Myosin heavy chain and Na^+,K^+-ATPase isoforms in equine skeletal muscle

Comparison of mRNA and protein expression profiles

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Myosine heavy chain en Na^+,K^+-ATPase isovormen in skeletspieren van het paard

Vergelijking van mRNA en eiwit expressieprofielen

(met een samenvatting in het Nederlands)

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Chapter 1

Introduction

Historically, horses were already used for sporting events, before they were used to work for men. The first horse races took place around 4500 BC in Central Asia, while the first evidence for the use of horses for transportation and agricultural work goes back to 2500 years BC. As a horse is a natural born athlete that can handle a lot of different sports, man has developed a lot of different sporting disciplines together with specific training programs. Current examples of equine sports include dressage, show jumping, endurance riding, three day events and racing. Although maneuverability, obedience and precision are important characteristics of the horse, its speed and endurance capacities are the most important requirements for the majority of equine sports.
At present, there is substantial knowledge about training horses [1]. Adaptations to training and exercise can be measured in blood, muscle, and bones and are also reflected in behavior. Since equine total muscle mass represents approximately 42% of total body weight [2], a major role for skeletal muscle in adaptation to training can be expected. The plasticity of skeletal muscle to adapt to changes in activity or in working conditions is extremely high. A comparison between expression profiles of proteins and their corresponding mRNAs in equine skeletal muscle during development, training or disease would provide us with valuable fundamental knowledge on how adaptations to these different stages or interventions take place in this tissue.

Myosin heavy chains

The myosin heavy chain (MyHC) is the backbone of the contractile machinery and it comprises 25% of the total protein content in skeletal muscle. The MyHC is part of a myosin complex comprising two MyHC and four myosin light chains. Myosins are called the thick filaments whereas actins form the thin filaments of a myofibril. Skeletal muscle fibers consist of a few hundred myofibrils. Muscle fibers contract due to the innervation by motor neurons. One motor neuron innervates a number of muscle fibers; all muscle fibers controlled by the same motor neuron are said to belong to one motor unit. All muscle fibers in a motor unit are of the same MyHC type (Figure 1).
Activation of the muscle fiber by depolarization causes the myosin heads to bind to actins. A conformational change occurs which draws the thin filament a short distance (~10 nm) along the thick filament. Then the connections break (for which ATP is needed) and reform further along the thin filament to repeat the process. As a result, the filaments are pulled past each other in a rattle like action. There is no shortening, thickening, or folding of the individual filaments themselves.

In mammalian striated muscle at least ten MyHC isoforms have been identified. Namely, 1) embryonic, 2) neonatal, 3) cardiac α, 4) slow type 1 (or cardiac β in cardiac muscle), 5) fast type 2a, 6) fast type 2d (also referred to as 2x), 7) fast type 2b, 8) extraocular, 9) mandibular or masticatory (m-MHC) and 10) slow tonic MyHC [3,4]. In adult mammalian locomotory muscles generally four of these MyHC isoforms are expressed, namely type 1, 2a, 2b and 2d MyHC [3,5,6]. However, type 2b MyHC expression can not be detected in human and equine locomotory muscles. In these species skeletal muscle comprises mostly type 1, type 2a, a hybrid form type 2ad and type 2d MyHC fiber types[6,7]. The contractile properties differ between the slow (type 1) and fast (type 2a, 2ad, 2d) MyHC fiber types. Shortening velocity in type 1 MyHC fibers is consistently about ten times lower than in 2d fibers, 2a fibers being intermediate, and hybrid fibers being intermediate between pure fiber types [8].
The ability to increase or decrease the number of sarcomeres (i.e. muscle size), together with an ability to alter isoform expression gives muscle the possibility to adapt to the different challenges that may be placed upon it [9]. Changes in size and fiber type occur due to growth, training (resistance, endurance), electrical stimulation, microgravity, denervation, nutritional interventions or environmental factors (hypoxia) [5]. One of the effects of changing MyHC fiber type composition is the existence of hybrid fibers, like type 2a/2d or 1/2a MyHC fibers.

Training
The MyHC fiber type composition of muscles varies according to the athletic capacities of an individual: marathon runners usually have a large number of MyHC type 1 fibers (slow), while the majority of fibers in leg muscles of a sprinter is of one of the type 2 MyHC isoforms (fast) [10]. It is possible to change the athletic abilities further by training. The adaptive changes in MyHC fiber type composition during training have been studied extensively, both in animals and man [1,5,9]. Depending on a particular training protocol muscle fibers may shift from a slow MyHC fiber type to fast MyHC fiber types or *vice versa*. Basically, we can distinguish two different types of training: endurance and resistance training. It is well established that endurance training, in general, shifts the MyHC profile towards slower MyHC isoforms in skeletal muscle, without changes in fiber cross sectional area (= total MyHC) and an increase of capillaries per fiber ratio [1,5]. Effects of resistance exercise are more complicated, because the outcome heavily

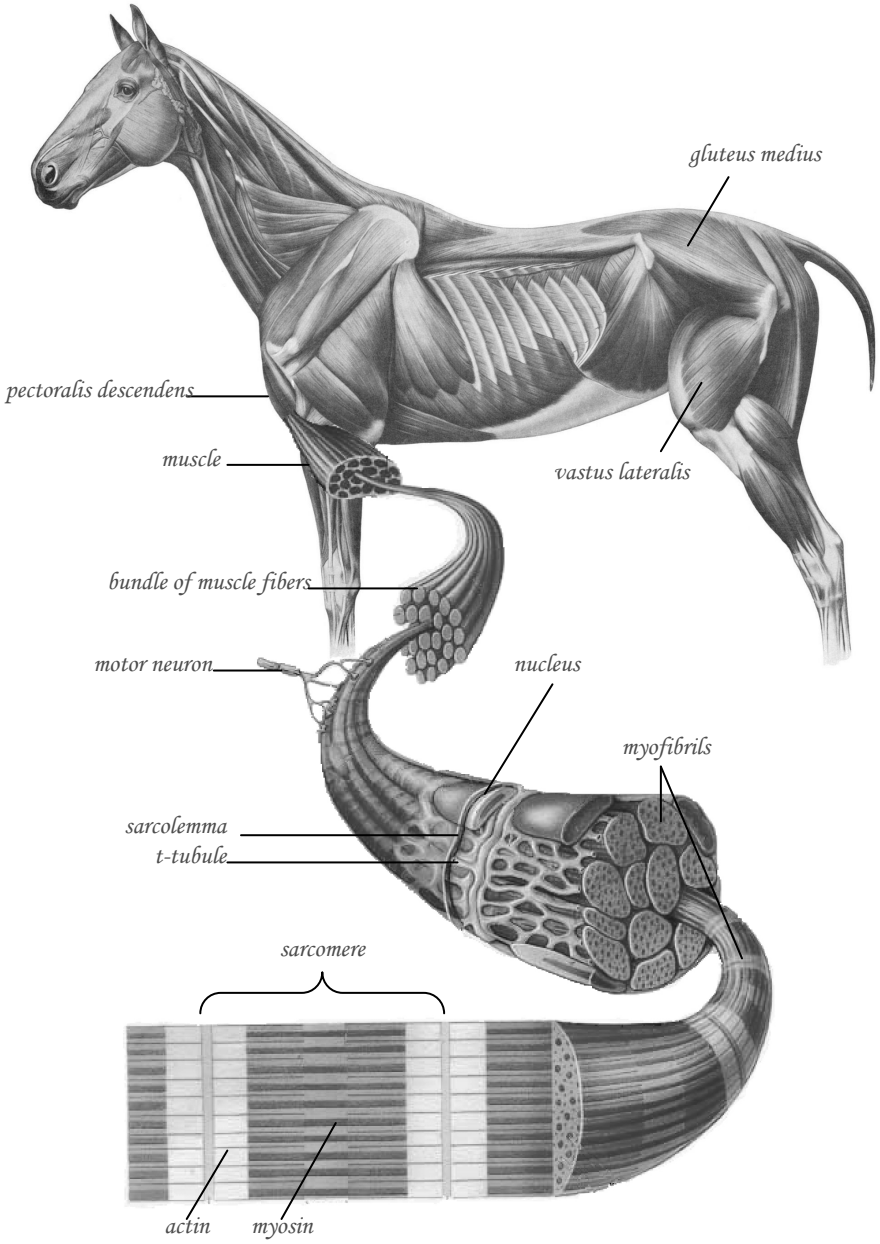

Figure 1.1. Anatomic model of the horse and a schematic model of complete muscle to molecular level. Pointed in the horse are the muscles used in this study.

depends on the specific training protocol. In general resistance exercise generates a shift towards fast MyHC isoforms, increases in fiber cross-sectional areas, and unchanged capillaries per fiber ratio [1,5,10].

Disease

A muscle disease is defined as a disorder leading to a structural or functional abnormality of skeletal muscle. Muscle diseases can be differentiated into diseases comprising mutations in skeletal muscle MyHC genes (for example, Laing early onset distal myopathy and myosin storage myopathy [11]) and diseases caused by abnormalities in the innervation pattern leading to changes in total MyHC and MyHC fiber type composition [12]. Lower motor neuron disease (LMND) is a disease that results in the loss of neural input via the neuromuscular junction, leading to a gradual denervation atrophy of affected muscles [13]. LMND has been found in horses suffering from equine motor neuron disease (EMND) and equine grass sickness (EGS). Equine motor neuron disease (EMND) is a sporadic acquired neuromuscular disorder of the somatic lower motor neurons in the ventral horns of the spinal cord and certain cranial nerve nuclei, with no involvement of upper motor neurons [14]. Clinically, EMND is characterized by weakness and muscle atrophy, but an important clinical feature in horses is the apparent tendency of the disease to ‘‘burn itself out’’ in many patients [15]. Due to the atrophy of the muscles horses become more disabled, because of the weakness it induces. Another phenomenon that causes weakness in horses with EMND is the decrease of MyHC type 1 fibers [16].

Molecular regulation

Myosin heavy chains are encoded by a highly conserved multigene family of which 10 isoforms are known in mammals. Adaptations of protein isoforms and contents towards a new stable situation can potentially be controlled by modifications in many steps from DNA to the composed translation products. All steps, namely pretranslational (including transcription), translational and posttranslational, are involved in the regulation of the skeletal muscle phenotype. Because hundreds or even thousands of proteins are differentially expressed in the different muscle fibers it is unlikely that a switch in the expression of a single protein controls the diversity of MyHC fiber types. It is well established that calcineurin plays an important role in MyHC fiber type control [17-19]. Furthermore, proteins like myocyte enhancing factor 2 (MEF-2) and nuclear factor of activated T cells (NFAT) have been shown to control slow fiber type specific genes. MyoD and myogenin are proteins important in controlling fast fiber type specific genes [18]. MyHC proteins are primarily

transcriptionally controlled [20]. In fibers that undergo fiber type transformation, changes in MyHC mRNA should precede changes in the corresponding protein products, leading to an at least temporary mismatch in the distribution of transcripts and corresponding protein products [21]. An explanation for this temporary mismatch can be found in the relatively long half-life of myofibrilar proteins, which is of 7-10 days for MyHC protein [21] and 1-3 days for MyHC mRNA [22].

Na^+,K^+-ATPase

The plasticity of skeletal muscle is evident following the onset of regular contractile activity where extensive adaptations can be observed at all levels of organization. Among the properties subject to regulation is the Na^+,K^+-ATPase, an integral membrane protein distributed throughout the sarcolemma and t-tubule, which functions to maintain high Na^+ and K^+ transmembrane gradients [23]. The Na^+,K^+-ATPase is a highly-conserved integral membrane protein that is expressed in virtually all cells of higher organisms. To indicate the importance of Na^+,K^+-ATPase, it has been estimated that roughly 23% of all cytoplasmic ATP is hydrolyzed by sodium pumps in a resting human body [24]. The Na^+,K^+-ATPase is a heterodimer composed of two protein subunits (Figure 2). The catalytic α subunit (~112 kD) that is involved in the splitting of ATP, binds both sodium and potassium ions, and contains the phosphorylation site. The smaller β subunit (~35 kD glycoprotein) is necessary for activity and transport of the pump [25]. Cation transport occurs in a cycle of conformational changes apparently triggered by phosphorylation of the pump. As currently understood, the sequence of events can be summarized as follows: The pump, with bound ATP, binds three intracellular Na^+ ions. ATP is hydrolyzed, leading to phosphorylation of a cytoplasmic loop of the pump and release of ADP. A conformational change in the pump exposes the Na^+ ions to the outside, where they are released. The pump binds two extracellular K^+ ions, leading somehow to dephosphorylation of the α-subunit. ATP binds and the pump reorients to release K^+ ions inside the cell.
Several isoforms of both α and β subunits have been identified, but aside from kinetic characterizations and tissue distribution, little is known regarding their differential physiologic importance. In mammals we can distinguish seven isoforms, four α isoforms (α1-α4) and three β isoforms (β1-β3). In skeletal muscle α1, α2, β1 and β2 isoforms are expressed, while only minor amounts of α3 and β3 have been found in human skeletal muscle [26-28]. The α4 isoform, identified in rats and humans appears isolated in the testis [29]. Re-

cently mRNA expression of the α4 isoform was also detected at a low level in human skeletal muscle [30].

Regulation of Na^+,K^+-ATPase expression as well as its activity is regulated in both acute and long term timeframes [25]. At first both subunits (α and β) are required. In muscle fibers excessive β subunits are present [31], making synthesis of the α subunits the limiting factor of regulating the amounts of pumps. The acute regulation of Na^+,K^+-ATPase finds its origin by action potentials, causing an influx of Na^+, followed by an efflux of K^+. This leads to rapid increases in the concentrations of Na^+ on the inner and K^+ on the outer surfaces of the sarcolemma, which is a strong stimulus for the activity of the Na^+-K^+-pump [25]. Furthermore acute regulation can be induced by hormones like adrenaline or insulin stimulating Na^+,K^+-ATPase activity in skeletal muscle [32]. Acute regulation is not directly associated with increasing total amounts of Na^+,K^+-ATPase in skeletal muscle.

The control of Na^+,K^+-ATPase in skeletal muscle is achieved by a number of factors that cause up-regulation or down-regulation of the total amount of Na^+,K^+-ATPase within a muscle. Besides hyperthyroidism [33], glucocortoid treatment [34] and insulin treatment [35] cause up-regulation, while inactivity [36,37], hypothyroidism [33] and heart failure [38] and aging [39] result in down-regulation. Training [25-28,40-43] is by far most often described as a factor for long term (up) regulation of Na^+,K^+-ATPase in skeletal muscle.

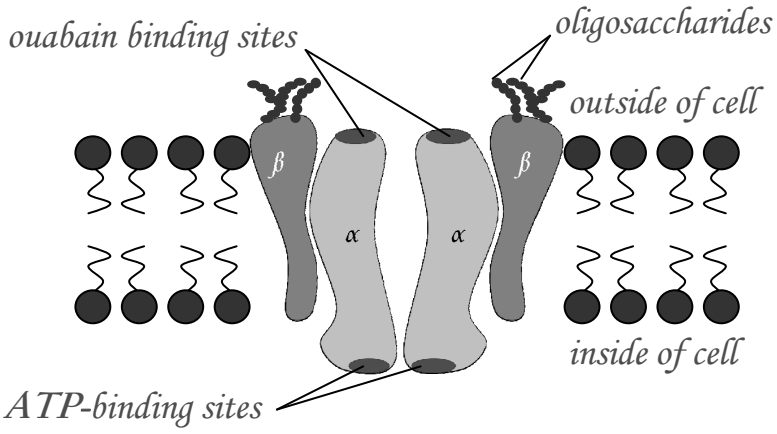

Figure 1.2. Na^+,K^+-ATPase consists of two α-isoforms and two β-isoforms, situated in the plasma membrane.

Training

Although the total amount of Na^+,K^+-ATPase is already high in untrained skeletal muscles (about 180000 billions of pumps per g wet weight [32]), training causes up-regulation of the total amount of Na^+,K^+-ATPase [25]. Depending on the fitness of the subject, the type of training and/or muscle function, up-regulation of Na^+,K^+-ATPase may vary between an increase of 2-45% in man and a variety of animals [25,32]. It has been shown that one short bout of exercise is not enough to increase total amount of Na^+,K^+-ATPase in a human muscle [26]. However, repeating an exercise 16 times within 16 hours [44] or training for several days [45] was required to increase the total Na^+,K^+-ATPase content in human muscle. In horses short term training of 10 days with moderate intensity was enough to increase Na^+,K^+-ATPase content by 23 % [41], while within 12 days of training Na^+,K^+-ATPase content increased by 32% in gluteus medius muscle of middle aged (10-16 years) horses [39].

On the mRNA level all Na^+,K^+-ATPase isoforms can increase due to short term exercise [26,30,46]. Long term training results in inconsistent mRNA expression levels. Three weeks of high intensity training in well-trained athletes up regulates α3- and β3-isoform mRNA [47], while 5.5 weeks of high intensity training does not stimulate isoform mRNA expression [27]. In endurance trained athletes, there is even down-regulation of the mRNA expression levels of α1, α3, β1 and β2 isoforms [48]. A reason for this down regulation after years of training might be found in the higher Na^+,K^+-ATPase content. The higher Na^+,K^+-ATPase content due to increase of the translation rate and/or efficiency and/or reduced degradation rate may induce a feedback mechanism for reduced mRNA expression via decreased transcription rate and/or efficiency and/or increased mRNA degradation [48]. On the protein level different studies showed increases in human muscle of some isoforms after repeated exercise [44] as well as after a training period [27,46,47]. Data on isoform specific expression levels and changes of mRNA as well as its protein due to exercise or training are inconsistent and incomplete.

Disease

Na^+,K^+-ATPase content in muscles is influenced by factors like the innervation pattern, fiber type, and muscular and neurodegenerative disorders [25,49]. However, no diseases are known that are directly related to a disorder of the Na^+,K^+-ATPase in skeletal muscle. LMND in horses is associated with denervation and reinnervation of muscle fibers (described in paragraph "Disease" for MyHC). It is not known how the combination of these processes influences the Na^+,K^+-ATPase content of affected muscles. Both de- and rein-

nervation would lead to relatively faster muscle fibers [50], and fast fibers usually show a higher Na^+,K^+-ATPase content [25].

Molecular regulation

The Na^+,K^+-ATPase belongs to a multigene family with different genes encoding four α-isoforms (α1-α4) and the three β-isoforms (β1-β3) [51]. Regulation of Na^+,K^+-ATPase occurs in many different ways. One of them is translocation of the pump from intracellular stores to the plasma membrane due to exercise [28,52], but also stimulation with insulin or *in vitro* contraction causes translocation of the pump [53]. For this translocation different signaling pathways can be activated. Na^+,K^+-ATPase translocation is sensitive to inhibition of PI3 kinase and atypical PKCs. Furthermore extracellular signal-related kinase 1/2 (ERK 1/2) is essential for translocation of the pump to the cell surface [53]. Recently, phospolemman was found to be an important player in the physiological regulation of Na^+,K^+-ATPase in skeletal muscle [54]. Although, the pump was discovered in 1956 [55], the mechanism governing Na^+,K^+-ATPase translocation in skeletal muscle is still incompletely understood.

Another form of molecular regulation is the synthesis of new pumps. In addition to the expression of different genes for α- and β subunits, several other mechanisms are involved in the generation of Na^+,K^+-ATPase isoforms [51]. It has been shown that increases of α- and β-subunits occur after a single bout of exercise on the mRNA level, but no changes in protein expression were measured [26]. Repeating a bout of exercise twice once per hour was enough to show increases in α2 and α3 protein isoform expression and a decrease in β3 protein isoform [44]. This indicates it is possible that repeated sessions of exercise are needed to induce translation of the transcripts of the different isoforms into proteins. The fact that different exercise studies show different adaptations in the isoforms, both at the mRNA and protein level complicates full understanding of the molecular regulation of the Na^+,K^+-pump.

Breeds

Although science has thus far been unable to come up with any breeding system that guarantees the birth of a champion, breeders over the centuries have produced an increasingly higher percentage of breeds that are successful at specific levels of equine sports. Due to these breeding programs and evolution, skeletal muscle parameters of equine breeds can vary a lot. For example the total muscle mass of a Thoroughbred (used for horse racing) comprises 53-57% of total body weight, while 42% is the average percentage of total muscle mass in the horse [2]. Several studies have shown that MyHC fiber type

composition varies in the different breeds [1,56-59]. For example, in untrained Standardbreds (used for harness racing) gluteus medius muscle consisted of around 20% type 1 MyHC fibers [58], while in Dutch Warmblood horses (used for dressage and show jumping) percentages were measured of 27% MyHC type 1 fibers [59]. Additionally, it has been shown that within breeds large variation in MyHC fiber typing is also possible [60]. Although not many breeds were used to measure Na^+,K^+-ATPase contents, it seems that that there are also differences between breeds for this parameter. In untrained Thoroughbreds and Dutch Warmblood horses of comparable age, concentrations of Na^+,K^+-ATPase (measured by [^{3}H]ouabain binding sites) in gluteus medius muscle were measured of approximately 80 and 100 pmol/gram wet weight [39,41,59]. Similar to the large variations in MyHC fiber type composition within a breed, it is possible that variations of Na^+,K^+-ATPase content exist in muscles from horses of the same breed.

Aim of the thesis

The aim of this thesis was to obtain a better understanding of the expression patterns of two major proteins involved in muscle contraction and resistance to fatigue and their adaptation to (patho)physiological processes. Therefore muscle biopsies were taken and analyzed as sections, intact biopsies and homogenates. The following questions were addressed:

1. What is the basal expression profile of MyHC and Na^+,K^+-ATPase in different equine locomotory muscles?
2. How do these proteins and/or their corresponding mRNAs respond to long-term training?
3. What are the effects of a single bout of exercise on Na^+,K^+-ATPase protein and mRNA expression patterns before and after training?
4. Do muscles of horses affected by lower motor neuron disease show changes in expression profile of MyHC and Na^+,K^+-ATPase isoforms?

All investigations were part of larger projects and therefore the selection of muscles was made within the framework of the different projects.

Outline of the thesis

In Chapter two the expression patterns of MyHC mRNA and protein isoforms were analyzed in equine gluteus medius muscle by *in situ* hybridization and immunohistochemistry on both transverse and longitudinal sections. The longitudinal sections were analyzed, because transverse sections may give a limited sampling of mRNA expression in case of uneven distribution of transcripts in a muscle fiber. These studies were performed in Dutch Warmblood horses.

In Chapter three the structural and functional adaptations in a fore limb (pectoralis descendens muscle) and hind limb (vastus lateralis muscle) of the horse after long-term training were investigated. MyHC fiber type composition, fiber size, capillary number and Na^+,K^+-ATPase content were measured and the responses of the two muscles compared. In addition, plasma thyroid hormone concentrations were determined before and after training, as thyroid hormone is a major determinant of MyHC profiling, capillarity, and Na^+,K^+-ATPase content. For this purpose, 2-yr old Standardbred horses were trained for 18 weeks combining endurance and interval training.

In Chapter four we describe in detail the effects of acute exercise and long-term training on Na^+,K^+-ATPase. Isoforms of the Na^+,K^+-ATPase were de-

tected in skeletal muscle, both on mRNA as well as protein level. The muscle samples for this study were obtained from the training experiment described in Chapter three, with an additional acute exercise test before and after the 18-week training protocol.

In Chapter five it was demonstrated that lower motor neuron disorder affected the mRNA expression levels of different Na^+,K^+-ATPase isoforms in muscles (pectoralis descendens and vastus lateralis muscle) of horses with EMND. In addition, it was found that EMND specifically reduced the size (cross sectional area) of MyHC type 1. Finally, we discussed whether quantification of Na^+,K^+-ATPase content and/or mRNA isoform expression levels would be useful to support the ante mortem diagnosis of LMND.

Chapter six provides an overview of the findings from the previous chapters and discusses the four aforementioned questions.

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Chapter 2

Myosin heavy chain isoforms in equine gluteus medius: comparison of mRNA and protein expression profiles

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Abstract
The major structural protein in skeletal muscle, myosin heavy chain (MyHC) is thought to be primarily transcriptionally controlled. We compared the expression of MyHC isoforms on the mRNA and protein level in biopsies from the m. gluteus medius from adult untrained horses. In transverse sections, the majority of fibers showed identical mRNA and protein expression patterns. However, co-expression of 2a and 2d/x MyHCs was substantially more common at the protein than at the mRNA level, suggesting a fine tuning of these two genes in normal muscle, not subjected to any training protocol. Since transverse sections give a limited sampling of mRNA expression in the case of uneven distribution of transcripts in a muscle fiber, we also analyzed longitudinal sections. We present, for the first time, that expression of MyHC mRNA and protein was equal along the length of the fiber. Hence, the mRNA expression is not regulated by differential expression of isoforms by separate myonuclei. It is concluded that the number of protein hybrid fibers in equine gluteus medius muscle is controlled by alternating the transcription pattern rather than by simultaneous transcription of genes.

Introduction

Skeletal muscle is composed of different types of myofibers, each expressing a distinct set of structural proteins and metabolic enzymes [1]. Fiber type is usually defined by the isoform of the present myosin heavy chain (MyHC). Like other structural muscle proteins, MyHCs are encoded by a highly conserved multigene family of which eight isoforms are known in mammals (2a, 2d (also referred to as 2x), 2b, embryonic, perinatal, slow/1/β, extraocular, and α), each with its own ATPase activity and each encoded by a separate gene [2,3]. In postnatal mammalian skeletal muscles in all species studied so far, at least three major fiber types are characterized: fibers expressing the slow/1/β (in this paper referred to as 1), and the fast 2a and 2d/x MyHC isoform. In addition, in rodents, rabbits, lamas and pigs the fast 2b MyHC isoform is present in fast muscles [4-6]. Depending on the type of muscle, a substantial number of fibers express two MyHC isoforms. Especially MyHC type 2a and type 2d protein hybrid fibers (2ad) are seen regularly, these fibers are regarded as a functional intermediate between the type 2a and type 2d/x fibers [7-11]. The regulation of the double MyHC expression is unclear.

The MyHC proteins are thought to be primarily transcriptionally controlled; the time course and threshold stimuli needed to trigger changes at the mRNA level are thus important aspects of gene regulation [12]. In fibers that undergo fiber-type transformation, changes in MyHC mRNA should precede changes in the corresponding protein products, leading to an -at least- temporary mismatch in the distribution of transcripts and corresponding protein products [13,14]. By comparing changes in expression of both MyHC mRNA and protein a clue can be obtained for the direction of change in MyHC gene expression. We previously studied the mRNA and protein expression in serial transverse sections of the gluteus medius muscle of the horse. We showed in one horse that the majority of the fibers analyzed had the same RNA and protein expression, while a minority of the fibers showed a mismatch. Most of these mismatches concerned the common type of hybrid fibers, expressing both MyHC type 2a and 2d protein but only a single MyHC RNA [15]. However, the mismatch between mRNA and protein MyHC expression observed in transverse sections, could be the result of non-homogenous distribution of the mRNA along the length of the fiber [16]. In the present paper we extend and confirm our previous observations by including data from more horses and demonstrate that MyHC expression in hybrid fibers is regulated at the transcriptional level. In order to test the homogeneity of mRNA and protein expressi-

on along the length of a fiber we analyzed, for the first time, longitudinal sections by in situ hybridization and immunohistochemistry.

Materials and methods

All chemicals were obtained from Merck (Amsterdam, The Netherlands) unless otherwise indicated.

Animals

Samples were taken from four adult, untrained healthy horses, two Dutch Warmblood (DW) mares (4 and 8 years old), one DW gelding (15 years old) and one Frisian stallion (26 years old). All procedures were reviewed and approved by the institutional animal care committee

Muscle biopsies

Percutaneous muscle biopsies from the gluteus medius were taken according to the protocol of Lindholm and Piehl [17 18]. The biopsies were taken on an imaginary line drawn from the coxal tuber to the sacral tuber, at one-third distance from the sacral tuber, perpendicular to the skin. They were taken as deep as possible (until resistance from the iliac wing was reached). The samples were rolled in talcum powder, mounted on cork blocks with the use of OCT embedding medium and oriented in such a way that the fibers could be sectioned either transversely or longitudinally and frozen in liquid nitrogen. All samples were stored at -80°C.

In Situ Hybridization

Transverse or longitudinal serial sections (10 μm) were made with a cryostat at -20°C, placed on Superfrost Plus slides, dried for 1 hour, fixed for 20 min. with 4 % paraformaldehyde in 1 × phosphate buffered saline (PBS, pH 7.4), washed in 1 × PBS, dehydrated and stored at –80°C. The in situ hybridization was performed as described previously 15, in brief: slides were returned to room temperature, rehydrated, rinsed and digested with 20 μg/ml of proteinase K at 37°C for 3 minutes. Acetylation was performed with 0.25 % acetic anhydride in 0.1 M triethanolamine for 10 min. After several rinses, refixation and several washes, the sections were completely dehydrated before hybridization. The cDNAs included MyHC 1 (276 bp) in pGEM-T, 2a (278 bp) in pGEM-T, 2d/x (282 bp) in pCRII-TOPO. Riboprobes were synthesized, according to the manufacturer's guidelines (Roche Molecular Biochemicals, Almere, The Netherlands)

and purified by a Qiagen RNeasy kitTM (Westburg, Leusden, The Netherlands). The riboprobes (500 ng/ml final concentration) were suspended in 40 % (deionized) formamide, 1 × SSC, 10 % dextran sulphate, 1 × Denhardt solution (0.02 % Ficoll, 0.02 % polyvynilpyrrolidine, 0.02 % bovine serum albumin (BSA)), 0.67 M NaCl, 0.1 µg/µl yeast tRNA and 0.1 µg/µl herring sperm DNA, heated at 80°C for 5 min.. Prehybridization was performed for thirty minutes. Approximately 30 µl of probe was used per slide, overlaid with a coverslip. Hybridization was performed overnight at 45°C in a humidified In Slide OutTM incubator (Boekel Scientific, Merck, Amsterdam, The Netherlands). Coverslips were removed, followed by two high-stringency steps at 60°C for 20 minutes in 0.5 × SSC and 20 % formamide and two rinses in 2 × SSC at room temperature. Unhybridized probe was digested with 1 µg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, at 37°C for 30 min, followed by 5 washes in 2 × SSC at room temperature and another high-stringency wash for 10 min. The sections were rinsed twice with 2 × SSC and maleic buffer (0.1 M Maleic acid 0.15 M NaCl, pH 7.5). Tissue sections were blocked with 5 % inactivated BSA in maleic buffer at room temperature. Blocking buffer was replaced with sheep anti-digoxigenin Fab-alkaline phosphatase conjugate (1:2000 in 1 % BSA in maleic buffer) overnight at room temperature. Several washes with the same buffer were followed by washes with 0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM MgCl2, 0.1 % Tween 20 at room temperature. Alkaline phosphatase activity was visualized by incubation with 0.18 mg/ml BCIP, 0.34 mg/ml NBT, in the buffer described above. The staining was allowed to develop for approximately 16 hours at room temperature, rinsed with distilled water and embedded in Aquamount.

Immunohistochemical staining

The monoclonal antibodies (Mabs) used were previously shown to cross-react with horse myosins [11]. Mab 219 reacts with type 1. Mab 333 reacts with type 2a, Mab 412 reacts with type 1 and 2d/x. Mab A4.74 is obtained from Biocytex (France) and recognizes MyHC type 2a. Transverse serial and longitudinal sections (10 µm) were obtained as described above. The protocol was followed as described previously [11,15]. Briefly: the slides were, rinsed in PBS, blocked in Teng-T (10 mM Tris, 5 mM EDTA, 0.15

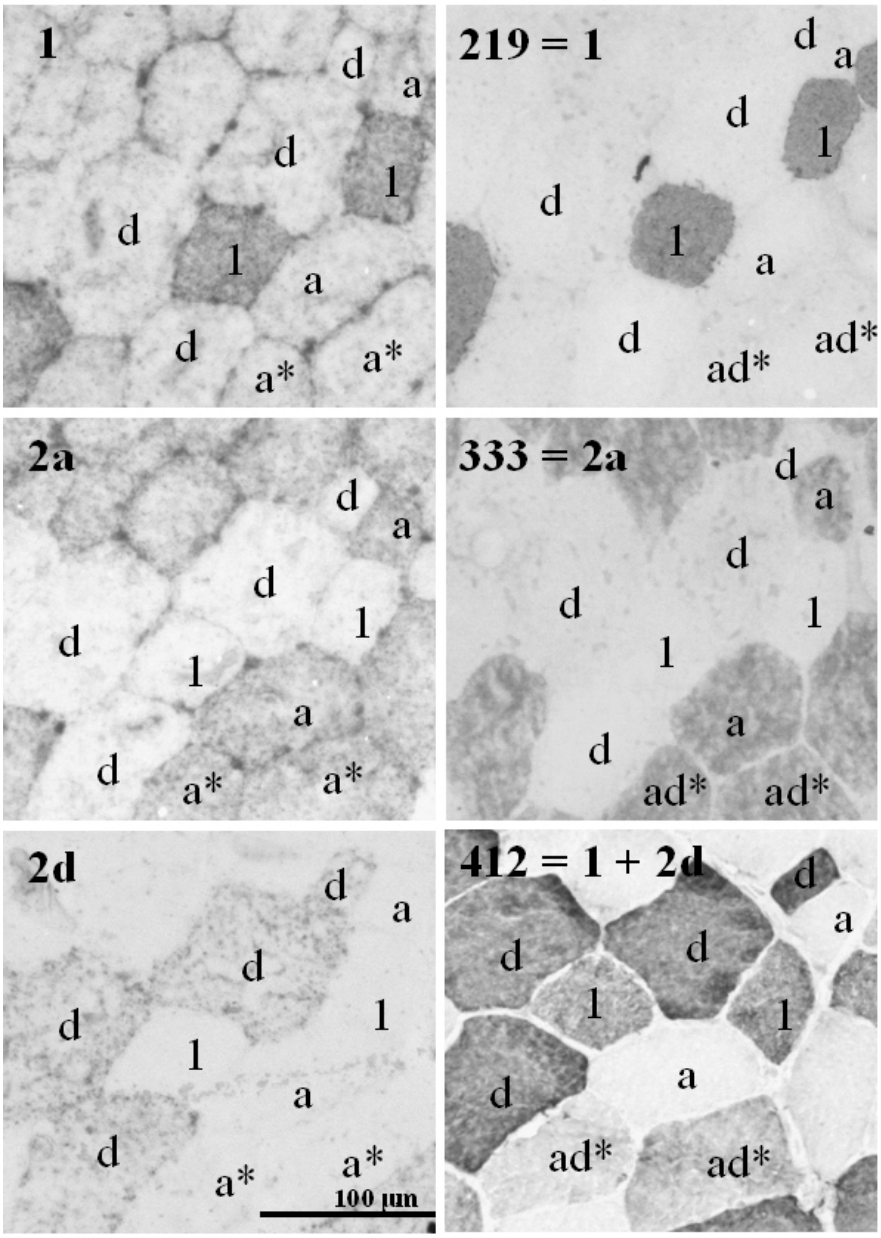

Figure 2.1. Identification of myofiber types by in situ hybridization using non-radioactive MyHC mRNA probes (left panels) and immunohistochemistry (right panels) in serial sections (from the same biopsy as table 1C). The specificity of the probes (left panels) and antibodies (right panels) are indicated. Fiber types are indicated according to the mRNA expression (left panels) or according to the protein expression (right panels).

M NaCl, 0.25 % gelatine and 0.05 % Tween 20; pH 8.0) for at least 15 minutes, followed by rinsing in PBS and incubation overnight at room temperature with the Mabs at a dilution of 1:10 (333), 1:25 (219, 412) or 1:50 (A4.74) in PBS. The slides were rinsed with PBS and incubated for 90 minutes with a biotinylated horse anti mouse polyclonal antibody (1:100 in PBS) (ABC-peroxidase staining kit Elite (Vector Labs., Burlingame, USA)), rinsed in PBS and incubated for 90 minutes with the components avidin (1:100 in PBS) and biotin (1:100 in PBS) of the ABC staining kit. After rinsing, visualized by incubation with 0.05 % 3,3'-diaminobenzidine tetrachloride in 30 mM imidazole and 0.09 % H2O2. The slides were subsequently embedded in DePeX. For Mab 412, the slides were rinsed in 0.1 M acetate buffer (pH 6.0) and di-ammonium nickel sulfate (2.5 % in acetate buffer) instead of imidazole was used.

Analyses
Between 146 and 296 fibers per biopsy were used for fiber typing and calculation of fiber type composition. The fibers were classified into type 1, type 2a, type 2ad and type 2d/x on basis of their reactions with the different in situ probes and the Mabs.

Results
Identification of myofiber types by in situ hybridization and immunohistochemistry

We analyzed the myofiber types of the gluteus medius muscle of four different horses by using in situ hybridization and immunohistochemistry on transverse serial sections. Figure 2.1 (left panels) shows serial sections from the equine gluteus medius muscle processed for in situ hybridization with mRNA probes specific for MyHC 1, 2a and 2d isoforms. Indicated is the fiber type according to the mRNA expression analyses. These classifications were compared to the corresponding fiber types according to the protein expression analyses (figure 1, right panels). Clearly, the staining pattern of the in situ hybridization and immunohistochemistry experiments show a high correlation. Very few of the total analysed fibers co-expressed two MyHC transcripts (Table 2.1), the majority expressed one mRNA isoform. The only co-expression detected was in a small minority of fibers, expressing 2a and 2d/x MyHC transcripts (type 2ad) (0 - 1.7%,

Table 2.1. Calculation of MyHC fiber types (%) present in the equine gluteus medius muscle as assessed by in situ hybridization (RNA) and immunohistochemistry (protein) on transverse serial sections. The numbers in the right column and the lower row indicate total numbers (%). n = number of fibers analyzed. A has been presented before (Eizema et al., 2003). A. Friesian stallion (n = 294), B. DW mare (n = 260), C. DW mare (n = 146), D. DW gelding (n = 189).

A

Protein / RNA	1	2a	2ad	2d/x	total
1	30.6	0	0.3	0	30.9
2a	0	8.2	19.7	0.3	28.2
2ad	0	0	0	1.7	1.7
2d/x	0	0	1.4	37.8	39.2
total	30.6	8.2	21.4	39.8	100

B

Protein / RNA	1	2a	2ad	2d/x	total
1	24.2	0.4	0	0.4	25.0
2a	0.4	29.3	10.0	1.5	41.2
2ad	0	0	0.4	0	0.4
2d/x	0	1.9	5.0	26.5	33.4
total	24.6	31.6	15.4	28.4	100

C

Protein / RNA	1	2a	2ad	2d/x	total
1	26.7	0	0	0	26.7
2a	0	28.1	20.0	0	48.1
2ad	0	0	0	0.7	0.7
2d/x	0	0	0.7	24.0	24.7
total	26.7	28.1	20.7	24.7	100

D

Protein / RNA	1	2a	2ad	2d/x	total
1	21.2	0	0	0	21.2
2a	0	23.8	16.4	0	40.2
2ad	0	0	0	0	0
2d/x	0	0.5	18.0	20.1	38.6
total	21.2	24.3	34.4	20.1	100

Table 2.1). On the protein level, however, many more hybrid fibers (15.4 - 34.4%)were detected, and they were all the type 2ad fiber type (Table 2.1).
Of all fibers analyzed, the majority had identical mRNA and protein expression, but roughly 20% showed a mismatch (Table 2.1). Most of these mismatches concerned fibers expressing type 2ad protein. In fact, except for one fiber (in horse B), type 2ad protein fibers never showed a match with 2ad mRNA. Two examples are seen in Figure 2.1, expressing type 2a mRNA but type 2ad protein (asterisks). Of the type 2ad fibers at the protein level, the majority expressed only type 2a mRNA, a minority only type 2d/x mRNA and 0.3% (1 fiber in one animal) expressed type 1 mRNA (Table 1). In total this results in a higher number of pure 2a fibers on the mRNA level as compared to the protein level, the number of pure 2d fibers are approximately equal. An exception was the result obtained with the 15 year old gelding (results in Table 1D), half of the type 2ad protein fibers expressed type 2a mRNA, the other half expressed type 2d mRNA.

Analysis of longitudinal sections
A hypothesis for the occasional appearance of a protein without its mRNA is that the entire fiber in question contains mRNA for more than one MyHC isoform but that each nucleus expresses only a single mRNA or expresses it in pulses as was demonstrated in murine muscle by Newlands et al. 16. In this case transverse sections would give a limited sampling of mRNA expression in a muscle fiber. In order to test this hypothesis we analyzed longitudinal sections by in situ hybridization and immunohistochemistry. Several biopsies were analyzed; figure 2.2 depicts a representative specimen. The left panels show the mRNA expression, the right panels the protein expression. The mRNA expression is slightly higher under the sarcolemma indicating the localization in the sarcoplasm, in agreement with the study of Russell 19. No differences in mRNA or protein expression level were seen along considerable stretches of fiber (approximately 500 μm), containing many myonuclei (distance between myonuclei approximately 20 μm) 20 (figure 2.2). Additionally, we analyzed 12 consecutive transverse 10 μm sections (120 μm total) stained with the different mRNA probes. Again fibers were positive in all 12 sections for the MyHC mRNA tested (tested for all three probes, results not shown) indicating consistent mRNA expression.

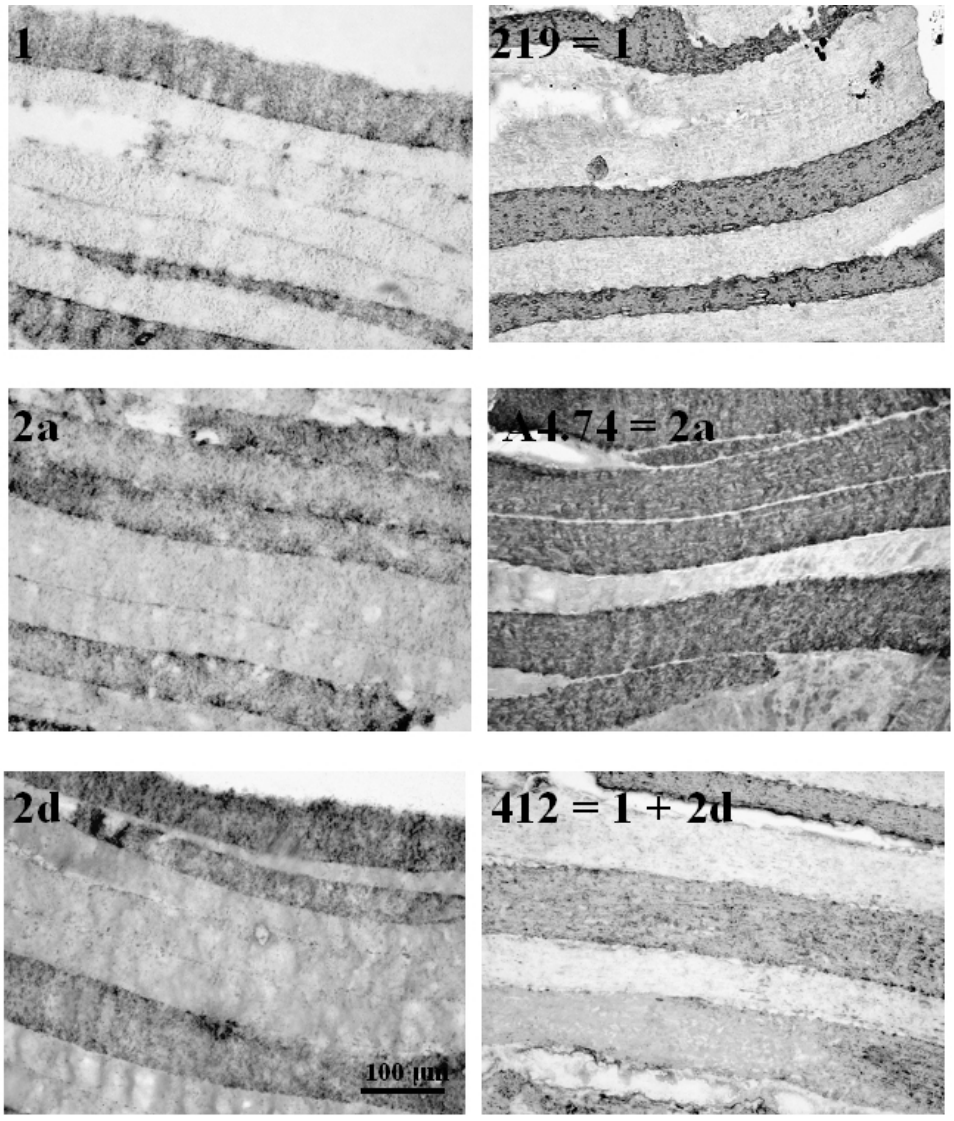

Figure 2.2. Part of a longitudinal section (from the same biopsy as table 1D) stained with the indicated mRNA probes (left panels) or with the indicated antibodies (right panels). Alignment of the sections is not possible. Clearly, the staining is equal along the visible fiber length.

Discussion

In the present paper we demonstrate conclusively that MyHC expression in hybrid fibers is regulated at the transcriptional level. The MyHC mRNA and protein is homogeneously expressed along the length of a fiber as analyzed, for the first time, using longitudinal sections assayed by in situ hybridization and immunohistochemistry.

The distribution of the MyHC transcripts was homogenous across the transverse section for all the probes tested (figure 2.1). This is in agreement with most other studies using a variety of techniques (electron microscopy, radioactive in situ hybridisation and non-radioactive in situ hybridisation) [7,19,21].

In this paper, we present for the first time longitudinal sections analysed for MyHC mRNA and protein expression. Unfortunately, comparison of different protein and mRNA expression within the same fiber was not possible since the fibers could not be aligned due to limitations in fiber diameter. We tried to use sections of 5 µm instead of 10 µm to improve the alignment, however these thin sections proved not suitable for in situ hybridisation experiments. The distribution of the transcripts along the length of the fiber (figure 2.2) was homogenous, switches of fiber type along the length of the fiber or localised areas with deviating MyHC expression as observed in human vastus lateralis fibers of very old subjects was never seen [22].

Most fibers analyzed expressed a single, corresponding, MyHC isoform on the mRNA and protein level but mismatches were the rule in hybrid fibers. In these, two MyHC protein isoforms were expressed, but the mRNA for only one of them (interestingly mostly 2a) was present. The low level of co-expression at the mRNA level and the homogenous expression along a considerable length of fiber suggests that a hybrid fiber synthesizes only one mRNA isoform at a time. Fibers containing two MyHC proteins, are most probably converting to the type corresponding to the expressed mRNA, the other isoform is no longer synthesized and will progressively disappear as a result of protein turnover. Hence, regulation of hybrid fibers is transcriptionally controlled in the equine gluteus medius muscle. Although, hybrid MyHC 2ad protein fibers are regarded as a ‘true’ type, because of their regular appearance, they can also be classified as transitory fibers as suggested by Pette and Staron (1997) and demonstrated in this paper [7-11]. The appearance of hybrid and mismatched

fibers indicates that fiber type transition occurs in presumably normal skeletal muscles not subjected to any training protocol.
Here we present the absence of hybrid 2ad fibers on the mRNA level in equine gluteus medius muscle. The same type of results were presented for longissimus and rhomboideus muscle of the pig, also a large animal [23]. Comparing studies in large (horse, pig and human) animals to studies using small (rat, rabbit) animals can indicated whether regulation of hybrid fibers types is identical.
In human vastus lateralis the vast majority of MyHC mRNA profiles, including hybrid fibers, corresponded with the protein isoforms detected [13,24-26]. In a comparative study of 14 different human muscles, up to 48% of the whole fiber population in certain samples co-expressed 2a and 2d MyHC transcripts. Additionally, up to 13% of the fibers co-expressed 1 and 2a MyHC mRNA. In contrast to the situation in the equine gluteus medius, around 60% of the hybrid 2ad fibers on the protein level were also 2ad hybrids on the mRNA level, approximately 30% expressed only 2a mRNA and 10% expressed only 2d mRNA [27,28]. Unfortunately, the authors did not state from which muscles these particular hybrid fibers were isolated.
In rabbit gastrocnemius, psoas and adductor magnus muscle single fiber analysis revealed the existence of 2b/2d MyHC hybrids (2a was not analyzed) [29]. Depending on the muscle, and in contrast with our results, the number of hybrids was equal or larger at the mRNA level than at the protein level. Another study [30] analyzed single fibers from rabbit rectus femoris, extensor digitorum longus, tibialis anterior and soleus muscle. Again hybrid fibers in mRNA and protein MyHC were detected. mRNA MyHC isoform patterns were consistent with MHC protein profiles in the majority of fibers from normal, untransforming muscle [30]. In rat, single fibers from the soleus muscle were analyzed; in this muscle, again, more hybrid fibers were detected on the mRNA level than on the protein level [31].
From the above mentioned studies it is clear that the detection of hybrids in normal, nontrained muscle is a general phenomenon. The number of hybrid fibers is however highly species and muscle specific. The studies using small animals show a higher number of hybrids on the mRNA level as compared to the protein level. The human muscle equal or lower numbers, the horse (this study) and pig muscles show (almost) no hybrid fibers on the mRNA level. However, comparison is hampered by the fact that different types of muscles were used. Since humans and horses do not

express 2b MyHC protein in their limb muscles it is conceivable that the existence of hybrid 2ad fibers is even more important for the fine-tuning of contraction properties as compared to animals who have three fast isoforms available [15,21,32].
Several protocols applied to induce transformation of muscle fibers show that upon transformation the amount of hybrid fibers increase, even up to the expression of three MyHC protein isoforms [33]. Upon this induced transformation, regardless the type of protocol applied, more mismatching between mRNA and protein expression is observed [13,25,30,31]. Clearly, analyzing muscle fibers (pure and hybrid) on the mRNA and protein level is important for understanding the steady-state situation and the transformation process. The regulation of muscle MyHC fiber type is a complex process, fine-tuned by transcriptional regulation of the expression of different MyHC mRNA's enabling the muscles to tune even with minimal changes in mechanical demands.

In conclusion, we compared the expression of MyHC isoforms on the mRNA level with the expression on the protein level on a fiber-to-fiber basis. Co-expression of MyHCs was more common at the protein than at the mRNA level and was mostly observed for 2a and 2d/x MyHCs, suggesting a fine-tuning of these two genes and a strong influence of their expression on myofiber plasticity. We show that expression of MyHC mRNA and protein is equal along the length of the fiber in equine gluteus medius muscle; therefore the mRNA expression is not regulated by differential expression of isoforms by separate myonuclei. Further research is necessary to establish and understand the importance of MyHC transcriptional and translational polymorphism in different muscles and different species of animals.

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Chapter 3

Structural and functional adaptations to combined endurance and interval training in equine vastus lateralis and pectoralis descendens muscle

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Abstract

Purpose: Both man and horses can improve their athletic performance by training. Most studies evaluating adaptations in skeletal muscle in humans are performed using vastus lateralis muscle, while in horses the gluteus medius muscle is primarily investigated. The present study examined the specific response to training in the equine vastus lateralis (hind limb) muscle, and compared the effects with those in the pectoralis descendens (forelimb) muscle.

Methods: Four two-year old Standardbred horses underwent combined endurance and interval training for 18 weeks. Biopsies were taken from the vastus lateralis and pectoralis descendens muscles both pre- and post-training. Myosin Heavy Chain (MyHC) isoform expression (contraction speed), fiber size (strength), and capillary number (oxidative capacity) were measured by immunohistochemistry. Na^+,K^+-ATPase content (excitability) was quantified by 3[H]ouabain binding. In addition, serum thyroid hormone parameters were determined pre- and post-training.

Results: Pre-training, vastus lateralis contained 65% more type 1 and 55% less type 2d fibers than pectoralis descendens muscle (both $P<0.05$). In 5 out of 8 muscle biopsies of pectoralis descendens muscle cardiac α MyHC was detected. Type 1 fiber area was larger in vastus lateralis than in pectoralis descendens muscle. The number of capillaries and Na^+,K^+-ATPase content were 15 and 24% higher, respectively, in pectoralis descendens muscle ($P<0.05$). Neither fiber type nor fiber size changed in either of the muscles in response to training. However, while vastus lateralis showed a 25% increase in Na^+,K^+-ATPase content ($P<0.05$), pectoralis descendens showed a 15% increase in capillary number ($P<0.05$) post-training. The serum levels of the active thyroid hormones exhibited no changes post- *vs* pre-training.

Conclusions: Equine vastus lateralis and pectoralis descendens muscle adapt differently to training, indicating the relevance of including muscles from different body parts in an equine training study. We also suggest to include parameters such as capillarity and Na^+,K^+-ATPase content, because they represent sensitive markers to evaluate muscle adaptations.

Introduction

Skeletal muscle adaptations to different training regimens designed to improve athletic performance have been studied widely in both man [1-5] and horses [6-10]. The type of adaptation depends on the training protocol: generally, resistance to fatigue is increased by endurance training while muscle mass is increased by strength training [4]. These adaptations may involve all aspects of muscle structure and function i.e. the Myosin Heavy Chain (MyHC) expression (contraction speed), fiber size (strength), capillary supply (oxidative capacity) and Na^+,K^+-ATPase content (excitability) [1].

Adult equine skeletal muscle contains four basic muscle MyHC fiber types: type 1 (slow twitch oxidative), type 2a (fast twitch oxidative/glycolytic), type 2d or 2x (fast twitch glycolytic, here referred to as 2d), and type 2ad (an intermediate fiber type containing both type 2a and type 2d) [11-14]. In a study by Peuker *et al* [15] involving adult rabbits, as well as studies in foals [14,16], cardiac-α MyHC (expression) has been found in skeletal muscle as an intermediate between type 1 and type 2a MyHC (expression). Fatigue resistance is highest in MyHC type 1 and decreases in the following order: type 1 > (cardiac-α >) type 2a > type 2ad > type 2d. High intensity training leads to a shift from type 2d MyHC fibers towards type 2a MyHC fibers in human muscle [3,4], whereas in horses no changes were measured in muscle fiber type [7]. Endurance training also causes transitions towards type 1 MyHC fibers [1] in human muscle, while in equine muscle shifts towards slower muscle fiber types (MyHC type 1 and 2a) were seen [7]. Combined interval and endurance training results in an increase in type 2a MyHC fibers and a decrease in type 2d MyHC fibers in equine skeletal muscle after four months of training [8].

An increase in muscle fiber size is characteristic for adaptation to strength. After heavy resistance training, in both man and horses, MyHC type 1 and type 2 fibers show hypertrophy [5,7]. Using a high intensity exercise protocol, training significantly increases the fiber size of gluteus medius muscle in horses [7]. Combined interval and endurance training causes decreases in the size of type 1 and 2d MyHC fibers of equine skeletal muscle [8]. In humans, short periods of combined training have produced hypertrophy in type 2 fiber areas, but longer periods are needed to increase fiber size in type 1 fibers in human skeletal muscle [5]. The reported effects of endurance training on fiber size in human skeletal muscle are equivocal [1,4].

Muscle capillarization represents the potential for exchange of respiratory gases, fuel and metabolites, and is related to endurance performance. Studies with several horse breeds at (young) adult ages [8,17,18] and in humans [19]

show reversible changes due to training and detraining. Capillaries per fiber ratios are higher after resistance as well as endurance training [20].

Apart from MyHC fiber type and capillarization the maintenance of excitability is an essential factor contributing to fatigue resistance. To maintain muscle excitability Na^+,K^+ gradients must be promptly restored during work [21]. Since this is the major function of the Na^+,K^+-ATPase, it is crucial that its activity and capacity are adequate. Several studies have shown that training induces an increase in Na^+,K^+-ATPase content in skeletal muscle in humans , rats and horses of several ages and breeds [6,22-24]. Some studies have also shown that the training-induced increase in Na^+,K^+-ATPase content of skeletal muscle is associated with improved physical performance in man [21].

Most studies on training in humans have been performed on the vastus lateralis muscle [2,4,5,25], while in horses the gluteus medius muscle is the most dominant subject of investigation [8-10,18,26,27]. Therefore, in addition to using gluteus medius muscle in equine training studies it is relevant to study equine vastus lateralis muscle to allow comparison of training effects with those in human subjects. Only a few studies have been performed on equine vastus lateralis muscle [28-30], including one examining the effects of training [31]. In addition, it is not well known whether muscles in different parts of the body, involved in different ways in a training regimen, show adaptations in the same direction and/or of comparable magnitude after training [18,31].

In the present study, we examined the effects of combined high intensity and endurance training in the vastus lateralis muscle (hind limb) and pectoralis descendens muscle (fore limb), in young Standardbred horses. This is a training combination that is often used by human athletes. We measured MyHC fiber type composition, fiber size, capillary per fiber ratio and Na^+,K^+-ATPase content. As both vastus lateralis muscle and pectoralis descendens muscle are involved in equine locomotion [32,33], we hypothesized that both muscles would have comparable adaptations to training. Finally, as thyroid hormone is a major determinant of MyHC profile [34], capillarity [35] and Na^+,K^+-ATPase content [21], we determined thyroid hormone concentrations before and after the training period, to see if systemic changes in hormone levels would occur as a result of the training [36].

Material and methods

Animals

Four Standardbred geldings aged 20 ± 2 months with a mean weight ± SD of 368 ± 28 kg were trained for 18 weeks. At the end of the training period the mean weight ± SD was 387 ± 26 kg. Horses were owned by the Faculty of Veterinary Medicine of Utrecht University, The Netherlands. The horses were individually housed; their diet consisted of grass silage supplemented with concentrate feed and vitamin supplements and with 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 Utrecht University. Furthermore, the procedures and the treatment of the animals conform to the ACSM animal care standards.

Training

All training sessions and exercise tests were performed on a high speed treadmill (Mustang 2000, Kagra A.G., Fahrwangen, Switzerland). The exercise intensity during the training was based on fixed percentages of the peak heart rate, as obtained in a previous study [21,37]. The training intensity was adjusted according to the heart rate (HR) measurements using a Polar heart rate monitor (Polar electro Nederland bv, The Netherlands) during the training sessions on a weekly basis. Each training session was preceded by 30 min warm-up in a hotwalker followed by 8 min warm-up (4 min at 1.6 m/s and 4 min at 3.0-4.0 m/s, no incline) at the treadmill. Each training session ended with a cooling down consisting of a 5 min walk on the treadmill followed by 30 min walk in a hotwalker. On rest days horses walked in a hotwalker for 60 min.

A standardized exercise test was performed on all horses every four weeks and on the final day of each period to monitor performance. The standardized exercise test was performed for other purposes and therefore not described in this context.

Acclimatization period (4 weeks): Prior to the start of the experiment, the horses were accustomed to the high-speed treadmill in the Equine Exercise Laboratory for four weeks. The acclimatization program during these four weeks consisted of the following: week 1, 30%HRmax for 20 min 3/week; week 2, 30% HRmax for 25 min 4/week; week 3, 40%HRmax for 30 min 4/week; and week 4, 50% HRmax for 35 min 4/week.

Training period (18 weeks): The training consisted of two types of exercise, endurance running and interval running, on alternate days. The endurance running included alternating 20-24 min of continuous level running at

60% estimated heart rate maximum (EstHRmax) (Tuesday) or 16-18 min at 75% EstHRmax (Friday). The interval training (Monday and Thursday) included three 3-min bouts at 80-90% EstHRmax (beginning of the training period), or four 2-min bouts at 80-90% EstHRmax (at the end of the training period) interspersed with 3-min (or 2-min) periods at 60% EstHRmax. The horses exercised 4 days/wk throughout the entire training period.

Muscle biopsies

Muscle samples were obtained pre- and post-training, before the standardized exercise test, after application under local anesthesia using a modified Bergström needle (diameter, 7 mm). A 5-cm-deep biopsy of the vastus lateralis muscle 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 muscle. A 4 cm deep biopsy of the pectoralis descendens muscle was taken at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle. Blood was carefully removed. Biopsies were divided in two parts of which one was embedded in embedding medium and frozen in cooled isopentane (-160°C) for immunostaining, while the other part was directly put into liquid nitrogen for biochemical analysis. All biopsies were stored at -80°C until analyzed.

Immunofluorescence and capillary staining

Monoclonal antibodies (Mabs) used were previously shown to cross-react with horse myosins [10]. Mab slow and fast (both Sigma-Aldrich, St. Louis) recognize slow (type 1) and fast (type 2) MyHC isoforms respectively. Mab A4.74 (Alexis, Kordia, Leiden, The Netherlands) recognizes MyHC type 2a[11]. Mab 412 reacts with MyHC type 1 and 2d and Mab 249 reacts with MyHC cardiac-α, both kind gifts from Prof. Moorman, Academic Medical Centre, Amsterdam, NL. Muscle biopsy specimens were cryosectioned (10 μm). The immuno-fluorescence protocol followed was as described previously [16]. Briefly: slides were rinsed in phosphate buffered saline (PBS pH 7.4), 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 and incubation overnight at room temperature with the Mabs at a dilution of 1:25 (412), 1:50 (A4.74, 249), 1:1000 (fast, slow) in PBS. Sections were rinsed in PBS and incubated with secondary antibody goat anti-mouse, highly cross-adsorbed whole antibody conjugate Alexa® Fluor 488 (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 fiber perimeter was performed with a Wheat Glutamin Antibody (dilution 1:500) directly coupled to Alexa® Fluor 594 (WGA594) (Molecular Probes, Invitrogen, Breda, The Netherlands). Incubation was performed together with secondary antibody.
The sections for capillary staining were fixed in ice cold acetone (-20°C) for 10 minutes at room temperature. All of the following steps were performed at room temperature. Sections were air dried for 10 minutes. The sections were incubated for 30 minutes in 0.3% H_2O_2 in methanol followed by hydration. Several washes with PBS were followed by incubation with Teng-T for 15 minutes. Overnight incubation was carried out with biotinylated lectin, 0.005 mg/ml in PBS. The next day sections were rinsed in PBS, followed by incubation with avidin and biotin-horse radish peroxidase (Vector Labs, Burlingame) for 45 minutes. Rinsing in PBS was followed by incubation for with 0.05% 3.3'diaminobenzidine tetrachloride, 0.01% H_2O_2. The reaction was stopped by rinsing in tap water. Finally the sections were dehydrated and mounted with Eukitt.
Analyses were performed with a Leica DMRE microscope and Leica image software (Database IM500 v1.2 release 19, Leica Microsystems AG, Heerbrugg, Switzerland). Fibers CSA and capillaries were measured by Leica software (standard v2.7, Leica microsystems imaging solutions LTD, Cambridge, UK). Fibers were classified into type 1, type 2a, type 2ad and type 2d and cardiac-α MyHC expression. Between 100 and 318 (mean 227) fibers per biopsy were used for fiber typing and calculation of fiber type composition.

Measurement of Na^+,K^+-ATPase in muscle
Na^+,K^+-ATPase content was quantified by measuring [^{3}H]ouabain binding capacity in presence of vanadate (VO_4) as described by Nørgaard *et al* [38]. This method allows the quantification of total content of Na^+,K^+-ATPase in small samples of muscle, corresponding to the total population of functional Na^+,K^+ pumps [21]. The method has previously been validated for the quantification of Na^+,K^+-ATPase content in muscles of young [6] and adult [24] horses. For the present study incubations were performed at 37 °C in buffer containing 0.6 μCi/ml [^{3}H] ouabain (Perkin Elmer, Boston) and unlabeled ouabain added to a final concentration of 10^{-6} M for 120 minutes under continuous gassing with air. Further processing was exactly as described in Suwannachot *et al* [6]. On the basis of the specific activity of [^{3}H]ouabain in the incubation medium, the amount of [^{3}H]ouabain taken up and retained in the muscle samples was calculated and after correction (for unspecific up-

take, isotopic impurity and the minute loss of specifically bound [^{3}H]ouabain during the washout) expressed as pmol/g wet weight (for details, see [6]).

Thyroid hormone determinations
Thyroid hormone levels were determined as described in [39]. In short: Plasma T4, free T4 (fT4) and T3 were measured by chemiluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, UK). Reverse T3 (rT3) was measured by RIA as previously described [40].

Statistics
All data are given as mean values ± SD. Statistical analyses were carried out with SPSS 12.0.1 for Windows using General Linear Model repeated measures with factor time (within) to demonstrate an effect of training. As a second factor muscle (within) was used to analyze differences between the pectoralis descendens and vastus lateralis muscle. For alpha = 0.05, the power of all tests was between 0.64 and 1.0. A P value of <0.05 was considered significant.

Results
MyHC fiber typing
Figure 1 shows the results of immunofluorescent staining of pre-training vastus lateralis muscle (left) and pectoralis descendens muscle (right) of the same horse. The figure shows serial sections stained with different antibodies to distinguish different MyHC fiber types as described. Table 3.1 (upper panel) displays the MyHC fiber type composition at both time points for the two muscles.
Equine vastus lateralis muscle and pectoralis descendens muscle displayed different MyHC fiber type compositions. Whereas vastus lateralis muscle contained 48% type 1 MyHC fibers (mean of all biopsies), pectoralis descendens muscle contained only 29% type 1 MyHC fibers ($P<0.05$). The amount of type 2d MyHC fibers was also different in both muscles (respectively 10% vs. 22% $P<0.05$). The percentages of type 2a (25% and 27% respectively) and 2ad (18% and 22% respectively) MyHC fibers did not differ between the vastus lateralis and pectoralis descendens muscles. There were no differences in MyHC fiber typing between pre- and post-training biopsies.

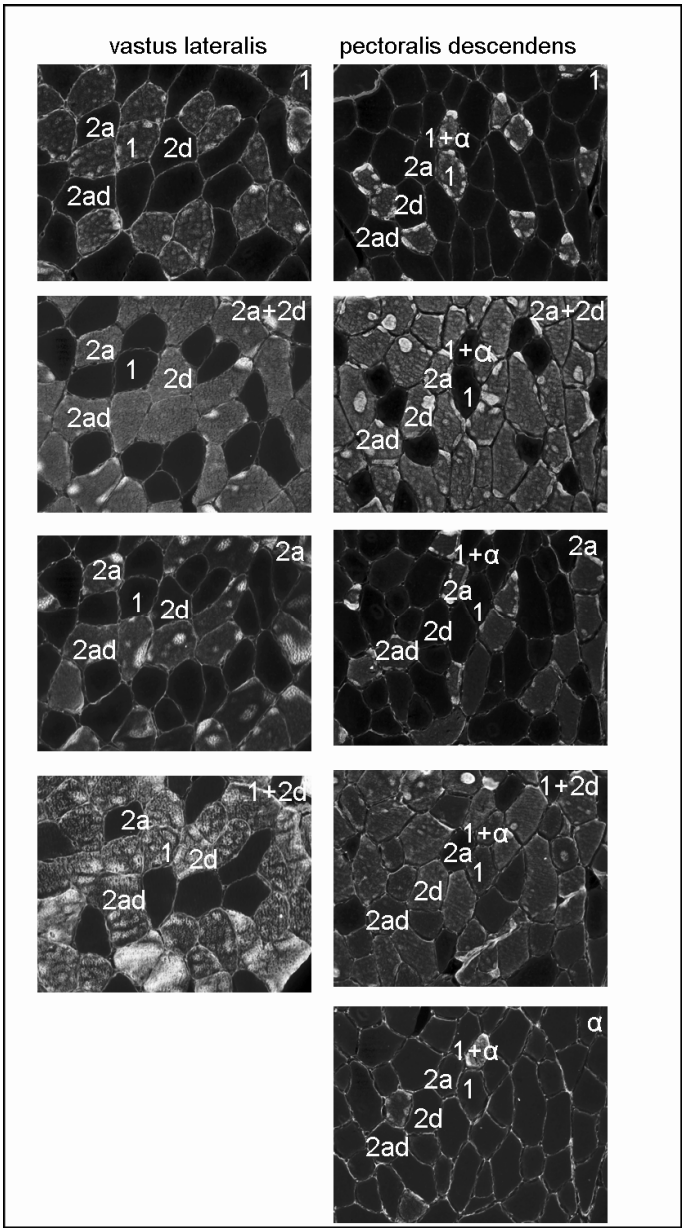

Figure 3.1. Identification of MyHC fiber types by immunofluorescence in serial sections of a pre training biopsy of the vastus lateralis (left) and pectoralis descendens (right) muscle of the same horse. Specificities of the antibodies are indicated. (Magnification 200x).

Table 3.1. MyHC fiber typing (%) and mean fiber size (μm^2) ± SD of vastus lateralis muscle and pectoralis descendens muscle before (pre) and after (post) 18 weeks of training. Fiber typing and fiber areas are given per MyHC fiber type (1, 2a, 2ad and 2d). No significant changes were measured between pre- and post- training biopsies. (a) in the table heading denotes a significant difference between type 1 MyHC and 2d MyHC fiber type and type 1 MyHC fiber area between vastus lateralis muscle and pectoralis descendens muscle.

		Fiber type (%)			
Muscle	**Biopsies**	**1** (a)	**2a**	**2ad**	**2d** (a)
vastus lateralis	**Pre**	49 ± 7	28 ± 8	18 ± 5	5 ± 4
	Post	46 ± 8	22 ± 3	18 ± 7	14 ± 13
pectoralis descendens	**Pre**	25 ± 2	25 ± 8	25 ± 10	25 ± 20
	Post	33 ± 10	28 ± 10	19 ± 3	19 ± 18
		Fiber area (μm^2)			
Muscle	**Biopsies**	**1** (a)	**2a**	**2ad**	**2d**
vastus lateralis	**Pre**	3533 ± 803	3468 ± 299	3805 ± 752	3349 ± 793
	Post	4746 ± 1953	3557 ± 1025	3635 ± 1152	3317 ± 1336
pectoralis descendens	**Pre**	2126 ± 401	3279 ± 691	3573 ± 843	4657 ± 813
	Post	2917 ± 1720	3932 ± 1391	3993 ± 914	4394 ± 1258

Furthermore, in 5 out of the 8 biopsies of pectoralis descendens muscle cardiac-α MyHC expression was detected (Figure 3.1). MyHC cardiac-α expression was found in 3 of the 4 horses. before the training period, whereas after 18 weeks of training only 2 of the 4 horses exhibited MyHC cardiac-α expression. The number of fibers expressing cardiac-α MyHC, in each of 5 biopsies, was 8±5 out of a mean of 273 fibers per biopsy. Cardiac-α MyHC was always co-expressed with type 1 MyHC. In the vastus lateralis muscle there was no cardiac-α MyHC expression detected.

Fiber size

There were fiber size differences between the muscles of type 1 MyHC fiber types (table 3.1, lower panel). In vastus lateralis muscle type 1 MyHC fibers were larger compared to pectoralis descendens muscle ($P<0.05$). The

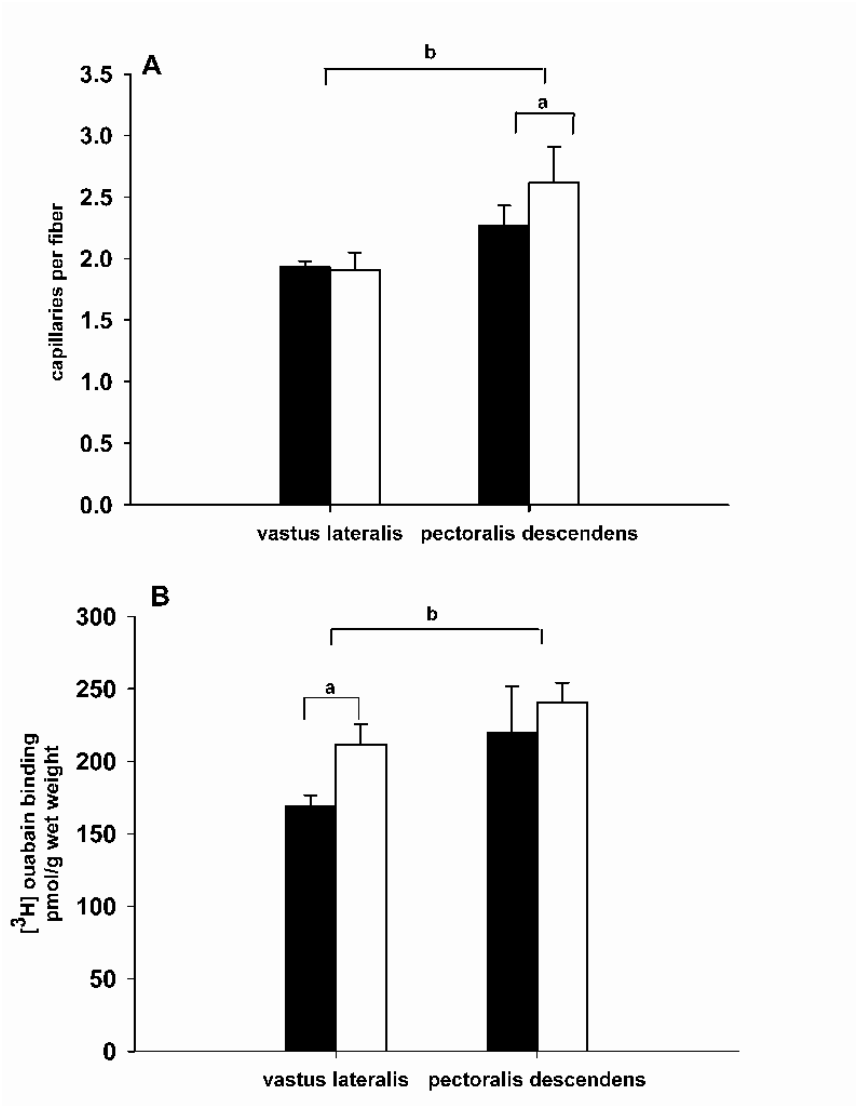

Figure 3.2. (A) Mean capillaries per fiber ratio ± SD before and after an 18 week training period in vastus lateralis muscle and pectoralis descendens muscle. (a) denotes a significant increase in pectoralis descendens muscle between pre- (black bars) and post training (white bars). At all time points pectoralis descendens muscle showed significantly higher amounts of capillaries per fiber (b).
(B) Na^+,K^+-ATPase content ± SD in vastus lateralis and pectoralis descendens muscle before and after training. (a) denotes a significant difference between pre- (black bars) and post training (white bars). At all time points pectoralis descendens muscle showed a significantly higher amount of Na^+,K^+-pumps (b).

other MyHC fiber types did not differ in size between the two muscles. Training did not induce changes in fiber size.

Capillaries per fiber ratio

The vastus lateralis and pectoralis descendens muscles had different capillaries per fiber ratios (figure 3.2a). The number of capillaries per fiber in vastus lateralis muscle was lower than in pectoralis descendens muscle ($p < 0.05$). In pectoralis descendens muscle a significant increase in the number of capillaries per fiber was seen after training (pre-training; 2.27±0.08 vs. post-training; 2.62±0.15 capillaries per fiber, $p < 0.05$).

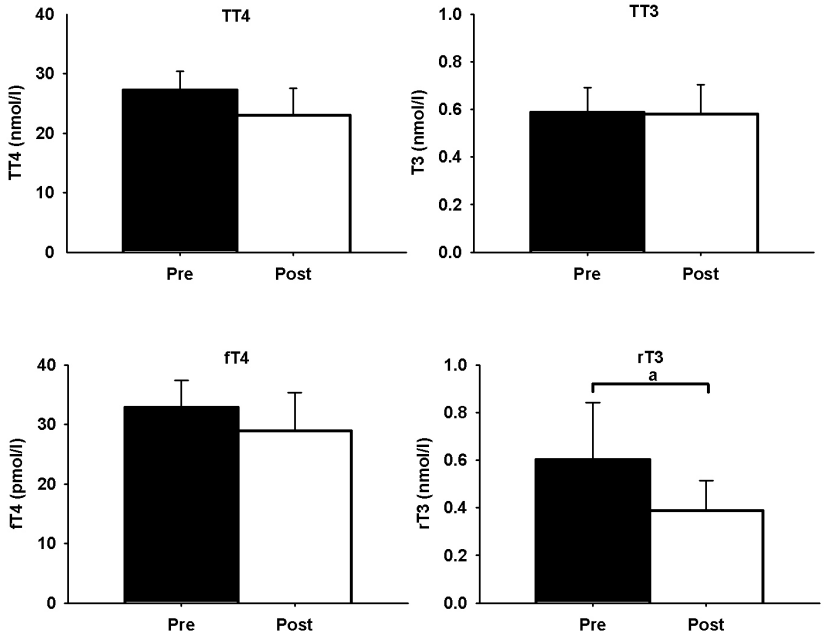

Figure 3.3. Thyroid hormone levels before and after an 18 week training period. Total T4 (TT4), free T4 (fT4), total T3 (TT3) and reverse T3 (rT3) levels are depicted pre (black bars) and post (open bars) training. The asterisk denotes a significant difference.

Na^+,K^+-ATPase

We quantified Na^+,K^+-ATPase content by [^{3}H]ouabain binding and found that vastus lateralis muscle contained fewer Na^+,K^+-ATPase pumps than pectoralis descendens muscle ($p < 0.05$) (Figure 3.2b). After training a significant increase in Na^+,K^+-ATPase content was measured (pre-training; 170 ± 4 pmol/gram wet weight vs. post-training; 212 ± 7 pmol/gram wet weight $p < 0.05$). In the pectoralis descendens muscle no significant changes were detected as a result of training.

Thyroid hormones determination

As can be seen in Figure 3, total T4, free T4 and total T3 concentrations were not changed after training, but the level of rT3 was significantly reduced (around 30%, $P<0.05$)

Discussion

The present study demonstrates that adaptations to training in equine vastus lateralis and pectoralis descendens muscles are different. Vastus lateralis muscle showed primarily increases in the Na^+,K^+-ATPase content, whereas in pectoralis descendens muscle the most striking change induced by training was an increase in capillaries. Both muscles showed no changes in MyHC fiber types and fiber areas upon training. The increase in Na^+,K^+-ATPase content was not due to an increase in thyroid hormone levels.

The study was performed with young adult Standardbred horses (around 2 years) that had not been subjected to any previous training protocol. In a recent study by our laboratory it was demonstrated that neither MyHC expression nor Na^+,K^+-ATPase content showed developmental changes after the age of two years [41]. This indicates that changes observed in the present study must have been adaptations due to training.

MyHC fiber type

In general one would expect that combined interval and endurance training would result in a more oxidative MyHC fiber type composition, e.g. a shift towards type 2a and 1 MyHC as previously found in horses [8,42,43] and humans [2,5]. However, we found no difference in MyHC fiber typing between pre- and post-training muscle samples. Possibly, the training stimuli applied was not sufficient to induce these changes. Due to our classification into four different MyHC fiber types (i.e. MyHC fiber types 1, 2a, 2ad, 2d), in stead of three (i.e. using ATPase staining delineating MyHC fiber types 1, 2a, 2b; usually corresponding to 2d using immunohistochemistry [10]) as in the study of Henckel [8], it is possible that we were not able to detect a

shift in fiber typing. In other equine studies with gluteus medius muscle training adaptations were found on parameters without finding changes in MyHC fiber typing [6,10,14,16].
The percentages of the different MyHC fiber types in vastus lateralis and pectoralis descendens muscle are in accordance with the study by van Dam *et al* [30] using Dutch Warmbloods. The MyHC fiber typing in that study was carried out using two antibodies and was not able to separately detect 2ad MyHC fibers.
This study showed, for the first time, expression of cardiac-α MyHC in young adult horses. In foals [14,16] cardiac-α MyHC expression was found in gluteus medius muscle and semitendinosus muscle, but expression of this MyHC protein disappeared a few months after birth. Expression was always detected in fibers co-expressing type 1 MyHC. Furthermore, there was only cardiac-α MyHC expression in the pectoralis descendens muscle. In the study by Peuker *et al* [15] with adult rabbits, cardiac-α MyHC expression has been found in skeletal muscle as an intermediate between type 1 and type 2a MyHC expression. These authors investigated muscles undergoing fast to slow transition by continuous chronic low frequency stimulation. The expression of MyHC cardiac-α in pectoralis descendens muscle could be an adaptation to training towards slower muscle fiber types (i.e. type 2a MyHC towards type 1 MyHC transition).
The percentage of type 1 MyHC fibers in vastus lateralis (Table 1) is in agreement with that found by Armstrong *et al* [29] in the deep vastus lateralis muscle. On the other hand the percentage of type 2d MyHC fibers we measured was more in agreement with the values found for the medial vastus lateralis muscle in the same study [29]. When comparing the MyHC fiber type composition of equine vastus lateralis muscle with its human equivalent it is comparable[2,44,45].

Fiber size
There were no adaptations in muscle fiber size of both muscles found during 18 weeks of training. The study of Henckel [8] demonstrated a transient decrease in fiber size of all MyHC fiber types in equine gluteus medius muscle after two months (9 weeks) of combined interval and endurance training, that merely disappeared during the following two months (total of 18 weeks, similar to this study) of training.
In pectoralis descendens muscle, the type 1 MyHC fibers are small compared to the type 2 MyHC fibers (Table 1). It was clear that the fiber size of the type 1 MyHC fibers of the vastus lateralis muscle was larger than the type 1 MyHC fibers of the pectoralis descendens muscle. In fact, the type 1

MyHC fibers of the vastus lateralis muscle are larger than most other type 1 fibers of different equine muscles studied [9,10]. An explanation for the different type 1 MyHC fiber size could be found in the potentially different function of both muscles. At present, it is tempting to speculate that vastus lateralis muscle besides having a locomotory role also plays an important role in stabilization thereby needing strength that requires large fibers. Also in human vastus lateralis muscle, the MyHC type 1 fibers have a large fiber area [2,45,46].

Capillaries per fiber ratio

As training generally increases the number of capillaries in trained skeletal muscle of both horses [17,18] and man [19] we expected to find an increase in the capillary to fiber ratio in vastus lateralis and pectoralis descendens muscle. This was indeed found in pectoralis descendens muscle but not in vastus lateralis muscle. An explanation for this difference is not evident, although, other studies reported no changes in the number of capillaries per fiber in muscles with large fibers [19,47].

In pectoralis descendens muscle total capillaries per fiber ratio is higher than in vastus lateralis muscle (Figure 2a). In our view this ratio is the result of a combination of function and demand of the muscle rather than determined by fiber size or fiber type [46]. The amount of capillaries per fiber in equine vastus lateralis muscle is comparable with human vastus lateralis muscle [45,48].

Na^+,K^+-pumps

The vastus lateralis muscle contained fewer Na^+,K^+-pumps per gram muscle than the pectoralis descendens muscle (Figure 2b). This observation fits with its smaller percentage of type 2 MyHC fibers, as it is known that muscles with more type 2 MyHC fibers contain a higher amount of Na^+,K^+-pumps [21]. Na^+,K^+-ATPase content increased during training in the vastus lateralis muscle but not in the pectoralis descendens muscle. The increase in Na^+,K^+-ATPase content corresponds in magnitude with training studies in human vastus lateralis muscle [21] and equine gluteus medius muscle [6,21]. On the other hand, Na^+,K^+-ATPase content of the equine vastus lateralis is not comparable with human Na^+,K^+-ATPase content [21]. It could be that there is a relationship between Na^+,K^+-ATPase content and the bodyweight of the species.

Unexpectedly, Na^+,K^+-ATPase content in pectoralis descendens muscle did not increase during training in this study. It is known that small bouts of exercise should be sufficient for an increase in Na^+,K^+-ATPase content [21].

This could suggest that pectoralis descendens muscle was not involved during training. This is however in contrast with the observed increase in capillaries. The amount of Na^+,K^+-pumps in pectoralis descendens muscle was already at a high level at the start of the training compared to values measured in other equine muscles [6,49].
It would be interesting to investigate whether the activity of the Na^+,K^+-ATPase in both, vastus lateralis muscle and pectoralis descendens muscle, increased during 18 weeks of training.

Thyroid hormone levels
The study by Graves *et al* (2006) [36] reported reduced levels of TT4, TT3 and rT3 after 5 days of a 424 km endurance ride in horses. We found no significant changes in TT4, fT4 and TT3 concentrations after 18 weeks of training and a significantly lower rT3 concentration. This all together suggests that peripheral thyroid hormone metabolism occurs at a lower rate in trained horses as both T3 and rT3 are derived from deodination of T4 [50]. Moreover, our data also indicate that the increase in Na^+,K^+-pump contents in trained vastus lateralis muscle is not due to a systemic rise in thyroid hormone levels, but rather to a local specific effect of the training itself. In general, the hormone levels are normal, indicating no adverse effects of the training [36].

Vastus lateralis muscle
When comparing human and equine vastus lateralis muscle, we can conclude that they are much alike with respect to the MyHC fiber type composition, fiber size and the number of capillaries per fiber. On the other hand, adaptations to training were not the same for all parameters tested. Adaptations were only comparable regarding the increase in Na^+,K^+-pumps. For human research it is interesting to study the effects of training on horses, since longitudinal training adaptations can be studied for outstanding long periods with the ability of repeatedly taking biopsies over time.

Conclusions
Vastus lateralis and pectoralis descendens muscle of two year old Standardbred horses adapt differently to training, indicating the relevance of including muscles from different body parts in an equine training study. We also recommend the inclusion of parameters such as capillarity and Na^+,K^+-ATPase content, because appear to be more sensitive markers to evaluate muscle adaptations than MyHC fiber properties.

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Chapter 4

Acute and long term exercise effects on Na^+,K^+-ATPase isoform mRNA and protein expression in equine muscles

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Abstract

Objective To investigate the effects of acute exercise and training on Na^+,K^+-ATPase specific isoforms on mRNA and protein level in equine muscle.

Animals Six Standardbred geldings aged 20 ± 2 months.

Procedure Horses performed a bout of exercise on a treadmill before and after 18 weeks of combined interval and endurance training. Muscle biopsies were obtained from vastus lateralis (VLM) and pectoralis descendens (PDM) muscles before and directly after exercise tests. Na^+,K^+-ATPase content and expression of specific isoforms on mRNA and protein level were determined using [^{3}H]ouabain binding, RT-PCR and Western blotting respectively.

Results Six Na^+,K^+-ATPase mRNA isoforms were present in equine muscle, but only α2 and β1 isoforms were detectable as proteins. Exercise before training resulted in increases of mRNA isoforms α1, α2, α3, β2 and α1, β3 in respectively VLM and PDM. Training increased mRNA resting levels of isoforms α3 and β1 in VLM and β3 in PDM. Na^+,K^+-ATPase [^{3}H]ouabain binding and proteins of α2 and β1 increased in VLM, whereas in PDM only α2 protein increased due to training. After training acute exercise effects on mRNA expression were no longer detectable.

Conclusions and Clinical relevance Equine muscle contains all Na^+,K^+-ATPase isoforms on mRNA level, but only α2 and β1 proteins could be detected. Expression of these isoforms changes as a result of acute exercise and long-term training, representing an adaptive response. Determination of Na^+,K^+-ATPase gene expression may be relevant for understanding alterations in excitability during neuromuscular diseases.

Abbreviations

VLM	Vastus lateralis muscle
PDM	Pectoralis descendens muscle
SET	Standardized exercise test
TBS-T	Tris-buffered saline Tween 0.05%
HR-max	Heart rate maximal
ECG	Electrocardiogram
BPM	Beats per minute

Introduction

Skeletal muscles have a high capacity to adapt to increases in loading, like during exercise and training. In history many studies have demonstrated up-regulation of structural proteins, capillaries and ion transport mechanisms in muscle after a single bout of exercise or prolonged training [1,2]. The Na^+,K^+-pump is one of the variables that show up-regulation with increased activity [2-4]. The Na^+,K^+-pump is a ubiquitous enzyme located in the plasma membrane of the muscle fiber. After an action potential the pump becomes activated to restore the concentrations of the Na^+ and K^+ ions in order to protect the resting membrane's potential, thereby maintaining excitability and force of the muscle [2,5]. The Na^+,K^+-pump comprises a catalytic α-subunit and a glycosylated β-subunit, that operate as a heterodimer. Four α-isoforms of the Na^+, K^+-ATPase can be distinguished (α1, α2, α3 and α4) and three β-isoforms (β1, β2 and β3). In mammalian skeletal muscle isoforms α1, α2, α3, β1, β2 and β3 genes and proteins are expressed [6,7]. The α4-gene transcript has only been detected at low levels in human muscle so far [8,9]. Numerous combinations with different isoforms can be provided, potentially meeting the specific demands of the working muscle cell [10].

Several studies in human skeletal muscle showed that these gene transcripts are very responsive to exercise. Six minutes of knee extensor exercise elevated the mRNA of α1, α2, α3, β1, β2 and β3 [7]. Another study showed that 15 minutes of intermittent exercise elevated α1, α2, β1 and β3 mRNA levels [9]. In rats, one hour of treadmill running elevated α1 and β2 mRNA levels, while isoforms α3 and β3 were not measured in this study [11]. This is in contrast with the protein expression of Na^+,K^+-ATPase isoforms, which does not respond as fast as Na^+,K^+-ATPase mRNA isoforms to acute exercise. One bout of exercise was not able to induce changes in Na^+,K^+-ATPase protein isoforms in humans [6,7,12]. On the other hand, the study by Green *et al*. (2007) showed increases of α2 protein after two bouts of 6 minutes cycling within a time span of one hour and six minutes [13].

A considerable number of studies have demonstrated up-regulation of the enzyme in skeletal muscle after training in a range of species by using quantification of Na^+,K^+-ATPase content by [^{3}H]ouabain binding, as reviewed by Clausen [2]. However, studies comparing both mRNA and protein isoform expression are scarce. One study reported that endurance trained athletes had lower resting mRNA expression levels for α1, α3, β2 and β3 isoforms than recreationally active persons, while the difference in Na^+,K^+-ATPase content was the other way around [14]. Another study found that 5.5 weeks of high intensity training did not result in any changes in mRNA expression levels [15]. Most training programs were never longer than 8 weeks and changes in pro-

tein expression of different isoforms due to training were often not consistent. Only Na^+,K^+-ATPase α2 isoform always showed an increased protein level due to training [15-19].
The Na^+,K^+-ATPase content in equine locomotory muscle has been found to increase in several training studies [3,4,20-22]. However, these data were thus far not accompanied by analysis of the different isoforms of Na^+,K^+-ATPase on mRNA and protein level. Thus the present study was designed to address the following questions: 1) do two equine muscles differentially involved in an exercise/training program also show a different response in Na^+,K^+-ATPase content; 2) are all Na^+,K^+-ATPase isoforms present in other mammalian species also detectable in equine muscle; 3) is the effect of training on Na^+,K^+-ATPase content associated with a proportional change in mRNA and protein isoform expression; 4) does acute exercise has an effect on mRNA and protein level of the Na^+,K^+-ATPase; and 5) is the effect of acute exercise on Na^+,K^+-ATPase isoforms still present after training?
In the current study 2yr old horses were subjected to an exercise test before and after 18 weeks of combined endurance and interval training. Biopsies were taken from VLM (hind limb) and PDM (fore limb), and analyzed for Na^+,K^+-ATPase content as well as the expression of the three α and the three β isoforms at mRNA and protein level.

Material and methods

Animals

Six Standardbred geldings aged 20 ± 2 months and weighted 374 ± 23 kg (mean ± s.d.) were trained for 18 weeks. At the end of the training period the mean weight ± s.d. was 388 ± 22 kg. Horses were owned by the Faculty of Veterinary Medicine of Utrecht University, The Netherlands. The horses were individually housed; 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 Utrecht University.

Training

All training sessions and exercise tests were performed on a high speed treadmill [a]. The exercise intensity during the training was based on fixed percentages of the peak heart rate, as obtained in a previous study [23]. The training intensity was adjusted according to the heart rate measurements with a

Polar heart rate monitor [b] during the training sessions on a weekly basis. In the current training study subjects acted as their own controls. Each training session was preceded by 30 min warm-up in a hotwalker followed by 8 min warm-up (4 min at 1.6 m/s and 4 min at 3.0-4.0 m/s, no incline) at the treadmill. Each training session ended with a cooling down consisting of a 5 min walk on the treadmill followed by 30 min walk in a hotwalker. On rest days horses walked in a hotwalker for 60 min.

A SET was performed in all horses at the beginning and the end of the training period to monitor performance on a high speed treadmill [a]. 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. Finally horses were allowed to cool down for 5 min at 1.5 m/s. Heart rate was measured using a heart rate meter [b] and continuous ECG monitoring [c]. 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). Horses were only allowed to trot. In the untrained horses this matched with a speed of 6.5-7.0 m/s, whereas after training horses trotted at a speed of 8.0-8.5m/s with a treadmill inclination of 1-2.5%.

The 18 weeks training period consisted of two types of exercise, endurance running and interval running, on alternate days. The endurance running included alternating 20-24 min of continuous running at 60% HRmax (Tuesday) or 16-18 min at 75% HRmax (Friday). The interval training (Monday and Thursday) included three 3-min bouts at 80-90% HRmax (beginning of the training period), or four 2-min bouts at 80-90% HRmax (at the end of the training period) interspersed with 3-min (or 2-min) periods at 60% HRmax. The horses exercised 4 days/wk throughout the entire training period.

Muscle biopsies

Muscle samples were obtained, before and directly after (= within a time frame of 15 minutes) the SET, at the beginning and the end of the training period, under local anesthesia (lidocain hydrochlorine (2%) without adrenaline) using a modified Bergström needle (diameter, 7 mm)[d]. A 5-cm-deep biopsy specimen of the VLM 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 muscle. A 4 cm deep biopsy of the pectoralis descendens muscle (PDM) was taken at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle. Blood was carefully removed. Biopsies were directly

put into liquid nitrogen for analysis. All biopsies were stored at -80°C until analyzed.

mRNA expression

Total RNA was extracted from 20 to 50 mg muscle, pulverized with a mortar and pestle, using the Qiagen RNeasy fibrous tissue kit[e], according to the manufacturer's recommendations. The RNA was eluted with RNase free water and stored at –80°C. Total RNA concentration was determined spectrophotometrically at 260 nm. For each sample, 1 µg of RNA was transcribed into cDNA using the iSCRIPT cDNA synthesis kit[f] in a total volume of 20 µl according to manufacturer's protocol.

Real Time-PCR (MyiQ cycler[f]) was run for 1 cycle (95°C for 3 min) and 40 cycles (95°C for 15 s, 57°C for 60 s). Fluorescence resulted from incorporation of SYBR Green (SYBR Green Supermix Mix[f]) to double stranded DNA and this fluorescence was measured after each repetitive cycle. Duplicate wells were run for each sample. Measurements included a no-template control. Primer sequence design was based on published equine Na^+,K^+-ATPase isoform sequences (table 1). Gene expression was quantified from fluorescence emission using a cycle threshold (C_T) method. The relative expression of the genes was calculated using the expression, $2^{-\Delta\Delta C_T}$, in which the expression of each gene was normalized for input cDNA using the housekeeping gene GAPDH [24].

Quantifying mRNA copynumbers

All Na^+,K^+-ATPase isoform cDNA fragments were generated by RT-PCR. By PCR partial sequences were isolated, cloned into a pGEM-T easy vector[g] and transformed into DH5α competent cells. Cloned partial sequences of the specific isoforms were confirmed by sequencing. Plasmid DNA was obtained by using the Qiagen Miniprep[e], following manufacturer's instructions. To quantify the amount of copynumbers of the isoform, specific plasmids were measured by spectrophotometry. The corresponding copy numbers were calculated by the following equation:

$$\frac{N_A \text{ (copies/mol)} \times \text{concentration (g/ml)}}{\text{(number of base)} \times (340 \text{ DA/base}) \text{ (g/mol)}} = \text{amount (copies/ml)}$$

(Where DA=Daltons and N_A=Avogadro's number). The copy numbers were calculated based on the molecular mass of the plasmid. A dilution series of 1 to 10^8 copies per reaction was made. This was used in realtime PCR where the expression of each isoform was plotted into a dilution curve.

Table 4.1-Na^+,K^+-ATPase gene α1–α3 and β1–β3 and GAPDH equine primer sequences used for mRNA analyses

gene	Genbank Accesion No.	Sense primer (5'-3')	Antisense Primer (5'-3')	bp
α1	EU_423853	TGCCGACAGAATTTGACC	CTTAGCCTTGATGAACTTCAG	149
α2	EU_423854	GGGAAGGAGAGAAGATGC	GATGAGTTATCCACCTTACAG	133
α3	EU_423856	CGAGATTGAGCACTTTATCC	ACAATGATGCCGATGAGG	129
β1	EU_423855	CGGCTACAAAGAGGGCAAACC	ACGGGCTGTACTTCATCACTGG	121
β2	EU_423857	CCAGAGCATGAATGTCACC	TTTGCCGTAGTAGGGGAAG	147
β3	EU_423858	GCACTCTTCTCGTTCACAATG	TGGATCAGACACACGGAATG	101
GAPDH	XR_036506.1	GCTGGTGCTGAATATGTTGTGG	AGCAGAAGGAGCAGAGATGATG	111

Measurement of Na^+,K^+-ATPase in muscle

Na^+,K^+-ATPase content was quantified by measuring [^{3}H]ouabain binding capacity in presence of vanadate (VO_4)[h] as described by Nørgaard *et al* [25,26]. This method allows the quantification of total content of Na^+,K^+-ATPase in small muscle samples, corresponding to the total population of functional Na^+,K^+ pumps [2]. The method has previously been validated for the quantification of Na^+,K^+-ATPase content in muscles of young [22] and adult [4] horses. For the present study incubations were performed at 37 °C in buffer containing 0.6 μCi/ml [^{3}H][i] ouabain and unlabeled ouabain added to a final concentration of 10^{-6} M for 120 minutes under continuous gassing with air. Further processing was exactly as described in Suwannachot *et al* [4,22]. On the basis of the specific activity of [^{3}H]ouabain in the incubation medium, the amount of [^{3}H]ouabain taken up and retained in the muscle samples was calculated and after correction (for unspecific uptake, isotopic impurity and the minute loss of specifically bound [^{3}H]ouabain during the washout) expressed as pmol/g wet weight (for details, see Nørgaard *et al* [26]).

Protein expression

Muscle samples (50 mg) were homogenized by pottering in a 1.5 ml tube in a 1:5 dilution with extraction buffer (25 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate, 5 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol and protease inhibitor). A portion of each sample was analysed for total protein content[j], with bovine serum albumin as the standard. The remaining samples were frozen at –80°C for immunoblotting.

SDS-PAGE (10% separating gel, 5% stacking gel) was performed and gels were loaded with 50 (β1) or 70 (α2) μg protein. Following electrophoresis (20 min, 100 V and 90 min, 150 V), the protein was transferred (45 min, 20

V) with a Trans-Blot Cell[j] to 0.45 µm nitrocellulose (β1) or 0.2 µm PVDF (α2) membrane, and blocked for 2 h with blocking buffer (5% non-fat milk[j] in TBS-T). Membranes were incubated overnight at 4°C in primary antibodies diluted in blocking buffer. Membranes were washed in TBS-T, and incubated for 1 h in horseradish peroxidase conjugated secondary antibodies (goat antimouse immunoglobulins or goat antirabbit immunoglobulins) diluted 1: 10 000 in TBS-T buffer. Following four washes in TBS-T, membranes were dried and treated with chemiluminescent substrate[k]. The signal was captured and imaged. Positive control samples included rat brain and kidney homogenates and these were run on each gel to assess the reactivity and specificity of the antibody (see below). The linearity of the blot signal *versus* protein loaded for our experimental conditions was established for each antibody.

Antibodies
Blots were probed with antibodies specific to each isoform: for α1, α6F[l]; for α2, AB9094[m]; for α3, MA3–915[n]; for β1, MA3–930[n]; for β2 catalog number 610914[o] and for β_3 catalog number 610992[o]. Briefly, this involved the use of additional antibodies (i.e. several other isoform specific antibodies were tested), control samples (i.e. equine brain, kidney and additional muscle samples), and BLAST analysis of the antigen sequence of each antibody (when known) to evaluate cross-reactivity. Blast analysis demonstrated that the antigen sequence of β2 and β3 (published by manufacturer) did not cross react with non Na^+,K^+-ATPase proteins, which corresponds to what was reported by Murphy *et al*. (2004) [7]. Other antigen sequences could not be found. Blast analysis of the equine Na^+,K^+-ATPase protein sequences of α1-α3 and β1-β3 demonstrated a homology with human Na^+,K^+-ATPase proteins of at least 97 %. Therefore, we decided to use the antibodies specific for human Na^+,K^+-ATPase protein isoforms to detect equine Na^+,K^+-ATPase isoforms. The selected antibodies recognized α1- α3 and β1- β2 isoforms in equine brain, and the β3 isoform in equine kidney. Since all antibodies used positively cross- reacted with rat tissues, as described by manufacturer (for α1, α2, α3, β1 and β2) or Murphy *et al* (2004) [7] (for β3), we ran rat brain and rat kidney homogenates in parallel to the equine muscle samples on the same gel to check antibody specificity.

Statistics
All data are given as mean values ± s.d.. Statistical analyses were carried out

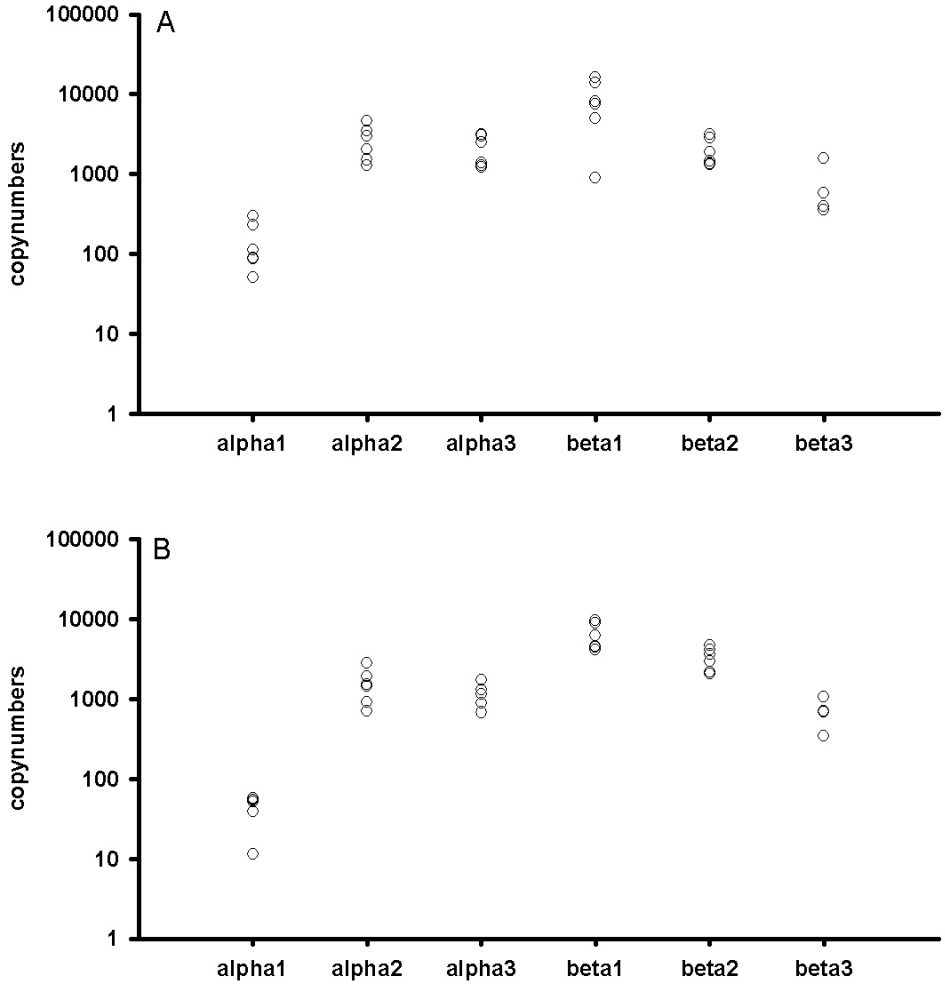

Figure 4.1. Expression of Na^+,K^+-ATPase subunit mRNA in resting untrained equine skeletal muscle. Biopsies were obtained from 6 geldings before SET1. Each biopsy was analyzed in duplicate. For each subject the average of the samples was converted to an absolute amount of molecules by using a standard curve generated by using partial sequences imported in a PGEM-T easy vector-system. Quantification of the mRNA copy-numbers revealed a comparable pattern of distribution of all isoforms in the vastus lateralis (A) and pectoralis descendens (B) muscle. Furthermore, within a muscle different levels of expression were found for three isoforms, namely α1, β1, β3 ($P<0.001$) and roughly equal expression for α2, α3 and β2 isoforms. Of the six isoforms β1 was most abundant, where for α subunits α2 and α3 were most dominantly expressed.

with SPSS 12.0.1 for Windows using a One-Way ANOVA with a Tukey's post hoc test to demonstrate differences in expression between Na^+,K^+-ATPase isoform mRNAs. General Linear Model repeated measures with factor time (within) was used to demonstrate an effect of exercise and/or training. As a second factor muscle (within) was used to analyze differences between the PDM and VLM.

Results

Na^+,K^+-ATPase mRNA levels in resting equine skeletal muscle

In VLM and PDM, three α (1-3) and 3 β (1-3) isoforms of Na^+,K^+-ATPase mRNA were found (figure 4.1). Quantification of the mRNA copynumbers revealed a comparable pattern of distribution of all isoforms in the two muscles. Furthermore, within a muscle different levels of expression were found for three isoforms, namely α1, β1, β3 ($P<0.001$) and roughly equal expression for α2, α3 and β2 isoforms. The most abundant α subunits were α2 and α3. The β1 isoform showed the highest mRNA expression level in both muscles compared to all other isoforms of Na^+,K^+-ATPase mRNAs.

Exercise induced changes in Na^+,K^+-ATPase mRNA levels

Acute exercise induced a significant increase at SET 1 (before training) in Na^+,K^+-ATPase mRNA expression of α1 (15.6 fold), α2 (2.7 fold), α3 (2.1 fold) and β2 (1.5 fold) in VLM (figure 4.2), whereas in PDM an increase in α1 (3.6 fold) and β3 (3.0 fold) mRNA expression was found (figure 3). After 18 weeks of training (SET 2) no significant effects of acute exercise could be detected in either of the two muscles.

Training induced changes in Na^+,K^+-ATPase mRNA levels

Training for 18 weeks significantly increased the basal level of Na^+,K^+-ATPase mRNA expression levels ($P<0.05$) of α3 (1.7 fold) and β1 (1.2 fold) in VLM and β3 (2.1 fold) in PDM biopsies (figure 4.2 and 4.3).

Na^+,K^+-ATPase protein expression

In contrast to the presence of three α and three β isoforms of the Na^+,K^+-ATPase at the mRNA level both in VLM and PDM, only α2 and β1 were detectable as proteins by Western blotting (figure 4). The other isoforms α1, α3, β2 and β3 were not detectable in equine skeletal muscle, although the antibodies against these isoforms were able to recognize the isoforms in horse tissues. These Na^+,K^+-ATPase protein isoforms were detectable in equine brain (α1, α3 and β2), or kidney (β3) (data not shown).

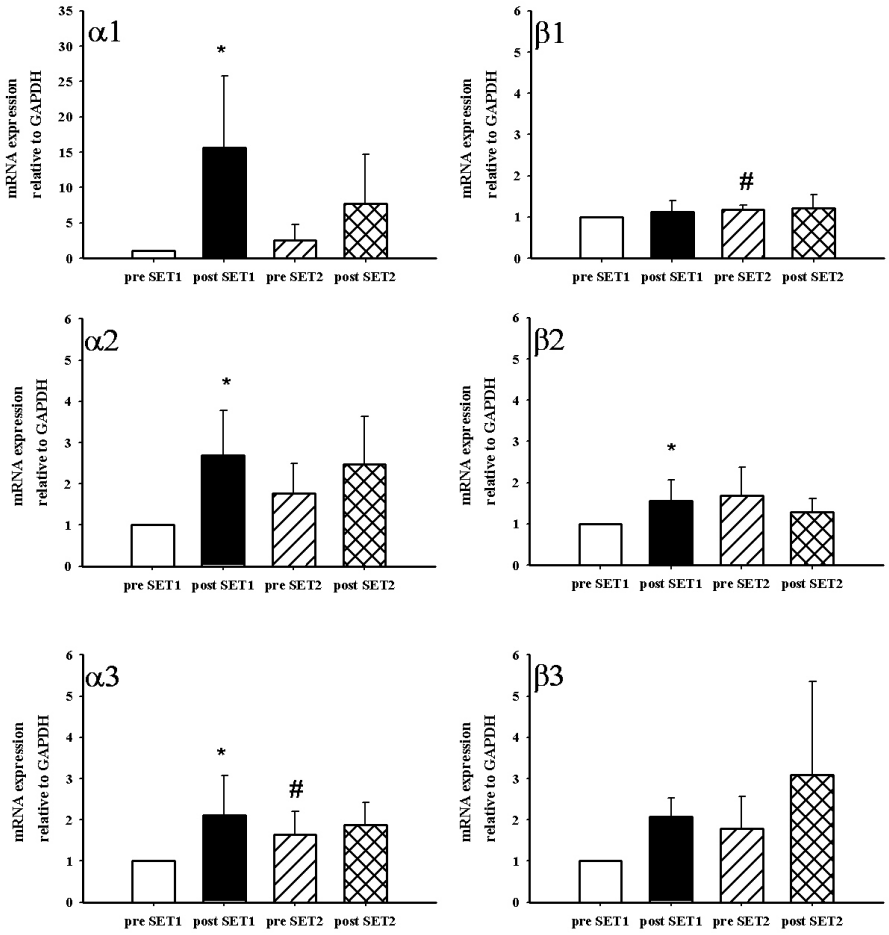

Figure 4.2. Na+,K+-ATPase mRNA isoforms expressed in vastus lateralis muscle at 4 time points (n=6). Graphs are displayed as mean ± s.d.. A (*) defines an exercise effect of SET1 ($P<0.05$) and a (#) defines an 18 week training effect between two pre SET biopsies ($P<0.05$). Each graph displays the measurements of one specific Na+,K+-ATPase isoform.

The basal protein level (pre SET 1) of both α2 and β1 Na^+,K^+-ATPase isoforms were significantly lower in VLM than in PDM ($P<0.05$) (figure 5). cute exercise did not induce changes in the expression level of α2 and β1 Na^+,K^+-ATPase proteins in either of the muscles before or after training (figure 4; compare values post SET with pre SET). On the other hand, significant increases of α2 Na^+,K^+-ATPase protein in both VLM (2.2 fold) and PDM (1.5 fold) were found after 18 weeks of training ($P<0.05$). The protein expression of the β1 was only increased in VLM after 18 weeks of training (1.7 fold, $P<0.05$).

Na^+,K^+-ATPase content

Na^+,K^+-ATPase content quantified by [3H]ouabain binding showed that VLM contained less Na^+,K^+-ATPase pumps than PDM ($P<0.05$) (figure 5). After 18 weeks of training a significant increase in Na^+,K^+-ATPase content was measured in VLM (pre-training; 170 ± 4 pmol/gram wet weight vs. post-training; 212 ± 7 pmol/gram wet weight $p < 0.05$). In the PDM no significant changes were detected as a result of training.

Discussion

This is the first study describing different isoforms of the Na^+,K^+-pump in equine skeletal muscle. The major findings of the present study are as follows: 1) six Na^+,K^+-ATPase mRNA isoforms measured were detectable in equine VLM and PDM, but only the α2 and β1 isoform could be detected as proteins in equine muscle. 2) One single bout of exercise increased mRNA expression of α1, α2, α3 and β2 and α1 and β3 Na^+,K^+-ATPase isoforms in VLM and PDM, respectively, without detectable changes in protein expression. 3) Basal levels of mRNA expression increased for α3 and β1 in VLM and β3 in PDM after 18 weeks of training. 4) A bout of exercise after 18 weeks of training induced no further increases of isoform mRNA expression levels. 5) The responses of the two muscles to acute exercise and long-term training were different.

Abundance of different isoforms in equine muscle

This study is the first to describe Na^+,K^+-ATPase isoform abundance in equine skeletal muscle. At the mRNA level α2, α3 and β1 were most abundant, while β2 was also expressed at a high level. Isoforms α1 and β3 were detected, but on a low level. The current findings of α2 and β1 being most dominantly expressed are in agreement with studies *in vivo* (i.e. human vastus lateralis muscle) [9] and *in vitro* (i.e. murine C2C12 cells) [27]. In contrast,

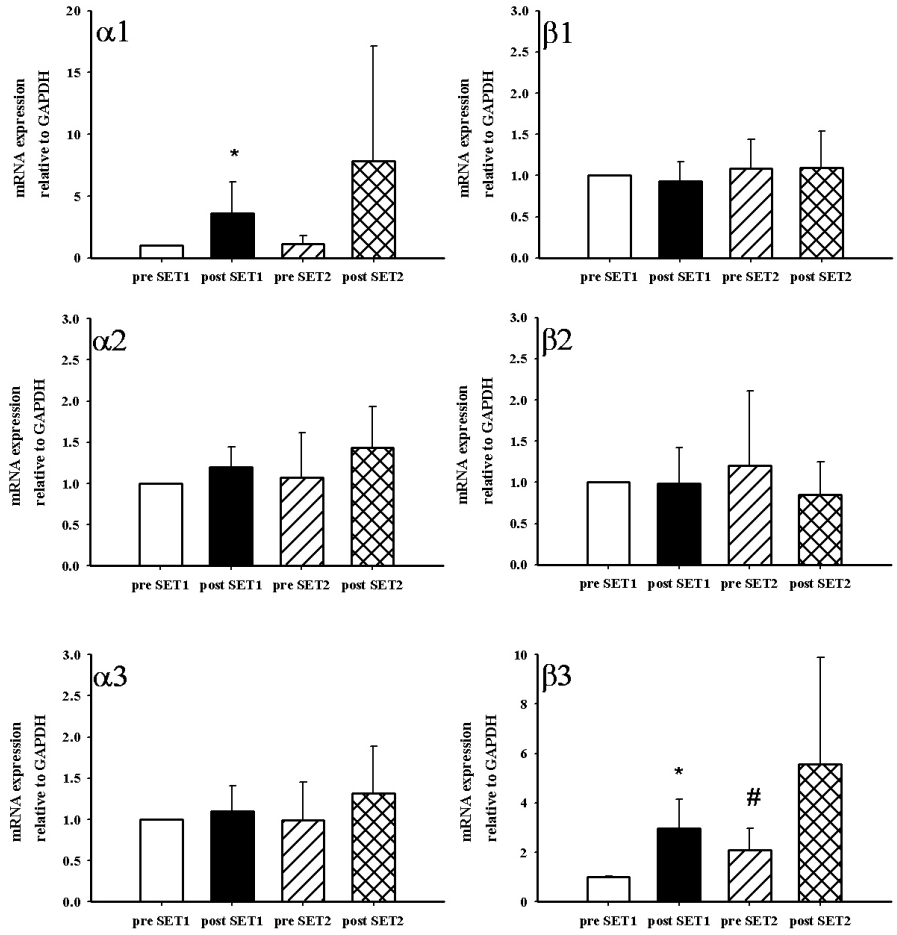

Figure 4.3. Na+,K+-ATPase mRNA isoforms expressed in pectoralis descendens muscle at 4 time points (n=6). Graphs are displayed as mean ± s.d.. A (*) defines an exercise effect between pre SET1 and post SET1 biopsies ($P<0.05$). And a (#) defines an 18 week training effect between the two pre SET biopsies ($P<0.05$). Each graph displays the measurements of one specific Na+,K+-ATPase isoform.

the finding of an equal amount of copynumbers for the α3 and the α2 isoform in equine muscle is different from what was found by Nordsborg *et al* in human muscle [9], who reported a much lower basal level of the α3 isoform mRNA. Unfortunately we were not able to detect α3 protein in equine muscle, and therefore the significance of our finding is difficult to discuss.
Although all six isoforms could be detected as proteins in other equine tissues and as mRNA in equine muscle, only α2 and β1 proteins could be detected by Western blotting in muscle samples. These two isoforms also showed the highest mRNA expression levels in the equine muscles. At this moment, we can only conclude that the abundancy of the α1, α3, β2, and β3 proteins in equine muscle is rather low under basal conditions. In spite of comparable expression of the mRNA Na^+,K^+-ATPase isoforms in VLM and PDM the abundance of the α2 and β1 proteins was higher in PDM. This is in agreement with the higher level of total Na^+,K^+-ATPase content in PDM measured by [^{3}H]ouabain binding as this ligand primarily detects the α isoform [25]. The difference between VLM and PDM can be explained by the observation that PDM contains a higher number of fast-twitch muscle fibers [28], which is associated with a higher number of Na^+,K^+-pumps [2].

Exercise effects on Na^+,K^+-ATPase mRNA and protein expression
As this is the first study to investigate the acute and long term effects of exercise on Na^+,K^+-ATPase mRNA and protein expression in horses, the results of our study can only be compared with those of studies in humans [6,7,9,13-15,29] and rats [11]. The relative increases in expression of the different isoform mRNAs in VLM in SET 1 are in accordance with other studies [7,9], apart from the 15 fold increase in α1 mRNA expression. PDM showed adaptations to acute exercise only for α1 and β3 isoform mRNA, while VLM additionally showed adaptations in mRNA expression of α1, α2, α3 and β2, and not for β3, indicating that the two muscles responded differently to exercise. This may be accounted for by differences in function or involvement in the exercise protocol. In a study with rats a different response to exercise was also found for rat red and white hind limb muscles [11].
The increase of α1 after exercise has been observed in all muscles studied [7,15,30] and its increase can theoretically easily be understood. Because of the low amount of copynumbers of α1 in resting muscle (see figure 1) a large increase in α1 mRNA may relatively easily be achieved as compared to the amount of copynumbers of other Na^+,K^+-ATPase isoform mRNA.
Unfortunately, we were not able to measure increases in β1 isoform mRNA in any of the muscles, although in several human studies, β1 isoform did not increase directly after exercise either [6,7,15]. On the other hand, Nordsborg *et al*

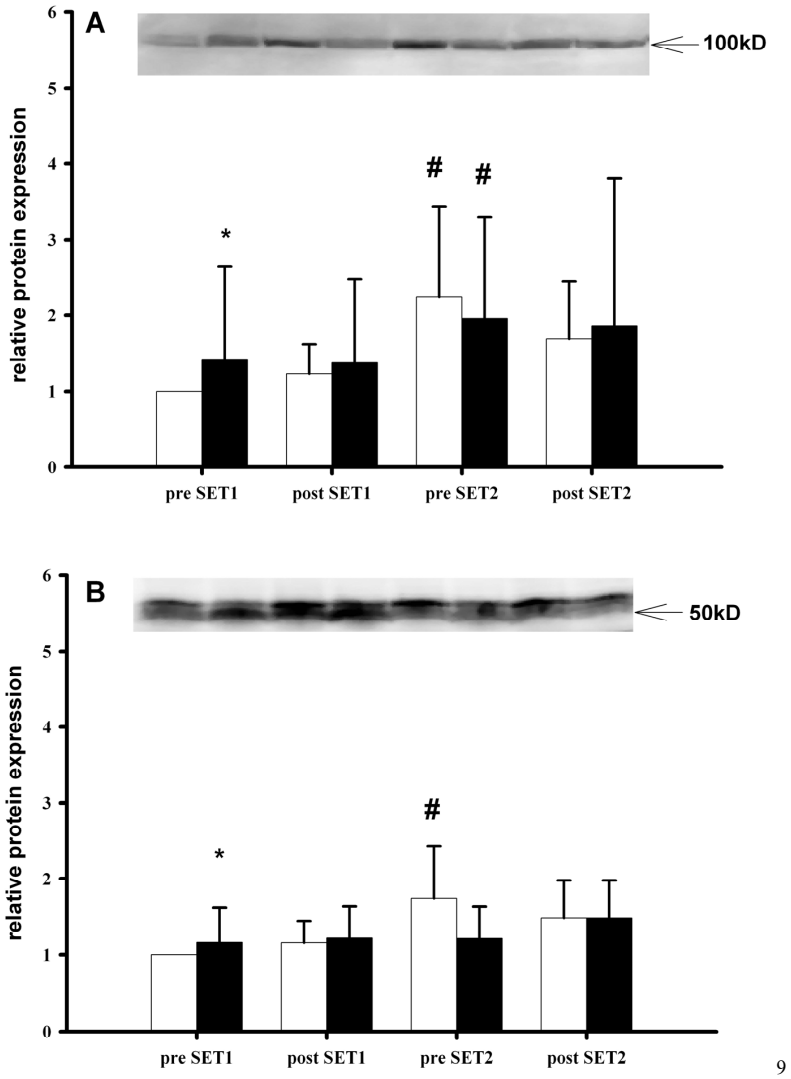

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Figure 4.4. Na+,K+-ATPase protein isoforms α2 (A) and β1 (B) expressed in vastus lateralis (open bars) and pectoralis descendens (closed bars) muscle at 4 time points (n=6). Graphs are displayed as mean ± s.d.. Samples were normalized against resting values of vastus lateralis muscle. (*) defines a difference in concentration of untrained α2 protein concentrations between vastus lateralis and pectoralis descendens muscle. (#) defines an 18 week training effect for VLM and PDM compared to the pre SET values biopsies of the same muscle ($P<0.05$).

described an increase of β1 isoform mRNA in vastus lateralis muscle, and Murphy *et al* [7] found positive effects on β1 when data from three biopsies taken at different time points after exercise were pooled. The increase of β2 isoform mRNA in VLM is in accordance with findings in other studies [6,7]. However, in one study β2 isoform did not increase due to exercise [9]. Presumably, differences in type, intensity and duration of exercise as well as function explain differences in isoform adaptations to exercise. Surprisingly, β3 isoform mRNA was threefold increased in PDM after a single bout of exercise, while VLM, that otherwise showed the largest increases in mRNA isoforms, showed no change. Also results of studies in humans are equivocal with respect to the changes of β3 isoform mRNA after exercise [6,9]. Murphy *et al* found only an increase after collecting results of biopsies taken at different time points after exercise [7].
The protein expression of α2 and β1 isoforms did not change due to a single bout of exercise in the horse. None of the studies in humans observed increases in isoform specific protein expression directly after a single bout of exercise, apart from one study that reported increases in α2 and β1 isoforms taken 24h after cessation of the exercise [6], indicating that translation of the mRNA into protein requires several hours. However, another study measured increases of α2 protein expression after 2 bouts of 6 minutes of cycling within a time span of one hour and six minutes [13]. It could be either the number of bouts or the time lag between cessation of exercise and biopsy sampling that determines whether changes in protein expression can be detected.
Unfortunately, we were not able to take biopsies 24 hours after exercise, because of the limitations within the project.

Training effects on Na^+,K^+-ATPase mRNA and protein expression
Although significant increases in several Na^+,K^+-ATPase isoform mRNA levels were found after exercise in the untrained state, they were apparently transient, as training only resulted in an increased mRNA resting level of α3 and β1 in VLM and β3 in PDM. The effect of training on the expression of the isoforms was in the same order of magnitude as those induced by a single bout of exercise. We did not observe down regulation of Na^+,K^+-ATPase mRNA as shown by Murphy *et al* [14], as this may be the result of several years of training in their study. On the other hand, well trained athletes showed no down regulation, but up-regulation of the mRNAs of some of the Na^+,K^+-ATPase isoforms after an additional 3 week period of training [16]. The results for β1 protein in VLM are in line with the results for β1 isoform mRNA.

Together with the increase of α2 isoform protein, the elevations of [^{3}H]ouabain binding after training corresponds well with results of other studies [13,14]. The rise of α2 protein isoform in PDM is not in line with the data on Na^+,K^+-ATPase content by measurement of [^{3}H]ouabain binding, although a small but non-significant increase of Na^+,K^+-ATPase content can be noticed in PDM (figure 5).

Exercise effects on Na^+,K^+-ATPase mRNA and protein expression after training

No effects of acute exercise were measured after 18 weeks of training on mRNA or protein expression of the Na^+,K^+-ATPase isoforms. The same workload in an exercise test after training for 18 weeks may not be sufficient to up regulate mRNA levels, as compared to the untrained state. Nordsborg *et al* [15] reported similar data, albeit after 5.5 weeks of high intensity training. Apart from the workload, it might be that further up regulation of mRNA expression levels in trained individuals occurs with a longer interval after the exercise test.

Mechanism of up-regulation

The question how the rise in mRNAs of Na^+,K^+-ATPase isoforms is achieved after acute exercise has been discussed in several papers [6,9,13,16,31]. The common observation of these studies was that the reduction in Na^+,K^+-ATPase activity after a short bout of exercise was correlated with increased Na^+,K^+-ATPase gene expression, suggesting a signal transduction role for the depressed enzyme activity occurring at fatigue. However, when Na^+,K^+-ATPase activity is increased after training, enzyme activity is still depressed after a short bout of exercise, albeit at a higher level. This would lead to a less stronger trigger for further up-regulation of mRNAs of Na^+,K^+-ATPase, as observed after training. Our results that the up-regulation of Na^+,K^+-ATPase gene expression was smaller after acute exercise in trained horses would be in agreement with this theory.

In summary, the present study shows that six of the Na^+,K^+-ATPase isoforms measured in human muscles are also detectable in equine skeletal muscle on mRNA level. On protein level only α2 and β1 were detectable with our method of Western blotting. Acute exercise up-regulates mRNA of Na^+,K^+-ATPase isoforms α1, α2, α3 an β2 in VLM and α1 and β3 PDM, implying that transcriptional regulation of these genes is important in relation to adaptation to training. This was also indicated by the adaptations of α2 protein in the two muscles and β1 in VLM after 18 weeks of training. Furthermore, up

regulation of isoform mRNA expression by acute exercise was no longer observed after training.

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Manufacturers' addresses:
[a] Mustang 2000, Kagra A.G., Fahrwangen, Switzerland
[b] Polar electro Nederland bv, The Netherlands
[c] Cardio perfect stress 4.0; Cardio perfect Inc, Atlanta
[d] Maastricht Instruments, Maastricht, The Netherlands
[e] Qiagen GmbH, Hilden, Germany
[f] Biorad, Hercules, CA
[g] Promega, Madison, WI
[h] Sigma-Aldrich, St. Louis
[i] Perkin Elmer, Boston
[j] BCA Assay Kit, Pierce, Rockford, Ill
[k] SuperSignal, Pierce West Pico, Ill
[l] Developmental Studies Hybridoma Bank developed by D. Fambrough under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa
[m] Chemicon International, Bio-connect BV, Huissen, The Netherlands
[n] Affinity Bioreagents, Golden, CO
[o] BD Biosciences Pharmingen, Franklin Lakes, NJ

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Chapter 5

Equine Motor Neuron Disease is associated with increased Na^+,K^+-ATPase mRNA expression in skeletal muscle

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Summary

Objective The study investigated whether changes in morphometry are associated with altered excitability, by measuring Na^+,K^+-ATPase content and Na^+,K^+-ATPase mRNA expression in muscles of horses with lower motor neuron disorder (LMND).
Animals 10 horses from the Dutch Warmblood breed (KWPN) were divided into 3 groups (A: LMND euthanized, B: LMND not euthanized and C: control) based on clinical examination, EMG analysis and/or post mortem neuropathology.
Procedures Myosin heavy chain (MyHC) fiber type composition and cross sectional area (CSA) were measured in biopsies of vastus lateralis muscle (VLM) and the Na^+,K^+-ATPase content and mRNA expression of different subunits were quantified in biopsies of VLM and pectoralis descendens muscle (PDM).
Results Group A showed a significant decrease in the number and size of type I fibers, with a concomitant increase in type IIad as compared to group C, demonstrating slow to fast MyHC transformation. Furthermore, group A showed increased mRNA expression of α1, α2 and α3 Na^+,K^+-ATPase subunits for VLM and α2, α3 and β3 for PDM. This was however not associated with significant changes of Na^+,K^+-ATPase content in VLM or PDM. Group B did not differ in any of the parameters from group C or A.
Conclusions and Clinical Relevance Slow to fast transition of MyHC in locomotory muscles of horses with LMND is associated with increased Na^+,K^+-ATPase mRNA expression. This study gives a first indication that measuring Na^+,K^+-ATPase mRNA expression can be used to support the diagnosis of LMND.

Abbreviatons

LMND	Lower motor neuron disorder
EMND	Equine motor neuron disease
EGS	Equine grass sickness
MyHC	Myosin heavy chain
CSA	Cross sectional area
PDM	Pectoralis descendens muscle
VLM	Vastus lateralis muscle

Introduction

Lower motor neuron disorder has been found in horses suffering from equine motor neuron disease and equine grass sickness. Equine motor neuron disease is an oxidative neurodegenerative disorder that affects the lower motor neurons in the brainstem and in the ventral horns of the spinal cord. The disease is characterized by weight loss despite a good appetite, muscle fasciculations, excessive sweating, abnormal gait and low position of the head [1]. In addition to the symptoms similar as seen in EMND, EGS also results in signs of gastrointestinal problems such as anorexia, dysphagia, intermittent colic or signs of ileus due to dystonomia, caused by mild lower motor neuron degeneration as well as degeneration of autonomic ganglia [2].

In affected muscles, degeneration of lower motor neuron results in the loss of neural input via neuromuscular junction, leading to a gradual denervation atrophy marked by angular atrophied fibers, predominantly MyHC type I fiber atrophy or type I and type II fiber atrophy [3,4]. In general, reduced neuromuscular activity induces a slow-to-fast transition of MyHC fiber types [5]. EMG analysis of horses with LMND reveals pathological spontaneous activity, as a result of membrane instability, and abnormal motor unit action potentials, suggesting that both de- and reinnervation occur in the diseased muscles [6,7]. Both processes would lead to slow to fast transition of MyHC [5].

Na^+,K^+-ATPase, a ubiquitous enzyme in the plasma membrane of the muscle fiber, becomes activated after action potentials, due to an influx of Na^+ through Na^+-channels, followed by an efflux of K^+ leading to an increase in intracellular Na^+ and extracellular K^+ concentration. Na^+,K^+-ATPase restores the concentrations of the Na^+ and K^+ ions to protect the resting membrane's potential, thereby maintaining excitability and force of the muscle [8,9]. The Na^+,K^+-ATPase comprises a catalytic α-subunit and a glycosylated β-subunit, that operate as a heterodimer. Four α-isoforms of the Na^+, K^+-ATPase can be distinguished (α1, α2, α3 and α4) and three β-isoforms (β1, β2 and β3). In mammalian skeletal muscle isoforms α1, α2, α3, β1, β2 and β3 are expressed, while the α4-gene transcript has also been detected at low levels in human muscle in two studies [10,11]. Na^+,K^+-ATPase content is influenced by multiple factors including training status, denervation, fiber type, and muscular and neurodegenerative disorders [9,12]. As described above, LMND in horses is associated with denervation and reinnervation of muscle fibers. It is not known how the combination of these processes influences the Na^+, K^+-ATPase content of affected muscles. Since both de- and reinnervation would lead to relatively more fast muscle fibers [5], and fast fibres usually show a higher Na^+,K^+-ATPase content [9], we hypothesize to find an increase in Na^+,K^+-ATPase content and mRNA in LMND.

The aim of the present study was to investigate (1) whether changes in contractility and morphometry are accompanied by altered Na^+,K^+-ATPase protein and mRNA content in muscle of horses with LMND, (2) whether measurement of Na^+,K^+-ATPase protein and/or mRNA content combined with muscle contractility and morphometry is useful to support the ante mortem diagnosis of LMND and (3) whether the alteration in Na^+,K^+-ATPase protein and mRNA content is general or muscle specific. We have chosen vastus lateralis and pectoralis descendens muscle for our study, because the former is often used for EMG analysis in horses [6], and the latter because it is easy accessible for biopsying. Biopsies were analyzed for MyHC fiber type composition, CSA, Na^+,K^+-ATPase content at the protein level by ^{3}H-ouabain binding capacity as well as on mRNA level by quantifying the different isoforms.

Material and methods

Animals

The total study group consisted of 10 Dutch Warmblood horses. Five horses showed signs of LMND based on clinical examination and EMG analysis. Clinical symptoms were progressive muscle atrophy, weight loss, muscle weakness, fasciculations and abnormal postural appearance for unknown reasons. The symptoms varied from moderate to severe. EMG analysis revealed muscular and neurogenic spontaneous activity along with different stages of denervation and/or reinnervation patterns. The post mortem neuropathology results showed peripheral and diffuse neuronal chromatolysis and eosinophilia of neurons with or without cytoplasmic inclusions or loss of neurons in the brain stem and spinal cord varying from slight to moderate. Three horses (Group A: age 8 ± 6 years, weight 429 ± 36 kg) were euthanized, because of the severe clinical symptoms. For this group the diagnosis was confirmed after additional post mortem neuropathological examination. Two horses (Group B: 15 ± 1 years, weight 519 ± 107 kg) showed moderate clinical symptoms and were therefore not euthanized. Five clinically healthy Dutch Warmblood horses were used as control horses (Group C: 11 ± 1 years, weight 591 ± 19 kg).

All procedures were reviewed and approved by the animal experiments committee (DEC) of Utrecht University, The Netherlands.

Muscle biopsies

All biopsies were taken percutaneously by the same person using a Bergström needle[a] with a diameter of 7.00 mm. Biopsies from the vastus lateralis muscle were taken on an imaginary horizontal line 15 cm ventrally

from the tuber coxae and at 10 cm caudally from the cranial border at 5 cm depth. Biopsies from the pectoralis descendens muscle were taken 20 cm caudal to a line extending through both shoulder joints in the middle of the muscle at 4 cm depth. All biopsies were taken under local anesthesia with lidocaïne (Lidocaïne 2% HCl). If horses were euthanized a surgical biopsy was taken from the same location. The muscle biopsies were frozen in isopentane that was precooled in liquid nitrogen. All samples were stored at –80 ºC until analyzed.

Immunohistochemistry

Immunohistochemistry on biopsies of the vastus lateralis muscle was performed with monoclonal antibodies (Mab) specific to MyHC isoforms in order to identify various MyHC isoforms and especially hybrid muscle fibers. Mab Slow[b] (1:2000, clone NOQ7.5.4D) reacts with type I, Mab Fast[b] (1:2000, clone MY-32) with type IIa and IId and Mab A4.74[c] (1:50) with type IIa. Mab 412-R1D5[d] (1:25) reacts with type I and IId.

Transverse serial sections (5 µm) were made with a cryostat at –20 ºC 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 Mabs, sections were rinsed in PBS and incubated with secondary antibody goat anti mouse, highly cross-adsorbed whole antibody conjugate Alexa® Fluor 568[e], at a dilution of 1:200 for 45 minutes (dark). Finally, sections were rinsed in large volumes of PBS, mounted in Fluorsave™Reagent[f], and left to dry at 37 °C (dark). Double staining of fiber perimeter was performed with a Wheat Glutamin Antibody[e] (dilution 1:500) directly coupled to Alexa® Fluor 350[e] (WGA350). Incubation was performed together with secondary antibody.

Analyses of MyHC fiber type composition and cross sectional area

A region of at least 200 contiguous fibers was taken for fiber typing, calculation of fiber type composition and cross sectional area measurements. The stained sections were digitized using a Nikon microscope and a Leica camera. The images were analyzed using Leica Qwin software[g, h].

The muscle fibers were classified into type I, type IIa, type IIad and type IId according to the reaction with the Mabs and cross sectional areas were measured.

Quantification of Na^+,K^+-ATPase

Na^+,K^+-ATPase content was quantified by measuring the [^{3}H]ouabain-binding capacity of small muscle samples in the presence of vanadate [13] in biopsies of the vastus lateralis and pectoralis descendens muscle. The obtained values correspond to the total population of functional Na^+, K^+ pumps [9,14], and has been validated for equine muscle [15]. Briefly, a ouabain concentration of 10^{-6} M was used, allowing saturation of the major part of the total number of ouabain binding sites [13]. Biopsies were incubated (120 min, 37°C) in buffer containing ^{3}H-ouabain (0.6 µCi/mL) and unlabeled ouabain (final concentration of 10^{-6} M). One set of biopsies was incubated with a ouabain concentration of 10^{-3} M to allow correction for the unspecific uptake of ^{3}H-ouabain. On basis of the specific activity of ^{3}H-ouabain in the incubation medium, the amount of ^{3}H-ouabain taken up and retained in the muscle samples was calculated and after correction (for unspecific uptake and isotopic purity) expressed as pmol/g wet wt.

mRNA expression

Total RNA was extracted from 20 to 50 mg muscle, pulverized with a mortar and pestle, using the Qiagen RNeasy fibrous tissue kit, according to the manufacturer's recommendations. The RNA was eluted with RNase free water and stored at –80°C. Total RNA concentration was determined spectrophotometrically at 260 nm. For each sample, 1 µg of RNA was transcribed into cDNA using the iSCRIPT cDNA synthesis kit[j] in a total volume of 20 µl according to manufacturer's protocol.

Real Time-PCR (MyiQ cycler[j]) was run for 1 cycle (95°C for 3 min) and 40 cycles (95°C for 15 s, 57°C for 60 s). Fluorescence resulted from incorporation of SYBR Green (SYBR Green Supermix Mix[j]) to double stranded DNA and this fluorescence was measured after each repetitive cycle. Duplicate wells were run for each sample. Measurements included a no-template control. Primer sequence design was based on published sequences (table 1). Gene expression was quantified from fluorescence emission using a cycle threshold (CT) method. The relative expression of the genes was calculated using the expression, $2^{-\Delta\Delta CT}$, in which the expression of each gene was normalized for input cDNA using the housekeeping gene GAPDH [16].

Statistics

Statistical analyses were carried out with SPSS 12.0.1 for Windows using an one-way ANOVA for differences between the three groups of horses. *Post-hoc* analyses used the Bonferroni test. Pearson's bivariate correlation was performed to test if Na^+,K^+-ATPase mRNA and protein expres-

sion of the vastus lateralis and pectoralis descendens muscle were correlated to each other. Mean values are expressed as mean ± s.d., a significant difference was accepted when $p \leq 0.05$.

Table 5.1- Na^+K^+-ATPase gene α1–α3 and β1–β3 and GAPDH primer sequences used for mRNA analyses.

gene	Genbank Accesion No.	Sense primer (5'-3')	Antisense Primer (5'-3')	bp
α1	EU_423853	TGCCGACAGAATTTGACC	CTTAGCCTTGATGAACTTCAG	149
α2	EU_423854	GGGAAGGAGAGAAGATGC	GATGAGTTATCCACCTTACAG	133
α3	EU_423856	CGAGATTGAGCACTTTATCC	ACAATGATGCCGATGAGG	129
β1	EU_423855	CGGCTACAAAGAGGGCAAACC	ACGGGCTGTACTTCATCACTGG	121
β2	EU_423857	CCAGAGCATGAATGTCACC	TTTGCCGTAGTAGGGGAAG	147
β3	EU_423858	GCACTCTTCTCGTTCACAATG	TGGATCAGACACACGGAATG	101
GAPDH	XR_036506.1	GCTGGTGCTGAATATGTTGTGG	AGCAGAAGGAGCAGAGATGATG	111

Results

The mean frequencies and CSA of the four MyHC fiber types, type I, IIa, IIad and IId of VLM the three groups of horses are shown in table 2 for vastus lateralis muscle.

Significant differences in the proportion of MyHC fiber type I and IIad were found between group A and C. The proportion of type I was lower in group A (11 ± 7 vs. 47 ± 3, $p<0.05$), while the percentages of type IIad was higher (40.7 ± 7 vs. 21.3 ± 6.0, $p<0.05$). The CSA of MyHC fiber type I was significantly smaller in group A compared to group C (1528 ± 202 vs. 4581 ± 555 µm², $p<0.05$). Na^+,K^+-ATPase content (figure 1) of VLM and PDM showed no significant differences between the 3 groups. On the other hand, a significant relationship between the two muscles was found for Na^+,K^+-ATPase content in the individual horses ($r = 0.78$, $p<0.01$).

Na^+,K^+-ATPase mRNA expression was significantly higher in group A compared to group C for α1, α2 and α3 in VLM (804%, 290% and 303% respectively, $p<0.05$). In PDM α2, α3 and β3 were significantly higher in Group A compared to Group C (337%, 363% and 465% respectively, $p<0.05$) (figure 2). Furthermore, significant relationships for α1($r = 0.75$ $p<0.05$) , α2 ($r = 0.82$, $p< 0.01$), α3 ($r = 0.84$, $p<0.01$) and β3 ($r = 0.96$, $p<0.01$) mRNA expression were found between the two muscles.

There were no differences in MyHC fiber type proportions, CSA, Na^+,K^+-ATPase protein content and Na^+,K^+-ATPase mRNA expression between group B and C and between group A and B.

Table 2- Group A: Lower motor neuron disorder (euthanized), Group B: Lower motor neuron disorder (not euthanized), Group C: Control, MyHC: Myosin heavy chain, fiber type classification based on reaction with different myosin antibodies, CSA: mean fiber cross sectional area, x: not present. Values are expressed as mean ± s.d.. In only one horse of group A type I/IIa fibers were detected. ND: not detectable. A (*) declines significant differences between group A and C of percentages type I and IIad MyHC fibers and CSA of Type I MyHC fibers, $p < 0.05$.

Table 2 Myosin heavy chain composition (MyHC) and cross sectional areas (CSA) in vastus lateralis muscle

		MyHC (%)					CSA (μm^2)				
Group no.		I	I/IIa	IIa	IIad	IId	I	I/IIa	IIa	IIad	IId
A	**mean**	**11***	**19**	**11**	**41***	**31**	**1528***	**3995**	**2863**	**2873**	**3406**
(n=3)	sd	12	-	9	12	10	350	-	1229	1254	1050
B	**mean**	**45**	**N.D.**	**25**	**24**	**6**	**3743**	**N.D.**	**3841**	**3588**	**3851**
(n=2)	sd	2		7	9	1	159		195	238	1592
C	**mean**	**47**	**N.D.**	**18**	**21**	**14**	**4581**	**N.D.**	**4734**	**4124**	**3984**
(n=5)	sd	7		7	6	11	1241		1327	704	978

Discussion

In the present study we investigated whether the changes in muscle histopathology of horses with the neuromuscular disorder LMND are associated with an altered muscular protein content and/or mRNA expression of Na^+,K^+-ATPase. The study included three groups of horses; A: LMND euthanized, B: LMND not euthanized and C: control horses. In agreement with previous studies [3,4], we found a transition in MyHC fiber type composition from slow to fast and atrophy of type I fibers. An increased mRNA expression of Na^+, K^+-ATPase isoforms was found in the vastus lateralis muscle ($\alpha 1$, $\alpha 2$ and $\alpha 3$) and pectoralis descendens muscle ($\alpha 2$ and $\alpha 3$ and $\beta 3$) of the horses of group A compared to group C. The horses of group B did not show any differences in any of the parameters compared to group A and C.

The MyHC composition of the vastus lateralis muscle of Group A demonstrated a transition from slow to fast by a decrease in type I fibers and concomitant increases of type IIad fibers compared to the control horses. In one horse with LMND the hybrid fiber I/IIa was present. Together with these changes in proportions there was an atrophy of type I fibers. Our results are in agreement with previously reported data for the gluteus medius and vastus

lateralis muscle of horses diagnosed with EMND [4,17]. The transitions from slow to fast in myosin isoform expression are induced when neuromuscular activity is reduced, including denervation and when motor units that do not match the original fiber type reinnervate denervated muscle fibers [5,18-20]. Probably, both events have occurred in horses of the present study, because the EMG analyses revealed denervation and reinnervation patterns (Inge Wijnberg, unpublished results).
A study in humans with lower motor neuron disorder demonstrated upregulation of the Na^+,K^+-ATPase[12]. Our data suggest the same, although the increase in Na^+,K^+-ATPase in LMND horses was not significant when meas-

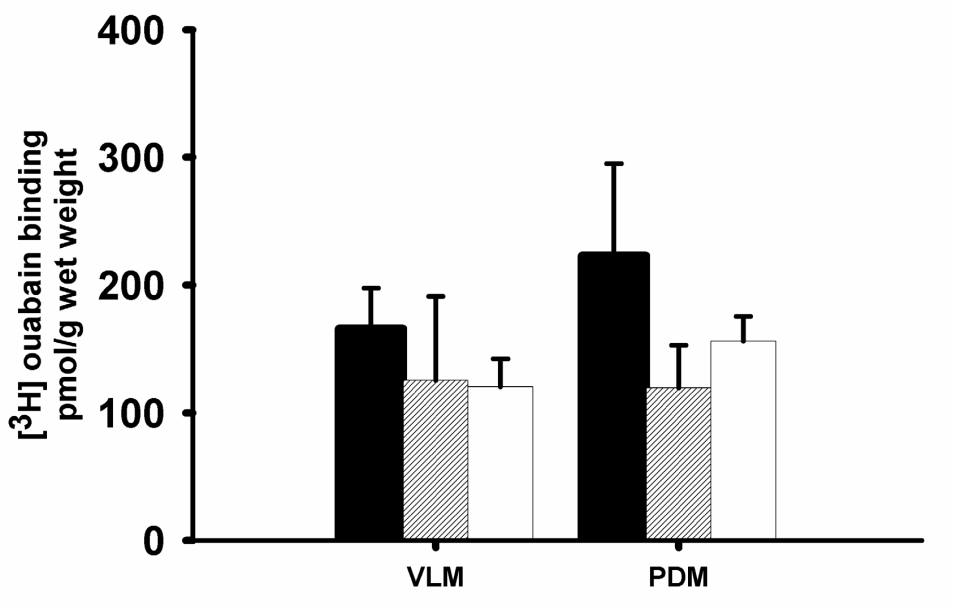

Figure 5.1- Values for Na^+,K^+-ATPase content in biopsies of the vastus lateralis muscle (VLM) and pectoralis descendens muscle (PDM) in horses with confirmed lower motor neuron disorder based on neuropathology examination (group A, black bars), horses suspected of lower motor neuron disorder (group B, striped bars) and control horses (group C, white bars). Values are expressed as mean ± s.d.. No significant differences between the groups were detected.

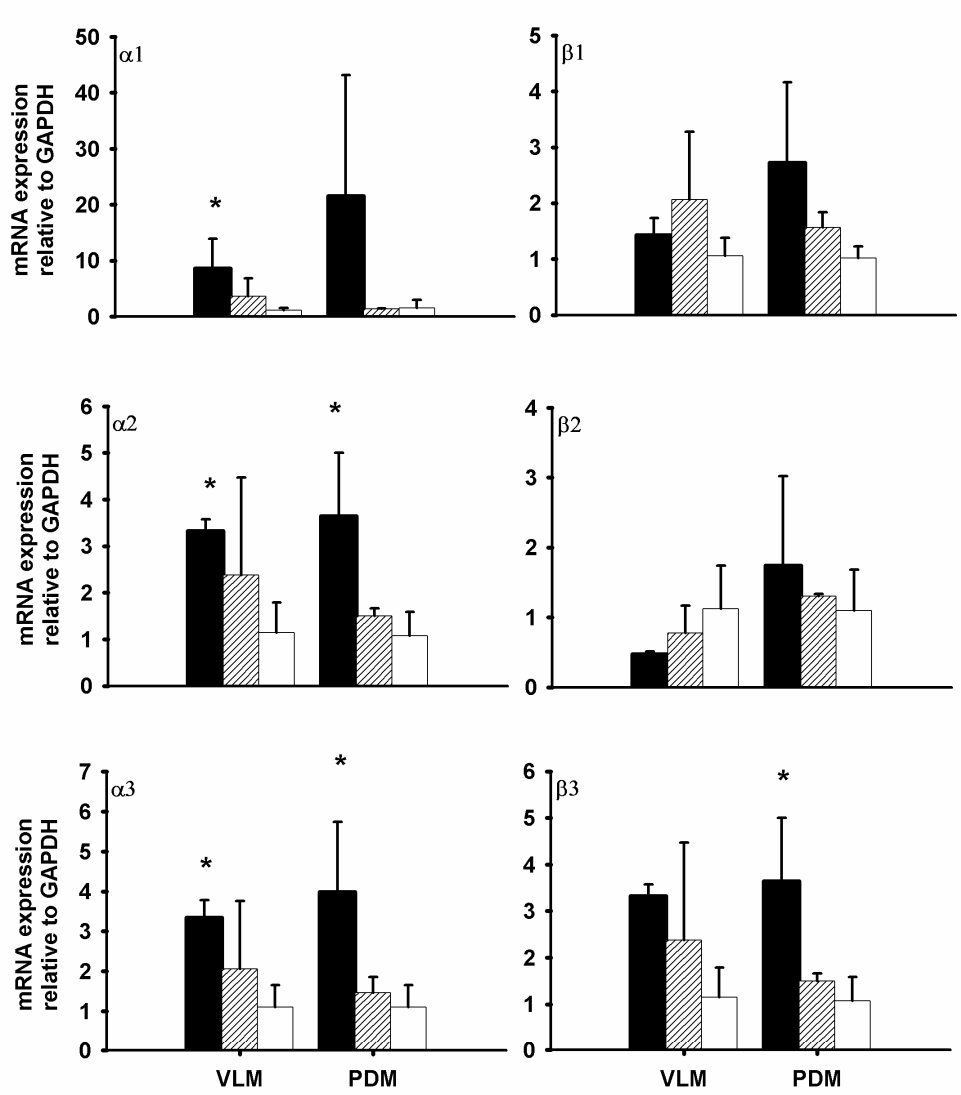

Figure 5.2. Values for Na^+,K^+-ATPase isoform mRNA expression in biopsies of the vastus lateralis muscle (VLM) and pectoralis descendens muscle (PDM) in horses with lower motor neuron disorder euthanized based on neuropathology examination (group A, black bars), horses with lower motor neuron disorder, not euthanized (group B, striped bars) and control horses (group C, white bars). Values are expressed as mean ± s.d. A (*) declares a significant difference within a muscle between control horses and horses with confirmed motor neuron disorder based on neuropathology examination ($p<0.05$).

ured at the protein level, but highly significant at the mRNA level. Moreover, this was observed in VLM and PDM, indicating a general effect of the disease on the locomotory muscles. An explanation for the increase in Na^+,K^+-ATPase protein and mRNA is probably the transition from slow to fast in MyHC fiber type composition. Most data indicate that type II fibers contain more Na^+,K^+-pumps than type I fibers, probably due to differences in passive Na^+, K^+-fluxes [9]. An additional explanation could be the larger demand that is put on the fast muscle fibers due to disappearance of slow fibers. The reason that we found a much larger increase in mRNA expression of the α subunits as compared to the β subunits of the Na^+,K^+-ATPase, could point to a larger "reservoir" of β subunits. In rat skeletal muscle the ratio α:β at the protein level is 0.23 in plasma membranes and 0.41 in intracellular membranes [21]. Probably, when new subunits are needed there is a larger demand for the production of α subunits.

Remarkably, the changes in MyHC fiber type composition, CSA, and Na^+,K^+-ATPase mRNA expression were not found in the horses of group B, although EMG analyses showed generalized neuropathy. One horse with LMND showed a decreased Na^+,K^+-ATPase content, which was not accompanied by changes in fiber type composition, CSA and Na^+,K^+-ATPase mRNA expression. There was no history of inactivity in this case, which could cause down regulation of Na^+,K^+-pumps [9]. The moderate symptoms also indicate that the horses of group B could suffer from EGS caused by mild lower motor neuron degeneration.

The results of group A and C could not be explained by the age differences between groups. It is known that age influences MyHC fiber type composition and Na^+,K^+-ATPase content [9,22,23]. However, in previous studies on the gluteus medius muscle it has been found that the MyHC fiber type composition and Na^+,K^+-ATPase content are stable from age two years onward[23,24]. To our knowledge, this age-dependency has not been investigated on mRNA expression level.

In summary, we have shown that the slow to fast transition in skeletal muscle of horses with LMND is accompanied by significant increases of among others, Na^+,K^+-ATPase isoforms α2 and α3 mRNA. This study gives the first indication that measuring Na^+,K^+-ATPase mRNA expression, a relatively easy measurement to perform, can be used to support the ante mortem diagnosis of LMND. However, further research with more LMND diagnosed horses is required. Furthermore it is preferable to carry out a longitudinal study with LMND diagnosed horses, in order to allow correlation of the severity of the symptoms with Na^+,K^+-ATPase mRNA expression.

Manufacturers' addresses:

a. Maastricht instruments, Maastricht, The Netherlands.
b. Sigma, Missourri, USA.
c. Alexis Biochemicals, Axxora Deutschland GmbH, Grunberg, Germany.
d. Gift from Prof. Moorman, Academic Medical Centre, Amsterdam, The Netherlands.
e. Molecular Probes, Invitrogen, Breda, The Netherlands.
f. Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany.
g. Database IM500 v1.2 release 19, Leica Microsystems AG, Heerbrugg, Switzerland.
h. Qwin standard v2.7, Leica microsystems imaging solutions LTD, Cambridge, UK.
i. Qiagen GmbH, Hilden, Germany
j. Biorad, Hercules, CA, USA

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Chapter 6

General Discussion

General discussion

This thesis describes the expression profiles of MyHC and Na^+,K^+-ATPase isoforms in equine skeletal muscles at the protein, as well as mRNA level. Besides basal expression levels, adaptations of the different isoforms due to exercise, training or disease were investigated. The data obtained could provide us with important knowledge on the outcome of training and the effects of disease, which in turn could help elucidate the mechanism behind these changes. Furthermore the results may be useful in the diagnostic analysis of a disease, such as LMND.

In this thesis the following questions were addressed:

1. What is the basal expression profile of MyHC and Na^+,K^+-ATPase in different equine locomotory muscles?
2. How do these proteins and/or their corresponding mRNAs respond to long-term training?
3. What are the effects of a single bout of exercise on Na^+,K^+-ATPase protein and mRNA expression patterns before and after training?
4. Do muscles of horses affected by lower motor neuron disease show changes in expression profile of MyHC and Na^+,K^+-ATPase isoforms?

What is the basal expression profile of MyHC and Na^+,K^+-ATPase in different equine locomotory muscles?

Myosin heavy chains

In this thesis three equine muscles were used to study MyHC expression profiles, namely gluteus medius muscle (Chapter two), vastus lateralis muscle (Chapters three and five) and pectoralis descendens muscle (Chapters three). In Chapter two, MyHC expression was compared at the mRNA and protein level, whereas in the other studies (Chapters three and five) MyHC was analyzed at only the protein level.

The expression profile of proteins in equine muscle finds its origin in the genetic background of the horse. Due to small differences in DNA sequences the MyHC profile varies per animal. In Chapter three we measured the MyHC fiber type composition of two muscles, namely the vastus lateralis muscle and the pectoralis descendens muscle, in four two years old geldings. The major difference between the two muscles was the two fold higher number of type 1 fibers in vastus lateralis muscle (Table 1). Although these Standardbred horses did not have any active history, the individual horses already differed enormously in their MyHC fiber type composition in both muscles, as indicated by the large standard deviation. Different studies show that MyHC fiber type composition depends on the type of muscle [1-3], breed [4], age [1,5,6] and even on sex [6]. Furthermore, as described by Rietbroek *et al* [7], large variation occurs even within one breed. Since our training study was based on a longitudinal design, we assumed that most of these factors would be less important for the outcome. Gluteus medius muscle showed roughly the same MyHC fiber type composition as pectoralis descendens muscle (Table 1). It is however difficult to compare the MyHC fiber type composition of the three muscles directly, because of their different involvement in locomotion. It has been established that muscle specific functioning is of great importance for MyHC fiber type composition [2,8,9]. Furthermore, within a muscle there can be a lot of variation in MyHC fiber typing [4,10], indicating that taking biopsies at the same sampling place is of great importance.

In Chapter five we used the vastus lateralis muscle to compare MyHC fiber type composition in healthy and LMND affected Dutch Warmblood horses. The (healthy) control horses showed a comparable percentage of type 1 MyHC fibers, as in vastus lateralis muscle of Standarbreds (Table 1). The other fiber types (type 2a, 2ad, and 2d) showed variation in the percentage of 2a and 2d MyHC fibers. These differences might be explained by differences in breed, age, sex and activity of the horses. Most

of the control horses used in Chapter five were mares, with a recreationally active history.
As the results in Chapter two showed around 23% of hybrid 2ad MyHC fibers (protein) without the presence of its corresponding mRNA we concluded that MyHC proteins are transcriptionally regulated. The shorter half life of mRNA as compared to protein [8,11] indicates that the mismatches between MyHC mRNA and protein expression are a normal pattern when muscle fibers undergo transition. Furthermore, it may explain why the differences in MyHC fiber type are mostly observed in the more extreme fast MyHC fiber types (i.e. 2a and 2d) in horses.
An interesting finding was the appearance of cardiac-α MyHC in pectoralis descendens muscle, but not in vastus lateralis muscle (Chapter three). Expression of cardiac-α MyHC isoform was also described in gluteus medius muscle of foals at both the mRNA and protein level [1,12]. This gene was thought to be silenced after birth [12]. This was confirmed by a study by Rivero *et al.*1996 [13] who could not detect cardiac-α MyHC in gluteus medius muscle of adult horses. The presence of cardiac-α in pectoralis descendens muscle was therefore an unexpected finding in adult equine muscles. In the study by Peuker *et al* [14], who investigated adult rabbit muscles undergoing fast to slow transition by continuous chronic low frequency stimulation, cardiac-α MyHC expression has been found as an intermediate between type 1 and type 2a MyHC expression. The expression of MyHC cardiac-α in pectoralis descendens muscle could represent the transition towards slower muscle fiber types (i.e. type 2a MyHC towards type 1 MyHC transition). As compared to the muscles of the hind limb the pectoralis descendens muscle is not only used for moving forward, but also for moving sideward. This may require more "fine-tuning" of the pectoralis descendens muscle than for muscles of the hind limb. An intermediate MyHC between type 1 and type 2a MyHC (i.e. cardiac-α MyHC), may be necessary to fulfill the locomotory demands of this muscle.

Na^+,K^+-ATPase
This thesis describes for the first time expression of Na^+,K^+-ATPase isoforms in two equine skeletal muscles: the vastus lateralis and the pectoralis descendens muscle (Chapters four and five). As in human and rat skeletal muscle the three α isoforms and three β isoforms could be detected at the mRNA level [15-18]. However, only α2 and β1 isoforms could be detected as proteins in muscle biopsies. Other Na^+,K^+-ATPase protein isoforms (i.e. α1, α3, β2 and β3) were detectable in the equine brain (α1, α3,

β2) or kidney (β3). The mRNA expression of most isoforms was in agreement with the resting levels in human muscle [18]. Remarkably, we found a high amount of α3 mRNA, but were not able to detect α3 protein by western blotting. At this point, we can only conclude that the abundance of the α1, α3, β2, and β3 proteins in equine muscle is rather low under basal conditions. Further investigations are necessary to confirm this conclusion. Compared to findings in gluteus medius muscle [19,20] or semitendinosus muscle [20], a high total Na^+,K^+-ATPase content in untrained horses was found in vastus lateralis and pectoralis descendens muscle (Chapters three, four and five). An explanation for the high Na^+,K^+-ATPase content in pectoralis descendens could be the higher number of type 2 (fast) MyHC fibers (Table 6.1), which is associated with a higher Na^+,K^+-ATPase content [21]. However, this does not explain the relatively high Na^+,K^+-ATPase content in vastus lateralis muscle, as its percentage of type 2 MyHC (fast) fibers was much lower than in gluteus medius muscle (Table 6.1). Another possible explanation is that Na^+,K^+-ATPase content rather depends on fiber size than on fiber type, since it has been shown in pigs that a smaller fiber size is related to more Na^+,K^+-pumps due to a larger total membrane area [22].

Table 6.1 Summarized results of basal MyHC fiber type composition of all Chapters. n.d. is not detectable.

				MYHC expression (%)					
				Standard MyHC fiber types					extra MyHC
Breed	**age (yrs)**	**Chapter**		**1**	**1/2a**	**2a**	**2ad**	**2d**	**cardiac-α**
Dutch Warmblood	4-15	Two	gluteus medius	24	n.d.	28	24	24	n.d.
	10-12	Five	vastus lateralis	47	n.d.	18	21	14	n.d.
Standardbred	2	Three	vastus lateralis	49	n.d.	28	18	5	n.d.
	2	Three	pectoralis descendens	25	n.d.	25	25	25	< 1%
Dutch Warmblood (EMND affected)	2-13	Five	vastus lateralis	11	19	11	41	31	n.d.

How do these proteins and/or their corresponding mRNAs respond to long-term training?

Myosin heavy chain

The effects of training on MyHC expression in equine skeletal muscle have been studied widely [2,4,23-28]. However, most of the studies were performed on gluteus medius muscle. Only few studies used other equine muscles to investigate the adaptations in MyHC fiber typing and fiber cross sectional areas [2,9]. In Chapter three we described the effects of moderate training in pectoralis descendens and vastus lateralis muscle. Although training did not result in changes in MyHC fiber typing or fiber size (=total MyHC), we saw an increase of total Na^+,K^+-ATPase (in vastus lateralis muscle) and increases of mRNA Na^+,K^+-ATPase isoform resting levels in both muscles (Chapters three and four). Furthermore the capillaries per fiber ratio increased in pectoralis descendens muscle (Chapter three). So it can be concluded that the exercise was intensive enough to initiate reactions in the muscles. The differences in MyHC fiber type composition are subject to quite an intra- and inter-individual variation [2]. It has been analyzed by Weijs *et al* (2002) [29], that a change of 10 % in MyHC fiber typing is necessary to find significant changes in a group of four horses. For future studies we recommend a more intensive training program compared to that used in our study described in Chapter three, or to include more horses to allow identification of adaptations smaller than 10% in MyHC protein expression. Finally, the results of thyroid hormone levels in blood plasma indicated no changes and thus no systemic adaptations. A decrease in thyroid hormone levels would have resulted in slower, but less oxidative, fibers while the reverse would be seen with increased thyroid hormone levels [30].

Na,K-ATPase

As described in Chapters three and four the training-induced adaptations in Na^+,K^+-ATPase included increases in both total Na^+,K^+-ATPase content and specific Na^+,K^+-ATPase isoforms at the protein and mRNA level (Table 2). Effects of training on total Na^+,K^+-ATPase content measured by [^{3}H]ouabain binding have been reported for a long time for a variety of species [21,31], while studies on specific isoform expression in skeletal muscle only began in 1996 [32]. Our study is the first to perform a longitudinal training study with horses over a period of 18 weeks and to analyze Na^+,K^+-ATPase at the isoform level as well as by [^{3}H]ouabain binding. Longitudinal studies with human subjects thus far covered maximum training periods of 3 to 5.5 weeks [15,33]. Only one study examined the ef-

fects of years of training, but this was not a longitudinal one [34]. The horse might be a good model for investigating longitudinal effects of exercise in two locomotory muscles in the body. Human arm and leg muscles make different movements, where horses are familiar with moving forelimb and hind limb simultaneous during trotting.

Training-induced increases in Na^+,K^+-ATPase content in vastus lateralis muscle (25%) are in accordance with values reported for equine gluteus medius muscle (23-32%) [19,35] and human vastus lateralis muscle (14-40%) [21,31]. The increases in α2 and β1 protein isoforms in vastus lateralis muscle after 18 weeks of training are in agreement with the observed increase in total Na^+,K^+-ATPase content measured by [^{3}H]ouabain. Also studies in humans [36,37] and rats [32] described a similar combination of results, indicating that this is a common effect of training.

Due to inconsistent results in other training studies where mRNA isoform resting levels increased [15], decreased [34], or did not change at all [33], we did not know exactly what we could expect. In Chapter four we found that α3 and β1 mRNA isoform resting levels increased after training in vastus lateralis muscle, while a trend was seen for elevated α2 mRNA (P=0.052). Thus, for both for the α2 and the β1, isoform expression of the protein is related to that of the mRNA.

From our data there is no indication for a possible down-regulation of the mRNA expression levels due to long-term training as suggested by Murphy *et al* [34], who reported lower mRNA expression resting levels in endurance trained athletes. However, it should be kept in mind that the latter study was not longitudinal, and the data were compared with those from recreationally trained men. Possibly more training is needed to induce or to detect elevated mRNAs of the isoforms that did not show an increase in mRNA expression level after training in our study.

At both the mRNA (β3) and protein (α2) level we measured increases in Na^+,K^+-ATPase isoforms in pectoralis descendens muscle after training. It was therefore unexpected that training did not have any effect on total Na^+,K^+-ATPase content in this muscle, although Suwannachot *et al.*(2005) described that from two muscles in the horse's hind limb one responds to training with an increase in Na^+,K^+-ATPase content, while the other does not [20]. Also, the amount of capillaries per fiber ratio increased as a result of training (Chapter three), indicating that pectoralis descendens muscle contributed to locomotion during trotting. The increase of α2 protein isoform suggests that formation of new functional Na^+,K^+-pumps was possible, since a "reservoir" of β isoforms is already present in skeletal muscle [38]. Finally, the intra-individual and inter-

individual variation, as mentioned for MyHC expression, could be a reason for the lack of a significant increase in total Na^+,K^+-ATPase content in pectoralis descendens muscle. The increase of β3 mRNA resting level after training is hard to explain. It could be that, due to the low number of copies (Chapter four), small increases can easily be measured. However, α1 mRNA has even less copy numbers in resting muscle and one would have expected to see an increase in that isoform too. Despite the fact that we found interesting results after training, it still remains unclear why some isoforms respond to training and some do not.

What are the effects of a single bout of exercise on Na^+,K^+-ATPase protein and mRNA expression patterns before and after training?
In Chapter four we described the effects of acute exercise on both Na^+,K^+-ATPase mRNA and protein expression. For interpretation of the data we had to make comparisons with human exercise studies [15,33,34,36,37]. An interesting finding was the specific differences between vastus lateralis and pectoralis descendens muscle. As both muscles are located in different parts of the equine body, different adaptations to exercise could be expected, as was also seen for the capillary to fiber ratio (Chapter three). On the other hand, both muscles were involved in locomotion as shown in Chapters three and four, which could be a reason to expect similar adaptations due to exercise. As mentioned above for the effect of long-term training, several other studies in horses described that muscle adaptations in Na^+,K^+-ATPase contents differed in specific muscles [20,35,39].

Table 6.2 Summarized results of total Na^+,K^+-ATPase content and specific Na^+,K^+-ATPase isoforms mRNA and protein expression. n.d. is not determined.

		[3H] ouabain	protein		mRNA					
			α2	β1	α1	α2	α3	β1	β2	β3
acute exercise	vastus lateralis	n.d.	↔	↔	↑ 16x	↑ 3x	↑ 2x	↔	↑ 0.5x	↔
	pectoralis descendens	n.d.	↔	↔	↑ 4x	↔	↔	↔	↔	↑ 3x
training	vastus lateralis	↑ 25%	↑ 2x	↑ 0.7x	↔	↔	↑ 2x	↑ 0.2x	↔	↔
	pectoralis descendens	↔	↑ 0.5x	↔	↔	↔	↔	↔	↔	↑ 2x
disease	vastus lateralis	↔	n.d.	n.d.	↑ 8x	↑ 3x	↑ 3x	↔	↔	
	pectoralis descendens	↔	n.d.	n.d.	↔	↑ 3x	↑ 4x	↑ 3x	↔	↑ 5x

The mRNA isoforms up-regulated due to acute exercise in untrained horses were different for both muscles (Table 2). Pectoralis descendens muscle showed adaptations to acute exercise only for α1 and β3 isoform mRNA, while vastus lateralis muscle additionally showed adaptations in mRNA expression of α1, α2, α3 and β2, indicating that the two muscles also responded differently to exercise. A suitable explanation for this difference may be found in their different involvement in locomotion. The effects of acute exercise seen in the two muscles before training, were no longer detectable after training, which might be explained by the intensity of the exercise [17,33]. During 18 weeks of training muscles adapted to increased movement. Therefore, the same exercise test after the training period may not be sufficient to up-regulate the mRNA expression levels of the isoforms. In a human study an exercise test after 5.5 weeks of training did not alter isoform mRNA expression levels either, while before training several mRNA isoforms were clearly up-regulated [33]. Differences in intensity of training and/or exercise might explain the different patterns of isoform expression found in human muscle [17,18] as compared to the exercise adaptations of mRNA isoforms described in our study (Chapter four).
The isoform protein expression did not change in both muscles due to a single bout of exercise before or after training (Table 2). In human studies a single bout of exercise did not alter isoform protein expression either [17,33]. Because the time-span between taking biopsies before and after the exercise test comprised approximately one hour, an increase in protein expression might have been possible. Green *et al* (2007) found that after two bouts of exercise within one hour and six minutes up-regulation of α2 and α3 protein could be measured in human muscle [37]. We conclude that repeating a short exercise within a time-span of one hour might be more effective to up-regulate protein isoforms than performing a long-lasting exercise for one hour.

Do muscles of horses affected by LMND show changes in expression profile of MyHC and Na^+,K^+-ATPase?
In contrast to Chapters three and four where we described the effects of exercise as well as training in horses, the effects of neuromuscular disorders on both MyHC and Na^+,K^+-ATPase isoform expression have not been studied to a great extent. In agreement with previous studies [40,41] we observed a transition of MyHC expression in vastus lateralis of EMND affected horses (Chapter five), which was evident from the decrease in

the number of type 1 MyHC fibers. In addition, the size of type 1 MyHC fibers decreased. As a consequence, transition from slow towards fast MyHC fibers was the result. However, the most remarkable results of the study described in Chapter five were the effects measured on Na^+,K^+-ATPase mRNA expression level. Just as increases of Na^+,K^+-ATPase isoform mRNA expression were measured after a single bout of exercise, we found exercise-like up-regulations in mRNA expression level in vastus lateralis and pectoralis descendens muscles of diseased horses. In both muscles the α2 and α3 mRNAs were the most abundant α-isoforms. These two isoforms were up-regulated at the mRNA level in both muscles in the diseased horses. Additionally, the α1 mRNA isoform was up-regulated in vastus lateralis muscle and the β1 mRNA isoform in pectoralis descendens. The reason that we found a much larger increase in mRNA expression of the α subunits as compared to the β subunits of the Na^+,K^+-ATPase, could point to a larger "reservoir" of β subunits. In rat skeletal muscle the ratio α:β at the protein level is 0.23 in plasma membranes and 0.41 in intracellular membranes [38]. Probably, when new subunits are needed there is a larger demand for the production of α subunits. Unfortunately, we did not have enough muscle sample to measure the effect on the protein expression at an isoform specific level. We only measured the effects on total Na^+,K^+-ATPase content. Due to a high variation in Na^+,K^+-ATPase content in the different muscle samples and a small number of EMND diseased horses we were not able to find a significant increase by measuring [^{3}H]ouabain binding capacity. But it might be considered that, due to atrophy of type 1 MyHC fibers and a transition towards a faster MyHC fiber type composition in both muscles, more Na^+,K^+-pumps are needed to maintain membrane potential and muscle excitability.

Conclusions and suggestions for future studies
The main conclusions drawn from the studies presented in this thesis are the following:

MyHC expression
All three muscles studied expressed four MyHC fiber types in a varying composition, with cardiac-α MyHC additionally present in pectoralis descendens. The expression of myosin heavy chains is transcriptionally controlled, as individual muscle fibers always express one mRNA isoform, but simultaneously can express two different proteins. This is the result of differences in half life of MyHC mRNA and protein. The muscle specific expression of cardiac-α MyHC needs to be further investigated. Sampling biopsies of different muscles and measuring the abundance of cardiac-α MyHC should give an indication for the relevance of cardiac-α MyHC in adult skeletal muscle.
It is complicated to define adaptations to exercise and training using the MyHC expression as a parameter, since MyHC expression in our studies did not change after long-term training. In future studies the intensity of training or the group size of horses must be increased to allow detection of adaptations in MyHC fiber type composition. Furthermore, it is suggested to measure, next to MyHC fiber type composition, other parameters that are more sensitive to training. This thesis showed that parameters such as capillaries per fiber ratio and Na^+,K^+-ATPase expression may be good candidates to test. Finally, we recommend taking biopsies from at least two muscles, because adaptations in muscles are not necessarily the same in different parts of the body. In contrast to training, measuring MyHC fiber type composition and fiber size is very useful to support the diagnosis of a neuromuscular disease like LMND.

Na^+,K^+-ATPase
This thesis showed that all Na^+,K^+-ATPase isoforms present in muscles of other mammalian species are also detected in equine muscle, at least at the mRNA level. Furthermore, the expression of α2 and β1 protein isoforms could also been demonstrated in muscle. Although the other isoforms (i.e. α1, α2, β2 and β3) were present in other tissues of the horse, we were not able to measure these isoforms in equine muscles. The mRNA isoform expression patterns are comparable for vastus lateralis and pectoralis descendens muscle, while protein expression of both α2 and β1 is higher in pectoralis descendens muscle. The Na^+,K^+-ATPase

isoforms at the mRNA level have been shown to be a good parameter to measure effects of acute exercise in both muscles of the horse, while measuring isoform protein expression is a solid parameter to confirm training adaptations. Just like the recommendations for measuring exercise and training induced adaptations in MyHC expression, we suggest to measure Na^+,K^+-ATPase isoforms as well as total Na^+,K^+-ATPase content in at least two muscles.

Finally, measurement of Na^+,K^+-ATPase isoform mRNAs in muscles of diseased horses was found to be a very sensitive method to detect changes in muscles of horses affected by LMND. Despite the fact that the group of diseased horses was small, increases in several Na^+,K^+-ATPase mRNA isoforms were measurable. Further investigation is required to determine whether the measurement Na^+,K^+-ATPase expression at both the mRNA and protein level could be a useful tool for diagnosing LMND in horses.

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Appendix

Nederlandse samenvatting

Nog voordat de mens het paard ging gebruiken om zwaar werk te verrichten, werd het al gebruikt in de sport. Er zijn gegevens bekend, dat dit al ongeveer 4500 jaar voor het begin van onze jaartelling voor het eerst plaatsvond. Het is niet vreemd dat het paard al zo lang voor de sport gebruikt wordt. Van nature heeft het paard een groot atletisch vermogen, geschikt om veel verschillende sporten te beoefenen en de daarbij behorende trainingsprogramma's te doorlopen. De belangrijkste sporten zijn dressuur, springsport, draverij en paardenraces. Naast eigenschappen zoals handig manoeuvreren, gehoorzaamheid en precisie, zijn de meest bepalende eigenschappen voor het paard in de sport vooral snelheid en uithoudingsvermogen.

Er is op dit moment veel kennis op het gebied van het trainen van paarden. De aanpassingen van het paard aan training zijn te meten in het bloed, het skelet, het gedrag en de spieren. Daar de spieren van een paard 42% van het totale gewicht uitmaken, kan men wel bedenken dat een groot deel van de aanpassingen plaatsvindt in dit deel van het paard. Het vermogen van spieren zich aan te passen aan verschillende activiteiten en inspanningen is erg groot. Het is interessant om te onderzoeken of de aanpassingen in enkele belangrijke eiwitten en de daarbij behorende mRNA-expressiepatronen vergelijkbaar zijn tijdens de postnatale ontwikkeling, training en ziekte. Het mRNA is een tussenstap die nodig is om vanaf het DNA eiwit aan te maken.

Daarnaast is het ook interessant te weten of aanpassingen die in een bepaalde spier plaatsvinden, ook plaatsvinden in een andere spier in het lichaam. Daarom hebben we in het paard in verschillende spieren naar veranderingen gekeken: een borstspier (pectoralis descendens), een bilspier (gluteus medius) en een dijbeenspier (vastus lateralis).

Myosine Heavy Chain

De Myosine Heavy Chain (MyHC) is de ruggengraat van het contractiele apparaat en omvat ongeveer 25% van de totale hoeveelheid eiwit in de spier. In alle spiervezels zitten grote hoeveelheden MyHC gerangschikt over de hele

vezel. Van het MyHC bestaan verschillende isovormen, die in verschillende spiervezels voorkomen: langzame spiervezels met het vermogen de spiercontracties lang vol te houden hebben type 1 MyHC; snelle spiervezels, die een krachtige, snelle inspanning kunnen leveren, maar wel snel vermoeid zijn hebben type 2d MyHC. Daarnaast bestaat een isovorm die tussen bovengenoemde MyHC typen in zit (type 2a). De karakteristieke eigenschappen van een spier worden uiteindelijk bepaald door de verhouding waarin de verschillende MyHCs in die spier voorkomen.

Aanpassingen door groei, training en ziekte kunnen de verhouding van de verschillende typen en de hoeveelheid MyHC (de grootte van de spier) veranderen. De aanwezigheid van het bijbehorende mRNA blijkt bepalend voor de mogelijkheid van aanpassingen van MyHC in een spiervezel. Dit is vooral terug te zien in spiervezels die een overgang vertonen tussen twee typen MyHC op eiwitniveau. De spiervezel bestaat dan tijdelijk uit twee typen MyHC (=hybride). In deze overgangsfase is het mRNA van het verdwijnende MyHC eiwit in de spiervezel snel verdwenen. Echter het daarbij behorende MyHC eiwit heeft langere tijd nodig om afgebroken te worden. Hierdoor kunnen we wel veel hybride vezels op eiwitniveau zien, maar zijn er zelden hybride mRNA vezels waar te nemen.

De manier van trainen bepaalt welke veranderingen er plaatsvinden in de verhouding van de MyHC isovormen in de spier. Daarnaast kan ook de totale hoeveelheid MyHC veranderen. Grote veranderingen in hoeveelheid zijn te zien aan een grotere omvang van de spier. Voor elke tak van sport kun je door een aangepast trainingsprogramma de MyHC samenstelling en de grootte van de spier zo ideaal mogelijk maken. Een opvallend MyHC type in één van de in dit proefschrift onderzochte spieren (de borstspier) is cardiac-α MyHC, een type dat gewoonlijk eigenlijk alleen bij de geboorte en vlak daarna aantoonbaar is in een spier. Een verklaring voor de aanwezigheid van dit type MyHC in een spier van het volwassen paard is nog niet gevonden.

Bij ziekten zien we vaak een ander beeld van de aanpassingen in de spieren. In plaats van positieve aanpassingen te creëren, verandert tijdens het ziekte-

verloop de MyHC samenstelling vaak zodanig dat het effect negatief is voor het uithoudingsvermogen en neemt daarnaast de grootte van de spieren dikwijls af. Bij paarden komt een ziekte voor die de zenuwen aantast waardoor een verminderde prikkeling van de langzame spiervezels vanuit het zenuwstelsel plaatsvindt. Bij deze ziekte (LMND= Lower Motor Neuron Disorder) zien we dat de langzame spiervezels afnemen in grootte en in aantal. Omdat de aanwezigheid van langzame spiervezels noodzakelijk is voor het uithoudingsvermogen van het paard, heeft afname ervan drastische gevolgen. Resultaat is dat het paard uiteindelijk niet meer kan staan. Het is gebleken dat deze ziekte niet specifiek op één spier aangrijpt, maar effect heeft op alle spieren van het paard. Zodoende kan bepaling van de MyHC samenstelling van een spier en het meten van de grootte van de spiervezels een belangrijke methode zijn in de diagnostiek voor paarden met LMND.

Na^+,K^+-pomp

Naast het MyHC eiwit is ook een eiwit bestudeerd dat een belangrijke functie heeft bij de handhaving van een lage intracellulaire concentratie Na^+ en een hoge intracellulaire concentratie K^+. Dit is de Na^+,K^+-pomp, ook wel Na^+,K^+-ATPase genoemd.

Wanneer een spier wil samentrekken is daar een prikkeling van een motorneuron vanuit het zenuwstelsel voor nodig. Deze prikkeling zorgt ervoor dat er een grote hoeveelheid Na^+ de spiervezel in loopt. Deze concentratieverhoging van Na^+ in de vezel en de daaropvolgende afgifte van K^+ naar de buitenzijde van de vezel zorgen voor het genereren van een actiepotentiaal, waarmee samentrekking van de spiervezels kan plaatsvinden. Er moet echter voor gezorgd worden dat de spier weer opnieuw kan samentrekken. Met behulp van energie (in de vorm van ATP) wordt de Na^+,K^+-pomp geactiveerd en worden de concentraties Na^+ en K^+ binnen en buiten de spiervezel weer hersteld. Tijdens deze actie worden eerst 3 Na^+ naar buiten en vervolgens 2 K^+ terug in de spiervezel gepompt. Om te zorgen dat deze herstelfase vlot verloopt kan men zich indenken dat een grote hoeveelheid pompen nodig is

in het buitenmembraan van de spiervezel. De Na^+,K^+-pomp bestaat uit vier componenten: twee identieke α-componenten en twee identieke β-componenten. Gebleken is dat in het paard van zowel de α- als de β-componenten 3 verschillende isovormen aanwezig zijn. Dit geldt in ieder geval op mRNA niveau. Op eiwitniveau blijken er in de spier van het paard tot op heden twee verschillende α-isovormen aantoonbaar te zijn en maar één β-isovorm. Wel zijn in andere organen van het paard de in spier niet waarneembare isovormen als eiwit aanwezig.

Over het algemeen heeft een spier van nature veel Na^+,K^+-pompen. Door te trainen maken de spieren echter nog meer pompen. Hierdoor kan de spier weer voldoen aan de eisen die gesteld worden aan de verhoogde inspanning. Deze veranderingen zijn meetbaar op verschillende niveaus. In dit proefschrift is gevonden dat er acute veranderingen optreden op mRNA niveau tijdens een enkele inspanning van een half uur wanneer een paard ongetraind is. Op eiwitniveau zijn op dat moment nog geen veranderingen waar te nemen. Door meerdere malen per week te trainen gedurende een periode van vele weken, blijkt dat de acute veranderingen op mRNA niveau niet meer waarneembaar zijn bij eenzelfde oefening die in het begin ook gedaan werd. Daarentegen is de hoeveelheid pompen op eiwitniveau wel behoorlijk gestegen tijdens de trainingsperiode. Hieruit blijkt dat een stijging van het aantal pompen vooraf wordt gegaan door een stijging van de hoeveelheid mRNA, maar dat mRNA stijging niet acuut wordt gevolgd door een stijging van het bijbehorende eiwit.

Zoals eerder vermeld zien we bij paarden met de ziekte LMND een afname van de langzame spiervezels. Blijkbaar is het verdwijnen van deze langzame spiervezels een signaal voor de spier zich aan te passen aan de vernieuwde omstandigheden. Het effect is dat de mRNA niveaus van verschillende isovormen van de Na^+,K^+-pomp hoger zijn dan bij gezonde paarden. Dit duidt op een soortgelijk effect als waarneembaar bij paarden die inspanningen hebben geleverd. Het is aannemelijk dat ook de hoeveelheid Na^+,K^+-pompen zal stijgen in de LMND-paarden, hoewel dat in deze studie niet significant

was. Het zou een gevoelige diagnostische methode kunnen zijn om paarden waarvan vermoed wordt dat ze LMND hebben, te kunnen testen op een verhoogde expressie van mRNA voor de Na^+,K^+-pomp. Verdere studie is daar echter wel voor vereist.

De MyHC en de Na^+,K^+-pomp zijn in de meeste experimenten van dit proefschrift steeds in twee spieren bekeken en vergeleken. Hoewel de aanpassingen in de twee spieren vaak overeenkomstige resultaten laten zien tijdens training en ziekte, zijn er ook verschillen waar te nemen. Het is daarom dan ook aan te bevelen om in toekomstige studies naar adaptaties in skeletspieren, zo mogelijk meer dan één spier te onderzoeken. Dit kan leiden tot beter inzicht in adaptaties aan veranderde omstandigheden en tot beter inzicht in de specifieke functie van een spier.

Curriculum Vitae

Maarten van den Burg werd geboren op 12 november 1977 te Breda. Hij behaalde in 1997 het VWO diploma aan het Cambreur College te Dongen. Aansluitend heeft hij de Hogere Laboratorium Opleiding, studierichting Biologie en Medisch laboratoriumonderzoek gevolgd. Tijdens deze studie heeft hij een periode van 12 maanden stage doorgebracht bij het ID-Lelystad BV te Lelystad, afdeling aviaire virologie. Na succesvolle afronding van zowel de stage als het afstudeerproject in februari 2001 heeft hij zijn diploma mogen ontvangen. Direct daaropvolgend is Maarten zijn werkloopbaan gestart, waar hij als student gestopt was bij het ID-Lelystad BV. Een half jaar later in augustus 2001 is hij aangenomen op de faculteit Diergeneeskunde te Utrecht, alwaar hij ging werken bij de toenmalige afdeling Anatomie, hoofdafdeling Anatomie en Fysiologie. Het opzetten van de *in situ* hybridisatie ten behoeve van aantonen van Myosine Heavy Chain mRNA expressie in de paardespier was zijn belangrijkste hoofdtaak. Door zijn grote interesse en het grote vertrouwen van zijn dagelijks leidinggevende, Karin Eizema, is in 2004 besloten dat Maarten een promotieonderzoek mocht starten. Mede door Marjanne Everts (hoogleraar Anatomie en Fysiologie) is het definitieve onderzoek opgezet zoals in dit boekwerk beschreven.

List of publications

2007 Differential Expression of Calcineurin and SR Ca2+ Handling Proteins in Equine Muscle Fibers During Early Postnatal Growth.
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J Histochem Cytochem. 55(3):247-54.

2006 Myosin heavy chain fibre type composition in foals: analyses at the mRNA and protein level
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2006 Overtraining induces an increase in Na,K-ATPase content of skeletal muscle, without changes in MyHC fibre type composition and fibre size.
van den Burg M.M.M., Joosten B.J.L.J., van Breda E., Wijnberg I.D., Keizer H.A., van der Kolk J.H., Eizema K., Everts M.E.
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2005 Myosin heavy chain isoforms in equine gluteus medius muscle: comparison of mRNA and protein expression profiles.
Eizema, K., van den Burg, M. M., de Jonge, H. W., Dingboom, E. G., Weijs, W. A. and Everts, M. E.
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Dankwoord

Zo zeg je “ja” tegen een promotieonderzoek en ruim vier jaar later lijkt het dan ook nog te gaan lukken. Ik kan het soms nog niet bevatten dat ik het voor elkaar heb gekregen. Terugkijkend op deze periode van vier, maar eigenlijk zeven jaar, zijn er een groot aantal mensen geweest die veel deuren voor mij hebben geopend en mijn weg naar de graad van doctor geëffend hebben. Een ieder die nog maar het kleinste bultje op mijn weg heeft weggepoetst wil ik dan ook hartelijk danken voor deze moeite. In de onderstaande tekst wil ik mijn bewandelde weg naar de dag van promotie zoveel mogelijk mensen persoonlijk bedanken die mij op één of andere manier geholpen, gestimuleerd of gesteund hebben.

Het moment dat ik besloot ooit te willen promoveren staat nog vers in mijn geheugen. Op het moment dat ik stage liep bij de afdeling virologie van ID-Lelystad, zat ik met Bart Kooi te praten over onderzoek en over wat Miriam Tacken (toen nog promovendus) nou toch precies deed. Ik stelde in mijn onwetendheid op een gegeven moment de volgende vraag: “Kan een analist van de HLO ook promoveren?” Bij het horen van het antwoord is de knop omgezet en is de eerste steen gelegd. Ik moest en zou ooit promoveren. Hoelang ik daarvoor nodig zou hebben, dat maakte me niet uit. Ik wil Bart en Miriam dan ook hartelijk danken voor het openen van de deur tot deze gedachte.

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De plaats van koffie drinken was in het eerste jaar nog het lab. Dat is wel vrij snel veranderd. Maar zelfs de koffie heeft bijgedragen aan het succesvol afronden van mijn promotieonderzoek. Sander is al die jaren wel de meest trouwe koffiedrinker in de ochtend gebleken. In het begin samen met Ellen, Ceriel, Jet en Rik. Later zijn er nog velen bijgekomen en ook weer gegaan. Sander, behalve een trouwe koffiedrinker, ben je ook een fijn kamergenoot en collega geweest en een heel goede gesprekspartner. Ik denk dat we heel veel samen hebben besproken en daarnaast ook veel gelachen hebben.

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Inge, Eric en Hans, hartelijk dank voor deze kans, het vertrouwen en voor de samenwerking van de afgelopen jaren.
Halverwege 2004 kreeg het promotieonderzoek zijn echte vorm. Als hoogleraar van de afdeling Anatomie en Fysiologie besloot Marjanne dat ik 100% promovendus zou kunnen worden. Het besluit mijnerzijds was snel genomen. Ik ben Marjanne erg dankbaar voor deze keuze. Hierdoor werd het traject in eens overzichtelijk en was er gelijk een mooi eindpunt in zicht. Onder de directe begeleiding van Karin en met medewerking van de afdeling Heelkunde paard heb ik een mooi steentje kunnen bijdragen aan een mooi project. Behalve de eerder genoemde namen die betrokken waren bij het MAUT project, wil ik ook Klien, Mireille, Cornelie, Henk, Peter, Andries, Karel, de overige paardentrainers, verzorgers en betrokken studenten bedanken. Persoonlijk ook nog Ellen Roelfsema. De manier waarop jij betrokken bent geweest bij het project heeft me enorm gemotiveerd en onze gesprekken waren zowel leerzaam als gezellig.
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Zoals gelezen doe je een promotieonderzoek zeker niet alleen. Vele stenen moeten gelegd worden. Samenwerken, contacten leggen/vasthouden en een goede communicatie zijn het halve werk. Bedankt allemaal!!