Power and fatigue related characteristics

of

equine locomotory muscle

Development, exercise and pathological conditions

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Kracht en vermoeidheid gerelateerde eigenschappen van de skeletspieren van het paard

Ontwikkeling, beweging en pathologische omstandigheden

(Met een samenvatting in het Nederlands)

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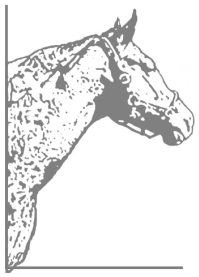

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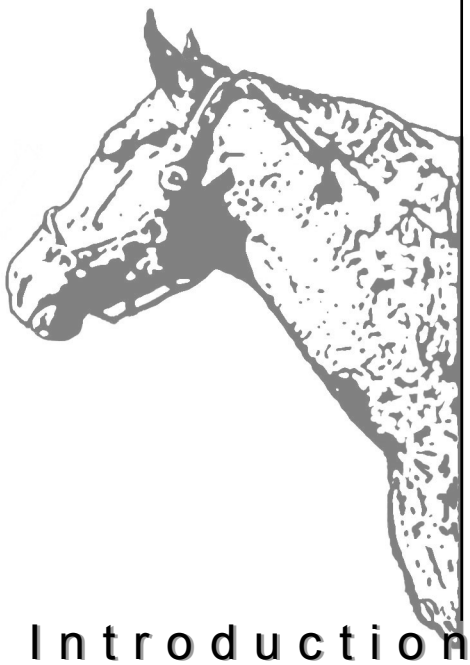
General Introduction



General Introduction

1 Introduction

During sport performances a horse needs an excellent combination of athletic ability, physical conformation and personality to compete at a high level. For efficient raising and training, it would be useful to predict the potential of a horse very early in life based on these traits. Previously, results were reported of studies on equine personality traits [1-4] and athletic ability [5-8], with the latter based on kinematic data. Another important determinant of the athletic ability is the musculoskeletal system [9]. A better understanding of the equine muscular system and its ability to adapt to different (patho)physiological conditions provides valuable knowledge to allow optimal preparation of the immature horse for performance at an adult age. Furthermore, it generates knowledge which will guide future research on prediction of the performance potential at a young age.

1.1 The properties of equine skeletal muscle

Skeletal muscles attach to the skeleton via their tendons. By acting across joints, they are able to move and/or to maintain the posture of an animal. Different muscles vary in their function, depending on the number and distribution of the different types of fibers. Fiber types are the result of coordinate expression of distinct sets of structural proteins and metabolic enzymes [10]. Muscle fibers are capable of responding to altered functional demands, such as changes in neuromuscular activity or mechanical loading, by changing their molecular, functional, and metabolic properties [11,12]. This combination of properties ensures the special characteristics of the muscle and enables to work in an energy efficient manner. The characteristics of the muscle can be described by parameters such as power output and resistance against fatigue.

1.1.1 Power output

Power output is the product of shortening velocity and the force generated by the muscle and is determined by the ratio of fast and slow fibers within a muscle [13,14]. The ability of the skeletal muscle to generate mechanical power is very important for sport performance. It determines the take-off during show jumping or the quality of the gaits during dressage [15,16].

1.1.1.a Contractile properties: Myosin heavy chain isoforms

Muscle fibers consist of thick and thin myofibrillar filaments, the myosins and actins, which are arranged in an orderly pattern. Myosin heavy chains (MyHC) are the major structural proteins of the thick filaments and, as a result of their ATPase activity, are able to convert chemical energy to mechanical energy required for muscle contraction.

Muscular contractions are generated by the cyclic formation and dissociation between myosin and actin. The myosin heads form cross-bridges with actin after the hydrolyzation of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and phosphate (P). The myosin heads undergo conformational changes when P is released, which results in strain on the actin filament: the power stroke of contraction. The dissociation of ADP at the end of the stroke and the subsequent binding of ATP reverses the conformational changes of the myosin head. The following binding of ATP leads to the dissociation of myosin and actin. The free myosin hydrolyzes ATP and the myosin head is rearranged for the next interaction with the thin actin filament.

Myosin heavy chains are expressed at high levels in at least four different isoforms in the mammalian limb muscles. These isoforms determine 80% of the mechanical contractile properties of a muscle fiber due to differences in their ATPase reaction speed [17-19]. The isoforms have been categorized as MyHC-I, MyHC-IIa, MyHC-IId and MyHC-IIb isoforms [10,20]. The co-expression of specific pairs of these major MyHC isoforms results in the formation of hybrid fibers [21]. In horses, only three main fiber types have been identified: type I, type IIa and type IId and a hybrid fiber type IIad [22-24] based on the reaction

with specific monoclonal antibodies (Mabs). In earlier reports the various MyHC isoforms were identified by using classical ATPase staining [23].

In general, the shortening velocity of the different fiber types increases from type I (slow) to type IIa to type IId (fast) [18]. Within the hybrid fibers, the variation in velocity of shortening is related to the proportion of MyHC-IIa and MyHC-IId [20].

1.1.1.b Force: Cross-sectional area

When a muscle shortens at a certain speed, the muscle develops force. The magnitude of the generated force depends on several factors, e.g. the shortening velocity of a fiber, the number of cross-bridges and the cross-sectional area (CSA) [14,25]. Fast muscles are able to maintain a higher speed when load increases, due to the force-velocity relationship [14,26] and muscle fibers with a large CSA produce more tension than small fibers [27], due to the greater number of contractile proteins.

In horses, the mean cross-sectional area of the different fiber types increases in the order I, IIa and IId (including the hybrid fiber IIad) [24]. Therefore, the force that is generated by the different fibertypes increases in the same order.

1.1.2 Resistance against fatigue

Fatigue is one of the limiting factors during prolonged contractile activity. It is often manifested as a reduction in speed of movement and power generation of the muscle [28]. To resist fatigue and to sustain power muscles depend on oxygen delivery by capillaries and on different enzymes for their energy metabolism and the maintenance of excitability [9,29].

1.1.2.a Oxidative capacity

Because only a small amount of ATP is present in the muscle, ADP has to be phosphorylated to ATP to prevent energy depletion during repeated contractions. There are two major pathways to resynthesize and maintain ATP concentrations: aerobic or oxidative metabolism and anaerobic metabolism.

Aerobic metabolism provides energy continuously and efficiently in the presence of oxygen during low intensity exercise throughout oxidation of pyruvate in the citric acid cycle (figure 1.1). After glucose or glycogen is transformed via the glycolytic pathway into pyruvate in the cytosol, pyruvate is converted by oxidative decarboxylation into acetyl CoA inside the mitochondria. Acetyl Co A enters the citric acid cycle and condenses with oxaloacetate to form citrate, catalyzed by citrate synthase. Besides carbohydrates, fatty acids are also an important fuel source in aerobic metabolism. However, acetyl CoA entering the citric acid cycleis now formed by beta-oxidation of fatty acids. Compared to carbohydrates, fatty acids yield more ATP per molecule, but more oxygen is needed and the rate of energy production is much lower. Finally, oxidative phosphorylation is the process that regenerates ATP as a result of the transfer of electrons to O_2 from the nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleootide ($FADH_2$), produced during glycolysis, fatty acid oxidation and in the citric acid cycle.

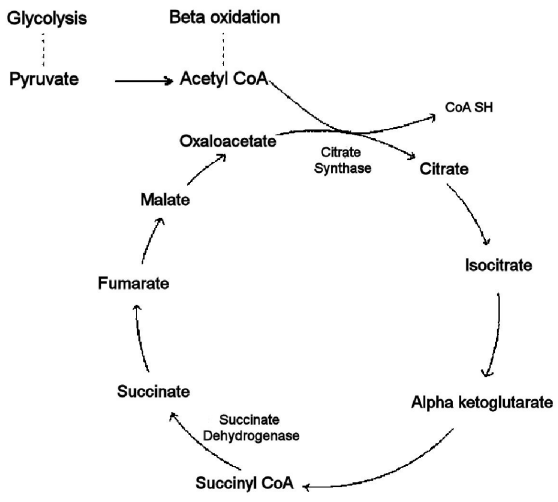


Figure 1.1

The citric acid cycle.

Anaerobic metabolism is mainly used during the early stages of exercise, during fast accelerations and short bouts of exercise. It is rather inefficient, because it yields little ATP per molecule of glucose or glycogen. During anaerobic metabolism three different reactions allow phosphorylation of ATP. The reaction involving phosphocreatine is used during the first few seconds of exercise, only because it is stored in limited amounts in the muscle and it can readily transfer its phosphoryl group to ATP. The myokinase reaction takes place when ATP is broke down very rapidly. In this reaction two ADP molecules are combined and one loses a phosphate group. The third reaction is the anaerobic phosphorylation of ADP from glucose or glycogen into lactic acid. Glucose is phosphorylation of ADP from glucose or glycogen into lactic acid. Glucose is converted to pyruvate via the glycolytic pathway and during intense dynamic exercise this is converted into lactic acid instead of entering the citric acid cycle. The lactic acid which is produced immediately dissociates into lactate and hydrogen ions. The free hydrogen ions induce acidosis and are partly responsible for the impairment of muscle or exercise performance [30].

The oxidative capacity of skeletal muscle can be measured by the activity of citrate synthase and succinate dehydrogenase. Citrate synthase catalyzes the condensation of oxaloacetate and acetyl coenzyme-A to form citryl CoA, which is then hydrolyzed to citrate and CoA. This is an important step in the citric acid cycle which determines the ability to meet the energy requirements. Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate. It is directly linked to the electron transport chain, because the $FADH_2$ produced does not dissociate from the enzyme [31].

The different fiber types based on the myosin isoforms have different metabolic properties. In horses, type I fibers, in general, depend on aerobic metabolism, while type IId fibers generate ATP via anaerobic metabolism. Type IIa fibers are able to provide their energy via both pathways [9]. However, a wide variation in metabolic enzyme activities has been found within the different fiber types [32,33].

1.1.2.b Capillary supply

The oxygen required for aerobic metabolism is delivered to muscle fibers by the capillary bed. The capillary bed is also important for the delivery of substrates, such as glucose and fatty acids, and the removal of waste products. Capillaries are designed to supply O_2 to meet at rates required at demands of maximal rates of oxidation but not to supply the substrates (glucose and fatty acids) at the rates required at high exercise intensities. Their availability is limited by the transport capacities of the sarcolemma [34]. To ensure adequate substrate supply at high work loads substrates are also stored within muscle fibers [35].

The capillary supply varies with MyHC fiber type composition, i.e. the number of capillaries surrounding oxidative fibers is higher than that surrounding glycolytic fibers [24]. The number of capillaries does not take into account the differences in area of the different fiber types and thus the diffusion distance of oxygen. Therefore, a physiologically more relevant description for capillarity should include muscle fiber area [36].

1.1.2.c Maintenance of excitability

Muscular contraction is initiated by the depolarization of the muscle fiber, caused by an action potential from a motor neuron. This elicits a rapid influx of Na^+, followed by an efflux of K^+. The subsequent increase in intracellular Na^+ and extracellular K^+ concentration stimulates Na^+, K^+-ATPase activity. The pump restores the concentrations of the Na^+ and K^+ ions and maintains the resting membrane's potential allowing ongoing contractions [29].

Sodium potassium-pumps are located in the sarcolemma and the T-tubules of the muscle fibers. They are composed of two α- and two β-subunits. The α-subunit contains binding sites for Na^+, K^+, ATP and digitalis glycosides and actually pumps Na^+ and K^+. The β-subunit is necessary for the transfer of the entire enzyme molecule from its site of synthesis in the endoplasmatic reticulum to its site of insertion in the plasma membrane. In each active Na^+, K^+-transport cycle one molecule of ATP is hydrolyzed and three Na^+ ions are extruded in exchange for two K^+ ions [29].

Sodium potassium-ATPase content in intact muscle biopsies can be quantified by the measurement of [^{3}H]-ouabain binding [37]. Ouabain binds to the α_2 - isoform of Na^+, K^+-ATPase [38], which is the most abundant isoform, at least in rat muscle [29]. The density of the Na^+, K^+-pumps determined from ouabain binding indicates that, at least in rat muscles, fast fibers contain about 20% more pumps than slow fibers [39]. This is considered to compensate the excitation-induced influx of Na^+, which is larger in muscles containing predominantly type II fibers than in muscles with predominantly type I fibers [39].

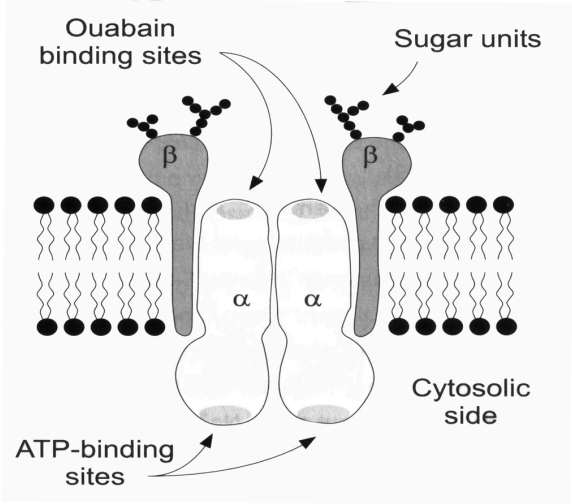


Figure 1.2

Schematic drawing of the Na^+, K^+-ATPase, redrawn from Stryer (1995). The Na^+, K^+-ATPase consists of two á- and two â-subunits, situated in the plasma membrane, with two ATP-binding sites on the cytosolic side and two ouabain binding sites on the extracellulair side.

1.2 Recruitment of muscle fibers

Variations in the range, power output or type of movement of the skeletal muscles are determined by the pattern of recruitment and the frequency of firing of different motor units. A motor unit consists of a motor neuron and the group of muscle fibers it innervates. All muscle fibers innervated by one single motor unit are of the same histochemical type.

However, the muscle fibers of one motor unit are interspersed among the muscle fibers of the other motor units.

The recruitment pattern of the different motor units occurs according to the size principle, i.e. from type I fibers with the slowest conduction velocity, through type IIa to type IId with the fastest conduction velocity [40,41]. However, there are number of observations where the orderly recruitment is not strictly remained to maximize contractile efficiency [42]. Type I fibers are recruited to maintain posture and at low speeds, type IIa fibers for movements requiring higher speed and force and type IId fibers for rapid accelerations and jumping [43,44]. In horses, hybrid fibers are recruited during the same exercise intensities as type IId fibers [45].

1.3 Muscular plasticity

The muscle fibers differentiate during the prenatal period as a result of neural, hormonal and genetic influences. Although equine skeletal muscle is terminally differentiated after birth, it is still able to adapt to different demands, such as altered neuromuscular activity as occurs during development, training and in neurodegenerative disorders [46,47].

1.3.1 Development

After the birth of the foal a period of rapid growth starts, during which many environmental factors, like exercise or management systems, influence its physical abilities. During the postnatal period the skeletal muscle develops relatively quickly, associated with changes in MyHC expression [23,33,48,49], fiber area [50,51] and oxidative capacity [51,52]. Until now, Na^+, K^+-ATPase and capillary supply have not been investigated in the early postnatal period in horses, but both have been shown to decrease in rat muscle [53-55].

1.3.2 Exercise and training

Most equine training programs aim at improving performance, diminishing the risk of injury and delaying the onset of fatigue. The basic principle of training is that a single

exercise session leads to fatigue, which in turn results in temporary adaptive responses. When exercise is performed regularly and training loads are increased gradually with sufficient rest periods it leads to an overall improvement of performance. However, the adaptations that take place in the muscle due to training or exercise depend on different factors including age, the original status of the muscle and of course the type of exercise [44].

In general, training enhances aerobic capacity, power output and excitability of a muscle by increasing the proportion of oxidative fibers (i.e. type I and/or IIa), CSA, capillarity and oxidative enzyme activity [9] and/or upregulation of Na^+, K^+-ATPase [29].

1.3.3 Selection

Many breeding organisations for sport horses want to improve performance in dressage and show jumping competition by genetic selection [56]. The Royal Warmblood studbook of the Netherlands (KWPN) has different breeding directions, such as dressage and show jumping. Selection is based on indices of sport specific performance traits [57].

Breeding selection processes must have an effect on the contractile and metabolic properties of the muscle, because it is known that there is a strong genetic influence on skeletal muscle fibers [58]. In previous studies, it was found that the MyHC fiber type composition is related to the sport the horses were selected for [59-61]. According to an earlier report the muscles of show jumpers and dressage horses are characterized by a balanced proportion of type IIa (36%) and IId (37%) [62]. In show jumpers, a high proportion of fast-twitch, oxidative fibers (IIa) was also found [63].

1.3.4 Pathological conditions

Lower motor neuron disorder (LMND) has been found in horses suffering from equine motor neuron disease (EMND) and equine grass sickness (EGS). 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 [64]. In addition to the symptoms demonstrated in cases of EMND, horses suffering EGS also shows signs of gastrointestinal problems such as anorexia, dysphagia, intermittant colic or signs of ileus [65].

In affected muscles degeneration of lower motor neurons results in the loss of neural input via the neuromuscular junction, leading to a gradual denervation atrophy marked by angular atrophied fibers, predominantly myosin heavy chain (MyHC) type I fiber atrophy or type I and type II fiber atrophy [66,67]. The reduced neuromuscular activity induces a transition of slow-to-fast myosin-based fiber types [21].

1.4 Aim, outline and experimental design

1.4.1 Aim of the thesis

The aim of this thesis was to obtain a better understanding of the mechanical and metabolic muscular properties and the adaptation to different (patho)physiological processes. Therefore, muscle biopsies were taken and investigated. The following questions were addressed:

1. What are the functional adaptations of equine muscle, reflected in power and fatigue resistance, during postnatal development?
2. Is exercise at a young age an important factor for the development of locomotory muscles and does it have any beneficial effects in performance ability?
3. Is the expected breeding value of a horse reflected in the contraction speed of its muscles and does this correspond with the requirements of the sport it is selected for?
4. How is the membrane excitability of a muscle affected by lower motor neuron disorder?

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

1.4.2 Outline of the thesis

In chapter two the effect of early training on the muscular parameters during postnatal growth was investigated. To achieve this MyHC fiber type compostion, fiber area, oxidative capacity and Na^+, K^+-ATPase content were studied in the gluteus medius muscle. The study was performed with Dutch Warmblood horses that were raised conventionally or subjected to jumping training from 6 months of age.

In chapter three the effect of three different exercise regimens (box rest, box rest supplemented with training and pasture exercise) on the oxidative capacity and capillary supply wasdescribed as well as the effects of cessation of the different regimens. Oxidative enzymes, citrate synthase and succinate dehydrogenase, and the capillary supply were studied in the gluteus medius muscle of Dutch Warmblood foals subjected to different exercise regimes at age 0, 22 and 48 weeks.

In chapter four it was determined whether the muscle characteristics were affected by the selection criteria used by the Royal Warmblood studbook and if these characteristics corresponded with the requirements of the sport the horses were selected for. To do this MyHC distribution, as a measure of contraction speed, and oxidative capacity, as a measure for the fatigue resistance, were compared in muscle biopsies from two groups of foals with different genetic background taken after weaning and up to one year of age.

In chapter five it was demonstrated that lower motor neuron disorder affected the Na^+, K^+-ATPase content in muscles of horses with EMND or EGS. Furthermore, it was investigated whether measurement of Na^+, K^+-ATPase content would be useful to support the ante mortem diagnosis of LMND.

Chapter six integrates all findings from the previous chapters and formulates the answers to the four main questions raised in this thesis.

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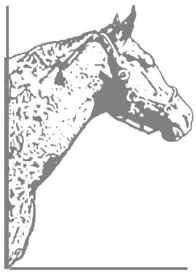
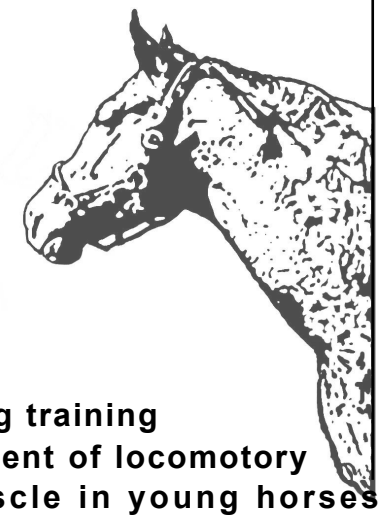


Chapter 2



Effect of show jumping training on the development of locomotory muscle in young horses

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Summary

Objective

To investigate whether training for show jumping that is commenced early after birth affects the characteristics of equine locomotory muscle.

Animals

19 Dutch Warmblood horses.

Procedures

Horses were assigned to a trained or not trained (control) group. After weaning, training (free jumping [2 d/wk] that was alternated with a 20-minute period of exercise in a mechanical rotating walker [3 d/wk]) was started and was continued until 3 years of age. Fiber type composition (determined from myosin heavy chain [MyHC] content), fiber area, diffusion index, citrate synthase activity, and Na^+, K^+-ATPase content were assessed in biopsy specimens of the deep gluteus medius muscle collected at 0.5, 1, 2, and 3 years.

Results

Developmental changes included an increase in MyHC fiber type IIa and decrease in type IIad; increases in fiber area, diffusion index, and citrate synthase activity; and a decrease in Na^+, K^+-ATPase content. The MyHC fiber types I and IId were present in high and low proportions, respectively. Training increased Na^+, K^+-ATPase content, but did not affect other variables.

Conclusions and Clinical Relevance

In horses, show jumping training at an early age resulted in increased Na^+, K^+-ATPase content of the deep gluteus medius muscle. The absence of training effects on the other muscle characteristics can partly be explained by the fact that an appropriate (aerobic) fiber type composition was already established at the start of training. These data have also suggested that the developmental changes in equine muscle represent sufficient adaptation to meet the demands of this specific training.

2.1 Introduction

The musculoskeletal system plays a major role in the athletic ability of horses. During show jumping, successful performance depends on an excellent combination of coordination, balance, and strength during the approach and take-off. Most of the force for take-off is provided by muscular power generated by the hind limbs.[1,2]

Muscular power is determined by fiber type composition and CSA of the muscle. Fiber type is usually defined by the MyHC isoforms present. The myosin isoform expressed is the major determinant of the maximum shortening velocity in muscle fibers.[3] Generally, muscle fibers are categorized as types I (slow oxidative), IIa (fast oxidative-glycolytic), IId (or IIx; fast glycolytic), and IIad (a hybrid fiber).[4-6] The CSA of each fiber type is a measure of power output of a muscle—muscle fibers with a large CSA generate more tension per CSA than muscle fibers with a small CSA.[7] In horses, the mean CSA of the fibers increases according to type: I < IIa < IId (including the hybrid fiber IIad).[6]

To maintain power output and prevent fatigue, a muscle depends on oxygen and on various enzymes for oxidative capacity and excitability. Oxygen is delivered to muscle fibers via the capillary bed. The capillary supply varies with MyHC fiber type composition; the number of capillaries surrounding oxidative fibers is higher than that surrounding glycolytic fibers.[6] A physiologically more relevant description for capillarity should include muscle fiber area, because the area provides a more realistic indication of diffusion distances. The oxidative capacity of skeletal muscle can be assessed by measurement of citrate synthase activity. Citrate synthase catalyses the conversion of acetyl coenzyme-A and oxaloacetate into coenzyme-A and citrate during energy production. For maintenance of excitability and force of a muscle, Na^+, K^+-ATPase is present in the plasma membranes of muscle fibers.[8] During excitation of the muscle fibers, action potentials are elicited by a rapid influx of Na^+, followed by an efflux of K^+. This leads to increases in intracellular Na^+ and extracellular K^+ concentrations that stimulate Na^+, K^+-ATPase activity. The pump restores the intra- and extracellular concentrations of the Na^+ and K^+ and protects the resting membrane's potential to maintain ongoing contractions.[9]

Skeletal muscle in horses develops quickly during the early postnatal period; the development is associated with changes in MyHC expression,[5,10-12] fiber area,[13,14] and oxidative capacity.[14,15] To our knowledge, evaluations of Na^+, K^+-ATPase and capillary supply during the early postnatal period in horses have not been performed, but the activity of that enzyme and the distribution of the capillary supply are known to decrease in rat muscle during that period.[16-18]

In general, training enhances aerobic capacity, power output and excitability of a muscle by increasing the proportion of oxidative fibers (ie, type I or IIa), CSA, capillarity, and oxidative enzyme activity[19] with or without upregulation of Na^+, K^+-ATPase.[9] Results of several studies[5,11,12,20-22] have indicated that training influences muscle characteristics of foals and adolescent horses, but those studies focused on training for sport purposes other than show jumping. Training of Warmbloods does not start before the horses are broken in at 3 years of age, and they are kept on pastures or in stables until that time. Previously, Santamaría et al[23] reported results of the effect of early jumping training on jumping technique. That study revealed that compared with untrained horses, trained horses jumped more efficiently as indicated by lower acceleration during hind limb push and lower velocity at take-off, and by the fact that the trained horses positioned their center of gravity at the apex of the jump beyond the fence less.

The purpose of the study reported here was to investigate whether training for show jumping that is commenced early after birth affects the characteristics of equine locomotory muscle. The MyHC fiber type composition, fiber area, oxidative capacity, and Na^+, K^+-ATPase content of the gluteus medius muscle (which has a major role in propulsion as extensor of the hip joint) were evaluated in Dutch Warmblood horses that were raised conventionally or underwent jumping training from the age of 6 months.

2.2 Material and Methods

2.2.1 Horses

The study group comprised 19 Dutch Warmblood horses that were expected to have reasonably good future jumping ability on the basis of the breeding values of their siresand dams. These study horses were part of a larger group that was used in a project to develop scientific criteria for selection and effective and injury-free training of show jumpers.[24] The horses were kept at the Animal Sciences Group, Wageningen University and Research Centre, the Netherlands. After weaning, the horses were assigned to a control or a training group. Eight horses (mean body weight ± s.e. at 6 months, 317 ± 12 kg; at 1 year, 418 ± 14 kg; and at 3 years, 552 ± 17 kg) were in the control group. Eleven horses (mean body weight at 6 months, 299 ± 10 kg; at 1 year, 400 ± 10 kg; and at 3 years, 531 ± 11 kg) were in the training group.

During winter, both groups of horses were housed in an open stable with an adjacent paddock. During summer, all horses were kept on pasture. The horses were fed concentrates in the morning and the afternoon and received additional straw and grass silage before noon. Water was available ad libitum. All horses were checked regularly by a veterinarian and received hoof care. All procedures were approved by the Institutional Care and Use Committee of Animal Sciences Group, Wageningen University and Research Centre, the Netherlands.

2.2.2 Training

Horses in the training group began training after weaning and this continued until the horses were 3 years of age. Training included free jumping (2 d/wk) and a 20-minute period of exercise in a mechanical rotating (hot) walker (3 d/wk) performed on alternate days. The free-jumping course consisted of a 3-fence combination: a cross rail, a vertical fence, and a parallel oxer. At the start of training after weaning, the height of these fences was 40, 50, and 60 cm (width, 60 cm), respectively; when the horses were 3-years old, the fence heights were 80 to 90 cm. The distances between the fences were 6 to 7 m.

During every session, the horses jumped the 3-fence combination 6 times; the mean speed per round of jumping was 5.6 m/s.

At the start of training after weaning, exercise in the mechanical walker (40-m in diameter) consisted of 6 sessions conducted within a 15-minute period. At 3 years of age, exercise in the mechanical walker consisted of approximately 18 sessions within a 20-minute period. In each session, horses walked (1.7 m/s) for 40 seconds, trotted (3.6 m/s) for 10 seconds, and cantered (5 to 5.6 m/s) for 10 seconds, followed by a period of walking when necessary.

2.2.3 Muscle biopsy procedures

At 0.5, 1, 2, and 3 years, muscle biopsy specimens were obtained percutaneously by use of a Bergström needle (inner diameter, 4.00 mm) from each horse by 1 individual (E.G.D.) according to the protocol of Lindholm and Piehl.[5,25] Specimens were obtained from the deep part of the gluteus medius muscle after horses received local anesthesia via injection with lidocaine hydrochloride.

To facilitate specimen collection, an imaginary line drawn from the coxal tuber to the sacral tuber; at a position one third of the distance from the sacral tuber, the biopsy needle was inserted perpendicular to the skin, as deep as possible (until resistance from the iliac wing was detected). All biopsy samples were stored at –80 ºC until analyzed.

2.2.4 Immunohistochemistry

Specific monoclonal antibodies were used to identify muscle fiber types according to their MyHC content.[5] Monoclonal antibody 219-1D1 (1:25) reacts with type I fibers. Monoclonal antibody 332-3D4 (1:10) reacts with type IIa and IId fibers. Monoclonal antibody 333-7H1 (1:10) reacts with type IIa fibers. Monoclonal antibody 412-R1D5 (1:25) reacts with type I and IId fibers. Transverse serial sections (10 mm) of biopsy specimens were obtained and further processed as described previously.[5,26]

2.2.5 Assessments of MyHC fiber type composition, fiber area, and capillary supply

From the gluteus medius muscle specimen, a region of 200 contiguous fibers was collected for fiber typing, determination of fiber type proportions, assessment of fiber area, and counting of capillaries. Fiber perimeters and capillaries were identified by means of the a-amylase periodic acid–Schiff method.[27] Images of the stained sections were digitized by use of a microscope and a camera and analyzed by use of computer software.

The muscle fibers of all 19 horses were classified as types I, IIa, IIad, or IId on the basis of monoclonal antibody reactions. The fiber areas for each type of muscle fiber and the number of capillaries were determined in a subgroup of the 9 horses (ie, 4 control and 5 trained horses).

The capillary supply was expressed by the diffusion index (mm^2/ capillary) and calculated from the digital measurements. It was calculated as follows:

$$\text{Diffusion index} = \frac{\text{CSA} \times \text{Fiber density}}{\text{Capillary density}}$$

2.2.6 Assessment of citrate synthase activity in gluteus medius muscle

A portion of each muscle biopsy specimen was disintegrated ultrasonically (three 15-second pulses; amplitude, 22 Hz) in a buffered solution containing 8.6% sucrose, 2.3mM EDTA, 0.01M Tris, 2M HCl, and 25000 units of heparin (pH, 7.4) and stored on ice. Citrate synthase activity was measured in duplicate by use of a spectrophotometer (25°C; 412 nm wavelength, measurementsperiod of 9 minutes). A solution containing 1mM 5,5'-dithio-2-nitrobenzoate in 1M Tris (pH, 8.1) mixed with 7.5mM acetyl co-enzyme A and 2% Triton X-100 was prepared; to establish background activity, this solution was equilibrated in the cuvette and homogenate (1%) was added and mixed. After 2 minutes, 5%

oxaloacetate (10mM in 0.1M Tris [pH, 8.0]) was added and measurement continued. Citrated synthase activity was measured as the amount of enzyme in 1 mL of homogenate that converted 1 nmol of oxaloacetate/min. To standardize results, enzyme activity was expressed as milli-units per milligram of protein content of the homogenate.[28]

2.2.7 Quantification of Na^+, K^+-ATPase

For 17 biopsy samples (control, n=8, and trained, n=9), Na^+, K^+-ATPase content was quantified by measuring the tritiated ouabain (^{3}H-ouabain)-binding capacity of small muscle samples in the presence of vanadate.[29] The values obtained corresponded to the total population of functional Na^+, K^+ pumps.[9,30] The method has also been validated for measurements in muscle biopsy specimens obtained from foals.[31] Briefly, a ouabain concentration of 10^{-6}M was used to allow saturation of most of the ouabain-binding sites.[29] Biopsy specimens were incubated for 120 minutes at 37°C in buffer containing ^{3}H-ouabain (0.6 ìCi/mL) and unlabeled ouabain (final concentration of 10^{-6} M). One set of biopsy samples was incubated with ouabain at a 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 retained in the muscle samples (pmol/g of tissue wet wt) was calculated after correction for unspecific uptake and isotopic purity.

2.2.8 Statistical analysis

Statistical analyses were performed by use of computer software using GLM repeated measurements with factors age (within) and group (control and training, between). An interaction between the factors age and group was considered indicative of an effect of early training. Data are expressed as mean ± s.e. A value of $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Development

From 0.5 to 3 years of age, there were significant changes in MyHC fiber type composition (specifically type IIa and IIad fibers) in horses of the control and training groups (Figure 2.1). Also, in both groups, CSA of each fiber type increased (Table 2.1). The changes in MyHC fiber type composition of the gluteus medius muscle from 1 to 2 years were

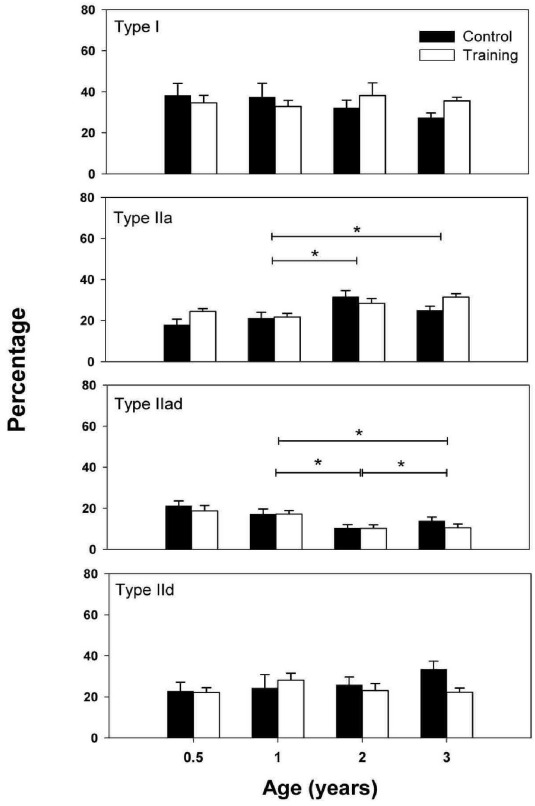

Figure 2.1

Myosin heavy chain composition of the gluteus medius muscle in control (n=8) and trained (n=11) horses, expressed as frequencies of type I, IIa, IIad,IId fibers at a 0.5,1,2 and 3 years. Mean ± s.e.,*p<0.05, within groups.

associated with an absolute increase (8%; $p < 0.05$) in type IIa fibers and a concomitant decrease (7%; $p < 0.05$) in type IIad fibers; from 2 to 3 years, changes in MyHC fiber type composition were associated with an increase (2%; $p < 0.05$) in type IIad fibers. These changes represented a relative change of 39%, 40% and 16%, respectively. The relative proportions of fiber types I and IId did not change during postnatal development. Type I fibers accounted for approximately 35% and type IId fibers accounted for approximately 25% of the MyHC fiber type composition at all ages. Diffusion index of the gluteus medius muscle was assessed in 5 trained horses and 4 control horses at 0.5, 1, and 3 years. At each time point, the diffusion index increased significantly ($p < 0.05$), compared with the previous value, in each group. At 0.5 years, the mean ± s.e. diffusion in the training and control groups was 0.88 ± 0.07 and 0.77 ± 0.11 mm^2/capillary (x 10^{-3}), respectively; at 1 year, 1.01 ± 0.12 and 1.16 ± 0.12 mm^2/capillary (x 10^{-3}), respectively; and at 3 years, 1.34 ± 0.12 and 1.20 ± 0.12 mm^2/capillary (x 10^{-3}), respectively.

Table 2.1 Mean cross-sectional areas (CSA) in μm^2 of MyHC fiber type I, IIa, IIad and IId of the gluteus medius muscle

	0.5 year		1 year		3 years	
	Control	Training	Control	Training	Control	Training
I	1295 ± 171	1122 ± 164	1676 ± 263	1738 ± 337 *	2229 ± 272	2316 ± 279 *
IIa	1505 ± 118	1586 ± 164	2030 ± 213	1874 ± 532	2788 ± 263	3115 ± 121 *
IIad	1661 ± 140	1818 ± 227	2234 ± 176	2648 ± 364 *	2880 ± 285	3086 ± 250 *
IId	2149 ± 246	2316 ± 204	3204 ± 393	3547 ± 299 *	3881 ± 244	4796 ± 275 *

Table 2.1

Mean cross sectional areas (CSA) of myosin heavy chain (MyHC) fiber type I, IIa, IIad and IId of the gluteus medius muscle in control (n=4) and trained (n=5) horses at a 0.5, 1 and 3 years. Mean ± s.e., *$p < 0.05$, compared tp previous age, within groups.

Citrate synthase activity of the gluteus medius muscle was assessed in 11 trained horses and 8 control horses at 0.5 and 3.0 years. At 3 years, citrate synthase activity was increased significantly ($p < 0.05$), compared with the value at 0.5 years, in each group. At 0.5 years, the mean ± s.e. activity in the training and control groups was 153 ± 12 and 151 ± 13 mU/mg protein, respectively; at 3 years, the values were 217 ± 14

and 198 ± 14 mU/mg protein, respectively.

The Na^+, K^+-ATPase contents of the gluteus medius muscle samples collected from trained and control horses were assessed (Figure 2.2). At 2 years, the values in both groups were significantly ($p < 0.05$) different from the corresponding value at 0.5 years.

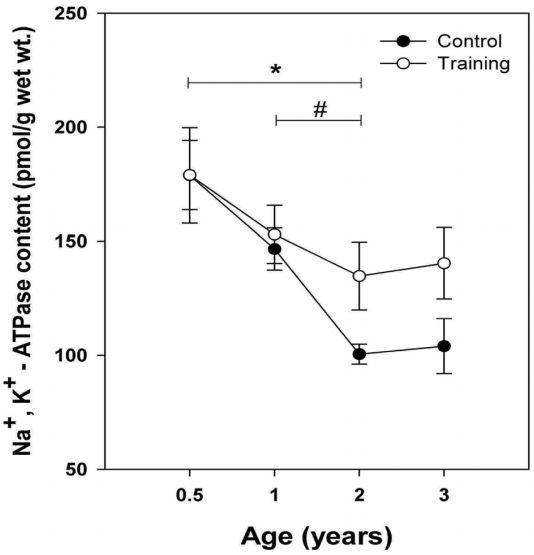

Figure 2.2

Na^+, K^+-ATPase content of the gluteus medius muscle, quantified as [^{3}H]ouabain binding capacity, in control (n = 8) and trained (n = 9) horses at a 0.5, 1, 2 and 3 years. Mean ± s.e., * $p < 0.05$, within groups. # $p < 0.05$, training effect (interaction age x group).

2.3.2 Exercise

From 1 to 2 years there was a significant effect of early training on Na^+, K^+-ATPase content (Figure 1). The age-dependent decrease was partly prevented in the training group. At 2.0 years, the content of Na^+, K^+-ATPase in the trained horses (n = 9) was 132 ± 14 pmol/g of wet weight of muscle; the content in the control horses (n = 8) was 105 ± 8 pmol/g of

wet weight of muscle. This difference was significant ($p < 0.05$). A similar difference in Na^+, K^+-ATPase content between the 2 groups was also detected at 3 years. Early training had no effect on the MyHC fiber type composition, CSA, diffusion index and citrate synthase activity of the gluteus muscle.

2.4 Discussion

In the present study, the effects of early training during postnatal growth on muscle characteristics in young Dutch Warmblood horses were investigated. We were interested in ascertaining whether previously identified effects of early training on jumping technique among young horses,[23] are also reflected in changes in muscular characteristics. With regard to developmental changes in the gluteus medius muscle, results of our study indicated that these included a change in MyHC fiber type composition (specifically, an increase in the proportion of type IIa fibers and a decrease in the proportion of type IIad fibers), an almost 2-fold increase in fiber area in all fiber types, increases in citrate synthase activity and diffusion index, and an age-dependent decrease in Na^+, K^+-ATPase content. In general, the MyHC fiber type composition included a high proportion of type I fibers and low proportion of type IId fibers. Surprisingly, there was no effect of early training on the different muscle variables, except for the Na^+, K^+-ATPase content.

The development-associated changes in MyHC fiber type composition were primarily detected from 1 until 3 years of age in IIa and IIad fibers; the proportion of type IIa fibers increased and the proportion of type IIad fibers decreased, whereas the proportions of type I and type IId fibers remained constant. This suggests a transition of type IIad fibers into type IIa fibers.[5,14] Evidence for this concept of fiber transition was provided by results of a study by Eizema et al,[32] which indicated that after birth, equine muscle develops towards a slower type of MyHC (according to the so-called nearest-neighbor rule for MyHC fiber type transitions). Additionally, it has been determined that in adult horses, fibers co-expressing IIa and IId MyHC protein predominantly express mRNA for the IIa isoform; these hybrid fibers are probably converting to the type corresponding

to the expressed mRNA.[33] The prevailing view is that hybrid fibers enable a muscle to fine-tune the wide range of forces and velocities it is required to generate.[34]

The proportions of type I (35%) and IId (25%) fibers in the horses of the present study are in contrast with results of other studies[12,14,35] involving horses of approximately the same age. In those studies, the proportion of type I fibers ranged from 15% to 20% and the proportion of type IId fibers ranged from 35% to 60%. An explanation for this discrepancy in type I fiber proportions could be a result of differences in tissue sample depth,[36,37] staining method (ATPase vs immunohistochemistry),[20] or breed. However, in a study,[20] involving horses of the same breed from which tissue samples were collected at the depth and stained by use of the same method as those of the present study, proportions of type I and type IId fibers were 20% and 38%, respectively, at 22 weeks. The differences between the findings of these similar studies could partly be ascribed to differences in genetic background between both study groups,[38] because the horses of the present study were especially selected for show jumping according to studbook criteria.

Other studies[23,39] have revealed that training results in a jumping technique that is more balanced and requires less force impulses generated by the hind limbs, compared with the jumping technique of inexperienced horses. By relating these results to the MyHC fiber type composition and knowing that adjustments to exercise always occur in the fast to slow direction,[40] we expected that early training would result in a more aerobic fiber type composition in the training group, compared with findings in the control group, in the present study. Also, previously reported results of the effects of training of horses at young ages have indicated that the proportion of MyHC fiber type I or IIa increases and that of fiber type IId decreases.[12,21,22] The training protocols used in those studies were designed to improve the physiologic adaptation to performance at high speed and were more intense (maximal speed, 16 m/s) than the protocol used in the present study (maximal speed, 5.6 m/s). However, the increase in Na^+, K^+-ATPase content of gluteus medius muscle in the horses of present study indicated that the jumping

training was intense enough to initiate a reaction in that muscle. Also in other studies of foals that were trained before weaning, the muscle content of Na^+, K^+-ATPase was higher in the trained group,[31] compared with the untrained group, whereas there were no changes in the MyHC fiber type composition.[5] The lack of training effects on MyHC fiber type composition can probably be explained, at least in part, by the adaptive range of each fiber type, which depends on the basal protein isoform profile and hence the position of that fiber type within the fast-slow spectrum.[41] At the start of the present study, the horses in both the training and control groups already had an aerobic fiber type composition; therefore, training could not induce an aerobic transition in the fiber types.

Although training did not lead to adjustments in CSA of fibers in the horses of the present study, development clearly resulted in an almost 2-fold increase in CSA of all fiber types. The increase in fiber CSA associated with development is similar to findings of previous investigations in horses.[12,14,35] When comparing the CSA of the individual fiber types of these studies with the findings of the present study, especially the type IId fibers in the present study are relatively small (3,000 to 7,000 μm^2 vs. 2,149 to 4,796 μm^2, respectively). This is probably a breed-related characteristic associated with the athletic ability of the breed used in the present study, because the large fiber size coincided with a high proportion of MyHC IId fibers and the small fiber size coincided with a low proportion of the IId fibers. Typically, training results in increases in fiber CSAs[19] and enhances power output. However, the results of early jumping training on the jumping technique,[23] indicated that trained horses generated less power at take-off than the untrained horses. This could suggest that fiber CSA would decrease, but this was not evident in the present study. Probably, the enlargement of fibers during natural maturation of muscle outweighed the effects of training.In the horses of the present study, the diffusion index also significantly increased (ie, the area that 1 capillary has to supply was increased) with age, but there was no effect of early training. The increase during development was likely caused by an increase in muscle size as capillary proliferation lags behind. This has been identified in rats, in which capillary density decreases during normal muscle growth.[17,18] Generally, in

adult horses, capillaries proliferate in muscle to improve oxygen supply and removal of waste products in response to training.[19,42] In the present study, training did not induce proliferation of capillaries. Again, we speculate that the maturation process outweighed the demand of exercise.

Similarly, in the horses of this report, citrate synthase activity in gluteus medius muscle increased with age but was not affected by training. The increase in citrate synthase activity reflects developmental changes towards a more pronounced aerobic fiber type composition. In a few studies,[43,44] a decrease in oxidative capacity of skeletal muscle of horses was detected, but this change occurred before the horses were 1 year old. Between 0.5 and 3 years of age, the oxidative capacity of gluteus medius muscle in the horses of the present study increased by 37%. This increase in activity cannot be explained by an increase in the proportion of oxidative fibers (types I, IIa, and IIad). Possibly, changes in oxidative capacity of the different fiber types would have been revealed by succinate dehydrogenase activity measurements, as in other studies.[14,21] Results of previous studies[15,19,21,22] on the effect of training in horses have indicated an enhancement of oxidative capacity. Therefore, a greater increase in citrate synthase activity was expected in the training group of the present study but the increase in citrate synthase activity during development was probably sufficiently large to supply energy for the increased demand associated with training.

The Na^+, K^+-ATPase content of equine gluteus medius muscle (measured as [^{3}H]ouabain capacity and expressed in pmol/ g of wet wt of muscle) in the present study was similar to findings of other studies[31,45] in foals (100 to 200 pmol/g of wet wt of muscle). During postnatal development, an age-dependent decrease in Na^+, K^+-ATPase content was detected in the foals of this report, which is in agreement with results of an earlier study in rats and mice.[16] This decrease can be explained by the enlargement of fiber CSAs, which resulted in larger fiber dimensions and subsequent reduction in surface-to-volume ratio.[9] Training partly prevented the age-dependent decrease in the Na^+, K^+-ATPase content. Training is considered a long-term regulatory factor for upregulation of

Na^+, K^+-pumps and is, in general, associated with a concomitant upregulation of the oxidative potential.[9] In the present study, the upregulation of the Na^+, K^+-pump content in the trained group was not associated with an increase in citrate synthase activity. However, one has to take into account that the horses were in a rapid growth phase and that, probably, the increase in citrate synthase during development was sufficiently large to supply energy for the increased training-associated demand. However, Na^+, K^+-pumps account for only 4% to 10% of the total energy turnover of muscle both at rest and during contraction[46]; thus, it is not surprising that we did not detect an increase in citrate synthase activity attributable to an increased activity of Na^+, K^+-ATPase in muscle samples from the horses of the present study.

Although early training of young horses induced an effect on the Na^+, K^+-pump content of locomotory muscle in our study, the question remains whether this effect is permanent and will be beneficial when conventional training starts at 3 years of age. It is known that the initial advantage in jumping technique derived from early training disappears when inexperienced group of horses also start training.[23] The Na^+, K^+-pump content in equine muscle increases by 20% to 50% with training at different ages[31,45,47] and it would be likely that the Na^+, K^+-pump content would also become upregulated in inexperienced horses by training that starts at 3 years of age. The question that remains is whether the horses that underwent early jumping training will have an additional increase in Na^+, K^+-ATPase content when training is continued at 3 years of age and will maintain a higher content of Na^+, K^+-pumps, compared with horses that have had no previous training prior to 3 years of age. If so, these early-trained horses would have an advantage in muscle excitability, compared with formerly untrained horses.

In addition to the effect on skeletal muscle, it is also relevant to know whether early training has a beneficial or detrimental effect on other components of the musculoskeletal system (eg, articular cartilage, bone, and tendons). In previous studies[48-50] of the effects of training at a young age in horses, it was concluded that a certain amount of exercise (when it is well balanced) is essential for development. However,

exercise should not be excessive as there are strong indications that too much or wrongly balanced exercise may be deleterious and may even have negative long-term effects.

The results of our study have indicated that training of show jumpers at a young age, which can result in a positive effect on jumping technique,[23] induces in an increase in Na^+, K^+-ATPase content of the gluteus medius muscle. It is known that those Na^+, K^+-ATPase pumps are important for the maintenance of excitability and force of the muscle. The absence of training effects in the other muscular characteristics that were evaluated can partly be explained by the presence of an appropriate (aerobic) fiber type composition of the muscle at the start of training. In addition, it also suggests that the developmental changes in muscle represent sufficient adaptation to meet the demands of the training. Finally, the importance of training for show jumping seems to be more on the level of coordination and balance than on the level of muscle characteristics.

2.5 Acknowledgments

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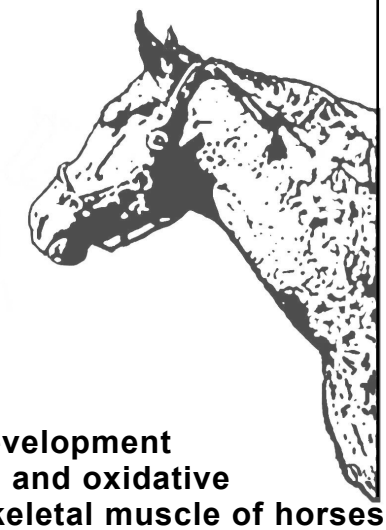
Chapter 3



Effect of exercise on development of capillary supply and oxidative capacity in skeletal muscle of horses

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Summary

Objective

To determine whether postnatal development of oxidative capacity and capillary supply of skeletal muscle is affected by different movement regimens in horses.

Animals

35 foals.

Procedures

Dutch Warmblood foals were allocated into 3 groups (box rest, box rest with training, and free pasture exercise).Training comprised an increasing number of gallop sprints from 1 week after birth to 22 weeks of age. From 22 to 48 weeks the 3 groups were combined and allowed to exercise freely. Capillary supply (diffusion index: the area supplied by 1 capillary), citrate synthase (CS) activity, and succinate dehydrogenase (SDH) activity were measured in biopsy specimens of deep gluteus medius muscle.

Results

During the first 22 weeks, diffusion index increased in all 3 groups (the training and pasture groups had a smaller increase, compared with the box rest group), total SDH activity increased in the training and pasture groups and decreased in the box rest group, and CS activity decreased in all groups. The effect of the different movement regimens on the diffusion index remained after the groups were combined.

Conclusions and Clinical Relevance

Withholding of exercise had a negative effect on the capillary supply (i.e., diffusion index increased) that remained after box rest was discontinued, and on oxidative capacity. Box rest with training prevented the negative effects and eventually had the same positive effect as pasture exercise.

3.1 Introduction

The musculoskeletal system plays a major role in the athletic ability of horses. During performance, muscle fatigue is a limiting factor and often causes exercise intolerance.[1] A measure of exercise tolerance is the maximum rate of oxygen consumption, determined by the interaction of mitochondrial oxygen supply and oxidative capacity.[2]

Muscle fiber type is determined by the isoform of the myosin heavy chain expressed by the fibers, which accounts for the shortening velocity of the fiber and therefore the contraction speed of the muscle.[3] Generally, muscle fibers are categorized as type I (slow oxidative), type IIa (fast oxidative-glycolytic), type IId (or IIx; fast glycolytic), and a hybrid fiber, IIad.[4-6]

Oxygen is delivered to muscle fibers by the capillary bed. The capillary supply varies with fiber type; that is, the number of capillaries surrounding oxidative fibers is higher than that surrounding glycolytic fibers.[6] A better physiologic description for capillarity should include muscle fiber area, because area gives a better indication of diffusion distances.

Oxidative capacity can be determined by measurement of activity of succinate dehydrogenase (SDH), which catalyses conversion of succinyl-CoA into succinate, and citrate synthase (CS), which catalyses conversion of acetyl coenzyme-A and oxaloacetate into coenzyme-A and citrate. Both enzymes are located in the mitochondria of muscle fibers. In general, oxidative capacity is higher in type I than type IId fibers.[6,7]

In the early postnatal period, equine muscle develops relatively quickly, which is associated with changes in Myosin heavy chain expression,[5,8-10] and oxidative capacity.[11,12] Capillary supply has thus far not been investigated in foals, but capillary density decreases postnatally in rat muscle.[13,14]

It is known that sprint training enhances performance capacity and fatigue resistance by an increase in capillarity and oxidative capacity along with the known anaerobic alterations, thereby improving exercise tolerance.[1,9,15] It is not known whether training or box rest at a young age is healthy or detrimental to capillary supply or oxidative

capacity during development of the equine muscular system. We previously studied the effect of different exercise regimens (box rest, box rest supplemented with training, and pasture exercise) on sodium potassium pumps and fiber type composition of the gluteus medius muscle of young horses.[5,16-18] These studies revealed a significant increase in content of sodium potassium pumps as an effect of training, whereas there was no effect on fiber type.

The purpose of the study reported here was to determine whether oxidative capacity and capillary supply are affected by different exercise regimens and whether any effects are longstanding.

3.2 Material and methods

3.2.1 Horses

Thirty five foals of the Dutch Warmblood breed (KWPN) were used. Foals were bred, raised, and trained for an experiment on the effects of exercise at an early age on the equine musculoskeletal system.[19] The foals housed in a box stall were fed fresh grass harvested from the same pasture in which the foals at pasture were confined.

Table 3.1 Mean body weight (kg) of foals of three exercise regimes

	n	0	22 weeks	48 weeks
Box	12	51 ± 2	266 ± 10	374 ± 13
Training	10	53 ± 2	252 ± 3	370 ± 7
Pasture	13	60 ± 2	254 ± 8	361 ± 7

Table 3.1

Mean ± s.e. values of body weight (kg) of the foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks.

3.2.2 Exercise regimens

After birth (day 1), all foals remained with their dams in a paddock for 1 week, after which they were randomly allocated into 3 groups that were subjected to different exercise regimens until weaning at 22 weeks.[19] Foals in $group_{box}$ were confined to a box stall (3 x 3.5 m). Foals in $group_{training}$ were kept in box stalls of the same size but given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a thick sandy top layer. The foals were trained by following the mares, which were chased between 2 persons at the far ends of the paddock. Training started when the foals were allotted to the training group (day 7) and consisted daily of 12 sprints of approximately 40 m each. From day 8 to day 24, 16 sprints were performed each day. From day 25 to day 38, 24 sprints were performed each day, and from day 39 until weaning at 5 months, 32 and 16 sprints were performed on alternate days, respectively. After the sprints, the foals were allowed an additional 0.5 hour of free exercise in the enclosure. Training was given 6 d/wk and mean velocity of the sprints was 6 m/s. Foals in $group_{pasture}$ were kept at pasture 24 h/d, with mares and their foals grazing together. Foals allowed free pasture exercise galloped for a mean of approximately 3.5 min/24h, divided over approximately 40 sprints.

At weaning, foals from each group ($group_{box,}$ n = 5; $group_{training}$, 5; $group_{pasture}$, 6) were euthanized for other research purposes. The remaining 19 foals were combined in a single group and kept in a open stable with access to a small paddock.

All procedures were reviewed and approved by the animal experiments committee of Utrecht University.

3.2.3 Muscle biopsy specimens

From each foal, biopsy specimens were taken percutaneously by the same individual, according to the protocol of Lindholm and Piehl,[5,21] at day 1, 22 and 48 weeks of age by use of a Bergström needle, with an inner diameter of 4.00 mm. Biopsies from the deep part of the gluteus medius muscle were taken on an imaginary line drawn from the tuber

coxae to the tuber sacrale, at one third of the distance from the tuber sacrale, perpendicular to the skin, as deep as possible (until resistance from the iliac wing was perceived). Biopsy specimens from the superficial portion of the semitendinosus muscle were taken on a line drawn from the tuber ischium to the popliteal area, at two thirds of the distance from the tuber ischium, at a depth that was reached just after the muscle fascia was penetrated. All biopsies were performed by use of local anesthesia with lidocaine 2% HCl. The specimens were rolled in talcum powder and mounted on cork blocks with O.C.T. (optimum cutting temperature) embedding medium. All samples were stored at –80 ºC until analyzed. Transverse serial sections (10 mm) were made with a cryostat at –20 ºC.

3.2.4 Capillaries

Sections were dried for 1 hour at room temperature (21ºC) and fixed in mixture of 35% methanol, 35% acetone, 5% acetic acid, and 25% distilled water for approximately 20 hours at –20 ºC, incubated in methanol with 0.3% H_2O_2 for 30 minutes, hydrated, incubated with pronase (1:100 in PBS solution) for 30 minutes, rinsed in PBS solution 3 times for 5 minutes, incubated in Teng-T (10mM Tris, 5mM EDTA, 0.15M NaCl, 0.25% gelatin, and 0.05% Tween 20; pH 8.0) for 15 minutes, and incubated with biotinylated lectin (0.005% for capillaries and 0.02% for cell membranes in PBS solution) overnight. The next day, the sections were rinsed in PBS solution for 5 minutes 3 times, incubated with avidin and biotin (in PBS solution) for 90 minutes, rinsed with PBS solution for 5 minutes 2 times, and stained with 0.05% 3,3-diaminobenzidine tetrachloride (DAB) with 0.01% H_2O_2. Sections for capillary staining were rinsed first in acetate buffer (0.1M, pH 6.0) and 0.2% imidazol was added to the DAB solution. Sections for membrane staining were first rinsed in Tris-HCl (0.05M, pH 7.4) and 2.5% ammonium nickel sulphate was added to the DAB solution. Rinsing in tap water stopped the reaction. Finally, sections were dehydrated and enclosed in embedding medium.

Stained sections were digitized. Images were analyzed by measuring capillary densities and fiber cross sectional areas with camera software. Capillaries were

measured twice in a frame of 0.44 mm^2 (to reduce the effect of variation in brightness) and at 5 fields in each section; mean values were used for analyses. Fiber areas were measured in digitized images (magnification, 400x) of adjacent sections.

Capillary density (number of capillaries/mm^2), fiber density (number of fibers/ mm^2), capillaries per fiber ratio (capillary density/ fiber density), mean fiber cross sectional area (CSA in mm^2/fiber) and diffusion index (mm^2/capillary) were calculated from the digital measurements. The latter was calculated as follows:

$$\text{Diffusion index} = \frac{\text{CSA x Fiber density}}{\text{Capillary density}}$$

Capillaries per fiber type were counted manually on the basis of fiber type identification according to the Myosin heavy chain content by use of specific monoclonal antibodies, as described,[5].

3.2.5 Citrate Synthase activity

Muscle biopsy specimens of all foals were disintegrated with a dismembrator in a buffered solution of 8.6% sucrose, 2.3mM EDTA, 0.01M Tris, 2M HCl and 25,000 units of heparin (pH 7.4) and stored on ice. Mitochondria were destroyed by freezing and thawing alternately to release the enzyme. CS activity was measured in duplicate with a spectrophotometer (25°C, 412 nm, 9 minutes). Measurements were done in 1mM 5,5'- dithio-2-nitrobenzoate in 1M Tris (pH 8.1) mixed with 7.5mM acetyl co-enzyme A and 2% Triton X-100, equilibrated in a cuvette. Homogenate (1%) was added and mixed, to establish background activity. Oxaloacetate (10mM in 0.1 M Tris, pH 8.0) was added after 2 minutes and measurement continued. CS activity is measured as the amount of enzyme in 1 ml of homogenate that converts 1 nmol of oxaloacetate/min. To standardize results, enzyme activity is expressed in mU/mg of protein content of the homogenate,[22].

3.2.6 Succinate dehydrogenase activity

Thirteen randomly chosen foals ($group_{box}$ day 1, n = 6; week 22, 6; week 48, 4; $group_{training}$ day 1, 3; week 22, 3, week 48, 1; $group_{pasture}$ day 1, 4; week 22, 4; week 48, 3) were used for determination of SDH activity. The SDH activity was measured in 4 fiber types, which were identified according to their Myosin heavy chain content with specific monoclonal antibodies.[5] Total SDH activity was calculated from the SDH activity per fiber type and the corresponding proportion of the myosin heavy chain fiber type composition as follows: $SDH_{total\ activity}$ = (SDH_{I} x Fiber $type_{I}$ [%]) + (SDH_{IIa} x Fiber $type_{IIa}$ [%]) + (SDH_{IIad} x Fiber $type_{IIad}$ [%]) + (SDH_{IId} x Fiber $type_{IId}$ [%]).

Transverse serial sections (10 mm) were made, adjacent to sections used for fiber type identification. The sections were incubated immediately after sectioning (within 24 hours after obtaining biopsy specimens), in 37mM sodium phosphate buffer, 74mM sodium succinate, and 0.4mM tetranitroblue tetrazolium at pH 7.6 for 30 minutes at 37°C. The reaction was stopped in 10mM HCl. Sections were washed with distilled water, embedded in glycerine-gelatin, and stored at 4°C in the dark until analysis.

Sections were studied with a microscope fitted with calibrated grey filters. The absorbance of the formed formazan deposit in the 4 fiber types was measured at 660 nm.[23] Duplicate measurements were made of 5 fibers of each type and these were pooled. Images were obtained with a 10X objective and a monochrome charge-coupled devises camera connected to an LG-3 frame grabber in a computer. Images were analyzed by use of a public domain NIH Image program.

3.2.7 Statistical analysis

Statistical analyses were performed by use of computer software,[j] using General Linear Model repeated measures with factors age (within groups) and group (between groups). An interaction between the factors age and group was considered indicative of an effect of the different exercise regimens. Data are expressed as mean ± s.e. For all comparisons, $p < 0.05$ was considered significant.

3.3 Results

3.3.1 Development

Mean body weights of foals in the 3 groups were determined at the 3 time points (Table 3.1). Typically, for all 3 groups combined from day 1 to 22 weeks there was a significant increase in diffusion index (Figure 3.1), a decrease in capillary density and fiber density, an increase in capillary-to-fiber ratio and cross sectional area (Table 3.2), and an increase in the number of capillaries per fiber for all Myosin heavy chain fiber types (Table 3.3). CS activity decreased approximately 30% ($p < 0.05$, for all groups combined) (Figure 3.2). Total SDH activity (Figure 3.3) did not change significantly (all groups combined), but increased in Myosin heavy chain type I and IIa fibers and decreased in Myosin heavy chain type IId fibers (Table 3.4).

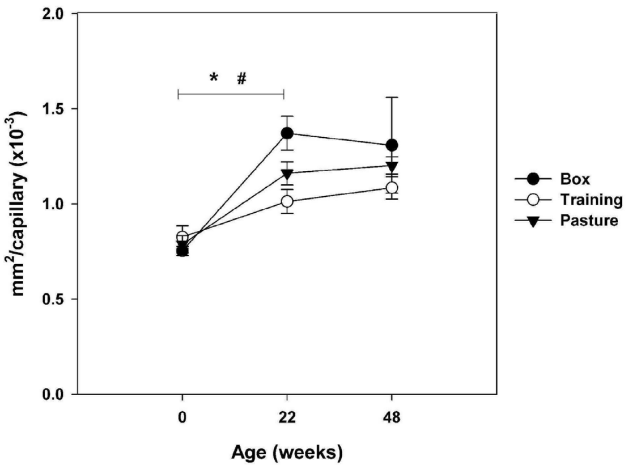

Figure 3.1

Mean ± s.e. values of the diffusion index (mm^2/capillary) of the gluteus medius muscle of foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks. * significant age-effect (p-value < 0.05). # significant exercise-effect (p-value < 0.05).

From 22 to 48 weeks there were fewer effects of development. There was an increase in capillary-to-fiber ratio (Table 3.2), an increase in total SDH activity (Figure 3.2), and an increase in SDH activity in type I fibers (Table 3.4).

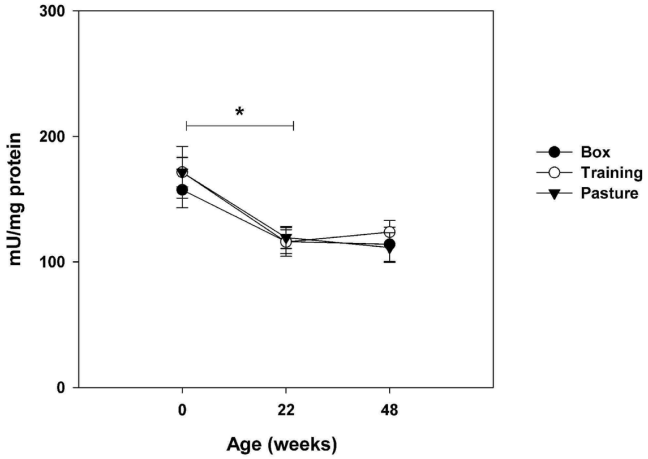

Figure 3.2

Mean ± s.e. values of citrate synthase activity (mU/mg protein) of the gluteus medius muscle of foals of three exercise regimes box rest (box, closed circle), box rest with training (training, open circle) and free pasture exercise (pasture, closed triangle) at age 0, 22 and 48 weeks. * significant age-effect (p-value < 0.05).

3.3.2 Exercise

From 0 to 22 weeks, diffusion index of $group_{training}$ and $group_{pasture}$ increased (22% and 47%, respectively), but the increase was significantly smaller than that of $group_{box}$ (83%). Total SDH activity (Figure 3.3) of $group_{training}$ and $group_{pasture}$ increased (39% and 29%, respectively), whereas that of $group_{box}$ decreased (11% [$p< 0.05$]). The SDH activity of the type IId fibers in $group_{box}$ decreased significantly (33% [$p< 0.05$]), compared with the other 2 groups (Table 3.4).

From 22 to 48 weeks, variables did not differ significantly among groups, except

for a significant increase in SDH activity in type IId fibers in $group_{box}$, compared with $group_{pasture}$ (because of low number of foals in $group_{training}$ at 48 weeks, no comparison was made with that group). The differences in diffusion index among the 3 groups that were detected in the first 22 weeks remained up to 48 weeks.

Table 3.2 Capillary density (CD), fiber density (FD), capillary to fiber ratio (CF) and cross sectional area (CSA) of the gluteus medius muscle

		0	22 weeks	48 weeks
CD	**Box**	1107 ± 40	696 ± 42	791 ± 101
(cap/mm2)	**Training**	1032 ± 59	924 ± 51 # *	875 ± 28
	Pasture	1110 ± 65	814 ± 36	773 ± 29
FD	**Box**	2144 ± 103	554 ± 42	465 ± 61
(fiber/mm2)	**Training**	1636 ± 160	605 ± 22 # *	492 ± 18
	Pasture	1911 ± 78	525 ± 22	453 ± 17
CF	**Box**	0,53 ± 0,02	1,29 ± 0,08	1,71 ± 0,14
(cap/fiber)	**Training**	0,62 ± 0,04	1,54 ± 0,09 *	1,78 ± 0,02 *
	Pasture	0,59 ± 0,03	1,57 ± 0,07	1,72 ± 0,09
CSA	**Box**	396 ± 19	1753 ± 135	2170 ± 404
(µm2/fiber)	**Training**	555 ± 57	1515 ± 54 # *	1930 ± 110
	Pasture	450 ± 21	1788 ± 80	2047 ± 58

Table 3.2

Mean ± s.e. values of capillary density (CD, cap/mm^2), fiber density (FD, fibre/mm^2), capillary to fiber ratio (CF) and cross sectional area (CSA, mm^2) of the gluteus medius muscle of foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks. * significant age-effect (p-value < 0.05). # significant exercise-effect (p-value < 0.05).

3.4 Discussion

The present study determined the effect of 3 exercise regimens on the capillarity and oxidative capacity of the gluteus medius muscle of young foals during the early postnatal period. Box stall confinement had an effect on diffusion index and SDH activity, which decreased, compared with both other regimens. Surprisingly, the negative effect on diffusion index was not restored after a period of free movement.

The diffusion index increased significantly with age in all 3 groups in the first 22

weeks. This was in agreement with the observations in rats that capillary density declines during normal muscle growth, because capillary proliferation lags behind the increase in muscle size.[13,14]

The diffusion index was also affected by the different exercise regimens. Normally, in adult horses, diffusion index decreases because of proliferation of capillaries as a muscular response to training to improve oxygen diffusion and removal of waste products.[1,24] In the present study, the conducted sprint training in $group_{training}$ did not induce the expected decrease in diffusion index compared to $group_{pasture}$, probably, because the effect of exercise was outweighed by maturation. However, the training was enough to prevent the large increase in diffusion index detected in $group_{box}$. The changes in the diffusion index induced by exercise were not caused by changes in the number of capillaries, but by the different responses in the growth of muscle fibers. Resuming normal activity (paddock exercise) after 22 weeks did not reverse the induced changes in muscle detected in $group_{box}$. To see if the lasting effect of the different regimens on the

Table 3.3 Capillary to fiber ratio per MyHC fiber type I, IIa, IIad and IId of the gluteus medius muscle

		0	22 weeks		48 weeks
I	**Box**	2,3 ± 0,2	4,1 ± 0,4	*	5,0 ± 0,4
	Pasture	2,4 ± 0,1	4,2 ± 0,7		4,4 ± 0,8
IIa	**Box**	2,5 ± 0,2	4,9 ± 0,5	*	6,4 ± 0,8
	Pasture	2,5 ± 0,3	5,1 ± 0,7		5,9 ± 1,0
IIad	**Box**	2,5 ± 0,3	5,0 ± 0,9		6,1 ± 0,3
	Pasture	2,8 ± 0,3	4,6 ± 0,5	*	5,7 ± 1,7
IId	**Box**	2,8 ± 0,2	4,5 ± 0,5	*	5,8 ± 0,5
	Pasture	2,8 ± 0,4	5,4 ± 0,7		5,6 ± 1,2

Table 3.3

Mean ± s.e. values of capillary to fiber ratio per MyHC fiber type I, IIa, IIad and IId of the gluteus medius muscle of foals of two exercise regimes; box rest (box) and free pasture exercise (pasture) at age 0, 22 and 48 weeks. * significant age-effect (p-value < 0.05).

gluteus medius muscle was a general phenomenon, it was compared with the diffusion index of another important propulsive locomotory muscle, the semitendinosus muscle. In that muscle too, free movement did not reverse induced changes (Figure 3.5).

Citrate synthase activity and SDH activity, although both indicators for oxidative capacity, responded differently with regard to age and exercise effects. With age, CS activity decreased, whereas total SDH activity increased. These findings were probably caused by differences in measurement methods. Results of previous studies on age effects indicated that of the aerobic metabolic enzyme activities in foals, CS and SDH activities decreased when measured on a tissue level (i.e. in whole muscle homogenates),[10,25,26], whereas SDH activity increased when measured on individual fiber level.[9,27] A possible explanation could be the disproportional increase of

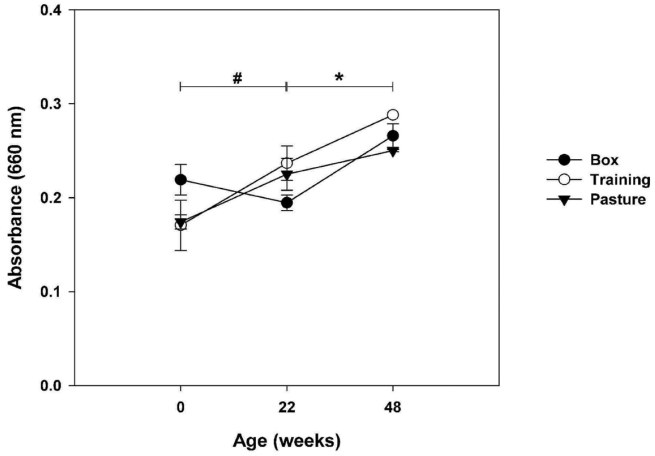

Figure 3.3

Mean ± s.e. values of succinate dehydrogenase activity (absorbance 660 nm) of the gluteus medius muscle of foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks. Total SDH activity was calculated from the SDH activity per fiber type and the corresponding proportion of the myosin heavy chain fiber type composition. * significant age-effect (p-value < 0.05). # significant exercise-effect (p-value < 0.05).

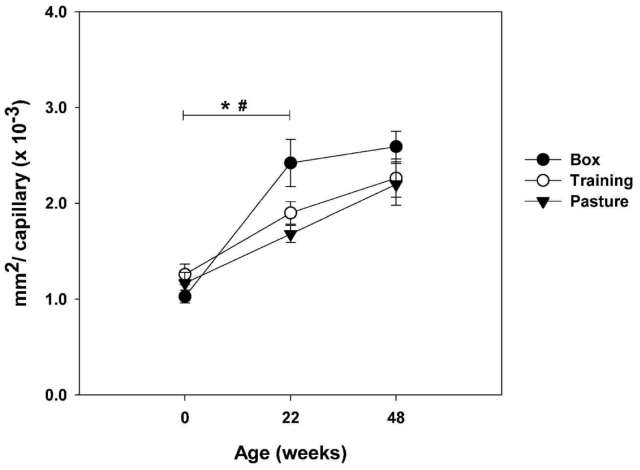

Figure 3.4

Mean ± s.e. values of the diffusion index (mm^2/capillary) of the semitendinosus muscle of foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks. * significant age-effect (p-value < 0.05). # significant exercise-effect (p-value < 0.05).

contractile proteins and CS during growth, which influences the results of oxidative capacity measurements on a tissue level in young horses. Thus, for horses in a rapid growth phase, oxidative capacity measurements on a fiber level will be more accurate. The increase in SDH activity of type I and IIa fibers was probably caused by the increasing demand on these fiber types during development. Also, analysis of myosin heavy chain composition, which has been reported,[18] revealed developmental changes that resulted in a more aerobic fiber type composition.

Exercise did not have an apparent effect on CS activity, whereas total SDH activity decreased in $group_{box}$. This effect on SDH activity was predominantly detected in type IId fibers. Apparently, measurement of whole muscle CS activity was not sensitive enough to detect an effect of exercise or an effect of exercise may have been masked by the large decrease in CS activity attributable to growth. The fiber-type-dependent differences in

Table 3.4	Succinate dehydrogenase activity per MyHC fiber type I, IIa, IIad and IId of the gluteus medius muscle							
		0		22 weeks		48 weeks		
I	Box	0,26 ± 0,02		0,27 ± 0,01		0,34 ± 0,02		
	Training	0,21 ± 0,03		0,31 ± 0,03	*	0,36		*
	Pasture	0,20 ± 0,01		0,31 ± 0,01		0,34 ± 0,01		
IIa	Box	0,28 ± 0,02		0,28 ± 0,01		0,30 ± 0,02		
	Training	0,22 ± 0,03	*	0,32 ± 0,02	*	0,31		
	Pasture	0,23 ± 0,02		0,31 ± 0,01		0,31 ± 0,01		
IIad	Box	0,27 ± 0,02		0,22 ± 0,01		0,24 ± 0,02		
	Training	0,21 ± 0,03	*	0,26 ± 0,03	*	0,27		
	Pasture	0,21 ± 0,01		0,27 ± 0,01		0,27 ± 0,00		
IId	Box	0,15 ± 0,01		0,10 ± 0,01	#	0,13 ± 0,01		#
	Training	0,13 ± 0,02		0,13 ± 0,02	*	0,15		
	Pasture	0,13 ± 0,01		0,12 ± 0,00		0,11 ± 0,01		

Table 3.4

Mean ± s.e. values of succinate dehydrogenase activity (absorbance 660 nm) per MyHC fiber type I, IIa, IIad and IId of the gluteus medius muscle of foals of three exercise regimes; box rest (box), box rest with training (training) and free pasture exercise (pasture) at age 0, 22 and 48 weeks.

* significant age-effect (p-value < 0.05). # significant exercise-effect (p-value < 0.05). (The effect of exercise from 22 to 48 weeks was only measured between the box group and the pasture group, because of the low number in the trained group at 48 weeks.)

oxidative capacity could have been caused by differences in recruitment of the different motor units, which depend on the activity level of the muscle.[1,6] It was expected, if an effect of the different exercise regimens was present, it would be due to training, because earlier studies with training in adults revealed an enhancement of oxidative capacity.[1] In the present study, the trained group ($group_{training}$) did not differ from the foals at pasture. However, training did have a positive effect because $group_{training}$ values were greater than $group_{box}$ values.

For both capillary supply and oxidative capacity, similar findings were obtained in $group_{training}$ and $group_{pasture}$. The training given can be classified as not excessively demanding. The maximum number of sprints during training equaled approximately 3

minutes of galloping, compared with 3.5 minutes of galloping in foals at pasture. However, in a previous study with the same experimental group of foals there were indications that tissue vitality and/or quality was less at 48 weeks with respect to articular cartilage, bone and tendon.[20] These findings were interpreted as a warning that the exercise regimen was detrimental, although, no conclusions can be drawn with respect to consequences for later performance.[20]

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Chapter 4

Muscle characteristics of Dutch Warmblood foals with different genetic background at ages 6 and 12 months

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Summary

Reasons for performing study

To obtain broader insight into the muscle of foals with different genetic background, muscle fiber composition, its postnatal development and citrate synthase (CS) activity of the gluteus medius were investigated.

Hypothesis

Because muscle properties are influenced genetically and related directly to performance ability, muscle characteristics should be affected by selection and correspond with the requirements of the sport selected for.

Methods

The foals were divided into Group A, (n = 16), considered as an average of the population, and Group B (n = 36), especially selected for show jumping. Biopsies were taken from the deep part of the gluteus medius muscle. Fiber type identification was performed immunohistochemically at ages 6 and 12 months Citrate synthase activity was measured at age 6 months.

Results

At age 6 months statistically significant differences were found between Groups A and B in the proportion of type I, IIad and IId fibers. Oxidative fibers (I and IIad) were more abundant in Group B, while fast-glycolytic fibers (IId) were more abundant in Group A. Except for IIa, the fiber type composition at age 12 months had become equal in the two groups. The difference in the sum of oxidative fibers (I + IIad + IIad) between Groups A and B at age 6 months was similar to the difference in CS activity at that age, although the latter was not significant.

Conclusions

At age 6 months the two groups had opposite proportions of type I and IId, but these differences had disappeared at age 12 months.

Potential relevance

The muscular response to training at an early age can be interpreted with knowledge of muscle characteristics and its postnatal development in foals selected for show jumping.

4.1 Introduction

An optimally developed musculoskeletal system is essential for the performance of the horse. Two of its characteristics, muscle fiber type composition and oxidative capacity are related directly to performance and athletic ability [1]. Moreover, they are partly determined at birth due to a predominant genetic influence [2].

The muscle fiber type composition is determined by the isoform of the myosin heavy chain (MyHC) expressed by the fibers, which accounts for the shortening velocity of the fiber and, therefore, the contraction speed of the muscle [3]. Equine muscle fibers are usually categorized as type I (slow oxidative), type IIa (fast oxidative-glycolytic), type IId (fast glycolytic) fibers, and a hybrid fiber IIad [4-6]. The postnatal development of muscle is associated with changes in innervation and therefore MyHC expression [5,7-13]. At mature age, the relationship between performance and athletic ability is expressed in the fastest sprinters by a high proportion of type II fibers, as seen in Quarterhorses and Thoroughbreds [7] and in elite endurance horses by a high percentage of type I and IIa fibers [14].

The oxidative capacity of the muscle is one of the factors that contributes to the resistance against fatigue. An indicator for the oxidative capacity of skeletal muscle is citrate synthase (CS), which catalyzes the conversion of acetyl coenzyme-A and oxaloacetate into coenzyme-A and citrate in the citric acid cycle during energy production. The activity of CS is correlated positively with the proportion of type I fibers and negatively related to type IId fibers [15,16].

The Dutch Warmblood horse is very popular in different sport disciplines. The Royal Warmblood studbook of the Netherlands (KWPN) has different selection directions, such as dressage and show jumping. Selection uses indices based on the linear scoring of certain conformational traits and sport specific performance traits [17]. Interpreting the effect of selection in young horses would be easier with knowledge of the muscle properties just after birth and during the first year of life.

The aim of the present study was to investigate the *gluteus medius* muscle of

the Dutch Warmblood foal, because of its major propulsive and powerful role during exercise, to determine (1) if muscle characteristics are affected by the selection criteria used in this studbook and (2) if these characteristics correspond with the requirements of the sport for which they are selected. Therefore, MyHC distribution as a measure of contraction speed during postnatal development and oxidative capacity as a measure for the resistance to fatigue were compared in muscle biopsies of two groups of foals with different genetic background after weaning until age one year.

4.2 Material and methods

4.2.1 Horses

All foals (n = 52) were from the Dutch Warmblood breed (KWPN). Group A (n = 16: mean body weight at age 6 months, 257 ± 20 kg and at age 12 months, 369 ± 25 kg) was used in a study focusing on the effects of exercise at a very early age on the equine musculoskeletal system [18]. Group B (n = 36: mean body weight at age six months, 304 ± 33 kg and at age 12 months, 403 ± 47 kg) participated in a project to develop scientific criteria for the selection and effective and injury free training of show jumpers and were, especially, selected based on their pedigree index for showjumping [19]. Both groups were housed at the research station for Animal Husbandry in Lelystad and were fed concentrates and grass silage. Water was available ad libitum. All procedures were reviewed and approved by the Animal Experiments Committee of Utrecht University.

4.2.2 Muscle biopsies

Biopsies were taken percutaneously from each foal by the same individual according to the protocol of Lindholm and Piehl [5,20] at age 6 and 12 months using a Bergström needle[1] with an inner diameter of 4 mm. Biopsies were taken from the deep *gluteus medius* muscle 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, as deep as possible (until resistance from the iliac wing). All biopsies were taken under local anaesthesia with

lidocaïne (Lidocaïne 2% HCl)[2]. All samples were stored at –80 ºC until analyzed.

4.2.3 Immunohistochemistry

To identify the fiber types according to their MyHC content specific monoclonal antibodies (Mabs) were used [5]. Mab 219-1D1 (1:25) reacts with type I. Mab 332-3D4 (1:10) reacts with type IIa and IId. Mab 333-7H1 (1:10) reacts with type IIa. Mab 412-R1D5 (1:25) reacts with type I and IId.

Transverse serial sections (10 mm) were made and further processed as described previously [5,21].

4.2.4 Analyses

A region of at least 200 contiguous fibers was chosen for fiber typing and calculation of fiber type composition. The muscle fibers were classified into type I, type IIa, type IIad and type IId according to the reaction with the Mabs.

4.2.5 Biochemical analyses of citrate synthase activity

Muscle biopsy specimens of all foals were disintegrated with a dismembrator,[f] in a buffered solution of 8.6% sucrose, 2.3mM EDTA, 0.01M Tris, 2M HCl and 25,000 units of heparin (pH 7.4) and stored on ice. Mitochondria were destroyed by freezing and thawing alternately to release the enzyme. CS activity was measured in duplicate with a spectrophotometer[g] (25°C, 412 nm, 9 minutes). Measurements were done in 1mM 5,5'-dithio-2-nitrobenzoate in 1M Tris (pH 8.1) mixed with 7.5mM acetyl co-enzyme A and 2% Triton X-100, equilibrated in a cuvette. Homogenate (1%) was added and mixed, to establish background activity. Oxaloacetate (10mM in 0.1 M Tris, pH 8.0) was added after 2 minutes and measurement continued. CS activity is measured as the amount of enzyme in 1 ml of homogenate that converts 1 nmol of oxaloacetate/min. To standardize results, enzyme activity is expressed in mU/mg of protein content of the homogenate,[22].

4.2.6 Statistics

Statistical analyses were carried out with SPSS 12.0.1 for Windows using GLM repeated measures with factors age (within) and group (between). Data are expressed as mean ± s.d.. $P < 0.05$ was accepted as a significant difference.

Analysis of error sources in fiber type counts was estimated according to Weijs *et al*. [23].

4.4 Results

Mean frequencies of the four fiber types, type I, IIa, IIad and IId of the gluteus medius of the two groups of foals at ages 6 and 12 months are shown in figure 4.1.

At age 6 months there was a significant difference between the two groups in the proportion of fiber type I, IIad and IId. The proportion of type I and IIad in Group B were significantly higher than in Group A (36.0 ± 13.9 vs. 24.4 ± 7.8 and 21.8 ± 8.5 vs. 15.5 ± 8.0 respectively), whereas the percentage of type IId fibers was higher in Group A than in Group B (37.8 ± 12.1 vs. 21.3 ± 11.2). At age 12 months MyHC composition of the two groups had become equal, except for the proportion of fiber type IIa, which was now higher in Group A than Group B (29.4 ± 9.9 vs. 21.2 ± 8.0).

Changes of the different fiber types age 6 to 12 months showed different pattern in the two groups. In Group A fiber types I and IIa increased significantly, while the proportion of type IId decreased. In contrast, Group B showed a significant increase of the percentage of IId fibers. Concomitant with this increase of IId, the IIad fibers showed a significant decrease in this period.

CS activity, expressed in mU/mg protein, showed no significant difference between the two groups (132.5 ± 38.5 in A vs. 153.8 ± 45.6 in B) at age six months.

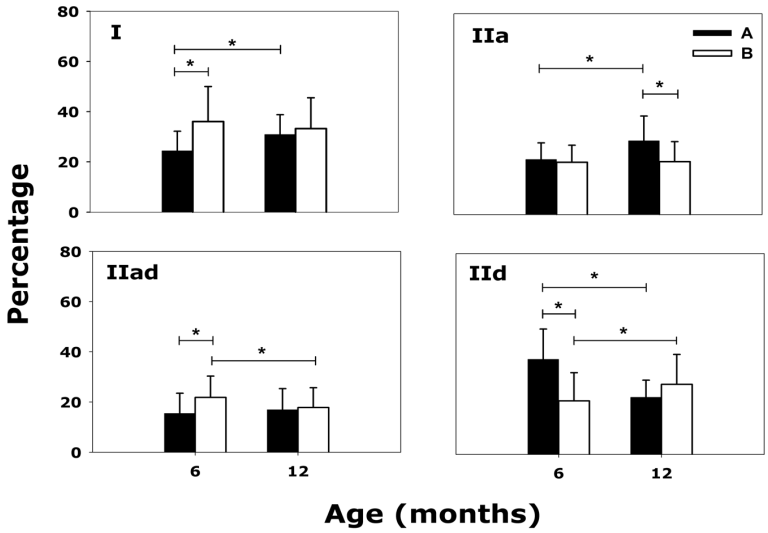

Figure 4.1

Muscle fiber composition of the *gluteus medius.* Mean ± s.d. Frequencies of type I, IIa, IIad and IId fibers at a half-year and one year of age. * $p < 0.05$.

4.5 Discussion

A remarkable finding in this study was the difference in muscle fiber type composition between the two groups at age six months, especially in the proportion of type I and IId fibers. Although belonging to the same breed, foals of Group B showed a more aerobic fiber type composition of the *gluteus medius* than foals of Group A. However, this difference in MyHC fiber type composition disappeared during the period to age 12 months.

The difference between two groups of the same breed in MyHC fiber type composition, especially in the proportion of type I, at age 6 months was surprising, because of the expected predominant genetic influence on skeletal muscle fiber composition, especially on proportion of type I muscle fibers [2]. Differences in the proportion

of type I fibers within a breed were previously reported in Thoroughbreds and Andalusian horses, of which the latter was due to a significant influence of the maternal bloodline of the breed [24,25]. However, based on previous studies [14,26,27], it was expected that if (genetic) differences were present, the MyHC fiber type composition at age 6 months would be in accordance with the sport for which the horses were selected. Group B, selected for show jumping based on their pedigree index, would be expected to have a high proportion of fast-twitch, oxidative fibers (IIa) [28] or a more balanced proportion of type I (27%), IIA (36%) and IIX (37%) [29], but they were found to have a high proportion of type I fibers. This discrepancy can probably be explained by the fact that the horses in the afore mentioned studies were mature. The high amount of type I fibers in Group B at age 6 months in the present study does not correspond with the sport selected for, but could still be a result of the selection based on traits.

It is possible that this same difference in selection accounted for the difference in postnatal development. For different equine locomotory muscles several authors describe an increase in the proportions of fiber type I and/or IIA (IIa) at the expense of the fiber types IIA/IIX (IIad) and/or IIB or IId/x fiber population in the early postnatal period and during the period of young maturity [7-13,30]. In the present study Group A showed the same pattern in development as in most studie, but Group B had a small increase in the proportion of type IId with a simultaneous decrease in the transitional fibers, type IIad. Also within the development of Thoroughbred foals, contradictory findings have been reported. Eto *et al.* [11] and Yamano *et al.* [12] found an increase of the IIA muscle fibers with a simultaneous decrease of the proportion IIA/IIX or IIX fibers, while Bechtel and Kline [31] and Thornton and Taylor [32] concluded that foals of the same breed enhanced their anaerobic, rather than their aerobic, component of muscle metabolism during the first months of life.

However, during the second half-year in the present study the difference in MyHC fiber type composition at age 6 months disappeared resulting in an almost equal fiber distribution at age 12 months. This might indicate that the fiber type composition at age

12 months is a distribution, optimal for the breed at that time. If the muscle continues to develop in this direction in Group B (an increase of IId) after age 12 months, optimal fiber distribution for their selected sport might be reached. Conversely, if consolidation of the MyHC fiber type composition at 6 months benefits future performance, the question arises of whether this can be achieved by interfering in the developmental process by training in this period regardless of the muscle fiber distribution expected.

The total oxidative capacity, reflected in CS activity, at age 6 months was not significantly different between the two groups, although there is a tendency for a higher value in Group B. Generally, type I and IIa show a higher oxidative capacity than type IId/x fibers [6,11,12,16,33]. Therefore, it was expected, due to the significant difference in muscle fiber distribution, that a significantly higher CS activity would occur in Group B. However, if the sum of all oxidative fibers (type I, IIa and IIad) is calculated for Groups A and B, a difference of around 16% appeared (Figure 4.2, left). This is in the same trend and range as the relative difference in CS activity between the groups (Figure 4.2, right).

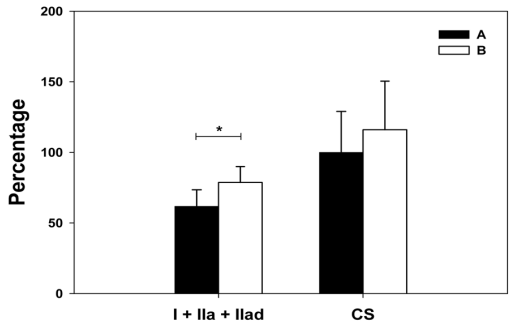

Figure 4.2

Mean of the sum of frequencies of the oxidative fibers (type I, IIa and IIad) and citrate synthase activity expressed as percentage of the mean of group A of the *gluteus medius.* * $p < 0.05$. Mean values for the citrate synthase activity, expressed in mU/mg protein, are 132.5 ± 38.5 (A) vs. 153.8 ± 45.6 (B).

In conclusion, there is a difference in MyHC expression and development within the Dutch Warmblood breed, suggesting an influence of the studbook selection, but at this young age not related to the sport selected for. However, if the muscle continues to develop in these directions after age one year optimal fiber distribution might be reached. Further research of older horses is necessary to understand fully the developmental changes in the gluteus medius of the Dutch Warmblood and whether it is possible to interfere with this natural process by training at young age.

4.6 Acknowledgements

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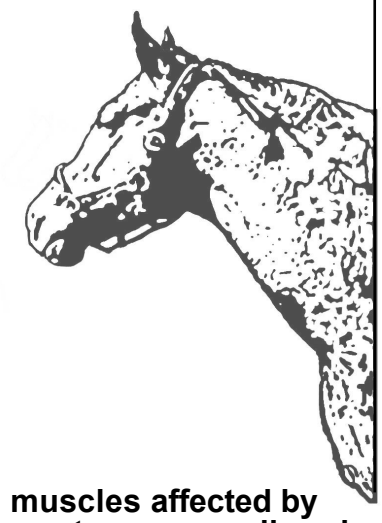
Chapter 5



Na^+, K^+, ATPase content in equine skeletal muscles affected by lower motor neuron disorder

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Submitted for publication

Summary

Objective

The study aimed to investigate whether changes in muscular contractility and morphometry are associated with altered Na^+, K^+-ATPase content in locomotory 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, severe symptoms and euthanized, B: LMND, moderate symptoms and 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 the vastus lateralis muscle and the Na^+, K^+-ATPase content was studied in biopsies of the vastus lateralis and pectoralis descendens muscle.

Results

An increased Na^+, K^+-ATPase content was found in the vastus lateralis muscle of the horses of Group A. This was accompanied by a significant transition in MyHC fiber type composition from slow to fast by a decrease in type I and a concomitant increase of type IIad together with an atrophy of type I fibers. Group B did not show any differences in any of the parameters compared to Group C.

Conclusions and Clinical Relevance

The slow to fast transition in muscular contractility in locomotory muscles of horses with LMND is associated with an increased Na^+, K^+-ATPase content. Measuring Na^+, K^+-ATPase content can be used to support the ante mortem diagnosis of LMND. It is recommended to repeat this study with an increased number of horses to explore further possibilities.

5.1. Introduction

Lower motor neuron disorder (LMND) has been found in horses suffering from equine motor neuron disease (EMND) and equine grass sickness (EGS). EMND 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 myosin heavy chain (MyHC) type I fiber atrophy or type I and type II fiber atrophy [3,4]. In general, the reduced neuromuscular activity is expected to induce a slow-to-fast transition of myosin-based fiber types [5]. Also, EMG analysis of horses with LMND reveals neurogenic abnormalities. This is demonstrated by pathological spontaneous activity, as a result of membrane instability due to denervation, and abnormal motor unit action potentials (MUP)[6,7].

The action potentials causing excitation of the muscle are elicited by an influx of Na^+ through Na^+-channels, followed by an efflux of K^+ leading to an increase in intracellular Na^+ and extracellular K^+ concentration. This activates Na^+, K^+-ATPase, a ubiquitous enzyme in the plasma membrane of the muscle fiber, that 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 content is influenced by multiple factors including denervation, fiber type, and muscular and neurodegenerative disorders [9,10]. As described above, LMND in horses is associated with denervation and reinnervation of muscle fibers and consequently with changes in MyHC fiber type composition and fiber area. It is not known whether this process influences the Na^+, K^+-ATPase content of

affected muscles.

The aim of the present study was to investigate (1) whether changes in muscular contractility and morphometry are accompanied by altered Na^+, K^+-ATPase content in muscle of horses with (suspected) LMND, (2) whether measurement of Na^+, K^+-ATPase content combined with muscle contractility and morphometry is useful as evidence to support the ante mortem diagnosis of LMND and (3) whether the alteration in Na^+, K^+-ATPase content is general or muscle specific. Therefore, MyHC fibre type composition and cross sectional area (CSA) were measured in biopsies of the vastus lateralis muscle and the Na^+, K^+-ATPase content was studied in biopsies of the vastus lateralis and pectoralis descendens muscle. All parameters were measured in horses diagnosed with LMND or suspected of LMND based on clinical examination, EMG analysis and/or neuropathology.

5.2 Material and methods

5.2.1 Animals

The total study group consisted of 10 Dutch Warmblood horses (table 5.1). 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) were euthanized, because of the severe clinical symptoms. For Group A the diagnosis was confirmed after additional post mortem neuropathological examination. Two horses (Group B) showed moderate clinical

symptoms and were therefore not euthanized. Five clinically healthy Dutch Warmblood horses were used as control horses (Group C).

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

Table 5.1 Details of all horses

Case no.	Group	Age (years)	Sex	Body weight (kg)	Clinical signs	
					(months)	degree
1	A	2	S	402	0,5	s
2	A	8	G	416	1	s
3	A	13	M	470	4	s
4	B	14	G	443	4	m
5	B	15	G	594	unknown	m
6	C	10	M	571	x	x
7	C	10	M	598	x	x
8	C	11	M	619	x	x
9	C	11	M	575	x	x
10	C	12	G	593	x	x

Table 5.1

Group A: LMND, severe symptoms and euthanized, Group B: LMND, moderate symptoms and not euthanized and Group C: Control., S: Stallion, G: Gelding, M: Mare, x: not applicable, s: severe, m: moderate.

5.2.2 Muscle biopsies

All biopsies were taken percutaneously by the same person using a Bergström needle[a] with an 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.

5.2.3 Immunohistochemistry

Immunohistochemistry on biopsies of the vastus lateralis muscle was performed with monoclonal antibodies (Mab) specific to MyHC isoforms in order to differentiate 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 mm) 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 fibre 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.

5.2.4 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.

5.2.5 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 [11] in biopsies of the vastus lateralis and pectoralis descendens muscle. The obtained values correspond to the total population of functional Na^+, K^+ pumps [9,12]. Briefly, a ouabain concentration of 10^{-6} M was used, allowing saturation of the major part of the total number of ouabain binding sites [11]. Biopsies were incubated (120 min, 37°C) in buffer containing ^{3}H-ouabain (0.6 ìCi/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.

5.2.6 Statistics

Statistical analyses were carried out with SPSS 12.0.1 for Windows using an independent t-test for differences between the three groups of horses. A GLM repeated measures was used for differences between muscles with factors muscle (within) and group (between). Pearson's bivariate correlation was performed to test if Na^+, K^+-ATPase content of the vastus lateralis and the pectoralis descendens muscle were correlated to each other. Mean values are expressed as mean ± s.e., a significant difference was accepted when $p = 0.05$.

5.3 Results

The mean frequencies and CSA of the four MyHC fiber types, type I, IIa, IIad and IId of the vastus lateralis of the three groups of horses are shown in table 5.2.

Significant differences in the proportion of MyHC fiber type I and IIad were found between Groups 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 5.1) of the vastus lateralis muscle was significantly higher in Group A compared to Group B (166 ± 18 vs. 120 ± 10 pmol/g wet wt, p<0.05). This significant difference was not found in the Na^+, K^+-ATPase content of the pectoralis descendens muscle. On the other hand, a significant relationship in Na^+, K^+-ATPase content was found between both muscles (r = 0.78, p<0.01).

There were no differences in MyHC fiber type proportions, CSA and Na^+, K^+-ATPase content between Groups B and C. In Group A compared to Group B the proportion of type I was lower, while the proportion of type IId was higher. The CSA of type I was significantly smaller.

Table 5.2 Myosin heavy chain composition (MyHC) and cross sectional areas (CSA) in vastus lateralis muscle

		MyHC (%)					CSA (μm²)				
Case no.		I	I/IIa	IIa	IIad	IId	I	I/IIa	IIa	IIad	IId
1	A	5	x	21	35	40	1186	x	1670	1895	2559
2	A	25	19	4	33	20	1886	3995	4126	4286	4581
3	A	2	x	8	55	35	1512	x	2792	2437	3079
4	B	44	x	20	31	5	3855	x	3703	3756	4976
5	B	46	x	30	18	6	3630	x	3979	3420	2726
6	C	55	x	29	12	4	4525	x	5535	4610	3896
7	C	36	x	14	21	29	4824	x	4651	3961	4715
8	C	51	x	15	21	13	6520	x	6532	5047	5074
9	C	48	x	20	29	2	3313	x	3443	3287	2573
10	C	47	x	9	23	21	3724	x	3509	3715	3664

Table 5.2

Group A: LMND, severe symptoms and euthanized, Group B: LMND, moderate symptoms and not euthanized and 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.

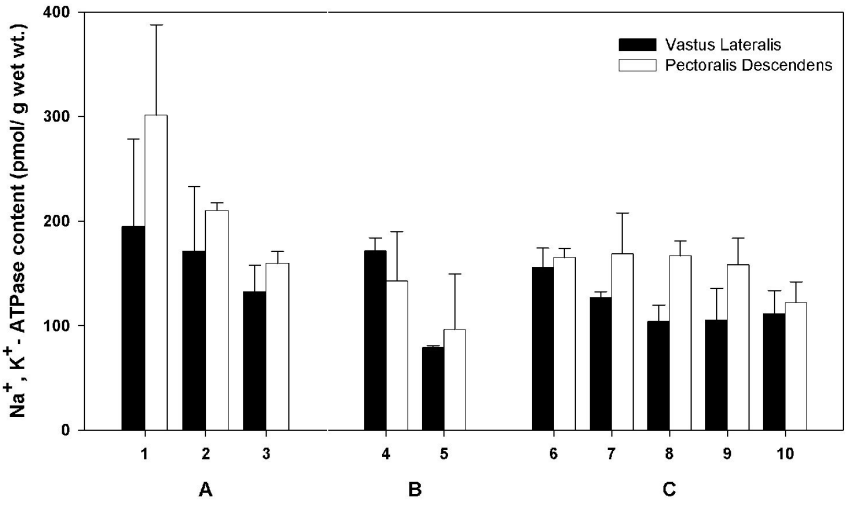

Figure 5.1

Individual values for Na^+, K^+-ATPase content in biopsies of the vastus lateralis muscle (black bars) and pectoralis descendens muscle (white bars) in Group A: LMND, severe symptoms and euthanized, Group B: LMND, moderate symptoms and not euthanized and Group C: Control. For each biopsy the Na^+, K^+-ATPase content was determined on 4 samples. Values are expressed as mean ± s.e per individual horse.

5.5. Discussion

In the present study we investigated whether the changes in muscle histopathology of horses with the neuromuscular disorder LMND is associated with an altered muscular Na^+, K^+-ATPase content. The study included three groups of horses; Group A: LMND, severe symptoms and euthanized, Group B: LMND, moderate symptoms and not euthanized and Group C: control horses. An increased Na^+, K^+-ATPase content was found in the vastus lateralis muscle of the horses of Group A compared to Group C. This was associated with a transition in MyHC fiber type composition from slow to fast and atrophy of type I fibers. Group B did not show differences in any of the parameters compared to Group C.

The MyHC composition of the vastus lateralis muscle of Group A in present study

demonstrated a transition from slow to fast by a decrease in type I fibers and concomitant increases of type IIad and IId 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 [4,13]. 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 [5,14-16]. Probably, both events have occurred in horses of the present study, because the EMG analyses revealed in both muscles denervation and reinnervation patterns.

An increased Na^+, K^+-ATPase content of approximately 40% accompanied the changes in muscular contractility and morphometry in the vastus lateralis muscle of Group A. This upregulation of the Na^+, K^+-ATPase content was also found in humans with lower motor neuron disorder [10]. An explanation for this increase 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]. The Na^+, K^+-ATPase content of the pectoralis muscle of Group A was also up regulated, although not statistically significant. However, the increase in Na^+, K^+-ATPase content in both muscles is around 40% compared to Group C. Together with the found correlation, this indicates a general effect on the locomotory muscles.

Remarkably, the changes in MyHC fiber type composition, CSA and Na^+, K^+-ATPase content were not found in the horses of Group B, although EMG analyses showed generalized neuropathy. One horse with LMND (case 5) showed even a decreased Na^+, K^+-ATPase content, which was not accompanied by changes in fiber type composition and CSA. There was no history of inactivity in this case, which could cause down regulation of Na^+, K^+-pumps [9]. Both horses were still active at the time of the experiment and hence it is possible that both horses were in a stage of the disease before changes in muscular parameters appeared. The moderate symptoms also indicate that they could suffer from

EGS caused by mild lower motor neuron degeneration.

The results of Groups A and B 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 [9,17,18]. 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 [18,19]. The Na^+, K^+-ATPase content remains around 100 pmol/g wet wt. from age two to sixteen years in the gluteus medius [19].

In summary, we have shown that the slow to fast transition in muscular contractility and morphometry is accompanied by an increased Na^+, K^+-ATPase content in muscle of horses with LMND. Measuring Na^+, K^+-ATPase content, a relatively easy measurement to perform, can be used to support the ante mortem diagnosis of LMND.

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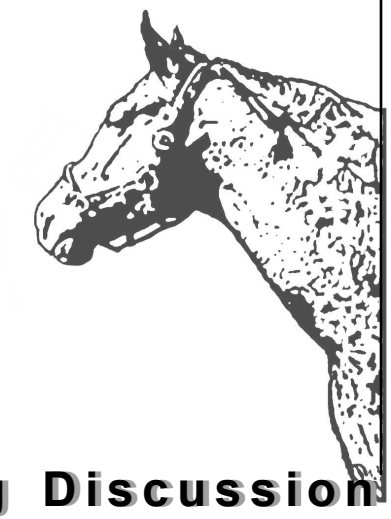
Chapter 6



Summarizing Discussion

6.1 General Discussion

After the birth of the foal, a period of rapid growth starts. The adaptations that occur in the locomotory muscles in this postnatal period depend on the original state of the muscle, which is partly determined by genetic influence [1,2]. Moreover, the postnatal development can be affected by various external influences including, among others, training.

The locomotory muscles are an important determinant of the athletic ability of the horse and hence the ability to compete at high level. A better understanding of the equine muscular system and its ability to adapt to different (patho)physiological conditions would provide information allowing optimal use of the potential of the horse. To expand the present knowledge on equine muscular properties the results of the previous chapters are integrated by answering four major questions.

1. What are the functional adaptations of equine muscle, reflected in power and fatigue resistance, during postnatal development?
2. Is exercise at a young age an important factor for the development of locomotory muscles and does it have any beneficial effects on performance ability?
3. Is the expected breeding value of a horse reflected in the contraction speed of its muscles and does this correspond with the requirements of the sport it is selected for?
4. How is the membrane excitability of a muscle affected by lower motor neuron disorder?

6.2 What are the functional adaptations of equine muscle, reflected in power and fatigue resistance, during postnatal development?

Power output is generated by a combination of velocity of shortening, i.e. contraction speed, and force of the muscle, determined by the myosin heavy chain (MyHC) fiber type composition and cross-sectional area (CSA), respectively, of the different fibers. From birth to age three years, the majority of the power enhancement of the muscle was caused by the almost six-fold increase in CSA of all fiber types (chapter 2 and 3). In particular, from birth to age one year the highest levels of growth rate (five-fold) occurred. The increase in CSA due to development is in line with previous publications [3-5]. This increase in force reflects the requirements for increased load due to the increase in body weight (figure 6.1).

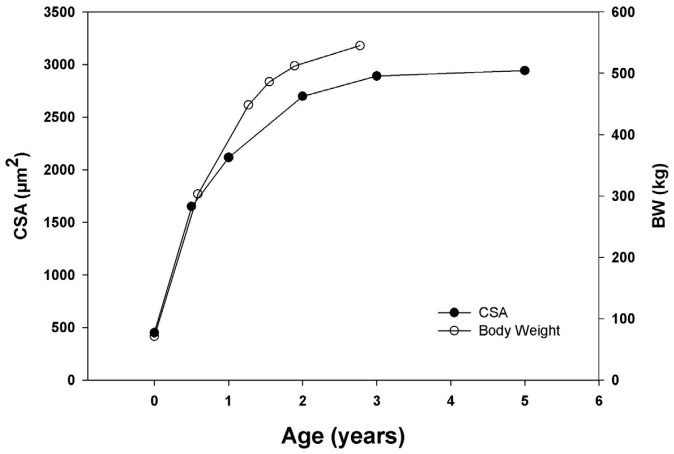


Figure 6.1

Mean fiber cross sectional area (left axis) of the gluteus medius muscle and mean body weight (right axis) of foals (n=20) of the Dutch Warmblood breed from birth until adulthood.

In the first year after birth the phase of extreme rapid growth was also reflected in the postnatal development of the MyHC fiber type composition, because adjustments occurred predominantly in the extreme fiber types, MyHC type I (slow-oxidative) and IId (fast-glycolytic) (chapter 4). However, opposing developmental patterns were found in different groups of foals. The aerobic enhancement in muscle fiber type composition from birth until age six months was reported in a previous study [6,7] From age six months to one year, an aerobic enhancement in MyHC composition was shown in foals used in an experiment focusing on the effects of exercise on the equine musculoskeletal system [8]. In this same age period, an anaerobic enhancement in fiber type distribution was found in foals used in a project for developing scientific criteria for selection and injury free training. This interesting difference in the development of the MyHC fiber type composition between foals could partly be explained by the stage of development, regarding the fast-slow spectrum, during the first year of life [1]. Apparently, differing age related functional requirements for the muscle can be met in different ways, because both developmental patterns resulted in an almost equal fiber type distribution at age one year.

From age one until three years, the changes in MyHC fiber type composition only affected the proportions of the intermediate fibers, type IIa and IIad (chapter 2). At the age of three years these adaptations resulted at age three years in a composition, which consisted of a large proportion of aerobic fibers without extensive loss of contraction speed, the MyHC type I and IIa fibers. Especially MyHC type IIa is considered an efficient fiber type due to its contractile and metabolic qualities, i.e. fast and aerobic. The shift towards an aerobic fiber type composition is in agreement with previous studies on developmental changes, which report an increase in the proportions of fiber type I and/or IIA (IIa) at the expense of the fiber types IIA/IIX (IIad) and/or IIB or IId/x fiber population in the early postnatal period and during the period of young adulthood for different equine locomotory muscles [3-5,9-12].

The presence of the hybrid fiber IIad in the MyHC fiber type composition and its

contribution to the developmental changes suggest transitions into type IIa or IId fibers according to the 'nearest-neighbour' rule for MyHC fiber type transitions [5,7,13]. The prevailing view is that hybrid fibers enable a muscle to fine-tune the wide range of forces and velocities it has to generate [14]. The transitions are probably regulated differently in the different age groups due to changes in innervation pattern or protein turnover rate. Evidence in support of this idea is provided by studies by Eizema et al. [15,16] who showed that hybrid fibers express both isoforms or just type IId mRNA in muscle of foals and express predominantly type IIa mRNA in muscle of adult animals. The hybrid fibers are probably converting to the type corresponding with the expressed mRNA and with increasing age this development proceeds more towards a slower and more aerobic type of MyHC. This is supported by the aerobic development seen up to age three years in MyHC composition in the present thesis.

The increase in power output of the muscle during development will, due to the larger energy demands, require adjustments in the properties that contribute to fatigue resistance. To resist fatigue and to sustain power output, the muscle depends on oxygen delivery by capillaries and on different enzymes for its energy metabolism and maintenance of excitability [17,18]. From birth until age three years the diffusion index of the muscle shows that due to the growth of fibers the area a single capillary has to supply is enlarged, because the proliferation of capillaries lags behind this increase in muscle size as reported in rats [19,20]. This implies a disadvantage for the oxygen supply. However, during this period the number of capillaries per fiber is also increased, together with an enhancement of another component of fatigue resistance: the activity of the mitochondrial enzymes, succinate dehydrogenase (SDH) and citrate synthase (CS). The improvements in oxidative capacity are in agreement with other developmental studies on young horses [12,21]. Although, these studies showed enhancement of SDH activity in all fibers, the present study showed decreased activity in type IId decreased. This fiber type dependent effect on the oxidative capacity could be due to differences in the recruitment of the different motor units, which depend on the activity level of the muscle [17,22]. Besides

enhancement in the enzyme activities for energy metabolism, there was also improvement in another component of fatigue resistance, the maintenance of excitability. From weaning the Na^+, K^+-ATPase content (measured as [^{3}H]ouabain capacity and expressed in pmol/g wet wt) decreased to a plateau at three years of age, which is in agreement with earlier studies in rats, mice and horses [23,24]. This decline can be explained by the increased CSA, reflecting the larger fiber dimension and ensuing reduction in surface-to-volume ratio [18]. Hence, when extrapolating this to the whole muscle the number of Na^+, K^+-pumps show an age-dependent rise [23].

In conclusion, equine muscle in general shows an improvement in power and fatigue resistance during postnatal development reflecting the increased load due to increased body weight. Remarkably, the adaptations in power output are hardly the result of changes in contractile activity, i.e. myosin heavy chain (MyHC) fiber type composition, but mainly due to the increase in force, i.e. cross sectional area (CSA). Fatigue resistance was enhanced by the proliferation of capillaries and improvement of oxidative capacity and membrane excitability.

6.3 Is exercise at a young age an important factor for the development of locomotory muscles and does it have any beneficial effects on performance ability?

In the present thesis the effects of different exercise regimens on the locomotory muscle were studied and compared to pasture exercise (chapter 2 and 3). Pasture is considered to be the optimal environment for development as previously reported for other components of the musculoskeletal system [25]. From birth to almost one year of age the importance of exercise for the muscular development was investigated. In addition, from weaning to three years, the influence of specific jumping training on the locomotory muscle and any possible beneficial effect on performance were studied.

During the first year of the postnatal period, withholding exercise, by confinement to a box stall, had profound effect on the development of the muscle. Concerning the

properties determining the power output, there was a larger gain in CSA than occurred during normal development, while there was no effect on the MyHC fiber type composition. The latter finding has already been reported in a previous study [6]. It is, however, questionable if the adaptation in CSA meant an actual enhancement in power output, because it was not associated with an increase in fatigue resistance. This diminished due to an increase in diffusion index (the area one capillary has to supply becomes larger) without a proportional increase in proliferation of fibers and a concomitant decrease in SDH activity, which was most evident in MyHC fiber type IId. Apparently, restriction to a box stall only places a demand on the postural role of the muscles, which requires the recruitment of the aerobic fibers [17,22,26]. The larger increase in diffusion index caused by withholding of exercise persisted when resuming a normal activity pattern by paddock exercise after 22 weeks. To determine if the (lasting) effect of the different regimes on the gluteus medius muscle was a general phenomenon, it was compared with the diffusion index of another important propulsive locomotory muscle, the semitendinosus muscle (chapter 3). Also in this muscle free exercise did not restore the previously induced changes. Nevertheless, the importance of exercise was emphasized by the results of the supplemented training. This prevented all the negative effects that occurred during box stall confinement and eventually showed the same results as pasture exercise.

Considering the findings that exercise is important for an optimal muscular development it was interesting what the effect would be on the locomotory muscles when foals are subjected to a special training protocol for a particular sport like show jumping for example. In general, training enhances power and fatigue resistance of a muscle [17,18], also, in foals or adolescent horses [3,12,21,27,28]. It has already been reported that specific jumping training resulted in a jumping technique that was more balanced and required less force impulses generated by the hind limbs compared to the technique of inexperienced horses [29]. Despite these findings the effect of training was not found on the muscular parameters, except for an enhancement in membrane excitability and maintenance of force. The latter findings provides evidence that the training was intense

enough to initiate a reaction in the gluteus medius muscle. The question that remains is whether the upregulation in Na^+, K^+-ATPase content is beneficial and will show an additional increase after conventional training has started at the age three years. If so, these horses would have an advantage in excitability compared to the previuosly untrained horses. However, it was shown that the initial advantage in jumping technique due to early training disappeared when the inexperienced group also started training [29]. The absence of training effects in the other properties could be because an aerobic fiber type composition was already present when training started or the adaptations due to the maturation process were sufficient to meet the demands of exercise. On the other hand, it was remarkable that the proportion of MyHC type IId showed a tendency to decrease due to training. Apparently, the intra- and inter-individual variation in the MyHC fiber type composition of horses of the studied breed is rather large. The broad genetic background of the breed due to crossbreeding can partly explain the variation between horses.

In summary, exercise is an important factor for normal muscle development, especially for fatigue resistance. Furthermore, specific jumping training at a young age, which resulted in a positive effect on the jumping technique, induces an increased Na^+, K^+-ATPase content, which is important for the maintenance of excitability and the force of the muscle. The absence of training effects in the other muscular parameters can partly be explained by the presence of an appropriate (aerobic) fiber type composition at the start of training. In addition, it also suggests that the developmental changes in muscle represent sufficient adaptation to meet the demands of the training. Finally, the benefit of training for show jumping seems to be gained at the level of coordination and balance rather than at the level of muscle characteristics.

6.4 Is the expected breeding value of a horse reflected in the contraction speed of its muscles and does it correspond with the requirements of the sport it is selected for?

An interesting finding was the difference in contraction speed between the muscles of two different experimental groups of six month old Royal Dutch Warmblood foals (chapter 4). One group of foals was especially selected for show jumping, based on their expected breeding value, above 110 (average value 100). This value is based on a 'four trait model' of sport specific performance traits of the horse's relatives and is used to estimate the genetic disposition for show jumping. The other group of foals was offspring of stallions that would not be selected for the studbook for breeding, because they could not meet the selection criteria to be an approved stallion. Therefore, the expected breeding value of these foals would probably be far below 100. In addition, all the stallions had osteochondrosis.

The difference in MyHC composition, a measure for contraction speed, at six months between the two groups of foals was most evident in the proportion of type I and IId fibers. This difference in composition within a breed was surprising because of the expected strong genetic influence on skeletal muscle fiber composition [2]. Differences in the proportion of type I fibers within a breed were also found in Thoroughbreds and Andalusian horses, of which the latter was due to a significant influence of the maternal bloodline of the breed [26,30]. However, based on previous studies [31,32], it was expected that if (genetic) differences were present, the MyHC fiber type composition at age six months would be in accordance with the sport the horses were selected for.

The horses that were especially selected for show jumping showed MyHC fiber type frequencies of type I (35%) and IId (25%) from weaning until age three years (chapter 2). In a previous study, it was reported that the MyHC fiber type composition of adult show jumping horses showed the following distribution of type I (27%), IIa (36%) and IId (37%) [33]. An explanation for this discrepancy could be a difference in sampling depth, the proportion of type I fibers increasing and type II fibers decreasing with depth [17] or training

status [33]. On the other hand, the suggestion that show jumpers need a high proportion of type IIa and/or IId need not necessarily be as important as presumed.

Another muscular feature for show jumpers, which is considered to be essential, is a large CSA of the fast fibers, because of the need of powerful and explosive contractions to clear a fence [33,34]. In this thesis the CSA of the individual fiber types especially the type IId fibers (2200 – 4500 ìm^2 vs. 3000-7000 ìm^2) of the horses was relatively small compared to other results [3-5] (chapter 2). Again, this could be due to differences in sampling depth [35] or training status [33]. It was reported, however, that specific show jumping training resulted in a jumping technique, that was more balanced and required less force impulses generated by the hind limbs at take-off compared to inexperienced horses [29,36]. Furthermore, the majority of the power during take-off comes from reutilization of the elastic energy in tendinous tissue [37,38]. This implies that large CSA's are not necessary for a good show jumping performance.

In conclusion, the expected breeding value used by the Royal Dutch Warmblood studbook is reflected in the contraction speed of the muscle at age six months. However, at an older age the selected horses can no longer be discriminated from the inferior population, because both groups developed an almost equal fiber type distribution. The MyHC fiber type composition and the CSA are not in accordance with earlier reported requirements for show jumping due to their aerobic character (slow and small). The muscular requirements for show jumping are probably more diverse than previously thought.

6.5 How is the membrane excitability of a muscle affected by lower motor neuron disorder?

Membrane excitability is affected by, amongst others, age and alterations in neuromuscular activity, including training and neurodegenerative disorders [18,39]. The influences of the first two factors, age and training, on equine muscle were already reported in this thesis (chapter 2) and earlier reports [6,24,28]. The effect of a

neurodegenerative disorder on the membrane excitability of equine muscle was described for the first time in the present thesis (chapter 5).

In muscles affected by lower motor neuron disorder (LMND), degeneration of lower motor neurons results in the loss of neural input via the neuromuscular junction. As a consequence the muscles investigated in the present thesis demonstrated an upregulation of the Na^+, K^+-ATPase content with a concomitant transition from slow to fast MyHC fiber type composition. The increase in Na^+, K^+-ATPase content was also found in muscles of humans affected by a lower motor neuron disorder [39]. The upregulation can be partly explained by the transition towards a fast fiber type, because most data indicate that type II fibers contain more Na^+, K^+-pump than type I fibers, probably due to differences in passive Na^+, K^+-fluxes [18]. On the other hand, the age-dependent rise in the number of Na^+, K^+- pumps was accompanied by an opposite shift towards a more aerobic fiber type composition (chapter 2). This discrepancy can probably be explained by the increased sodium permeability due to denervation of fibers [40,41] in muscles affected by LMND, which causes depolarization of the membrane and subsequently an increased effort to maintain the resting membrane potential. The observed transition in myosin isoform expression in LMND affected muscles was in agreement with earlier reported results of LMND [42]. The transition from slow to fast are induced when neuromuscular activity is reduced, including denervation and when motor units that do not match the oiginal fibre type reinnervate denervated muscle fibers[13,43-45].

In conclusion, muscles affected by lower motor neuron disorder show an increase in membrane excitability together with an increase in contraction speed. These alterations in muscular properties cannot explain all the symptoms demonstrated by horses suffering from EMND or EGS. However, measuring Na^+, K^+-ATPase content can be used to support the ante mortem diagnosis of LMND.

6.6 Concluding remarks

This thesis demonstrates that the muscle of an immature horse is a heterogeneous tissue that is formed by a process of functional adaptation. Muscle biopsies are an important complementary tool for ante mortem diagnosis of lower motor neuron disorders, but are of limited value for the prediction of athletic potential, because the distinctive characteristics at a young age disappear throughout later development.

During development the muscle shows an enhancement in power and fatigue related properties. For an optimal development exercise in terms of movement is considered to be essential, especially during the first year after birth. On the other hand, training a horse before conventional training starts does not have huge benefits for muscle performance. Apparently, the developmental adjustments measured by the present variables represent sufficient adaptation to meet the demands of training. However, the enhancement in membrane excitability and maintenance of force proved that the training the foals were subjected to was intense enough to initiate reactions in the muscle. This means it is possible that alterations occur at another level than measured in the present thesis. Therefore, it would be very interesting to further explore the adaptations to (patho)physiological conditions on the molecular level where subtle changes can be detected, i.e. the expression of mRNA for the relevant proteins.

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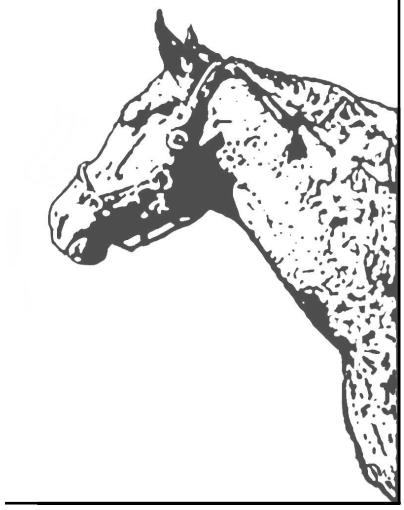
Samenvatting

Summary



Summary

The locomotory muscles represent an important determinant of the athletic potential of the horse and hence the ability to compete at a high level. For efficient raising and training, it would be useful to predict the potential of a horse early in life based on the characteristics of the locomotory muscles. These characteristics are partly determined at birth by genetic influences, but still undergo changes due to different demands, such as altered neuromuscular activity as occurs during development and training as well as in neurodegenerative disorders. Therefore, the aim of the present thesis was to obtain a better understanding of the equine muscular system and its ability to adapt to different (patho)physiological conditions. This would provide valuable knowledge to explore the possibilities for predicting the potential of the horse at a young age or to allow optimal preparation of the immature horse for performance at an adult age. For this purpose small biopsies were taken and investigated from various muscles from three different groups of horses at certain time points allowing (longitudinal) comparison of alterations induced by different exercise regimens or neuromuscular disease.

The characteristics of skeletal muscle in general can be described by parameters such as power output and resistance to fatigue. Power is the product of contraction speed and force generated by the muscle and is reflected in the myosin heavy chain (MyHC) fiber type composition and the cross-sectional area (CSA) of the different fibers. Resistance to fatigue of a muscle depends on oxygen delivery by capillaries (quantified as diffusion index: i.e. the area one capillary has to supply) and on a number of enzymes for maintenance of energy metabolism (citrate synthase (CS) and succinate dehydrogenase (SDH)) and excitability (Na^+, K^+–ATPase). The biopsies of the different horse muscles were analyzed at the individual fiber level for MyHC composition, CSA, diffusion-index and SDH activity. Measurements of Na^+,K^+-ATPase content were performed on intact biopsies, while the biopsy material was homogenized to determine CS activity.

From birth to age three years, the majority of the power enhancement of the muscle results from the increase in force due to the almost six-fold increase in CSA of all fiber types (Chapters 2 and 3). In particular, the interval from birth to age one year is interesting, because in that period the highest levels of growth rate (five-fold) occurred. This means that the muscles must produce considerably more force to bear and move the increasing body weight. Remarkably, the adaptations in power were not associated with changes in MyHC fiber type composition (Chapters 2-4). The increase in power output was accompanied by an enhancement in fatigue resistance as a result of the proliferation of capillaries and increase of enzymes of the energy metabolism and membrane excitability, indicated by the decrease in diffusion index and increases in SDH activity and Na^+,K^+-ATPase contents.

Training of adult horses generally aims at improving performance and delaying the onset of fatigue. Nevertheless, the outcome of training programs is not neccessarily the same as muscular adaptations depend on factors including the type and intensity of exercise, the original status of the muscle and the age of the animal. In young horses pasture is considered to be the optimal environment for development, as previously reported for other components of the musculoskeletal system, in particular bone and cartilage. In the present thesis the effects of different exercise regimens on locomotory muscles in young horses were studied and compared to pasture exercise (Chapters 2 and 3). It was found that exercise is essential for normal muscle development, especially for fatigue resistance. This was evident from the profound negative effect of box stall confinement on the diffusion index as compared to pasture exercise during the first year of the postnatal period. Withholding exercise resulted in a large increase of the diffusion index, which persisted after resuming a normal activity pattern. The supplemented training prevented all the negative effects that occurred during box stall confinement and eventually showed the same results as pasture exercise. When foals were subjected to a special training protocol for show jumping, training only resulted in a significant increase in Na^+,K^+-ATPase content, without effects on MyHC fiber composition, CSA, diffusion index or CS activity. This could partly be explained by the presence

of a sufficient aerobic fiber type composition (more than 30% slow-oxidative fibers) in this group of horses already at the start of training.

Genetic selection is used by breeding organizations to improve performance in dressage and show jumping. The strong genetic influence on skeletal muscle determines to a certain extent the contractile and metabolic properties of the muscle. When comparing two different experimental groups of Royal Dutch Warmblood foals: one group was especially selected for show jumping and one group was offspring of stallions that could not meet the selection criteria to be an approved stallion, there was a remarkable difference in contraction speed at age six months (Chapter 4). However, at an older age the two groups could no longer be distinguished, because both groups had developed an almost equal fiber type distribution. In contrast to previous studies the muscle fibers of the horses selected for show jumping in present study showed relatively small CSAs. This suggests that the muscular requirements for show jumping are probably more diverse than previously thought.

A variety of pathological conditions influence locomotory muscle. An example is the lower motor neuron disorder (LMND), a neurodegenerative disorder, which has been found in horses suffering from equine motor neuron disease (EMND) and equine grass sickness (EGS). In horses affected by LMND, degeneration of lower motor neurons results in the loss of neural input via the neuromuscular junction. As a consequence, the muscles (pectoralis descendens and vastus lateralis muscles) investigated demonstrated an upregulation of the Na^+, K^+- ATPase content with a concomitant transition from slow to fast MyHC fiber type composition (Chapter 5). The upregulation could be partly explained by the transition towards a fast fiber type, because most data from literature indicate that fast fibers contain more Na^+, K^+- pumps than slow fibers. Another explanation could be the increased sodium permeability due to denervation of fibers in affected muscles , which causes depolarization of the membrane and subsequently requires an increased effort to maintain the resting membrane potential. The increase in Na^+,K^+-ATPase contents in two muscles of around 40% suggested that measurement of Na^+, K^+-

ATPase content could support the ante mortem diagnosis of LMND.

In conclusion, this thesis demonstrates that the muscle of an immature horse is a heterogeneous tissue that achieves its adult form by a process of functional adaptation. Muscle biopsies are an important complementary tool for ante mortem diagnosis of lower motor neuron disorders, but are of limited value for the prediction of athletic potential, because the distinctive characteristics at a young age disappear throughout later life. During development the muscle shows an enhancement in power and fatigue related properties. For an optimal development exercise is considered to be essential, especially during the first year after birth. Training a horse before conventional training starts does not have huge benefits for muscle performance. Apparently, the developmental adjustments measured by the present variables represent sufficient adaptation to meet the demands of training. The enhancement in membrane excitability and maintenance of force indicated that the training the foals were subjected to was intense enough to initiate reactions in the muscle. This means it is possible that alterations occur at another level than measured in the present thesis. Therefore, it would be very interesting to further explore the adaptations to (patho)physiological conditions on, for example, the molecular level where subtle changes can be detected, i.e. the expression of mRNA for the relevant proteins.

Nederlandse samenvatting

Door de toenemende populariteit van de paardensport op nationaal en internationaal niveau wordt het steeds interessanter om een paard met een uitzonderlijk talent te fokken en te trainen. Opfokken en trainen is echter een langdurig proces waarvan de uitkomst pas bekend is als het paard al deelneemt aan de competitie. Het zou veel effectiever zijn om reeds op veulenleeftijd het talent van een paard in te schatten aan de hand van verschillende objectieve eigenschappen, zoals bijvoorbeeld de spieropbouw, omdat dit voor een groot deel het atletisch vermogen van het paard bepaalt.

De skeletspieren zorgen voor de voortbeweging door een balans tussen samentrekken en ontspannen. Dit wordt gestuurd door prikkelingen vanuit het centrale zenuwstelsel. De spieren bestaan uit meerdere soorten vezels die in bundels in de spier liggen. De combinatie van deze soorten spiervezels zorgt er voor dat het dier een minimale hoeveelheid energie nodig heeft om te bewegen. Het functioneren van de spiervezels wordt bepaald door de snelheid waarmee ze samentrekken (contractie eigenschappen) en de manier waarop ze hun energiehuishouding regelen (metabole eigenschappen). Grofweg bestaan er drie soorten spiervezels. De eerste groep bestaat uit smalle vezels waarbij het samentrekken en ontspannen relatief langzaam verloopt. Deze vezels maken gebruik van zuurstof om suikers en vetten om te zetten in energie (aëroob metabolisme). Deze eigenschappen geven de vezel een groot uithoudingsvermogen, maar weinig kracht. Het is een ideale vezel voor inspanningen waarbij langdurige activiteit wordt gevraagd van de spier. De tweede groep bestaat uit grotere vezels die in staat zijn om snel samen te trekken. Zij breken suikers af zonder zuurstof (anaëroob metabolisme). Deze vezel is krachtig en in staat om snel en explosief te reageren, maar is o.a. door het snel opraken van de energievoorraad snel vermoeid. Tussen deze twee uitersten zit nog een derde groep vezels die qua snelheid van samentrekken tussen de eerste twee groepen zit en voor zijn energiehuishouding zowel van het aërobe als het anaërobe metabolisme gebruik maakt. Het gevolg is dat deze vezels ondanks het snelle samentrekken relatief

goed bestand zijn tegen vermoeidheid.

De vezelsamenstelling van een spier en dus het specifieke karakter van een spier is voor een groot deel al bepaald tijdens de geboorte. De spier is echter in staat om zich gedurende de verdere ontwikkeling aan te passen aan verschillende omstandigheden zoals bijvoorbeeld tijdens training of te veranderen ten gevolge van een spieraandoening. Meer kennis over de opbouw van de spieren van het paard en zijn aanpassingsvermogen zou meer inzicht geven in de voorspellende waarde van een spieronderzoek op jonge leeftijd. Voor het onderzoek van dit proefschrift zijn er kleine spierbiopten genomen uit verschillende voortbewegingsspieren bij verschillende groepen paarden op verschillende tijdspunten onder variërende omstandigheden. Hierbij is specifiek gekeken naar de verandering in spieropbouw met de leeftijd, de invloeden van verschillende bewegingsregimes, fokdoeleinden en een pathologische zenuwaandoening op kracht, uithoudingsvermogen en prikkelgevoeligheid.

Ontwikkeling

Direct na de geboorte maakt het veulen een snelle ontwikkeling door met de grootste gewichttoename in het eerste levensjaar. In deze periode wordt in de spier een zelfde sterke toename in kracht en uithoudingsvermogen gevonden (hoofdstuk 2 en 3). Deze krachttoename wordt veroorzaakt door een toename in de doorsnede van de vezels.

Training

Trainen van volwassen paarden heeft als doel de prestatie en het uithoudingsvermogen te verbeteren en de kans op blessures te verminderen. De reactie van de spier op training is afhankelijk van meerdere factoren waaronder de leeftijd, de samenstelling van de spiereigenschappen bij de start van de training en natuurlijk het type training. Voor jonge paarden is weidegang al voldoende voor de normale ontwikkeling van bot en kraakbeen. Ook voor de spierontwikkeling blijkt bewegen belangrijk (hoofdstuk 2 en 3). Het maakt hierbij niet uit of dit bewegen in de wei wordt gedaan of door middel van

training. Het bewijs hiervoor wordt voornamelijk gevonden door het negatieve effect van beperkte bewegingsvrijheid (door boxrust) op de spierdoorbloeding en het niet optreden hiervan wanneer de dieren beweging krijgen. Trainen (aanvullend op boxrust) blijkt hetzelfde effect te hebben als weidegang. Wanneer getraind wordt voor een specifieke sport als springen (aanvullend op weidegang) is er een toename in de prikkelgevoeligheid van de spiervezel te vinden. De aanpassingen in kracht en uithoudingsvermogen ten gevolg van de leeftijdsontwikkeling zijn blijkbaar meer dan voldoende om aan de vraag tijdens springtraining te voldoen.

Fokkerij

Veel stamboeken proberen door middel van speciale fokprogramma's het ras te verbeteren zodat het paard beter kan presteren in verschillende sporten. Door de genetische invloed op de spier is zo'n selectieprocedure voor een deel bepalend voor de verschillende eigenschappen van de spier. Bij een vergelijking tussen twee onderzoeksgroepen, waarbij één groep veulens speciaal geselecteerd was voor springen volgens het fokprogramma van het stamboek en één groep veulens nakomelingen waren van hengsten die niet voldeden aan de selectiecriteria van het stamboek, was dit verschil in selectie duidelijk aanwezig in de spiereigenschappen op zes maanden leeftijd (hoofdstuk 4). Door de verschillende spierontwikkeling in het tweede half jaar was dit verschil echter verdwenen op éénjarige leeftijd. De spiereigenschappen waren blijkbaar specifiek voor het ras op dat moment. Het was dus uiteindelijk niet meer mogelijk deze twee groepen van elkaar te onderscheiden op spierniveau.

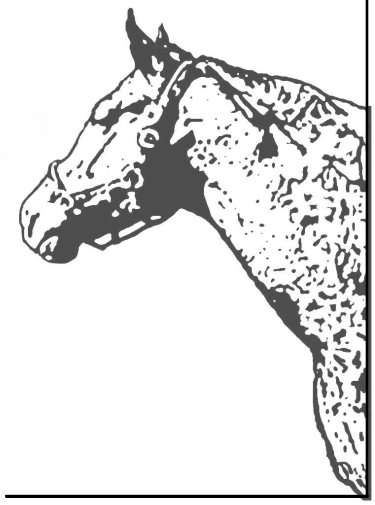
Pathologische omstandigheden

Verschillende zenuw- en spieraandoeningen zijn van invloed op de spieren. Bij het paard is equine motor neuron disease (EMND) een bekend voorbeeld van een neurodegeneratieve aandoening. Hierdoor is er een verminderde prikkeling van de spieren door een aantasting van zenuwen in de hersenstam en het ruggemerg. Het gevolg

voor de spier is een verhoogde prikkelgevoeligheid en een verschuiving van de contractiesnelheid van langzaam naar snel (hoofdstuk 5). De verhoogde prikkelgevoeligheid kon deels verklaard worden door het verschil in prikkelgevoeligheid van de vezels; snelle vezels zijn gevoeliger dan langzame vezels, maar ook door de aantasting van de vezelwand door verminderde signaalprikkeling, waardoor een verhoogde gevoeligheid nodig is om de vezels nog te laten reageren op prikkeling vanuit het centrale zenuwstelsel. Omdat de effecten in de twee onderzochte spieren even groot waren en dus alle spieren in dezelfde mate aangetast worden door de aandoening, biedt het meten van prikkelbaarheid van de spier de mogelijkheid om een bijdrage te leveren aan de diagnose van EMND.

Wanneer de bevindingen ten aanzien van groei, de invloeden van verschillende bewegingsregimes, selectie en een pathologische aandoening op de kracht, uithoudingsvermogen en prikkelgevoeligheid van de spier op een rij gezet worden kan geconcludeerd worden dat de locomotiespieren van het jonge paard aan grote veranderingen onderhevig zijn.
Spieronderzoek met behulp van spierbiopten is een belangrijke aanvulling op de diagnose EMND, maar is van beperkte waarde voor de voorspelling van het talent op jonge leeftijd. Dit komt omdat de verschillen in de spiereigenschappen die aanwezig zijn op jonge leeftijd bij de verschillende paarden tijdens de verdere ontwikkeling verdwijnen.
In de eerste levensjaren van het paard ontwikkelen de spieren zich door een toename in kracht en uithoudingsvermogen en daarbij is bewegen een cruciale factor. Extra bewegen door gericht trainen in deze periode heeft weinig invloed op de al aanwezige ontwikkeling van de spieren. De veranderingen in prikkelbaarheid laten wel zien dat de training intensief genoeg is geweest om de spier aan te zetten tot veranderingen. Het zou dus heel goed mogelijk kunnen zijn dat er wel veranderingen hebben plaatsgevonden, maar op een ander niveau dan gemeten in dit onderzoek. Het is dan ook interessant om de (patho)fysiologische invloeden verder te onderzoeken op moleculair niveau waar eventuele subtiele veranderingen meetbaar zijn, zoals bijvoorbeeld de expressie van mRNA's van de onderzochte eiwitten.

Curriculum vitae



Dankwoord

Dankwoord

En dan is het zo ver. Het boekje ligt klaar voor de drukker en de datum voor de verdediging is bevestigd. Het werk zit er (bijna) op. Nu nog het dankwoord. Al die jaren dat je bezig bent met je onderzoek denk je wel eens aan de dag dat het klaar is en je je dankwoord kan schrijven, maar als het moment eenmaal daar is, is het een beetje onwerkelijk. Ook omdat het voor mij een afsluiting is van tien jaar 'Anatomie'. Een enerverende periode.

Terug kijkend op die periode is er één iemand die als een rode draad door deze tijd liep, mijn dagelijks begeleider en co-promotor Liesbeth Dingboom. Zowel tijdens mijn onderwijs werkzaamheden en later tijdens mijn onderzoek was zij mijn grote steun en toeverlaat. Ze heeft me door de (vier) periodes gesleept die je als promovendus meemaakt. Elke keer lukte het haar om me weer enthousiast te krijgen en me vol goede moed verder te laten gaan. Lieve Lies, onze besprekingen en discussies zijn voor altijd in mijn geheugen gegrift en ik denk er met heel veel plezier (en een traan) aan terug. Samen begonnen we aan een onbekende klus, jij als begeleider en ik als promovendus, en hebben we een woelige zee bevaren. Ik weet dat mijn eigenwijze karakter niet altijd even makkelijk voor je was, maar je geduld was gelukkig groot genoeg. Bedankt voor je vertrouwen en je begeleiding op meer wegen dan alleen het onderzoekstraject. Met Bert en Rob als mijn paranimfen is het feestje compleet.

Met alleen een co-promotor kom je er natuurlijk niet. Ook mijn (beoogd) promotoren zijn van grote invloed geweest op mijn weg in onderzoeksland. Het begon met Wim Weijs, die mij voor dit onderzoek heeft aangenomen. Mijn onderwijskwaliteiten waren inmiddels bekend, maar mijn lab ervaring nog niet. Hij vroeg dan ook voor de zekerheid of ik wist wat laboratoriumwerk in hield, maar met de practica biochemie en histologie nog vers in het geheugen kon ik daar (in mijn ogen) een positief antwoord op geven. Bij het echte werk ontdekte ik dat het wel meer inhield dan een practicum. Wim wist ook de eerste onzekerheid over de meerwaarde van een promotie voor een dierenarts op rustige en overtuigende wijze weg te nemen. Hij had gelijk. Na twee jaar had Marjanne Everts de

moeilijke taak om een lopend onderzoek over te nemen. Haar frisse blik op het onderwerp zorgde vrij snel voor een eerste publicatie en een presentatie op de ICEEP, maar ook voor een succesvolle uitbreiding van de spierparameters. Marjanne, je heldere manier van denken hebben me geholpen om alles goed op een rij te krijgen. Ook je hulp bij het schrijven was voor mij onmisbaar en zorgde er voor dat mijn gedachtenkronkel ineens duidelijk geformuleerd was. Ik wil je bedanken voor alle tijd die je er in hebt gestoken om het tot een goed einde te brengen. Uiteindelijk lag er voor Ab Barneveld de uitdaging om het onderzoek in het licht van de paardensport te houden. Ab, je las in een sneltreinvaart al mijn stukken door en je wist door je vragen mij aan het denken te zetten en zo het werk weer in de oorspronkelijke vorm te gieten. Spieren als voorspellers. Het was voor mij een goede stimulans om de laatste stukken te schrijven. Dank je wel.

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Lieve Robbie en Helen, mijn allerliefste kleine dondersteentjes. Ik houd van jullie.

Curriculum vitae

Nancy Rietbroek werd op 7 juli 1973 geboren te Alphen aan den Rijn. In 1992 behaalde zij haar eindexamen van de middelbare school ‘Albanianae’ te Alphen aan den Rijn. In dat zelfde jaar begon zij aan haar studie diergeneeskunde aan de Universiteit Utrecht, die zij in 2002 afrondde. Direct na haar afstuderen startte zij haar promotieonderzoek bij de toenmalig hoofdafdeling Veterinaire anatomie en fysiologie, thans het Departement Pathobiologie, waar zij al tijdens haar studie werkte als toegevoegd docent voor het vak topografische anatomie. Sinds juni 2007 werkt zij als junior docent bij het Department Landbouwhuisdieren.

Nancy Rietbroek was born on 7th july 1973 in Alphen aan de Rijn. In 1992 she passed her exams at the Albanianae in Alphen aan den Rijn. In that same year she started her studies of veterinary medicine at University Utrecht and graduated in february 2002. On 1st April 2002 she started working as a PhD student at the Department of Veterinary Anatomy and Physiology, now the Department of Pathobiology, where she was already employed as a ‘toegevoegd docent’ for the Anatomy division during her studies. Since June 2007 she works at the Department of Farm animal health.