Equine Locomotory Muscles

Postnatal development and the influence of exercise

Liesbeth G. Dingboom

Utrecht - 2002

Equine Locomotory Muscles

Postnatal development and the influence of exercise

Locomotiespieren van het paard

Postnatale ontwikkeling en de invloed van beweging

(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

There has always been a great interest in the equine musculoskeletal system, focusing on parameters related to the ability to perform at high sport levels. Functional muscle properties play a major role in the capacity to perform and sport competition creates the pressure for improvement. For this reason, many studies to the effects of exercise and training on muscle properties were performed on (young) adult horses. Exercise was shown to have a beneficial effect on the functional properties of muscles, such as power and resistance against fatigue. The question is, if further benefits can be achieved when very young foals are subjected to exercise. In the first months after birth muscles are sensitive to changes in workload and adaptation occurs. It can be expected that, particularly in this dynamic period, exercise influences the normal maturation pattern. This, in turn, might effect the capacity for later athletic performance as better possibilities may be created for further adaptation during the conventional training period later in life.

Functional muscle properties

Muscle fibre types

The ability for athletic performance is partly determined by the combination of adequate contraction speed and power and sufficient resistance against fatigue of the skeletal muscles. These properties are determined by the relative presence of muscle fibre types with different properties. Skeletal muscle fibre diversity was recognised as early as 1873 when Ranvier distinguished 'white' and 'red' muscles. Only just in the early sixties of the previous century it became clear how this distinction is based on the existence of different fibre types (see for review, Pette and Staron 1990).

Histochemical fibre typing

Initially, in mammalian skeletal muscle, two fibre types were classified, type I and type II fibres (Engel 1962). Later, Bárány *et al.* (1965) demonstrated that slow and fast muscles contain myosins with different ATPase activities, correlating with differences in speed of contraction (Bárány 1967). Type I fibres were classified as slow contracting, and type II fibres as fast contracting.

The observation that slow and fast myosins have different stabilities with alkaline and acid preincubations (Sreter *et al.* 1966; Seidel 1967) formed the basis for the elaboration of more refined methods for myosin-ATPase based fibre typing. Studies of the pH stability of mammalian myosins revealed the oversimplification of the distinction into only two fibre types and led to the delineation of fast fibre subtypes, i.e. IIA, IIB and IIC fibres (Brooke and Kaiser 1970). Modifications and additional procedures for classification of fibres based on myosin ATPase activity confirmed the existence of major fast fibre subtypes, but have also identified more than one slow fibre type (I and IC) (Staron and Pette 1986).

Based on the also found existence of different formaldehyde sensitivities of myosin ATPase (Stein and Padykula 1962), some investigators combined the pH and formaldehyde sensitivities of myosin ATPase. This led to another classification system, where one slow fibre type (β) and two fast fibre types (α and αβ) were distinguished (Guth and Samaha 1969; Samaha *et al.* 1970).

Immunohistochemical fibre typing

The detection of antigenic differences between myosins (e.g. Gröschel-Stewart and Doniach 1969; Masaki 1974) initiated immunohistochemical analyses of muscle fibres with antibodies (e.g. Billeter *et al.* 1980; Danieli-Betto *et al.* 1986). Responsible for the ATPase activity is a major structural component of the thick filaments of the myosin molecules, the Myosin Heavy Chain (MHC). With help of monoclonal antibodies raised against MHC and gene identification (e.g. Robbins *et al.* 1986), it was shown that in mammals MHC occurs in different isoforms i.e. MHC type I (Weeds and Burridge 1975; Gauthier and Lowrey 1979) or □ (Lompré *et al.* 1984), IIa and IIb (Dalla Libera *et al.* 1980; Billeter *et al.* 1981) and Cardiac-□ (Bredman *et al.* 1991). An additional fast fibre type (IIx) has been identified in muscles of small mammals by using monoclonal antibodies (Schiaffino *et al.* 1985; Gorza 1990); another fast myosin heavy chain isoform was (independently) identified by gel electrophoreses in various skeletal muscle of rat (Bär and Pette 1988). This isoform was named IId because of its abundance in the diaphragm. Immunoblot analyses demonstrated that IIx and IId are identical (Schiaffino *et al.* 1989). In muscle fibres from fetal and newly born individuals, the MHC isoforms 'embryonic' and 'neonatal' (or 'Developmental' or 'fetal') are expressed (Butler-Brown and Whalen 1984; Butler-Browne *et al.* 1988; d'Albis *et al.* 1986). MHC isoform 'Developmental' is also expressed in muscle fibres of regenerating muscles (Sartore *et al*. 1982).

Multiple expression of MHC's is the rule in some muscle fibres (Danieli-Betto 1986; Biral *et al.* 1988), but occurs particularly under conditions of induced fibre transformation (Staron *et al.* 1987; Maier *et al.* 1988), for example during growth or after a change in training regimen (Termin *et al.* 1989; Schiaffino *et al.* 1986, 1989, 1990; Gorza 1990; Talmadge *et al.* 1995).

All the isoforms differ in their ATPase reaction speed and consequently engender differences in sarcomere contraction speed. Contraction speed increases and oxidative capacity decreases in the order, I, Cardiac-α, IIa, IId and IIb (e.g. Kwa *et al.* 1995 (rabbit); Rome *et al.* 1990 (horse)). The position of the Developmental type in this order is yet unknown.

Metabolic enzyme-based fibre classification

In comparative studies (Bass *et al.* 1969 a and b 1970; Staudte and Pette 1972) activities of 'metabolic oxidative enzymes' were shown to be extremely useful for distinguishing different fibre types. Three major fibre types were derived from differences in these enzyme activities, high, intermediate and low oxidative fibres (Ogata and Mori 1964, Padykula and Gauthier 1967; Gauthier 1969, 1974). The combination of metabolic enzyme-

based fibre classification and myosin ATPase histochemistry resulted in the classification of three major fibre types in muscles of guinea pig and rabbit, slow-twitch oxidative (SO), fast-twitch oxidative / glycolytic (FOG) and fast-twitch glycolytic (FG) fibres (Barnard *et al.* 1971; Peter *et al.* 1972). A method of depleting glycogen in single motor units made it possible to establish a relationship between metabolic properties, speed of contraction and fatigability (Edström and Kugelberg 1968; Kugelberg and Edström 1968). It was shown that motor units composed of FG fibres are fast fatiguable and that motor units composed of FOG or SO fibres are fatigue resistant (Burke *et al.* 1971, 1973, 1974; Burke 1981).

Compatibility of the classification methods

Taken together, a variety of fibre types can be distinguished in a given muscle by myosin ATPase-based histochemistry, immunohistochemistry or by metabolic enzyme-based fibre classification, but the existence of these different classification schemes raises the question of compatibility. For example Green *et al*. (1982) showed complete correspondence between ATPase determined type I and fibres, but significant variations between the fast fibre subtypes IIA and IIB on the one hand and and on the other hand.

Since the work of Bárány (1967) it is generally accepted that the contraction speed of given muscle fibres relates to the ATPase activity of the MHC part of myosin molecules. The rate of myosin ATPase activity is different in the different MHC isoforms and correlates well with the resistance against acid and alkaline preincubation. Therefore, myosin-ATPase-based fibre typing and immunohistochemically-based classification produce similar results. However, this correlation is not perfect. Some fibres designated as IIB with ATPase techniques express both IIa and IIb MHC's. On the other hand, it occurs that fibres characterised as intermediate between IIA and IIB with the ATPase (type IIA/B fibres) contained only type IIa MHC isoform (Danieli-Betto *et al.* 1986; Rivero *et al.* 1996). Furthermore, the other MHC isoforms that occur in skeletal muscle (IId, Cardiac-α, Developmental), could not be accounted for by the standard ATPase techniques.

Also the assumption that metabolic properties should correspond to properties related to different myosin ATPase activities may not be justified. In some mammals, the slow contracting, fatigue resistant type I fibres are the most oxidative (e.g. human and cat) whereas in other mammals, the type I fibres are intermediate between IIA (or IIa) and IID (or IId) in their oxidative potential (e.g. rat, guinea pig and horse) (Barnard *et al.* 1971; Peter *et al.* 1972; Prince *et al.* 1976; Reichmann and Pette 1982; Essen-Gustavsson 1986). Furthermore, after quantifying the histochemically assessed aerobic oxidative metabolism in the different fibre types, it became clear that no discrete levels exist but an entire spectrum of metabolic activity (Nemeth and Pette 1981; Pette and Tyler 1983; Reichmann and Pette 1982, 1984, White and Snow 1985), overlapping the fibre types.

For the above mentioned reasons, it can be concluded that classification systems do not appear to be entirely compatible and should therefore not be interchanged.

Energy production and fatigue

Muscular fatigue often causes exercise intolerance. For a proper muscle function, fatigue must be prevented. As under normal conditions, fatigue is usually based on lack of ATP, substrate needs to be converted into energy (Stryer 1995). In figure 1, the energy production in cells or fibres is shown schematically. Three distinct stages in the breakdown of substrates can be distinguished, glycolysis (in the cytosol), the Krebs cycle (in the mitochondrial matrix) and oxidative phosphorylation (in the mitochondrial inner-membrane). These three metabolic pathways ensure a constant energy supply, but the relative contributions of the pathways depend on the level of muscle work.

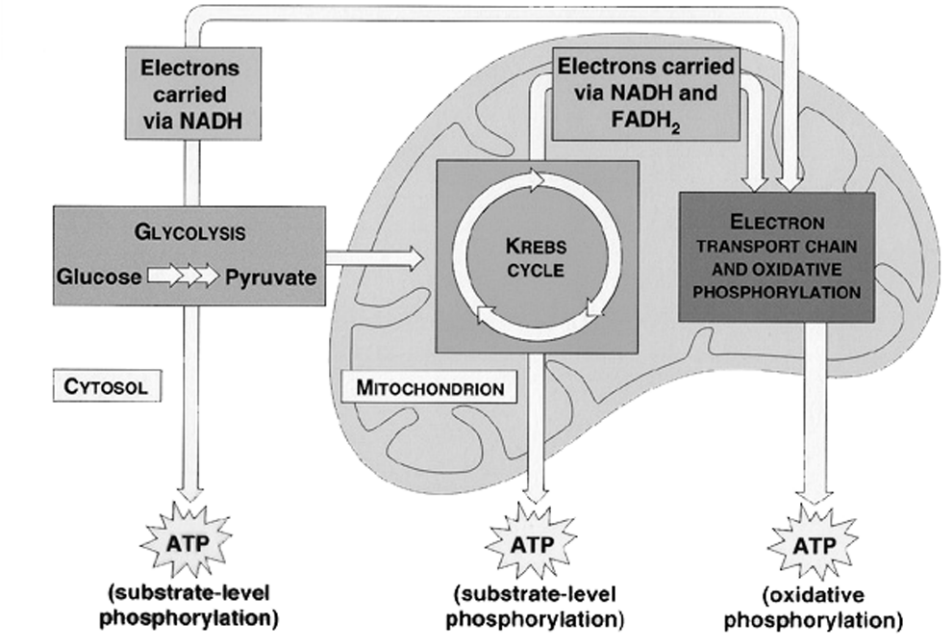

Fig. 1 Energy production in cells and fibres

Glycolysis is the anaerobic breakdown of glucose to pyruvate with the production of ATP. In the presence of oxygen it is possible to produce more energy because pyruvate can than be further degraded via the eight steps of the Krebs cycle (figure 2). During this process, much of the energy is stored as high-energy electrons. Although the Krebs cycle itself does not use oxygen, it is required to transport the high-energy electrons along the 'electron transport chain' in the inner membrane of the mitochondrion. Protons follow the electrons and will be transported to the intermembranal space. Re-entering of the protons, back into the mitochondrial matrix, takes place through channels in the ATP synthase

enzyme complex of the inner-membrane and is coupled to ATP synthesis (oxidative phophorylation).

Indicative for resistance against fatigue is the oxidative capacity. This is the capacity of muscle fibres to use oxygen in the production of the necessary energy for contraction and relaxation. It can be estimated by the activity of oxidative enzymes from the Krebs cycle, but also by the capillarity (blood supply) of the muscle tissue.

Oxidative enzymes

Energy for the muscle (ATP) can be most efficiently produced in the muscle fibres with the help of oxygen. The activity of enzymes, involved in the Krebs cycle, predict the maximum rate of oxygen consumption and therefore correlate with energy production (van der Laarse *et al.* 1989). This activity can therefore be considered to reflect the capacity to resist fatigue. Pette *et al.* (1962) described a positive correlation between the amounts of various oxidative enzymes present in single fibres, making the choice of the enzyme to be measured

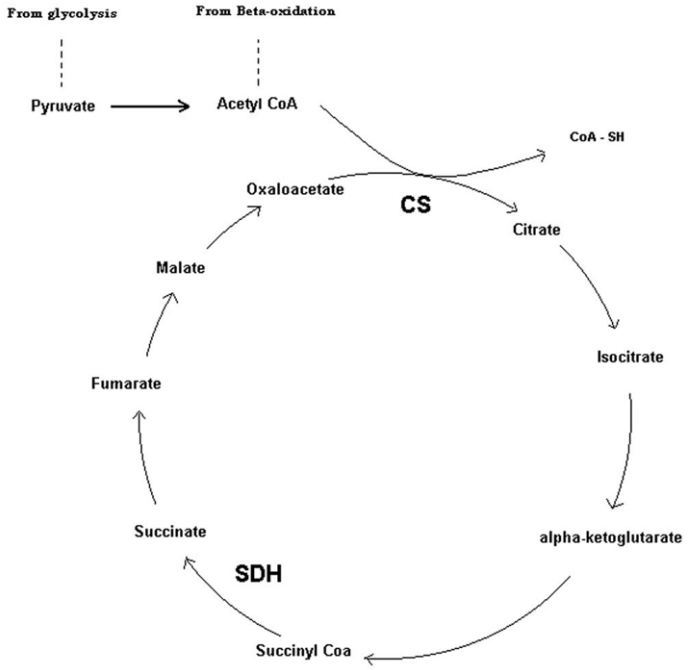

Fig 2. Krebs cycle

less important. We choose the activity of citrate synthase (CS) and succinate dehydrogenase (SDH) to estimate oxidative capacity. CS participates in the first step of the Krebs cycle (conversion of Acetyl coenzyme-A and oxaloacetate in coenzyme-A and citrate) and SDH catalyses the conversion of succinyl-CoA in succinate (figure 2).

Capillarity
In order to function properly, muscle fibres need a sufficient blood supply, since oxygen and substrates for energy production (i.e. carbohydrates and fatty acids) must be delivered and metabolites (i.e. lactate and carbon dioxide) must be released by the bloodstream. The capillary supply, or capillarity, can be expressed as the amount of capillaries per mm^2 (capillary density) or per muscle fibre (capillary to fibre ratio). In both cases, the results depend on the cross-sectional areas of the muscle fibres involved. Therefore, it is more accurate to describe the capillarity by a parameter indicating the muscle fibre area one capillary has to supply (diffusion index) (Andersen and Hendriksson 1977; Rivero *et al.* 1995). This parameter can be used as an indicator of fatigue resistance.

Postnatal development

After birth, muscles assume their normal function. During growth, muscle fibres change in properties while adapting to their new function. Age effects on muscle characteristics as MHC expression, oxidative capacity and blood perfusion, depend on the type of muscle, species and breed.

MHC expression

That dynamic properties of fast and slow skeletal muscles change in the course of age was already described by Close (1964). He observed in developing rat muscles that the process of differentiation into fast and slow muscles can be attributed to an increase in the speed of contraction of the fast muscles. The MHC expression pattern of a single muscle fibre, depends on its development stage and on the origin of its myogenic precursor cells. It is affected by neural and hormonal influences (Schiaffino and Reggiani 1996).

After birth, the earliest age effect is the disappearance of juvenile MHC isoforms like Developmental and Cardiac-α MHC. Several studies showed that Developmental MHC was present after birth in the masseter muscle (e.g. d'Albis *et al.* (1986) and locomotory muscles (e.g. Butler-Brown and Whalen 1984) of small mammals. The neonatal isoforms persisted until a few weeks after birth in the locomotory muscles, but the disappearance in the masseter muscle was delayed. The Cardiac-α MHC, characteristic for heart atrium, was already found in cranial muscles, like masticatory, extra-ocular and hyoid muscles (Bredman *et al.* 1991), but the occurrence of α MHC in mammalian locomotory muscles in the first weeks after birth has not yet been described.

Although age effects depend on the type of muscle, most authors describe for different locomotory muscles an increase of fibre type I and / or IIA (IIa) percentages at the expense of the fibre type IIB (IIb) or IId/x fibre population in the early postnatal period and during the period of young adulthood, both in small (e.g. Goldspink and Ward 1979) and large (Suzuki and Cassens 1980 (pig); Essén *et al.* 1980; Essen-Gustavsson *et al.* 1983; Henckel 1983; Roneus *et al.* 1991; Roneus 1993; Rivero *et al.* 1993 (horse)) mammals. In

contrast, it was found that the percentage of type I fibres decreases in the biceps brachii (hamsters, Goldspink and Ward 1979) and extensor digitalis longus muscles (sheep, Finkelstein *et al.* 1992).

The simultaneous increase of type IIA (IIa) fibres and decrease of type IIB (IIb) or IID/X (IId/x) fibres suggest that type IIB (IIb) or IID/X (IId/x) fibres turn into IIA (IIa) fibres. If this were the case, it should most probably happen via transitional fibres, expressing both MHC types. There is indeed a population of such fibres. Rivero *et al.* (1996) found frequencies of 10-15 % in the gluteus medius of 2 and 3 years old horses. They stated that these fibres may not only reflect fibre type transformation, but also may form a biologically important fibre type in horses.

Metabolic profile

The metabolic difference between fast and slow muscles in adult mammals was found to be absent in the newborn; it becomes visible during postnatal development (Bass *et al.* 1970; Margreth *et al.* 1970; Hudlicka *et al.* 1973; Dalrymple *et al.* 1974; Zuurveld *et al.* 1985). Most likely, this change results from the adaptation to changing usage patterns. In muscle tissue of different mammals it was found that the oxidative enzyme activity decreases in the first postnatal weeks (rat, Fratacci *et al.* 1996; cat, Wada *et al.* 1995). In equine muscle the activity of the anaerobic glycolytic enzymes, rather than the aerobic oxidative enzymes was found to increase during the first year of life (Bechtel and Kline 1987; Kline and Bechtel 1990).

Also at the level of the muscle fibres, the metabolic heterogeneity increases during postnatal development (Dangain *et al.* 1987; Nemeth *et al.* 1989; Ishihara and Inoue 1989). In contrast to the biochemical studies with muscle tissue, it was found that the oxidative capacity of the different fibre types increases, suggesting a progressive adaptation to the extra-uterine environment, but the fibres show different degrees of increase. Some authors describe that the oxidative enzyme activity of IIB (IIb) fibres decreases (e.g. Smith *et al.* 1988) in the postnatal period.

Blood supply

Mammals are born with a certain capillary supply, which changes in the course of time after birth due to growth of the muscle fibres. Growth results in a decrease of fibre density; because of the larger distance between the surrounding capillaries there is also a decrease in capillary density (e.g. Sillau and Bachero 1977; Ripoll *et al.* 1979; Kurnoth *et al.* 1994). Several investigators found in different muscles of small mammals an increase in the number of capillaries around the fibres during growth (e.g. Welt *et al*. 1975; Ripoll *et al.* 1979). However, since the total area of the fibres in the growing muscle is also increasing, the diffusion index (i.e. the area that one capillary has to supply) was found either to remain constant (Welt *et al*. 1975) or to decrease (Smith *et al.* 1989).

Muscles and performance

Many studies show that sport capacities are correlated with muscle properties such as muscle fibre type composition and enzyme activities (human athletes, e.g. Costill *et al.* 1976a and b; Costill *et al.* 1987; horse, Bechtel and Kline 1987; Roneus *et al.* 1991; Roneus 1993). Basically, endurance capacity is correlated with high percentages of slow contracting, oxidative, fatigue resistant type I fibres (e.g. Rivero *et al.* 1993), whereas sprint capacity is correlated with high percentages of fast contracting, glycolytic, fatiguable type II fibres (e.g. Barrey *et al.* 1999).

Human studies have shown that muscle fibre type composition is highly genetically determined (Komi 1977; Komi and Karlsson 1979; Simoneau and Bouchard 1995). Studies performed on animals also demonstrate this correlation between genetical background and muscle fibre type composition (e.g. horse, Lopez-Rivero *et al.* 1991, Rivero *et al.* 1996; pig, Szentkuti and Schlegel 1985). This would imply that, for muscle, the ability to perform, in a certain sport and at a certain level later in life, is partly determined already at birth. To change muscle fibre type percentages in a muscle, exercise must be given at a level of high intensity and for a long time. For example, in a study with human athletes the fibre type composition changed after 3 months of intensive strength- and interval-training (Anderson *et al*. 1994). On the other hand, dynamic parameters as capillarity (e. g. Hudlicka *et al*. 1977) and enzyme activity (e.g. Henriksson and Reitman 1977) can adapt more rapidly to the imposed workload.

Training

Most of the knowledge about the effect of training on muscle properties is derived from human literature. (See for review, Wilmore and Costill 1994). The problem of interpreting training results from studies with horses is that often the training regimen is not described clearly enough. Furthermore, some studies with apparently equal training regimens produce inconsistent results. Therefore, it is hard to make detailed inferences about the influence of training on the equine muscle characteristics based on literature.

There are many ways to design training protocols, but basically, training can be divided in ‘endurance training’ and ‘sprint training’. Endurance training focuses on the increase of resistance against fatigue of the muscle, by increased aerobic metabolism. In general it can be stated that endurance training enhances the oxidative capacity of all fibre types, stimulates the conversion of type IIB or IID into IIA and increases the cross-sectional area of type I fibres (e. g. horses, Hodgon and Rose 1987; Lopez Rivero *et al.* 1991; Rivero *et al.* 1993; Tyler *et al.* 1998). Sprint training focuses on the increase of the ability for fast and powerful short contractions. Energy will be primarily supplied through the anaerobic metabolism, but due to the enhancement of the oxidative capacity of the fast contracting fibres as a result of the training, energy will also be supplied through the improved aerobic metabolism. In studies with humans it appeared that sprint (or power training) increases the relative area, occupied by the type II population (Dawson *et al.* 1998; Jansson *et al.* 1990).

Because of the higher speed of this fibre population, muscles are able to generate more power (i.e. force times speed) during a single contraction.

Restricted mobility

Most studies concern the effects of detraining, which are the reversal of the training effects (e.g. human, Mujika *et al*. 2000; Mujika and Padilla 2001; Winters and Snow 2000; e.g. horse, Serrano *et al.* 2000; Guy and Snow 1977; Snow and Guy 1979; Essen-Gustavsson *et al.* 1989; Foreman *et al.* 1990; Tyler *et al.* 1998). These studies show that the effects of detraining are a decreased capillary density (within two to three weeks of inactivity) and a rapid and progressive reduction in oxidative enzyme activities. Although, in some cases, the muscle characteristics of detrained individuals remain above sedentary values, usually they return to baseline values in shortly-trained individuals. The fibre type composition remains unchanged during the initial weeks of inactivity, but the number of oxidative fibres may decrease in human endurance-trained athletes and increase in sprint-trained athletes within eight weeks of detraining (Mujika and Padilla 2001). Muscle fibre cross-sectional area declines rapidly in sprint-trained athletes, and in briefly endurance-trained subjects, whereas it may increase slightly in athletes trained for endurance for a longer period.

Effects from immobility are comparable with, but much more dramatic than the effects of detraining (Wilmore and Costill 1994). Forced immobility (for example bed rest) causes muscle atrophy, accompanied by losses in muscle strength and power. Haggmark *et al.* (1986; human) described a substantial decrease in type I fibre percentages when sudden immobilization occurs after a period of intensive sport training. At this moment it is not clear, how and to what extent restricted mobility can affect the normal maturation of muscle characteristics in the young individual.

Design of the study

In the horse, there has always been a great interest in the muscle characteristics and the effect of exercise on muscle properties. Most of the earlier studies were performed on adult or young adult horses. The first objective of this thesis is to obtain better fundamental insights in the normal growth and maturation of equine locomotory muscles in the first year of life. Three parameters related to the ability to perform at high sport levels are studied, muscle fibre type composition, oxidative enzyme activities and capillarity. The second aim of this study was to learn to what extent exercise interacts with the postnatal development of muscle.

Animals

Subjects of investigation in our studies were Dutch warmblood foals. The Dutch warmblood horse (KWPN) is a relatively new race, defined in 1958, and is used for

different sport purposes as diverse as dressage and show jumping. The race is a mixture of many different breeds (25 – 35 % thoroughbred) and has a broad genetical background. For this reason, it is impossible to standardise the test group completely. We must therefore be aware that this, in our study uncontrollable, variation can mask potential group effects on the test results.

The investigation was performed in a group of 38 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system (see for overview, The EXOC project, Osteochondrosis and musculoskeletal development in the foal under the influence of exercise. Equine Veterinary Journal, supplement 31 (1999)). In this experiment, the sires and 4 broodmares were proven (by radiological examination) to suffer from osteochondrosis i.e. a cartilage development disorder in different joints of the locomotory system. In our studies we only used clinical healthy foals with no signs of lameness.

After birth, all foals remained with the mares in a paddock for one week, after which they were randomly divided into 3 groups that were subjected to different exercise regimens until weaning at 5 months. Two groups (boxrest and training group) were individually housed with the mare in box stalls. The third group was kept at pasture with the mare 24 h a day (pasture group). At 22 weeks of age 22 foals were euthanised for other purposes (7 foals from boxrest and training groups and 8 from the pasture group). The remaining 16 foals (6 males and 10 females) were joined in a single group which was kept in a loose house with access to a small paddock until 48 weeks of age. Then these animals were also euthanised.

Exercise protocol

Our study group was divided in three subgroups. Until 22 weeks, the boxrest group was kept in the box stall (3 x 3.5 meter) for 24 h/day. Most of the time, the foals stood still or walked a little bit. The training group was also kept in box stalls, but was given an increasing number of gallop sprints in a paddock of 48 x 15 meter, with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals would follow the mares.

Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 22 weeks 32 and 16 sprints on alternating days. Exercise was given 6 days a week. The sprints lasted for about 6 seconds (" 6.5 m/s) and a pause was kept after each sprint to prevent exhaustion. After the sprints, the foals were allowed an additional 0.5 hours of free exercise in the enclosure.

With this intensity and weight, the exercise can best be described as moderate (anaerobic) sprint exercise. The guideline for the exercise level was, that it should be the maximal effort possible with minimum risk for injury. The level of exercise could be

adapted to the individual foals and was scrutinised regularly by a veterinarian. Unfortunately, no information exists concerning the exercise tolerance of the foals. After the exercise, the foals were gasping for air.

The pasture group received voluntary movement. This meant that the foals walked and trotted an undefined amount of time and galloped approximately 3 minutes a day.

After 22 weeks up to 48 weeks, none of the foals were trained, so all got the same exercise regimen

Muscles

Two locomotory muscles (i.e. gluteus medius and semitendinosus muscle (figure 3) were chosen as a model because of their importance in propulsing the horse. The gluteus medius is a muscle of exceptional size and power. It is primarily an extensor of the hip, so its main function is propulsion. Secondly, it has a major function as stabiliser of the hip joint during weight bearing. The semitendinosus muscle is part of the hamstring group. The actions and uses of the hamstring muscles are complex. When the hind limb bears weight they extend the hip and knee joint, propulsing the animal. These two muscles are subjects of investigation in many studies, mainly because of their propulsive role, but also because taking biopsies from these muscles is relatively easy.

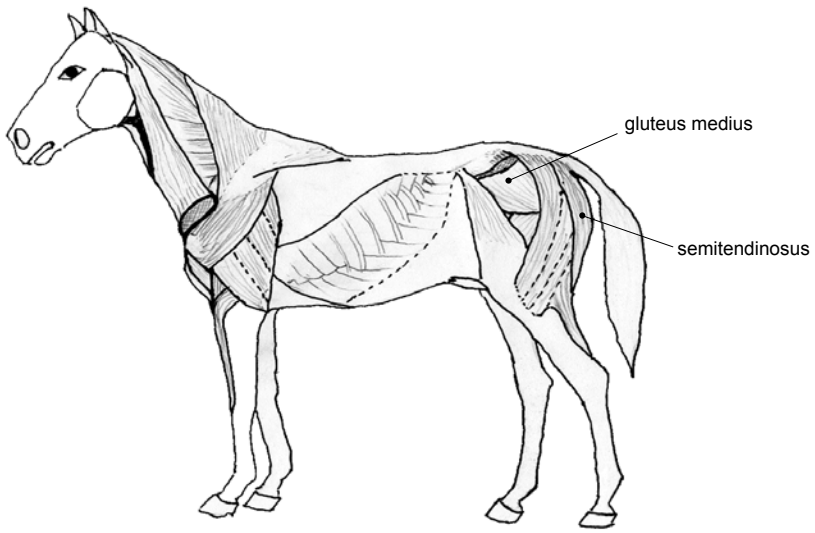

Fig. 3

Biopsies

In order to limit variance due to measurement error, percutaneous muscle biopsies were always taken by the same person, aiming to obtain material from identical sites in the muscles according to the protocol of Lindholm and Piehl (1974). This was done in the first week after birth (day 3 on average), at 2, 4, 8 and/or 22 weeks of age depending on the study, plus, when possible, also at the age of 48 weeks.

The 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. They were taken as deep as possible (until resistance from the iliac wing) because it was expected that at this site, the percentage type I fibres was high enough to demonstrate age- and / or exercise effects. Biopsies from the superficial semitendinosus were taken on a line drawn from ischiadic tuber to popliteal area, at two third distance from the ischiadic tuber, at a depth that was reached just after the muscle fascia was penetrated. This location was chosen because of the expected homogeneous type II population on this site (e. g. Barrey *et al*. 1995).

Projects

In chapter II the myosin heavy chain isoform expression in the gluteus medius muscle of 16 foals (divided over the boxrest and the boxtraining group) at 0, 2, 4, 8 and 22 weeks and of 4 foals at 48 weeks of age are demonstrated and discussed. The muscle fibre types were determined using monoclonal antibodies discriminating against the MHC isoforms type I, IIa, IId, Cardiac-α and Developmental. The purpose was to establish the presence of fetal MHC's and of possible rapid changes after birth in a limited study group.

In chapter III the myosin heavy chain isoform expression in both the gluteus medius and the semitendinosus muscle of 38 foals (divided over the boxrest, boxtraining and pasture group) at 0 and 22 weeks and of 16 foals at 48 weeks of age is demonstrated, compared and discussed. The muscle fibre types were determined using monoclonal antibodies specific for the MHC isoforms type I, IIa and IId. Sections from 18 biopsies were also stained histochemically (classical ATPase technique) to study the possible discrepancies between the results of both methods. The purpose was to establish the age and exercise effects on the presence of adult fibre types in two different muscles in the total study group.

Chapter IV describes the following error sources in fibre type counts of biopsies from the gluteus medius muscle, error due to (1) positioning of the biopsy needle (2) variation within a single biopsy (3) variation in the observers assessment of fibre type. These errors are compared with fibre type distribution patterns as seen in muscle blocks that were obtained post-mortem. The magnitude of the error variance determines the resolution of the method in detecting age and exercise effects on the fibre type composition. The resolution

determines the necessary number of animals in cross-sectional and longitudinal study designs

In chapter V age and exercise effects on the activity of two oxidative enzymes were determined. The citrate synthase (CS) activity of both the gluteus medius and the semitendinosus muscle of 37 foals (divided over the boxrest, boxtraining and pasture group) at 0, 4 and 22 weeks and of 16 foals at 48 weeks of age was measured in muscle homogenates. The CS activity was determined spectophotometrically and expressed in mU per mg protein content of the homogenate. Measurements of whole muscle CS activity do not provide information about the distribution of the oxidative capacity among the fibre types. For this reason, the oxidative capacity is also studied at the muscle fibre level by measurement of the SDH activity in cross sections of the gluteus medius muscle in 13 foals (divided over the three training groups) at the age of 0 and 22 weeks. The SDH activity was determined microdensitometrically and expressed in absorbance at 660 nm. This way, we were able to distinguish the SDH activity in the four major occurring muscle fibre types (I, IIa, IId and IIad).

In chapter VI the capillarity in both the gluteus medius and the semitendinosus muscle of 36 foals (divided over the boxrest, boxtraining and pasture group) at 0 and 22 weeks and of 14 foals at 48 weeks of age is demonstrated, compared and discussed. The endothelium of the capillaries and the membranes of the muscle fibre were stained with lectine at two different concentrations. In the sections, the capillary density (CD), the fibre density (FD), the capillary to fibre ratio (C/F), the mean cross sectional muscle fibre area (CSA) and the diffusion index (area supplied by one capillary) were measured with the help of a computer based image analysis system.

In chapter VII describes the general discussion with the main conclusions from the preceding chapters. The findings from the different chapters are interrelated and used as a basis for a better view on equine muscle developments in the first year of life including the importance of movement for the normal maturation.

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Chapter 2

Postnatal muscle fibre composition of the gluteus medius muscle of Dutch warmblood foals; maturation and the influence of exercise

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Summary

The fibre type composition of the deep gluteus muscle was studied in biopsies of Dutch warmblood foals from birth until 48 weeks of age. Half the foals were given box rest, the other half received exercise consisting of an increasing number of gallop sprints. The muscle fibre types were determined using monoclonal antibodies discriminating against the following myosin heavy chain (MHC) isoforms: type I, IIa, IId, Cardiac-α and Developmental. During the first 48 weeks there was a consistent increase of fibres expressing IIa MHC, replacing fibres expressing IId MHC. This change was reflected in the presence of a quite large population of fibres co-expressing MHC IIa and IId. The difference between the exercised (training-) and non-exercised (boxrest-) group was small but suggested that the increase of IIa fibres was larger in the training group. It appeared that after birth a significant number of fibres co-express either Developmental and type IIa-MHC or Cardiac-α and type I-MHC. The Developmental isoform disappears during the first 10 weeks after birth and almost all the α isoform expression during the first 22 weeks. It is concluded that a fast turnover of fibre types takes place in the deep gluteus medius in the first months of life. Potentially, exercise could have an effect on the rate of change of these fibre types.

Keywords: horse, fibre type, immunohistochemistry, growth, myosin heavy chain, cardiac myosin

Introduction

Mammalian skeletal muscle fibres can be subdivided into different types. Histochemical staining for mATPase after acid and alkaline preincubation identifies type I, IIA and IIB fibres with increasing ATPase activity and speed of contraction (Brooke and Kaiser, 1970; Bárány, 1967). Another classification system (slow oxidative, fast oxidative/glycolytic and fast glycolytic) was used by Peter *et al.* (1972) in which the mATPase stain was combined with oxidative and glycolytic enzyme activities in muscles of guinea pigs and rabbits. Their study showed a correlation between these fibre types and oxidative capacity. The latter is indicative for resistance against fatigue. Although in most small animals the I and IIA fibres are highly oxidative and the IIB fibres have little oxidative capacity, this relationship does not hold for all muscles and species. For example, it is well known that high oxidative capacity also can be found in IIB fibres in muscles of both humans, horses and wild animals.

With help of monoclonal antibodies against myosin heavy chain (MHC), the main determinant of contraction speed, type I, IIA and IIB fibres were shown to express specific isoforms of MHC, e.g. MHC I, IIa and IIb. However, this correlation is not perfect. Some fibres designated as IIB with the ATP-ase method seem to express both IIa and IIb MHC's and nearly all type IIA/B fibres contained only type IIa MHC isoform (Danieli-Betto *et al.* 1986; Rivero *et al*. 1996a). Furthermore, various other MHC isoforms were discovered in skeletal muscle, including type IId (or IIx) (Bär and Pette 1988, Schiaffino *et al*. 1989, Gorza 1990) and cardiac-α (Bredman *et al*. 1990). In fetal and muscle fibres from newly born individuals, the MHC isoform Developmental is expressed by the genome (d'Albis *et al*. 1986; Butler-Browne *et al*. 1988). Multiple expression of MHC's is the rule in some muscles (Biral *et al*. 1988), but occurs particularly during growth and after a change in training regimen (Termin *et al*. 1989; Talmadge *et al.* 1995). All the isoforms differ in their ATP-ase reaction speed and consequently engender differences in sarcomere contraction speed. Contraction speed increases and oxidative capacity usually decreases in the order: I, cardiac-α, IIa, IId and IIb. The position of the Developmental type in this order is yet unknown.

In the horse, there has always been a great interest in the muscle fibre composition and the effect of exercise on muscle properties. Most of these studies were performed on adult or young adult animals. They show that trained horses have a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres in the locomotory muscles (e.g. Henckel, 1983; Lovell and Rose, 1991).

In the first months after birth, mammalian muscle fibres undergo changes in innervation and MHC expression, so it can be expected that external factors like the amount of exercise in this period may have a strong effect on the muscle fibre composition and hence on the capacity for later athletic performance as it may create better possibilities for further adaptation during the conventional training period later in life.

This study is the first to describe the expression of MHC isoforms in the gluteus medius muscle of Dutch warmblood horses during the first year of life and the effect of exercise on this MHC expression.

Materials and methods

Foals
The investigation was performed in a group of 16 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system (van Weeren, P.R. and Barneveld, A. (submitted). All foals were individuallly housed with the mare in 3 x 3.5 m box stalls. They were fed freshly cut grass ad libitum, along with the mother's milk. One week after birth (day 7) the group was divided in 2 subgroups (boxrest and training group). The boxrest group consisted of 4 males and 4 females (mean weight at birth: 51 kg; at 22 weeks: 271 kg; at 48 weeks: 376 kg) and the training group of 2 males and 6 females (mean weight at birth: 53 kg; at 22 weeks: 256 kg; at 48 weeks: 367 kg).

Exercise protocol
The boxrest group was kept in the box stall for 24 h/day. The training group was kept in box stalls of the same size, but was given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals would follow the mares. Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 5 months 32 and 16 sprints on alternating days. exercise was given 6 days a week from monday to saturday. After weaning, the foals were joined in one single group which was kept in a loose house with access to a small paddock. None of these foals was trained, so all got the same exercise regimen.

Muscle biopsies
From each foal percutaneous muscle biopsies were taken by the same person, according to Lindholm and Piehl (1974) in the first week after birth (day 3 on average) and at the age of 2, 4, 8 and 22 weeks. From four horses (3 from the boxrest group, 1 from the training group) biopsies were also taken at the age of 48 weeks. The 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. They were taken as deeply as possible (until resistance from the iliac wing). Duplicate biopsies were not taken because in earlier studies the analysis of variance of gluteus medius muscle biopsies showed that the interindividual variation was greater than the intra-individual variation (Snow, 1983). The samples were rolled in talcum powder, mounted on cork blocks with the

use of OCT embedding medium and oriented in such a way that the fibres could be sectioned transversely. All samples were stored at -80°C.

Antibodies
To identify the fibre types according to their MHC content we used specific monoclonal antibodies (Mabs). They were prepared according to the procedure of Fazekas de St. Groth and Scheidegger (1980). Mab 249-5A4 (raised against human atrial myosin) reacts with cardiac-α MHC (de Groot *et al*. 1989; Wessels *et al*. 1991). Mab 219-1D1 (raised against chicken heart myosin) reacts with type I (Wessels *et al*. 1991; Bredman *et al*. 1991). Mab 332-3D4 (raised against rabbit eye muscle) reacts with type IIa and IId. Mab 333-7H1 (raised against protein extract from muscle tissue of adult rabbit anterior tibialis) reacts with type IIa (Bredman *et al*. 1991). Mab 412-R1D5 (raised against myosin isolated from rabbit psoas muscle) reacts with type I, IId and α (Bredman *et al*. 1991). Mab Developmental (raised against hind limb muscle of 7 days old rats (Novocastra Labs. Ltd., Newcastle upon Tyne, UK) recognises a Developmental type MHC present during the embryonic and neonatal period in the development of muscle (Butler-Browne *et al*. 1988).

Immunohistochemical staining
Transverse serial sections (10 μm) were made with a cryostat at - 20°C. Overnight fixation took place at -20°C in a 35% methanol, 35% acetone and 5% acetic acid solution. The slides were rinsed in 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by incubation in pronase (1:100 in PBS) for 30 minutes. After rinsing in PBS the slides were incubated in Teng-T (100 mM Tris, 50 mM EDTA, 1.5 M NaCl, 2.5 % gelatine and 0.5 % Tween 20; 1:10 in aqua dest.; pH 8.0) for at least 15 minutes, followed by rinsing in PBS and incubation overnight at room temperature with the Mabs at a dilution of 1:10 in PBS. After this incubation the slides were rinsed with PBS and then incubated for 90 minutes with a biotinylated horse anti mouse polyclonal antibody (1:100 in PBS; ABC-peroxidase staining kit Elite, Vector Labs., Burlingame, USA). The slides were again rinsed in PBS and subsequently incubated for 90 minutes with the components avidin (A; 1:100 in PBS) and biotin (B; 1:100 in PBS) of the ABC staining kit. Both components (A and B) were mixed at least 30 minutes before use. After rinsing the immunoreaction was visualised by incubation with 0.05 % 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, USA) in 30 mM imidazole (Janssen Chemica, Beerse; Belgium) and 0.09 % H_2O_2 (Merck, Darmstadt, Germany). The slides were subsequently stained with Harris hematoxylin for 45 seconds.

This protocol was followed for the Mabs 249-5A4, 219-1D, 332-3D4 and 333-7H1. For Mab 412-R1D5 basically the same protocol was followed. The difference is that no pronase was used, the dilution of Mab 412-R1D5 was 1:25 and instead of imidazole, di-ammonium nickelsulphate (2.5 % in acetate buffer) was used. Before visualisation with 3,3'-diaminobenzidine tetrachloride the slides were rinsed in 0.1 M acetate buffer (pH 6.0; 5-10 minutes) and were stained in Harris for only one second.

For Mab Developmental the protocol differed at the following points: no fixation took place and the procedure started with the incubation with the Mab Developmental at 37 °C for 60 minutes (dilution 1:20 in PBS). From here the procedure was the same as for Mab 412-R1D5.

Analyses

In the sections, a group of at least 200 contiguous fibres (Snow and Guy, 1980) were used for fibre typing and calculation of fibre type composition. The muscle fibres were classified into type I, type IIa, type IId, type IIa/d and fibres also containing Cardiac-α or Developmental MHC isoforms on basis of their reactions with the Mabs.

Analysis of variance (ANOVA) was used to test the effects of age and exercise. Thereby, time was used as within subject factor and exercise as between subject factor. Only data up to 22 weeks were used for this analysis. The data from the 48 weeks old foals were used to demonstrate the continuity of the trends.

Results

Muscle fibre characterisation

The monoclonal antibodies against MHC used in this study allowed us to distinguish seven frequently occurring fibre types in the deep gluteus medius muscle of the juvenile horses (Table 1). Like Rivero *et al*. 1996b), we could not find IIb fibres using immunohistochemical methods. Using combined ATPase- and immunohistochemistry, ATPase IIB fibres appeared to contain MHC IId. In addition to the types mentioned in table 1 a few rarer (< 3 %) mixed fibre types were observed.

Table 1. Fibre types in the equine gluteus medius muscle and their reactions with the panel of monoclonal antibodies that was used in this study.

	I	IIa	IIa/d	IId	I + α	IIa + Dev	IIa/d + Dev
332-3D4	-	+	+	+	-	+	+
219-1D1	+	-	-	-	+	-	-
333-7H1	-	+	+	-	-	+	+
412- R1D5	+	-	+	+	+	-	+
249-5A4	-	-	-	-	+	-	-
Dev	-	-	-	-	-	+	+

Figure 1 illustrates the results of the immunohistochemical staining. Figure 1A shows the reaction with Mab 332-3D4 and demonstrates an overview of the fibre types occurring in this section. Fibres reacting positively with Mab 219-1D1 (Fig. 1B) were identified as fibres expressing MHC type I. Fibres reacting positively with 249-5A4 (Fig. 1C) were identified as fibres expressing MHC type α and are usually fibres also expressing type I (83 %) and in some cases IIa MHC. Fibres reacting positively with 333-7H1 (Fig. 1D) and negatively with 412- R1D5 (Fig. 1E) were identified as fibres expressing MHC

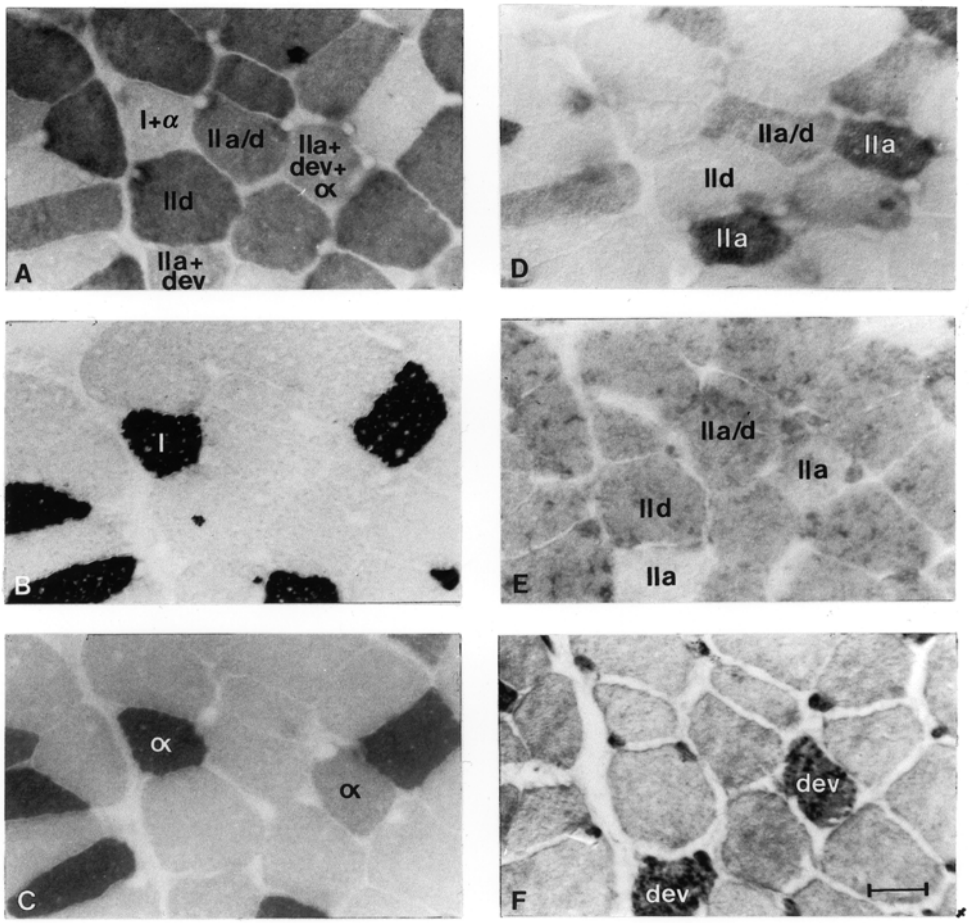

Fig. 1. Results of immunohistochemical staining. Transverse serial sections (10μm) of a biopsy from the deep part of the gluteus medius muscle of a Dutch warmblood foal of 4 days old. **A**: Mab 332-3D4; **B**: Mab 219-1D1; **C**: Mab 249-5A4; **D**: Mab 333-7H1; **E**: Mab 412-R1D5; **F**: Mab Developmental. Original magnification: x 601.4 (bar = 16.63 μm).

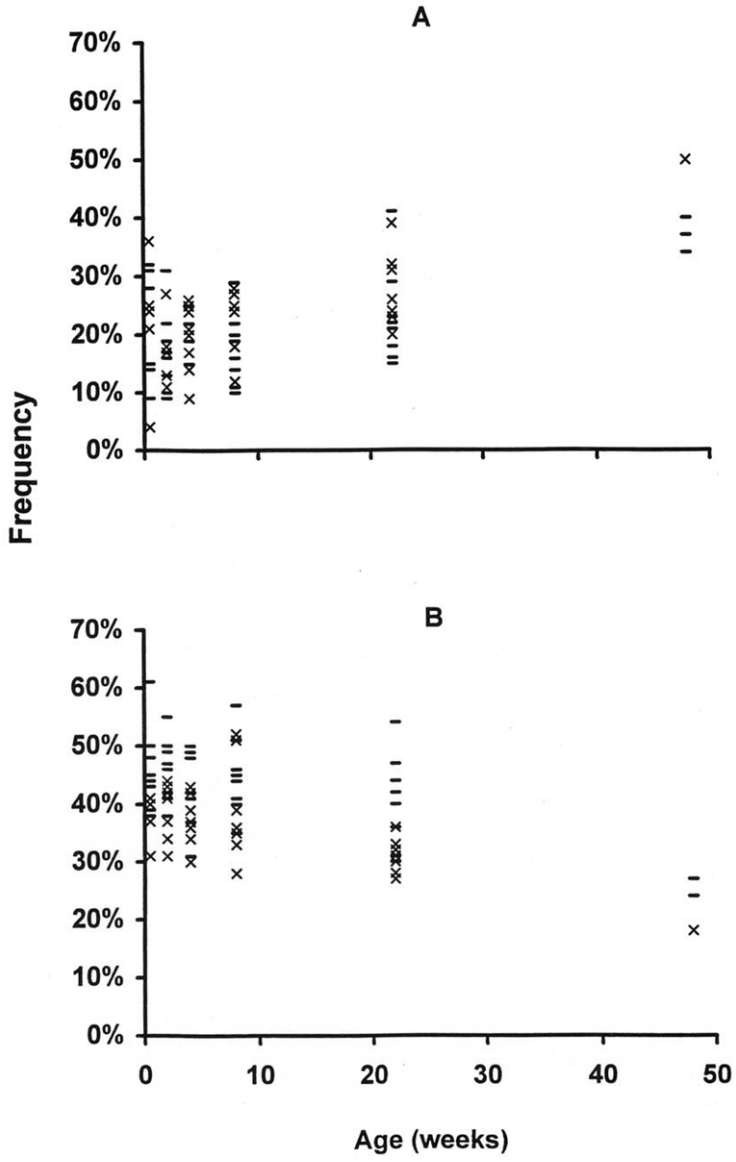

Fig. 2. Scatterplot of fibre type IIa (A) and IId (B) during aging (in weeks). Each marker is a biopsy taken from one horse. Line (–) markers are from the boxrest and cross (✖) markers are from the training group.

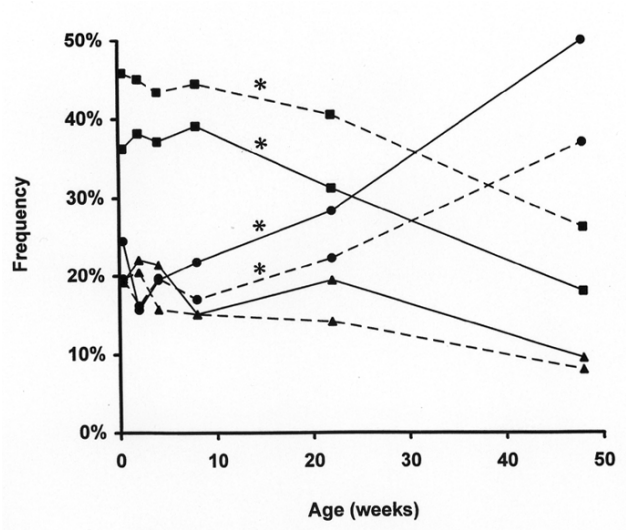

Fig. 3. Mean frequencies of fibre type IIa (●), type IId (■) and type IIa/d (▲) during aging (in weeks) from the boxrest (- - - - -) and the training group (———). *p < 0.05.

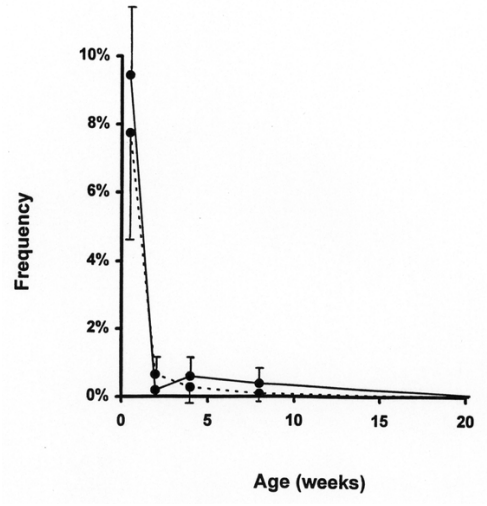

Fig. 4. Mean frequencies (+/- SEM) of fibres expressing Developmental MHC during aging (in weeks) from the boxrest (- - - - -) and the training group (———).

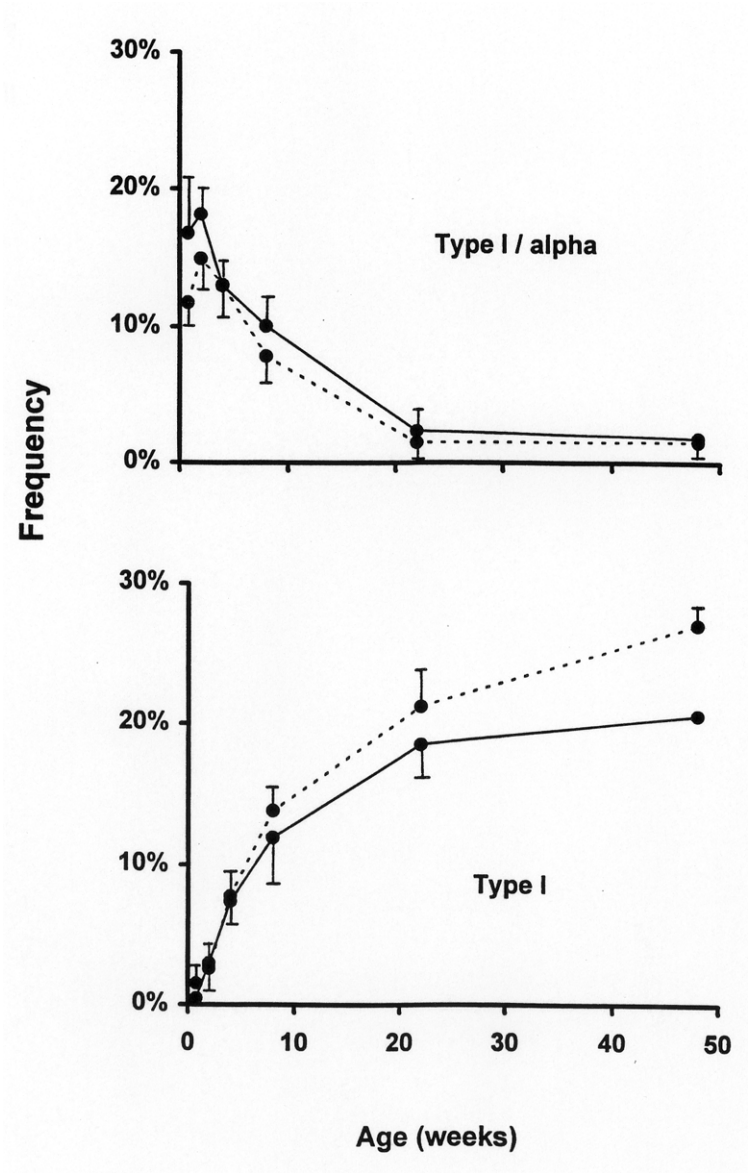

Fig. 5. Mean frequencies (+/- SEM) of type I/α and type I fibres during aging (in weeks) from the boxrest (- - - -) and the training group (———).

type IIa, the ones reacting positively with 333-7H1 and 412- R1D5 were identified as fibres expressing MHC type IIa/d and the ones reacting positively with 412- R1D5 and 332-3D4 but negatively with 333-7H1 and 219-1D1 were identified as fibres expressing MHC type IId. Fibres reacting positively with Mab Developmental (Fig. 1F)) were identified as fibres expressing MHC type Developmental and are usually fibres also expressing type IIa (53.3 %) or type IIa/d MHC (38.6 %).

Muscle fibre development

Fast fibres The frequencies of type IIa and IId fibres in individual biopsies of the deep gluteus medius muscle in the two groups of foals are plotted in Figure 2. The figure shows the range of interindividual variation. There are clear trends of an increase of the IIa frequency and a decrease of the IId frequency during growth.

The mean frequency of type IIa fibres is increasing in both groups during the first year of life (Fig. 3). The two other fast fibre types, IId and IIa/d decrease in mean frequency in both groups**.** Analysis applied on the data from 0 to 22 weeks (see material and methods) showed that the increase of type IIa fibres and the decrease of type IId fibres are statistical significant ($p < 0.05$). The decrease of IIa/d fibres groups is not statistically significant ($p = 0.08$).

Exercise did not significantly influence the rate of change of the fast fibre types. Figure 3 suggests a difference between the boxrest and the training group in the increase of IIa fibres. Although the increase in the training group is higher, the difference does not reach the level of statistical significance. Figure 3 shows that the percentages of IId fibres are higher in the boxrest group, but this difference was already present at birth and did not increase.

Fibres containing Developmental MHC Developmental MHC is present during the embryonal and neonatal period of skeletal muscle development in some IIa and IIa/d fibres and disappears in both boxrest and training group at the same time (Fig. 4), before 10 weeks of age.

Slow fibres Two slow fibre types are seen: fibres expressing solely type I MHC and fibres co-expressing type I and α MHC. Type I and type I / α fibres combined increase slightly in numbers during growth, from about 15 to 25 %, but this effect does not reach the level of statistical significance ($p = 0.06$). Strikingly, at birth almost all slow fibres are type I / α. In the first 22 weeks α disappears almost completely from these fibres (Fig. 5). No exercise effect was found on the disappearance of these fibres.

Discussion

Age effect

The age effect on muscle fibre composition depends on the type of muscle, the species, breed and even on sex. Suzuki and Cassens (1980) found in pigs that the percentage of type I muscle fibres increased from birth to 8 weeks of age in several muscles. In contrast, in

hamsters it was found that the percentage of type I fibres decreases in the biceps brachii, and increases in the soleus (Goldspink and Ward, 1979). Lindholm and Piehl (1974) reported a higher percentage fast twitch, high oxidative fibres (FOG) in the middle gluteal muscle in adult horses compared with 6 months old foals. The percentage fast twitch, low oxidative fibres (FG) became lower over time. Others (Essen-Gustavson *et al*. 1983; Henckel, 1983) also found increasing percentages of fibre type IIA and decreasing percentages of fibre type IIB from 6 month up to older ages. On the other hand Bechtel and Kline (1987) reported that foals, during the first 6 months of life enhance their anaerobic, rather than their aerobic components of muscle metabolism.

In this study we showed that the age effect on deep gluteus medius muscle fibre composition in the first year of life is dramatic in both groups. There is a simultaneous increase of type IIa fibres and decrease of type IId fibres. This suggests that type IId fibres turn into IIa fibres. If this is the case, it should most probably happen via transitional fibres, expressing both type IIa and IId MHC (IIa/d). There is indeed a population of such fibres. Essén *et al.* (1980), investigating standardbred trotters, found that the increase of type IIA fibres and the decrease of IIB fibres continued to the age of 3-4 years. So, probably the transition in our study is not complete yet. Indeed, at the age of 11 months the frequency of type IIa/d fibres is still 10 % in both groups. Rivero *et al*. (1996a) found frequencies of 10-15 % in the gluteus medius of 2 and 3 years old horses. These fibres, coexpessing IIa and IId, may not only reflect fibre type transformation, but also may form a biologically important fibre type in horses in the first place.

The occurrence of α MHC in the gluteus medius muscle is a new finding. This myosin, characteristic for heart atrium, was already found in cranial muscles, like masticatory, extraocular and hyoid muscle, but not in trunk and limb (Bredman *et al*. 1990).Rivero *et al*. (1996b) claimed that this myosin was not present in equine gluteus medius muscle. This contradictory finding may be explained by the fact that the horses used in their study were not young enough to demonstrate the expression of Cardiac-α MHC. In contrast with earlier studies (Bredman *et al*. 1991), in this study Cardiac-α MHC did not appear as the only MHC in a muscle fibre, but always in combination with other MHC isoforms, usually with type I MHC.

The increase in the slow fibre (I plus I/α) frequency was not statistically significant. Except for the disappearance of fibres co-expressing MHC I and Cardiac-α, the slow fibre population is stable in the first year of life. However, the loss of Cardiac-α from these fibres probably implies that they become slower contracting (van Buren *et al*. 1995; Kwa *et al*. 1995).

Another age effect is the disappearance of Developmental MHC expression in the first 2 months after birth. In an electrophoretic study d'Albis *et al*. (1986) showed that Developmental MHC was present after birth in mice and rats. In rats these isoforms also persisted until the age of 2 months.

Our foals were not trained after weaning (22 weeks). Nevertheless, the trends of type I and IIa increase and IId decrease continued until the age of 48 weeks. In older, less active, standardbred trotters (>10 years) it was found that the IIA/IIB fibre ratio was still

high (Essén *et al*. 1980). Therefore it may be assumed that the muscle fibre composition created in the early phase of life is the basis for further adaptations during the conventional training period later in life.

Effect of exercise
The gluteus medius, as one of the main propulsive muscles of the body, is expected to be well adaptable to exercise. Earlier studies show that trained horses, especially trained for endurance, have a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres (now called type IId) in the locomotory muscles (*e.g*. Rivero *et al.* 1995). However, in our study exercise did not significantly influence the rate of change of the fibre types. The question arises if our exercise protocol provided enough stimulation to induce changes in the muscle. The paper of Suwannachot *et al*. (1999) in this issue shows a significant increase in the total concentration of Na^+,K^+-ATPase in the deep gluteus medius muscle of the training group. This means that the exercise was intensive enough to initiate some reaction in this muscle. The mean difference in fibre composition is subject to a quite large intra-individual (e.g. Essen-Gustavsson *et al.* 1989; Lopez-Rivero *et al.* 1992) and interindividual variation (e.g. Snow 1983). Exercise induced effects will therefore only be measurable if they are large. If the effect is small it can only be demonstrated using larger groups. The higher percentages of IId fibres in the boxrest relative to the training group at birth must be explained by the small group size.

In conclusion this study is the first to demonstrate the presence of Cardiac-α and Developmental MHC in equine skeletal muscle. It also demonstrates a high degree of MHC co-expression, especially occurring during the first months after birth. Furthermore, the results of the present study show that there is an age effect in the MHC expression. As type I and type IIa fibres generally have a better fatigue resistance, the muscle apparently becomes increasingly resistant against fatigue. This conclusion is opposite to the one drawn by Bechtel and Kline (1987). The transition of fibre types can possibly be influenced by exercise. However, we have yet not been able to demonstrate that exercising young foals can produce such a change in fibre composition.

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Chapter 3

Changes in fibre type composition of gluteus medius and semitendinosus muscles of Dutch warmblood foals and the effect of exercise during the first year of life

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Summary

In order to obtain broader insights in the equine musculoskeletal system, we studied the fibre type composition of two locomotory muscles in biopsies from Dutch warmblood foals taken at three different ages in the first year of life. The muscle fibre types were determined histochemically as well as immunohistochemically. ATPase characterized IIB fibres appear to express either IId or type IIa plus IId myosin heavy chain (MHC). A high percentage of fibres classified as IIA with ATPase expressed both fast types of MHC. The type I classification by the two methods matched almost completely. There was an increase with age of fibres expressing I and IIa MHC in the deep gluteus medius. At the same time there was a decrease of fibres expressing IId MHC and fibres co-expressing MHC IIa and IId. The MHC expression of the semitendinosus muscle did not change in time at first, but from 22 to 48 weeks there was decrease of the percentage type IId fibres. In general, the deep gluteus medius contained more type I fibres but less type IId fibres compared to the superficial semitendinosus. At most ages the fibre type compositions of both muscles correlated with one another. To examine the effect of exercise, one third of the foals were given box rest, one third received exercise and one third was kept at pasture during the first 22 weeks of life. The three exercise groups differed in their fibre type composition, however, these differences could not be attributed to the effect of exercise.

Keywords: horse, fibre type, immunohistochemistry, exercise, myosin heavy chain

Introduction

Mammalian skeletal muscle fibres can be subdivided into different types. Histochemical staining for ATPase after acid and alkaline preincubation identified type I, IIA and IIB fibres with increasing ATPase activity and speed of contraction (Bárány 1967; Brooke and Kaiser 1970). Another classification system (slow oxidative (SO), fast oxidative/glycolytic (FOG) and fast glycolytic (FG)) was used by Peter *et al*. (1972). They combined the ATPase staining method with oxidative and glycolytic enzyme activities in muscles of guinea pigs and rabbits. Their study showed a correlation between the fibre types and oxidative capacity, which is also an indication for resistance against fatigue. Fibres with the highest oxidative capacity are the most resistant against fatigue. Although in most small animals the type I and type IIA fibres are highly oxidative and the IIB fibres have little oxidative capacity, this relationship does not hold for all muscles and species. For example, it is well known that high oxidative capacity can also be found in IIB fibres in muscles of horses (e.g. Hodgson and Rose 1987; Valberg *et al*. 1988; Roneus *et al.* 1994). With help of monoclonal antibodies against myosin heavy chain (MHC), the main determinant of contraction speed, type I, IIA and IIB fibres were shown each to express a specific isoform of MHC, e.g. MHC I, IIa and IIb (e.g. Rivero *et al*. 1996a) (note that capitals indicate ATPase determined fibre types and lower cases indicate immunohistochemically determined fibre types). However, this correlation is not perfect. Some fibres designated as IIB with ATPase techniques express both IIa and IIb MHC's. On the other hand, nearly all fibres characterized as intermediate between IIA and IIB with the ATPase (type IIA/B fibres) contained only type IIa MHC isoform (Danieli-Betto *et al*. 1986; Rivero *et al*. 1996a). Multiple expression of MHC's is the rule in some muscles (Biral *et al*. 1988), particularly during growth and after a change in training regimen (Termin *et al*. 1989; Talmadge *et al*. 1995). Furthermore, various other MHC isoforms, not accounted for by ATPase techniques, were discovered in skeletal muscle, including type IId (or IIx) (Bär and Pette 1988, Schiaffino *et al*. 1989; Gorza 1990). For these reasons, fibre typing on basis of Mab's is considered superior to the classic methods, using ATPase techniques.

All the isoforms differ in their ATP-ase reaction speed and consequently engender differences in sarcomere contraction speed. Contraction speed increases and oxidative capacity usually decreases in the order: I, IIa, IId and IIb (e.g. Kwa *et al.* 1995 (rabbit); Rome *et al.* 1990 (horse)).

The Dutch warmblood horse (KWPN) is a relatively new race, defined in 1958. This race is a mixture of many different breeds and has therefore a broad genetical background. The Dutch warmblood horse is used for different sport purposes, which demand different properties of the locomotory muscles. In the horse, there has always been a great interest in the muscle fibre composition and the effect of exercise on muscle properties. Most of the studies were performed on adult or young adult animals of different breeds. They showed that muscle fibre compositions of different breeds were different and correlated with their sport capacities (Bechtel and Kline 1987; Roneus *et al.* 1991; Roneus 1993) and performance (e.g. Rivero and Henckel 1996). Furthermore, studies showed that

trained horses had a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres in the locomotory muscles (e.g. Essen *et al*. 1980; Henckel 1983; Lovell and Rose 1991: Roneus *et al.* 1992).

In the first months after birth, mammalian muscle fibres undergo changes in innervation and MHC expression (e.g. Dingboom *et al*. 1999). It can therefore be expected that external factors, like the amount of exercise, in this period have a major effect on the muscle properties and hence on the capacity for later athletic performance, as it may create better possibilities for further adaptation during the conventional training period later in life. To investigate this we choose the gluteus medius and the semitendinosus muscle because of their important role in locomotion. The gluteus medius is a muscle of exceptional size and power. It is primarily an extensor of the hip, so its main function is propulsion. Secondly, it has a major function as stabilizer of the hip joint during weight bearing. The semitendinosus muscle is part of the hamstring group and also serve for propulsion.

In a previous report (Dingboom *et al*. 1999) we demonstrated for the first time the early postnatal changes in MHC expression in the gluteus medius muscle, especially the expression and disappearance of the foetal isoforms ("-Cardiac and Developmental). The present study describes the expression of adult MHC isoforms in and the correlation between the gluteus medius and the semitendinosus muscle of the Dutch warmblood horse in the first year of life and the influence of exercise on this expression. We used the classic ATPase method along with the more modern immunohistochemical method, to make the data comparable with data from older studies and to demonstrate that type IIB fibres in equine muscle are fibres expressing IId MHC.

Materials and methods

Foals

The investigation was performed in a group of 38 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system as described by van Weeren and Barneveld (1999). After birth, all foals remained with the mares in a paddock for one week, after which they were randomly divided into three groups that were subjected to different exercise regimens until weaning at five months. Two groups (boxrest and training group) were individually housed with the mare in 3 x 3.5 m box stalls. They were fed freshly cut grass ad libitum, along with the mother's milk. The third group was kept at pasture with the mare 24 h a day (pasture group). At 22 weeks of age 22 foals were euthanized for other experimental purposes (7 foals from boxrest and training group each and 8 from the pasture group). The remaining 16 foals (6 males and 10 females) were joined in one single group which was kept in a loose house with access to a small paddock. Before euthanasia, the boxrest group consisted of 5 males and 8 females (mean weight at birth: 51 kg; at 22 weeks: 258 kg; at 48 weeks: 369 kg). The training group consisted of 6 males and 5 females (mean

weight at birth: 53 kg; at 22 weeks: 254 kg; at 48 weeks: 369 kg) and the pasture group of 6 males and 8 females (mean weight at birth: 53 kg; at 22 weeks: 251 kg; at 48 weeks: 361 kg).

Exercise protocol
Until 22 weeks, the boxrest group was kept in the box stall for 24 h/day. The training group was also kept in box stalls, but was given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals would follow the mares. Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 22 weeks 32 and 16 sprints on alternating days. Exercise was given 6 days a week. The sprints lasted for about 6 seconds (6.5 m/s) and a pauze was kept after each sprint to prevent exhaustion. After the sprints, the foals were allowed an additional 0.5 hours of free exercise in the enclosure. The pasture group received voluntairy movement. After 22 weeks up to 48 weeks, none of the foals was trained, so all got the same exercise regimen.

Muscle biopsies
From each foal percutaneous muscle biopsies were taken by the same person, according to the protocol of Lindholm and Piehl (1974) in the first week after birth (day 3 on average) and at the age of 22 weeks. From half the foals, biopsies were also taken at the age of 48 weeks. The 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. They were taken as deep as possible (until resistance from the iliac wing). Biopsies from the semitendinosus were taken on a line drawn from ischiadic tuber to popliteal area, at two third distance from the ischiadic tuber, at a depth that was reached just after the muscle fascia was penetrated. The samples were rolled in talcum powder, mounted on cork blocks with the use of OCT embedding medium and oriented in such a way that the fibres could be sectioned transversely. All samples were stored at -80°C.

Histochemical staining
Transverse serial sections (10 µm) were made with a cryostat at - 20°C. Sections of 18 biopsies were stained for myofibrillar adenosine triphosphatase (ATPase) after both alkaline (pH 10.3) and acid (pH 4.2 (0 weeks) or 4.3 (22 and 48 weeks)) preincubation (Brooke and Kaiser 1970).

Antibodies
The monoclonal antibodies were prepared according to the procedure of Fazekas de St. Groth and Scheidegger (1980). Mab 219-1D1 (raised against chicken heart myosin) reacts

with type I (Wessels *et al*. 1991; Bredman *et al*. 1991). Mab 332-3D4 (raised against rabbit eye muscle) reacts with type IIa and IId. Mab 333-7H1 (raised against adult rabbit anterior tibialis muscle) reacts with type IIa (Bredman *et al*. 1991). Mab 412-R1D5 (raised against rabbit psoas muscle) reacts with type I and IId (Bredman *et al*. 1991).

Immunohistochemical staining

Transverse serial sections (10 ：m) were made with a cryostat at - 20°C. Overnight fixation took place at -20°C in a 35% methanol, 35% acetone and 5% acetic acid solution. The slides were rinsed in 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by incubation in pronase (1:100 in PBS) for 30 minutes. After rinsing in PBS the slides were incubated in Teng-T (100 mM Tris, 50 mM EDTA, 1.5 M NaCl, 2.5 % gelatine and 0.5 % Tween 20; in aqua dest 1:10 ; pH 8.0) for at least 15 minutes, followed by rinsing in PBS and incubation overnight at room temperature with the Mabs at a dilution of 1:10 in PBS. After this incubation the slides were rinsed with PBS and then incubated for 90 minutes with a biotinylated horse anti mouse polyclonal antibody (1:100 in PBS; ABC-peroxidase staining kit Elite[1]). The slides were again rinsed in PBS and subsequently incubated for 90 minutes with the components avidin (A; 1:100 in PBS) and biotin (B; 1:100 in PBS) of the ABC staining kit. Both components (A and B) were mixed at least 30 minutes before use. After rinsing the immunoreaction was visualised by incubation with 0.05 % 3,3'-diaminobenzidine tetrachloride[2] in 30 mM imidazole[3] and 0.09 % H_2O_2[4]. The slides were subsequently stained with hematoxylin for 45 seconds and embedded in DePeX[5].

This protocol was followed for the Mabs 219-1D, 332-3D4 and 333-7H1. For Mab 412-R1D5 basically the same protocol was followed. The difference is that no pronase was used, the dilution of Mab 412-R1D5 was 1:25 and instead of imidazole, di-ammonium nickelsulphate (2.5 % in acetate buffer) was used. Before visualization with 3,3'-diaminobenzidine tetrachloride the slides were rinsed in 0.1 M acetate buffer (pH 6.0; 5-10 minutes) and were stained in hematoxylin for one second.

Analyses

A group of at least 200 contiguous fibres (Snow and Guy 1980) were used for fibre typing and calculation of fibre type composition. The muscle fibres were classified into type I, type IIa, type IId, type IIa/d on basis of their reactions with the Mabs and classified into type I, type IIA, and type IIB on basis of their acid stability.

General linear model-repeated measures (SPSS 8.0 for Windows) was used to test the effects of age and exercise. Thereby, time was used as within subject factor and exercise as between subject factor. Factor time was defined at 3 levels (0, 22 and 48 weeks). The first 2 levels to establish the effect of exercise and the last level to see if exercise effects persist. Analysis of variance (ANOVA) was used to test if there were differences already existing at birth. ‘Muscle’ was used as a second within subject factor to analyze if the differences between the muscle fibre composition of the gluteus medius and the semitendinosus muscle were significant. To test if the muscle fibre composition of the gluteus medius and the semitendinosus muscle are correlated with each other, we

performed Spearman's bivariate correlations. In all tests, differences were accepted when $p < 0.05$.

Results

Muscle fibre characterisation

The monoclonal antibodies against MHC used in this study allowed us to distinguish 4 frequently occurring fibre types in muscle of the juvenile horses (Table 1). In addition to the types mentioned in Table 1, a few rarer (< 1 %) mixed fibre types were observed. Rivero *et al*. (1996b) have shown that IIb MHC is not expressed in equine gluteus muscle. Accordingly, when we combined the results from the ATPase- and immunohistochemistry staining, fibres typed as IIB by ATPase appeared to express IId MHC. The results from the comparison of the immunohistochemistry with the ATPase method are demonstrated in Table 2.

Table 1. Fibre types present in equine muscle and their reactions with the panel of monoclonal antibodies that was used in this study.

	I	II a	II a/d	II d
332 - 3D4	-	+	+	+
219 - 1D1	+	-	-	-
333 - 7H1	-	+	+	-
412 - R1D5	+	-	+	+

Table 2. Percentages of similarity in the comparance of the results from the immunohistochemistry with the ATPase method. Number of fibres compared in 18 biopsies of the deep part of the glutus medius muscle. fibre type I : 740, fibre type IIa : 860, fibre type IIa/d : 580, fibre type IId : 1600

%	IHC			
ATPase	**I**	**IIa**	**IId**	**IIad**
I	99.5	-	-	-
IIA	-	72.4	2.5	25.1
IIB	0.5	1.8	81.6	16.1

Figure 1 illustrates the results of the histochemical- and immunohistochemical staining. Figure 1A shows the results of the myofibrillar ATPase staining at pH 4.3 and demonstrates an overview of the fibre types occurring in this section. Fibres reacting positively with Mab 219-1D1 (Fig. 1B) and negatively with Mab 332-3D4 (Fig. 1C) were identified as fibres expressing MHC type I. Fibres reacting positively with 333-7H1 (Fig.

1D) and negatively with 412- R1D5 (Fig. 1E) were identified as fibres expressing MHC type IIa, the ones reacting positively with 333-7H1 and 412- R1D5 were identified as fibres co-expressing MHC types IIa and IId, and the ones reacting positively with 412- R1D5 and 332-3D4 but negatively with 333-7H1 and 219-1D1 were identified as fibres expressing MHC type IId.

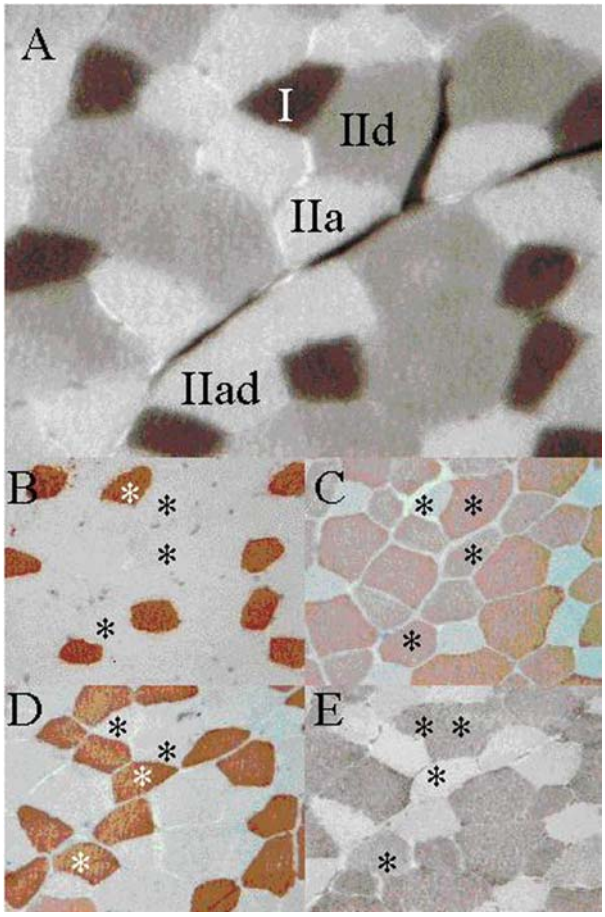

Fig. 1. Results of immunohistochemical staining. Transverse serial sections (10 ：m) of a biopsy from the deep gluteus medius muscle of a Dutch warmblood foal of 22 weeks old. A: ATPase staining at pH 4.3; B: Mab 219-1D1; C: Mab 332-3D4; D: Mab 333-7H1; E: Mab 412-R1D5.

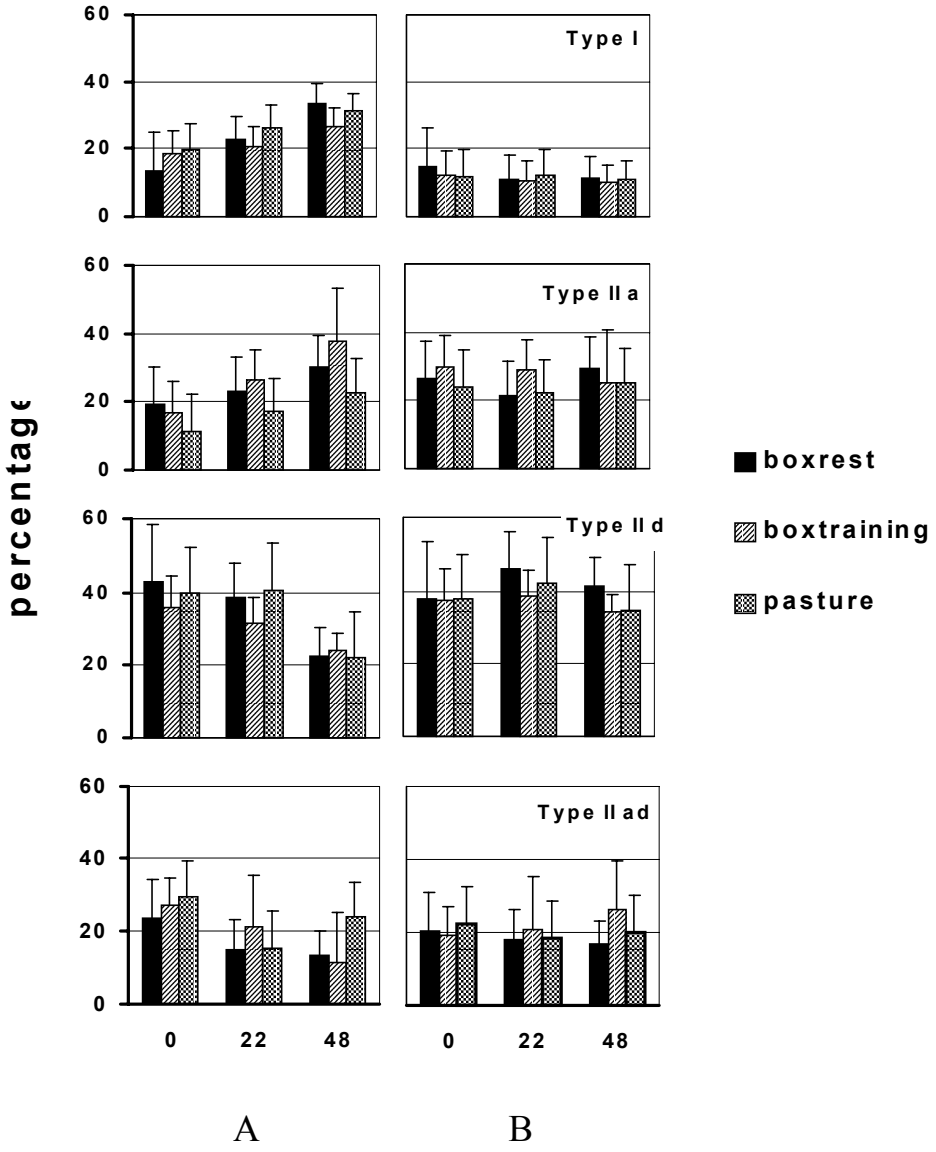

Fig. 2. Mean frequences of the fibre types from the gluteus medius muscle (A) and semitendinosus (B) in the 3 training groups during aging (in weeks). The Y-error bars represent the SD's in positive direction. At age 0 and 22 weeks : n = 38 (boxrest group 13; training group 11; pasture group 14). At age 48 weeks : n = 16 (1 group).

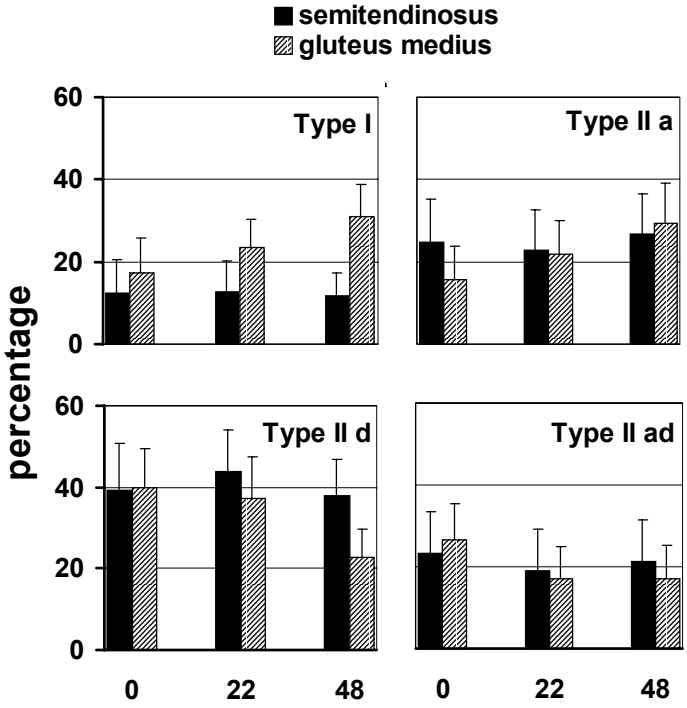

Fig. 3. Mean frequences of the main four fibre types at the age of 0, 22 and 48 weeks of age in the gluteus medius muscle (grey bars) and semitendinosus (black bars) from the total group. The Y-error bars represent the SD's in positive direction. At age 0 and 22 weeks : n = 38 ; at age 48 weeks : n = 16

Fibre type composition of the deep gluteus medius muscle

The mean frequencies of the fibre types from the deep gluteus medius muscle in the three groups of foals are given in Figure 2A. Statistical analyses showed that the three groups (boxrest-, boxtraining- and pasture group) differed in their fibre type composition ($p < 0.002$). This is a group effect, mainly caused by the difference in the amount of type IIa fibres between the three groups ($p < 0.01$) from 0 to 22 weeks of age. The difference already existed at birth ($p < 0.05$); because we found no significant interaction between group and time, the group effect could not be explained by exercise. Since there was no exercise effect, the three training groups were considered as one single group. Figure 3 demonstrates the percentages of the four different fibre types occuring in the total group (grey bars). From this Figure it is evident that the MHC expression of the gluteus medius muscle changed in time (age effect). From 0 to 48 weeks there was an overall statistically significant ($p < 0.001$) increase in type I and IIa fibres and a decrease in type IId and IIad fibres.

Fibre type composition of the superficial semitendinosus muscle

The mean frequencies of the fibre types from the semitendinosus muscle in the three groups of foals are given in Figure 2B. From 0 to 22 weeks, the three groups differed in their fibre type composition ($p < 0.02$). Like in the gluteus medius muscle, this group effect was mainly caused by the difference in type IIa ($p < 0.001$). Again, this difference already existed at birth ($p < 0.03$) and there was no statistical significant interaction with time. This means that also in the superficial semitendinosus muscle a clear exercise effect could not be demonstrated.

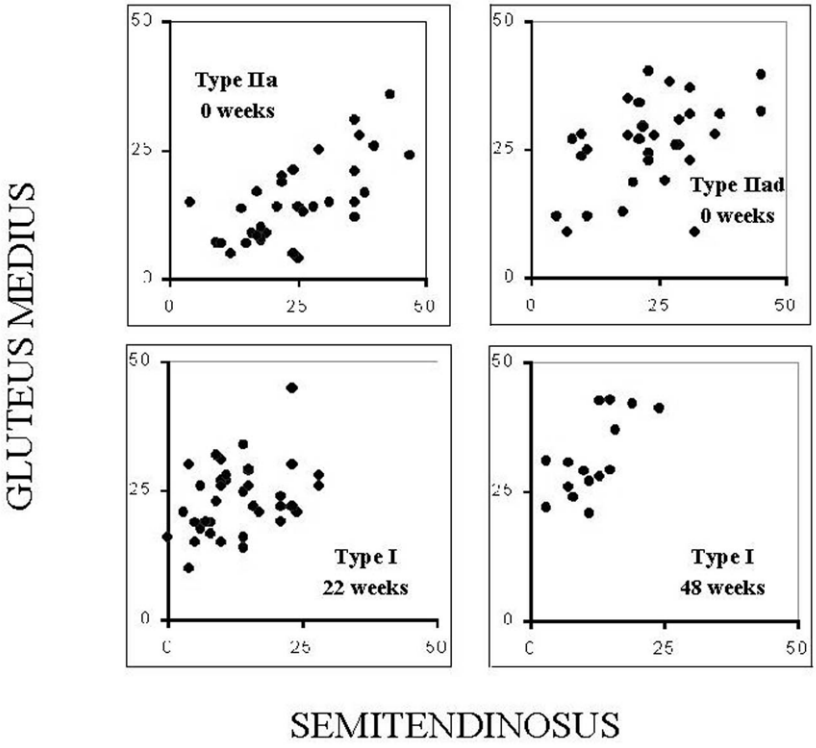

Fig. 4. The significant correlations between gluteus medius and the semitendinosus muscle of the type I, IIa and IIad percentages fibres at different ages

Figure 3 demonstrates the percentages of the four different fibre types occuring in the total group (black bars). The MHC expression of the superficial semitendinosus muscle did not change in time from 0 to 22 weeks of age. From 22 to 48 weeks there was a small age effect, due to a statistically significant decrease ($p < 0.05$) of the percentage type II d fibre in this period.

Comparison of the semitendinosus with the gluteus medius muscle
The fibre type composition of the superficial semitendinosus differed from that of the deep gluteus medius muscle (Fig. 3). In the gluteus medius muscle the percentages of fibre type I were higher ($p < 0.001$) and type IId lower ($p < 0.01$) as compared to the semitendinosus. The difference in percentages of fibre type IId could first be detected at the age of 22 weeks and increased up to the age of 48 weeks. The difference in percentages of fibre type I could already be detected at birth ($p < 0.02$). Although they were different, the fibre type compositions of both muscles did correlate with one another, at most ages (Fig. 4). At birth, the correlation coëfficient for the percentage of fibre type IIa was 0.69 ($p < 0.001$), the correlation coëfficient for fibre type IIad was 0.48 ($p < 0.005$). The percentages of fibre type I were not correlated at birth, however at 22 and 48 weeks the correlation coëfficients were 0.37 ($p < 0.03$) and 0.64 ($p < 0.01$) respectively. The percentages of fibre type IId were not correlated at any age.

Discussion

Immunohistochemical versus ATPase staining method
Several investigators compared the ATPase staining method with the immunohistochemistry and found a mismatch in the subclassification of mainly the type II fibres. Fibres, classified as IIB with the ATPase (later IID or IIX), are often expressing both type IIa and IId MHC and are therefore better classified as fibre type II D/A or IIX/A (Staron 1991; Anderson *et al*. 1994; Rivero *et al*. 1996b). In the equine gluteus medius muscle, nearly all fibre types classified as IIAB by the ATPase method contained only the type IIa MHC isoform, as shown immunohistochemically (Rivero *et al*. 1996b). Linnane *et al.* (1999) found in the type II subclassification a mismatch of 4.2 % in the superficial and 41 % in the deepest parts of the gluteus medius. The difference in mismatch they found, was most likely due to the greater amount of hybrid fibre types in the deeper parts of the muscle. Likewise, in this study we showed that IIB fibres are fibres either expressing IId or, in 16 % of the cases, co-expressing type IIa and IId MHC. We also found that a high percentage (25 %) of fibres classified as IIA expresses both fast types of MHC (IIa and IId). Fibres, classified as type I with the ATPase usually match 100 % with the immunohistochemistry (e.g. Rivero *et al*. 1996b; Linnane *et al*. 1999). This was confirmed in our study (match 99.5 %).

We can conclude that muscle fibre characterisation with the use of monoclonal antibodies raised against myosin heavy chain (the main determinant of contraction speed) is

more accurate and therefore preferable, especially in characterising hybrid fibres. ATPase is satisfactory for a subdivision of fibres into type I and type II fibres.

Fibre type composition of the gluteus medius and semitendinosus muscle

Our study is the first to describe the correlation and the difference between the fibre type compositions of two locomotory muscles in the horse. These correlations, although not present at all ages, were evident. The data show that if a horse has e.g. a relatively slow gluteus medius, the semitendinosus is slow as well. This suggests that horses may have an overall slow, or fast make-up of their locomotory muscles**.** In the deep gluteus medius muscle the percentages of fibre type I were higher and type IId lower as compared to the semitendinosus. The gluteus medius muscle is a propulsive but also a postural muscle (especially the deeper parts of the muscle). Even when a horse is at rest, the muscle has to be active to stabilise the hip joint. The semitendinosus muscle is only active in a moving horse. Therefore it is imaginable that the deep gluteus medius muscle has to have a higher oxidative capacity to be more fatigue resistant than the semitendinosus.

The fibre type composition of the deep gluteus medius muscle at birth is comparable with that in adult horse breeds with good sprint capacities (high percentage type IId; low percentages fibre type IIa and I). This confirms data of Ronéus and Essén-Gustavsson (1986) who, in the middle portion of gluteus medius, also found a relatively high percentage of fast muscle fibres in very young foals. At the age of 48 weeks, the composition has developed towards a more endurance type of composition (low percentage type IId; high percentages fibre type IIa and I) (e.g. Snow and Guy 1980; Bechtel and Kline 1987).

In the superficial semitendinosus muscle we found a steady 13 % type I fibres. Barrey *et al*. (1995) found a mean of 2 % type I fibres. On the other hand, Essén *et al*. (1980) detected 12-15 % and some investigators (e.g. Aberle *et al.* 1976) mentioned even higher percentages type I fibres (up to 30 %). Investigators did not always mention their sampling depths while the muscle fibre composition depends on it (e.g. Karlström *et al*. 1994)**.** This might explain the discrepancies. Bodine *et al*. (1982), for example, found that samples from superficial parts of the semitendinosus of the cat had approximately 1 % type I fibres, whereas samples from deep parts of the muscle had approximately 23 % type I fibres.

Age effect

Age effects seen on muscle fibre composition depends on the type of muscle, the species, breed and even on sex. Suzuki and Cassens (1980) found in pigs that the percentage of type I muscle fibres increased from birth to 8 weeks of age in several muscles. In contrast, in hamsters it was found that the percentage of type I fibres decreased in the biceps brachii, and increased in the soleus (Goldspink and Ward 1979). Roneus *et al*. found in horses that the percentage of type I fibres and the type IIA/IIB ratio increased in the course of age (1 to 6 years) in the gluteus medius musle of thoroughbreds (Roneus *et al.* 1991) and standardbreds (Roneus 1993). Essén *et al*. (1980) also described that the type IIA/IIB ratio

in the semitendinosus muscle was higher in horses over 5 years of age than in younger horses. Rivero *et al*. (1993) found in Andalusian and Arabian horses that the percentages of type I and type IIA increased and the percentages of type IIB decreased in the middle gluteal muscle up to the age of 6 years. Lindholm and Piehl (1974) reported a higher percentage fast twitch, high oxidative fibres (FOG) in the middle gluteal muscle in adult horses compared with 6 months old foals. The percentage fast twitch, low oxidative fibres (FG) became lower over time. Others (Essén-Gustavsson *et al*. 1983; Henckel 1983) also found increasing percentages of fibre type IIA and decreasing percentages of fibre type IIB from 6 months up to older ages. On the other hand, Bechtel and Kline (1987) reported that foals, during the first 6 months of life enhance their anaerobic, rather than their aerobic components of muscle metabolism in the gluteus medius.

In this study we showed that the age effect on the gluteus medius muscle fibre composition in the first year of life is dramatic. The type I fibre population increased together with a simultaneous increase of type IIa fibres and decrease of type IId fibres. This suggests that type IId fibres turn into IIa fibres. If this is the case, it should most probably happen via transitional fibres, expressing both type IIa and IId MHC (IIa/d) (Pette and Staron 1997). Indeed we found a population of such fibres (at birth 26 %). They were also found in human (Biral *et al.* 1988) and rat muscle (Galler *et al*. 1994) and Rivero *et al*. (1996a) found frequencies of 10 to 15 % in the gluteus medius of 2 and 3 years old horses. Essén *et al*. (1980), investigating standardbred trotters from 2 months of age and older (11-28 years), found that the increase of type IIA fibres and the decrease of IIB fibres continued to the age of 3-4 years. So, probably the transition in our study is not complete yet. Indeed, at the age of 11 months the frequency of type IIa/d fibres is still 18 %.

Like Essén *et al*. (1980), we found a decrease in type IId fibre type percentages in the semitendinosus muscle (from 22 to 48 weeks), but the other fibre type percentages did not show age changes. The mean percentage of the transitional fibres (IIad) in the semitendinosus at birth was 22 % and remained at that level up to the age of 48 weeks. These hybrid fibres may hence not only reflect fibre type transformation, but also may form a biologically important fibre type in the first place.

It can be concluded that the gluteus medius muscle becomes slower and fatigue resistant in the course of time. Because of the postural role of the deeper parts of the gluteus medius muscle during stabilising the hip joint, like Lopez-Rivero *et al*. (1992) we think this is most likely caused by the adaptation to weight bearing. Kernell and Hensbergen (1998), for example, have shown that indeed there is a relationship between the percentage of slow type I fibres and percentage duty time in cat leg muscles. The muscle fibre composition of the semitendinosus muscle is rather stabile.

Effect of exercise

The gluteus medius and the semitendinosus are expected to adapt to training. Earlier studies showed that trained horses, especially trained for endurance, had a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres (now called type IId) in the locomotory muscles (e.g. Rivero *et al.* 1995). However, in our study, exercise did not

significantly influence the rate of change of the fibre types. The question arises whether our exercise protocol provided enough stimulation to induce changes in the muscle. However, Suwannachot *et al*. (1999) showed, in the same horses, a significant increase in the total concentration of Na+,K+-ATPase in the deep gluteus medius muscle of the training group. This means that the exercise was intensive enough to initiate some reaction in this muscle. The mean difference in fibre composition is subject to a quite large intra-individual (e.g. Essén-Gustavsson *et al.* 1989; Lopez-Rivero *et al*. 1992) and interindividual variation (e.g. Snow 1983). Exercise-induced effects will therefore only be measurable when they are large.

The exercise performed can best be described as a moderate intensity sprint exercise. This kind of exercise first may affect the muscle fibre areas and the oxidative capacity of the existing fibres before a change in MHC expression occurs (e.g. Hodgson *et al*. 1985). We observed no exercise effect with respect to the fibre type composition of both muscles. The difference between the three training groups in the muscle fibre composition of both muscles must be caused by other factors like the genetical background.

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Chapter 4

Experimental analysis of error sources in fiber type counts of biopsies

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Summary

The contribution to total variance of different error sources in fibre type counts of equine gluteus medius muscle biopsies was determined to quantify and possibly improve the resolution of the method. Fibre types were defined on basis of myosin heavy chain immunostaining. Errors were determined at levels: (1) positioning the insertion channel, (2) positioning the needle tip (3) biopsy heterogeneity (4) observers interpretation. Errors at levels 1 and 2 were considerable. Confidence intervals for individual observations were ± 10-15 %. In longitudinal studies a group size of 4 animals is necessary to resolve fibre composition changes of 10 %, in cross-sectional studies 7 animals per treatment group appear neccessary. Comparison with multiple counts on post mortem specimen showed that local muscle fibre heterogeneity is responsible for about one third of the error variance. Variance is effectively reduced by processing 3-4 shavings from the same insertion channel.

Keywords: gluteus medius, muscle, biopsy, fibre type, horse

Abbreviations

|d|	95% error margin
SD	Standard deviation
V_o	Total variance
V_e	Error variance
V_t	True variance

Introduction

Skeletal muscle fibres can be subdivided into a number of histochemically distinct types differing in speed of contraction and resistance against fatigue. Therefore assessment of relative numbers of these types with the help of muscle biopsies is a widely used technique to estimate contraction properties of muscle, e.g. to assess the effects of disease, disuse and training. Classically, fibres have been subdivided into type I (slow) and type IIA (fast, fatigue resistant) and type IIB (fast, fatiguable) with the help of ATPase histochemistry. More recent immunohistochemical methods subdivide the fibres on basis of their expression of Myosin Heavy Chain (MHC): in adult humans and horses three MHC isoforms are found, e.g. type I, type IIa and type IId, and fibres with these MHCs correspond to the ATPase fibre types I, IIA and IIB, respectively [5]. A fourth, intermediate fibre type that co-expresses IIa and IId MHC can be distinghuished.

Biopsy sampling is an invasive procedure causing discomfort; the fibre typing process is time consuming. For these reasons the number of biopsies is usully kept to a minimum. In most studies it is assumed that a single biopsy (counting only a few hundred musle fibres) gives a representative picture of the musculature. However, even in human vastus lateralis muscle, showing little evidence of regionalisation in fibre type composition [6] correlations as low as 0.40 – 0.67 between biopsies of the left and right muscle have been reported and standard deviations of fibre type proportion were as high as 7-12%. [2,11,24]

The first error source is the mosaic distribution pattern of the fibre types. The type of a specific muscle fibre depends on both its own differentiation history and on properties of the neurons that contact it.[13] Fetal skeletal muscle fibres are temporarily polyneurally innervated; furthermore, dynamic processes of denervation and reinnervation continue during postnatal life. The result of these processes is not always a random spatial distribution of the fibre types. With the help of different methodologies it has been shown that in healthy man the fibre types are distributed more regularly than random at early age, random from juvenile to middle age and clustered at higher age. [17,28] The degree of non-randomness, *e.g.* apparent by clustering of fibre types, determines the necessary sample size for biopsies (for data on elderly people see [19]). A further complicating factor is the non-random arrangement of fibre types within muscle fascicles (i.e. fibre bundles surrounded by a connective tissue sheet). It has been shown that type IIB fibres are situated preferentially at the periphery and type I fibres in the middle of the fascicles. [20,26]

The second source of error is formed by the regional gradients of fibre type composition, e.g. from superficial to deep, obvious in many muscles. Errors in navigation to the biopsy site will then lead to additional variance in the fibre counts.

This paper estimates the various sampling errors in the determination of fibre type composition of the deep part of the equine gluteus medius muscle, using the immunohistochemical classification. The muscle is known to be heterogeneous: the proportion of type I fibres increases from superficial to deep, from cranial to caudal and from dorsal to ventral.[3] It can serve as a model for a large human muscle with a gradient in fibre type composition, where a biopsy from a single, predefined site must be taken. The

purpose of this study is to evaluate the significance of the errors made during the various steps of the procedure.

Errors in assessing the fibre type composition of a predefined target area were estimated by repeated measurements. Four sources were distinguished.
(1) inaccuracies in biopsy needle placement, leading to error because of *regional variation* within the muscle; assessment by comparing biopsies of the left and right muscle of the same animal.
(2) variation in sampling area at the tip of one needle insertion channel, leading to error due to *local variation* (e.g. in the order of a few cm^3); assessment by comparing four biopsies from the same insertion channel, at the same depth.
(3) variation in selection of the counted area within a biopsy, leading to error due to *intra-biopsy variation* (at the mm^3 level); assessment by comparing two fields in a single biopsy
(4) inconsistencies in assignment of fibre type, leading to error due to *inter-observer variation* in interpretation; assessment by comparing a single field count by three observers.

An observed measurement is taken to consist of a "true" part and an "error" part. If repeated measures are summed and it is assumed that the true parts and the error parts are unrelated, the variance, V_o , of these sums over the horses equals:

$$V_o = V_e + V_t$$

where V_e is the variance of the sums of the error parts and V_t the variance of the sums of the true parts. The latter two variances are between the horses as well. V_e can be estimated from the repeats with a reliability analysis. From V_e , the confidence interval for a single measurement can be determined and from this it is possible to determine how many repeats are necessary at each level of the analysis for an accurate determination of fibre type composition.

On basis of the description of the regional intramuscular variation[3] it is hypothesised that the main source of error is the inaccuracy of biopsy needle placement. If this is true, V_e should be maximal at level I (regional variation). Furthermore, we tested the hypothesis that V_e at levels (II) and (III) is caused by sampling error of non-randomly distributed fibre types at the local level. For this purpose the data were compared with multiple counts in single cross-sections of approximately 1 cm^2 of large biopsies taken post mortem from the studied region.

Materials and methods

Materials
Biopsies were taken from 22 weeks old Dutch warmblood foals of both sexes. These animals were bred, raised and trained for the EXOC project (levels I, IV) [29] and a follow-up project (JUMPEX, levels II,III). All procedures had been approved by the animal experiments committee (DEC) of the School of Veterinary Medicine (Utrecht). Table 1

gives the numbers of animals. The raising conditions were described before.[29] From each foal percutaneous muscle biopsies from the left and right gluteus medius muscles were taken by the same person with the help of a 7 mm diameter biopsy needle, under suction. Bony landmarks served for orientation; the muscle is about 10 cm thick at the biopsy site and the samples were taken from the deepest part; for further details, see. [4]

Table 1 Number of observations on the five levels of analysis

level	n (horses)	k (repeats)
I (regional)	13	2
II (local)	9	4
III (intra-biopsy)	12	2
IV (inter-observer)	3	3
V (post mortem)	4	3

Fibre typing

The biopsies were rolled in talcum powder, frozen in liquid nitrogen and stored at -80°C. They contained 200-600 cross-sectioned muscle fibres. The post-mortem biopsies had a cross-sectional area of 1 cm^2. To identify the fibre types according to their MHC content we used specific monoclonal antibodies (Mabs): Mab 219-1D1 (raised against chicken heart myosin) reacts with type I.[2,30] Mab 332-3D4 (raised against rabbit eye muscle) reacts with type IIa and IId. Mab 333-7H1 (raised against protein extract from muscle tissue of adult rabbit anterior tibialis) reacts with type IIa.[2] Mab 412-R1D5 (raised against myosin isolated from rabbit psoas muscle) reacts with type I, IId. [2]

Transverse serial sections (10 μm) were fixed overnight in a 35% methanol, 35% acetone and 5% acetic acid solution, treated with pronase and then incubated with the Mabs. The slides were then incubated with a biotinylated horse anti mouse polyclonal antibody and subsequently incubated with the components avidin and biotin. The immunoreaction was visualised by incubation with 3,3'-diaminobenzidine tetrachloride in 30 mM imidazole and 0.09 % H_2O_2 . The slides were subsequently stained with Harris hematoxylin for 45 seconds. The procedure was described in detail previously. [4]

In each biopsy 200 adjacent fibres were classified on basis of their myosin heavy chain (MHC) expression into one of four fibre types, i.e. fibres expressing MHC I, IIa, IId and IIa+IId, with the help of the Mabs. The percentage of type I, IIa, IId and IIad fibres was determined in each sample. Note that the lower case a and d indicate fibre typing on basis of MHC, not on ATPase

Experimental design

The measurements were carried out in random order by three trained observers, unaware of the exact source of the biopsies.

Level I Regional intramuscular variation

To determine the effect of the inaccuracy in placement of the biopsy needle in 13 animals a biopsy was taken from the right and from the left muscle.

Level II Local intramuscular variation

In nine horses unilateral biopsies were taken as follows: after insertion of the needle to the prescribed depth, suction was applied and one biopsy taken. Suction was then removed, the needle somewhat retracted and rotated around its longtitudinal axis, reinserted, and suction was reapplied and a second biopsy taken at the same depth. The procedure was repeated another two times, yielding four biopsies from the same channel.

Level III Intra-biopsy variation

In each of 12 biopsies from different horses two separated fields of 200 adjacent fibres were counted to assess the variation within a mm^2 area.

Level IV Inter-observer variation

In three biopsies from different horses a fibre classification was performed independently by three trained observers.

Post mortem analysis (level V)
In 4 foals that were euthanised at 22 weeks of age, large samples were taken from the designated area of the muscle. Per sample three separate areas, separated by an average distance of 0.5 cm and containing 200 fibres, were counted.

Statistics
As the subjective ease of assessment of the four fibre types differed (type IIad being the most difficult to classify) the reliability of the measurements was determined separately for the percentages of type I, IIa, IId and IIad fibres. The analysis was carried out for each of the four levels (I-IV) of the in vivo biopsy analysis and the post mortem analysis (V). At each level, a matrix of k (repeats) x n (horses) was available for each fibre type. Table 1 gives the numbers of horses and repeats. These matrices were subjected to a reliability analysis using the strict parallel scale model. This model assumes that the means and standard deviations of the repeated series are equal. Inspection of the data did not yield any evidence to the contrary.

As an indication for the quality of the data the correlation between the repeats is calculated first. In case more than one repeat is available, the combined correlation ('common inter-item correlation') is looked at next. Finally, for single measurements the

total (or measured) variance V_o, the error part variance V_e, and the reliability were estimated. The reliability of the measurement is defined as:

$$Rel = V_t / V_o \text{ or } Rel = (V_o - V_e)/ V_o$$

It was assumed that the errors are normally distributed with $\mu = 0$ and $\sigma = \sqrt{(V_e)}$ so boundaries of a 95 % confidence interval of a single measurement X were determined by:

$$X \pm 1.96*\sqrt{(V_e)}$$

The analyses were carried out with the Statistical Package for the Social Sciences (SPSS 10.0 for Windows).

Results

General

The estimated mean correlation between the k repeats, the error variance, the reliability, mean and the confidence margin of a single measurement are shown in Table 2.
All data are expressed as percentages.

The inter-repeat correlations in the first column can be seen as an indication of the closeness of the repeats. In case of the inter-observer and post mortem levels they are based on few data. The general trend is that the correlations increase from level I through IV. For fibre type IIad, the correlation between the repeats for levels I, II and IV was poor (even negative between one pair of repeats). It was concluded that for those levels further calculations were useless.

The error variance in column 2, expressed in $(\%)^2$ varies strongly between the levels and the fibre types. The values are based on different groups of horses that may, by random effects, differ in their within group means and variance. V_e decreases from level I/II to IV, and as a result, the estimated reliability of the sampling method increases from level I/II to IV. It was expected that the error on level I would be higher than on level II, as on level I a new insertion channel had to be made. In contrast, for fibre types I and IId the level II variation is clearly higher, and for IIa fibres the two levels show the same error variance.

Reliability values, which can be found in column 3, are high mostly. They generally increase from level I/II to IV.

For a single measurement X %, the 95 % confidence interval indicates the boundaries between which the true value is located with 95 % probability. To determine these boundaries for a particular observed value X % the margins found in column 4 should be subtracted and added to X %. For example, if in a single biopsy the gluteus medius muscle the type IIa fibres constitute 23 % of the total count, after subtracting and adding 10.1 % for level I one can say that there is a 95 % probability that the true value is in the range of 12.9-33.1 %.

The table shows that despite the high reliability of the data, the 95 % confidence margins of the means are large. The given percentages should be compared with the mean percentages of these fibre types. These means are given in column 4 of table 2.

Differences between fibre types

The estimation of type IIad fibres is clearly poorer than that of the other fibre types. It is only on the intra-biopsy level that repeats agree sufficiently for further calculations. It should be kept in mind that type IIad is identified by positive reaction to two different antibodies.

Variance within post-mortem biopsies

The biopsies were collected to estimate the amount of local heterogeneity in the area were the biopsies are normally taken. The counts from single post mortem samples (V) showed an error variance, intermediate between levels I/II at the one and III at the other hand. To play it safe one should use the highest error found for each fibre type. For fibre types I, IIa and IId highest errors are respectively 60.1, 26.5 and 65.2 $\%^2$ (table 2). Within the post mortem biopsies, however, errors were found of respectively 5.1, 10.4 and 30.9 $\%^2$ for these fibre types. This means that roughly one third of the variance of the biopsy method can be attributed to heterogeneity of fibre type composition at the level of the post mortem biopsies, i.e. within a cross-sectional area of 1 cm^2.

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*Estimated common inter-repeat correlation

[1]Expressed in $(\%)^2$

[2]The 95 % confidence margin of one measurement X %: X % ± 1.96*$\sqrt{}$(V_e) %

Table 2 Reliability and confidence margins of single measurements of fibre type percentages

level	Correlation*	Ve [1]	Reliability	mean (%)	95 % margin[2]
Fibre type I					
I (regional)	.57	14.1	.72	18.7	7.3
II (local)	.89	60.1	.97	37.5	15.2
III (intra-biopsy)	.95	3.7	.98	28.7	3.8
IV (inter-observer)	.99	0.3	.99	14.9	1.1
V (post mortem)	.95	5.1	.98	27.0	4.4
Fibre type IIa					
I (regional)	.59	26.5	.74	17.9	10.1
II (local)	.38	25.4	.71	13.4	9.9
III (intra-biopsy)	.84	3.3	.84	15.0	3.6
IV (inter-observer)	.92	2.0	.97	31.2	2.8
V (post mortem)	.40	10.4	.67	37.0	6.3
Fibre type IId					
I (regional)	.89	11.1	.94	42.4	6.5
II (local)	.80	65.2	.94	22.3	15.8
III (intra-biopsy)	.90	15.7	.95	26.5	7.8
IV (inter-observer)	.97	3.1	.99	39.5	3.5
V (post mortem)	.60	30.9	.94	27.0	10.9
Fibre type IIad					
I (regional)	.23	-	-	20.6	-
II (local)	.12	-	-	26.7	-
III (intra-biopsy)	.64	14.4	.78	30.2	7.4
IV (inter-observer)	.25	-	-	14.5	-
V (post mortem)	.73	4.4	.96	9.0	4.1

Discussion

Resolving power and implications for experimental design

In horses, fibre type distributions have been described as a function of race,[10] age, [4,8,10,23] training groups [23] and performance groups.[22] In contrast to changes in capillarisation, changes in fibre type composition often appear to be absent or, at least, stay below the detection level.[5,7] This study shows that the detection level for changes of fibre type composition may be rather high. Assume, a single biopsy is taken in two identical horses that have undergone different exercise regimens. The error of the method is V_e. Before one can say these horses are different, the absolute value of the difference d in % fibres of a certain type must exceed the 95% confidence margins of error for such difference. These margins |d|, indicating the resolving power of the method, can be found by multiplying the 95% confidence margin for a single measurement by √2; the horses are different if

$$|d| > 1.96 * \{\sqrt{(V_e)}\} * \sqrt{2} \qquad (1)$$

In this study, for fibre types I, IIa and IId the highest margins for single measurements for levels I and II range from 10.1 - 15.8 % (table 2). Hence, the worst resolving power of the difference in a single animal test equals √2 times these values, 14-22 % (mean 19 %). Consequently, the biopsy technique has a very limited value if applied to a single individual. The situation is not better in four human muscles, where standard deviations for biopsy sites across the entire muscle were estimated to be 9-16 % [6] , and the 95% margins will be twice that figure. In a study of a single biopsy site in the (non-regionalised) human vastus lateralis, standard deviations of 5 – 6 % were found, and even larger values for samples of the left and right muscles[1]; in that muscle correlations between left and right muscles were as low as 0.40 - 0.67.[24]

It should be noted, that our repeat data on fibre type IIad fibres were not reproducible enough to warrant further evaluation of errors. This may be related to the fact that these fibres are small and the type is difficult to classify. In literature, relatively large observer errors were likewise found in the subclassification of ATPase determined type II fibres.[1]

Hence, failure to find statistically significant changes in fibre type composition, both in human and equine muscle, may be due to the lack of resolving power.

A strategy to increase the resolving power is to take 2-4 biopsies from the same animal and treat their average as input data. The $\sqrt{(V_e)}$ values will decrease with a factor √2 - √4, and the resolving power will increase accordingly. Our results suggest that a considerable gain can already be reached by taking consecutive shavings inside the same insertion channel, avoiding the damage caused by completely reinserting the needle. On basis of the finding that the percentage of type I fibres increased from superficial to deep in the gluteus medius muscle[18] a comparable strategy was adopted of collecting biopsies from two or three depth levels.[21]

The difference between two measurements in a single individual (n = 1) has a 95% confidence margin of 19 % (mean of 3 fibre types, see above). In case of longitudinal studies of treatment effects, the 95% confidence margin decreases in proportion to the square root of the number of animals. In figure 1 the relationship between the width of the confidence interval and number of animals in a longitudinal study has been plotted with the mean of 19 % as a starting point for a single individual (thick line). It takes 4 animals to reduce the detection level to 10 %, *e.g.* to resolve a change from 25 to 35 %. A detection level of 5 % requires 15 animals.

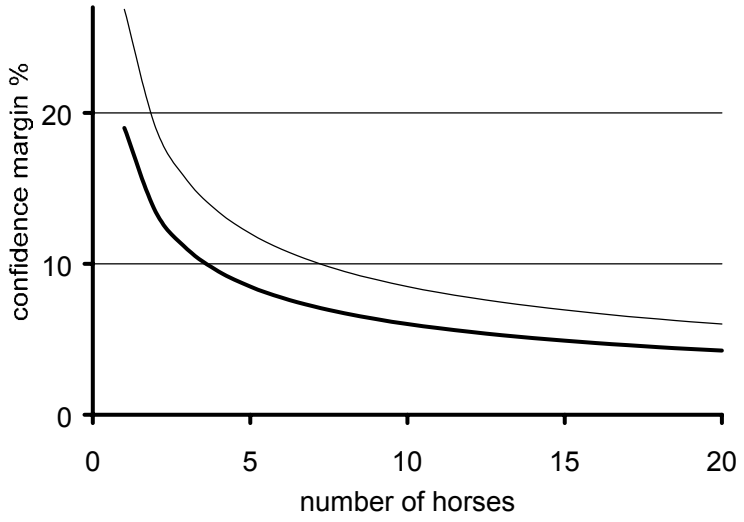

Fig. 1. Confidence margins of the difference d between the mean fibre type percentages of two sets of observations (|d|, see discussion) and the number of animals (n). In a longitudinal study (thick line) d is the difference between a single group of n animals measured a two moments. In a cross-sectional study (thin line) d is the difference between two groups of n horses. For n = 1, the confidence margin equals 19 % in a longitudinal study, and 19 * √2 = 26 % in a cross-sectional study (see discussion).

In cross-sectional studies formula (1) for the margins of the difference between two individuals also applies, and so does, in case of group size n, the decrease of these margins by a factor √n. However, in this case the total variance V_e equals:

$$V_e = V_{e\ (method)} + V_{e\ (interindividual)}$$

As V_e was about twice $V_{e(method)}$ (data not shown) the error margin wil be a factor √2 larger.

Consequently, in a comparison of two animals the confidence margin for d of 19 % would be increased with a factor √2, *i.e.* to 27 %. In groups of size n the confidence margin of d is found by dividing the confidence margin of 27 % by √n. The consequences for the resolving power are given in fig.1 (thin line). Eight animals would be needed to reach 10 % resolving power, a power of 5 % is not feasible. In human vastus lateralis, comparable numbers of subjects would be needed in a cross-sectional study, as the inter-individual variation is large: the standard error of the type I fibre % in 215 young adult Caucasian males was 13% (error margin = 26%).[25]

It should be noted that shifts of 10 % on a mean of *e.g.* 25 % constitute a significant biological effect. However, following previous studies[1], we have chosen not to express results in percentages of the mean, as the magnitude of the errors seemed independent of the means, and the means are mutually dependent, adding up to 100 %.

Sources of error

The primary cause of the measuring error appears to be neither the difference in interpretation of fibre type between observers (with the possible exception of type IIad), nor the number of fibres counted in a single biopsy. However, significant error variance is introduced at regional and local levels in the muscle.

The first factor significantly contributing to this error is heterogeneity at a local scale. It is generally agreed that counts of more than 200 adjacent fibres do not increase accuracy.[2,6,27] However, differences of 40% or more in type I fibre type % are seen between separate areas in the same biopsy.[6] This may be caused by unequal distributions of fibre types between the periphery and the centre of fascicles, as demonstrated in porcine muscle,[14] but also in some human [20,26], bovine,[9] rabbit and rat[12] muscles. Measurements on local variation of fibre type proportions of human vastus lateralis in an autopsy study[15,16] show that within a region of 60-100 mm^2 the type I fibre proportion in individuals varies greatly, with a standard deviation of 4-10 % on a mean of about 50 %.

Our data for type I fibres on the post mortem biopsies taken from comparable areas show lower absolute standard deviations ($\sqrt{V_e}$ = 2.2 %, table 2, level V). Nevertheless, the error variance for fibre types I, IIa and IId, seen in repeated measurements within the 1 cm^2 post mortem biopsies also reaches levels of about one third of the largest observed (either regional or local) error variance (V_e) seen in in vivo biopsies. Hence the observed errors in biopsy data may for a significant part be the result of tissue heterogeneity at the 1 cm^2 level.

The remaining variance must be due to a positioning error of the biopsy needle, in combination with the fact that the fibre types are not homogeneously distributed over the entire muscle. Although the insertion point of the biopsy needle is easy to reproduce, the angulation of the insertion channel, reaching a depth of 8 cm or more may lead to a positioning error in the site of the biopsy of several centimeters. However, we have no explanation for the finding that the error at level I (left/right comparison) is not larger than at level II (shavings from the same insertion channel). Apparently, both procedures lead to the harvest of biopsies from areas at comparable distances apart.

In an autopsy study of the equine gluteus medius it was shown that besides a gradient in fibre type composition with depth, there were also gradients in cranio/caudal and dorso/ventral directions.[3] For the area that was sampled in this study, the data (table 1, A3-5) show that the mean percentage of type I fibres may differ up to 5-15 % over distances of 5 cm at a single depth level. Our standard deviations ($\sqrt{V_e}$; 3.8 and 7.6 % for regional and local levels), are within this range. We conclude that the fraction of observed variation that could not be accounted for by local heterogeneity must be produced by inaccuracies in positioning of the biopsy needle.

In conclusion, needle positioning errors and local non-random distribution of fibre types contribute both to a rather large inaccuracy in the determination of fibre type composition in the deep part of the equine gluteus muscle. As the localisation error accounted for two thirds of the variance and the error is already apparent in biopsies taken from the same insertion channel, taking multiple shavings from this channel seems an appropriate way to reduce the error. The error described for human vastus lateralis, soleus, triceps and biceps brachii muscles seems to be about as large as in equine gluteus. In the vastus lateralis regional variation is absent and local heterogeneity accounts for all of the error.[16] In such cases counting more fibres leads to a significant reduction in error. In muscles showing regional gradients of fibre type composition (e.g. m. triceps brachii [6]) a strategy of taking multiple biopsies by a single insertion channel might be the best option to reduce error.

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Chapter 5

Postnatal development of the oxidative enzyme activity in equine locomotion muscles and the influence of exercise

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Summary

To study the effect of age and exercise on the citrate synthase (CS) activity, we took biopsies from the deep gluteus medius and superficial semitendinosus muscle at four ages in 37 Dutch warmblood foals, divided over three training groups. The CS activity is higher in gluteus medius than in semitendinosus muscle. At birth, the CS activity in gluteus medius and semitendinosus muscle is positively correlated. CS activity decreases from 0 to 22 weeks. An exercise effect was not detected. The succinate dehydrogenase (SDH) activity was studied in four fibre types of the gluteus medius in biopsies of 13 foals, divided over the training groups, at birth and 22 weeks. At birth, SDH activity in IId fibres is lower than in other types. At 22 weeks SDH activity in IIad fibres is lower than in IIa and I, but still higher than in IId fibres. SDH activity increases in I and IIa and decreases in IId fibres. The decrease in IId fibres is largest in the boxrest group. We conclude that the normal capacity of equine locomotory muscle to use oxygen for energy production for contraction and relaxation decreases in the postnatal period. Restricted movement results in underdevelopment.

Keywords: horse, citrate synthase, succinate dehydrogenase, gluteus medius, semitendinosus, exercise, biochemistry, histochemistry

Introduction

Muscular fatigue often causes exercise intolerance in horses (Valberg 1996). For a proper muscle function, fatigue must be prevented and therefore, substrate needs to be converted into energy. Energy for the muscle comes in the form of adenosine triphosphate (ATP), which can be most efficiently produced by oxidative phosphorylation. The activity of succinate dehydrogenase (SDH) predicts the maximum rate of oxygen consumption (oxidative capacity) and therefore correlates with energy production (van der Laarse *et al.* 1989). Pette (1962) described a proportional relation between oxidative capacity-related enzymes. The activity of citrate synthase (CS), another oxidative enzyme, can also be used as a measure of tissue energy production.

Citrate synthase is an enzyme in the mitochondria which catalyses the conversion of acetyl coenzyme-A and oxaloacetaat in coenzyme-A and citrate in the citric acid cycle. SDH is an enzyme which is also located in the mitochondria and catalyses the conversion of succinyl-CoA in succinate in the citric acid cycle. Pool *et al.* (1979) have developed a quantitative histochemical method to measure succinate dehydrogenase activity in individual muscle fibres.

Age and exercise are known to increase the oxidative capacity in the muscles of horses (Guy and Snow 1977; Snow and Guy 1979; Hodgson *et al.* 1986; Roneus *et al.* 1992; Roneus 1993). It is also known that in horses, type I and IIa fibres generally show a higher oxidative capacity than type IId fibres (e.g. Valberg *et al.* 1985, 1988) but that this can vary markedly within one type of the different fibres. Especially in the population of type IId fibres, investigators found oxidative enzyme activities varying between low and as high as in type IIa fibres (Valberg and Essen-Gustavsson 1987). Most of these studies were performed on adult or young adult race horses. Some authors describe a decrease in oxidative capacity in the first months after birth due to decreasing percentages of oxidative fibres (Bechtel and Kline 1987, Kline and Bechtel 1990). However, direct information about the oxidative capacity in the muscles of new-born horses and the development during the first year of life is still lacking.

The aim of this study was to investigate enzyme activities, as predictors of the oxidative capacity, in locomotory muscles of Dutch warmblood foals in the first year of life. Furthermore, we wanted to investigate if exercise, given in such an early phase of life, could influence the normal pattern of age related maturation of the oxidative capacity.

Material and methods

Foals

The investigation was performed in a group of 37 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system (in particular osteochondrosis) as described by van Weeren and Barneveld (1999). All procedures were approved by the

animal experiments committee (DEC) of the Faculty of Veterinary Medicine (Utrecht, The Netherlands). After birth, all foals remained with the mares in a paddock for one week, after which they were randomly divided into three groups that were subjected to different exercise regimens until weaning at five months. Two groups (boxrest, n = 12 and training, n = 11) were individually housed with the mare in 3 x 3.5 m box stalls. They were fed freshly cut grass ad libitum, along with the mother's milk. The third group was kept at pasture with the mare 24 h a day (pasture group, n = 14). At 22 weeks of age 21 foals (7 foals from each group) were euthanized for other purposes. The remaining 16 foals were joined in one single group which was kept in a loose house with access to a small paddock. All foals were subject of investigation in the determination of the CS activity but, due to the labour intensiveness of the method, only 13 (boxrest n = 6, training n = 3, pasture n = 4) of them were used for the determination of the SDH activity.

Exercise protocol

Until 22 weeks, the boxrest group was kept in the box stall for 24 h/day. The training group was also kept in box stalls, but was given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals followed the mares. Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 22 weeks 32 and 16 sprints on alternating days. Exercise was given 6 days a week. The sprints lasted for about 6 seconds (" 6.5 m/s) and a pause was kept after each sprint to prevent exhaustion. After the sprints, the foals were allowed an additional 0.5 hours of free exercise in the enclosure. The pasture group received voluntary movement. After 22 weeks up to 48 weeks, none of the foals was further trained, so all got the same exercise regimen.

Muscle biopsies

Two locomotory muscles (i.e. gluteus medius and semitendinosus muscle) were chosen as a model. These two muscles are subjects of investigation in many studies, mainly because of their important propulsive role, but also because taking biopsies from these muscles is relatively easy.

Percutaneous muscle biopsies were taken from each foal according to the protocol of Lindholm and Piehl (1974) in the first week after birth (day 3 on average) and at the age of 4 and 22 weeks. From half the foals, biopsies were also taken at the age of 48 weeks. To cause as little as possible discomfort, the young foals were handled by their caretakers in the presence of their mares and sedation was not necessary. After local anaesthesia, biopsies were taken as described by Dingboom *et al* (in press). Biopsie from the deep gluteus medius muscle were taken on an imaginary line drawn from the coxal tuber to the sacral tuber, at one third distance from the sacral tuber, perpendicular to the skin. They were taken as deep as possible (until resistance from the iliac wing) because it was expected

that at this site, the percentage type I fibres was high enough to demonstrate age- and / or exercise effects. Biopsies from the superficial semitendinosus were taken on a line drawn from ischiadic tuber to popliteal area, at two third distance from the ischiadic tuber, at a depth that was reached just after the muscle fascia was penetrated. This location was chosen because of the expected homogeneous type II population on this site. A part of each sample (for CS activity determination) was frozen and stored at –80°C. For SDH activity determination, a part of the samples from the gluteus medius muscle were rolled in talcum powder, mounted on cork blocks with the use of OCT embedding medium and oriented in such a way that the fibres could be sectioned transversely. Transverse serial sections (10 µm) were made with a cryostat at - 20°C.

CS measurement

Work reagens 1.0 ml 5,5'-dithio-2-nitrobenzoaat (DTNB; 1 mM: 0.79 mg DTNB in 2.0 ml 1 M Tris (pH 8.1)), 400 µl acetyl coenzyme A (7.5 mM) and 250 µl Triton X-100 (2 %) was put together in 7.6 ml H_2O. The pH was between 7.4 and 9.0.

Homogenate The muscle biopsy was defrozen in cooled SETH (4.3 % sucrose ; pH 7.4; osmolarity between 280 and 310 mOsm / kg) buffer (10 %: 10 mg muscle tissue in 100 µg SETH) and was kept cool on ice. The defrozen sample was cut with a pair of siccors and desintegrated ultrasonically to become a homogenate. To destroy the mitochondria, in order to release the enzyme, 50 µl of the homogenate was frozen (in liquid nitrogen) and defrozen (in a waterbath of 25 ^{o}C) twice and kept on ice until the spectrophotometical measurement.

Spectrophotometry measurements of CS activity was done with a spectrophotometer[1] connected to an Olivetti computer, with Ultro spec III (enzyme kinetics) software was used. Duplicate measurements were carried out at 25 ^{o}C. The absorbance was measured at 412 nm. The measuring time was set to 7 minutes. The work reagens (925 µl) was put in the calibrated spectrophotometer and equilibrated to 25 ^{o}C. Then, the homogenate (25 µl) was added and mixed carefully with the workreagens before measuring the absorption. To establish the zero activity, we followed the absorption for 90 seconds. Next, we added 50 µl oxaloacetate (10 mM in 0.1 M Tris (pH 8.0)) and followed the absorption for at least 5 minutes. The CS activity is measured as mU/ml, which is the amount of enzyme, in 1 ml homogenate, that converts 1 nmol oxaloacatate per minute. To standardise the results, the enzyme activity is expressed in mU/mg protein content of the homogenate (protein measurements according to Lowry).

SDH measurement

Staining sections (10 µm) for muscle fibre type determination were incubated with monoclonal antibodies (Mab's) raised against certain myosin heavy chain isoforms (Dingboom *et al.* 1999). Adjacent sections (10 µm) for histochemical SDH staining were incubated, immediately after sectioning (within 24 hours after taking the biopsies), in 37 mM sodium phosphate buffer, 74 mM sodium succinate and 0.4 mM tetra nitro blue tetrazolium (TNBT) at pH 7.6 for 30 minutes at 37 ^{o}C; a formazan deposit is formed. The reaction was stopped by immersing the sections in 0.01 N HCL, followed by two rinses

with water. The sections were mounted in glycerin-gelatin. Stained sections were stored at 4 ^{o}C in darkness until measurement (Pool *et al.* 1979).

In series of Mab- and SDH stained sections of one biopsy, muscle fibres were typed in I, IIa, IId or IIad fibres with a certain SDH staining intensity. The muscle fibres were classified into type I, type IIa, type IId, type IIa/d on basis of their reactions with the Mabs (Dingboom *et al*. 1999). The SDH activity of the four different fibre types was determined by measuring the absorbance of the TNBT formazan deposit by means of microdensitometry at 660 nm (van der Laarse *et al.* 1989). The mean staining intensities of duplicate measurements of five fibres from each type were pooled. The sections were studied with a Leica DMRB microscope[2] fitted with calibrated grey filters. The images were obtained with a 10x objective and a monochrome Charge Coupled Devises camera[3], connected to a LG-3 frame grabber[4] in an Apple Macintosh computer. Images were analysed using the public domain NIH Image 1.61 program[5].

Statistics

For both CS and SDH activity, the program for general linear model-repeated measures (SPSS 10 for Windows) was used to test the effects of age and exercise. Time was used as within subject factor and exercise as between subject factor and was defined at four levels (0, 4, 22 and 48 weeks) for CS or at two levels (0 and 22 weeks) for SDH. Analysis of variance (ANOVA) was used to test if there were differences already existing at birth. We analysed the data from 0 to (4 and) 22 weeks to establish the effect of exercise and from 22 to 48 weeks to see if exercise effects persisted. To analyse if the differences between the gluteus medius and the semitendinosus muscle in CS activity at 4 ages were significant, 'Muscle' was used as a second within subject factor.

To test if the CS activity of the gluteus medius and the semitendinosus muscle is correlated, we performed Spearman's bivariate correlations.

ANOVA was used to test if the four fibre types are different with respect to their SDH activity at birth and at 22 weeks.

In all tests, differences were accepted when $p < 0.05$.

Results

CS activity

Table 1 shows the results of the CS activity (expressed in mU/mg protein) in the gluteus medius and semitendinosus muscle in the three training groups separately. Figure 1 shows the results of the CS activity in the total group. From this table and figure it can be observed that the standard deviations are rather large.

In general, at all four ages, the CS activity in the gluteus medius muscle is higher than in the semitendinosus muscle ($p < 0.001$). At birth, the CS activity in the gluteus medius muscle is positively correlated with the CS activity in the semitendinosus muscle (figure 2; correlation 0.63, $p < 0.001$).

In both muscles there is a statistically significant decrease ($p < 0.02$) of the CS activity in the postnatal period, but the time course of the decrease differs. In the semitendinosus the CS activity decreases immediately after birth (from 0 to 4 weeks of age); in the gluteus medius it occurs later, in the period from 4 to 22 weeks. After 22 weeks, the CS activity remains stable in both muscles.

An effect of exercise could not be detected.

Table 1. The mean (+/- standard deviation) of the CS activity (expressed in mU/mg protein) from the deep gluteus medius and superficial semitendinosus muscle in the three training groups and the total group at 0, 4, 22 and 48 weeks of age. (boxrest n = 12; training group n = 11; pasture group n = 14). * age effect compared to the previous age-colomn ; p < 0.001.

		0	4	22	48
Gluteus medius	Boxrest	158.4 (49.7)	181.1 (58.6)	117.1 (36.7)	114.0 (33.3)
	Boxtraining	171.5 (38.8)	191.4 (74.6)	115.9 (38.0)	123.7 (18.7)
	Pasture	166.7 (78.0)	192.4 (81.9)	119.4 (31.6)	114.9 (30.6)
	Total	**165.6 (55.5)**	**188.3 (71.7)**	**117.5 (35.4) ***	**117.5 (27.5)**
Semitendinosus	Boxrest	104.4 (21.3)	76.5 (23.6)	68.5 (26.6)	72.6 (7.7)
	Boxtraining	115.3 (32.2)	76.0 (28.1)	116.4 (22.5)	101.7 (29.0)
	Pasture	153.9 (70.9)	121.2 (43.3)	81.7 (24.6)	81.8 (12.5)
	Total	**124.5 (41.5)**	**91.2 (31.6) ***	**88.8 (24.6)**	**85.4 (16.4)**

SDH activity

Figure 3 shows an example of the combination of the results from the muscle fibre classification and the histochemical SDH staining, in order to determine the SDH activity in the four different fibre types.

Figure 4 shows the results of the SDH activities (expressed as the absorbance at 660 nm) in the four different fibre types in the gluteus medius muscle at 0 and 22 weeks of age in the boxrest, training and pasture group and in the three groups together (total). At birth, the SDH activity in type IId fibres is low compared to the other three types. At 22 weeks of age the SDH activity in type IIad fibres is also lower than in type IIa and type I fibres, but remains higher than in type IId fibres.

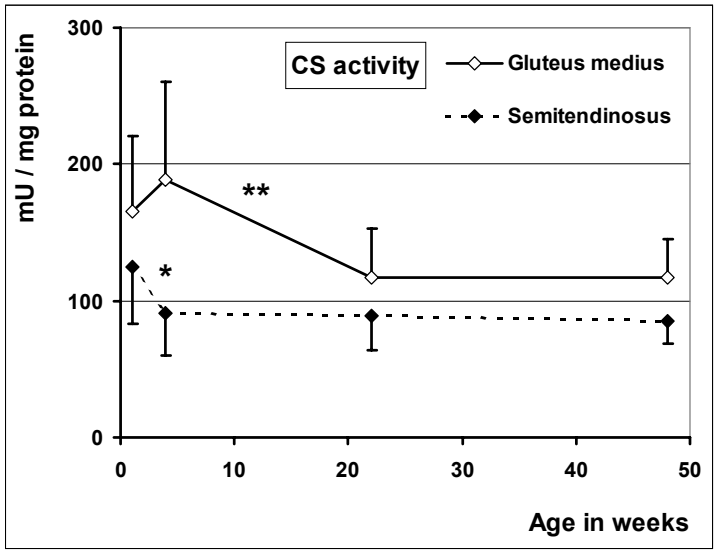

Fig. 1. The mean and standard deviations (y-error bars) of the CS activity from the gluteus medius and semitendinosus muscle in the total group at 0, 4, 22 and 48 weeks of age (n = 37). * age effect; $p < 0.02$. ** age effect; $p < 0.001$.

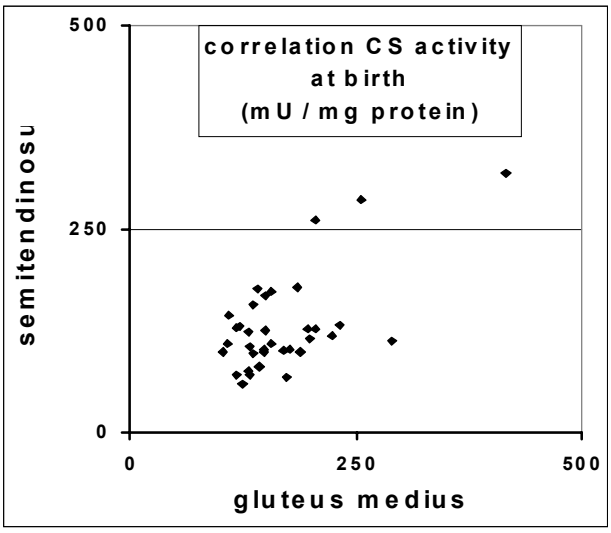

Fig. 2. Scatter plot of individual CS activities from gluteus medius and semitendinosus muscle that show a statistically significant correlation at birth. Correlation 0.627, $p < 0.001$.

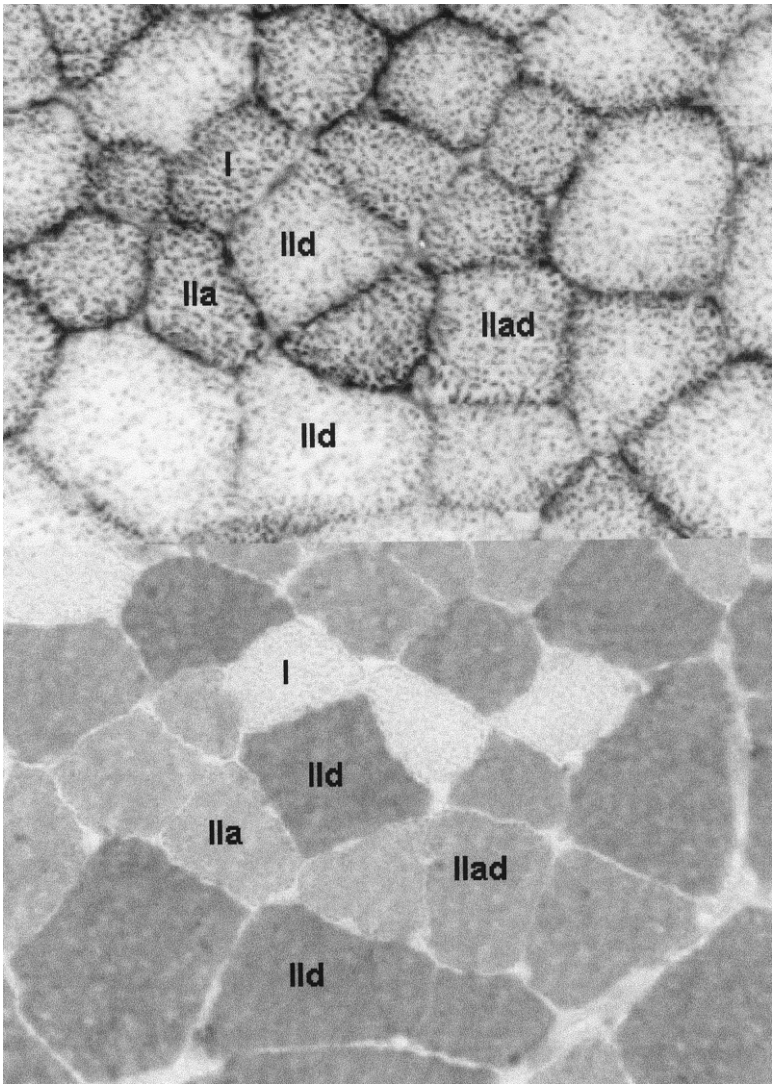

Figure 3. An example of the combination of the results from the histochemical SDH staining (top) and the muscle fibre classification after immunohistochemical MHC staining (bottom), in order to determine the SDH activity of the four different fibre types. Gluteus medius muscle of a 22 weeks old foal. The type I and type IIa fibres stain dark, the IIad fibres stain intermediate and the type IId fibres stain light.

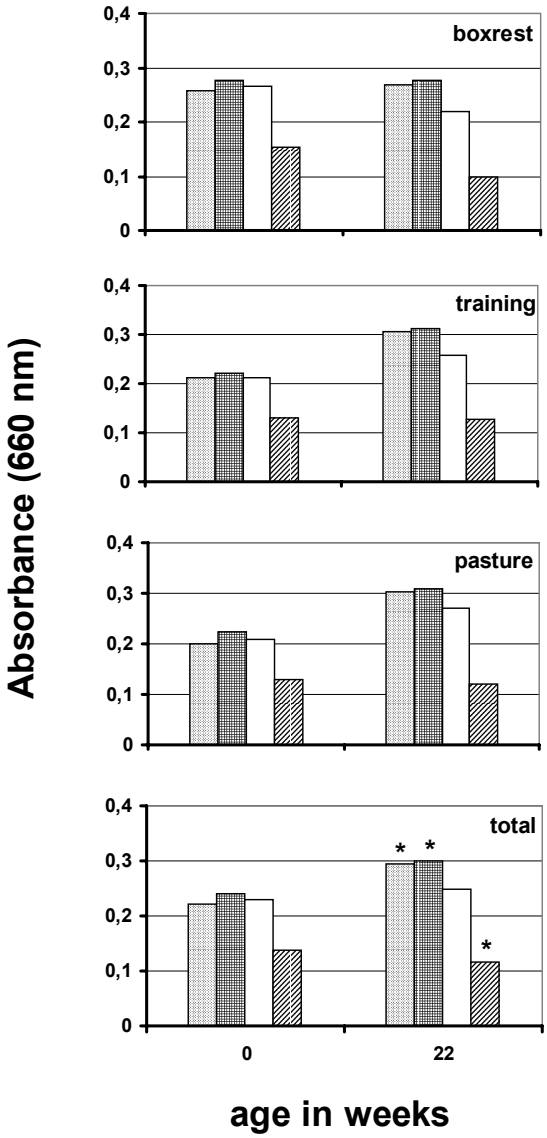

Fig. 4. The means of the SDH activities in the four different fibre types (grey bar for type I, blocked bar for type IIa, white bars for type IIad, striped bar for type IId) in the gluteus medius muscle at 0 and 22 weeks of age in the boxrest- (n = 6), in the training- (n = 3), in the pasturegroup (n = 4) and in the three groups together (total). * age effect; $p < 0.01$.

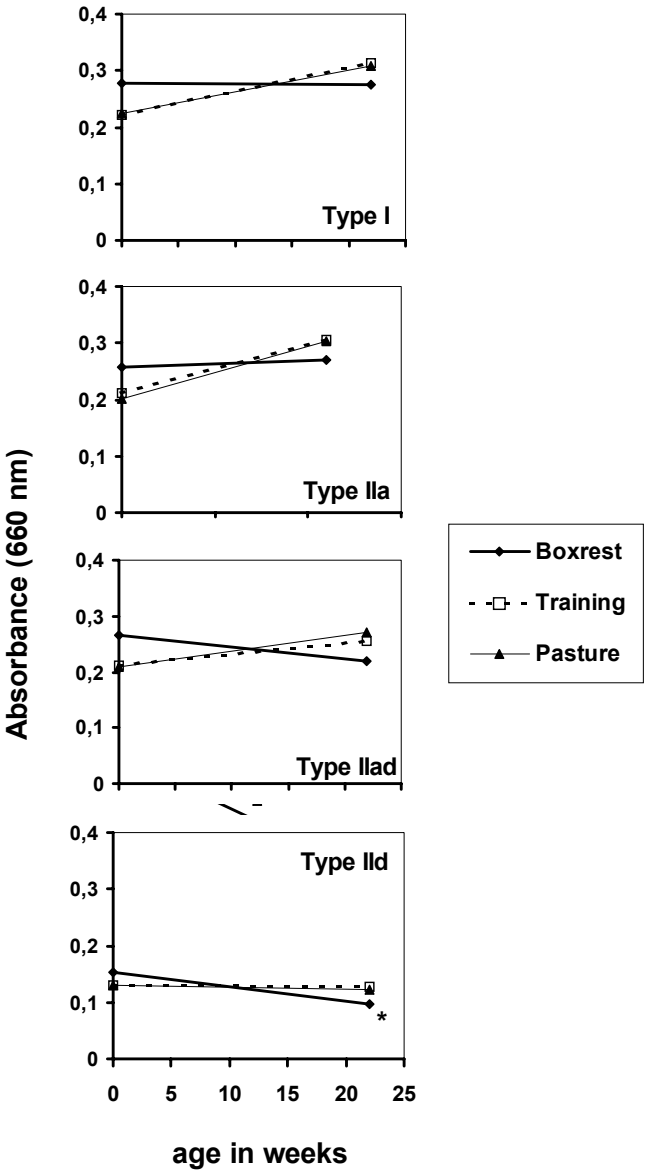

Fig. 5. The means of the SDH activities of the glutus medius mucle in the boxrest- (bold solid lines, n = 6), in the training- (dotted lines, n = 3) and in the pasture group (thin solid lines, n = 4) demonstrated per fibre type. * exercise effect, $p < 0.04$.

From the graph of the total group (figure 4) we conclude that from 0 to 22 weeks there is a significant age effect ($p < 0.01$) i.e. an increase of SDH activity in fibre type I and IIa and a decrease in fibre type IId.

Figure 5 reviews the changes in the mean SDH activities per fibre type and per training group. The increase of SDH activity in type I and IIa fibres appears not to be present in the boxrest group. The effect of age on the SDH activity in fibre type I and IIa seems to be counteracted by the lack of exercise but this is not statistically significant ($p = 0.069$). Furthermore, we see that the overall decrease of SDH activity in type IId fibres is caused by the decrease in the boxrest group. This exercise effect is statistically significant ($p = 0.032$).

Discussion

CS activity

General: The CS activity in the gluteus medius muscle is higher than in the semitendinosus muscle which is in line with the results of other investigators (e.g. Essen *et al*. 1989) but may also be due to the dissimilarity of the sampling site in both muscles. From the gluteus medius, the biopsies were taken as deep as possible and from the semitendinosus muscle as superficial as possible. From other studies we know that these sites differ in their muscle fibre type composition (aerobic versus anaerobic; e.g. Dingboom *et al.* in press), capillarity (high versus low; unpublished data) and presumed function (postural role plus propulsion versus solely propulsion). The higher CS activity in the gluteus medius corresponds with the high oxidative demand on the deep portion of this muscle. The CS activities of the two muscles show a significant positive correlation at 0 weeks of age. Such correlations were previously found in the same group of animals for muscle fibre type composition and capillarity. Not only do the average values for a number of parameters differ between the two muscles, they also correlate positively across the individuals. This suggest that this variation is genetic. Results from other investigators also point in this direction. For example, (Lopez) Rivero *et al*. (1991, 1996) found evidence that proportions of type I and type II fibres are highly stable within a given horse breed and significantly correlate with genetic factors. Other investigators found that the fibre type composition of human muscle (Simoneau *et al.* 1995), pig muscle (Szentkuti and Schlegel, 1985) and mice muscle (van der Laarse *et al*. 1984) is also mainly determined by the genetical background.

The large standard deviations found for the CS activities in both muscles are also described by others (e.g. Valberg *et al*. 1988). These authors showed that the CS activity in the muscle fibres of different horses could vary as much as twofold.

Age effect: The CS activity decreased in the first five months after birth. Apparently, the need for oxidative enzymes decreases in the course of time. A possible explanation is that equine muscles grow excessively during the first months of life and therefore need sufficient enzyme activity to deliver the substrates and energy for the biosynthesis of muscle proteins. After the growth of the fibres has levelled off, the enzyme activity is

allowed to decrease. In our study group the peak growth rate appears before 20 weeks of age (unpublished data). Similar effects were found for capillarity parameters in the same group of foals (unpublished data). The fact that the CS activity in the semitendinosus muscle decreases immediately after birth while in the deep gluteus medius the decrease occurs later, might be related to the postural role of the latter. It has been stated that foals have a higher glycolytic and a lower oxidative capacity (and therefore higher resting lactate levels) than older horses (Lindholm and Piehl 1974; Essen *et al*. 1980). Apparently, later in life the oxidative capacity increases again, probably when the horse begins to work.
Exercise effect: The decrease of the CS activity in this study was the same for the three training groups, so an exercise effect could not be demonstrated. Other investigators concluded that training (e.g. Essen-Gustavsson *et al.* 1989: five-week regime of controlled intensive daily training on a high-speed treadmill) rapidly increases the CS activity in the gluteus medius muscle tissue of (young) adult horses. Therefore, despite the younger age of our horses, the absense of an exercise effect was unexpected. A reliability test on our data revealed that, to be detectable, an exercise induced difference must exceed 14 %; possibly a small exercise effect was present but undetectable.

Measurements of whole muscle CS activity do not provide information about the distribution of the oxidative capacity among the fibre types. Different fibre types belong to different motor units (Burke *et al.* 1973; Nemeth *et al.* 1986) and their recruitment depends on the activity level of the muscle (Henneman, 1981). It is therefore possible that our exercise regimen led to a fibre-type-dependent effect on the oxidative capacity. It is for this reason that, when a subtle exercise effect must be demonstrated, investigation at the level of the fibres is recommended.

SDH activity
General: The higher oxidative enzyme activity in type I and IIa fibres compared to the activity in IId fibres is an expected finding (e.g. Valberg *et al.* 1988) but the similar result for the activities in type I and IIa fibres is rather surprising. Although in some studies (e.g. Rivero *et al.* 1998; rat) other orders are found, in most species, type I fibres have the highest oxidative capacity and type IIa fibres are usually intermediate between type I and IId (or IIB in older studies) (Essen *et al*. 1975 (human); Essen-Gustavsson and Henrikson, 1984 (human); Reichmann and Pette, 1984 (mouse and rabbit); Valberg and Essen-Gustavsson, 1987 (horse)). Our foals are very young. Maybe, the mature order becomes visible later in life.
Age effect: Further diversification between the fibre types I and IIa on the one and IId on the other hand occurs during growth: increase of the SDH activity per surface unit in type I and IIa fibres and decrease in type IId fibres. It is conceivable, that differential growth of the four types leads to different degrees of ‘dilution’ of the enzyme activity. To check whether this is a possible explanation for the different age effects in the fibre types, the increase in cross-sectional area was compared (table 2, unpublished data). The growth factors of the fibre types did not differ much. Therefore, growth differences cannot be held responsible for the different age effects.

Table 2. The mean cross-sectional area's (in : m^2) of the four different fibre types at 0 and 22 weeks of age. The growth factor was calculated from the increase.

	0	22	Growth factor
Type I	351.7	1256.4	3.6
Type IIa	334.9	1291.4	3.9
Type IIad	386.8	1573.1	4.1
Type IId	797.0	2983.4	3.7

Table 3. VO_{2max} and cross-sectional area (CSA) from the four different fibre types of the gluteus medius muscle at 22 weeks of age. Determination VO_{2max} from the SDH activity; thickness of the slide: 10 : m; incubation time: 1800 seconds. (van der Laarse *et al.* 1998)

	SDH activity (abs. 660 nm)	Absorbance . : m^{-1}. s^{-1}	VO_{2max}	CSA (: m^2)
Type I	0.30	1.67 . 10-5	0.10	1260
Type IIa	0.30	1.67 . 10-5	0.10	1290

Type IIad	0.25	1.39 . 10-5	0.08	1573
Type IId	0.12	0.67 . 10-5	0.02	2983

The type I and IIa fibres become more and the type IId fibres less oxidative. Type IId fibres occupy the largest relative area in the deep gluteus medius muscle (55 – 59 %), so their oxidative capacity contributes most to the results for the whole muscle tissue. The decrease of the earlier mentioned CS activity is in line with the decrease of the SDH activity in the type IId fibres.

Van der Laarse *et al*. found a linear relationship between the SDH activity (expressed in absorbance units per ∶m per second of incubation time) and the maximum velocity of oxygen consumption (VO_{2max}) (van der Laarse *et al.* 1989). Furthermore, they described a hyperbolic relationship between VO_{2max} and the cross-sectional area of the muscle fibres (van der Laarse *et al*. 1998). We determined for our foals the VO_{2max} in relation to the cross-sectional area of the four different fibre types at 22 weeks of age (table 3) and concluded that the VO_{2max} is relatively low. This means that, at 22 weeks, the fibres are still immature and it can be expected that, in the course of age, the CSA and / or oxidative capacity will increase.

Exercise effect: Previous studies on the same group of animals have shown that the exercise did not change the muscle fibre type composition (Dingboom *et al.* in press). However, an effect of (the lack of) exercise on the development of the SDH activity from 0 to 22 weeks of age (further decrease in the low oxidative IId fibres) was found in the population of type IId fibres. Apparently, this fibre type is the most sensitive for exercise regimen. When movement was allowed (training and pasture group), the oxidative capacity in these fibres remained stable. In case of restricted movement, the oxidative capacity of type IId fibres declined, possibly because they were not recruited sufficiently (Henneman, 1981).

We conclude that the oxidative capacity of the locomotory muscles of horses decreases during the first postnatal months and this decrease is stronger when movement is restricted. This subtle effect of (non) exercise could only be made visible with the method which analyses the muscle at the level of the muscle fibres, and not with biochemical assessment of CS activity of the muscle tissue.

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Chapter 6

Age-induced changes in capillary supply of equine locomotory muscles and the importance of movement in early life

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Summary

Effects of age and exercise on capillary supply in the deep gluteus medius and the superficial semitendinosus muscles of Dutch warmblood horses were studied on biopsies from 36 foals, in 3 training groups, at ages 0, 22 and 48 weeks, trained from 1-22 weeks. In lectin stained sections capillary density (CD), fibre density (FD), capillary to fibre ratio (C/F), mean cross sectional fibre area (CSA) and diffusion index (DX, area supplied per capillary) were measured. In the gluteus CD, FD and C/F were higher and CSA and DX lower than in the semitendinosus. At birth, CD and DX of the two muscles correlated positively. At 22 weeks this was also the case for C/F. From 0-22 weeks CD and FD decreased and C/F, CSA and DX increased in both muscles, but more so in the gluteus. From 22 to 48 weeks no changes occurred, except for continued increase of gluteus C/F. Lack of exercise caused a higher diffusion index in both muscles. *Conclusions*: the deep gluteus medius has a better blood supply than the superficial semitendinosus; capillary supply decreases due to growth; lack of exercise enhances this decrease and this effect is not restored later in life; lack of movement affects the gluteus less than the semitendinosus.

Keywords: horse, capillarity, gluteus medius, semitendinosus, exercise

Introduction

In order to function properly, muscle fibres need a sufficient blood supply, since substrates for energy production (i.e. carbohydrates and fatty acids) must be delivered by the bloodstream.

Mammals are born with a certain capillary supply, which changes in the course of time after birth due to growth of the muscle fibres. The growth changes result in a decrease in capillary density (e.g. Sillau and Bachero 1977; Ripoll *et al.* 1979; Kurnoth *et al.* 1994). During growth there is an increase in the number of capillaries around the fibres (e.g. Ripoll *et al.* 1979). Furthermore, in most cases the number of capillaries around type I fibres is higher than around type II fibres (Romanul 1965; Karlström *et al*. 1991).

The capillary supply of muscles is of importance for the exercise tolerance in horses (e.g. Gunn 1981; Karlstrom *et al.* 1991). Furthermore, it is well-known that, when higher demands are requested, muscle tissue reacts with vasodilatation, followed by the growth of more capillaries. Studies about the effect of exercise on the capillarity of locomotory muscles of different horse breeds at (young) adult ages (e.g. Hodgson *et al.* 1985, 1986; Hodgson and Rose 1987; Sinha *et al.* 1993; Rivero *et al.* 1995) and humans (Andersen and Hendriksson 1977) show reversible changes due to training and detraining. However, the capillarity of equine locomotory muscles at birth and in the first postnatal months and the influence of exercise in such an early phase of life is unknown.

The capillary supply, or capillarity, can be expressed as the amount of capillaries per mm^2 (capillary density) or per muscle fibre (capillary to fibre ratio). In both cases, the results are depending on the cross-sectional areas of the muscle fibres involved. Therefore, it is more accurate to describe the capillarity by a parameter indicating the muscle fibre area one capillary has to supply (diffusion index) (Andersen and Hendriksson 1977; Rivero *et al.* 1995).

We have studied the capillarity of two muscles. The first muscle is the semitendinosus muscle. This muscle is part of the hamstring group. The actions and uses of the hamstring muscles are complex. When the hind limb bears weight they extend the hip and knee joint, propulsing the animal. When the hoof is raised from the ground, they flex the knee joint. The second muscle is the gluteus medius, a muscle of exceptional size and power. This muscle is primarily an extensor of the hip, so its main function is propulsion. However, it also has a major postural role, because of its function as stabiliser of the hip joint during weight bearing. Because of this extra postural role, one can expect that the gluteus muscle is better vascularised, will change in a different way with age and will react differently to exercise than the semitendinosus.

The purpose of our study is to determine the capillary supply in locomotory muscles of Dutch warmblood foals at birth and the development in the first months of life. Further, we investigated whether external factors, like the amount of exercise, can affect the normal development of the capillarity, especially in the dynamic postnatal period where it can be expected that the sensitivity for phenotypic influence is high. At the same time, it can be expected that each individual horse is born with a certain characteristic capillarity

which is defined for all locomotory muscles. For this reason, we have also investigated if the capillarity parameters of the gluteus medius muscle correlate positively with those of the semitendinosus.

Material and methods

Foals

The investigation was performed in a group of 36 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system, as described by van Weeren and Barneveld (1999). All procedures had been approved by the animal experiments committee (DEC) of the Faculty of Veterinary Medicine (Utrecht). After birth, all foals remained with the mares in a paddock for one week, after which they were randomly divided into three groups that were subjected to different exercise regimens until weaning at five months. Two groups (boxrest, n = 12, and training group, n = 11) were individually housed with the mare in 3 x 3.5 m box stalls. They were fed freshly cut grass ad libitum, along with the mother's milk. The third group was kept at pasture with the mare 24 h a day (pasture group, n = 13). At 22 weeks of age 22 foals were euthanized for other experimental purposes. The remaining 14 foals groups (boxrest, n = 5; training, n = 4; pasture, n = 5) were joined in one single group which was kept in a loose house with access to a small paddock.

Exercise protocol

From 0 to 22 weeks, the boxrest group was kept in the box stall for 24 h/day. The training group was also kept in box stalls, but was given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals would follow the mares. Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 22 weeks 32 and 16 sprints on alternating days. Exercise was given 6 days a week. The sprints lasted for about 6 seconds (6.5 m/s) and a pause was kept after each sprint to prevent exhaustion. After the sprints, the foals were allowed an additional 0.5 hours of free exercise in the enclosure. The pasture group received voluntary movement. After 22 weeks up to 48 weeks, none of the foals was trained, so all got the same exercise regimen.

Muscle biopsies

Percutaneous muscle biopsies were taken from each foal according to the protocol of Lindholm and Piehl (1974) in the first week after birth (day 3 on average) and at the age of 22 weeks. From the remaining foals, biopsies were also taken at the age of 48 weeks. To cause as little discomfort as possible, the young foals were handled by their caretakers in

the presence of their mares and sedation was not necessary. After local anaesthesia, 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. They were taken as deep as possible (until resistance from the iliac wing). Biopsies from the superficial semitendinosus were taken on a line drawn from ischiadic tuber to popliteal area, at two thirds distance from the ischiadic tuber, at a depth that was reached just after the muscle fascia was penetrated. The samples were rolled in talcum powder, mounted on cork blocks with the use of OCT embedding medium and oriented in such a way that the fibres could be sectioned transversely. Transverse serial sections (10 µm) were made with a cryostat at - 20°C.

Histochemical staining

The sections were mounted on cover slips and dried for 1 hour at room temperature. Subsequently they were fixed in a mixture of methanol 35%, acetone 35%, acetic acid 5% and aqua destilata 25% for about 20 hours at –20 degrees. The next day the sections were incubated for 30 minutes in 0.3% H_2O_2 in methanol followed by hydration and incubation with pronase (1:100 in Tris buffered Saline (TBS) for 30 minutes. The next step was rinsing 3 times 5 minutes in TBS again. Incubation with Teng-T (100mM TBS, 50mM EDTA, 1.5 M NaCl, 2.5% gelatine and 0.5% Tween; 1:10 in aqua dest.; pH 8.0) for 15 minutes followed. The sections were incubated overnight with biotinylated lectin[1], 0.005 mg/ml in TBS for capillaries and 0.02 mg/ml in TBS for cell membranes. The next day the sections were rinsed 3 times 5 minutes in TBS, followed by incubation with avidin and biotin[2] (both components 1:100 in TBS, mixed at least 30 minutes before use). Peroxidase was coupled to biotin and 3.3'diaminobenzidine tetrachloride[3], the staining agent. After rinsing in Tris-HCl (Tris buffered in HCl, pH 7.4), 3 times for 5 minutes, visualisation took place with 0.05% 3.3'diaminobenzidine tetrachlorid, 0.01 % H_2O_2[4] for capillaries (brown staining) and 2.5% ammoniumnickelsulphate[5] in Tris-HCl for fibre membranes (black staining). The process of visualisation was controlled by microscopy and lasted for about 10 minutes. The reaction was stopped by rinsing in aqua dest. Finally the sections were dehydrated and enclosed in DePeX[6].

Measurements

The stained sections were digitised at 100 x magnification using a Nikon microscope and a LEICA DC 100 CCD-camera. The microscope magnification resulted in a computer display magnification of 400 x. The images were analysed using LEICA QWIN 2.3 software. A program to measure capillary densities and fibre cross-sectional area was developed. The software counted twice (in order to reduce the effect of the variations in brightness of the microscope lamp) the number of capillaries in a measuring frame of 0.44 mm^2. From each section 5 fields were measured, because earlier experiments showed considerable variation in capillary count between different fields in one section. The average of the 5 fields was used for analysis.

Adjacent sections, stained for fibre area measurements, were digitised at 400 x magnification and 3 fields were measured. For greater accuracy, in the sections from the newborn foals, 5 fields were measured with a computer display magnification of 800 x. The total number of fibres counted in each field was at least 100. The mean fibre area was computed with the average of 3 respectively 5 fields. This way, nearly the total area of the sections was involved in the CD and CSA measurements.

From the computer measurements, we calculated capillary density (CD expressed as capillaries/mm^2), fibre density (FD expressed as fibres/mm^2), capillaries per fibre ratio (C/F, equalling CD/FD), mean fibre cross sectional area (CSA in mm^2) and diffusion index (DX). The latter was calculated as follows:

$$DX = \frac{CSA \times FD}{CD} \quad (mm^2 / capillary)$$

Statistical analyses

General lineair model-repeated measures (SPSS 10.0 for Windows) was used to test the effects of age and exercise. Thereby time was used as within subject factor and exercise as between factor. Factor time was defined at three levels (0, 22 and 48 weeks). The first two levels to establish the effect of exercise and the last level to see if exercise effects persisted. Analysis of variance (ANOVA) was used to test if there were differences already existing at birth. 'Muscle' was used as a second within subject factor to analyse differences between the gluteus medius and the semitendinosus muscle. To test if the muscle fibre composition of the gluteus medius and the semitendinosus muscle are correlated with each other, we performed Spearman's bivariate correlation tests. To test gender differences we used the independent t-test. In all tests, differences were accepted when $p < 0.05$.

Results

General

Figure 1 gives the results for capillary density (CD), fibre density (FD), capillary per fibre ratio (C/F) and mean cross sectional fibre area (CSA). The diffusion index, as more important parameter for the capillarity, is displayed separately (figure 2). In these figures, the column graphs demonstrate the difference between the two muscles and the age effects. Exercise effects are visualised in the line graphs at the right, separately for the two muscles. In table 1, the capillary parameters are given for the three training groups and the total group. Figure 3 illustrates the detected correlations between the two muscles.

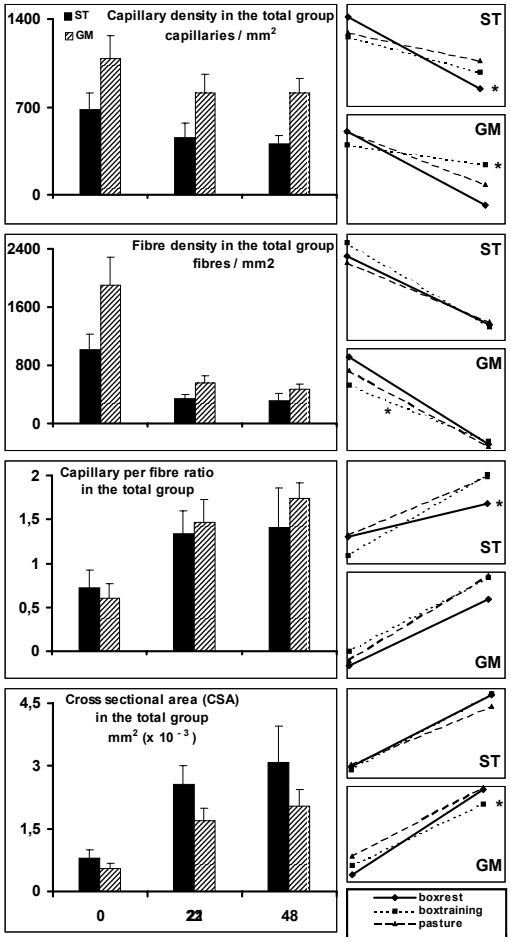

Fig. 1. The means (column graphs) and standard deviations (y-error bars) of the CD, the FD, the CF and the CSA in the total group at three different ages (0, 22 and 48 weeks) from the semitendinosus (black colums) and the gluteus medius (grey colums). n = 36 (at 0 and 22 weeks) or 16 (at 48 weeks). The line graphs at the left demonstrate the means of the CD, the FD, the CF and the CSA in the three training groups at two different ages (0 and 22 weeks) for the semitendinosus (ST) and the gluteus medius (GM) separately. * Significant exercise effect ($p < 0.01$).

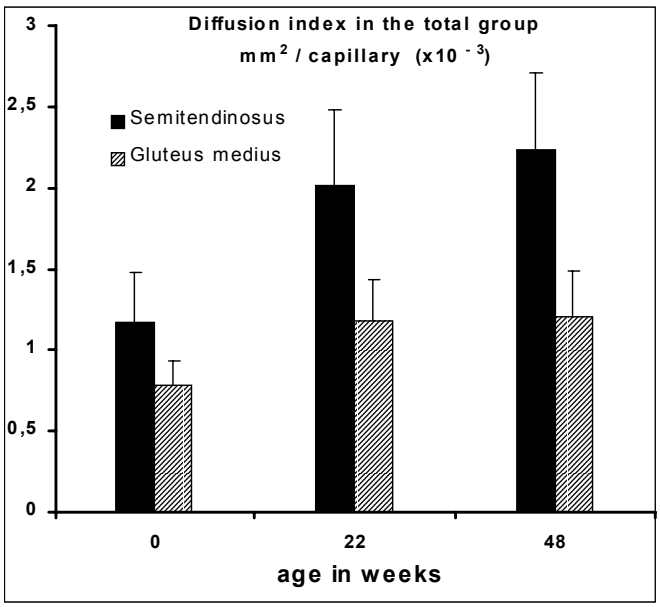

Fig. 2. Results for the diffusion index. For explanation see legend to figure 1

Muscle effect

In the gluteus medius muscle capillary density (CD), fibre density (FD) and capillary to fibre ratio (C/F) were higher and cross-sectional area (CSA, fig. 1) and diffusion index (DX, fig. 2) lower than in the semitendinosus muscle ($p < 0.01$). At birth, CD and DX of the gluteus medius correlated positively with CD and DX of the semitendinosus muscle (fig. 3; $p < 0.03$). At 22 weeks not only CD and DX, but also C/F of the two muscles were positively correlated (fig. 3; $p < 0.05$).

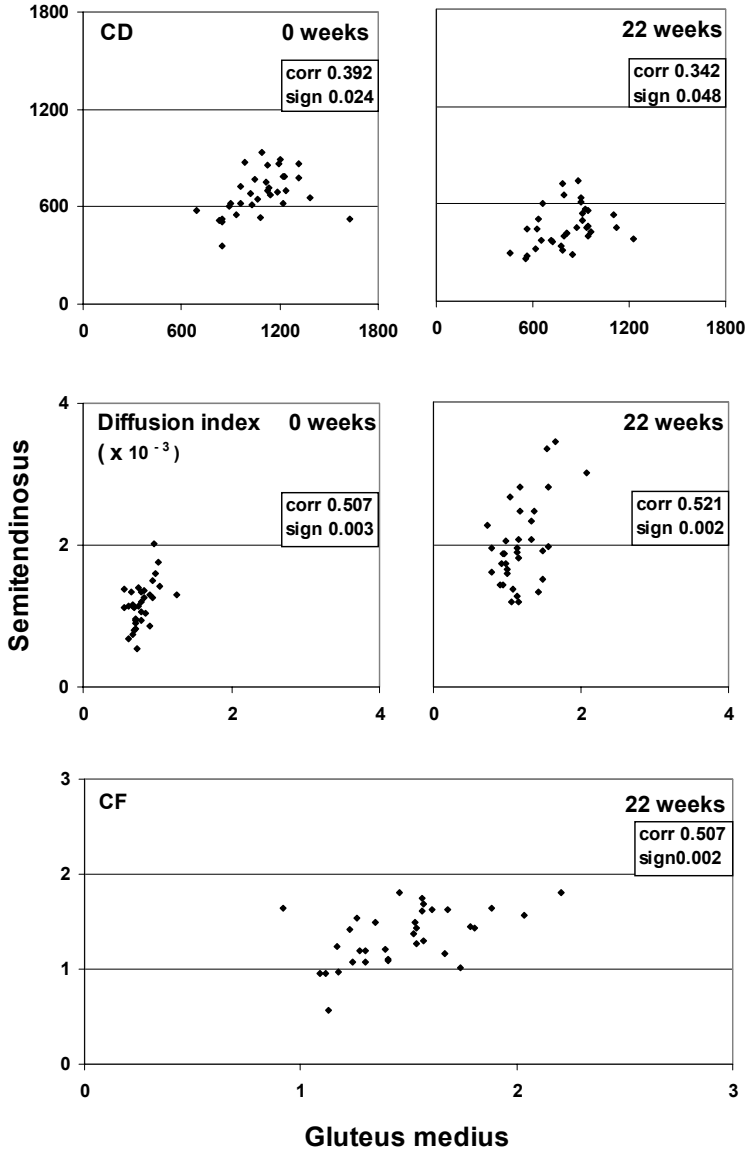

Fig. 3. Scatter plots of capillarity variables of gluteus medius and semitendinosus muscle that show a statistically significant correlation.

Age effect
In both muscles there was a statistically significant age effect from 0 to 22 weeks ($p < 0.001$) e.g. a decrease in CD and FD and an increase in C/F, CSA (fig. 1; table 1: total) and DX (fig. 2; table 1: total).

For all parameters except the CD the age effects in the two muscles had different slopes in the period from 0 to 22 weeks ($p < 0.001$). We found a significant stronger decrease of FD and a stronger increase of C/F in the gluteus medius than in the semitendinosus muscle. On the other hand, the increase of CSA and DX was stronger in the semitendinosus muscle.

From 22 to 48 weeks there were no significant changes except in C/F in the gluteus medius which continues its course of increment (fig. 1; table 1: total; $p < 0.001$).

Exercise effect
In the gluteus medius muscle a significant difference ($p < 0.02$) was seen between the boxrest and the training group concerning the CSA, and consequently also the FD, already existing at birth. This unexpected finding is most probably caused by an error of measurement.

In both muscles there were statistically significant effects of exercise in the period from 0 to 22 weeks ($p < 0.01$; fig. 1 and 2 (line graphs); table 1). In the semitendinosus muscle the decrease of CD and the increase of DX were more pronounced in the boxrest group. Furthermore, the increase of C/F was less pronounced in the boxrest group. In the gluteus medius muscle the decrease of CD and FD and the increase of CSA were less pronounced in the training group. The increase in DX was, like in the semitendinosus muscle, stronger in the boxrest group.

The two muscle did not react equally to the exercise regimen. In the first place, exercise suppressed the growth of the muscle fibres (CSA) in the gluteus medius muscle but not in the semitendinosus muscle. Secondly, in the boxrest group, the area one capillary had to supply (DX) enlarged relatively more in the semitendinosus than in the gluteus medius muscle. Both effects were statistically significant ($p< 0.001$).

From 22 to 48 weeks (no exercise) no statistically significant interactions were found between (previous) exercise regimen and time. In other words, the courses in time of the capillary parameters in the three training groups were parallel (table 1). Consequently, the between-group differences remained present.

Discussion

General
Mammals, growing from birth to subadult size, show an interesting similarity in the capillary density and capillary to fibre ratio. The CD decreases from 1500 to 250 per mm^2 in rat muscle (Ripoll *et al.* 1979; Kurnoth *et al.* 1994), from 1000 to 250 per mm^2 in pig muscle (Kurnoth *et al.* 1994) and from 1085 to 400 per mm^2 in equine muscle (this study).

In young adults humans CD has a value of 250-425 per mm^2 (Andersen 1975; Andersen and Hendriksson 1977; Brodal *et al.* 1977). The C/F, given by the same authors, change from 0.5 to 3.0 in rat muscle, from 0.5 to 1.2 in pig muscle and from 0.6 to 1.7 in equine muscle. The C/F in young, adult human muscle varies between 1.7 and 2.5. It appears that the size of the animal is unimportant in determining these characteristics. At first sight this is remarkable as the maximum oxygen uptake capacity ($VO_{2\ max}$ in O_2/min/kg bodyweight) depends on the size of the animal and tends to decrease with increasing body size (Hoppeler *et al.* 1987, Tayler *et al.* 1981). However, the horse is an exception of this rule as the maximum rate of oxygen uptake capacity in horses is comparable with that of rats. Even the very best of human endurance athletes do not reach these levels (Saltin and Gollnick 1983). As CD and C/F values are comparable in these species, this shows that these parameters are not sufficient in describing O_2 uptake capacity of muscles.

Muscle effect

It could be hypothesised that the capillarisation of muscles in individual animals is subject to a genetically determined variability. This would imply that the capillarity of a particular muscle in an animal is determined by the muscle itself (fibre type composition, use) but also by the individual make-up of the animal. This idea is supported by our data for the gluteus medius and the semitendinosus muscle. The capillarity parameters of these two muscles show significant positive correlations at 0 and 22 weeks of age. However, we did not find this at 48 weeks. This might due to the fact that the experimental group size had became too small to demonstrate a correlation.

Systematic differences in capillarity between the muscles might be attributed to their differences in fibre type composition. The deep gluteus medius muscle has a higher percentage of slow contracting, oxidative, fatigue resistant type I fibres. The superficial semitendinosus muscle, has a high percentage of fast contracting, glycolytic, fast fatigable type II fibres (Dingboom *et al.* 2002). Other investigators (e.g. Karlström *et al.* 1991) found that type I fibres are surrounded by relatively more capillaries than type II fibres. For this reason it can be expected that the amount of capillaries per mm^2 (CD) and the amount of capillaries surrounding a fibre (C/F) will be higher, the mean cross sectional area of the fibres (CSA) smaller and the diffusion index lower in the gluteus medius muscle as compared to the semitendinosus. This is exactly what has been found.

Age effect

Like in other mammals (e.g. Sillau and Bachero 1977; Ripoll *et al.* 1979; Kurnoth *et al.* 1994), the capillary supply of equine muscles changes due to increase in girth of the fibres (CSA increases) and simultaneous increase in the number of surrounding capillaries (C/F increases). Despite the appearance of new ones, the capillaries become more separated from each other (CD decreases). Although at the age of 22 weeks more capillaries surround the fibres than at the time of birth, these capillaries have to supply a larger cross-sectional area (diffusion index increases). Apparently, the need for blood supply decreases during growth. A possible explanation is that equine muscles grow excessively during the first months of

life and therefore need a sufficient bloodsupply to deliver the substrates and energy for muscle protein synthesis. After the growth of the fibres has levelled off, the diffusion index would be allowed to increase. To which extent this happens may depend on the workload. The relatively smaller increase of CSA, decrease of C/F, and smaller decrease of the diffusion index in the deep gluteus medius muscle compared to the semitendinosus muscle might be the result of the presumed postural role of the former.

From 22 to 48 weeks the C/F in the gluteus medius continues its increase. Despite the greater number of capillaries per fibre, there was no statistically significant change in the CD and the diffusion index. This is especially strange, taken in account that the CSA remained the same. Most probably this must be explained methodologically. The small group size for this period and the large variation preclude demonstration of all but very large differences. Up to what age the C/F will continue to increase is unknown.

Whether the muscles have reached an adult state of capillarity cannot be determined from this study. Karlström *et al.* (1991) performed their study with older standardbred trotters (2-9 years) and found in the gluteus medius muscle a mean CD from 725 capillaries per mm^2 and a mean C/F of 2. These results are similar to our results at the age of 48 weeks (table 1), suggesting that our horses had reached capillary maturity. Henckel (1983) investigated the muscle capillarity in the gluteus medius from two and three years old standardbred horses. He also did not find an age effect on this ratio in this period.

Exercise effect

The capillarity of locomotory muscles has a major influence on sport capacity (e.g. Gunn 1981). A relationship exists between the maximal O_2 uptake and the capillary density (e.g. Armstrong *et al.* 1992; swine and rats). The capillarity of locomotory muscles and the influence of exercise have been investigated in humans (e.g. Protector *et al.* 1995; McCall *et al.* 1996), horses (e.g. Henckel 1983; Essen-Gustavsson *et al.* 1985 and 1989; Rivero 1996; Tyler *et al*. 1998) and other mammals (e.g. Parsons *et al.* 1985; dog). In general it was concluded that exercise enhances the oxidative capacity by an increase of the capillary density. One has to take in consideration that this is not only caused by the proliferation of capillaries surrounding the fibres but, especially after a certain period of endurance training, by reduction of the mean CSA of the fibre types. McCall (1996) showed in humans, that also in resistance training, where the CSA usually increases, an increase of the C/F takes place, however insufficient to lower the diffusion index. Parsons *et al.* (1985; dogs) and Nimmo *et al.* (1982; horses) saw no exercise effect whatsoever and attributed this to the insufficient length or intensity of the training period. This emphasises the importance of the correct and clear definition of the training regimen.

In our study, daily exercise influenced the different capillarity parameters in the two locomotory muscles in an equal direction, leading eventually to a higher diffusion index in case of no exercise (e.g. boxrest) compared to the situation where the horses were free to move in the pasture. In box-kept foals only 5 to 10 minutes of daily exercise were sufficient to reach the same effect. Lack of movement affects the deep gluteus medius

muscle less then the semitendinosus muscle, which emphasises the extra postural function of the deep gluteus medius.

From 22 to 48 weeks of age, the situation, created by the lack of exercise is sustained, despite of the introduction of (free) movement in this period. We conclude that in the first half year of life some form of movement, free or forced, is necessary for the development of a normal capillarity. The poor perfusion because of the lack of movement will not be restored by normal movement later in life.

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Chapter 7

Summarising discussion

Speed of contraction (Myosin Heavy Chain expression)

General

From the study, described in chapter III, we concluded that to investigate muscle fibre type composition, muscle fibre characterisation with the use of monoclonal antibodies raised against myosin heavy chain is more accurate and therefore preferable above the ATPase staining method. Therefore, in the following paragraphs we only discuss the results from the immunohistrochemistry.

The study described in chapter II is the first to demonstrate the presence of Cardiac-α and Developmental MHC in equine skeletal muscle. It appeared that after birth a significant number of fibres of the gluteus medius muscle co-expresses either Developmental and type IIa-MHC or Cardiac-α and type I-MHC. Cardiac-α MHC, characteristic for heart atrium, was previously found in cranial muscles, like masticatory, extraocular and hyoid muscles, but not in trunk and limb muscles (Bredman *et al*. 1990). Rivero *et al*. (1996b) found this myosin to be absent in equine gluteus medius muscle. This contradictory finding may be explained by the fact that the horses used in their study were too old to demonstrate the expression of Cardiac-α MHC. In contrast to the situation in rabbit masseter muscle (Bredman *et al*. 1991), Cardiac-α MHC did not appear as the only MHC in a muscle fibre, but always in combination with other MHC isoforms, usually with type I MHC. The presence of Developmental MHC in equine locomotory muscles is a new finding but was already described for developing (e.g. Butler-Brown and Whalen 1984) and regenerating muscles (Sartore *et al*. 1982) of other mammals.

In the deep gluteus medius muscle the percentages of fibre type I were higher and type IId lower than in the superficial semitendinosus. The deep gluteus medius muscle serves for propulsion but is also a postural muscle. Even when a horse is at rest, the muscle has to be active to stabilise the hip joint. The superficial semitendinosus muscle is only active in a moving horse. Therefore it is imaginable that the gluteus medius muscle needs a higher number of slow contracting, oxidative type I fibres to be more fatigue resistant than the semitendinosus.

In chapter III a possitive correlation is described between the fibre type compositions of the gluteus medius and semitendinosus muscle. This suggests that individual horses may be characterised by an overall slow, or fast make-up of their locomotory muscles**.**

Age effect

From chapter II and III it can be concluded that the MHC expression of the semitendinosus muscle did not change in the first year of life. We found a constant composition of on average 13 % type I, 23 % type IIa, 40 % IId and 20 % IIad fibres. Other investigators found different percentages, depending on sampling depths (e.g. Aberle *et al*. 1976; Barrey *et al*. 1995; Essén *et al*. 1980; Karlström *et al*. 1994). On the other hand, a fast turnover of

fibre types in the deep gluteus medius muscle takes place in the first months of life. The Cardiac-α isoform expression decreases after birth and can no longer be demonstrated after the age of 22 weeks. The decrease was accompanied by a consistent increase of numbers of fibres expressing solely type I MHC. The data of chapter II suggest that the slow fibre population is stable in the first year of life, except for the disappearance of fibres co-expressing MHC type I and Cardiac-α. However, after study of an increased number of animals (n = 38 in chapter III, versus n = 16 in chapter II) a significant increase was found in the percentage of fibres expressing MHC isoform type I. The Developmental isoform expression disappears during the first 10 weeks after birth. In locomotory muscles of small mammals these isoforms also do not persist longer than until a few weeks after birth (Butler-Brown and Whaler 1984; d'Albis *et al*. 1986). Furthermore, in the fast fibre population there was a increase of fibres expressing IIa MHC, replacing fibres expressing IId MHC. This change was reflected in the presence of a quite large (approximately 25 % at birth) population of fibres co-expressing MHC IIa and IId (see also Biral *et al.* 1988; Galler *et al*. 1994; Pette and Staron 1997). Rivero *et al*. (1996a) found frequencies of type IIad fibres of 10 - 15 % in the gluteus medius of 2 and 3 years old horses. This suggests that, in our study group, the transition had not been completed. Indeed, at the age of 11 months the frequency of type IIa/d fibres was still 18 %.

In our study group, the fibre type composition of the deep gluteus medius muscle at birth is comparable to that in adult horse breeds with good sprint capacities, showing a high percentage type IId and low percentages of type IIa and I fibres. At the age of 48 weeks, the composition had developed towards a more endurance type of composition, showing a lower percentage type IId fibres (20 %) and higher percentages type IIa and I fibres (both 30 %) (see for percentages in different races e.g. Snow and Guy 1980; Bechtel and Kline 1987).

The finding of the increase in type I and type IIa fibre percentages is in line with the results of other studies with horses of different ages (e.g. Essén *et al*. 1980; Essen-Gustavson *et al*. 1983; Henckel 1983; Lindholm and Piehl 1974; Rivero *et al*. 1993; Roneus *et al.* 1991; Roneus 1993). As type I and type IIa fibres generally have a better fatigue resistance, the deep gluteus medius muscle apparently becomes increasingly resistant against fatigue. However, we found a decrease rather than an increase of the oxidative capacity in the gluteus medius muscle (see paragraph 'resistance against fatigue: Age effect'). This inconsistency can be explained by the fact that, although in our study group, the muscle fibre type composition of the gluteus medius muscle changed, the relative cross-sectional area occupied by the different fibre types remained equal from 0 to 22 weeks of age (unpublished data). Changes in the oxidative capacity of the gluteus medius muscle of our foals are therefore do not result from changes in fibre type composition, but from changes in the oxidative enzyme activities in the individual fibre types.

Exercise effect

Although the gluteus medius and the semitendinosus muscle, two important propulsive muscles of the body, are expected to be well adaptable to training, we did not observe an exercise effect with respect to the fibre type composition of both muscles. The question arises whether our exercise protocol provided enough stimulation to induce changes in the muscle. The exercise performed can best be described as a moderate intensity sprint exercise. This kind of exercise may affect the muscle fibre areas and the oxidative capacity of the existing fibres before a change in MHC expression occurs (e.g. Hodgson *et al*. 1985). The exercise was intensive enough to initiate a reaction in this muscle (see paragraph 'resistance against fatigue: Exercise effect'). Also Suwannachot *et al*. (1999) showed, in the same horses, a significant increase in the total concentration of Na+,K+-ATPase in the deep gluteus medius muscle of the training group. The mean difference in fibre type composition is subject to a quite large intra-individual variation. From chapter IV it appears that in the gluteus medius muscle the confidence intervals for individual observations were 10 - 15 %. Exercise induced effects will therefore only be measurable if they are large; small effects can only be demonstrated in large groups. In cross-sectional studies a group size of 8 (chapter II) and 12 (chapter III) animals should be enough to resolve fibre type composition changes of 8 - 9 %. Potentially, exercise could have an effect on the rate of change of these fibre types, but in our studies the exercise effect, if present, must have been smaller than 8 – 9 %.

Resistance against fatigue

General

From chapter II, III and IV it appeared that the deep gluteus medius muscle has a higher type I / type IId ratio than the semitendinosus. As type I fibres generally posess higher levels of oxidative enzyme activities, it can be expected that the gluteus medius muscle is more resistant against fatigue. It can also be expected that this muscle is better vascularised because type I fibres are usually surrounded by relatively more capillaries than type II fibres (e.g. Karlström *et al*. 1991). Indeed we found that the gluteus medius has higher oxidative enzyme activity (chapter V; citrate synthase (CS)) and better vascularisation (chapter VI) than the semitendinosus. This finding is in line with the results of other investigators (e.g. Essen-Gustavsson et al. 1989).

Earlier, we suggested that horses may have an overall slow, or fast make-up of their locomotory muscles because of the finding of significant positive correlations between the fibre type compositions of the two locomotory muscles. Because such correlations were also found for CS activity (at 0 weeks) and capillarity parameters (at 0 and 22 weeks) it can be hypothesised that, in general, functional properties of muscles in individual animals are subject to a genetically determined variability.

Maximum oxygen uptake capacity

The maximum oxygen uptake capacity of muscle fibres ($VO_{2\ max}$; expressed in O_2/min/kg bodyweight) depends on the size of the animal and tends to decrease with increasing body size (Hoppeler *et al.* 1987). The horse is an exception to this rule as the maximum rate of oxygen uptake capacity in horses is comparable to that of rats. The $VO_{2\ max}$ is hyperbolically related to the cross-sectional area of the fibre types (van der Laarse *et al*. 1998) because diffusion distance is a limiting factor for the usability of oxygen for energy production. In order to attain a $VO_{2\ max}$ comparable to that of rats, the size of equine muscle fibres must be approximately equal that of muscle fibres of rats. In a muscle, the average size of the fibre cross-sectional area depends on the muscle fibre type composition, but in general, the mean CSA in adult rat muscle is 2000 – 6000 $:m^2$ (Ripoll *et al.* 1979) and adult horses 2400 – 5300 $:m^2$ (Gunn 1995; Rivero *et al*. 1993).

In studies with swine and rats a relationship was demonstrated between maximal oxygen uptake and capillary density (Armstrong *et al.* 1992). Despite the diversity in maximum oxygen uptake capacity, the muscle capillarity of other (non-equine) mammals (including human) show an interesting similarity with our data for horses in the capillary density and capillary to fibre ratio (see chapter VI for references). This shows that capillarity alone is not sufficient in predicting oxidative capacity of muscles. Because a linear relationship exists between the maximum oxygen uptake capacity and succinate dehydrogenase (SDH) activity (van der Laarse *et al.* 1989) it is possible to estimate the VO_{2max} by measuring the SDH activity of the muscle fibres. By this method we estimated the VO_{2max} of the four different fibre types of our foals at 22 weeks of age and plotted these values against the cross-sectional areas. We concluded that the VO_{2max} of our foals is low in relation to the muscle fibre size. This means that, at 22 weeks of age, the fibres are still immature and it can be expected that in the course of time, the oxidative capacity must increase to mature quantities.

Age effect

In both gluteus and semitendinosus muscle, the oxidative capacity decreased during the first five months after birth. This was apparent as a decrease of oxidative enzyme activity (CS in muscle tissue and SDH in the type IId fibres) and as a decrease of vascularisation relative to the mean fibre area. This can be interpreted as a diminishing need for high resistance against fatigue in the first year of life. An alternative explanation is that in early life, the growing muscles need sufficient blood supply and oxidative enzyme activity to deliver the substrates and energy for the biosynthesis of muscle proteins. After the growth of the fibres has levelled off, the oxidative capacity would be allowed to decrease. In our study group the peak growth rate was reached before 20 weeks of age (unpublished data). To what extent and at what time this decrease starts may depend on the function of the muscle. The relatively smaller decrease of the diffusion index in the deep gluteus medius muscle and the

fact that in this muscle the CS activity decrease occurs later than in the semitendinosus muscle might be the result of the presumed postural role of the former.

Whether the muscles have reached an adult state of oxidative capacity cannot be determined from these studies. It has been stated that adult horses have a higher oxidative capacity than young foals (Lindholm and Piehl 1974; Essen *et al*. 1980). Apparently, later in life the oxidative capacity increases, probably when the horse begins to work. Studies of the capillarity in muscles of older horses (e.g. Karlström *et al.* 1991) show results that are similar to our results at the age of 48 weeks. This suggests that at this age, our horses have already reached a mature state of capillary supply.

Together with the decrease of SDH activity in the type IId fibres, we observed an increase in type I and IIa fibres. This shows that changes in oxidative capacity can occur in opposite directions in different fibre types and consequently in different types of motor units. Type IId fibres occupy the largest relative area in the deep gluteus medius muscle (55 – 59 %), so their oxidative capacity contributes most to the obtained results for the whole muscle tissue. The decrease of the SDH activity in the type IId fibres is in line with the decrease of the CS activity in muscle tissue.

Exercise effect

The effect of exercise on the oxidative capacity in our studies might better be described as the effect of non-exercise because it was the boxrest group that distinguished itself from the other two groups. In the boxrest group, movement was restricted. The SDH activity in the IId fibre population decreased more strongly and the capillarity remained underdeveloped in this group. For normal maturation of the oxidative capacity the muscle needs to be stimulated. Lack of movement affects the deep gluteus medius muscle less then the superficial semitendinosus muscle, which emphasises the extra postural function of the deep gluteus medius.

Of major concern are the signs that the situation, created by the lack of exercise, will not be restored by normal movement later in life. Despite of the introduction of (free) movement in the period from 22 to 48 weeks of age, the poor perfusion of the locomotory muscles was sustained.

The lack of exercise effect on CS activity was an unexpected finding. Most previous studies describe a rapid increase of the CS activity as a reaction to exercise (e.g. Essen-Gustavsson *et al*. 1989). In the interpretation of the data, it must be considered that measurements of whole muscle CS activity do not provide information about the distribution of the oxidative capacity among the fibre types. Different fibre types belong to different motor units (Burke *et al*. 1973; Nemeth *et al*. 1986) and their recruitment depends on the activity level of the muscle (Henneman, 1981). It is therefore possible that our exercise regimen led to a fibre-type-dependent effect on the oxidative capacity. Apparently, the fibre type IId population is the most sensitive to the exercise regimen. When movement was allowed (training and pasture group), the oxidative capacity in these fibres remained

stable. In case of restricted movement, the oxidative capacity of type IId fibres declined, possibly because they were not recruited sufficiently (Henneman, 1981).

Conclusion

The first objective of this thesis was to obtain better fundamental insights in the normal growth and maturation of equine locomotory muscles in the first year of life. Three parameters related to the ability to perform at high sport levels were studied: muscle fibre type composition, oxidative enzyme activities and capillarity. It was demonstrated that juvenile MHC isoforms (Cardiac-α and Developmental) are expressed in equine skeletal muscles in the first weeks after birth. Furthermore, it is concluded that at birth the deep gluteus medius is slower than the superficial semitendinosus muscle and becomes, due to an increase of percentage type I and IIa fibres and a decrease in type IId fibres, even slower in the course of time. The fibre type composition of the semitendinosus muscle is stabile. In both muscles, the oxidative capacity (oxidative enzyme activity and capillarity) decreases during the first postnatal months.

The second aim of this study was to learn to what extent exercise interacts with the postnatal development of muscle. We observed no measurable exercise effect with respect to the fibre type composition of both muscles, but concluded that muscles needs to be stimulated for normal maturation of the oxidative capacity. Therefore, in the first half year of life some form of movement, free or forced, is necessary. The poor perfusion because of the lack of movement will not be restored by normal movement later in life.

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Main conclusions

In this summarising chapter the main conclusions from the preceding chapters of this thesis are listed (in italics) and briefly discussed.

Chapter II *It is concluded that a fast turnover of fibre types takes place in the deep gluteus medius in the first months of life. Potentially, exercise could have an effect on the rate of change of these fibre types.*

During the first 48 weeks there was a consistent increase of fibres expressing IIa MHC, replacing fibres expressing IId MHC. This change was reflected in the presence of a quite large population of fibres co-expressing MHC IIa and IId. The difference between the exercised (training-) and non-exercised (boxrest-) group was small but suggested that the increase of IIa fibres was larger in the training group. It appeared that after birth a significant number of fibres co-express either Developmental and type IIa-MHC or Cardiac-α and type I-MHC. The Developmental isoform disappears during the first 10 weeks after birth and almost all the α isoform expression during the first 22 weeks.

Chapter III *It is concluded that the gluteus medius muscle is slower than the semitendinosus muscle and becomes more slow in the course of time. The muscle fibre composition of the semitendinosus muscle is stabile. We observed no exercise effect with respect to the fibre type composition of both muscles.*

In general, the gluteus medius contained more type I fibres but less type IId fibres compared to the semitendinosus. At most ages the fibre type compositions of both muscles correlated with one another. There was an increase of fibres expressing I and IIa MHC in the gluteus medius. At the same time there was a decrease of fibres expressing IId MHC and fibres co-expressing MHC IIa and IId. The MHC expression of the semitendinosus muscle did not change in time at first, but from 22 to 48 weeks there was decrease of the percentage type IId fibres.

Chapter IV *It is concluded that the contribution of different error sources to the total variance in fibre type counts of the equine gluteus medius muscle is large and especial from the regional and local level considerable. Local muscle fibre heterogeneity is responsible for one third of the error variance.*

Due to the large error variance, the confidence intervals for individual observations were 10 - 15 %. For this reason, in longitudinal studies a group size of 4 animals is necessary to resolve fibre type composition changes of 10 %, in cross-sectional studies 7 animals per group appear necessary. The resolution of the method can be improved by processing 3-4 shavings from the same insertion channel.

Chapter V *It is concluded that the oxidative enzyme activity is higher in the gluteus medius compared to the semitendinosus muscle and, at birth in the gluteus medius positively correlated with the oxidative enzyme activity in the semitendinosus muscle. The oxidative capacity of the locomotion muscles of horses decreases during the first postnatal months due to a decrease of the oxidative capacity in the type IId fibres. The decrease is more evident when movement is restricted.*

The CS activity is higher in the gluteus medius compared to the semitendinosus muscle. Furthermore, at birth, the CS activity in the gluteus medius muscle is positively correlated with the CS activity in the semitendinosus muscle. In both muscles, the CS activity decreases from 0 to 22 weeks of age. An exercise effect could not be detected. At birth, the SDH activity in type IId fibres is low, compared to the other three types. At 22 weeks of age the SDH activity in type IIad fibres is also lower than in type IIa and type I fibres, but remains higher than in type IId fibres. From 0 to 22 weeks there is a significant age effect i.e. an increase of SDH activity in fibre type I and IIa and a decrease in fibre type IId. The decrease of SDH activity in type IId fibres is more dramatic in the boxrest group.

Chapter VI *It is concluded that the gluteus medius muscle has a better blood supply than the semitendinosus muscle. Each individual has a characteristic capillarity. Furthermore, the capillary supply changes dramatically due to growth. In the gluteus medius muscle this age effect is more evident. For normal maturation of the capillarity, the muscle needs to be stimulated. The poor perfusion because of the lack of movement will not be restored by normal movement later in life. Lack of movement effects the gluteus medius muscle less than the semitendinosus muscle. This emphasises the extra postural function of the gluteus medius.*

In the (deep) gluteus medius muscle the CD, the FD and the C/F were higher and the CSA and the diffusion index were lower than in the (superficial) semitendinosus. At birth, the CD and diffusion index in the gluteus medius correlated both positively with the CD and the diffusion index in the semitendinosus. At 22 weeks this was, along with the CD and the diffusion index, also the case for the C/F. In both muscles there was an age effect from 0 to 22 weeks e.g. a decrease in the CD and the FD and an increase in the C/F, the CSA and the diffusion index. In the gluteus medius muscle this age effect was more evident, due to a larger increase in the C/F combined with a relatively lesser growth of the fibre diameter. This led to a smaller increase of the diffusion index in the gluteus medius muscle compared to the semitendinosus muscle. From 22 to 48 weeks there were no changes, except in the C/F in the gluteus medius. The latter value continued its increase. Exercise influenced the different parameters in the two locomotion muscle in an equal direction. As a result, the diffusion index reached a higher value, when there was no exercise as compared to the situation where the horses were exercised or free to move in the pasture.

Nederlandse samenvatting

Het Nederlandse warmbloedpaard (KWPN) is een ras dat in de diverse takken van de paardensport veel gebruikt wordt. Het prestatievermogen van de dieren is afhankelijk van factoren als karakter, uithoudingsvermogen en de kwaliteit van het voortbewegingsapparaat (skelet en locomotiespieren). Deze factoren zijn voor een deel reeds bij de geboorte gedefinieerd en in de navolgende maanden van groei en rijping ontwikkelen zij zich volgens een van nature aanwezig patroon.

Het prestatievermogen is naast deze natuurlijke aanleg te verbeteren met behulp van goede training. Er is bij mensen en (jong)volwassen paarden veel studie gedaan naar de invloed van training en beweging op functionele spiereigenschappen als kracht en weerstand tegen vermoeidheid. Deze studies hebben het positieve effect van training meerdere malen bewezen. De vraag is echter of er nog meer voordeel gehaald kan worden wanneer jonge veulens al in het eerste levensjaar aan geforceerde beweging worden blootgesteld. De verwachting is dat in de periode na de geboorte (= postnatale periode), waarin de spieren zich moeten aanpassen aan de eisen van de buitenwereld, het bewegingpatroon van invloed is op de normale ontwikkeling. Misschien kan dan, doormiddel van extra beweging, al in het eerste levensjaar, een betere basis voor verdere aanpassing tijdens de 'normale' trainingsperiode in een latere levensfase gecreëerd worden.

In dit proefschrift wordt een groepsbeschrijving gegeven van 38 Nederlandse warmbloedpaarden uit het EXOC project (EXamination OsteoChondrosis) welke onze faculteit in 1996 uitvoerde in samenwerking met het proefdierstation Waiboerhoeve te Lelystad. Het was voor ons een unieke kans om een dermate grote groep paarden te kunnen onderzoeken. De groep werd al in de eerste levensweek verdeeld in 3 'trainingsgroepen'. De eerste groep was de Boxgroep. De dieren uit deze groep werden, samen met de moeder, permanent op stal gehouden. De tweede groep was de Boxtrainninggroep. Deze groep werd ook op stal gehouden, maar onderging daarnaast een dagelijks sprintprogramma. De derde groep was de Weidegroep. Deze dieren hadden een onbeperkte bewegingsvrijheid. Op de leeftijd van 22 weken is de helft van de dieren geëuthanaseerd voor andere onderzoeksdoeleinden dan de onze. De andere helft werd, als groep, in een paddock gehuisvest waarin iets vrije beweging mogelijk was.

Er zijn op meerdere leeftijden tussen 0 en 48 weken, van 2 belangrijke voortbewegingsspieren (gluteus medius en semitendinosus) biopten genomen. Deze zijn geanalyseerd op kracht en snelheid (uitgedrukt in spiercontractie-eigenschappen) en weerstand tegen vermoeidheid (uitgedrukt in doorbloeding en oxidatieve enzymactiviteiten). De analyses werden gedaan volgens moderne methodes zoals het gebruik van monoclonale antilichamen en beeldanalysesystemen.

Onze studie is de eerste studie die de aanwezigheid van twee 'afwijkende' spiercontractie-eiwitten in de locomotiespieren van pasgboren veulens beschrijft Deze spiercontractie-eiwitten zijn gewoonlijk aanwezig in het hartspierweefsel of in spierweefsel herstellend van een beschadiging. Uit onze studies kan verder geconcludeerd worden dat, tijdens het eerste levensjaar, door groei en rijping van de voorbewegingsspieren, de 'prestatie gerelateerde' spiereigenschappen sterk veranderen. KWPN paarden worden, qua spiercontractie-eigenschappen, geboren als 'sprinter' en ontwikkelen zich gaandeweg verder tot 'duursportatleet'. De weerstand tegen vermoeidheid is groot bij de geboorte, neemt af gedurende de volgende 22 weken en is daarna stabiel. Als de jonge veulens, tijdens de zoogperiode, beperkt worden in hun bewegingsvrijheid (Boxgroep) dan zal de doorbloeding en het vermogen van de spieren om efficiënt energie te produceren 'onderontwikkeld' blijven. Beweging (geforceerd of door middel van weidegang) is noodzakelijk voor de normale groei en rijping van de voortbewegingsspieren. Van extra voordeel door geforceerde beweging in deze levensfase is uit onze studies niets gebleken. Aanbevolen wordt dan ook de jonge dieren, met name in de zoogperiode, zoveel mogelijk weidegang te geven.

Dankwoord

Tja, waar zal ik mee beginnen ? Ik voel me dankbaar. Dankbaar dat het me gelukt is, dat het af is en dat het ook nog een samenhangend geheel geworden is. Dankbaar dat mijn gezin er niet al te veel onder geleden heeft (denk ik), dat ik me nu ook weer op andere dingen kan gaan concentreren. Ik ben dankbaar en tevreden. Maar voordat ik ga uitrusten wil ik met u, de lezer, eerst nog wat filosoferen over de totstandkoming van dit boekje.

In september 1996 kwam ik als dierenarts, nog behoorlijk naïef, bij de afdeling Anatomie te werken als junior docent / onderzoeker. Dat dat mij mogelijk gemaakt werd, daar moest ik allereerst *Henk van Dijk* voor bedanken. Hij attendeerde de baas op mij en mij op de baan. Ik gaf hem een fles wijn met de woorden dat ik hem niet zou teleurstellen en vol goede moed stortte ik mij op de wetenschap. Ik wilde vooral laten zien hoe goed ik dat kon. Helaas kon ik het niet goed en ik zocht mijn troost in het geven van onderwijs. Een en ander dreigde geheel te ontsporen doordat een aantal mensen hun twijfels niet onder stoelen of banken staken en mij regelmatig met diep fronzende blik op de tekortkomingen van de proefopzet, de begeleiding en de uitvoering van dit stukje onderzoek wezen. Als ik vertwijfeld en lichtelijk onzeker gas probeerde terug te geven werd er diep zuchtend gereageerd met de woorden: “de tijd zal het leren”. Nou ….. de tijd heeft het geleerd en deze mensen worden bedankt voor het vertrouwen. *Wim Weijs* nam me bij de hand en leidde me, soms wat onvoldoende bewust van mijn hypersensitiviteit, terug naar het pad der wetenschap. Ik ben hem daar dankbaar voor. Erg dankbaar, want ik heb me wat vaak voor het bordje “vluchtgang” gestaan, de neiging onderdrukkend er gehoor aan te geven. Maar ik bleef ….. en de eerste stukjes van de puzzel die ik moest oplossen begonnen op zijn plaats te vallen ….. en het ging steeds beter, ik begon het weer leuk te vinden.

Gaandeweg vormde zich een beeld van de groep jonge paarden, waarvan een vriezer vol materiaal was verzameld. Hiervoor waren, een jaar lang, mijn collega’s *Edwin Enzerink*, *Andries Klaarenbeek*, *Hans van Oudheusden* en *Grietje Dijkstra*, keer op keer uitgerukt richting Lelystad. Bij het verzamelen konden zij rekenen op perfecte assistentie. Hier wil met name *Henri*, *Marieke*, *Johan Knaap en zijn vrouw* voor bedanken. Aan *Grietje* de eer van het uitzoeken van de methodieken en het toepasbaar maken van de protocollen.

In de daarop volgende jaren moest de vriezer gaandeweg leger worden. Het bleef echter akelig lang vol doordat met name de vezeltypering enorm veel tijd in beslag nam. *Grietje*, *Hans* en ik zwoegden ons door de voorraad en we bleken in staat om 3 artikelen vol te “scoren”. Tussendoor en gaandeweg is middels de noeste arbeid van *Grietje* in Amsterdam en van *Hans*, *Karin Eizema* en *Ellen van der Wiel* in Utrecht, de activiteit van de oxidatieve enzymen in het spierweefsel gemeten (artikel 4). Dankzij het werk van onze enthousiaste diergeneeskunde studenten (*Simon Schuurman*, *Fleur Kloppenberg* en *Wendy Workel*) werd de doorbloeding in kaart gebracht (artikel 5). *Nancy Rietbroek* zette, middels een

diepgaande literatuurstudie, de mogelijke effecten van verschillende soorten training op de besproken spiereigenschappen op een rij. Zie hier…de opsomming van een top team.

Het naar buiten brengen van informatie moest natuurlijk voor het grootste deel voor mijn eigen rekening komen. Ik heb dan misschien een grote mond, maar op congres, in mijn eentje…hmmm. Een buitenlands congresbezoek was beduidend minder eng toen mijn fijne collega *Pisit Suwannachot* erbij was. Ik wil hem langs deze weg alsnog bedanken voor zijn steun bij mijn eerste 'echte' presentatie en voor het plezier wat ik dankzij en met hem gehad heb. *Sander Gussekloo*, *Wim Kersten* en *Claudia Wolschrijn* zijn mensen waar ik vooral vakinhoudelijk veel aan heb gehad. Regelmatig ben ik, mede dankzij hun indrukwekkend wetenschappelijke denken, op een goed spoor gekomen bij het schrijven van de verschillende artikelen.

Ceriel Maas en *Tanja Hoekstra* wil ik bedanken omdat zij in het 'schrijfjaar' mijn coördinatietaken in het onderwijs hebben overgenomen. De manier waarop zij dat gedaan hebben was zo bijzonder goed dat ik bang ben dat het voor velen een terugval zal zijn als ik zelf weer ten tonele verschijn. Nou … so be it, want ik kom toch en heel graag weer terug.

Dan wil ik op deze plaats *al mijn collega's* als groep bedanken. Ik heb een stel prima collega's. Dankzij hen, voel ik mij op mijn plek bij de Anatomie. Mijn angst om buiten de groep te vallen als gevolg van mijn gehaaste en zelfgerichte houding in 'het laatste jaar' is ongegrond gebleken. Zij toonden hun verdraagzaamheid met de, altijd weer negatief beantwoorde vraag of ik mee ging lunchen of met de lekkere extra bak middagkoffie. Een lekker potje lachen op het lab (of huilen bij *Henk*) heeft mij menigmaal nieuwe spirit gegeven om door te kunnen gaan. Bedankt lieve mensen.

En toen kon dit boekje gemaakt worden. Het was dankzij *Harry Otter* dat binnen afzienbare tijd, een stapel tekst, figuren en tabellen was omgetoverd in dit staaltje vakmanschap. Toen ik het zag, werd ik voor het eerst overspoeld door een overweldigend gevoel van trots. Met een brok in mijn keel dank ik hem voor de mooie afronding ons aller inspanning.

Aan *Bert*

Wat heb je een hoop te stellen gehad met mij. Om te promoveren moet je egoïstisch kunnen zijn en ik ben enorm dankbaar voor de ruimte die jij me daarvoor gegeven hebt. Om het gezinsleven op een gezonde manier door te laten gaan heb jij, vaak in je eentje, hard aan de kar moeten trekken. Ik wil dat je weet dat ik, in de tijd dat het leek dat ik constant de andere kant opkeek, nimmer blind was voor jou onbaatzuchtige toewijding. Bedankt, bedankt, bedankt. Op de een of andere manier zal ik het weer goed met je maken.

Liesbeth

Curriculum vitae

Elizabeth Dingboom was born on the 4th of December 1965 in Hendrik Ido Ambacht. In 1984 she passed her final exams at the grammar school Oude Hoven in Gorinchem. Until 1986 she followed the in-service education for nurse in the Roman Catholic Hospital in Dordrecht. In 1986 she started the course in Veterinary Medicine at the State University Utrecht. In 1994 she finished her studies and started teaching anatomy at the department of Functional Morphology of the Faculty of Veterinary Medicine, State University Utrecht. This was a part-time job and she combined it with veterinary work in several large and small animal practices in the country. In September 1996 she started her work as PhD student for 4 days a week at the department of Veterinaire Anatomy and Physiology, Utrecht University.

Liesbeth Dingboom is geboren in Hendrik Ido Ambacht op 4 december 1965. In 1984 deed zij eindexamen Atheneum-B op het 'Ouden Hoven' in Gorinchem. Tot 1986 volgde zij de in-service opleiding voor A-verpleegkundige in het Rooms Katholieke ziekenhuis in Dordrecht. Na drie keer meeloten startte zij in 1986 als nageplaatst student met de studie diergeneeskunde aan de Rijksuniversiteit van Utrecht. In 1994 studeerde zij af en begon haar carriere als toegevoegd docent op de toenmalige afdeling Functionele Morfologie van de Faculteit Diergeneeskunde, Rijksuniversiteit Utrecht. Dit betrof een parttime baan welke zij combineerde met veterinair werk in meerdere landbouw- en gezelschapshuisdieren praktijken in het land. In september 1996 startte zij haar functie als junior docent / onderzoeker bij de Veterinaire Anatomie en fysiologie, Universiteit Utrecht.

Stellingen

behorende bij het proefschrift

Equine Locomotory Muscles

Postnatal development and the influence of exercise

Liesbeth Dingboom

Utrecht
7 maart 2002

I De natuur is een wondelijk iets, dat moet je niet altijd proberen te begrijpen.

II Recent onderzoek toonde aan dat het eten van vet voedsel bepaalde spiereigenschappen in dezelfde richting beinvloedt als training. Wat een opluchting.

III Training zorgt er slechts voor dat de schadelijke effecten van beweging sneller hersteld worden.

IV "Houden van" is een werkwoord (voor diegenen die denken dat het vanzelf gaat).

V Waar je voor zorgt, daar ga je van houden.

VI IIB or not IIB, that's the question (Hamlet: *Shakespeare* 1601; *Dingboom et al*. 1999).

VII De ware genezende kracht komt vanuit je eigen onderbewustzijn.

VIII Onvoldoende beweging tijdens de jeugd leidt tot een onderontwikkeld vermogen om op een efficiente manier energie voor spierarbeid te produceren.

IX Iedere dwaas kan de waarheid spreken. Het vereist enig verstand om te weten hoe je goed liegt (*Samuel Butler*).

X Rokers kunnen zich het niet veroorloven om aan de gezondheidsrisico's te denken. Als ze dat namelijk wel doen, wordt hun zelfs de illusie dat ze er van genieten afgenomen (*Allen Carr* 1995).