as seen in equine LMND is associated with enhanced glucose metabolism and GLUT-4 protein expression in vastus lateralis muscle. The whole body basal glucose metabolism on the average was increased 2.7 times in the LMND-affected horses which is in agreement with the results of a previous study[24]. The glycogen content of the lateral vastus muscle in LMND-affected horses was decreased similar to that of gluteus medius muscle in LMND-affected horses as reported earlier[17] indicating net muscular glycogen loss.

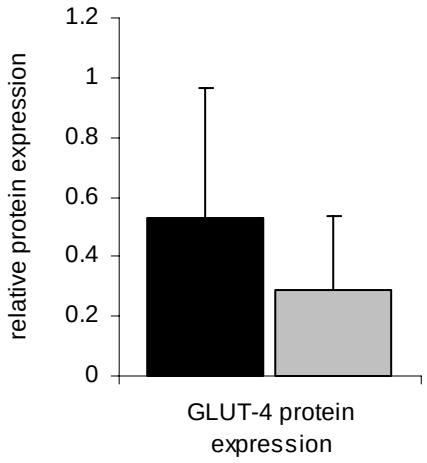


Figure 7.5
GLUT-4 protein expression
Western blotting results of GLUT4 in LMND and control. Optical density (OD) of GLUT-4 protein bands are expressed relative to a positive control from rat gastrocnemicus muscle (O.D. set at 1.0). No significant increase in membrane bound GLUT-4 expression (p=0.07) was observed in LMND patients (black bar) compared to healthy controls (gray bar)

In addition, the activity of citrate synthase in LMND-affected horses, an indicator mitochondrial density and oxidative metabolism, was significantly lower in gluteus medius muscle[17] as well as lateral vastus muscle in this study compared to healthy controls. In addition, the current study revealed a decreased activity of 3-Hydroxy-acyl dehydrogenase (HAD), an indicator of mitochondrial ß-oxidation in lateral vastus muscle in LMND-affected horses too. As a consequence, it can be concluded that anaerobic muscle metabolism predominates in horses suffering from LMND.
Equine LMND was associated with lower than normal plasma glucose values during an oral and IV glucose tolerance test[7]. Because no abnormalities in intestinal glucose uptake were apparent, this must be due to an increased clearance rate of plasma glucose[1, 24]. Skeletal muscle is of major importance in whole body glucose uptake and its uptake is mainly regulated by glucose transporter 4 (GLUT-4)[19]. We hypothesized an increased expression and localization to the plasma membrane of the insulin sensitive muscle specific GLUT-4 protein. However, no significant increase of GLUT-4 protein

expression was observed in the M-fraction with Western Blotting. GLUT-4 is most potently increased in membranous fractions after stimulation of either insulin or exercise[10]. In LMND- affected horses translocation of GLUT-4 to the plasma membrane might not be sufficient to elicit changes in membrane fractions of LMND patients. Immunohistochemical analysis of GLUT-4 expression did not reveal fibre type related increases in LMND-affected horses. To the best of our knowledge, the fibre type specific expression of the glucose transport protein GLUT-4 in LMND-affected mammals has not been studied before. In addition, the literature also revealed no previous studies investigating glucose metabolism in humans suffering from PSMA. GLUT-4 protein expression in lateral vastus muscle in LMND-affected horses in the current study obviously did not comply with the increased whole body basal glucose metabolism, thereby possibly facilitating net muscular glycogen loss. It is worthwhile to mention from an earlier report, that in LMND-affected horses neither pancreatic insulin secretion nor insulin sensitivity revealed deficiencies[24]. Further research is necessary to assess GLUT-4 protein expression in various muscles from both equine LMND and human progressive spinal muscle atrophy patients.

It has been previously reported that chronic reduction in neuromuscular activity induced by LMND resulted in a slow to fast transition in MyHC expression and a corresponding increased muscle glycolytic capacity as reflected by higher activities of lactate dehydrogenase and glycerol-3-phosphate dehydrogenase[17]. As mentioned before, increased protein expression of GLUT-4 was shown in fast glycolytic muscle fibres compared to slow oxidative muscle fibres in horses[23]. Due to the marked shift in muscle fibre type from slow/oxidative to fast/glycolytic in LMND-affected horses a relative increase in GLUT-4 expression might indeed be assumed. Gluteus medius muscle in LMND-affected horses had lower capillary-to-fibre ratio, which might indicate a decreased muscle perfusion[17]. This can also be a cause of the predominance of anaerobic metabolism (especially seen in type II fibres) over aerobic glucose and fat metabolism (seen in the slow/oxidative type I fibres) in LMND-affected horses.

A second aim of this study was to test the hypothesis that the increase in whole body basal glucose metabolism is secondary to denervation and reinnervation processes. In LMND-affected horses, histopathological findings of denervation atrophy (i.e., angular atrophy) involved predominantly type I fibres[22] or both type I and type II fibres[17]. In the current study, histological evidence of neurogenic atrophy was present in the lateral vastus type I fibres, and associated with an increase in muscle fibres expressing multiple MyHC isoforms in LMND-affected horses. In a previous study on LMND-affected horses, no features of reinnervation like target fibres and fibre-type grouping were noticed[17]. Fibre type grouping is considered a convincing evidence of neurogenic atrophy facilitating the diagnosis, but this does not always occur in large animals[13]. Histopathological evaluation of muscle biopsies did reveal slight neurogenic changes such as grouped atrophy or angular atrophy. However, type grouping being considered the gold

standard of reinnervation, was lacking. In this study, a tentative diagnosis of LMND was made by quantitative EMG analysis at an early stage of the disease (3-8 weeks disease history) in contrast to the situation in man, in which this type of examination is often performed after a long history of disease (2 months to 20 years of complaints)[3, 9, 14]. More recent studies in man showed that a time course of 12-18 months was needed to confirm the diagnosis by means of quantitative EMG analysis sometimes without associated type grouping[3, 8]. In the present study, this is illustrated by one horse, which was re-examined after 5 months. EMG analysis elucidated neurogenic abnormalities on both examinations, whereas histopathological examination of the muscle biopsy demonstrated neurogenic atrophy only convincingly in the second biopsy after 5 months without the presence of type grouping. Clinically, the disease seemed to have stabilized and the horse's weight had increased by 15%. Post mortem histopathology demonstrated the presence of LMND in this horse, suggesting that neurogenic EMG changes precede histopathological changes in LMND.
In the current study, quantitative EMG analysis in horses with LMND revealed reinnervation also without convincing evidence of muscle fibre type grouping. In neurogenic atrophy, the muscle fibre that looses its innervation is reinnervated by collateral sprouting from an adjacent nerve fibre associated with either a type I or a type II neuron. In muscles with an extreme predominance of one fibre type, it might be assumed that a denervated muscle fibre of the least predominant fibre type will be reïnnervated by a nerve fibre of the most predominant group. As a consequence, the mean group size of the least predominant fibre type diminishes in neurogenic atrophy. This hypothesis might be illustrated by the increased number of solitary type I fibres in the majority of LMND-affected horses. In addition, grouping of muscle fibres of the same type following reinnervation implies shorter distances between these muscle fibres that are in agreement with current morphometric findings. The reduction in the NND of type I fibres in LMND-affected horses might have been due to reduction in size of type II fibres or to reinnervation. As no correlation was found between the size of type II fibres and the NND, reinnervation of fibres might be an alternative explanation. Both the increase in MUP duration and the increase in number of phases and number of turns indicate larger motor units also consistent with reinnervation. Finally, an increase in hybrid muscle fibres is associated with both denervation[21] and reinnervation[18].
Equine LMND-patients showed increased whole body basal glucose metabolism associated with increased glycolytic capacity. This is particularly interesting as some human patients suffering from ALS show a state of hypermetabolism and mitochondrial abnormalities that might play a role in this phenomenon[5]. It is worth mentioning that evidence of mitochondrial abnormalities was present in histological staining in 3 affected horses in this study. Furthermore, patients with muscle wasting due to neuromuscular disease are prone to develop hypoglycaemia during fasting conditions[16].

Our findings in equine LMND show anaerobic changes in muscle metabolism in affected lateral vastus muscle secondary to a shift in muscle fibre type from I to II as based on both morphometry and metabolic analysis. On the basis of EMG analysis we conclude that both denervation and reinnervation cause this shift in muscle fibre type, although no direct morphometric evidence was found for reinnervation. GLUT-4 protein expression in lateral vastus muscle in LMND-affected horses in the current study obviously did not comply with the increased whole body basal glucose metabolism, thereby possibly facilitating net muscular glycogen loss. Neurogenic changes in skeletal muscle seem to precede myogenic changes and therefore EMG-analysis is seen as an attractive tool for early identification of neuromuscular changes due to denervation.

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Chapter 8

General Discussion

Training affects many organ systems in mammals, and adaptation to training is more than the sum of the individual organ adaptive changes induced by training. Between species, adaptation to training shows many similarities as well as numerous dissimilarities. These differences are mainly due to differences in the functional physiological parameters between species. Skeletal muscle is one of the major tissues subjected to adaptations to training loads, and adaptation to training shows similar patterns between mammals. Similar to human skeletal muscle, but in contrast to some rodent skeletal muscles, equine skeletal muscle displays a heterogeneous muscle fibre type[62]. A different fibre type distribution has, based on the physiological characteristics of the individual fibre types, consequences for the metabolic function of the entire muscle[21, 45].
The presence of homologous proteins in different species can shed more light on the metabolic function of skeletal muscle. In the present thesis we have shown a similar expression and localisation of transport proteins GLUT-4 and FAT/CD36 in equine skeletal muscle as compared to human skeletal muscle (Chapter 3)[7, 43]. Another important feature was the finding of the expression of monocarboxylate transporters (MCT1, MCT2 and MCT4) in equine skeletal muscle (Chapter 4), resembling another important similarity between human and equine skeletal muscle. This implicates a similar function of these proteins in human an equine skeletal muscle.
Skeletal muscle is a heterogeneous tissue with an enormous plasticity and the ability to adapt to various stimuli[23]. Most equine skeletal muscles display a muscle fibre type distribution, type 1, 2a and 2x muscle fibres comparable to humans[57, 63, 62]. Muscle fibres are classified in according to the myosin heavy chain expression. A remarkable feature is the occurrence of hybrid muscle fibres, with characteristics of two or more fibre types. The Standardbred trotters as used in the studies in the present thesis also displayed these three main muscle fibre types[71]. The different muscle fibre types have a specific (metabolic) protein expression pattern that accompanies their functional demands[21].
Results from our laboratory showed that EMG profiles in the horse follow the general principles of motor unit alterations as described previously in man[75, 74]. Later in this discussion we will show that EMG analysis in training, overtraining and/or pathological horses can play a valuable diagnostic role.

Muscle substrate transport proteins
In this thesis, we describe fibre type specific expression of glucose transporter 4 (GLUT-4) and fatty acid translocase/CD36 (FAT/CD36) proteins in equine skeletal muscle. We have shown that GLUT-4 is mainly expressed in type 2x and type 2a muscle fibres and to a lesser extend in type 1 muscle fibres. This is in line with some human studies but contrasts sharply with rodent studies that reported high GLUT-4 expression in type 1 muscle fibres,[7, 12, 29]. Although an explanation for these differences it is not easy to provide, it has been suggested that GLUT-4 expression is not coupled to muscle fibre type necessarily, but mainly on the functional load and activity

pattern of the muscle[7, 12, 13]. Additionally, type 2x muscle fibres in equine muscles have a high capacity for anaerobic glycolysis which, in turn, demands a high potential for plasma glucose uptake[58].
FAT/CD36 expression is limited to type 1 muscle fibres and especially to sarcolemmal aggregates near capillaries. This is in agreement with observations in rodents[3] and humans[43] and corresponds with the primarily oxidative nature of this muscle fibre type. In rodents FAT/CD36 has been found to correlate with long chain fatty acid uptake[17]. This also seems to hold for horses since intramuscular triglycerides are mainly found in type 1 muscle fibres[19]. Therefore, the expression of FAT/CD36 for facilitative LCFA uptake in equines is not surprising. Additionally, fat supplementation to equine diets has shown to increase oxidative enzyme activity and upregulates skeletal muscle enzymes associated with fatty acid oxidation in ponies[28]. Based on these findings a functional role for FAT/CD36 in LCFA transport is expected in horse skeletal muscle. Equines, however, derive a major portion of the required energy from hindgut fermentation of cellulose resulting in a high availability of volatile fatty acids and short chain fatty acids. Taking these physiological characteristics into mind it is reasonable to suggest that horses may relay less on LCFA for energy production. This, in turn, sheds a different light on the function of FAT/CD36, which may also be involved in the transport of short chain fatty acids.
Another family of transport proteins are the monocarboxylate transporters. To date, 14 MCT isoforms have been identified that are involved in the transport of lactate, short chain fatty acids, pyruvaat, ketone bodies, and branched-chain ketoacids[34]. It has been reported that, depending on the metabolic properties of each muscle, MCT proteins are expressed in different quantities in porcine muscles[65]. Of all 14 isoforms discovered so far, MCT1, MCT2 and MCT4 are the most abundantly expressed in human and rat skeletal muscle[6, 22, 36, 65]. The function of MCT4 in skeletal muscle is associated with the outward transport of the lactate anion with a proton[35, 54]. Since lactate can also be oxidized by liver, heart muscle and inactive skeletal muscle, cellular inward transport of the lactate anion has to be facilitated as well. The inward transport of lactate is, at least in skeletal muscle, mainly dependent on MCT1[4].
Here, to the best of our knowledge, we demonstrated for the first time in skeletal muscle of Standardbred trotters, the expression of MCT1, MCT4 and relative high levels of MCT2 expression compared to rat gastrocnemius muscle. Previously, MCT2 expression has been demonstrated in rodent[36], human[6] and porcine skeletal[65] muscle and is proposed to aid in the transport of both lactate and other monocarboxylates, and possibly volatile fatty acids in reindeer liver[35, 65]. In human and rodent skeletal muscle this protein is expressed in low abundance in comparison with MCT1 and MCT4[6]. Since horses rely for a large part on hindgut fermentation and the subsequent release of short chain fatty acids from the gastrointestinal system we suggest that the high levels of MCT2, described in Chapter 4, may fit the

evolutionary make up of the horse, and aid in short chain fatty acid transport in horse skeletal muscle.

Effects of acute exercise

Exercise has a pronounced effect on skeletal muscle metabolism. In order to provide oxygen and substrates for metabolism cardiac output and blood flow to active muscles is increased. During the initial minutes of exercise, skeletal muscle energy is primarily provided by ATP and creatine phosphate. Subsequently, exercise intensity and muscle fibre type recruitment determine substrate oxidation[69].

During exercise of low intensity small motor units consisting of type 1 muscle fibres are recruited. These fibres rely for a large part on fatty acid oxidation for energy provision. As exercise intensity increases, larger motor units consisting of type 2 muscle fibres are gradually recruited, and glucose metabolism becomes increasingly important in energy provision[78].

The increase in energy demand during acute exercise results in parallel increases in substrate transport across the sarcolemma. For this purpose glucose and fatty acid transport proteins are recruited. Both GLUT-4 and FAT/CD36 have been shown to translocate from an intra-cellular domain to the plasma membrane in several species, including humans, rodents and for GLUT-4 also in horses after exercise or muscle contraction[5, 29, 48, 59].

Since we were interested in energy metabolic adaptations during acute exercise, we focussed on enzymatic activities as markers for changes in muscle metabolism. In our experimental studies we applied a bout of 20 minutes sub-maximal exercise at approximately 80% of the estimated maximal heart frequency (HF_{max}).

Our results showed a significant increase in hexokinase activity in untrained (SET 1) and trained (SET 2) Standardbred trotters after acute exercise. These findings suggests an increased glucose uptake, because hexokinase is involved in the intracellular trapping (phosphorylation) of glucose derived from blood. This is accompanied by findings of a significant decrease in glycogen concentration in skeletal muscle of our Standardbred trotters. An increase in glucose metabolism during exercise is a well described phenomenon in horses[33, 49]. Finally, increased sarcolemmal glucose transport also suggests an increase in membrane associated GLUT-4 in Standardbred trotters due to exercise, although we were, due to technical difficulties, not able to confirm such translocation process.

During sub-maximal exercise, fatty acid oxidation provides a large part of energy provision[39]. We, however, could not find an increase in 3-hydroxy-acyl dehydrogenase (HAD) due to acute exercise, which suggests that mitochondrial β-oxidation activity was not increased after sub-maximal exercise. This does, at first sight, not comply with physiological principles. It is generally accepted that fatty acid oxidation is increased during sub-maximal exercise[67]. We must take into consideration however, that fatty acid entrance into the mitochondria by carnitine palmitoyl transferase1 (CPT-1) seems to be more tightly regulated than β-oxidation enzymes[67]. Increases in

glucose oxidation inhibit CPT-1 through malonyl-CoA accumulation and therefore decrease fatty acid entrance into the mitochondria [67, 76]. Furthermore, it is also possible that allosteric inhibition of HAD is reduced during acute exercise, but is increased directly after exercise when energy expenditure is reduced. Finally, another explanation for our findings is that volatile fatty acids such as acetate and butyrate that can make up of two thirds of total energy sources in horses. These substrates are not metabolized through HAD catalyzed reactions in the β-oxidation pathway, and therefore do not affect HAD activity[27].
In conclusion, we found that an acute bout of sub-maximal exercise in Standardbred trotters lead to increases in glucose phosphorylation and an increased glycogenolysis. This is in line with numerous results from studies in others species[30, 50]. Data on metabolic effects of acute sub-maximal exercise in Standardbred trotters is, however, scarce and our results provide more insight in metabolic adaptation to acute sub-maximal exercise in untrained and trained Standardbred trotters.

Training

A prolonged period of physical training leads to adaptations in skeletal muscle in order to comply with the increases in metabolic demand. The most common adaptations in all species subjected to endurance training are; 1) increases in capillarization, 2) increases in substrate storage in skeletal muscle, and 3) increases in key metabolic enzyme activity. With moderate intensity endurance training the oxidative enzymatic pathways are upregulated, whereas with high intensity training glycolytic pathways are upregulated[55]. We have shown that Standardbred trotters subjected to an 18 week training program with combined endurance and interval training only showed a decrease in hexokinase activity. This indicates a decrease in glucose metabolism. It is well known from human studies that trained subjects use a larger portion of fatty acids during exercise at a fixed exercise intensity compared to untrained subject[40, 72]. This is also supported by observations of a so-called "glycogen-sparing" effect after training[39], that is also apparent in horses[49]. In the current study, no further increases in CS and HAD enzyme activity or glycogen storage were found.
Several factors can explain the lack of expected training induced changes in skeletal muscle. First, glycogen stores are largely dependent on diet and the previous exercise bout. In our experimental setup the last training session was carried out 24 hours before the SET, and therefore glycogen stores may not have been completely replenished. This notion is supported by Hyppa et al.[41] who showed that in contrast with humans the glycogen repletion in horses may take up to 72 hours. A second explanation for the lack of expected increase in glycogen storage and enzyme activity could be the absence of sufficient overload in the training program. It is well known that adaptation towards physical training necessitates regular overload of the physiological systems[46, 53]. A change in muscle fibre type distribution has previously been observed after a period of training, and is considered a

common adaptation to training[66, 68]. We were not able to show changes in muscle fibre type after the training period in the vastus lateralis and pectoral descendens muscles these horses[71]. This is another indication that the overload principle was, at least to some extends, not always met in these muscles. It is also possible that vastus lateralis and pectoral descendens muscles do not participates as much in exercise as we expected. Most research in equine skeletal muscle focuses on medial gluteus muscle, but muscle related differences have been reported in enzymatic activity[21, 31, 45]. In the young Standardbred trotters used in this study, also growth may have influenced our results. Ageing is associated with a fast to slow shift in muscle fibre type[58] but from research in horses it ha been concluded that growth does not affect metabolic changes related to training in skeletal muscle[37, 64, 77]. Finally, it can not be excluded that a considerable part of adaptation had already occurred in the first four weeks of phase one. This phase was included in the study in order to accommodate the horses to the high speed treadmill. Increases in enzyme activity have been reported within several weeks of training[20, 31] in horses and this could have influenced our pre-trained baseline measurements at SET 1.

Measurement of motor unit potentials (MUPs) by needle EMG can also provide valuable information about training adaptation[26]. For instance, increases in muscle strength without evident hypertrophy are considered the result of neural involvement in acquisition of muscular strength[26]. Additionally, neuromuscular excitation is improved in well trained, non-fatigued endurance athletes[52, 51]. The increase in MUP amplitude and decrease in MUP phase and turns after our training program, reported in Chapter 6, suggests recruitment of larger motor units and synchronisation of MUPs. This phenomenon is considered an adaptation of the motor unit to training[26].

The absence of alterations in biochemical properties of skeletal muscle but the changes in muscle EMG parameters after training indicates that needle EMG analysis is potentially a sensitive tool in measuring effects of training in equine skeletal muscle. We hypothesize that neuromuscular changes precede, and possibly induce, biochemical and structural skeletal muscle adaptation to training.

Intensified training

In this thesis we used a controlled training program with a period of intensified training to evaluate skeletal muscle (mal)adaptation to increased metabolic stress levels. In a previous study from our group horses only showed signs of overtraining after removal of resting days in combination with an increase in exercise intensity[8]. One of the aims of the present study was to identify adaptation in substrate transport proteins, enzyme activity and neuromuscular properties in skeletal muscle. Another aim was to identify early markers for skeletal muscle mal-adaptation and possible precursors for overreaching.

In human subjects, a clear sign of overtraining is the loss of performance despite unchanged or even increased training load. In this thesis we did not find a decrease in performance after six weeks of intensified training. In human athletes, overreaching and overtraining are accompanied by increases in oxygen consumption and a decrease plasma in blood lactic acid accumulation at sub-maximal exercise25, 42, 46. This implicates that changes in energy supply may be involved in the development of overreaching and/or overtraining. More specifically, if blood lactate levels are lowered, the suggestion can be made that the outflow of lactate anions from active muscles is hampered. If this occurs, intracellular pH drops which eventually inhibits muscle contraction. It could also be possible that changes in MCT expression are involved in this process. Hypothetically, a down regulation of MCT4 would decrease the magnitude of lactic acid clearance from the muscle cell and may therefore induce a premature intracellular pH drop.

Based on the activity of key enzymes we could not find any mal-adaptation in the intensified trained horses compared to horses that remained on a balanced training regimen. An increase in enzyme activity of CS, HAD and hexokinase was apparent in both groups. This increase was more pronounced in the intensified trained group, however. As mentioned previously, increases in the activity of these key enzymes is indicative of a positive trainings effect[20, 31, 33, 38]. Therefore, we conclude that the training protocol used in this study did not put too much stress on skeletal muscle metabolic parameters for mal-adaptation to occur.

Studies concerning the use of neuromuscular excitability (NME) to recognize overtraining in athletes, report that an increase in stimulus was required to produce a single contraction of reference muscle after rigorous training[52, 51]. This can lead to a change in muscle fibre recruitment pattern. Small motor units composed of mainly oxidative type 1 muscle fibres will have to produce a higher force and provide more energy because less type 2 motor units are recruited due to the decrease in NME. In humans they observed this decrease of NME after 6 weeks of overtraining and after 2 weeks of regeneration this effect was even more obvious[52]. As a result of the decrease in NME, we would expect recruitment of smaller motor units resulting in lower amplitude MUPs at a certain stimulus in intensified trained horses compared to control horses. In addition, chronic overload training may lead to ultrastructural damage and myopathology which can also be analyzed by EMG[32, 45, 47, 73]. Therefore, we applied needle EMG analysis as potential tool for early identification of overreaching or overtraining. Instead of low amplitude MUPs, we found increases in amplitude and decreases in phase, number of turns and duration of the MUP. These results are associated with the recruitment of larger motor neurons and synchronization of the individual muscle fibres contributing to the MUP, which belong to normal physiological adaptations to training[51]. Therefore, no evidence of neuromuscular overtraining was apparent in this study, but rather an increased training induced adaptation compared controls. In

addition, instead of evidence of myopathies we detected evidence of neurogenic pathology in 1 couple horses.
Although many physiological variables may be linked with overreaching or overtraining our results of EMG analysis and biochemical analysis indicate a training effect in the intensified trained horses rather than overreaching or overtraining. Endocrine variables, etiological observations of increased stress levels and increased irritability and disturbances in sleep patterns may be helpful in the early detection of overreaching or overtraining[1, 61]. These parameters are currently under investigation by our research group.

Pathology, biochemistry and EMG
Neural innervation has a pronounced effect on skeletal muscle. Training and exercise increase neural input in skeletal muscle, whereas equine lower motor neuron disease (LMND) is associated with the degeneration of lower motor neurons in the spinal cord. The denervation and decreased neural input is associated with clinical signs of trembling, muscle atrophy and muscle fasciculations[11, 15]. Changes in muscle fibre type distribution[56] and whole body glucose uptake[2, 70] have been reported in this disease. In depth knowledge of this pathological condition is not only beneficial for equine medicine practice, but is also valuable as a model to study metabolic effects of denervation. The disease could also shed a new light on human neuromuscular diseases like amyotrophic lateral sclerosis (ALS) and progressive spinal muscle atrophy (PSMA). Hypermetabolic states have been reported in both human patients and in equine LMND patients and a general characteristic is the tendency to "burn oneself out"[14, 16]. Surprisingly, very little research has focused on skeletal muscle metabolic adaptations in such conditions. In this thesis, we have addressed these metabolic issues by measuring muscle fibre distribution, GLUT-4 expression and key enzymatic activities in equine LMND.
Previously, it has been shown that the chronic reduction in neuromuscular activity in LMND resulted in a slow to fast transition in myosin heavy chain (MyHC) isoform expression[56]. In humans, denervation of skeletal muscle also leads to decreases in the expression of MyHC 1 and an increase in the MyHC 2x[9]. In studies employed in this thesis we observed a significant reduction in type 1 muscle fibres in LMND affected horses compared to controls. Additionally, we observed an increase in the hybrid type 2a/2x muscle fibre type, and in one subject hybrid fibre type 1/2a. This suggests that denervated motor neurons innervating type 1 fibres are reinnervated by neighbouring motor neurons innervating type 2 fibres[60]. We can, however, not exclude that the shift in muscle fibre distribution is attributable to a relative change in fibre type distribution.

In line with the decrease in oxidative fibres, oxidative key enzyme activity is decreased in LMND subjects as well. This implies an increased anaerobic glycolysis in LMND affected muscles. More evidence for this assumption comes from the following additional findings:

1. Glycogen concentration and oxidative enzyme activity is decreased in LMND, suggesting higher glucose metabolism (present thesis).
2. Lactate dehydrogenase activity is increased in these animals[56].
3. Whole body glucose uptake is increased in LMND, and skeletal muscle is one of the most important sites for post-prandial glucose uptake[44, 70].
4. LMND is associated with lower than normal plasma glucose values during an oral and intra-venous glucose tolerance test[2, 15].

As suggested above the metabolic adaptations in LMND muscles put more stress on the glucose metabolic system. Analysis of immunohistochemical GLUT-4 expression in LMND muscles revealed no differences in fibre type specific expression and localisation compared to healthy controls (see Chapter 7). Due to the higher GLUT-4 expression in glycolytic type 2a and type 2x muscle fibres in equine muscle (see Chapter 3) an increase in total GLUT-4 availability in LMND patients is suggested due to the increased number of glycolytic muscle fibres. Western blotting analysis of membranous fractions for GLUT-4 did not show significant increases in membrane bound GLUT-4, despite the almost double membrane associated GLUT-4 levels. In order to increase glucose uptake, GLUT-4 has to fuse with the plasma membrane[24]. To date, these processes are still not fully understood, although much research is being conducted in this field. One explanation for the lack of an increase in membrane associated GLUT-4 is the large individual variance between LMND subjects, which, in turn, probably relates to disease duration in these animals. Therefore, a physiological role for increased membrane bound GLUT-4 in whole body glucose uptake and metabolism can not be excluded in LMND affected animals with a longer disease history.

The tentative diagnosis of LMND was made by quantitative EMG analysis at an early stage of the disease (3-8 weeks disease history).Quantitative EMG analysis in LMND patients indicated neurogenic abnormalities originating from instable muscle fibres (observed as fibrillation potentials, positive waves, and complex repetitive discharges) and instable neurogenic tissue (observed as doublet, triplet, and multiplet MUPs and neuromyotonia) pathologic spontaneous activity. These results are indicative of reinnervated motor units. Histopathological evaluation of muscle biopsies did reveal slight neurogenic changes such as grouped atrophy or angular atrophy, however fibre type grouping, considered the gold standard of reïnnervation was lacking. Eisen (1999) and Chio (1999) however, showed in man that a time course of 12-18 months was needed to confirm the diagnosis based on quantitative EMG by means of histopathological analysis [10, 18]. Due to the

relative short disease history in our LMND patients, histopatholgical changes like fibre grouping may not have been already present.
A decrease in neuronal drive, as seen in LMND, results in a shift in muscle fibre type due to degeneration and reinnervation of oxidative muscle fibres. The increase in glucose metabolism indicated by multiple studies[2, 56, 70] is most likely also function of a shift in muscle fibre type. On the basis of the reported findings (see also Chapter 7), we suggest that both denervation and reinnervation occur in skeletal muscle of LMND patients. Insights in the pathophysiology of equine LMND may provide knowledge about muscular changes due to decreased neuronal drive and further insights in human neuronal pathologies like ALS and PSMA.

Summarizing conclusions

In the present thesis we focused on finding proper markers of adaptation to training and intensified training. We found that expression of several selected substrate transport proteins are conserved between species. This, however, can not be generalized to physiological functioning. For instance, we have shown that the expression of glucose-transport protein GLUT-4 and the long-chain fatty acid transport-protein FAT/CD36 are conserved between man and equines. This could suggest similar energy metabolic functions during training and acute exercise. Our training protocol based on daily training practice of Standardbred trotters, failed to induce all expected adaptations. A deviant metabolic characteristic of equines compared to humans and rodents, is the large availability of volatile fatty acids. Although it is suspected that these substrates passively diffuse through the plasma membrane, the expression of FAT/CD36 and MCT2 in equine skeletal muscle implicates that these, or another facilitative transport mechanisms, may be involved in fatty acid transport in skeletal muscle.
In Standardbred trotters, and probably in other horse breeds as well, the monocarboxylate transporter 2 (MCT2) is highly expressed compared to rat skeletal muscle, and is suggestive for a different energy metabolism and adaptation of energy metabolism to training compared to humans and rodents. MCT2 could be involved in the transport of short chain fatty acids in skeletal muscle, that are derived from the evolutionary hindgut fermentation that plays a key role in the food processing of equids.
In this study, six weeks of intensified training did not lead to identification of neuromuscular or biochemical markers associated with increased stress. Most of the adaptations we found were related to normal physiological training adaptations even in the intensified training period. Moreover, horses that were trained according to our intensified training schedule followed physiological adaptation more closely than the horses in the control group. The most feasible explanation for these findings is that, at least at muscle metabolic level, the intensified training was the only stimulus that induced the overload needed for training adaptation. This notion was supported by our EMG analysis that showed an increased training adaptation after intensified training without evidence of alterations in neuromuscular

excitability as expected in the case of overtraining. We must conclude that equine skeletal muscle is resistant to the amount of stress induced by the intensified training protocol used in this study.
Another cause for the relative low training adaptations may be gait during the training and exercise tests. The Standardbred trotters were only allowed to trot during training and exercise tests, and this pattern of gait may put insufficient stress on the metabolic parameters of skeletal muscle.
We have also shown in this thesis that EMG analysis in horses is a highly sensitivity marker for the evaluation of training adaptation and neuropathy (LMND). This marker is probably more sensitive than muscle biopsies since it precedes the biochemical and histopathological markers for training, neurogenic atrophy and reinnervation. Furthermore, we have to conclude that, based on the selected parameters, training adaptation at the level of muscle is likely preceded by endocrine, neurological and etiological parameters. Such studies are currently being executed.
Finally, the expression of GLUT-4, FAT/CD36 and MCT proteins in horse skeletal muscle is an interesting finding. If these proteins are as important in equine muscle metabolism and function as in humans and rodents, it is certainly worth exploring these proteins in horses. Unfortunately, due to technical drawbacks, we were not able to perform an in depth analysis of these proteins after training and intensified training. Therefore, analysis of these proteins in future equine research can provide valuable information about skeletal muscle metabolism and functioning in horses. In order to effectively explore these proteins, horse specific antibodies raised against proteins of interest would simplify analysis greatly.

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Nederlandse samenvatting

De relatie tussen paard en mens bestaat al vele millennia. In de eerste periode dat mensen paarden temden, werden paarden vooral gehouden als vleesvoorraad en lastdier. Al snel ontwikkelde het paard zich echter tot een gewaardeerde kracht vanwege zijn atletische vermogen. In een later stadium werden oorlogsvoering en de jacht belangrijke redenen om paarden te trainen. Maar paarden werden ook al voor de eerste Olympische spelen gebruikt om te racen. De oorsprong van de moderne paardenraces dateert echter uit de middeleeuwen, toen Engelse kruisridders terugkeerden uit Jeruzalem met snelle Arabische paarden. Door deze ontwikkelingen heeft het trainen van paarden tot het optimale prestatieniveau een steeds belangrijkere plaats ingenomen in de relatie tussen paard en mens.
Het induceren van fysiologische veranderingen en het verbeteren van het prestatieniveau voor een bepaalde taak is het belangrijkste doel van training. Een effectieve training moet minimaal rekening houden met twee principes:

1. overbelastings principe
2. specificiteits principe

Het overbelastings principe houdt in dat een trainingsessie zwaarder moet zijn dan het huidige prestatieniveau. Het lichaam wordt dan gedwongen zich aan te passen aan de hogere belasting van bijvoorbeeld de spieren, de botten en het hart en vaatstelsel. 3-7 dagen na een trainingsessie zijn volledig herstel en aanpassingen meestal compleet.
Het specificiteits principe houdt in dat een training taakspecifiek moet zijn om het prestatieniveau te verhogen. Voor een endurance prestatie moet vanzelfsprekend een andere training toegepast worden dan voor een spring prestatie.

Regelmatige inspanning (of training) in mensen en paarden wordt verder geassocieerd met verscheidene positieve effecten op de gezondheid, zoals een verhoogde levensverwachting, hogere kwaliteit van leven, vermindering van mentale problemen en een positief effect op bijvoorbeeld hart- en vaatziekten. Te veel en te zware training wordt echter juist geassocieerd met gezondheidsproblemen en blessures. Indien de trainingsintensiteit chronisch hoger is dan de trainingscapaciteit kan er maladaptatie optreden. Dit kan tot een verminderd prestatieniveau, en zelfs tot gezondheidsproblemen leiden. Als deze situatie inderdaad tot gezondheidsproblemen leidt, wordt er ook wel van overtraining gesproken. Er is reeds veel onderzoek uitgevoerd naar de adaptatie en maladaptatie door training en intensieve training. Het belangrijkste probleem bij de identificatie van signalen voor overtraining is het gebrek aan een goed model en de vergelijkbaarheid van analyse methoden. Ook is het maar de

vraag hoe goed een gebruikt trainingsprogramma tijdens een wetenschappelijk onderzoek aansluit bij de praktijk.
Het doel van het onderzoek dat beschreven is in dit proefschrift is tweeledig. Ten eerste is in deze studies onderzocht hoe belangrijke eiwitten voor transport van glucose, vetzuren en lactaat tot expressie komen in skeletspieren van paarden. Ten tweede is in deze studies onderzocht of er met een gecontroleerd trainingsprogramma dat gebaseerd is op training in de praktijk ook duidelijke adaptatie in skeletspieren optreedt. Hierbij onderzoeken we ook de effecten van een periode van geïntensiveerde training op spiermetabolisme en neuromusculaire aansturing.

Tijdens inspanning zijn glucose en vetzuren de belangrijkste brandstoffen voor spieren. Deze energiebronnen zijn deels opgeslagen in de spier, glucose als glycogeen en vetzuren als vetdruppeltjes, maar worden ook voor een groot deel opgenomen uit het bloed. Zowel voor glucose als voor vetzuren bestaan hiervoor in spieren specifieke transporteiwitten. Deze eiwitten functioneren als een kanaaltje door de spiercelwand dat alleen glucose of vetzuren doorlaat. Het belangrijkste transporteiwit voor glucose is GLUT-4 en voor vetzuren is dat FAT/CD36. Over dit belangrijke onderdeel van spiermetabolisme is nog maar erg weinig bekend bij paarden. Uit de analyses die we gedaan hebben bleek dat deze transporteiwitten ook in paardenspieren aanwezig waren, en een gelijk expressiepatroon vertoonden als bij mensen. Daaruit blijkt dat het spiermetabolisme van mensen en paarden op dit niveau gelijkenissen vertoond.
Als de inspanning lang duurt, of als de intensiteit van de inspanning erg hoog wordt, wordt er als product van het spiermetabolisme ook steeds meer lactaat (melkzuur) geproduceerd. Ophoping van lactaat in de spier leidt tot "verzuring" van de spieren en vermoeidheid. Een belangrijk doel van training is het zo lang mogelijk uitstellen van deze verzuring van de spieren. In de afgelopen decennia, zijn er ook transporteiwitten voor lactaat gevonden in spieren van mensen en knaagdieren. Deze transporteiwitten spelen mogelijk een belangrijke rol in de lactaathuishouding in spieren. In ons onderzoek zijn wij er voor het eerst in geslaagd om deze eiwitten ook in spieren van paarden aan te tonen. Deze eiwitten vertonen echter een afwijkend beeld in vergelijking met mensen en knaagdieren. Twee soorten transport eiwitten waren minder aanwezig in vergelijking met ratten, terwijl een ander transport eiwit juist veel meer voorkwam. Dit wijst juist op verschillen in spiermetabolisme tussen paarden en andere dieren en mensen. In tegenstelling tot in mensen, werd er in paarden geen effect van training op de expressie van deze lactaat transporteiwitten gevonden.

De energie uit glucose en vetzuren wordt verkregen door een serie van chemische reacties, het spiermetabolisme. De snelheid waarmee deze reacties verlopen wordt bepaald door verschillende enzymen, en de snelheid van deze enzymen wordt verhoogd door frequente training. Het spiermetabolisme wordt verder voor een groot deel bepaald door de

neuromusculaire impulsen vanuit het zenuwstelsel die voor de spiercontractie zorgen. Bijvoorbeeld tijdens inspanning zorgen meer en sterkere elektrische impulsen vanuit het zenuwstelsel voor meer contracties en krachtontwikkeling in spieren, en dus ook een hogere energie vraag. Deze elektrische impulsen kunnen in spieren gemeten worden door middel van elektromyografie (EMG). Eén enkele zenuw stuurt impulsen naar minimaal 20 tot maximaal enkele honderden spiercellen. De zenuw en de spiercellen die impulsen ontvangen van de zenuw wordt een motorische eenheid (ME) genoemd. Door training veranderen er verschillende eigenschappen van de ME die gemeten kunnen worden met behulp van EMG. Uit het onderzoek is gebleken dat de aanpassingen van de ME tot de eerste gevolgen van training behoren.

Voor verdere studies naar de effecten van training op spiermetabolisme is er op 4 momenten een klein stukje spierweefsel afgenomen bij paarden: in rust, na acute inspanning, na een periode van normale training en na een periode van intensieve training. In dit stukje spierweefsel hebben we de activiteit van bepaalde enzymen die een maat zijn voor de maximale capaciteit van het spiermetabolisme gemeten. Verder werd de voorraad glucose in de het stukje spierweefsel gemeten. In de spieren van het paard werd ook het EMG gemeten om neuromusculaire aanpassingen te bestuderen.

Na 18 weken gecontroleerde, op de praktijk gebaseerde training bleken er nauwelijks veranderingen in het spiermetabolisme te zijn opgetreden. Na deze periode van training bleken er wel veranderingen te zijn opgetreden in het EMG. Hieruit blijkt dat EMG mogelijk een gevoeliger experiment is om adaptatie door training te meten, dan het meten van enzymactiviteit.
Na de eerste trainingsperiode werd de helft van de paarden intensiever en langer getraind, terwijl de andere helft van de paarden het normale trainingsprogramma bleef volgen. In beide groepen steeg de activiteit van de metabole enzymen, wat duidt op een aanpassing van de spieren. En hoewel er geen significante verschillen waren tussen de intensieve en de normale training, was de adaptatie van het spiermetabolisme aan de intensieve training in de getrainde paarden sterker dan in de normaal getrainde paarden. In het EMG werden vergelijkbare aanpassingen aan intensieve training gevonden. Wij concluderen hieruit dat de training niet de juiste overbelasting toediende om duidelijke metabole aanpassingen te vinden. De intensievere trainingsperiode leidde wel tot adaptatie, maar niet tot tekenen van overbelasting.

Ten slotte, nadat we de effecten op spiermetabolisme tijdens een verhoging van de spieractiviteit bestudeerd hadden, onderzochten we het spiermetabolisme ook in paarden met een verlaagde spieractiviteit. Deze paarden lijden aan een ziekte, lower motor neuron disease (LMND). Een belangrijk kenmerk van deze aandoening is de degeneratie van neuronen in

het ruggenmerg die de spieren aansturen. Daardoor krijgt een deel van de spiervezels geen impulsen meer van het zenuwstelsel en sterven de spiercellen af. Een alternatief is dat de spiercellen vervolgens impulsen krijgen van naastgelegen ME's, dit noemt men reïnnervatie. Uit eerder onderzoek was al bekend dat dieren met deze aandoening een verhoogd glucose metabolisme hebben. In dit onderzoek bleek een verhoogd glucose metabolisme ook uit de bepalingen van de enzymactiviteit. Deze verhoging van het glucose metabolisme bleek voort te komen uit het afsterven van spiercellen die slecht glucose verbranden. Een deel van deze spiervezels werd vervolgens gereïnnerveerd als spiercel die juist voornamelijk glucose verbrandt om in zijn energiebehoefte te voorzien. Dit proces van reïnnervatie was reeds in een vroeg ziektestadium aan te tonen met behulp van het EMG.

Samenvattend kan worden gesteld dat transporteiwitten voor glucose, vetzuren en lactaat in skeletspieren van paarden waarschijnlijk een belangrijke functie vervullen. Verder wilden we in dit proefschrift een gecontroleerd, op de praktijk gebaseerd trainingsprogramma onderzoeken. Het bleek dat de spieren zich niet op een manier aanpasten zoals we verwacht hadden. Een periode van 6 weken verzwaarde training leidde op spierniveau niet tot tekenen van maladaptatie, maar juist een versterkte trainingadaptatie. Wij concluderen hieruit dat de training niet de juiste overbelasting toediende om duidelijke metabole aanpassingen te vinden. De intensievere training periode leidde wel tot deze adaptatie, maar niet tot tekenen van overbelasting.
In een tegengestelde situatie met een verlaging neurale impulsen, LMND, bleek EMG een gevoelig instrument om veranderingen in de spier te meten. Deze veranderingen leken vaak een voorspeller te zijn voor veranderingen in spiermetabolisme.
De kennis over de metabole aanpassingen in spieren als gevolg van training in paarden, heeft de afgelopen decennia een grote vlucht genomen. Desondanks blijven er nog veel onduidelijkheden over de vraag of andere aspecten (hormonaal en/of mentaal) ook niet een grote rol spelen in (mal)adaptatie van skeletspieren als gevolg van training.

Curriculum Vitae

Klien Gerard van Dam werd op 2 mei 1979 geboren te Winsum (Gn). In 1997 behaalde hij zijn VWO diploma op het Coenecoop College te Waddinxveen. In hetzelfde jaar startte hij met de studie Gezondheidswetenschappen aan de Universiteit Maastricht. In het laatste jaar van de studie liep hij stage bij de capgroep Bewegingswetenschappen en hielp hij als assistent mee met testen om het prestatieniveau te bepalen. Nadat hij in januari 2002 afstudeerde in de Gezondheidswetenschappen met als afstudeerrichting Bewegingswetenschappen, heeft hij een half jaar bij het Academisch Ziekenhuis Maastricht (AZM) bij de afdeling Pulmonologie als onderzoeksassistent gewerkt. Hier werd door het meten van verschillen in zuurstof isotopen in ingeademde en uitgeademde lucht gekeken of de perifere diffusie weerstand voor zuurstof beïnvloed werd door training. In september 2002 werd begonnen met het promotie onderzoek, beschreven in dit proefschrift, aan de Faculteit Diergeneeskunde onder leiding van Prof. dr. A. Barneveld, Dr. J. H. van der Kolk, Dr. I. D. Wijnberg en Dr. E. van Breda. Deze promotie periode werd hij vanuit de Hoofdafdeling Gezondheidszorg Paard gedetacheerd bij het Nutrition and Toxicology Research Institute Maastricht (NUTRIM), binnen de capgroep Bewegingswetenschappen.

Klien Gerard van Dam was born on 2 may 1979 in Winsum (Gn), The Netherlands. In 1997 he completed secondary school on the "Coenecoop College" in Waddinxveen, The Netherlands. In the same year, he started his study Health Sciences at the Maastricht University. In the final year of the study he did an internship at the department of Movement Sciences and worked as an assistant in the exercise lab. After obtaining his MSc in January 2002, he worked for 6 months in the Academic Medical centre Maastricht (AZM) at the department Pulmonology as a research assistant. He performed a research that measured differences in oxygen isotopic fractionation in inhaled and exhaled air to evaluate if peripheral diffusion resistance for oxygen was influenced by training. In September 2002 he started his PhD, described in this thesis, at the Faculty of Veterinary Medicine, Utrecht University. This research was performed under supervision of A. Barneveld, J.H. van der Kolk, I. D. Wijnberg and E. van Breda. During his PhD he was seconded from the Department of Equine Sciences to the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) within the department of Movement Sciences.

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Klien van Dam

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